Kinase activity profiling in pediatric brain tumors

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STELLINGEN

Behorende bij het proefschrift

Kinase activity profiling in pediatric brain tumors: a novel strategy for the identification of druggable targets.

1. Generating a network view on kinase activity will provide critical perspectives on cancer therapy, either by allowing the selection of accurate targets or to devise the most optimal therapy for the individual patient. *(This thesis)*

2. Combining the old SRC (Surgery, Radiotherapy, Chemotherapy) with the new Src (sarcoma tyrosine kinase inhibitors) holds great promise for the treatment of pediatric brain tumors. *(Adapted from de Groot J., Milano V., et al. J Neurooncol)*

3. Tyrosine kinase activity screening allows the generation of clinically relevant kinase activity profiles enabling rapid screening for potential druggable targets in a broad range of malignancies. *(This thesis)*

4. Variability in substrate motif recognition explains the apparent contradictory interpretations of the forces that drive kinase substrate selection. *(This thesis)*

5. Insufficient ways to predict the efficacy of inhibiting specific kinase activity gives rise to a discrepancy between the evaluation of aberrant cell signaling and the currently available ensemble of highly specific targeted treatment strategies. *(This thesis)*

6. Counteracting effects in response to activation of different Eph receptors reflects a tightly controlled balance in relative expression of the various receptors, which is ultimately decisive for the phenotype. *(This thesis)*

7. Phos-Tag SDS-PAGE allows simultaneous assessment of protein phosphorylation at multiple phosphorylation sites. *(This thesis)*

8. Ofschoon notoir pathologisch; alcohol blijft onontbeerlijk in de pathologie.

9. To regret growing older is to regret a privilege denied to many. (*Unknown)*

10. Curiosity is the essence of human existence. (*Eugene A Cernan)*

Arend H. Sikkema
2 november 2011

Cmb.

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Chapter 1

General introduction and thesis outline

Introduction to the thesis

Cancer is the most common cause of non-accidental death among children and young adults [1;2]. Although the 5-year survival rates have increased dramatically over the last 50 years, the proportion of cancer deaths due to brain and other nervous system tumors increased from 17.8% in 1975 to 25.7% in 2006 [3]. This is indicative for a disproportionate lack of improvement of clinical outcome compared to other malignancies. Furthermore, the survivors commonly pay a high price in treatment related morbidity. Brain malignancies are the second most common type of cancers occurring at childhood age with an incidence of 3.2 in 100,000 (2004) [4], representing 17.4 percent of all malignancy cases during childhood (fig. 1A). In the Netherlands approximately 120 children are diagnosed with a brain tumor each year. Roughly 60% of all childhood brain tumors are of infratentorial origin and arise in the posterior fossa (fig. 1B, C). The most common of these are: medulloblastoma, low-grade astrocytoma of the cerebellum (primarily pilocytic astrocytoma) and ependymoma. These will be briefly addressed in the following sections.

Current treatment modalities comprise neurosurgical resection, intensive chemotherapy and craniospinal radiotherapy. The fact that improvement in therapeutic outcome has been relative limited for pediatric brain tumors indicates little evidence that more effective use of available cytotoxic agents will be sufficient for substantial progress in treatment. The tremendous boost in high-throughput assaying technology to rapidly screen aberrant cell biology promises to generate rapid insight in key targets for novel treatment options. The current thesis will focus on application of high-throughput screening of aberrant kinase activity in pediatric brain tumors to identify new molecular targets for therapy.

Medulloblastoma

The medulloblastoma is a malignant and invasive tumor resembling embryonal origin, which develops in the cerebellum primarily from the vermis area [5]. It consists of malignant invasive embryonal cells from the primitive neuro-ectoderm (WHO grade IV). Three histopathological variants can be identified: classical, desmoplastic and large cell/anaplastic medulloblastoma. The tumors located in the cerebellar hemispheres are mainly of desmoplastic/nodular nature and confer a better

prognosis [6]. Medulloblastoma is a typical pediatric tumor with a peak age at presentation of 7 years where 70% of the cases occurs in children under 16. The tumors are highly proliferative and exhibit the propensity for leptomeningeal dissemination and local spread [7-9].

Besides histopathological subtypes, a classification can be made on cell biological basis as well. Cell signaling pathways implicated in medulloblastoma progression are the Hedgehog (Shh) pathway, Wingless (Wnt) signaling pathway and the Notch pathway. Notch and Shh are known to play a central role in the development of the formation of the external granular layer (EGL) during embryonal development. Within the Shh pathway the Smoothened (Smo) gene has been identified as a key player. Mice with constitutively activated Smo typically generate medulloblastomas in more than 90% of the cases [10]. Notch and Wnt are believed to balance proliferation and differentiation by regulating neurogenic transcription factor activity [11-13]. Frequent mutation of the Wnt signaling member b-catenin and elevated levels of Notch signaling can be appreciated in medulloblastoma [14;15]. Recent gene expression studies identified 5 molecular subclasses in the overall population with distinct gene-expression profiles and clinical profile [16]. Distinct Wnt and Shh expression signatures have been identified in two of these subgroups. Differences in patient characteristics and tumor histology and M-stage could be appreciated. Isochromosome 17q (i[17q]) is the most frequent chromosomal abnormality in medulloblastoma, occurring in 30-60% of cases in karyotype analyses [17-19].

Ependymoma

Ependymomas are slow growing tumors that mainly occur in children and adolescents [20]. It is a relatively rare tumor that in children is primarily localized infratentorially along the ventricular system and the spinal canal [21]. Cytogenetic changes are common in ependymoma with copy number changes at chromosomes 1, 3, 6q, 9q and 22 reported most frequent [22;23]. Although genetic amplification is not very common, many ependymomas possess overexpression of EGFR [24]. High expression of RAF1, MMP12, PSAP, CLU and IGF2 has been found in ependymomas as well [25]. Interestingly, supratentorial tumors show high expression of members from the EphB, NOTCH and CDK kinase families [25;26].

The WHO III grade counterpart known as the anaplastic ependymoma is characterized by accelerated growth and unfavorable prognosis. Gains of chromosome 1q correlate with anaplastic features [27].

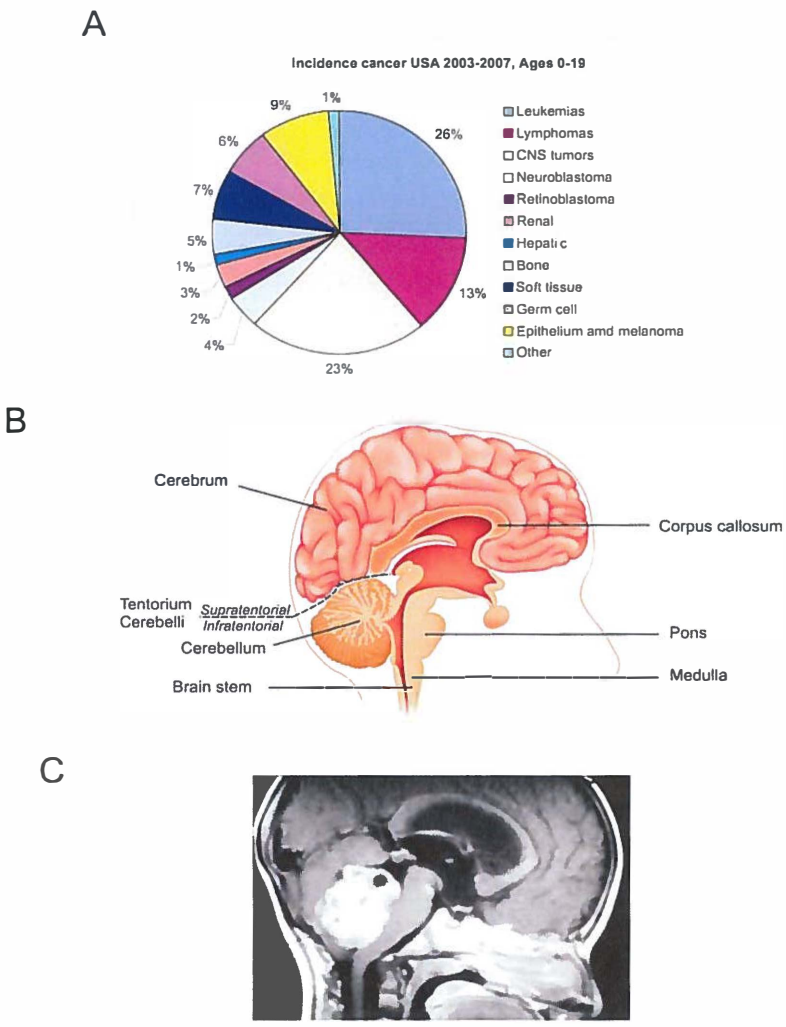

Figure 1: Pediatric brain tumor incidence and intracranial localization. Tumors of the central nervous system constitute the second most common cancer type and the primary cause of solid tumor malignancies among children (A). Although pediatric brain tumors can occur throughout the entire brain, the most common localization is the cerebellum (B,C).

Low-grade/pilocytic astrocytoma

Pilocytic gliomas comprise of a very heterogeneous group of CNS tumors which are relatively well circumscribed (WHO grade I) [28]. Whereas in adults the high grade glioma is predominant, in children the pilocytic astrocytoma is the predominant representative. Although pilocytic astrocytoma can be localized throughout the neuraxis, infratentorial localization is most common. In contradiction to medulloblastoma, the pilocytic astrocytoma grows slowly and is relatively well circumscribed. Furthermore it has a remarkably rich vasculature [29]. Unlike other gliomas the pilocytic variants are stable in their WHO status, indicating a high degree of genetic stability. This has been confirmed by cytogenetic studies, although comparative genomic hybridization analyses have indicated genetic alterations. Evidence for involvement of specific cell signaling in the tumor phenotype has not been generated so far. Activity of Ras and overexpression of ErbB3 has been reported for (sporadic) pilocytic astrocytoma [30-32].

Signal transduction and cancer

In part, due to the small number of clinical trials aimed at treatment of pediatric brain tumors and pediatric solid tumors in general, progress in treatment regimens has been relatively limited. In addition, there is little evidence that more effective use of cytotoxic approaches will result in substantial progress on short notice [3]. Thus, novel innovative treatment options improving treatment outcome are warranted. Since treatment focus is shifting away from cytotoxic therapeutic approaches a tremendous boost in research aimed at identifying perturbed cell signaling driving the tumor phenotype.

Cellular signal transduction activity is tightly controlled by the balance between phosphorylating and dephosphorylating action by kinase and phosphatase activity, respectively. Posttranslational modification through phosphorylation of a protein can be either activatory or inhibitory depending on the phosphorylation site. Src kinase is a characteristic example of a kinase that depends on the ratio between phosphorylation at multiple activatory and inhibitory phosphorylation sites that determines ultimate activity [33;34]. Sequencing of the whole human genome showed that the genome encodes approximately 500 kinases [35]. Furthermore, it is

estimated that the human proteome contains around 100,000 potential phosphorylation sites [36]. So far only 2000 of these sites have been validated, underscoring the complexity of these processes and interactions.

Aberrant kinase activity has been identified as being the driving force behind the onset of cancer and maintenance of the malignant phenotype. Irrespective of tumor type, neoplastic growth is characterized by an altered signal transduction network, highly influencing the cellular response to both, extracellular as well as intracellular stimuli [37]. Hence, focusing on increased understanding of the cell signaling pathways that enhance tumor growth-related cellular processes such as proliferation, survival and migration is necessary to gain insight in potential therapeutic targets [38]. The key to obtaining effective kinase-targeted therapeutic approaches is identifying those cell signaling mediators that are vital to the tumor phenotype. These oncogenic dependencies create the Achilles heel that has to be identified and subsequently exploited for anticancer therapy.

Elucidating the cell signaling network

Druggable aberrations in signal transduction have been identified for a number of malignancies already. The first breakthrough was the approval of the Abl kinase inhibitor Imatinib for treatment of BCR-Abl positive Chronic Myeloid Leukemia (CML) [39]. Furthermore, successful clinical introductions have been achieved for inhibitors with EGFR and VEGFR signaling activity as their primary targets [40]. Many FDA-approved and pre-clinical inhibitors have been tested in phase 1-3 clinical trials for the treatment of malignant glioma (chapter 2, table 1). Unfortunately, application in treatment of pediatric brain tumors has been very scarce so far. Recently, a clinical trial on application of the VEGF monoclonal antibody Bevacizumab for treatment of pediatric brain tumors has commenced [41;42]. New potential targets for mechanism-based pediatric brain tumor treatment strategies remain to be identified.

Kinase activity profiling: obtaining the network view on cell signaling

Tumor-specific changes in individual cell signaling kinases or pathways have lead to an increased understanding of the phenotype a cancer cell displays. However, key to improving targeted anti-cancer therapy is identifying those cell signaling mediators

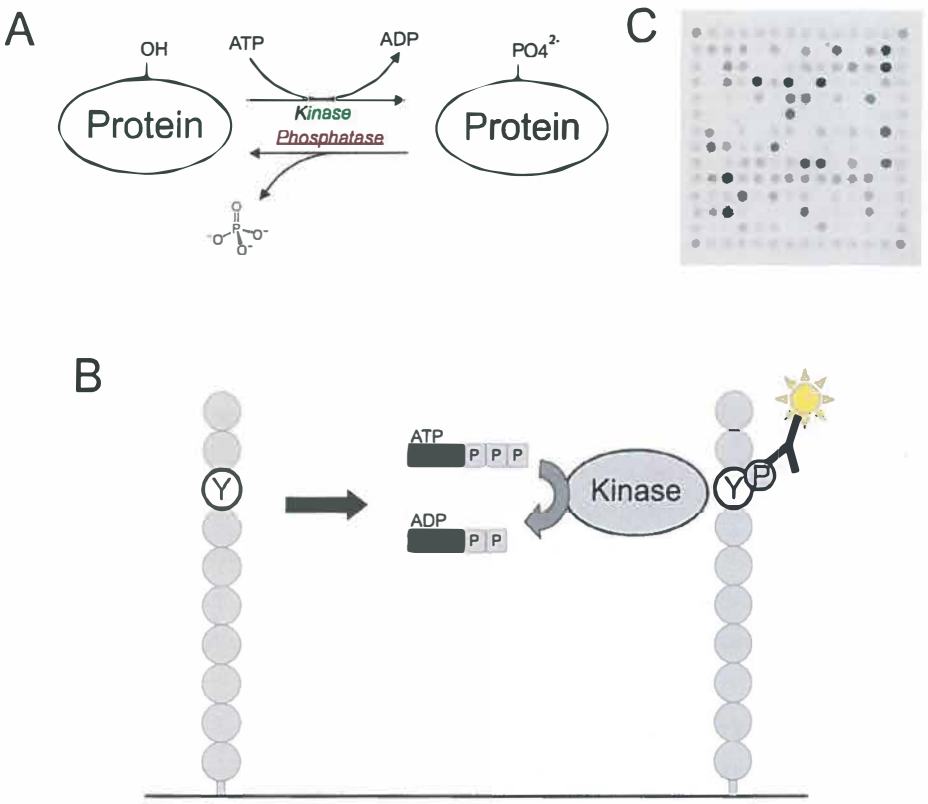

Figure 2: Measurement of cell signaling activity by assessing kinase activity. Protein phosphorylation and dephosphorylation constitutes one of the most essential posttranslational controlling mechanisms of cell signaling activity. The phosphorylation status of a cell signaling protein ultimately determines the extent and nature of signal transduction leading up to a phenotype. Kinases and phosphatases influence the activity of cell signaling proteins by catalyzing the phosphorylation and dephosphorylation of signaling proteins, respectively (A). The activation status of a kinase at a given time thus determines downstream cell signaling activity. This thesis describes application of a high-throughput peptide-based kinase activity profiling technique assessing kinase activity by measuring the phosphorylation of short-amino acid substrates representing key phosphorylation motifs. In the presence of ATP, tissue lysate is incubated with the array. Depending on the kinase activity present in the sample, the substrates become phosphorylated (B). A P-Tyr recognizing antibody visualizes the extent of phosphorylation (C).

that exert a pivotal function in the response to intra- as well as extracellular stimuli resulting in neoplastic tumor progression. Obtaining network views on the protein kinase signaling network has become a synonym to obtaining insight in the phosphorylation status of the signaling network: the phosphoproteome. However, the net signaling activity not only depends on the phosphorylation status of proteins at specific sites but involves intricate interplay of a variety of posttranslational modification events. Hence, research on aberrant cell signaling is gradually shifting towards quantitative measurement of kinase activity. In this thesis a high-throughput peptide substrate-based approach was adopted to gain insight in tyrosine kinase activity of pediatric brain tumors (fig. 2). Here, short 12-amino acid substrates were employed in an array-based fashion. Although the tertiary structure of the protein of origin is largely lost in this system, we were able to generate interpretable and reproducible kinase activity profiles that rendered an interesting primary screen of the cell signaling network in pediatric brain tumors. A number of potential targets for new therapeutic angles were subsequently validated in further functional studies.

Aim and outline of the thesis

Gradually, the identification of new potential targets for therapeutic options to counteract tumor progression is shifting from measurement of absolute expression levels to direct measurement of protein kinase activity. The aim of the current study is to generate comprehensive insight in the activity of cell signaling mediators in pediatric brain tumors by means of high-throughput screening of kinase activity. Subsequently, validation studies are performed to assess the functional relevance of number of these potential therapeutic targets. Our ultimate goal is identifying druggable cell signaling mediators, critical to pediatric brain tumor progression.

Chapter 2 provides an overview of recent progress in the development of high-throughput systems biology approaches in dissecting the tumor cell signaling network. Although the bulk of tumor cell signaling research focuses on determining the protein phosphorylation status, recent developments open up the possibility of assessing the net activity of cell signaling kinases. The value of these techniques in either target discovery or patient stratification based on the kinase activity profile will be discussed.

To obtain an indication of the cell signaling profile a broad screen of tyrosine kinase activity was performed on a selection of primary pediatric brain tumor samples from each of the previously discussed tumor types. **Chapter 3** describes the results of this initial screen, supplemented with a validation study of a tyrosine kinase that was found to be highly active in all three pediatric brain tumor types, c-Src. This chapter provides an optimized protocol for screening kinase activity in primary (brain) tumor tissue samples. Furthermore, it presents the minimal kinome approach as a valuable tool to structure the peptide phosphorylation data and as a means for data interpretation. Validation experiments were performed to confirm the activity of Src kinase. Functional studies with Src kinase inhibitors, including the FDA approved Bcr-Abl/Src kinase inhibitor Dasatinib, provide insight in the applicability of Src as a therapeutic target.

Upon application of pilocytic astrocytoma samples we observed kinase activity on a number of VEGFR derived peptide substrates in our kinase activity screen. Since the literature on VEGFR activity in pilocytic astrocytoma is highly inconsistent we decided to further study the VEGFR activity and localization in this tumor type applying a number of novel approaches. **Chapter 4** describes the application of an adapted proximity ligation protocol in determining the presence of phosphorylated VEGFR2. Furthermore, this chapter presents data on the localization of VEGFR expression and activity in pilocytic astrocytoma, as determined by laser microdissection.

In **chapter 5** a study on the activity and function of Eph/Ephrin signaling in medulloblastoma is presented. The kinase activity profiling screen showed activity on Eph receptor derived peptides upon application of medulloblastoma lysate. We explored the presence of expression and activity of Eph receptors and Ephrin-B ligands in medulloblastoma. Based on these data we continued with functional studies on a number of medulloblastoma cell lines, assessing the role of Eph/Ephrin signaling in *in vitro* cell proliferation, migration and adhesion. Furthermore, we determined the role of EphR promoter methylation in the control of expression and the effects of Eph receptor stimulation on downstream signal transduction activity.

The molecular determinants driving amino acid composition of substrate motifs for kinases in proteins remain essentially unresolved. To gain insight in this issue we decided to explore the sensitivity and selectivity of the various peptide substrates present on the substrate array, as we employed in our kinase activity screening

studies. In **chapter 6** a number of recombinant kinases were applied and peptide phosphorylation was assessed. To interpret the validity and resolution of the kinase activity profiling data the results are linked to the current status of knowledge about kinase-substrate interactions in literature. Furthermore, indications for a direct interaction between VEGFR2 and Aminopeptidase A, as well as InsR and Syk, were studied in more detail. Kinetic data on the buildup of the phosphorylation signal was studied to determine potential patterns in the sensitivity of peptide substrates towards the applied recombinant kinases.

In conclusion, **chapter 7** provides a detailed summary of the studies delineated above and discusses the value of these data in the context of the current status of research on the (pediatric brain) tumor cell signaling. Furthermore, we provide suggestions for future research efforts on the identification of essential cell signaling activity in pediatric brain tumors and describe future perspectives on optimizing the treatment of these tumors.

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Chapter 2

Optimizing targeted cancer therapy: towards clinical application of systems biology approaches

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Abstract

In cancer, genetic and epigenetic alterations ultimately culminate in discordant activation of signal transduction pathways driving the malignant process. Pharmacological or biological inhibition of such pathways holds significant promise with respect to devising rational therapy for cancer. Thus, technical concepts pursuing robust characterization of kinase activity in tissue samples from cancer patients have been subject of investigation. In the present review we provide a comprehensive overview of these techniques and discuss their advantages and disadvantages for systems biology approaches to identify kinase targets in oncological disease.

Recent advances in the development and application of array-based peptide-substrate kinase activity screens show great promise in overcoming the discrepancy between the evaluation of aberrant cell signaling in specific malignancies or even individual patients and the currently available ensemble of highly specific targeted treatment strategies. These developments have the potential to result in a more effective selection of kinase inhibitors and thus optimize mechanism-based patient-specific therapeutic strategies. Given the results from current research on the tumor kinome, generating network views on aberrant tumor cell signaling is critical to meet this challenge.

