Molecular characterization of tumor vascular phenotype and pharmacology of antiangiogenic therapy
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Chapter 7

Summary, discussion & future perspectives
SUMMARY, DISCUSSION & FUTURE PERSPECTIVES

Gaining insight into a drug’s actual mechanism of action before it enters clinical trials is the ideal route to antiangiogenic drug development and increases the likelihood for clinical success. In daily practice, the opposite route is however taken. In fact, many anti-angiogenic drugs are used without a precise understanding of how they work - which target cells they hit, what molecular processes they alter and which of these events are resulting in antitumor therapeutic efficacy [1]. This is illustrated by the current status of antiangiogenic clinical trials: although VEGF inhibitor therapy evidently has the ability to induce functional changes in the vasculature of patients with metastatic colorectal cancer [2, 3], the tumor response is less dramatic than could be expected from preclinical studies [4]. In various clinical trials, the survival benefits are relatively modest, averaging several months [5-9]. A better understanding of the molecular make up underlying the process of angiogenesis and of the response of the various cell types within the complex tumor tissues to drug treatment forms a first step toward a more rational design of antiangiogenic treatment regimens. It enables identification of molecular markers of targets that may be used for the design of effective (multi)targeted treatment strategies or serve as biomarkers either as read out for drug effects or for selecting patients that will respond to the drug. As introduced in Chapter 1, the research described in this thesis focuses on identifying the molecular nature of the angiogenic phenotype of the vasculature of pre-clinical tumors, and the changes in this phenotype that are associated with effective tumor growth inhibition by antiangiogenic therapy. This chapter summarizes the results described in this thesis, discusses them in a broader context and highlights the future perspectives for the field of antiangiogenesis research.

Tumor vascular heterogeneity

Besides introducing the objectives and approach of the research described in this thesis, in Chapter 1 we briefly introduced the basic principles of the regulation of tumor angiogenesis and the current concepts on the mechanism of action of anti-VEGF therapies aimed at inhibiting tumor angiogenesis. Central to our work was the hypothesis that the vasculature in tumors is characterized by a large degree of heterogeneity. Tumor vascular behavior, in terms of e.g., morphology and gene expression, may vary from tumor type to tumor type, in relation to the host environment in which the tumor grows, and with tumor growth stage. In addition, even cell-to-cell variation within a single vessel may occur. Tumor vascular heterogeneity is highly likely to influence the efficacy of antiangiogenic therapy. For example, while tumor growth progresses, the vasculature
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goes through different stages of angiogenic activation, sprout formation and vascular maturation. The level of vessel maturity has been clearly linked to its susceptibility to antiangiogenic therapy in preclinical studies [10]. Already more than 10 years ago, Bergers et al. demonstrated that tumors in more advanced stages of growth respond differently to angiogenesis inhibitors than early-stage tumors [11]. Moreover, even on a single cell basis, variations in angiogenic activation status may influence the responsiveness of endothelial cells to therapy. In Chapter 2, we reviewed the current knowledge on tumor vascular heterogeneity and we discussed our current understanding of how antiangiogenic drugs affect tumor endothelial behavior in more detail.

Molecular characterization of the tumor vascular phenotype

We hypothesized that differences in angiogenic status of endothelial cells during tumor outgrowth would be reflected in variations in vascular morphology, and in the underlying local expression patterns of angiogenesis- and vascular maturation-regulating genes. In Chapter 3, we demonstrated that morphology of the vasculature in the subcutaneously (s.c.) growing B16.F10 melanoma changed drastically during tumor growth. In contrast to our expectations, the accompanying profile of angiogenesis-related genes did not change. We applied laser microdissection to isolate the tumor vasculature from its pathophysiological environment, and compared gene expression in the vasculature from large tumors with that in quiescent non-angiogenic endothelium from healthy kidney venules. This revealed that numerous pro-angiogenic genes such as integrin β3, Nur77 and Ang2 exhibited a gain of function in the tumor vasculature, while loss of function was observed for vessel stability genes such as Ang1 and Tie2. As this pattern was not different in early versus late stage of tumor outgrowth, we concluded that B16.F10 tumor vasculature continues to actively engage in angiogenesis using the same specific pro-angiogenic repertoire, even in the later stages of tumor growth.