1. Introduction

Cancer is defined as an uncontrolled proliferation of clonally derived cells. This malignant transformation is characterized by changes in the expression and activity of key mediators of signal transduction coordinating proliferation, migration and cell death.[1] The tremendous significance of changes in cell signaling activity in the malignant transformation of cells has only been fully recognized in the last decade.[2-4] The identification of disease-related signal transduction effectors culminated into the acknowledgement of protein kinases as one of the most important classes of potential drug targets to date.[5] Significant advances in the development of small molecule inhibitors to counteract aberrant cell signaling promoting tumor cell proliferation have been made since then. Kinase signaling, because of the excellent specific druggability of the ATP-binding pocket, as well as the general importance in virtually all signal transduction pathways, holds great promise here. The first breakthrough was the approval of the Abl kinase inhibitor Imatinib for treatment of BCR-Abl positive Chronic Myeloid Leukemia.[6] Application of this inhibitor is at present part of first-line treatment for Chronic Myeloid Leukemia and has improved patient outcome dramatically.

Despite the successful application of kinase inhibitors for the treatment of specific malignancies, tumor resistance due to acquired mutations in downstream effectors of the targeted molecule and toxicities as a result of limited agent specificity remain important challenges to overcome.[7-13] Insufficient ways to predict the efficacy of inhibiting specific kinase activity gives rise to a discrepancy between the evaluation of aberrant cell signaling and the currently available ensemble of highly specific targeted treatment strategies. Hence, providing better ways to predict the efficacy of kinase-targeted small molecule inhibitors in the tumor- or patient-specific situation will be one of the most important goals of cancer research in the upcoming decade. Accurate identification of tumor type-specific or even patient-specific aberrations in cell signaling activity that are crucial for tumor progression, is required. In this review we provide an overview of recent developments in kinase activity screening concepts focused on achieving that goal. An introduction concerning aberrant protein kinase activity as the essence of the oncogenic phenotype will be followed by a discussion of the gap in knowledge on anomalous cell signaling in specific malignancies that is becoming ever more evident, thereby severely hampering the implementation of

kinome-targeted cancer therapies. After a description of the most apparent challenges that have emerged in recent research, we will address the most encouraging strategies aimed at dissecting the cancer-specific signal transduction network. Promising research focused on allowing us to obtain a better grip on the selection of effective and specific kinome-targeted cancer treatment strategies will be evaluated. Future investigational topics will be discussed.

2. Molecular biology of cancer cells: targeting signaling kinases

Up until the last decade, cancer research essentially relied on the expression of messenger RNA and, to a lesser extent, protein to elucidate changes in cell signaling.[14] Microarray applications have made it possible to simultaneously study the expression of a tremendous number of genes, thus providing a network view on gene expression. The concept of personal genomics in individualized medicine is aiming at the assessment of risk and the selection of therapeutic approaches based on the individual genetic signature.[15-17] Successful identification of gene expression phenotypes mirroring a clinically relevant genotype has been reported for numerous malignancies. In medulloblastoma, gene expression profiling resulted in the identification of five (recently re-defined as four) medulloblastoma subtypes with a distinct gene expression profile.[18-20] However, a link with patient outcome has not been established yet. In breast cancer gene expression signatures have been proven useful in the prediction of clinical outcome.[21-24] A gene expression profiling approach recently made it to clinical practice in the risk stratification of patients to evaluate the application of adjuvant systemic therapy in breast cancer treatment.[25-27] Furthermore, Her2 and estrogen receptor expression levels in addition to BRCA mutational status are well established prognostic markers for breast cancer treatment. Nonetheless, the consistency of the gene expression profiles deals with a tremendous interpatient variability preventing stable patient stratification.[28,29] Moreover, gene expression only partially reflects the expression and activity of cell signaling mediators.

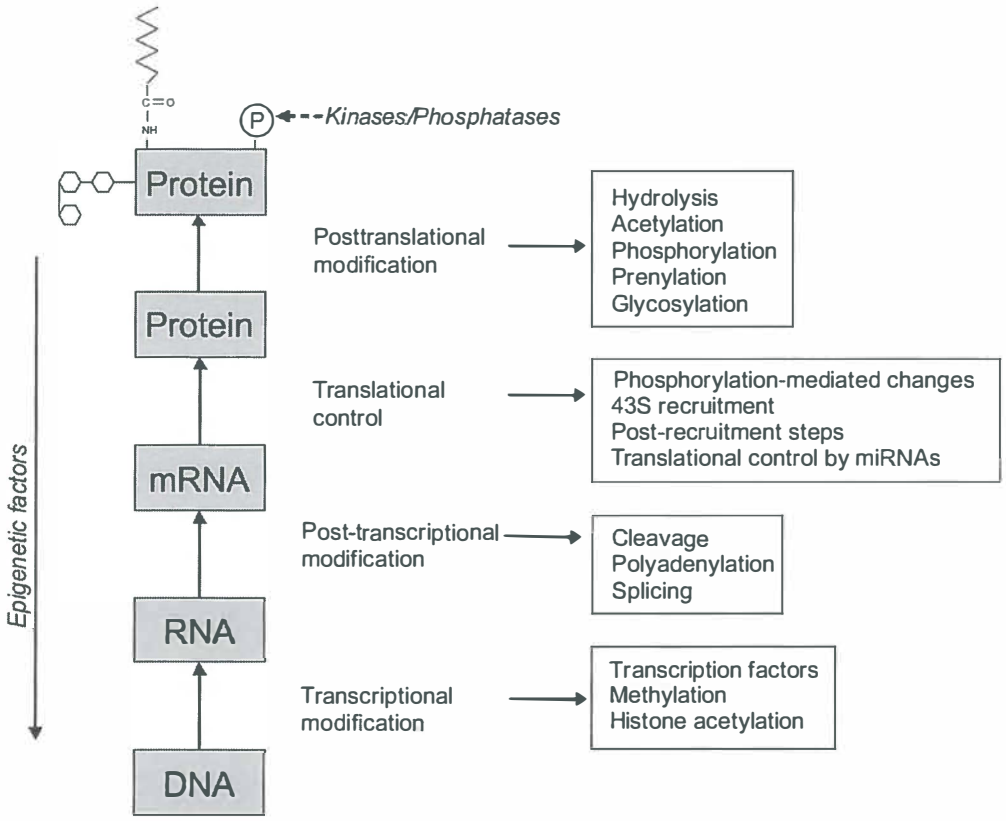


Figure 1: Rate determining factors in protein kinase activity. Kinase activity depends on multiple factors, genetic, epigenetic as well as environmental. All these factors exert their influence on various regulatory levels. Roughly, four levels can be identified: transcriptional modification[157], post-transcriptional modification[158], translational control[159] and posttranslational modification.[160] Examples of key cellular processes that can be brought into play at a specific regulatory level have been listed.

Not only gene and protein expression levels but rather the posttranslational modification of proteins is the determining factor in the eventual phenotype.[30] During or after translation modification of proteins can occur on multiple levels such as glycosylation, acetylation, lipid attachment, cleavage of protein segments and phosphorylation (fig. 1). These modifications are decisive for downstream events by inducing major changes in protein conformation and charge.[31,32] The phosphorylation status of cell signaling proteins forms the basis of rapid and specific response to

extracellular stimuli and is determined by the balance between kinase and phosphatase activity, either phosphorylating or dephosphorylating specific protein phosphorylation sites, respectively[33] (fig. 2).

Genome- and transcriptome wide studies lead to the discovery of anomalous proteins or expression profiles for a multitude of malignancies. However, the effects of these changes in genome and gene expression on the cellular signaling network often remain unclear. In addition, absolute expression levels of protein kinases are not always predictive for the eventual signaling activity of this protein or its role in the signaling network. As pointed out by Irish et al., delicate changes in gene transcription can have tremendous effects on the signal transduction phenotype.[34,35] This results in a highly heterogeneous response to extracellular stimulatory factors, rate-determining in tumor cell proliferation. Although these patients are all diagnosed with AML the signal transduction potential can vary greatly. Similar findings have been recently presented on breast cancer protein expression.[36] These results support the assumption that subtle adjustments in the signaling through canonical as well as non-canonical signaling routes provide the mechanistic basis for neoplastic growth. This indicates that inter-tumor heterogeneity in genome, transcriptome and ultimately (phospho)-proteome can result in a highly varying signal transduction activity signature. In addition, key mutations in cell signaling proteins such as BCR-Abl can result in constitutive activation independent of external stimuli.

The relative importance of the individual changes in cell signaling is in a lot of cases still a mystery. Weinstein et al. introduced the concept of oncogene addiction, claiming that cancers depend on only one or a few genes to maintain the malignant phenotype.[37,38] Recent publications also provide evidence supporting this now well accepted theory.[39,40] Although the oncogene addiction theory suggests oncogenic signaling to be dependent on a highly specific part of the cell signaling, the extent of redundancy in signal transduction pathways and signaling interplay has been shown to aid the oncogenic phenotype. Bertotti et al. pointed out that a subset of Met-activated pathways is sufficient to sustain oncogene (Met) addiction.[40] If no other receptor tyrosine kinases were able to take over activation of these essential downstream pathways the cancer cells went into cell-cycle arrest. Otherwise, the phenotype remained unchanged.

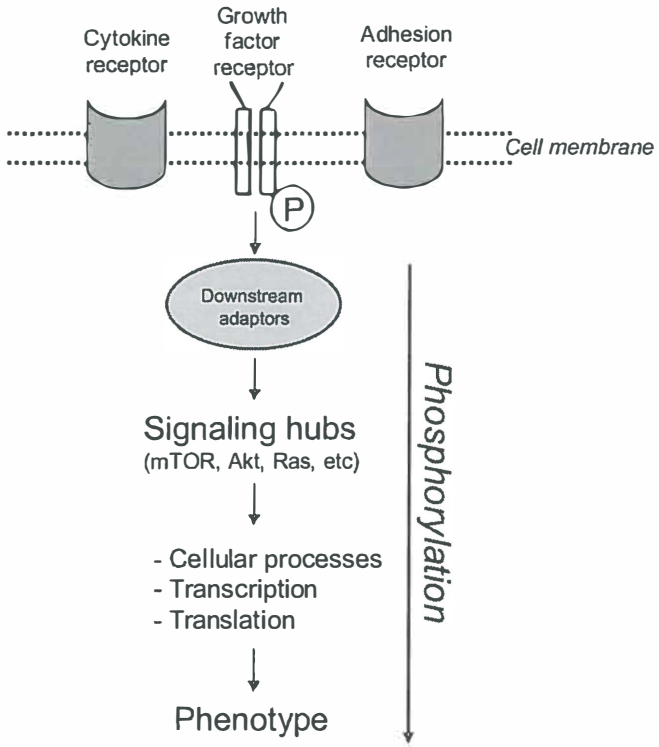


Figure 2: Schematic representation of the cell signaling network. The extent of protein phosphorylation is rate determining in all downstream cell signaling ultimately resulting in a phenotype. Essentially, extracellular stimuli result in receptor activation that, in turn, conveys the signal to downstream adaptor proteins. It is believed that multiple of these signals converge into a smaller set of signaling hubs such as mTOR, Akt and Ras that ultimately influence key cellular processes that result in a phenotype. Throughout this multi-layered signal transduction network protein phosphorylation is rate determining.

In AML simultaneous activation of multiple pathways confers a poor prognosis, suggesting that a combinatorial approach in cell signaling inhibition is necessary to obtain the desired effects.[41] Therefore, kinases located at key signaling pathways like mammalian target of rapamycin (mTOR), which functions as a central hub conveying signals downstream, are popular drug targets[42,43] (fig. 2). Although these kinases are

rarely mutated and often not oncogenic at all, upon inhibition they can result in a suppressed tumor progression or even tumor cell death. This vulnerability can be the result of sudden alterations in the signaling network making certain signaling pathways critical for cell survival that were initially not critical for survival at all.[44-46] The extent of this synthetic lethality as well as the nature of the oncogenic signaling can vary tremendously between individual patients since it depends on mutational status. Thus, identification of the essential signaling proteins could result in a tremendous improvement in therapy selection. This exemplifies that uncovering the dominant and recessive nodes within a severely affected oncogenic signaling network is essential to obtain therapeutic responsiveness.

3. Application of kinome-targeted therapies: opportunities and challenges

Broad spectrum kinase inhibition tends to narrow the therapeutic window of compounds because of serious side effects on non-malignant tissues.[47] Nevertheless, a therapeutic window proves to exist for an increasing number of compounds with a side effect profile that challenges that of the traditional cytotoxic agents.[48] Examples are Imatinib and the relatively broad-spectrum BCR-Abl and Src-family kinase inhibitor Dasatinib, which has been approved for treatment of chronic myeloid leukemia and is tolerated in patients remarkably well.[49,50] Third-generation equivalents such as Nilotinib have recently been marketed. Supplementary table 1 (provides a list of kinases that are currently targeted in running clinical trials for treatment of brain tumors.

Despite these hopeful developments, challenges like drug resistance, a wavering cell selectivity and patient unresponsiveness illustrate the necessity for a more rational means of selecting (combinations of) therapeutic agents. The current pharmaceutical pipeline of compound development is aimed at the application of highly selective kinase inhibitors in order to limit adverse effects. Strikingly, up until now the clinically most effective kinase inhibiting compounds are small molecule inhibitors with a relatively broad off-target inhibitory spectrum.[51] As shown by Fabian et al. by means of a small molecule kinase interaction map, clinically applied kinase inhibitors are generally active at an array of protein kinases, which suggests that the selection of the most optimal target for the individual patient is lagging behind.[47]

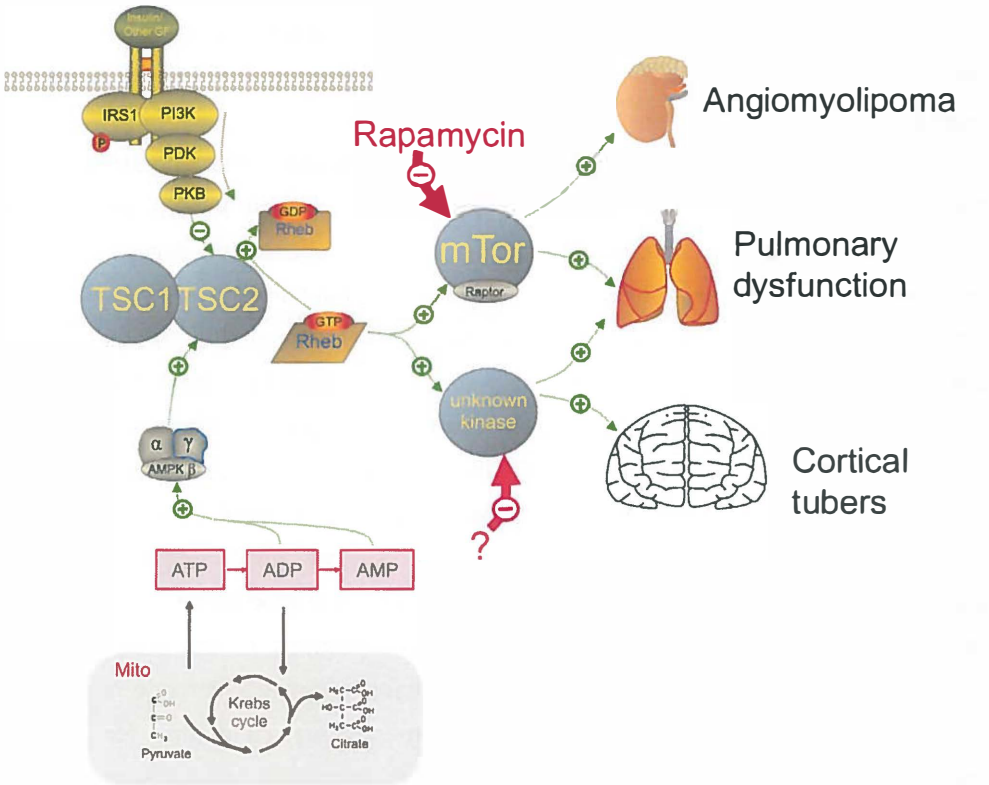
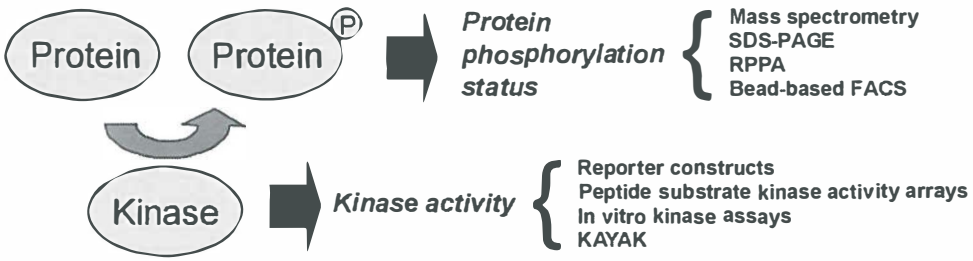


Figure 3: The efficacy of mTOR signaling inhibition varies in different tuberous sclerosis complex manifestations, illustrating the relevance of cell signaling context. Recently a prospective study on rapamycin proved safe and effective for the treatment of tuberous sclerosis complex-associated renal angiomyolipoma. Unfortunately, however, other manifestations of tuberous sclerosis complex, especially lung function and neurological symptoms do not or only partially react to prolonged rapamycin treatment. The potential of prolonged rapamycin treatment to provoke full mTOR inhibition is undisputed. Hence we are forced to conclude that part of the tuberous sclerosis complex clinical manifestations arise from Tsc1/Tsc2-induced mTOR-independent non-canonical signal transduction. This illustrates that the potential efficacy of inhibitors largely depends on the cell-biological context of the chosen target, which shows the importance of identifying technology with just this capability.

Kinases functioning as central hubs conveying signals downstream like mTOR and Ras are popular drug targets.[42,43] Nevertheless, the cell biological context in which these signaling hubs function often remain decisive for an eventual clinical response. An example of this phenomenon has recently been reported for mTOR inhibition as a treatment strategy for tuberous sclerosis complex-associated renal angiomyolipoma. The genetics of tuberous sclerosis complex[52] are now well known and involve autosomal dominant mutations in tsc1 and tsc2.[53,54] These two proteins form a complex which is part of an intracellular canonical signal transduction pathway that integrates cellular nutritional status(via the LKB/AMPK signaling module)[55] and cellular survival signaling (mediated by the PI3-kinase/PKB signal transduction module)[56-58] and delivers its output to the Rheb GTPase (fig. 3). When Rheb is GDP-bound, the protein is inactive, its GTP loading, however, leads to activation of mTOR.[59,60] The latter protein is an important regulator of cellular metabolism and biochemistry and its inhibition by rapamycin is now firmly established as a therapeutic option in various diseases, especially transplantation medicine and various forms of cancer. The observation that mutations in tsc genes produce exaggerated activation of its canonical downstream target mTOR made rapamycin-treatment of tuberous sclerosis complex an obvious candidate for rational medicine.[61] In a prospective study by Bissler et al. rapamycin was proven safe and effective for the treatment of tuberous sclerosis complex-associated renal angiomyolipoma, suggesting that this medication is indeed useful for the clinical management of tuberous sclerosis complex.[62] Unfortunately, however, other manifestations of tuberous sclerosis complex, especially lung function and neurological symptoms do not or only partially react to prolonged rapamycin treatment.[62,63] The potential of prolonged rapamycin treatment to provoke full mTOR inhibition is undisputed. Hence we are forced to conclude that part of the tuberous sclerosis complex clinical manifestations arise from Tsc1/Tsc2-induced mTOR-independent non-canonical signal transduction (as also stated in an editorial published in New England Journal of Medicine[64]). This indicates that the potential efficacy of inhibitors largely depends on the cell-biological context of the chosen target, which shows the importance of identifying technology with just this capability. While for a number of specific malignancies, such as chronic myeloid leukemia, insight in the cell biological state of the tumor has implications for patient treatment,

this is generally not the case.[6] A network view on cancer signal transduction cascades ultimately should provide the information necessary to target the appropriate signaling effectors in order to normalize the cellular phenotype or sensitize the cancer cells for more traditional anticancer therapies.

A



B

	Technique	High throughput	Time requirement	Quantitative	Bottleneck
Phosphoproteomics	Mass spectrometry	No	High	No	Coverage
	SDS-PAGE	No	Intermediate	No	Antibodies
	RPPA	Yes	Intermediate	Yes	Antibodies
	Bead-based FACS	No	Intermediate	Yes	Antibodies
Kinase activity screening	Reporters	No	High	Yes	Specificity
	Peptide substrate kinase activity arrays	Yes	Low	Yes	Annotation
	In vitro kinase assays	No	High	Yes	Requires transfection
	KAYAK	Yes	High	Yes	Annotation

Figure 4: The pros and cons of the described proteomics techniques aimed at assessing protein kinase activation. The various approaches to dissect the kinase activity network can be divided in two classes (A): quantitative phosphoproteomics, measuring the phosphorylation status of cell signaling mediators, and quantitative kinase activity profiling, measuring the actual kinase activity. The characteristics of the most generally applied techniques aimed at assessing both conceptual paradigms are summarized (B).

In conclusion, insufficient ways to predict the efficacy of inhibiting specific kinase activity gave rise to a discrepancy between the evaluation of cell signaling aberrations and the currently available ensemble of specific kinase-targeted treatment strategies. This has led to substantial uncertainty as to whether clinically applied inhibitors will reach the desired effect. Therefore, a more thorough understanding of signaling activity through specific signal transduction pathways is warranted to provide a rationale for treatment aimed at tumor-specific and patient-specific tumor cell biology. Next, an overview will be given of concepts and techniques aimed at obtaining the network view on tumor cell signaling, i.e. the phosphoproteome (fig. 4).