We next zoomed in on the different vascular morphological phenotypes that were observed in large tumors: large lumen-containing vessels existed next to small vascular profiles without a lumen. Surprisingly, these two morphologically different types of vessels were not associated with a different angio-gene expression profile, or with a different proliferative capacity of the surrounding tumor cells. Yet, we found that large lumen-containing vessels were associated with pericytes that were strongly positive for αSMA, while small vascular profiles were devoid of αSMA expressing pericytes. The difference in αSMA positivity may reflect a difference in pericyte activation, or even vascular maturation status [12], that in this model, however, has no relation to the transcriptional activity of the genes studied, even though these genes are nowadays considered key players in angiogenesis and vascular maturation [13-15].
As we could not identify molecular determinants of vascular heterogeneity throughout B16.F10 tumor growth, we were unable to address the broader aim of our study, i.e., to establish the molecular nature of tumor vascular heterogeneity during tumor outgrowth. Extension of our study to genome wide transcriptional profiling is necessary to identify if other genes than the ones under study are underlying the observed differences in vascular phenotype. Such studies were thus far not feasible due to the low yield of material during laser microdissection, especially from small tumors. On the other hand, one could argue that the three different stages of B16.F10 tumor growth studied in fact represent one single stage of exponential growth, in contrast to the clinical observation that tumor progression in humans is often characterized by variations in growth kinetics. Hence, vascular heterogeneity is likely to present a greater issue in the clinic. This is also illustrated by differences in vascular phenotype in relation to angiogenic behavior, tumor grade, or even invasiveness in clinical tumor specimens of various cancers [16-22]. Nevertheless, the relevance of our data for current angiogenesis research is found in the observation that the molecular control of neovascularization, as concluded from studies in transgenic animal models that either overexpress specific genes or have a full gene knock-out, differs from that endorsed by endogenously controlled mechanisms. For example, while murine knock-out models and CAM assays suggested a role for VEGF in lumen formation [23, 24], VEGF was expressed at equal levels in small vascular profiles versus large lumen-containing vessels. This emphasizes that each pre-clinical angiogenesis model needs to be appreciated for its own molecular repertoire underlying the angiogenesis process, and that care should be taken in extrapolating observations from one model to the other.

**An in vitro approach for in vivo pharmacology**

Heterogeneity in VEGFR2 phosphorylation status within tumors, as demonstrated in e.g., human colorectal carcinoma [25], suggests that during tumor outgrowth endothelial cells are present in different stages of angiogenic activation. Engagement in VEGF signaling in vivo may render the receptor differentially responsive to VEGFR2 inhibition. This issue is neglected in the majority of studies that evaluate efficacy of these drugs in vitro, as these are conducted in quiescent endothelial cells [26-28]. The possible influence of a pre-existent status of angiogenic activation on responsiveness to therapy remains therefore unknown. In Chapter 4, we mimicked this in vivo situation in vitro, by investigating whether the efficacy of VEGFR2 inhibitor treatment is changed when the endothelial cells are exposed to the inhibitor after angiogenic activation with VEGF. We made use of the receptor tyrosine kinase inhibitors vandetanib (ZD6474), vatalanib (PTK787/ZK222584), and SU6668, that all bind to the ATP-binding pocket of VEGFR2.
and thereby prevent dimerization and autophosphorylation [26, 29, 30]. In addition to VEGFR2, these inhibitors have affinity to other tyrosine kinase receptors (Chapter 4, Table 1), and they are currently in Phase I, II and III clinical trials for various types of cancer [31-33]. VEGFR2 inhibitor treatment after angiogenic activation less efficiently blocked expression of the VEGFR2-responsive genes EGR3, NR4A1, NR4A3 and COX-2 than VEGFR2 inhibitor treatment prior to angiogenic activation. This demonstrates that the pharmacological efficacy of VEGFR2 inhibitors is impaired in endothelial cells that are already engaging in VEGF-signaling. We restricted our analysis until now to induction of gene expression. Further studies should evaluate late VEGFR2 inhibitor treatment for its ability, or lack thereof, to inhibit endothelial cell function, such as proliferation, migration and tube formation.