4. Dissecting the human phosphoproteome

4.1 Quantitative phosphoproteomics

Phosphoproteomics studies using mass spectrometry techniques already provided us with a wealth of insight in reversible phosphorylation events that occur in the mammalian cell. The number of potential phosphorylation sites is estimated to be several hundred thousand in the human proteome, representing a large combinatorial repertoire for proteome regulation.[65] This level of complexity makes it hard to reliably quantify specific phosphorylation states in order to interpret the signaling activity through a pathway of interest. Recent advances in the isolation of phosphopeptides from complex protein samples have made it possible to generate accurate relative quantifications of the abundance of specific phosphorylated proteins.[66-69] These techniques generally function by stable labeling of living cells *in vitro* involving stable-isotopes that detectably alter protein mass[70-73] or after lysis by labeling of phosphorylated peptides[74-76], followed by assessing the relative abundance of specific phosphopeptides by mass spectrometry. Recent efforts substantially expanded the number of kinase phosphorylation sites that can be assessed simultaneously in a comparative profiling format using stable labeling.[77] Furthermore, intrinsic validation methods tend to improve quantitative accuracy.[78] A number of studies on phosphopeptide abundance in solid tumor tissue have been published for a.o. low grade astrocytoma[79], hepatocellular carcinoma[80], prostate cancer[81] and lung cancer[82]. Furthermore, several studies applied quantitative phosphoproteomics to study the effects of specific cancer therapy on the

phosphoproteome.[83] The human studies concerned post-resection labeling since prior stable isotopic labeling is not an option.

Despite promising first results, the sensitivity of mass-spectrometry techniques remains problematic due to limited abundance of signaling proteins.[84] Thus, detecting delicate changes in protein phosphorylation status in complex protein samples is still challenging. New techniques for studying subproteomes in complex protein samples are still being developed.[85,86] In essence these techniques are based on covalent linking of small-molecule compounds, followed by a sorting function to allow for isolation of captured (phospho-)proteins. Here, a major drawback is the requirement for fairly large amounts of cells or tissue to obtain sufficient peptide enrichment.[87]

The key to understanding the relevance of specific signaling is to identify and understand the relevance of individual phosphorylation sites. Mass spectrometry already provided invaluable insights regarding this issue and with the innovative relative quantification methods like mass-tag will most likely continue to do so.[88-90] However, for each phosphorylation event a (group of) protein kinase(s) is ultimately responsible. For a little over one-third of all the phosphorylation sites identified thus far a kinase has been pinpointed.[91] Obtaining this information is essential for a thorough understanding of the cell signaling network as a whole. Developments in phosphoproteomics strategies and computational algorithms such as NetworKIN prove to be highly effective in predicting *in vivo* substrates for a kinase of interest.[92,93] Recent application of protein selection by chemical labeling showed the possibility of quantifying kinase activity based on the availability of the active site.[94,95] This technique relies on the availability of activity-based kinase probes. Currently these probes are mainly targeting the ATP-binding site. Upcoming application of neoplastic tissue should point out the usability of these approaches in generating comprehensive insight in cancer cell signaling.

The main drawback of SDS-PAGE based analyses of kinase activity and protein phosphorylation remains that it allows detection of only one phosphorylation site at a time. While multi-blot systems[96] and multicolor FACS approaches[34] have been able to raise the number of phosphoproteins assessed and limit the required material, the number of proteins to be studied simultaneously remains limited. The key to

achieving a network view on signal transduction is to obtain information on signaling at different regulatory levels. Recently the development of reverse phase protein arrays (RPPA) has made it possible to study the protein phosphorylation status on a large panel of protein lysates simultaneously.[97-100] RPPA is a sensitive functional proteomics technique that extends the power of immunoblotting by allowing simultaneous measurement of extensive sample sets. All sample lysates are simultaneously spotted onto an array slide followed by probing with one antibody per array slide. Each array only requires the protein equivalent of 200 cells, making the analysis of a large number of proteins possible for small samples. Application of thoroughly validated phospho-specific antibodies thus can clarify kinase activation in a high number of samples at once. Multiple applications of RPPA in studying functional (phospho-) proteomics in solid tumor patient material have been described[101-105] (reviewed in [106]). More recently, RPPA screening has been proven possible in hematopoietic stem cells and primary leukemic specimens.[107,108]

In analogy to the array-based phospho-proteomics, bead-based protein phosphorylation profiling proves to be a robust alternative and has recently been applied successfully to study tyrosine kinase activity in 130 cancer cell lines.[109] In this approach a panel of kinase-specific antibodies has been coupled to polystyrene microspheres, each with a specific color. After mixing the bead-antibody mix with a whole protein lysate a biotinylated anti-phosphotyrosine is added to the mixture. Flow cytometric analysis measuring both the bead color as well as the streptavidin R-phycoerythrin conjugate signal thus allows quantification of the relative (phospho)protein abundance. Inverse approaches encompassing an antibody microarray platform have been applied successfully as well, accommodating all of the previously mentioned advantages.[110]

Although these strategies only allow detection of previously identified proteins and protein phosphorylation sites, a detailed picture of the phosphoproteome can still be obtained in a high-throughput fashion using limited specimen amounts. A potential drawback of this technology is the demand for selective antibodies that in many cases are still not available. Furthermore, the protein phosphorylation state is not always representative for kinase activity since multiple phosphorylation events work in concert to balance signaling and additional posttranslational events can modify net signaling response.

4.2 Direct selective measurement of kinase activity

4.2.1 Reporter constructs

Short amino acid sequences have been applied as kinase substrates in various different configurations. A quantitative and dynamic measurement of kinase activity in living cells can be achieved using bioluminescent reporter molecules. Bioluminescent reporter assays to measure kinase activity *in vivo* in real time such as fluorescence resonance energy transfer (FRET) have been around for quite some time already.[111-113] (reviewed in [114]). Only recently by means of the application of substrates derived from key tyrosine phosphorylation sites, more kinase specificity is added. Zhang et al. applied this approach in the measurement of PKB/Akt kinase activity *in vitro* as well as *in vivo* by using a reporter construct that contains an 18 amino acid long sequence representing a key phosphorylation site derived from Akt.[115] Thus, factually this reporter measures activity of upstream kinases of Akt such as PI3K. In the unphosphorylated form the reporter has luciferase kinase activity. Upon phosphorylation of the substrate the luciferase protein becomes disrupted due to a conformational alteration upon which bioluminescence is abolished. Loss of luciferase activity indicates an increased phosphorylation of the Akt peptide. Similar reporter constructs have been applied successfully in the non-invasive monitoring of PKA and PKC kinase activity.[116,117] Although the reporter contains a substrate of only 18 AA in length, substantial changes in kinase activity can be observed upon stimulation, suggestive for reasonable kinase specificity.

The kinase activity reporter can provide a sensitive and reliable means of measuring specific kinase activity. In that sense it has developed into a useful tool in cancer research. However, due to the laborious establishment of transduced cell lines, this technique is not suitable for high-throughput measurement of kinase activity. Furthermore, it does not allow measuring kinase activity in primary patient material.

4.2.2 Peptide substrate-based kinase activity arrays

Short amino-acid sequences have been used as kinase substrates for decades already. Only recently, this principle has been made suitable for high throughput application. Array based kinase activity screens provide an excellent opportunity to simultaneously screen for kinase activity on a high number of kinase substrates.

Substantial progress has been made over the recent years in the way the peptides are immobilized onto a surface or chip, thus making the substrates much more sensitive for phosphorylation.[118-122] Recently the selection of suitable substrates for broad spectrum measurement of specific kinase activity has taken a leap forward. Diks et al. showed that by application of radioactively labeled ATP (33P-γ-ATP) it is possible to visualize phosphorylation on 192 varying substrates spotted onto glass in an array format.[123] Confidence in the usefulness of peptide array technology for studying signal transduction came from western blot analysis of lipopolysaccharide-stimulated cells, which corroborated the signals obtained using peptide arrays, as well as from the demonstration that kinase inhibitors effected peptide array phosphorylation patterns consistent with the expected action of the inhibitors.

To allow a more comprehensive description of kinase activity the platform was expanded into the kinase I PepChip containing 1024 peptide substrates derived from key phosphorylation sites in signal transduction, selected from the Phosphobase resource.[124] In functional studies, a comprehensive description of cell signaling by PepChip kinase activity profiling has been proven possible.[125] More recently a new peptide-based kinase activity screening platform characterized by a continuous flow-through of the reaction mix through the porous array membrane containing the spotted peptides was developed. This approach results in a substantial reduction of the background signal.[126,127] Furthermore, it allows for a quantitative assessment of the signal buildup over time, providing a means for studying enzyme kinetics. The Pamchip array contains 144 tyrosine kinase-derived peptides containing tyrosine phosphorylation sites known to be involved in key phosphorylation events *in vivo*. The possibility to screen for serine and threonine kinase activity has become available on this platform as well, thereby allowing for assessment of cell signaling activity on multiple signaling levels.[128,129]

Phosphorylation of a peptide may indicate an activation process taking place in the biological system of interest. However, the peptide phosphorylation essentially depicts the activity of kinases for which the peptide functions as a substrate. Although the substrate is aimed to represent the cell biological substrate *in vivo*, the tertiary structure and spatiotemporal regulatory mechanisms which normally control enzyme specificity, are largely lost. Therefore other kinases can have potential activity on substrates as well, whereas *in vivo* this site is not a target for this kinase at

all.[130] Hence we conclude that for reliable peptide array data interpretation taking into account all potential upstream kinases of a substrate is preferable. To prevent the list of potential upstream kinases becoming incomprehensibly large, a basic requirement is a detailed annotation of potential upstream kinases of a particular phosphorylation site. The knowledge on protein interactions has increased tremendously over the last years, as can be deduced from the rapid expansion of the Phospho-Elm database that inventories all the known phosphorylation interactions (http://phospho.elm.eu.org). In addition, computational analysis algorithms like Scansite (http://scansite.mit.edu) can be used to identify candidate substrates of kinases of interest based on phosphorylation consensus sequences. Increasing knowledge of *in vivo* phosphorylation events will continue to improve the interpretability of peptide array data in the coming years. This will result in a view on cell signaling that will gain in reliability as well as in comprehensibility.

4.2.3. Quantitative measurement of kinase activity

The key to exact measurement of kinase activity is using substrates which are sensitive to specific kinase activity and which, despite the loss of numerous kinase regulatory mechanisms, retain selectivity. A common substrate of choice for *in vitro* kinase assays is a peptide of approximately 20 amino acids in length containing a key phosphorylation site from a downstream target protein. Multiple research papers describe the strength and selectivity of protein binding to specific phosphorylation motifs.[131-133] In addition, recent results also show that, even in case of high kinase homology, a workable sequence specificity remains when using short amino-acid substrates.[134] Nevertheless, a much discussed potential problem in *in vitro* kinase assays is the fact that the regulatory constraints that normally control protein phosphorylation are lost.[135] Regulatory mechanisms playing a role in determining the selectivity of protein interactions include distal docking sites in the tertiary protein structure, presence of adaptor- and scaffolding proteins and spatio-temporal constraints. The relative importance of either the primary amino-acid sequence of individual phosphorylation sites or contextual constraints in determining the kinase selectivity can vary substantially.[92,136] The application of purified kinases is a potential solution to exclude a-specific substrate phosphorylation.[137] Yet, obtaining these samples has potential drawbacks of contaminating the assays with other enzymes

that affect results. Thus, new ways have to be found to deal with wavering substrate specificity. The kinetic buildup of the phosphorylation signal is a parameter that should not be disregarded. Detailed understanding of the fundamental factors driving selectivity and sensitivity of protein-protein interactions is an essential prerequisite. Possibilities to harness the variation in substrate specificity have to be explored to devise new angles on treatment of disease by means of small molecule inhibitors.[138]

Recent publications confirmed that taking into account the phosphorylation of multiple potential substrates can boost the interpretability of the data tremendously.[139] However, thorough screens of substrate specificity with purified and/or recombinant kinases ideally have to be performed in advance. Selecting suitable substrates for each of the ~500 kinases in the human genome[140] is gradually becoming more consistent since the knowledge on important protein phosphorylation sites playing a role in the kinase activity is increasing rapidly. Even so, one has to keep in mind that generating hypotheses is currently the limit of what peptide-based kinase activity screens can provide.

From a technical perspective important criteria for obtaining reproducible data are a short post-operative delay of tissue freezing (preferably below 30 minutes) as well as performing tissue lysis shortly before initiating the assay.[141] The requirement for only minute amounts of tissue is a prominent advantage over mass spectrometry, where larger amounts of tissue need to be readily available.

Yu et al. explored the possibility to study kinase activity on peptide substrates reading out phosphorylation in a quantitative fashion using mass spectrometry.[142,143] Their protocol, kinase activity assay for kinome profiling (KAYAK), encompasses incubation of tissue lysates with 90 different peptides followed by phosphorylation readout with mass spectrometry. The gain in quantitative and site-specific measurement of peptide phosphorylation comes at a cost since the high-throughput feature, characteristic for the peptide array-based kinase activity profiling, is largely lost.

A commonly discussed potential disadvantage of mass-spectrometry applications is the limited sensitivity of the technique due to high sample complexity and the presence of highly abundant proteins. Since array based kinase activity profiling measures kinase activity instead of relative protein abundance it has the potential to measure kinase activity present in only limited amounts, for instance in subsets of cells.[144] This opens up possibilities to study complex spatial and temporal cell

signaling dynamics. Subdivision of complex tissue structures by laser microdissection will become a valuable addition to these studies. Laser microdissection (LMD) procedures have already been applied successfully to dissect tumor tissue from tumor-enriched tissue specimens in order to assess the protein phosphorylation status using RPPA.[145,146] Successful application of LMD to study enzymatic activity in subsets of cells within a tumor cell population has not yet been shown since retaining kinase activity has not been possible.

4.3 Peptide array-based kinase activity profiling to assess aberrant signaling in disease and neoplasia

Translating patient-specific knowledge about kinase activity driving signal transduction networks into the clinic constitutes the most important challenge of cancer treatment in the near future. Array based kinome profiling technology offers an excellent opportunity to study aberrant kinase activity in human disease. Recently a number of interesting studies have been published in the field.

Van Baal et al. describe the application of kinase activity profiling in characterizing Barrett's Esophagus.[147] By successfully generating kinase activity profiles they were able to construct and ratify provisional signal transduction schemes showing the differences in cellular signaling between Barrett's Esophagus and the surrounding non-malignant epithelium. This paper pioneered data interpretation by means of constructing signal transduction schemes by combining changes in phosphorylation of peptides representing multiple members of signal transduction pathways of interest.[148]

De Borst et al. applied kinase activity profiling in mapping aberrant kinase activity occurring as a result of angiotensin II-mediated hypertensive renal damage in a rat model.[149] This phenotype resulted in increased kinase activity of p38 MAPK and PDGFRβ. The increased activity of p38, which is in line with previously published literature showing increased p38 phosphorylation in renal disease, underscores that the kinase activity profiles represent a comprehensive description of the cell signaling status.

Schrage et al. performed kinase activity profiling on a selection of chondrosarcoma cell lines and primary cultures[150], thus identifying a number of interesting potential targets for systemic treatment. Validation studies confirmed activity of specific

kinases as indicated by the kinase activity profiling and *in vitro* cell survival studies revealed the Src kinase family as a druggable target in chondrosarcoma.

Bratland et al. were able to show and validate osteoblast-induced EGFR activation in androgen-sensitive prostate carcinoma cells, based on kinase activity profiling data.[151]

Jinnin et al. detected VEGFR2 activity in infantile hemangioma applying Pamchip arrays.[152] Here, the signal intensities of most VEGFR-derived peptides corresponded with the phosphorylation levels as detected by immunoblotting, evincing the validity of these substrates in mirroring VEGFR kinase activity.

Diks et al. suggested the use of peptide array based kinase activity profiling to devise a minimal eukaryotic phosphoproteome.[153] We recently proposed the application of this concept to construct a minimal kinase activity profile of pediatric brain tumors.[141] With the aid of the Phospho-Elm database we were able to determine the potential upstream kinases of most peptide substrates, providing a list of kinases potentially active in various types of pediatric brain tumor. This list corresponded with numerous publications on aberrant kinase activity, confirming the validity of the kinase activity profiling data. Furthermore, it presented new insights in potential targets for treatment of these malignancies (see also table 1).

The next logical step in the application of array based kinase activity profiling is to assess the clinical validity of the profiles. Based on the kinase activity profiles on Pamchip tyrosine kinase arrays Folkvord et al. were able to predict preoperative chemoradiation tumor response with a high degree of certainty.[154] Additional articles comparing kinase activity profiles with clinical data will most likely be published shortly.

5. Conclusions and future perspectives: bridging the gap

Effective mechanism-based cancer treatment strategies specifically target cell signaling mediators which are critical to cell survival and tumor progression. Increasing numbers of kinase-targeted small molecules with high specificity are currently becoming available. Despite the successful application of relatively broad spectrum kinase inhibitors for the treatment of specific malignancies (table 1), insufficient ways to predict the efficacy of inhibiting specific kinase activity gives rise to a discrepancy between the evaluation of aberrant cell signaling and the currently

available ensemble of highly specific targeted treatment strategies. Bringing together these two pillars in novel cancer drug development has escalated into one of the most important challenges in cancer research. The current status of research on the tumor kinome is telling us that generating network views on aberrant tumor cell signaling is vital to meet this challenge. In the current review we provided an overview of the recent developments in generating this network view on aberrant cell signaling in human malignancies. Identifying the key players in specific malignancies proves to be highly challenging due to signaling redundancy and inter-patient variability. A shift in focus can be observed from the measurement of the phosphorylation status of signal transduction mediators as a surrogate for kinase activation to direct quantitative measurement of kinase activity. High-throughput array-based kinase activity screens provide insight in essential signal transduction pathways by showing activation of specific kinases. Hypotheses generated upon kinase activity profiling have been validated by conventional techniques in multiple independent studies. This underscores the potential of peptide array based kinase activity profiling in the discovery of new therapeutic targets. Generating a network view on kinase activity will provide critical perspectives on cancer therapy, either by allowing the selection of accurate targets or to devise the most optimal therapy for the individual patient.

To overcome the heterogeneity in the response to small molecule inhibitors insight in the molecular profile of a tumor can provide a better patient stratification. Potentially, this can function as a diagnostic marker for selecting agents that result in the most optimal inhibition of tumor growth for each tumor subtype or individual patient by pinpointing vital signal transduction pathways. Possibly this predictive application can be expanded in the future with monitoring treatment response on cell biological level over time, provided that repeated tumor biopsies are an option. Furthermore, the extent to which peptide phosphorylation is altered when performing kinase activity screening in the presence or absence of specific kinase inhibitors is likely to improve the identification of active kinases functioning as dominant nodes in oncogenic signaling.

Diagnostic applicability of kinase activity profiling in cancer treatment depends on whether a number of basic requirements can be met. First of all, tumor material frozen with a short post-operative delay has to be obtained, thus sufficiently

retaining kinase activity. In addition the profiles have to be understandable in such a way that comprehensive insight in the cell signaling allows the selection of the most appropriate set of inhibitors. Up until now the latter criterion proves to be most challenging. Increasing knowledge on the mechanisms determining the selectivity of *in vivo* kinase-substrate interactions has become critical in the selection of optimal substrates for kinase activity assaying. Computational advances already provided a wealth of information on potential *in vivo* kinase substrates. Nevertheless, our understanding of kinase action does not yet permit pinpointing kinase substrates based on the primary amino acid sequence of signaling proteins. Identification of the most sensitive and selective peptide substrates for each kinase can improve the interpretability of peptide-based kinase activity profiling technology to a great extent. Thus, empirical methods to select suitable and selective substrates are essential to obtain a high throughput measurement of specific kinase activity in complex protein samples. Incorporating genomics as well as antibody based total-protein and phospho-specific protein technology, however, allows the construction of complex network views of aberrant cell signaling. Consequential prioritization of essential cell signaling processes already provided a remarkably effective selection of targets for more functional studies, as discussed previously.

Futreal et al. started collecting data suggesting a causal involvement of specific genes implicated in oncogenesis which resulted in a census of human cancer genes based on the currently known data.[155] Possibly such a census could be initiated on protein phosphorylation and activity as well, providing us with a means of selecting key factors in carcinogenesis and maintenance of malignancy. Overall, we suggest further implementation of peptide-based kinase activity profiling in overcoming the heterogeneity in patient response upon kinase-targeted therapies by targeting cell signaling pathways that play a decisive role in tumor progression. In combination with proteomics approaches like reverse phase protein arrays, peptide-based kinase activity screening can aid in providing a complete view on aberrant cell signaling.[156] This promises to facilitate a more effective selection of kinase inhibitors that provide the most optimal mechanism-based tumor- and patient-specific therapeutic strategy.