To understand the molecular events underlying the impaired responsiveness of endothelial cells engaged in VEGF signaling, we investigated the effect of vandetanib treatment on the kinetics of phosphorylation of VEGFR2 and its downstream kinases extracellular signal-regulated kinase (ERK), p38 mitogen activated kinase (MAPK) and Akt. Vandetanib treatment of quiescent cells inhibited VEGFR2 phosphorylation for the first 5 minutes after administration, thereby inhibiting phosphorylation of ERK and p38 as well, but not of Akt. Yet, p38 and ERK remained inhibited for the full 30 minutes under study, while VEGFR2 phosphorylation gradually increased even under VEGFR2 inhibitor pressure. In contrast, vandetanib treatment of angiogenically active endothelial cells did not inhibit VEGFR2, p38 and Akt phosphorylation, but significantly decreased ERK phosphorylation only at >10 minutes after the drug was added.

The observation that vandetanib did not induce any detectable change in phosphorylation at the surface receptor level, while remarkably affecting downstream ERK was surprising. As discussed in Chapter 4, this may involve direct inhibition of ERK by vandetanib [34]. Another explanation is that small changes in VEGFR2 activity could result in major changes in ERK phosphorylation, although to our knowledge, this has not been reported so far. Yet it should be taken into account that ELISA-based detection of phosphorylated ERK is more powerful to detect small changes in a reproducible manner than Western Blot-based detection of phospho-VEGFR2, p38 and Akt. Hence, we cannot rule out that similar changes as detected in ERK phosphorylation upon drug exposure are in fact occurring in VEGFR2, p38 and Akt phosphorylation, but remain undetectable with Western Blot. Quantifying phosphorylation levels of VEGFR2 and p38 using the same ELISA set-up is at present impossible due to the unavailability of these assays.

The observed delay in ERK inhibition upon late VEGFR2 inhibitor treatment suggests that VEGF activation renders the cell refractory for drug treatment for a short period of
time. This may involve redistribution of VEGFR2 within the cell and trafficking of a new pool to the cell surface. An important new question arising from this observation is how long a receptor in ligand-bound condition remains refractory for drug treatment? And once the drug is bound to the receptor, how long does it prevent autophosphorylation of the receptor? How does the drug affect the various pathways downstream of VEGFR2 and their kinetics of activation? Finding an answer to these questions requires the availability of good antibodies recognizing phosphorylated epitopes of VEGFR2 and its various downstream kinases in combination with fluorescent confocal or even live-cell imaging techniques. Furthermore, future studies should extend the analysis to other VEGFR2-regulated kinases, for example using signal transduction arrays [35] or kinome profiling [36, 37]. Importantly, the in vitro established knowledge on the kinetics of VEGFR2 signal transduction in response to VEGFR2 inhibitor treatment should be validated in vivo in animal models and ideally in patient material, for example by intravital or multiphoton excitation laser scanning microscopy [38]. Intravital imaging using paramagnetic, RGD-modified quantum dots that recognize αvβ3 integrin has been used to visualize angiogenic blood vessels in a tumor [39, 40]. If these quantum dots were to recognize VEGFR2 and its phosphorylated epitope, the effect of VEGFR2 inhibitor treatment on kinetics of VEGFR2 phosphorylation in vivo could be imaged in real-time. Also the proximity ligation assay could be used to study phosphorylation of these kinases and even interactions between these kinases, both in animal tissues and in clinical tumor specimens [41]. To obtain a complete picture of the molecular pharmacology of VEGFR2 inhibitors in vivo, the observed effects of VEGFR2 inhibitor treatment on kinase activation should be related to changes in the transcriptome, and to eventual changes in vascular behavior that lead to the antitumor effect.