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Supplementary Table 1

primary target	agent	cancer type	Key references
Abl, Lyn	Bafetinib	glioma, ependymoma, oligodendroglioma	-
Akt	Perifosine, KRX-0401	glioma	-
CDK	Flavopiridol, Alvocidib	refractory tumors	-
EGFR	Cetuximab	pontine tumors and high-grade astrocytomas	161,162
EGFR	Erlotinib	glioma	163,164
EGFR	Gefitinib	glioma	165,166
EGFR	MAb-425	glioma	-
EGFR	MDX-447	glioma	-
EGFR, HER2	Lapatinib	CNS tumors	167
EGFR, VEGFR	AEE788	glioblastoma	-
ErbB	PF-00299804	glioblastoma	-
Flk-1, VEGFR2	SU5416	astrocytoma, glioma	168
Flt3, PDGFR,	Tandutinib	glioma	-
Fms, Kit, Flt3	PLX108-01	solid tumors	-
HGF/SF	AMG 102	glioma	169
IGF-1R	IMC-A12	pediatric solid tumors	-
MEK	AZD6244	low-grade glioma	-
MET	PF-02341066	solid tumors	-
MET, VEGFR2	XL184	glioma	-
mTor	Temsirolimus	primary or metastatic brain tumors	170
mTor	Everolimus	glioma	171

mTor	Deforolimus	glioma	172,173
panHER	AC480	glioma	-
PDGFR	CP-868,596	glioma	174,175
PDGFR	SU101	glioma	-
PDGFR, BCR-Abl	Nilotinib	glioma	-
PDGFRa	IMC-3G3	glioma	-
PI3K	XL147	glioma	-
PI3K, mTor	XL765	glioma	-
Raf, VEGFR	Sorafenib	glioma	177
Src, cKit, PDGFR	Imatinib	glioma	178,179
various	Suramin	glioma	180
VEGF	Bevacizumab	glioma, medulloblastoma, ependymoma, low-grade glioma	181,182
VEGF	Aflibercept	glioma	-
VEGF production	PTC299	CNS tumors	-
VEGFR	Pazopanib	refractory solid tumors	183
VEGFR	Vandetanib	glioma	184,185
VEGFR	PTK787/ZK 222548	glioma, hemangioblastoma	186,187
VEGFR2	Cediranib, AZD2171	brain metastases, CNS tumors	188
VEGFR2	CT-322	glioma	-

Chapter 3

Kinome profiling in pediatric brain tumors as a new approach for target discovery

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Abstract

Progression in pediatric brain tumor growth is thought to be the net-result of signaling through various protein kinase mediated networks driving cell proliferation. Defining new targets for treatment of human malignancies, without *a priori* knowledge on aberrant cell signaling activity, remains exceedingly complicated. Here we introduce kinome profiling using flow-through peptide microarrays as a new concept for target discovery.

Comprehensive tyrosine kinase activity profiles were identified in 29 pediatric brain tumors using the PamChip kinome profiling system. Previously reported activity of EGFR, c-Met and VEGFR in pediatric brain tumors could be appreciated in our array results. Peptides corresponding with phosphorylation consensus sequences for Src-family kinases showed remarkably high levels of phosphorylation compared to normal tissue types. Src activity was confirmed applying Phos-Tag SDS-Page. Furthermore, the Src-family kinase inhibitors PP1 and Dasatinib induced substantial tumor cell death in nine pediatric brain tumor cell lines but not in control cell lines.

Thus, this study describes a new high-throughput technique to generate clinically relevant tyrosine kinase activity profiles, as has been shown here for pediatric brain tumors. In the era of a rapidly increasing number of small molecule inhibitors, this approach will enable us to rapidly identify new potential targets in a broad range of human malignancies.

Introduction

Cancers arise by an evolutionary process as somatic cells mutate and escape the restraints that normally withhold them from unlimited expansion. To forestall unlimited cell division, multiple mechanisms work in concert. Neoplastic progression is the net-result of uncontrolled signaling through various protein kinase-mediated networks driving cell proliferation. In neoplasms, cell signaling involving these pathways can be affected by numerous factors; genetic, epigenetic and environmental.[1] In theory these signals converge into a limited number of downstream effectors. The most prominent signal transduction pathways implicated to mediate these growth and suppressive signals include the JAK/STAT, PI3K/AKT and the RAS/Erk and PKCα pathways.[2] In pediatric brain tumors, research mainly focused on the potential involvement of specific protein kinases like EGFR, PDGFR, PI3K and JAK in cell proliferation and survival.[3-8] The overall complexity and co-regulation of the various signaling networks, however, has not been unraveled yet. Therefore, high throughput procedures, such as kinome profiling, which generates comprehensive insight in cellular signaling without *a priori* assumptions, would enable us to directly assess a broader range of targets for future treatment strategies.

Microarray based kinome profiling approaches have been subject of development over the last years.[9-13] In the present study, this approach has been employed for the detection of aberrant kinase activity in neoplastic tissue for the first time. Global tyrosine kinase activity profiles of pediatric brain tumors were identified applying a newly developed peptide array system (PamGene).[14]

In the PamChip tyrosine kinase profiling system a sample is constantly pumped up and down through a porous microarray of 144 kinase substrates. The phosphorylation is determined by measuring the association of a fluorescently labeled anti-phosphotyrosine antibody to the substrates phosphorylated by the kinase activities in the sample.

This procedure generated a comprehensive set of kinase induced peptide phosphorylation profiles of all tumor samples included in this study. Peptide array data were validated by a combination of conventional and state-of-the-art techniques such as cell survival assays and Phos-Tag SDS-Page, thereby establishing Src kinase as a primary target for therapeutic intervention in pediatric brain tumors.

The results demonstrate the usefulness of kinase activity profiling as a powerful approach to identify new potential targets for treatment.

Materials and Methods

Patient samples and cell lines

All tissue was obtained by surgical resection. After resection the tissue was subjected to a short period of room temperature (RT) to transfer the tissue from operation room to the pathology department (mean time 10 minutes). Subsequently, the tissue samples were snap frozen in liquid nitrogen and stored at -80 °C.

Tissue material was histologically evaluated and graded according to WHO classification 2007.[15] Written informed consent and local Ethics Committee approval was granted for use of the patient material.

Tyrosine kinase activity profiles of 29 pediatric brain tumors (9 ependymomas, 7 pilocytic astrocytomas and 13 primitive neuroectodermal tumors (PNETs) of which 10 medulloblastomas), were generated (supp. table 1). Wilms tumor (n=5) and colon carcinoma (n=5) tissue was included to study tumor specificity. Normal kidney (n=2) and colon tissue (n=5) samples were included as control tissue.

We used the cell lines Uw-402, Uw-426, Uw-473, Res-256 (medulloblastoma), Res-186, Res-259, Uw-467 (low grade astrocytoma) Res-196 (ependymoma) (Dr. Michael S. Bobola, Seattle Children's Hospital Research Institute), DAOY (medulloblastoma), DLD-1 (colon carcinoma), 293T (human embryonic kidney), TF-1 (acute myeloid leukemia), K-562 (chronic myeloid leukemia) (American Type Culture Collection) and HL-60 (acute myeloid leukemia) (German Collection of Micro organisms and Cell Cultures). DAOY and 293T were cultured in DMEM (Lonza, Breda, the Netherlands) supplemented with 10% fetal bovine serum (FBS; Bodinco, Alkmaar, the Netherlands). The other brain tumor cell lines were cultured in DMEM-F12 (Invitrogen, Breda, the Netherlands) containing 5% FBS. HL-60, TF-1 and DLD-1 were grown in RPMI-1640 (Lonza) with 10% FBS. Cell cultures contained 100 U/mL penicillin and 100 µg/mL streptomycin (PAA laboratories, Pasching, Austria). GM-CSF (Novartis, Basel, Switzerland) was supplemented to the TF-1 cultures (10 ng/mL).

Tyrosine kinase activity profiling using PamChip® peptide arrays

Kinase activity profiles were determined using the PamChip tyrosine kinase microarray system (PamGene International B.V., 's Hertogenbosch, the Netherlands). This microarray consists of 144 unique peptide sequences spotted onto a porous membrane enabling constant flow-through of the reaction mixture (www.PamGene.com). Each peptide represents a 15 amino acid sequence corresponding to a putative endogenous phosphorylation site, which functions as a tyrosine kinase substrate (supp. table 2). Phosphorylation is visualized by measurement of the end-point fluorescent signal emitted as a consequence of fluorescein-labeled anti-phosphotyrosine antibody binding (PY20).

Tissue was lysed in M-PER reagent containing phosphatase- and protease inhibitors (Pierce, Rockford IL). The reaction mixture consisted of 1x ABL buffer (Westburg, Leusden, the Netherlands), 100 µM ATP (Sigma Aldrich, Zwijndrecht, the Netherlands), 20 µg/mL PY20 (Exalpha, Maynard MA) and 5 µL lysate. Prior to loading the reaction mixture onto the array a blocking procedure was performed with 0.2% Bovine Serum Albumin (Calbiochem, Breda, the Netherlands). The experiment was repeated 8 times for each tissue sample to study technical reproducibility. After loading the cell lysates onto the PamChip arrays, incubation was commenced for 60 cycles utilizing the PamStation96 followed by washing and FITC fluorescence measurement of all peptide spots.

The optimal protein concentration was determined by adding samples with various protein concentrations to the array. Between 0.5 and 1.5 µg/µL, variation in the total amount of protein applied to the array did not influence signal intensity (data not shown). Protein concentrations were measured in the first experiments to standardize the tissue procedure (12 slices of 5 um of a tissue block measuring 5x5 mm).

Tissue-Tek O.C.T. Compound (Sakura Finetek B.V., Zoeterwoude, the Netherlands), used in the tissue freezing procedure, did not affect kinase activity.

Immunoblotting of phosphorylated Src

The immunoblotting procedure was performed basically as described previously.[16] Frozen tissue was lysed in 250 µL RIPA buffer containing Complete™ solution (Roche Diagnostics, Penzberg, Germany) and 1 mM Na_3VO_4. Cultured cells were washed with

ice cold PBS and scraped in 500 µL RIPA buffer. Laemmli sample buffer was supplemented and the lysate was boiled for 5 minutes.

To visualize the levels of phosphorylated Src a newly developed immunoblotting technique was applied using a phosphate-binding molecule (Phos-Tag AAL-107; NARD Institute, Amagasaki, Japan).[17] Together with 0.2 mM $MnCl_2(H20)_4$, 0.1 mM Phos-Tag AAL-107 ligand was added to the sodium dodecyl sulphate/ polyacrylamide (SDS) gel (7.5%). Protein was blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Etten-Leur, the Netherlands). Blots were incubated at 4 ^{0}C in 5% bovine serum albumin containing a primary antibody against total Src (2 µg/mL stock; 1:1000; 36D10 Rabbit mAb; Cell Signaling, Danvers MA) overnight, followed by incubation with swine-anti-rabbit peroxidase-conjugated antibodies (1:3000; P0217; Dako, Denmark) at RT for one hour. Antibody binding was visualized by enhanced chemiluminescence. Beta-actin was probed as a protein loading control (200 µg/mL stock; 1:3000; sc-47778 mouse mAb; Santa Cruz Biotechnology, Santa Cruz CA).

Cell survival assays

A WST-1 colorimetric viability assay protocol was performed following the procedures recommended by the manufacturer (Roche). Cells were seeded at a density of $4x10^4$ cells per well in medium (1% FBS) and subjected to 0, 0.1, 0.5, 1, 2 and 20 µM of 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine (PP1; BioMol International L.P., Exeter, UK), or 0, 0.05, 0.1, 0.5, 1, 5, 10, 50 and 100 nM Dasatinib (BMS-354825; Bristol Myers Squibb, Princeton NJ) for 48 hours (6 replicates for each concentration).

Cell proliferation assays

5-Bromo-2-deoxyuridine (BrdU) cell proliferation assays were performed following procedures recommended by the manufacturer (Cat. No. QIA58; Merck Biosciences, Darmstadt, Germany). Cells were seeded at a density of $2x10^4$ cells per well and incubated with either 0, 2 or 20 µM PP1 in medium containing 1% FBS (6 replicates for each concentration). BrdU was added after 0, 24 or 48 hours incubation with PP1 followed by another 24 hours of incubation.

Data analysis and statistics

Quantification of spot intensities was conducted using Bionavigator software (PamGene, 's Hertogenbosch, the Netherlands).

Background was determined by plotting all signals against the relative standard error of mean (RSE) over the replicates. The threshold signal intensity was read from the trend line at an RSE of 1/e (~37%), above which signals were considered to reflect a genuine phosphorylation event. Phosphorylation values with an RSE exceeding 37% were excluded from further analysis. Data analysis was performed in BRB-Array Tools version 3.5.0..[18] Data normalization was achieved by determining the median signal over each array. Differences in peptide phosphorylation were statistically assessed with non-parametric Mann-Whitney U tests in SPSS (release 14.0.2).

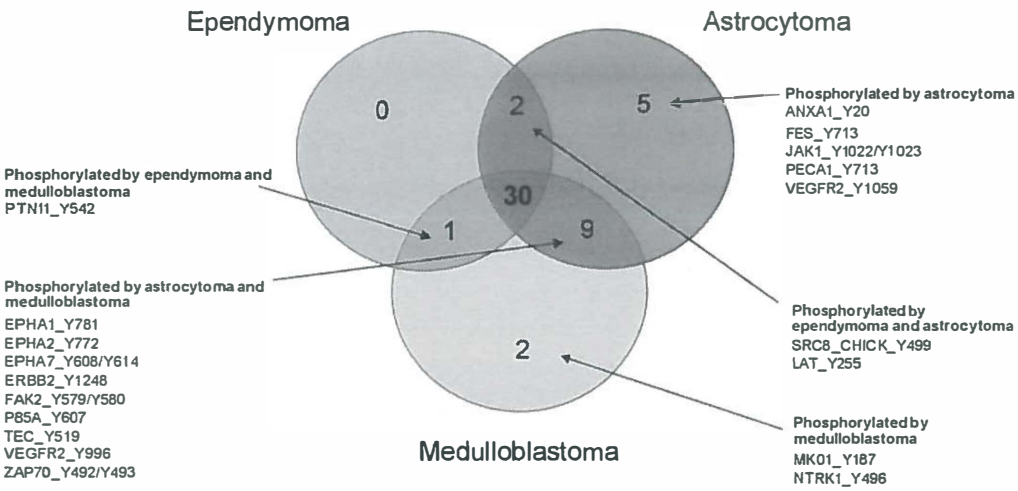


Fig. 1. Peptides phosphorylated by astrocytoma, medulloblastoma and ependymoma tissue lysate. The number and identity of the peptides phosphorylated by more than 90% of the samples within one tumor type are displayed in a Venn diagram comparing peptide phosphorylation between the different pediatric brain tumor types. 49 of the 144 peptides present on the array displayed signal intensities above the threshold value. A remarkable thirty peptides were phosphorylated by more than 90% of each type of pediatric brain tumor, whereas peptides preferentially phosphorylated by lysate of a specific tumor type can be appreciated as well.

Differences in BrdU incorporation levels were statistically assessed with Kruskal-Wallis one-way analysis of variance (SPSS).
The Phospho-ELM BLAST tool (Phosphobase resource release 7.0) was applied to determine the kinases potentially responsible for phosphorylation of a specific peptide (http://phospho.elm.eu.org/pELMBlastSearch.html).

Results

Tyrosine kinase activity profiles of pediatric brain tumors

The flow-through feature of the PamChip tyrosine kinase profiling system resulted in a low and evenly distributed background signal, making it possible to measure kinase activity in high resolution. Measuring the signal intensities of highly phosphorylated peptides at multiple time points indicated that the phosphorylation signals did not reach the plateau during the run.
To study the reproducibility of the peptide array data we generated kinome profiles in separate assay-runs on the same day and on separate days. Hierarchical clustering showed comparable lists of phosphorylation values in both cases. Furthermore, regression analysis comparing the phosphorylation values of identical patient tissue, prepared and analyzed on separate days rendered an R^2 value of 0.79 (supp. fig. 1).

To determine the time window for measuring tyrosine kinase activity (time between tissue resection and freezing during operation), kinase activity profiles of tumor tissue lysed after either, 2, 5, 10, 20 or 30 minutes incubation at RT were established. No differences in kinase activity profiles were observed up until 30 minutes of RT with an R^2 of 0.91 comparing the values after 2 and 30 minutes of incubation (supp. fig. 2). Thus, we can conclude that kinase activity is sufficiently stable to allow analysis according to our experimental setup.
Next, kinase activity profiles of pediatric brain tumor lysates were established. We used two methods to analyze the phosphorylation data. First, phosphorylation intensities of the peptides were compared between the different brain tumor subtypes in accordance with the minimal kinome approach described by Diks et al. (fig. 1).[19] The minimal kinome approach determines the set of substrates which phosphorylation is shared by more than 90 percent of all samples within one tumor

type. The CNS PNET samples were excluded from this analysis. Second, differences in phosphorylation levels were assessed by class comparison.

On average, the ependymoma, astrocytoma and medulloblastoma tumor lysates elicited phosphorylation of 70, 73 and 72 peptides respectively (fig. 2; supp. table 3). Phosphorylation of 30 substrates appeared to be common to all pediatric brain tumor types. Within this set of 30 peptides (supp. table 4), suggested to represent the minimal tyrosine kinome of pediatric brain tumors within the current set of substrates, high levels of kinase activity was observed on peptides corresponding with phosphorylation consensus sequences derived from ERBB2/HER2 receptor and c-Met/HGF-receptor kinases. Furthermore, with the aid of the Phospho-ELM database, we identified 20 peptides which could potentially be phosphorylated by Src-family kinases. Seven of these peptides were present in the established minimal tyrosine kinome of pediatric brain tumors.

Differential phosphorylation of the VEGFR2 derived peptides was also observed. VEGFR2_Y1059 (DIYKDPDYVRKGD) was phosphorylated by all of the included medulloblastomas whereas some ependymoma and astrocytoma samples lacked phosphorylation of this peptide. Furthermore, VEGFR2_Y996 (EEAPEDLYKDFLT) was phosphorylated by all astrocytomas and medulloblastomas but not all ependymomas. Class comparison showed a significantly higher phosphorylation of the peptide LAT_Y200 (MESIDDYVNVPES) in astrocytomas compared to ependymomas. In addition, the phosphorylation of CBL_Y700 (EGEEDTEYMTPSS) by astrocytoma lysate was higher compared to medulloblastomas.

Overall, these results imply substantial overlap in kinase activity, whereas some phosphorylation events could be appreciated as potential druggable targets.

Tyrosine kinase activity profiles of neoplastic tissue and its normal counterpart

In the light of the observed similarities in kinase activity in three pediatric brain tumor types we wondered whether these similarities are tumor specific. Therefore, we performed an experiment comparing kinase activity profiles of three diverse types of solid tumor.

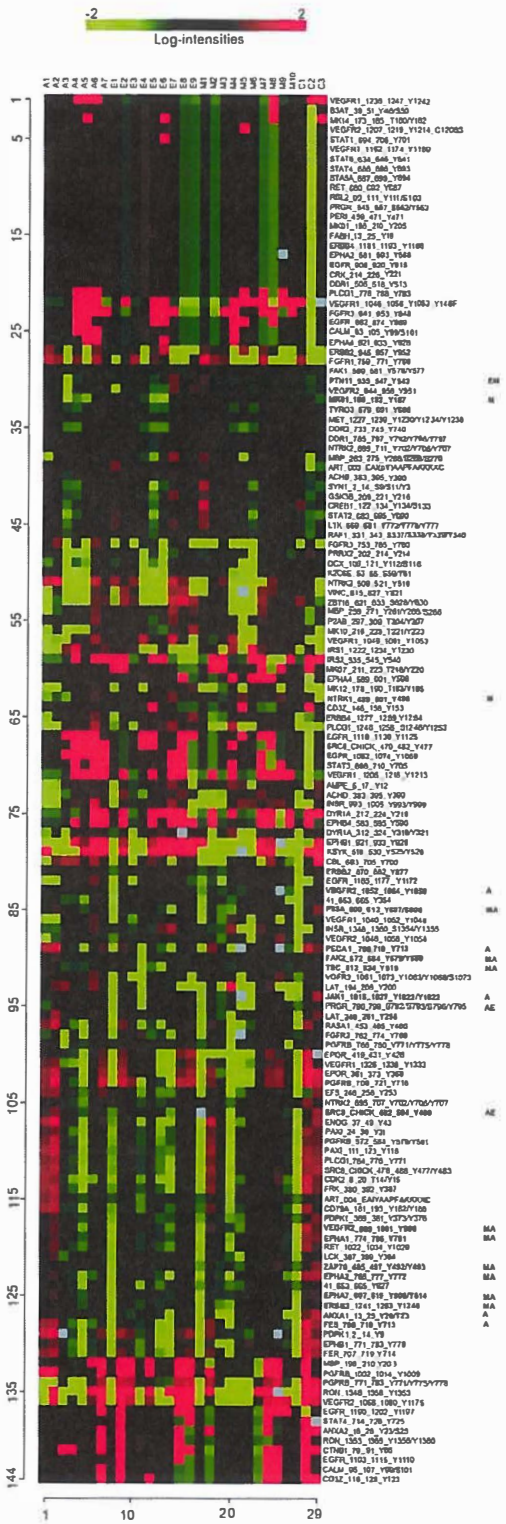

-2
2
Log-intensities
A1 A2 A3 A4 A5 A6 A7 E1 E2 E3 E4 E5 E6 E7 E8 E9 M1 M2 M3 M4 M5 M6 M7 M8 S10 C2 C3
1
5
15
25
35
45
55
65
75
85
95
105
115
125
135
144
1
10
20
29

Fig. 2. Supervised clustering of normalized peptide phosphorylation signals. The 68 peptides which were phosphorylated by more than 60% of all brain tumor lysates are displayed here. Red and green spots represent high and low signal intensities, respectively. The sample labels correspond with supplementary table 1. A stands for astrocytoma, E for ependymoma, M for medulloblastoma and C for CNS PNET. The peptide names correspond with the phosphorylation consensus sequences of the kinase they originated from (supp. table 2). Highlighted peptides (bold) represent peptides that are phosphorylated by more than 90% of the samples within one tumor type, as listed in figure 1.