**Molecular effects of VEGFR2 inhibitor therapy on the tumor vasculature in vivo**

Detailed knowledge on the molecular effects that antiangiogenic drugs exert on the tumor vasculature in vivo, and that culminate in an antitumor effect, is largely lacking. In Chapter 5 we therefore analyzed the compartmentalized effects of treatment with the VEGFR2/EGFR inhibitor vandetanib on gene expression and morphology of the vasculature of a s.c. growing Lewis Lung Carcinoma (LLC). Prior to gene expression analysis, we isolated tumor vascular cells by enzymatic digestion followed by anti-CD31-conjugated magnetic beads. In Chapter 6, we compared the changes in tumor vascular gene expression upon vandetanib treatment in LLC with those in B16. F10 melanoma by employing laser microdissection-assisted isolation of the tumor vasculature.
The data presented in Chapter 5 indicate that vandetanib exerts its antitumor effect through the induction of vascular stabilization. Efficient tumor growth inhibition by vandetanib was accompanied by a significant increase in the mRNA levels of vascular stability molecules Ang1, Tie2, Notch3 and PDGFRβ, and the pericyte markers desmin and αSMA in the vasculature [15, 42]. Concomitantly, the mRNA levels of the pro-angiogenic molecules integrin β3 and Robo1 decreased [43-45]. The increased pericyte mRNA levels in the isolated vascular cell preparations upon vandetanib exposure did not result from an increase in the percentage of vessels covered by pericytes. Instead, they pointed to enhanced endothelial-pericyte interactions upon vandetanib treatment, that were rendered less sensitive to separation by enzymatic digestion. This conclusion was supported by the elevated mRNA levels of N-cadherin after therapy, a molecule expressed at the endothelial-pericyte junction that is required for cell-cell adhesion between endothelial cells and pericytes [46, 47]. Some of the genes that responded to long-term vandetanib treatment were up-regulated (N-cadherin) or downregulated (integrin β3) immediately after start of therapy. Moreover, vandetanib rapidly shifted the balance of Ang2/Ang1 toward Ang1, with the initial downregulation of Ang2 at 6h and 26h after a single dose being followed by an upregulation of Ang1 after long-term treatment. These data suggest that the process of enhanced vascular stabilization and endothelial-pericyte adhesion was initiated immediately after treatment was started and continued throughout the 10-day treatment period. Validation of the increased expression of Ang1, PDGFRβ, Notch3 and N-cadherin at the protein level will be necessary to confirm the observed shift toward vascular stabilization.

In Chapter 6, we compared the molecular pharmacology of vandetanib in the s.c. growing LLC with that in the s.c. growing B16.F10 melanoma, and employed laser microdissection to isolate the vasculature of these tumors prior to gene expression profiling. Although in both models tumor growth was effectively inhibited, the effects of vandetanib on tumor vascular behavior was different. While the vascular density in B16.F10 tumors markedly decreased upon therapy, vandetanib did not affect vascular density of LLC. Furthermore, vandetanib increased the expression of Tie2, N-cadherin and P-selectin, while decreasing the expression of Ang2 and integrin β3 in Lewis Lung Carcinoma, confirming the shift toward vascular stabilization observed in Chapter 5. Yet in B16.F10 vasculature, vandetanib did not affect Tie2 and N-cadherin, but upregulated eNOS next to P-selectin. This demonstrates that while the outcome of therapy can be the same, the underlying molecular changes that orchestrate the antitumor effect can be different for each tumor type. It is not unlikely that human tumors will respond to therapy with an even larger degree of heterogeneity, emphasizing the notion that successful evaluation
of therapeutic efficacy in the clinic will require identification and validation of specific biomarkers of response for each individual tumor type.

To investigate the possibility that vandetanib targets different cellular compartments in LLC and B16.F10, we assessed the localization of the major targets for vandetanib. Both VEGFR2 and EGFR expression were restricted to the vasculature, implying that pharmacological targeting of the endothelium alone can be sufficient to induce an effective antitumor effect. Furthermore, given the 10-fold higher affinity of vandetanib to VEGFR2, and the observation that expression of EGFR was much less intense than that of VEGFR2, we concluded that the observed changes in vascular gene expression are predominantly mediated by VEGFR2 inhibition. A striking observation was that in both tumor models vandetanib reduced protein levels of VEGFR2, and to a lesser extent of EGFR, while leaving mRNA levels unaffected (Chapter 5 and 6). A role for the recently identified VEGFR2 protein-controlling microRNA-296 [48] in the vandetanib-induced loss of VEGFR2 protein was however excluded. The extent of VEGFR2 and EGFR loss should be quantified by ELISA or Western Blot, this was however far unfeasible due to the small amounts of tumor tissue obtained from the vandetanib-treated tumors. A highly relevant question that should be addressed in future studies is how drug-induced VEGFR2 loss will affect the antitumor outcome if treatment would be continued for longer time periods. Moreover, validation of our observations in human tumor biopsies is required. If tyrosine kinase inhibitor-induced loss of target protein were to occur in clinic, this may represent a major mechanism of resistance [49] and may (partly) account for the limited efficacy of VEGFR2 inhibitor therapy in the clinic. Furthermore, the observed VEGFR2 loss emphasizes the likely need for therapeutic strategies that target more than one molecule, as will be discussed below.