Kinase activity profiles of 9 pediatric brain tumors (A2, A6, A7, E2, E8, E9, M7, M8, M9), 5 Wilms tumors and 5 colon carcinomas were compared to normal kidney (n=2) and colon tissue (n=5). Since there is no possibility of getting healthy normal brain tissue and post mortem brain tissue is not applicable due to the slow decrease in body temperature after death resulting in autolysis, we decided to compare the various brain tumor tissues with a small panel of normal tissues to see if the resolution of the technique is sufficient to discern differences in kinase activity. After data processing, 88 peptides showed signal intensities below the threshold value. Twenty-eight peptides, including substrates for Wee1, Src, Ret, Pak and PDGFRB proved to be phosphorylated by lysate of all three tumor types (fig. 3, supp. table 4).
For the two tissue-normal counterpart combinations, the neoplastic tissue displayed a different number of phosphorylated peptides, reflecting altered tyrosine kinase activity in neoplastic cells. The colon carcinomas rendered an average of 75 phosphorylated peptides compared to 58 peptides applying normal colon tissue. For Wilms tumors this concerned 62 peptides compared to 66 in the normal kidney counterpart. Colon carcinoma lysate showed a significantly higher kinase activity on 5 peptide residues compared to normal counterpart tissue. These peptides correspond with consensus sequences of Src, RET and FAK tyrosine kinases.
Applying the minimal kinome approach, the colon and kidney tumors together produced unique phosphorylation of 11 peptides compared to 1 in their normal counterparts (fig. 3). Within this set of 11 tumor-specific peptides, phosphorylation of CDK2_Y15 (EKIGEGTYGVVYK) and MET_Y1230/Y1234/Y1235 (RDMYDKEYYSVHN) can be appreciated. These peptides are also present in the established minimal pediatric brain tumor tyrosine kinome. Thus, although phosphorylation of a significant number

of substrates is shared between tumors of different origin, also clear germ line specific and tissue specific phosphorylation patterns can be appreciated in cancers of different origin.

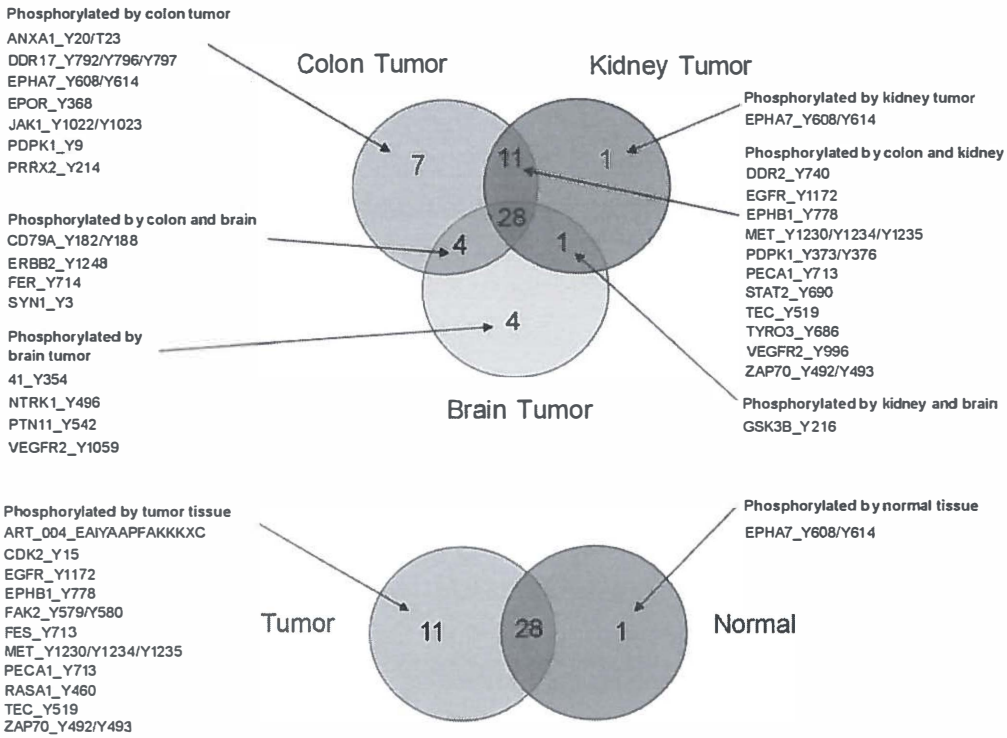


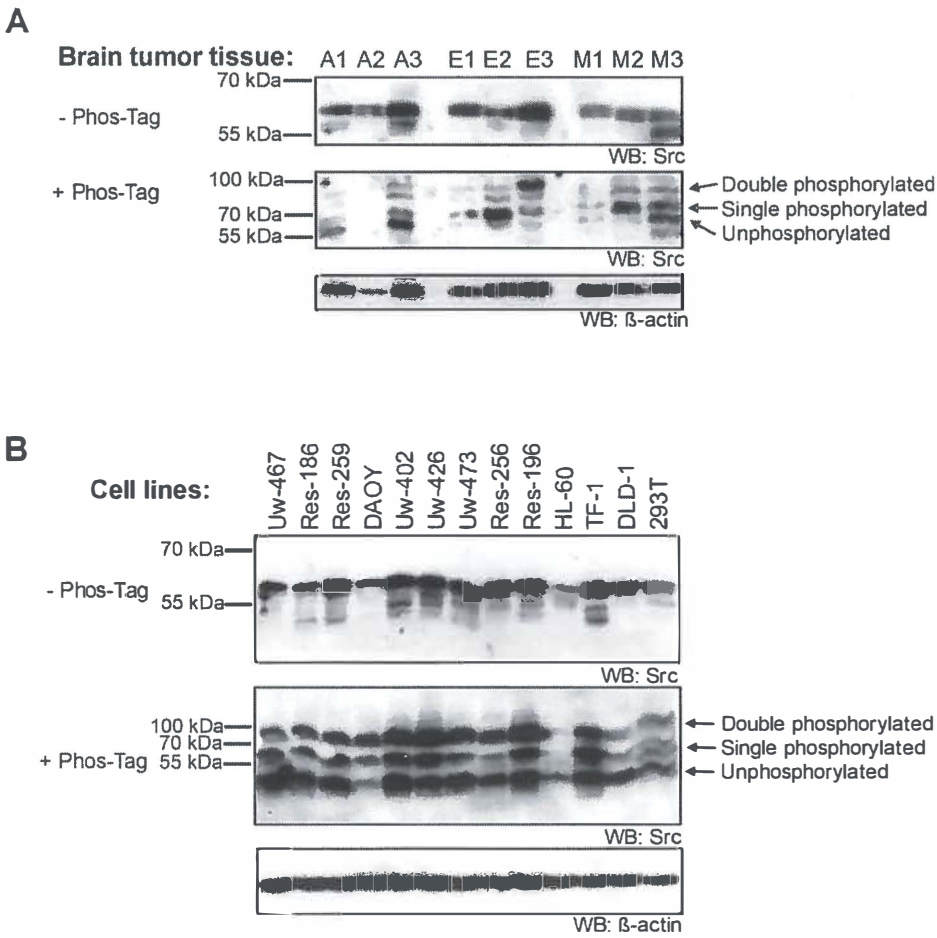
Fig. 3. Tumor and tissue specificity of kinase activity profiles. Tyrosine kinase activity profiles of three diverse types of solid tumor were generated: colon carcinomas, Wilms tumors and pediatric brain tumors. Normal colon and kidney tissue was included as normal counterpart. The number and identity of the peptides phosphorylated by more than 90% of the samples within one tumor type are displayed in a Venn diagram. A list of peptides commonly phosphorylated by all three tumor types can be found in supplementary table 4.

The lower panel displays a Venn diagram of the peptides phosphorylated by either tumor or normal counterpart lysate. 40 peptides were phosphorylated by more than 90% of the tumor lysates, normal tissue samples or both. Peptides preferentially phosphorylated by a specific tissue type have been specified in the figure.

Differences in the phosphorylation status of Src related peptides between pediatric brain tumor- and normal tissue samples were assessed by class comparison. Interestingly, brain tumor lysate showed a significantly higher phosphorylation of ten of the twenty potential Src substrates (supp. fig. 3): ANXA1_Y20 (IENEEQEYVQTVK; P=0.044), EFS_Y253 (GGTDEGIYDVPLL; P=0.007), ENOG_Y43 (SGASTGIYEALEL; P=0.005), P85A_Y607 (NENTEDQYSLVED; P=0.012), PAXI_Y118 (VGEEEHVYSFPNK; P=0.009), PAXI_Y31 (FLSEETPYSYPTG; P=0.007), PDPK1_Y9 (ARTTSQLYDAVPI; P=0.014), PDPK1_Y373/Y376 (DEDCYGNYDNLLS; P=0.005), SRC8_CHICK_Y477/Y483 (EYEPETVYEVAGA; P=0.016), SRC8_CHICK_Y499 (YQAEENTYDEYEN; P=0.009). These results imply Src activity to be a prominent characteristic of pediatric brain tumor growth.

Src mediated signaling might be important in pediatric brain tumor progression

Src phosphorylation levels were determined in three tissue samples of each brain tumor type, applying the newly developed Phos-Tag SDS-Page approach. This technique makes use of the affinity of phosphorylated proteins for the Phos-Tag ligand, resulting in retention of phosphorylated proteins, separating them from their unphosphorylated counterpart during electrophoresis. In theory this will result in two or more distinct protein bands representing the phosphorylated and unphosphorylated status of the protein. Src possesses two key phosphorylation sites determining activity: the stimulatory site Tyr-416 and the inhibitory site Tyr-527. Phosphorylation of both tyrosine residues renders the enzyme hyperactive.[20,21] Thus, three possible phosphorylation states can be appreciated: non-phosphorylated, single phosphorylated at Tyr-527 or Tyr-416 and double phosphorylated at both Tyr-527 and Tyr-416. On the Phos-Tag gels we observed all three phosphorylation states, confirming Src kinase activation in tissue samples of all three brain tumor types as well as in each of 9 pediatric brain tumor cell lines (5 medulloblastomas, 3 astrocytomas, 1 ependymoma) (fig. 4). We observed a less active phosphorylation ratio of Src in the control cell lines HL-60, DLD-1 and 293T.



To verify the functional role of Src kinases in pediatric brain tumor growth, cell survival assays were performed using Dasatinib and PP1 as potent Src-family kinase inhibitors.

PP1 induced a dose dependent decrease in cell survival in all brain tumor cell lines with LC50 (50 percent decline in cell survival) concentrations ranging from 1.1 µM to 9.7 µM (fig. 5). This was in agreement with the concentrations necessary to inhibit Src kinase activity (data not shown). Hardly any decrease in cell survival was seen in two leukemic cell lines (HL-60 and TF-1, LC50 > 20 µM) suggesting other pathways to take over cell-survival signaling in these cell lines. A substantially lower response to PP1 was also found in the colon carcinoma cell line DLD-1 (LC50 > 20 µM) and the human embryonic kidney cell line 293T (LC50 > 20 µM).

Fig. 4. Phospho-specific SDS-Page confirms Src kinase activity. Src phosphorylation levels were determined in three tissue samples of each brain tumor type (A) and in each of the 9 pediatric brain tumor cell lines (B) (medulloblastoma (DAOY, Uw-402, Uw-426, Uw-473, Res-256), astrocytoma (Res-186, Res-259, Uw-467) and ependymoma (Res-196)) using Phos-Tag SDS-Page. Phosphorylated proteins have affinity for the Phos-Tag ligand, resulting in separation of phosphorylated proteins from their unphosphorylated counterparts. On the Phos-Tag blots three phosphorylation states representing unphosphorylated as well as single phosphorylated and double phosphorylated Src can be identified. Src kinase phosphorylated at both Tyr-416 and Tyr-527 renders a hyperactive isoform. Hence, the blotting results confirm substantial Src kinase activation.
Although the expression of Src is lower in three of the four control cell lines (DLD-1, 293T and HL-60), a lower ratio of active versus inactive Src can be observed. The phosphorylation status of Src in TF-1 is comparable to that of the pediatric brain tumor cell lines. The images have been cropped and background has been subtracted to improve clarity.

Also Dasatinib decreased cell survival in all pediatric brain tumor cell lines except for the low-grade astrocytoma cell line Uw-467 (fig. 6). Three cell lines reached LC50 within the applied concentration range: Uw-473, Res-186 and Res-196 (65, 20 and 17 nM respectively). No effect could be observed on the control cell lines except for the positive control K-562. The high apoptosis rate of K-562 upon treatment with Dasatinib is accompanied by an attenuation of the levels of phosphorylated Bcr-Abl, c-Src, Lyn and CrkL indicating that this effect is not only the result of Src inhibition in this specific cell line.[22]
Summarizing, Src inhibitors are able to induce tumor cell death in pediatric brain tumor cell lines whereas no effect can be observed in the controls.

The effect of Src inhibition on cell-cycle arrest was determined by performing BrdU proliferation assays with the medulloblastoma cell line Uw-473 (supp. fig. 4). Cell survival assays performed in parallel indicated a strong dose dependent decrease in Uw-473 cell survival with PP1 added ($P<0.001$). A significant dose dependent decrease in Uw-473 cell proliferation rate upon Src inhibition was observed as well ($P<0.01$).

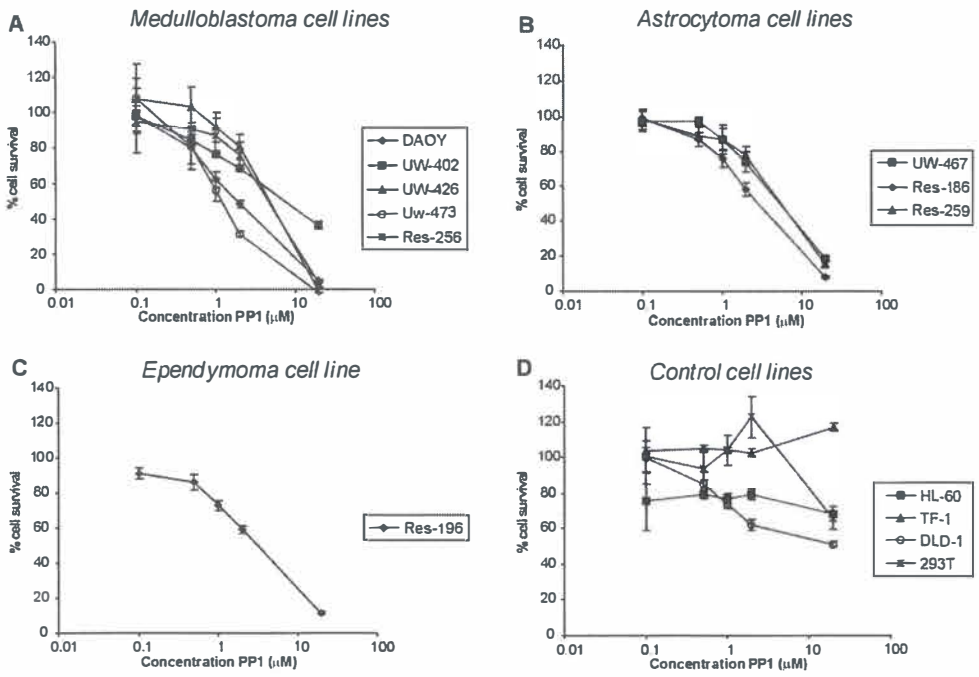


Fig. 5. Decreased tumor cell survival in response to Src inhibition with PP1. The cell survival percentage has been plotted against the concentration of PP1 (μM) for all medulloblastoma (A), astrocytoma (B), ependymoma (C) and control (D) cell lines. Error bars represent the standard deviation of six replicate measurements. All pediatric brain tumor cell lines show decreased cell survival.

Discussion

In this study we successfully applied a novel high throughput technique to generate tyrosine kinase activity profiles of pediatric brain tumors and show its usefulness in target discovery. We identified and validated Src activity as a potential target in pediatric brain tumors for therapeutic intervention.

The tyrosine kinase activity profiles of different pediatric brain tumor types showed considerable overlap. Utilizing the minimal kinome approach, a set of 30 out of 144 substrates proved to be phosphorylated in more than 90 percent of all brain tumor lysates. Eleven substrates showed tumor specific activity in a comparison of various tumor tissues, such as pediatric brain tumors, Wilms tumors and colon carcinomas,

with normal tissue types. Within these 11 tumor-specific substrates, CDK2, c-Met and EGFR derived peptides were present.

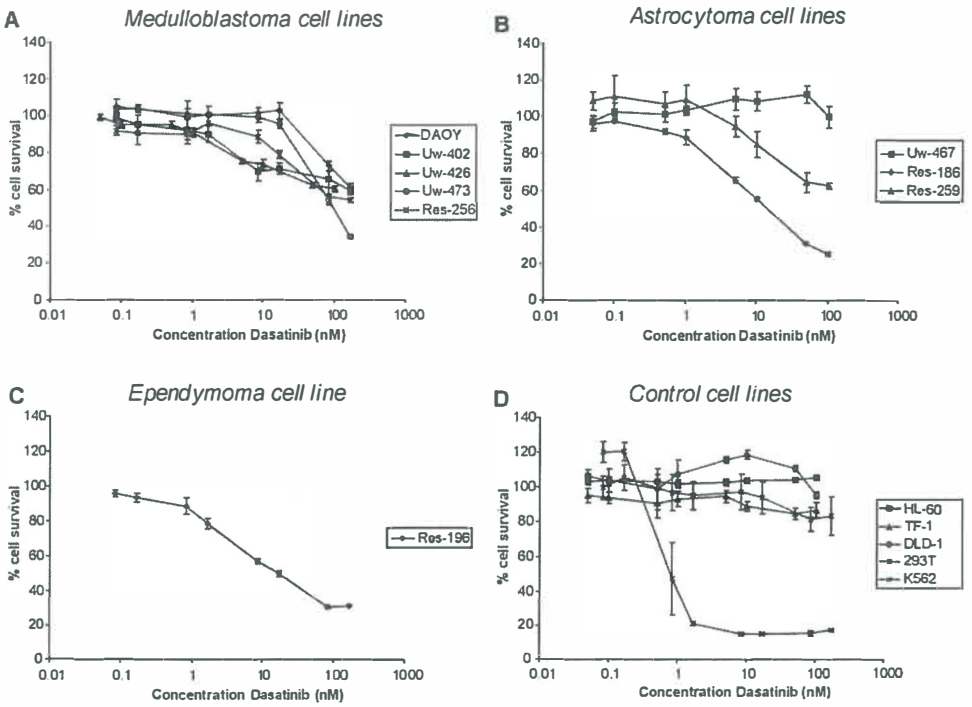


Fig. 6. Decreased tumor cell survival in response to Src inhibition with Dasatinib.
The cell survival percentage has been plotted against the concentration of Dasatinib (nM) for all medulloblastoma (A), astrocytoma (B), ependymoma (C) and control (D) cell lines. The cell line K-562 functioned as a positive control. Error bars represent the standard deviation of six replicate measurements. With the exception of Uw-476 all pediatric brain tumor cell lines show decreased cell survival.

The importance of CDK2 related to malignant progression has already been recognized. CDK2 is the major cell cycle kinase, which activation represents an increase in mitotic index.[23-27] C-Met is the tyrosine kinase receptor of Scatter Factor/Hepatocyte Growth Factor (SF/HGF) and has been shown to promote migration, invasion and angiogenesis in gliomas followed by a decrease in prognosis.[28-31] Interestingly, the SF/HGF:c-Met pathway has been shown to require

CDK2 activation to induce cell cycle progression in medulloblastoma.[32] The presented results suggest a more common role for c-Met induced tumor progression involving CDK2 activation.

In all tumor types, substantial phosphorylation of several peptide motifs related to epidermal growth factor receptor (EGFR)-family members was observed. EGFR signaling has been linked to various malignancies including childhood medulloblastoma.[33,34]

Summarizing, the observed tumor specific kinase activity profile overlaps with previously published tyrosine kinase activation, proving the validity of the peptide array results. Moreover, this kinase activity profile is able to give us a view into the complexity and the subtlety of the cellular signaling networks that drive tumor cell proliferation in favor of tumor cell death.

On the peptide array twenty potential Src substrates are present, of which ten showed a significantly higher level of phosphorylation in pediatric brain tumor tissues compared to normal tissues. With the aid of Phos-Tag SDS-Page we were able to confirm an activatory phosphorylation status of Src in pediatric brain tumor lysates whereas this was not the case in control cell types. The increased sensitivity of pediatric brain tumor cell lines compared to several tumor control cell lines upon increasing dosages of a Src-family kinase inhibitor, points to a potentially important role of Src signaling in pediatric brain tumors. The successful application of Phos-Tag SDS-Page demonstrates its potential in studying protein phosphorylation.

Src-family kinases are controlled by various classes of receptor pathways including protein–tyrosine kinases, integrin receptors and G-protein coupled receptors. Src signals to a variety of downstream effectors including cytoskeletal proteins (FAK, paxillin) and pathways involving Stat, PI3K and Erk, thus exerting effects on cell survival, proliferation and differentiation.[35] We showed a decreased pediatric brain tumor cell viability and proliferation rate upon Src inhibition. The LC50 concentrations of Dasatinib were within nanomolar range. Dasatinib has been shown to decrease tumor cell survival and proliferation in breast cancer, non-small cell lung cancer and neuroblastoma.[36-39] Within the brain, Src-family kinases are known to play a critical role in cerebellar cell migration and histogenesis during embryonic

development.[40-42] Recent publications have shown that Src signaling is also involved in brain tumor cell invasion. By preventing Src-family kinase recruitment to EGFR or CD95 a decrease in glioblastoma cell invasiveness could be established.[43,44] Furthermore, protein phosphatase activity of PTEN has been shown to control glioma cell migration by inhibition of Src-family kinases.[45] PTEN is mutated in one third of all glioblastomas. Our data provides the first evidence that Src signaling is an interesting potential target for pediatric brain tumor treatment. Preliminary data already showed that detectable concentrations of Dasatinib can be reached in the cerebrospinal fluid.[46]

Current mass spectrometry techniques and novel proteomics approaches like antibody microarrays determine protein phosphorylation levels rather than the enzymatic activity resulting from it. Measurement of kinase activity using the peptide microarray provides a direct view on the extent of enzymatic activity leading to specific signal transduction. The validity of the PamGene tyrosine kinase array data has been studied previously by Lemeer et al..[47] They were able to confirm the peptide array phosphorylation data with anti-tyr(P) immunoprecipitation followed by mass spectrometry-based proteomics.