Our data and those published by others suggest that promotion of vascular stabilization renders vessels less dependent on VEGF, and thus less sensitive to VEGFR2 inhibitor therapy [10, 50]. Nevertheless, promotion of vascular stabilization may be beneficial, as it results in vascular normalization, a restoration of the balance between pro- and anti-angiogenic signaling that may lead to the formation of more regularly shaped vessels that are characterized by reduced permeability and improved blood flow [3, 51]. As such, normalization of the vasculature may improve delivery of chemotherapeutic agents to the tumor tissue, as recently demonstrated by Eichhorn et al. [52]. In contrast, others reported a decline in the uptake of contrast agents upon VEGFR2 inhibitor therapy [53, 54], which could also be a consequence of inhibition of VEGF-induced permeability in tumor vasculature [55], thereby challenging the improved delivery concept.
Isolation of endothelial cells

Profiling of whole tumor RNA reflects the status of both tumor and host cells, making it difficult to dissect molecular events within specific cellular compartments in the tumor microenvironment. Analysis of the compartmentalization of gene expression is necessary to unravel the local balances of gene expression that control endothelial behavior. Its importance was illustrated in Chapter 3, by our finding that the ratio between the pro-apoptotic gene Tsp-1 and the anti-apoptotic Bcl2 was 1.9 in the tumor tissue, but 13.6 in the tumor vasculature. Thus, analysis of the compartmentalization of gene expression revealed that a relatively higher degree of Tsp-1 induced pro-apoptotic signaling may occur in the tumor vasculature compared to the non-vascular tumor compartment.

In Chapter 3, we initially microdissected B16.F10 tumor vasculature that was morphologically visualized with hematoxylin [56]. As such, our isolation was restricted to vessels that could be recognized by their visible lumen. Over the past years, we developed a protocol for visualization of the vasculature using fluorescently labeled lectin or antibodies to Collagen IV that could be used for laser microdissection while maintaining RNA integrity. This enabled us to microdissect also small vascular profiles without a visible lumen, such as in LLC, and to separately analyze vascular segments with different morphology (Chapter 3 and 6). A next step will be to separately laser microdissect endothelial subsets by their immunohistochemically identified phenotype [57, 58]. The current status is that these techniques face certain limitations, not only with regard to the availability of antibodies that allow sensitive detection of specific markers while maintaining RNA integrity, but also with regard to the RNA and protein yield of laser microdissected tissue. Buckanovich et al. successfully combined laser microdissection of immunohistochemically detected ovarian cancer endothelium with amplification of mRNA and Affymetrix microarray [59] and identified tumor vascular markers specific for ovarian tumors [60, 61]. Development of more sensitive techniques for gene expression profiling that require an even lower input, such as the Illumina BeadArray platform, will broaden the possibilities of combining laser microdissection with genome-wide screens in the future. If such array techniques were to allow input of laser microdissected endothelial subsets that are differentially activated as observed by e.g., gain or loss of (phosphorylated) VEGFR2 or αvβ3 [25, 62, 63], unraveling the true spatiotemporal heterogeneity of endothelial behavior during ongoing tumor growth in small (clinical) tissue specimen would come within reach.

In addition to laser microdissection (Chapter 6), we isolated endothelial cells from Lewis Lung Carcinoma using the widely employed method of enzymatic digestion combined with CD31-magnetic bead-based single cell selection [64-66] (Chapter 5),
Figure 1: Comparison of two methods to isolate endothelial cells. Endothelial cells from Lewis Lung Carcinoma were isolated either by enzymatic digestion combined with magnetic bead-based selection for CD31\(^+\)-cells or by laser microdissection of tumor vasculature that was visualized by fluorescent Collagen IV staining. A-C: Enrichment of mRNA of the vascular markers CD31 (A) and VE-cadherin (B) and the pericyte marker \(\alpha\)SMA (C) in isolated vascular cell fractions compared to whole tumor. Values depict mRNA levels in vascular cells adjusted to GAPDH and expressed as fold increase versus whole tumor. Each dot represents a sample. D: Vandetanib-induced changes in mRNA expression of angiogenesis and endothelial behavior-associated genes in LLC vasculature isolated by the two different methods. Values represent mRNA levels in treated tumors adjusted to GAPDH and expressed as fold change versus untreated tumors. LLC-bearing mice were treated with vandetanib or vehicle for 10 days, starting at day 6 after tumor inoculation. *p<0.01 in microdissected vascular cells from vandetanib versus vehicle-treated mice, \(n = 6\); \(p<0.01\) in magnetic bead-isolated endothelial cells from vandetanib versus vehicle-treated mice; \(n = 4\). ND\(_{veh}\) = not detectable in vehicle-treated mice, but clearly detectable in vandetanib-treated mice.
both with the aim to investigate the changes in endothelial gene expression upon vandetanib treatment. Both methods introduce important advantages and disadvantages. Isolation of endothelial cells by enzymatic digestion combined with magnetic beads yields a high purity sample, as concluded from the high enrichment of vascular marker mRNA (150-1000 fold for CD31; 50-175 fold for VE-cadherin; Fig 1A-B). However, the incubation with collagenase at 37°C, and also the dissociation from their natural surroundings and the detachment of integrins from ECM components, may induce changes in the transcriptome of these cells. On the other hand, one could argue that both the experimental and the control group underwent the same procedure, hence the observed differences in gene expression must be induced by the treatment. In contrast, laser microdissection-isolated endothelial cell fractions are characterized by a lower yield and a lower degree of purity as judged from the lower enrichment in endothelial marker mRNA (12-38 fold for CD31; 18-52 for VE-cadherin), yet the cells are isolated in a frozen state, ensuring that the tumor-imprinted endothelial transcriptome is maintained during the procedure. Moreover, laser microdissection offers the important advantage that knowledge regarding the localization and (morphological) phenotype of the cells under study is maintained.

Using laser microdissection, we identified a set of 5 vandetanib responder genes (Ang2, Tie2, N-cadherin and P-selectin with \( p<0.01 \), and PDGFRβ with \( p=0.106 \)) in LLC vasculature. Analysis of magnetic bead-isolated vascular cells confirmed three of these genes (Tie2, N-cadherin and P-selectin) and identified ten additional ones, predominantly pericyte-related, as responders to vandetanib treatment (Fig 1D). These differences likely find their origin in the different endothelial cell isolation methods employed. Both methods lead to the concomitant isolation of adhered pericytes, as demonstrated by the enrichment in αSMA (Fig 1C). Yet, only when the endothelial cells were isolated using magnetic beads, we observed an increase in the pericyte mRNA content upon vandetanib treatment. This difference could be explained by the mechanical nature of the isolation procedure. In the first, co-isolation of adhered pericytes depends on the strength of endothelial cell-pericyte interactions, while in the latter, dissecting vessel fragments with a laser is not influenced by cell-cell adhesion. Hence, the stabilized endothelial-pericyte interactions as a result of vandetanib treatment caused a significant increase in the generally low number of pericytes that are co-isolated during the magnetic bead procedure.

**Mouse models for cancer**

A serious limitation of the rapidly growing xenografts or syngeneic mouse tumors implanted in avascular pockets is that they fully depend on angiogenesis for their growth. Yet, various types of human cancer have been reported to acquire a vasculature by other
mechanisms as well, such as vascular co-option and mimicry [67-71]. Moreover, the blood vessels in these s.c. growing models are present in a synchronized stage of outgrowth and thus homogenously sensitive to VEGF inhibition [72]. In contrast, blood vessels in human tumors may be present in distinct stages of development, as demonstrated by spatial differences in VEGFR2 expression and phosphorylation status [25, 62].

As such, these animal models not only underestimate the complexity of human tumor growth and associated vascular behavior, but also overestimate the sensitivity to antiangiogenic therapy. This complicates extrapolation of data from these models to the clinical situation. Indeed, vandetanib treatment significantly delayed growth of LLC tumors, most likely by inhibition of sprouting angiogenic activity. Previous studies furthermore showed that in MEL57 tumors growing in the highly vascularized brain, vandetanib also successfully inhibited angiogenesis. Yet in this model, inhibition of sprouting angiogenesis did not result in inhibition of tumor growth, as the tumor switched to a vessel co-opting phenotype enabling tumor progression [73]. Thus, vandetanib-induced vascular stabilization may only result in effective tumor growth inhibition in models that rely on synchronized vessel outgrowth without an optional switch for other neovascularization modes, and may be much less effective in tumor types that contain a more heterogeneous vasculature. Animal tumor models based on orthotopic "spontaneous" tumor outgrowth, such as the Rip-Tag2 model for pancreas carcinoma, the MT/ret melanoma model, and the mutated adenomatous polyposis coli model for intestinal adenoma [10, 11, 74], may overcome part of the extrapolation problems encountered with the artificial models. As recently reviewed by Ellis and Fidler, improved models should take into account the chronic and metastatic, rather than acute and expansive, character of most human cancers, in addition to the microenvironment-driven control of vascular behavior as occurs in the orthotopic location [75]. Importantly, therapy should be initiated after tumors are already established, to better reflect the large tumor burden observed in most patients. Such an approach in models of multistage carcinogenesis, where VEGF-dependent and independent tumor growth occurs in parallel, is likely of considerable added value for antiangiogenesis research, given the biological resilience of human cancers to treatment.