A potential drawback is the variation in the number of potential upstream kinases for the various substrates, as determined using the Phospho-ELM database. In our view, the decreased kinase specificity of a peptide can be compensated by taking into account all substrates potentially sensitive to a kinase of interest, thereby enhancing the interpretability of the results. The annotation of specific peptide substrates has gained reliability over the last years as the Phospho-ELM database has expanded dramatically. Still, determining the sensitivity of a substrate for specific kinases will prove to be of tremendous value in future application of the peptide array. Most likely, future application of recombinant and/or purified kinases will tell us more about the specificity of individual peptides, thus making it easier to pinpoint specific kinases based on the obtained peptide array results. Therefore, generating hypotheses is the limit of what is possible at the current developmental stage of peptide microarray technology.

Overall, we successfully employed a peptide microarray based tyrosine kinase activity screening technique, identifying new potential targets for pediatric brain tumor treatment. The obtained kinase activity profiles suggest the existence of a more tumor specific kinase activity profile including CDK2 and c-Met, confirming earlier reports showing activation of these kinases. In the era of a rapidly increasing number of small molecule inhibitors this tyrosine kinase activity profiling method will be of tremendous value by enabling us to rapidly screen for potential druggable targets in a broad range of malignancies.

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Supplementary table 1. Patient and tumor characteristics

tumor	sex	age (months)	tumor localization	grade	primary or recurrence	survival	follow-up (months after diagnosis)
ependymoma 1	M	32	infratentorial	2	P	Yes	7
ependymoma 2	F	58	infratentorial	3	2nd R	Yes	45
ependymoma 3	M	72	infratentorial	3	2nd R	Yes	63
ependymoma 4	F	286	infratentorial	2	P	Yes	36
ependymoma 5	F	40	infratentorial	2	R	Yes	45
ependymoma 6	M	65	supratentorial	2	P	Yes	78
ependymoma 7	F	139	supratentorial	2	P	Yes	28
ependymoma 8	F	56	infratentorial	3	P	No	17
ependymoma 9	M	14	infratentorial	2	P	Yes	63
medulloblastoma 1	F	112	infratentorial	3	P	Yes	118
medulloblastoma 2	F	72	infratentorial	3	P	Yes	97
medulloblastoma 3	M	126	infratentorial	3	P	Yes	77
medulloblastoma 4	F	136	infratentorial	3	P	Yes	81
medulloblastoma 5	F	19	infratentorial	3	P	No	12
medulloblastoma 6	F	105	infratentorial	3	P	Yes	60
medulloblastoma 7	M	136	infratentorial	3	P	Yes	37
medulloblastoma 8	M	49	infratentorial	3	P	Yes	38
medulloblastoma 9	F	106	infratentorial	3	P	No	5
medulloblastoma 10	M	114	infratentorial	3	P	No	8
CNS PNET 1	M	8	supratentorial	3	P	No	5
CNS PNET 2	M	33	supratentorial	3	P	No	0
CNS PNET 3	F	71	supratentorial	3	P	No	10
astrocytoma 1	F	69	infratentorial	1	P	Yes	58
astrocytoma 2	F	135	infratentorial	1	P	Yes	34
astrocytoma 3	M	108	supratentorial	1	P	Yes	7

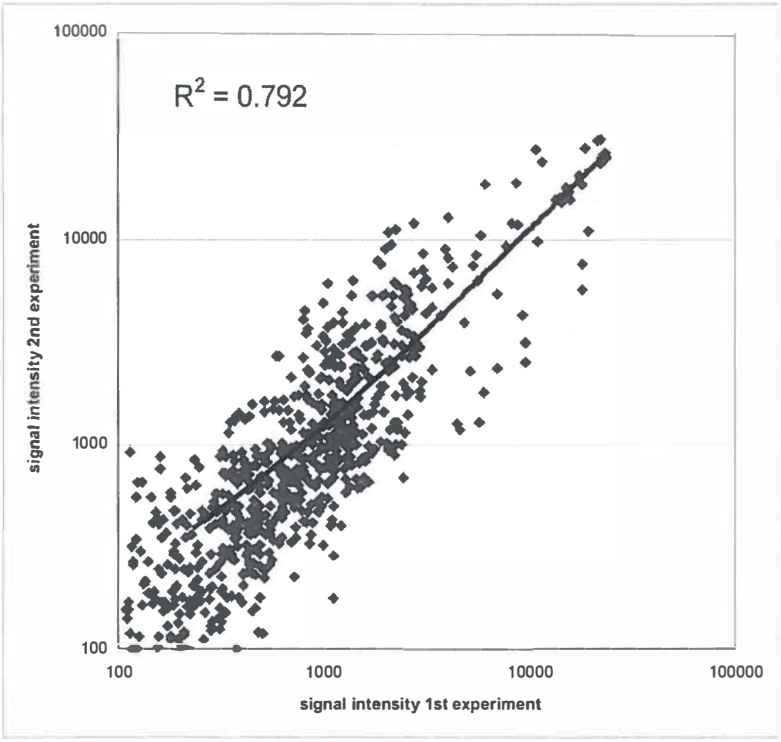
astrocytoma 4	M	101	infratentorial	1	P	Yes	97
astrocytoma 5	F	91	infratentorial	1	P	Yes	32
astrocytoma 6	M	63	infratentorial	1	P	Yes	33
astrocytoma 7	F	143	supratentorial	1	P	Yes	24

Supplementary table 2. Peptide annotation (http://cancerres.aacrjournals.org/). This table contains all the peptide sequences together with the protein of origin, the Swiss-Prot accession numbers and the gene identifiers. Furthermore, the potential upstream kinases of each peptide according to the Phospho-ELM database are listed.

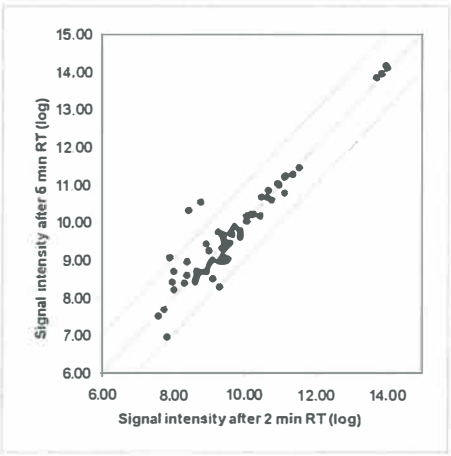
Supplementary table 3. Raw data values (http://cancerres.aacrjournals.org/).

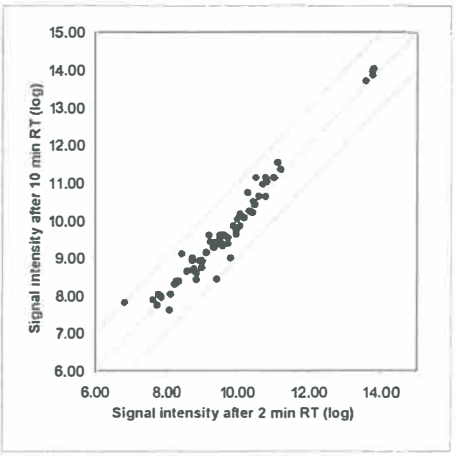
Supplementary table 4. Minimal tumor kinome (http://cancerres.aacrjournals.org/). Following the minimal kinome approach according to Diks et al. the set of substrates whose phosphorylation is shared by more than 90 percent of all pediatric brain tumor samples has been determined.

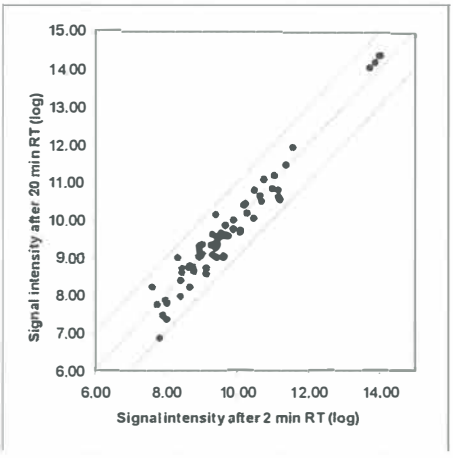
Supplementary figure 1. Reproducibility of PamChip tyrosine kinase profiling results. Regression analysis comparing kinase activity profiles of identical samples obtained on different days.

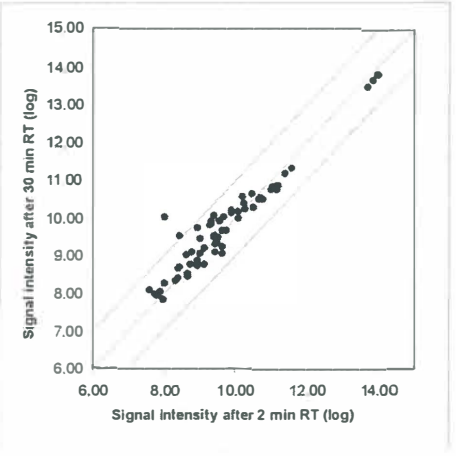

100000
10000
1000
100
R^2 = 0.792
signal intensity 2nd experiment
100
1000
10000
100000
signal intensity 1st experiment

Supplementary figure 2. Stability of tyrosine kinase activity during tissue preparation. Tumor tissue samples were collected from surgery and subjected to 2, 5, 10, 20 or 30 minutes of room temperature (RT) prior to snap freezing and tissue storage. Subsequently kinase activity was determined by performing peptide microarray profiling. Comparison of phosphorylation levels of individual peptides after increasing periods of RT shows that kinase activity is generally stable. The peptides that show a more than two-fold difference between different periods of RT do not include potential Src substrates.

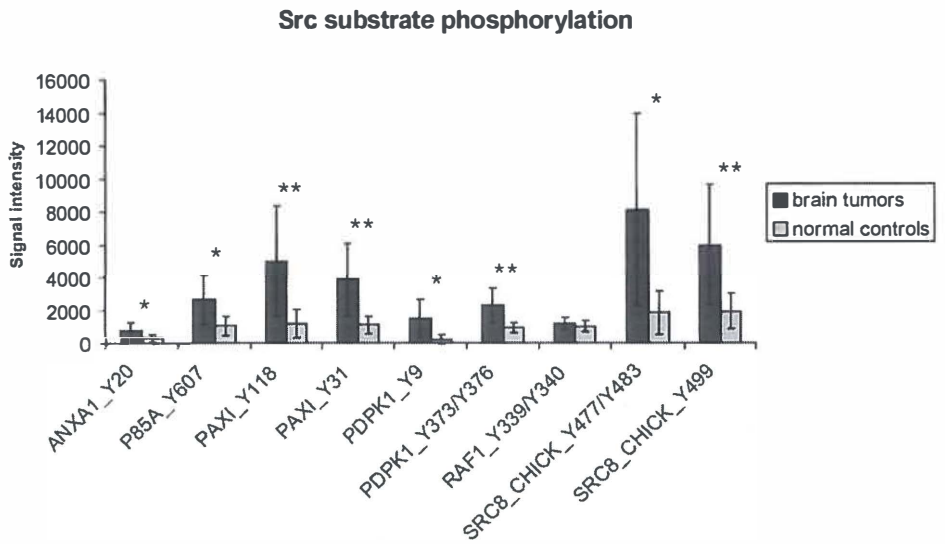


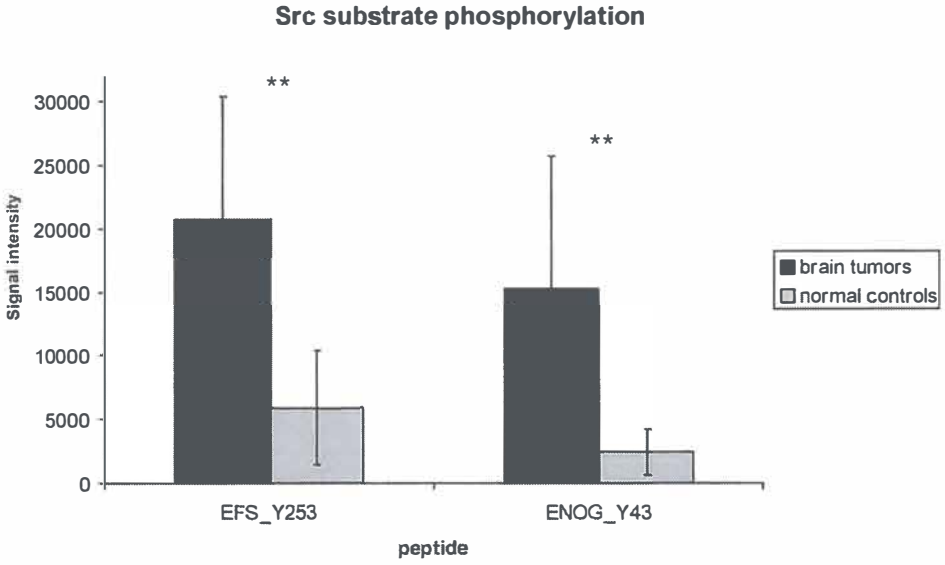




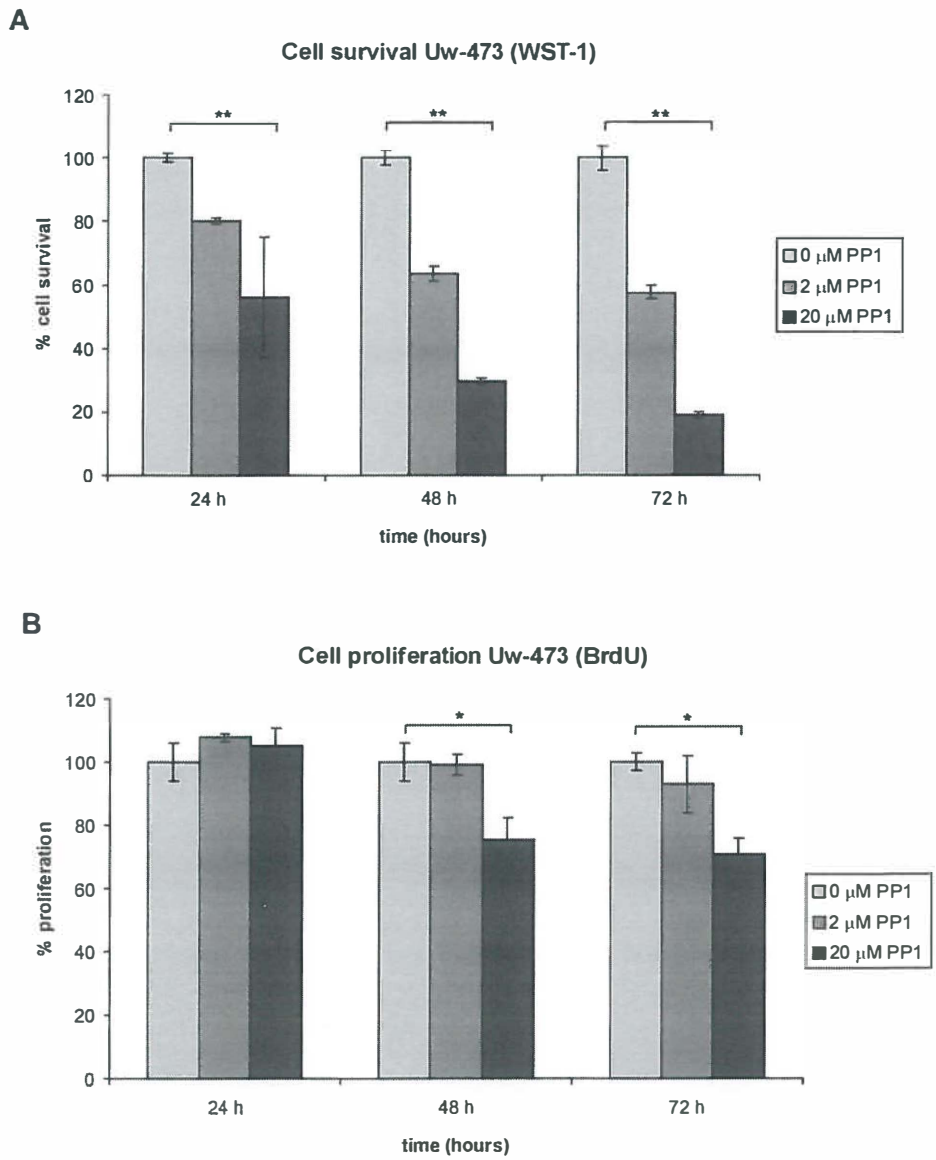


Supplementary figure 3. Histograms comparing phosphorylation levels of Src related peptides induced by pediatric brain tumor and normal control tissue lysate. The remaining Src related peptides did not reach threshold signal intensity in both groups (*P<0.05, **P<0.01).





Supplementary figure 4. Decreased cell proliferation after at least 24 hours of incubation with PP1. Uw-473 medulloblastoma cells were cultured in the presence of either 0, 2 or 20 μM PP1. Cell proliferation was assessed by BrdU incorporation. A dose dependent decrease in cell survival (A) is accompanied by a decreased cell proliferation (B) after at least 24 hours of incubation (*P<0.01, **P<0.001).



Chapter 4

VEGFR-2 signalling activity in paediatric pilocytic astrocytoma is restricted to tumour endothelial cells

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Abstract

Aims: For enhanced tumour cell survival and progression tumours depend on angiogenesis. Vascular endothelial growth factor receptor (VEGFR) signalling plays a major part in this process. Previously, we evaluated tyrosine kinase activity in paediatric brain tumour tissue lysates using a peptide microarray containing 144 different tyrosine kinase peptide substrates. When applied to paediatric pilocytic astrocytoma tissue, this analysis revealed extensive phosphorylation of VEGF receptor-derived peptides. The aim of the current study was to validate this result and determine the presence of VEGFR-2 activity in paediatric pilocytic astrocytoma as the main VEGF receptor in terms of mitogenic signalling. In addition, the localization of VEGFR1-3 mRNA expression was assessed.

Methods: VEGFR-2 phosphorylation was determined adopting a proximity ligation assay approach. Enrichment of endothelial markers and VEGF receptors in tumour endothelium was determined by qPCR analysis of laser microdissected blood vessels.

Results: Proximity ligation assays (PLA) on tumour cryosections showed the presence of phosphorylation of VEGFR-2, which primarily localized to vascular endothelium. qPCR analysis of endothelial markers and VEGF receptors showed a 13.6-fold average enrichment of VEGFR-2 expression in the laser microdissected endothelium compared to whole tumour. Also the expression of VEGFR-1 and -3 was highly enriched in the endothelium fraction with an average fold-enrichment of 16.5 and 50.8 respectively.

Conclusions: pVEGFR-2 could be detected on endothelial cells in paediatric pilocytic astrocytoma. Furthermore, endothelial cells are the main source of VEGFR1-3 mRNA expression. This suggests a crucial role for VEGF/VEGFR-induced angiogenesis in the progression and maintenance of these tumours.

Introduction

Pilocytic astrocytomas, also known as grade I astrocytomas, constitute a specific class of brain tumours exclusively occurring in children and young adults. They are characterized by slow growth and little peritumoural oedema.[1,2] Current treatment of choice comprises gross total resection, which results in a 5-year survival rate of 90%.[3-5] Nevertheless, treatment related morbidity and difficulties in treatment due to tumour localization emphasize the urge for new treatment modalities.[6] Pilocytic astrocytomas are highly different from their high grade counterpart the glioblastoma (grade IV glioma) in their growth rate.[7] Also differences in the blood vessel architecture have been reported. Conversely, the vessel maturation status and the angiogenic activity of pilocytic astrocytoma and glioblastoma show remarkable overlap.[8] This seeming discrepancy urges further study of the expression and activity of key players in tumour angiogenesis and vasculogenesis.

Cancer cells are characterized by aberrant activation of protein kinases thereby catalyzing essential processes related to tumour progression like proliferation, migration and angiogenesis.[9] Over the past decades the targeting of anomalous kinase activity has become an increasingly important anti-cancer strategy. Importantly, however, clinically successful kinase inhibitors are neither very specific with respect to actual tyrosine kinases they inhibit nor do they show a specific preference for target cancer cells over other cell types.[10] To be able to devise and select the most optimal therapy against specific tumour types, a thorough understanding of altered kinase signalling is imperative. Recently, we explored kinase activity of paediatric brain tumours by means of high throughput kinase activity assays.[11] Application to pilocytic astrocytoma tumour lysates revealed the phosphorylation of a number of vascular endothelial growth factor receptor 2 (VEGFR-2) derived peptides. The aim of the current study was to validate and localize VEGFR-2 activity in paediatric pilocytic astrocytoma.

Protein expression of VEGFRs and VEGF-family ligands has been reported previously for pilocytic astrocytoma.[7,12-14] However, the localization and extent of active VEGFR signalling activity in these tumours still remains unclear. To assess the relevance of tumour angiogenesis in the progression of these highly vascular tumours a thorough description of the angiogenic phenotype is crucial. Using proximity ligation assays we

were able to show that active VEGF-VEGFR signalling is taking place in pilocytic astrocytoma. By assessing the expression of VEGFR mRNA in laser microdissected tumour endothelium, a selectively vascular VEGFR-2 mRNA expression can be appreciated. This holds true for VEGFR-1 and -3 as well. Thus, not the actual tumour cells but the tumour vasculature is the major source of active VEGF-VEGFR-2 signalling in paediatric pilocytic astrocytoma.

Materials and methods

Tumour tissue

Pilocytic astrocytoma tumour tissue (N=17) was obtained by surgical resection (supplementary table 1). After resection the tissue was directly transferred to the pathology department, snap frozen in liquid nitrogen and stored at -80 °C.[11] Tissue material was histologically evaluated and graded according to WHO classification 2007 as grade I tumours.[1] Written informed consent and local Ethics Committee approval was granted for use of the patient material.