**Future perspectives**

Recent preclinical studies unveiled that instead of promoting survival, antiangiogenic therapy may increase tumor angiogenesis or the occurrence of metastasis. Two independent groups demonstrated that anti-VEGF therapy with the VEGFR2 blocking antibody DC101 and with the tyrosine kinase inhibitor sunitinib may elicit malignant
progression of tumors by increased local invasion, and accelerate metastasis [76, 77]. It has been suggested that this involves hypoxia-driven upregulation of hepatocyte growth factor (HGF)/c-Met signaling that promotes tumor cell motility and aggressiveness [78, 79]. In addition, Reynolds et al. demonstrated that low nanomolar concentrations of αvβ3 inhibitors can paradoxically stimulate angiogenesis and tumor growth [80]. At low concentrations, these RGD-mimetics promote the recycling of VEGFR2 to the cell membrane, thereby enhancing VEGF responsiveness. These intriguing findings demonstrate that switching to different metastatic programs, but also posttranslational processes are important adaptive responses of cells to therapeutic intervention. Insight into a drug’s actual mechanism of action, or of side-effect, is warranted before it enters clinical trials.

A more complete view of the mechanism of action of antiangiogenic therapy can only be obtained by looking at the total picture of molecular and cellular events that determine the outcome of therapy. Important questions to be answered are which vessels do respond to therapy and which do not? How do changes in VEGFR2 signaling, activation of downstream kinases and transcription factors, and subsequent changes in the transcriptome and proteome upon treatment relate to an efficient antitumor effect? As described below, the answers to these questions will aid in the identification of biomarkers for efficacy and the rational design of multitarget antiangiogenic therapeutic strategies. To answer these questions, kinetics of VEGFR2 phosphorylation in response to treatment should be analyzed, and kinome and transcriptome profiling should be performed at various time points throughout the course of treatment. If imaging of VEGFR2 phosphorylation were to be combined with laser microdissection of the specific vascular segments that responded to VEGFR2 inhibition, identification of the downstream consequences of therapy for gene expression would come within reach. Such analyses should be performed in vivo, in a tumor model in which antiangiogenic therapy has proven effective, and in a model in which antiangiogenic therapy is unable to inhibit tumor growth, ideally with the molecular target being on the same cellular compartment, i.e., the vasculature. Comparison of these data sets will provide comprehensive insight in the molecular processes that are involved in success and failure. Most importantly, these analyses should be performed in tumor models that accurately mimic human tumor growth, as described earlier. Subsequent validation of the observed preclinical findings in the clinic is necessary, and requires the availability of clinical tumor specimens, preferably taken at different time points throughout the treatment period. As such, validation of preclinical data in human specimen faces certain limitations, not only because such biopsies are difficult to acquire, but also because periodic sampling is limited for ethical
and medical reasons. For example, surgical resection of the tumor is only performed six weeks, i.e., three half-lives, after the last Bevacizumab dose, hence complicating the interpretation of the results of such analyses.

Novel techniques for imaging of tumor angiogenesis that combine analysis of in vivo gene expression with analysis of vessel function open up new possibilities to monitor response to antiangiogenic therapy throughout the course of treatment [81]. Targeted ultrasound has been demonstrated feasible for the longitudinal molecular profiling of tumor angiogenesis and for the sensitive assessment of therapy effects in vivo. Such techniques monitor all perfused vessels by assessing stimulated acoustic emissions from disintegrating microbubbles. By linking these microbubbles to e.g., VEGFR2 or ανβ3 binding ligands, functionality of different vessel fractions, before and after start of antiangiogenic treatment, can be monitored [82, 83]. Furthermore, scintigraphical imaging and positron emission tomography has rapidly developed as a suitable method for non-invasive imaging of e.g., VEGF-A expression localization using radiolabeled bevacizumab as a tracer [84], or imaging of VEGF receptors using single chain VEGF probes that can be labeled with contrast agents [85].