Tyrosine kinase activity profiling using PamChip peptide arrays

Kinase activity profiles were generated as described previously.[11] Briefly, tumour tissue lysates (N=7) were incubated with an array containing 144 unique peptide substrates in the presence of ATP and protease- and phosphatase inhibitors. Phosphorylation was visualized by measurement of the end-point fluorescent signal emitted as a consequence of fluorescein-labelled anti-phosphotyrosine antibody binding (PY20). The experiment was repeated 8 times for each tissue sample to study technical reproducibility.

Laser microdissection and quantitative PCR[15]

Frozen tumour tissue (N=11) was sectioned (9 um) onto polyethylene terephthalate (PET) membrane frame slides (Leica) followed by staining with fluorescein-labelled Ulex Europaeus Agglutinin I (UEA1, diluted 1:100, Vector Laboratories) to visualize endothelial cells. Endothelial cells from capillaries, arteries and veins were isolated by laser microdissection and collected directly into lysis buffer (RNeasy micro kit, Qiagen). Tumour cell areas lacking visible vasculature were dissected as well. All laser

microdissection work was performed using a Leica LMD6500 microdissection system (Leica Microsystems BV). Total RNA isolation and cDNA synthesis were performed as described previously.[16,17] To exclude RNA degradation the quality of the extracted RNA was assessed on the Experion capillary electrophoresis system using RNA HighSens chips (Bio-Rad Laboratories). Gene expression levels were determined by quantitative reverse transcription-polymerase chain reaction (qPCR) using Taqman assay-on-demand primer-probe sets (Applied Biosystems) for CD-31 (Hs00169777_m1), vascular endothelial (VE)-cadherin (Hs00174344_m1), CD-34 (Hs00156373_m1) VEGFR-1 (Hs01052936_m1), VEGFR-2 (Hs00176676_m1) and VEGFR-3 (Hs01047677_m1). Reverse transcription qPCR was performed according to manufacturer's instructions in an ABI PRISM 7900HT Sequence Detector (Applied Biosystems). Relative mRNA expression was determined using the delta-delta-CT method, normalizing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (Hs99999905_m1).[18] All qPCR reactions were performed in duplicate and differences between separated cell populations were statistically assessed by paired t- tests.

VEGFR-2 protein detection by immunofluorescence

Tissue cryosections (6 µm) were fixed in ice-cold methanol for 15 minutes and washed in PBS (N=12). Sections were blocked in 3% bovine serum albumin (BSA) for 1h followed by incubation with primary anti-VEGFR-2 antibody (2 µg/ml, AF357, R&D Systems) for 2h. Sections were washed in PBS and incubated with Alexa-conjugated secondary antibodies (Molecular Probes) and FITC-labelled UEA1 for 1h, followed by nuclear staining with Hoechst 33342. Sections were mounted using Fluoromount-G (Southern Biotech) and analyzed by confocal microscopy (LSM 510 META, Zeiss).

VEGFR-P in situ proximity ligation assay (PLA)

The VEGFR-P proximity ligation was conducted using the Duolink™ in situ proximity ligation assay (OLINK) according to the manufacturers instructions.[19-21] Briefly, sections (6 µm) were fixed in methanol 10 minutes on ice (N=3), and then blocked in Duolink™ block buffer for 30 minutes at 37°C. Sections were then incubated with primary antibodies at 4°C over night. Anti-VEGFR-2 antibodies (2 µg/ml, AF357, R&D) were combined with either anti-VEGFR-2-pTyr1175 (1:100 dilution, 19AA10, Cell

Signalling) or pan-pTyr (1µg/ml, 4G10, Upstate Biotechnology) antibodies. Then, sections were washed and incubated with anti-goat PLUS PLA probes combined with anti-rabbit MINUS PLA probes or anti-mouse MINUS PLA probes for 120 minutes at 37°C, followed by 15 minutes incubation in hybridization buffer. Sections were then incubated in duolink ligation buffer for 15 minutes, followed by rolling-circle amplification (RCA) detected by the use of Duolink™ detection kit 613. Endothelial cells were stained with fluorescein-labelled UEA1. As a negative control, anti-VEGFR-2 antibodies were added alone (omitting the antibodies detecting phospho-tyrosine), resulting in no detectable PLA signal (data not shown).

	A1	A2	A3	A4	A5	A6	A7
VEGFR1_Y1048	58.6	57.1	111.0	162.0	114.9	333.3	197.2
VEGFR1_Y1053/Y148F	0.0	0.0	0.0	131.6	66.8	95.1	0.0
VEGFR1_Y1053	0.0	32.7	0.0	75.8	52.3	103.3	80.6
VEGFR1_Y1169	0.0	0.0	0.0	0.0	0.0	111.0	0.0
VEGFR1_Y1213	0.0	0.0	0.0	39.2	54.5	129.9	51.7
VEGFR1_Y1242	0.0	0.0	0.0	49.3	19.8	340.0	0.0
VEGFR1_Y1333	339.7	364.9	87.2	97.5	0.0	596.5	435.7
VEGFR2_Y1054	112.9	187.7	200.5	315.6	292.7	655.2	345.5
VEGFR2_Y1059	223.1	272.3	235.1	295.0	227.5	495.2	322.0
VEGFR2_Y1175	0.0	164.3	0.0	44.2	0.0	355.7	215.5
VEGFR2_Y1214/C1208S	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VEGFR2_Y951	187.5	224.2	0.0	171.3	168.0	319.5	235.5
VEGFR2_Y996	1225.4	1367.2	575.4	582.0	330.3	1465.4	1240.8
VGFR3_Y1063/Y1068/ S1073	108.7	148.9	130.0	147.8	84.9	242.3	174.6

Table 1. Kinase activity profiling data of seven pilocytic astrocytomas showed phosphorylation of a number of VEGFR-derived peptides. Kinase activity profiles of tumour tissue lysates were generated applying Pamchip kinase activity arrays. Phosphorylation signal intensities for all VEGFR-derived peptides present on the PamChip array are displayed here. The highlighted values are considered to represent genuine phosphorylation events. Samples taken for laser capture microdissection are A1, A2, A3, A4, and A7. VEFGR2-P proximity ligation assays were performed on A1, A3 and A6.

Results

Kinase activity on VEGFR-derived peptide substrates

Pamchip tyrosine kinase arrays were applied to study kinase activity in pilocytic astrocytoma tumour tissue. The applied PamChip array contained 12-amino acid peptide substrates of all VEGFR-2 phosphorylation sites currently considered to be the most critical for downstream signalling. Pilocytic astrocytoma tissue lysate displayed kinase activity on most of these substrates (table 1). The highest phosphorylation levels were measured on the peptide containing the Y996 phosphorylation site. Furthermore, phosphorylation was observed on peptides containing the tyrosines Y951, Y1054 and Y1059. These results suggest the presence of VEGFR-2 activity in pilocytic astrocytoma tissue. In addition, phosphorylation of a number of peptides representing VEGFR-1 and VEGFR-3 phosphorylation sites could be observed, indicating activity of these kinases as well.

VEGFR-2 expression and kinase activity localize mainly to vessels

Immunohistochemical staining of VEGFR-2 total protein in pilocytic astrocytoma tissue indicated that VEGFR-2 is highly expressed on the endothelial cells lining the tumour vasculature (figure 1A). The majority of the UEA1 positive blood vessels stained positive for VEGFR-2 (60-100%). Limited VEGFR-2 total protein staining could be observed on cells that were not in close proximity of endothelium. To determine whether these receptors possess signalling activity, a novel VEGFR-P proximity ligation assay strategy was adopted. This technique enables direct observation of protein complex formation in situ, at a single molecule level.[19-21] We adopted this strategy to visualize the VEGFR-2 phosphorylation status by applying the antibody combinations VEGFR-2/pan-pTyr (figure 1B) and VEGFR-2/VEGFR-2-pTyr1175 (figure 1C). The former antibody combination elicits a signal when the VEGFR-2 protein is phosphorylated or a phosphorylated downstream signalling effector is bound to the receptor, whereas the latter antibody combination intrinsically provides the most specific information on the VEGFR-2 phosphorylation status. The results confirmed the presence of phosphorylated VEGFR-2 in the tissue sections. In addition, it revealed that phosphorylated VEGFR-2 staining was mainly localized on endothelial cells with occasionally some VEGFR-2 positivity in the peri-vascular regions. These

data indicate that VEGFR-2 is active in these tumours, and that it is primarily located at and engaged in cell signalling in the tumour endothelial compartment.

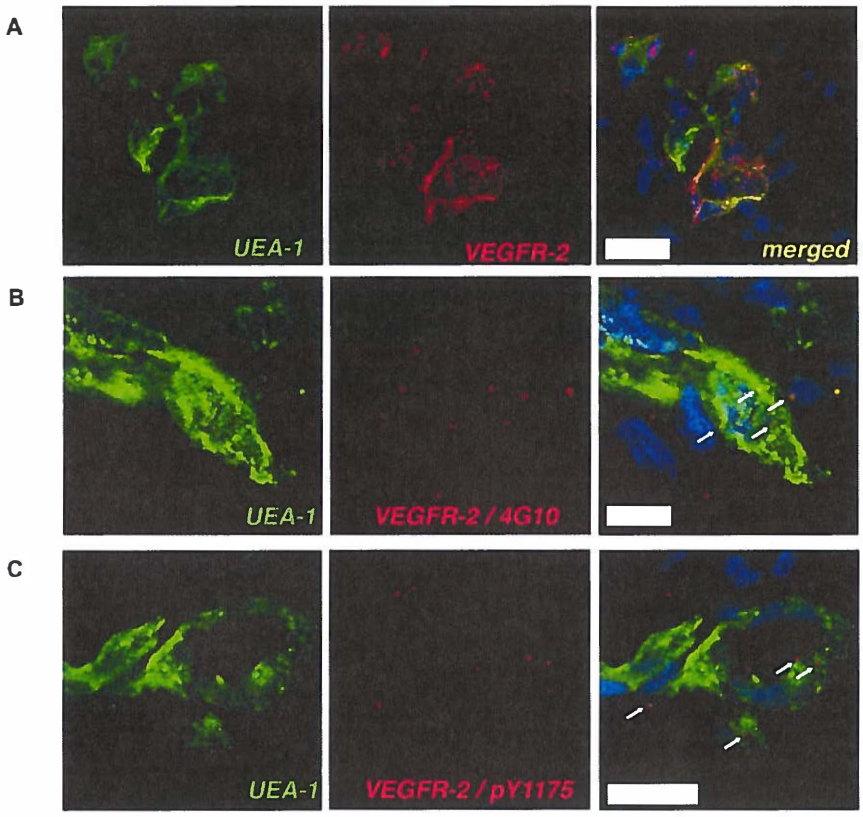


Fig. 1. Expression and phosphorylation of VEGFR-2 is primarily present on tumour endothelium. Immunofluorescent tissue stainings showing expression of VEGFR-2 (red), primarily on UEA1 positive vascular endothelium (A). Also some VEGFR-2 positivity can be appreciated outside the UEA1 stained regions. UEA1-FITC (green) staining visualizes the endothelial cells and counterstaining with DAPI (blue) was applied to visualize the cell nuclei (bar, 20 μm).

Proximity ligation assays indicated phosphorylation of VEGFR-2 in pilocytic astrocytoma tissue. The stainings (red) show phosphorylated VEGFR-2 on single molecule level. Two antibody combinations were applied: VEGFR-2/pan-pTyr (B) and VEGFR-2/VEGFR-pY1175 (C). In both cases phosphorylated VEGFR-2 (arrows) is present on the tumour endothelium. UEA1-FITC (green) staining visualizes the endothelial cells and counterstaining with DAPI (blue) was applied to visualize the cell nuclei (bars, 10 μm).

VEGFR-2, as well as R-1 and R-3, are highly and selectively expressed on vascular endothelium

Enrichment of endothelial cells in laser microdissected vasculature compared to whole tumour was validated by determining the mRNA expression of three endothelial markers CD-31, CD-34 and VE-cadherin. This indicates an average fold-enrichment of endothelial cells of 11.0-fold for CD-31, 30.4-fold for CD-34 and 15.3-fold for VE-cadherin (figure 2). The enrichment of endothelium enabled us to assess the local expression of signal transduction mediators functioning as key players in tumour angiogenesis. VEGFR-2 expression turned out to be 13.6-fold higher in endothelium compared to whole tumour (figure 3). Moreover, VEGFR-2 expression in UEA1 negative cells was lower than measured in whole tumour and for four tumours even non-detectable, indicating that the VEGFR-2 expression is almost exclusively present on the UEA1 positive tumour vasculature in pilocytic astrocytoma. Also the expression of VEGFR-1 and -3 was highly enriched in the tumour endothelium fraction with an average 16.5-fold and 50.9-fold enrichment, respectively (figure 3). Hence, these results demonstrate that VEGFR1-3 are expressed in a highly compartmentalized fashion, i.e. in the tumour endothelial cells of pilocytic astrocytoma.

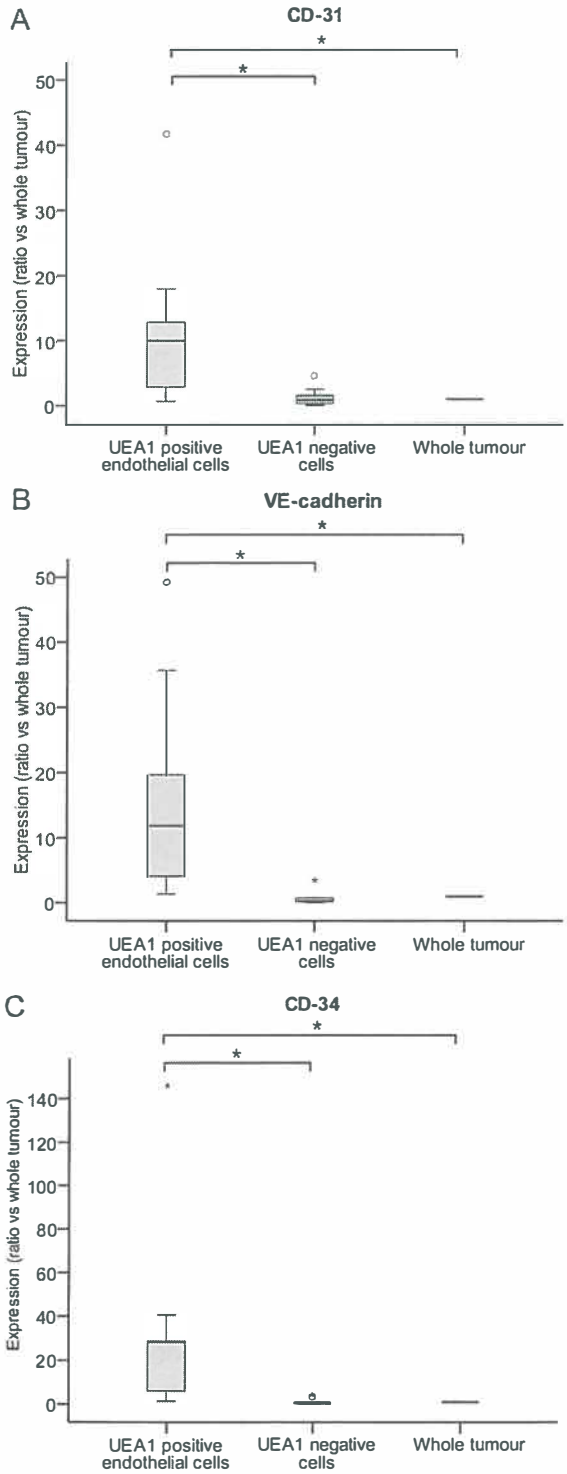
Discussion

Although total protein expression of VEGF and VEGFRs has been described previously for pilocytic astrocytoma, the presence and localization of active VEGFR signalling activity in these highly vascular tumours still remains subject of much debate.[7,13,14] In the current study, proximity ligation assays showed that phosphorylated VEGFR-2 is present in pilocytic astrocytoma and is primarily localized on the tumour endothelium. By laser microdissection we were able to separate UEA1 positive tumour endothelium from the whole tumour, rendering an average 19-fold enrichment, as calculated based on the CD-31, CD-34 and VE-cadherin expression. qPCR analysis showed a 13-fold higher mRNA expression of VEGFR-2 in the endothelium fraction compared to whole tumour. Also the expression of VEGFR-1 and -3 was highly enriched in the tumour endothelium fraction (15-fold and 50-fold enrichment, respectively). Hence, we were able to demonstrate that pVEGFR-2 is

present in a highly compartmentalized fashion, i.e. in the tumour endothelial cells of pilocytic astrocytomas. Limited pVEGFR-2 staining could be observed outside the UEA1 positive vascular regions, mainly in near proximity of highly vascular areas. Possibly this is due to VEGFR-2 signalling activity in pericytes, inflammatory cells and potentially some tumour cells. Another explanation could be that small tumour blood vessels, characterized by a tremendous heterogeneity,[22,23] do not stain positive for UEA1 and therefore do not show up as endothelial cells in this assay.

Anti-angiogenic therapies targeting VEGF/VEGFR signalling gradually become a realistic treatment option in an increasing number of malignancies. Also in recurrent high grade glioma the efficacy of anti-angiogenic therapy in combination with a cytotoxic agent has been proven to be beneficial in phase II studies.[24-27] The addition of an anti-VEGF monoclonal antibody bevacizumab resulted in markedly increased activity of the chemotherapy. There is less experience with the application of bevacizumab in children but early clinical trials indicate that it is relatively well tolerated.[28,29] Recently the response to combination of chemotherapy with bevacizumab has been assessed in a small group of patients with low grade gliomas.[30] Although this study contained only two patients with a pilocytic astrocytoma, an objective response was observed in these patients, with a relatively good tolerance.

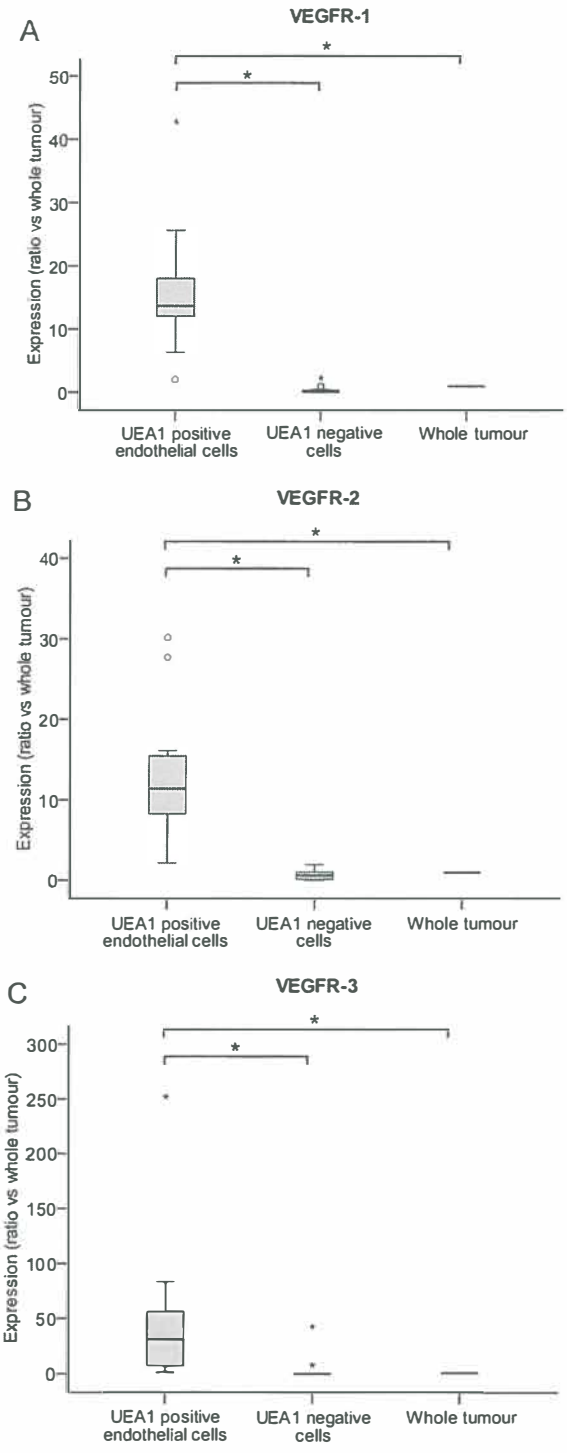
Fig. 2. Expression of endothelial markers in the laser microdissected cell populations shows enrichment of endothelium in the UEA1 positive cell fraction. Enrichment of endothelium was determined by assessing the mRNA expression of the endothelial markers CD-31 (A), VE-cadherin (B) and CD-34 (C) by qPCR. Signals were normalized for GAPDH expression. Expression is displayed as the ratio against the expression in whole tumour tissue. The data indicate an enrichment of endothelial cells of at least ten-fold in the UEA1 positive samples compared to whole tumour ($P<0.01$).


A
CD-31
Expression (ratio vs whole tumour)
50
40
30
20
10
0
UEA1 positive endothelial cells
UEA1 negative cells
Whole tumour
B
VE-cadherin
Expression (ratio vs whole tumour)
50
40
30
20
10
0
UEA1 positive endothelial cells
UEA1 negative cells
Whole tumour
C
CD-34
Expression (ratio vs whole tumour)
140
120
100
80
60
40
20
0
UEA1 positive endothelial cells
UEA1 negative cells
Whole tumour

Despite differences in turnover and vessel architecture, more specific vessel diameter, the vascular profile of pilocytic astrocytoma and glioblastoma overlap to a great extent.[8] Recent studies describe an expression profile of VEGFR-1, -2 and the ligand VEGF-A that is comparable between low and high grade astrocytomas (grade II to IV).[13] This implies a strong angiogenic signalling taking place in a paracrine as well as autocrine fashion on both tumour cells and endothelial cells. Although VEGFR-2 was initially described as an endothelial cell specific protein,[31] autocrine VEGFR signalling has been implied in tumour progression for a number of solid tumours including high grade glioma.[13,32-36] Here we show that active VEGFR-2 signalling is essentially limited to the tumour vasculature in pilocytic astrocytoma. It would be interesting to see whether these findings hold true for diffuse- or even high-grade gliomas as well. We conclude that these results provide no indication of an autocrine VEGF-VEGFR signalling in pilocytic astrocytoma tumour cells.