More insight into the molecular changes occurring under pharmacological VEGFR2 inhibitor pressure will enable identification of biomarkers of response [86]. To our knowledge, currently no validated biomarkers exist for appropriately selecting patients with cancer for antiangiogenic therapy, nor are biomarkers available for monitoring drug response [87]. Our data in Chapter 5 identified Angiopoietin-2, integrin β3, N-cadherin and VCAM as early responder genes to vandetanib treatment in LLC, while Ang1, Tie2 and Notch3 responded after long-term treatment. Provided that the same changes occur at the protein level, these may represent potential biomarkers for antitumor efficacy of vandetanib. Yet, applicability of these molecules as biomarkers in the clinic is only feasible if they can be measured in serum, which requires these molecules to be shed or in any other way released from the cells in the blood stream. Our findings need verification in serum of treated animals followed by assessment in patient serum, and their association with clinical outcome should be evaluated.

In addition to biomarker development, more insight into the molecular changes induced by pharmacological interference will contribute to the identification of novel targets that may be used in combination treatment strategies. Such strategies may overcome resistance to antiangiogenic therapy, caused by activation of alternative pro-angiogenic signaling and/or loss of VEGF-dependency [49, 88]. Several combination treatment regimens have been pursued, for instance, anti-VEGF therapy has been combined with inhibitors of Tie2 signaling [89], inhibitors of Dll4-Notch1 signaling...
Our observation that vandetanib inhibits pro-angiogenic sprouting and promotes vascular stabilization in Lewis Lung Carcinoma tumors strengthens the concept that dual targeting of both endothelial cells and pericytes might be more efficient [91], although the added benefit of targeting both endothelial cells and pericytes may vary per tumor type [92, 93].

Finally, it is noteworthy to mention that antiangiogenic combination treatment strategies may find a promising future application in a metronomic ‘low’ dosing protocol, as has been proposed in combination with chemotherapeutics [94]. As our data in Chapter 4 suggest, an active angiogenic status may render cells refractory to drug treatment for a short period of time. Metronomic dosing of multiple inhibitors targeted at different neovascularization stages ensures that temporal fluctuations in sensitivity of the endothelial cells will be overcome by a continuous exposure to the angiogenesis inhibitors at a dose that is well remaining within the effective therapeutic window.

Conclusion

The research described in this thesis aims to molecularly characterize the tumor vascular phenotype in vivo, and the changes therein in response to antiangiogenic therapy. We demonstrated that the vasculature of a B16.F10 tumor does not change its repertoire of pro-angiogenic gene expression during tumor outgrowth, and that changes in vascular morphology are associated with a different pericyte activation status, but have no relation to the angio-genes studied. In addition, endothelial cells that are actively engaging in pro-angiogenic signaling experience an impaired response to pharmacological inhibition of VEGFR2. Lastly, we demonstrated that VEGFR2 inhibitor treatment of s.c. growing Lewis Lung Carcinoma shifts the molecular phenotype of the vasculature toward enhanced vascular stabilization, while in B16.F10 melanoma a different molecular response underlies the anti-tumor effect. In both models, VEGFR2 inhibitor treatment induced loss of expression of its target receptor at the protein level.

Altogether, our research illustrates that one of the largest hurdles that needs to be taken in antiangiogenesis research is development of improved models. Each angiogenesis model, either in vitro or in vivo, is a model in itself and as such not a good reflection of the clinical situation. Endothelial cell cultures in vitro lack the capacity to recapitulate the tumor microenvironment-driven control of endothelial behavior. Furthermore, the molecular control of angiogenesis as defined in chorioallantoic membrane assays or murine knock-out models differs from endogenously-controlled angiogenesis as observed in B16.F10 melanoma growth. This emphasizes that each preclinical model needs to be appreciated for its own molecular repertoire underlying angiogenesis. Still,
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these models poorly reflect the complexity and heterogeneity of angiogenic behavior in clinical tumors, complicating extrapolation of molecular and pharmacological findings to the clinic [75]. Thus, the challenge lies in the development of mouse tumor models that more precisely mimic human tumor growth [95]. Given the variation between tumors and their molecular response to pharmacological intervention, successful antiangiogenic therapy will likely require a personalized medicine approach. Such therapy regimens can only be established by a multidisciplinary effort to create better pre-clinical tumor models, identify and validate biomarkers for efficacy and develop imaging techniques that serve as read-out and monitoring tools for pharmacological effects of antiangiogenic therapy in patients.

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