The results showed expression and kinase activity of VEGFR-3 as well as VEGFR-1 and -2. VEGFR-3, the receptor for VEGF-C and -D has been demonstrated to play a central role in vessel formation, lymphangiogenesis as well as tumour spread and metastasis.[37-43] A recent study by Grau et al. pointed out that expression of VEGFR-3, which is not expressed in adult normal brain endothelium, correlates with tumour grade in glioma on mRNA and protein level.[44-46] Only very moderate expression levels of VEGFR-3 were observed on grade II astrocytomas. Here, we show expression of both VEGFR-2 and VEGFR-3 on vascular endothelium of pilocytic astrocytomas. It is tempting to speculate that VEGFR-2 and -3 collaborate in mediating tumour angiogenesis by forming heterodimers, as has been observed previously.[47-49] Recently a proximity ligation approach showed VEGFR-2/-3 heterodimerization in angiogenic sprouts, thus positively regulating sprouting activity.[50] Further study should clarify the significance of VEGF-C-VEGFR-3 signalling and presence of VEGFR-2 and -3 interplay in pilocytic astrocytoma angiogenesis.

Fig. 3. Enriched VEGFR expression in laser microdissected tumour endothelium versus whole tumour. Enrichment of endothelium as indicated by the enriched endothelial markers (fig 2) is accompanied by an enrichment of VEGFR-1 (A), -2 (B), and -3 (C) mRNA expression ($P<0.01$). Signals were normalized for GAPDH expression. Expression is displayed as the ratio against the expression in whole tumour tissue. This suggests a selective expression of VEGFR in the tumour endothelial cells.


A
VEGFR-1
Expression (ratio vs whole tumour)
50
40
30
20
10
0
*
*
UEA1 positive endothelial cells
UEA1 negative cells
Whole tumour
B
VEGFR-2
Expression (ratio vs whole tumour)
40
30
20
10
0
*
*
UEA1 positive endothelial cells
UEA1 negative cells
Whole tumour
C
VEGFR-3
Expression (ratio vs whole tumour)
300
250
200
150
100
50
0
*
*
UEA1 positive endothelial cells
UEA1 negative cells
Whole tumour

Laser microdissection allows for specific assessment of the molecular status of tumour endothelial cells without interference from their physiological environment. As already posed by Langenkamp et al., assessment of local expression profiles of signal transduction mediators critical for tumour angiogenesis was hitherto not possible, but now has come within reach.[23] Laser microdissection (LMD) procedures have already been applied successfully to dissect tumour tissue from tumour-enriched tissue specimens in order to assess the protein phosphorylation status using reverse phase protein microarray technology.[51] However, so far successful application of LMD to study enzymatic activity in subsets of cells within a tumour cell population has not been shown yet. Yet, rapid technological advancement likely will enable us to study kinase activity in dissected endothelium as well as in other cell populations in the near future. This will increase our understanding of the molecular basis of tumour vascular heterogeneity as well as of tumour endothelial responsiveness to anti-angiogenic therapies.[52]

High throughput kinase activity measurement techniques, such as array based kinase activity profiling, enable measurement of kinase activity on hundreds of different substrates at once. The presence of peptides derived from multiple phosphorylation sites allows for phospho-site-specific measurement of kinase activity. Kinase activity profiling results indicated the presence of kinase activity on a number of VEGFR-2 derived peptides containing key phosphorylation sites for downstream signalling. Phosphorylation was observed on tyrosine residues Y951 and Y996 which are known to mediate cell migration and vascular permeability through phospholipase C gamma (PLC-γ) and TSAd as important downstream signalling effectors.[53] Furthermore, we observed phosphorylation of peptides containing tyrosines known to increase downstream signalling activity of VEGFR-2 upon phosphorylation (Y1054, Y1059).[54,55] This indication for active VEGFR-2 signalling taking place in paediatric pilocytic astrocytomas could be confirmed in the current study.

Response to treatment of brain tumours is generally assessed by magnetic resonance imaging (MRI). Increasing evidence suggests that anti-angiogenic therapies, at least in part, result in a pseudo response due to rapid loss of contrast in MRI imaging.[56] Thus, alternatives are warranted to facilitate a reliable assessment of the angiogenic status of brain tumours. High throughput measurement of enzymatic activity of key players in angiogenesis induction, like VEGFR-2, could facilitate a rapid assessment of the

angiogenic profile. Furthermore, new targeted imaging techniques might be helpful in the near future. Application of radiolabelled Bevacizumab PET in ovarian cancer xenograft studies already underscored the applicability of angiogenesis imaging using VEGF as a target.[57,58] Conceivably, this opens up possibilities to predict the potential biological treatment response, for instance of anti-angiogenesis targeted therapies in a clinical setting.

In conclusion, this report shows the presence of phosphorylated VEGFR-2 in pilocytic astrocytoma. Furthermore, evidence was presented for a selective expression and activity of VEGFRs in the (peri-)vascular regions, indicative for an active VEGFR signalling taking place in the tumour endothelium of paediatric pilocytic astrocytoma. Taken together, these results justify further (pre-)clinical assessment of the therapeutic potential of anti-angiogenic therapies aimed at VEGF/VEGFR signalling for treatment of this malignancy.

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Supplementary table 1. Patient and tumour characteristics

tumour	sex	age at diagnosis (months)	tumor localization
1	f	225	infratentorial
2	m	72	infratentorial
3	m	81	infratentorial
4	f	160	infratentorial
5	f	94	infratentorial
6	m	85	optic chiasm
7	m	89	infratentorial
8	f	75	infratentorial
9	m	47	third ventricle
10	f	69	infratentorial
11	f	135	infratentorial
12	m	108	optic chiasm / third ventricle
13	m	101	infratentorial
14	f	91	infratentorial
15	m	63	infratentorial
16	f	143	optic chiasm
17	m	81	infratentorial

Chapter 5

EphB2 activity plays a pivotal role in pediatric medulloblastoma cell adhesion and invasion

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In revision for Neuro-Oncology

Abstract

Eph/Ephrin signaling has been implicated in various types of cancer enhancing key processes like migration, proliferation and angiogenesis. In medulloblastoma invading tumor cells characteristically lead to early recurrence and a decreased prognosis. Based on kinase activity profiling data published recently, we hypothesized on a key function of the Eph/Ephrin signaling system in medulloblastoma invasion.

In primary medulloblastoma samples a significantly higher expression of EphB2 and the ligand Ephrin-B1 was observed compared with normal cerebellum. Furthermore, medulloblastoma cell lines showed high expression of EphA2, EphB2 and EphB4. Stimulation of medulloblastoma cells with Ephrin-B1 resulted in a marked decrease in *in vitro* cell adhesion and an increase in the invasion capacity of cells expressing high levels of EphB2. The cell lines which showed an Ephrin-B1 induced phenotype possessed increased levels of phosphorylated EphB2 and, to a lesser extent, EphB4 after stimulation. Knockdown of EphB2 expression by shRNA completely abolished Ephrin ligand induced effects on adhesion and migration. Analysis of downstream signal transduction identified Akt, Erk, STAT5, Paxillin and p27 as downstream signaling mediators potentially inducing the Ephrin-B1 phenotype.

Culturing of cell lines in the presence of demethylating agents resulted in an increased EphB receptor expression. Promoter methylation levels of EphB receptors, however, did not show an increased Eph promoter methylation, indicating that epigenetics plays an indirect role in the regulation of Eph expression.

In conclusion, the observed deregulation of the Eph/Ephrin expression in medulloblastoma enhances the invasive phenotype, suggesting a potential role in local tumor cell invasion and the formation of metastases.

Introduction

The Eph receptors constitute the largest family of receptor tyrosine kinases identified so far. Receptor binding of either glycosylphosphatidylinositol (GPI)-linked or transmembrane Ephrin ligands results in signaling which can be bi-directional at sites of cell-cell contact.[1] Eph receptor forward signaling is known to be essential for neural development, mediating processes like cytoskeleton dynamics, guided migration, cell proliferation and angiogenesis.[1-5] Ephrins can also transduce a reverse signal into the cell. This PDZ-dependent reverse signaling has recently been implicated in neural development as well by mediating axon guidance.[6]

Eph receptor and Ephrin expression has been observed in various benign human tissues.[7] The role of Eph/Ephrin signaling from a tumor perspective can be divided in effects on the tumor stroma or by direct effects on the tumor cells through induction of migration or proliferation. A key role for reverse signaling downstream of Ephrin-B2 in tumor angiogenesis has recently been established. Upon binding of EphB, Ephrin-B2 directly activates VEGFR2 and VEGFR3 resulting in angiogenesis even in the absence of VEGF.[8-10] Furthermore, Eph/Ephrin forward, as well as reverse signaling have been implicated in a number of malignancies including glioma, enhancing key cancer promoting processes like tumor cell migration and proliferation.[11-15]

The complexity of the Eph/Ephrin signaling is intriguing since both tumor promoting as well as suppressing effects of Eph/Ephrin signaling on tumor growth have been reported.[16-20] This seeming discrepancy likely depends on the relative expression signature of the Eph/Ephrin family members.[21] Furthermore, strict selectivity in the activation of downstream signaling pathways has tremendous influence on the phenotype.[22] Recently generated kinase activity profiles of primary pediatric brain tumor tissue suggested the presence of Eph receptor kinase activity in medulloblastoma.[23] In the current study we aimed to determine whether aberrant Eph/Ephrin signaling plays a role in medulloblastoma progression from a tumor cell perspective.

Medulloblastoma comprises the most frequent malignant brain tumor occurring during childhood. Local recurrence due to tumor cell migration and invasion is a characteristic feature of medulloblastoma. Furthermore, it exhibits the propensity for leptomeningeal dissemination and local spread.[24-26] Up until now the potential role of

Eph/Ephrin signaling in relation to medulloblastoma tumor cell behavior is unknown. Here, we demonstrated overexpression of EphB2 and its corresponding ligand Ephrin-B1 in medulloblastoma. Stimulation with Ephrin-B1 ligand resulted in a marked decrease in *in vitro* cell adhesion and an increase in invasion of medulloblastoma cell lines expressing high levels of EphB2. This implicates a selective expression of Eph/Ephrin family genes in favor of tumor cell migration. It is tempting to speculate that the observed deregulation of the Eph/Ephrin expression and function may underlie the invasive character of medulloblastoma.

Materials and Methods

Patient samples and cell lines

All tissue was obtained by surgical resection. Tissue material was histologically evaluated and graded according to WHO classification 2007.[27] Written informed consent and local Ethics Committee approval was granted for use of the patient material.

The following medulloblastoma cell lines were included in the study: DAOY, Res-300, Res-256, Uw-426, Uw-473 and Uw-402. DAOY was derived from the American Type Culture Collection (LGC Standards, Wesel, Germany). The other medulloblastoma cell lines were characterized (immunohistochemistry) and made available by Dr. Michael S. Bobola (Seattle Children's Hospital Research Institute).[28]

All cell lines were cultured in DMEM-F12 (Invitrogen, Breda, the Netherlands) supplemented with 5% FBS. All cell cultures contained 100 U/mL penicillin and 100 µg/mL streptomycin (PAA Laboratories, Pasching, Austria).

qRT-PCR

Eph/Ephrin expression levels were determined in 11 primary medulloblastoma samples and in 6 medulloblastoma cell lines. RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Venlo, the Netherlands) and cDNA was prepared as described previously.[29] qRT-PCRs (quantitative reverse transcriptase polymerase chain reactions) were performed on the Bio-rad MyiQ Thermal Cycler platform using SYBR Green I for DNA visualization (Bio-rad, Veenendaal, the Netherlands). Custom primers were designed for all currently identified Eph receptors and Ephrin-B ligands

(Supplementary Table 1; Invitrogen, Breda, the Netherlands). The mRNA expression levels were quantified relative to the expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase using the ΔΔCt method.
Expression in medulloblastoma was compared with expression levels in normal cerebellum tissue (N=4). Expression in cell lines was compared with commercially available pooled mRNA samples of adult cerebellum (#636535; Clontech, Mountain View, CA, USA) and fetal brain (#636106; Clontech, Mountain View, CA, USA). Expression differences were statistically assessed by two-tailed Student's t-tests.

Antibody arrays

Changes in cell signaling were assessed by performing Proteome Profiler antibody arrays (Catalog Number ARY001 and ARY003; R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. ARY001 contains antibody probes for a panel of receptor tyrosine kinases, including most of the Eph receptors. ARY003 was applied to identify changes in phosphorylation of downstream signaling mediators. After protein binding phosphorylation was visualized using a pan-pTyr antibody. Scanned images were processed using Image J software (version 1.41) followed by relative quantification of signal intensities using ScanAlyze array analysis software (***Eisen*** Lab, Stanford University, Stanford, CA, USA).

Cell adhesion assay

Culturing dishes were coated with laminin, or collagen for two hours at 37°C. Cell lines were serum starved for 4 hours in medium containing 0.5% FCS. After trypsinization the cells were treated with 2 μg/mL Recombinant Mouse Ephrin-B1 Fc Chimera (R&D Sytems, Minneapolis, MN, USA), 2 μg/mL Recombinant Mouse Ephrin-A1 Fc Chimera (R&D Sytems, Minneapolis, MN, USA) or 2 μg/mL ChromPure Mouse IgG, Fc-Fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and plated on the coated wells in quadruplicate. After 30 minutes at 37°C the wells were washed and the adhered cells were fixed with methanol followed by cell staining with crystal violet (1%). Cell adhesion was quantified by cell counting at high power fields (2 fields per well) and statistically assessed by two-tailed Student's t-tests.

Cell invasion assay

Cell invasion assays were based on the Boyden's chamber technique described previously.[30] Transwell inserts (8 µm pore size; Corning Life Sciences, Amsterdam, the Netherlands) were coated with collagen (10 µg/cm^2 in 50 µL) for 2 hours at 37°C. The transwell inserts were adjusted to cell culture medium for 30 minutes prior to cell seeding. Cells were serum starved (0.5% FCS) for 4 hours. After trypsinization the cells were treated with 2 µg/mL Recombinant Mouse Ephrin-B1 Fc Chimera (R&D Sytems, Minneapolis, MN, USA), 2 µg/mL Recombinant Mouse Ephrin-A1 Fc Chimera (R&D Sytems, Minneapolis, MN, USA) or 2 µg/mL ChromPure Mouse IgG, Fc Fragment (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 15 minutes prior to plating in the top compartments of the transwell system. The lower compartments contained 5% FCS to create a gradient. After 2.5 hours the cells in the upper compartment were removed with cotton swabs and the invaded cells were fixed in methanol followed by staining with crystal violet (1%). Cell invasion was quantified by cell counting at high power fields (2 fields per well) and statistically assessed by two-tailed Student's t-tests.

Cell proliferation assay

Proliferation was assessed performing Bromodeoxyuridine (BrdU) proliferation assays (QIA58) according to manufacturer's instructions (Merck Chemicals, Darmstadt, Germany).

DNA bisulfite treatment and methylation analysis

DNA was modified with sodium bisulfite. In summary, 2 µg of DNA were used for bisulfite treatment. DNA was denatured in 0.2N NaOH at 37 °C for 10 min and incubated with 3M sodium bisulfite at 50 °C for 16 h, purified with the Wizard cleanup system (Promega, Madison, WI, USA) and desulfonated with 0.3N NaOH at 25 °C for 5 min, then DNA was precipitated with ammonium acetate and ethanol, washed with 70% ethanol, dried, and re-suspended in H_2O. For the analysis of DNA methylation, bisulfite pyrosequencing was used. For pyrosequencing, we performed polymerase chain reactions. The degree of methylation was calculated using the PSQ HS 96A 1.2 software (Biotage AB, Uppsala, Sweden). The methylation status of EphB1-4, EphA2, EphA4-7 and EphA10 in medulloblastoma (N=15) was compared to

the status in normal cerebellum control tissue (N=4). Primer sequences for bisulfite polymerase chain reaction and pyrosequencing are shown in supplementary table 1. DNA quality was assessed by performing a multiplex PCR according to the BIOMED-2 protocol. Only DNA samples with PCR products of at least 600 bp in size were included in this study.[31]

5-Aza-2'-deoxycytidine demethylation treatment

Medulloblastoma cell lines were cultured in the presence of 5 μM 5-Aza-2'-deoxycytidine during 72 hours. Changes in EphB receptor mRNA expression were assessed by qRT-PCR as described earlier.

EphB2 gene expression knockdown

Early passage DAOY, Res-256 and Uw-402 cells were stably transfected with pLKO.1 Mission shRNA vectors (Sigma-Aldrich, Zwijndrecht, the Netherlands) against EphB2 using Fugene HD Transfection Reagent (Roche, Woerden, the Netherlands) according to the manufacturer's instructions. Transfected cells were selected and maintained in culture medium containing 1.0 μg/mL puromycin. pLKO control vector 1 was used as a vector control. Knockdown was validated by measuring EphB2 mRNA levels by means of qRT-PCR.

Results

Eph receptor and Ephrin ligand expression

Previously generated kinase activity profiling data of pediatric brain tumors indicated high kinase activity on Eph receptor-derived peptides (Supplementary Fig. S1). Although the phosphorylated Eph peptides were derived from specific EphA and EphB receptors, high homology in the primary amino-acid sequence of the Eph receptor phosphorylation sites could indicate activity of other EphA and EphB receptors as well. This urged us to address the relevance of the various members of the Eph/Ephrin protein family in an unbiased approach. To assess whether aberrant Eph receptor signaling potentially plays a role in medulloblastoma cell behavior we assessed the mRNA expression of Eph/Ephrin family genes in medulloblastoma primary tissue samples and compared it to expression levels in normal cerebellum

(Fig. 1A). Expression of all Eph receptors and Ephrin-B ligands was observed. A significantly higher expression was observed for the B-type Eph receptor EphB2 and one of its corresponding ligands, Ephrin-B1 ($P<0.05$) (Fig. 1B). The A–type Eph receptors EphA3 and -A8 were significantly higher expressed in medulloblastoma as well. No significant differences between normal cerebellum and medulloblastoma tissue were measured for the other receptors and ligands.

Expression levels in medulloblastoma cell lines were compared with the levels in normal cerebellum and fetal whole brain, indicating an enhanced expression of EphA2, EphB2 and EphB4 in most cell lines (Fig. 1C). Interestingly, in correspondence with the expression data of the medulloblastoma tissue a number of cell lines demonstrate a consistent overexpression of EphB2. Furthermore, multiple cell lines displayed a decreased expression of EphA3 through -A8, EphB1, -B3, and -B6.

Functional effects of Eph receptor stimulation on adhesion and invasion

Based on the Eph/Ephrin mRNA expression results we focused on dissecting the functional role of EphB2 forward signaling. To determine the relative importance of the EphB and EphA receptor families, 3 medulloblastoma cell lines with highly differential EphB2 receptor expression levels were stimulated with Fc-conjugated Ephrin-A1 or Ephrin-B1 upon which the invasion and adhesion capacity was assessed.

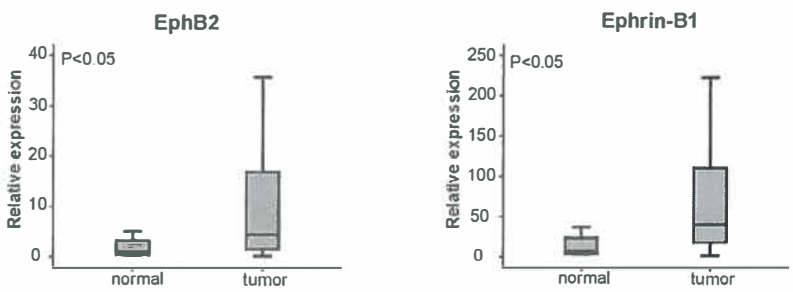
Figure 1. Significantly increased mRNA expression of EphB2 and Ephrin-B1 in a cohort of primary pediatric medulloblastomas. Relative mRNA expression levels of Eph receptors and Ephrin-B ligands was determined in a panel of 11 primary medulloblastomas (Mbl) and 4 normal cerebellum samples (NC) (A). Significantly higher expression of EphB2 and Ephrin-B1 could be appeciated in the tumor tissue compared to the normal control cerebelli (B). Overexpression of EphB2 was also present in a number of medulloblastoma cell lines (C) Here, expression levels of Eph receptors and Ephrin-B ligands were compared with a pooled adult normal cerebellum sample (AC) and a pooled fetal normal brain sample (FB). For each gene the samples with the highest expression have been highlighted.

A

	EphA receptors								EphB receptors					EphrinB ligands		
	EphA1	EphA2	EphA3	EphA4	EphA5	EphA6	EphA7	EphA8	EphB1	EphB2	EphB3	EphB4	EphB6	EfnB1	EfnB2	EfnB3
NC1	0.44	3.05	0.58	0.66	0.47	0.14	1.00	8.55	1.44	5.01	21.49	11.44	0.15	36.02	0.19	1.97
NC2	1.15	0.48	0.19	0.72	0.43	0.21	0.52	0.63	1.54	0.28	45.91	1.18	1.39	3.47	0.14	0.64
NC3	0.24	0.69	0.26	4.91	1.15	1.58	0.69	1.22	3.93	0.45	84.08	1.68	0.12	2.59	0.21	0.08
NC4	3.67	5.66	0.46	0.53	2.62	1.50	1.79	2.42	2.31	1.33	72.29	2.26	2.17	10.06	ND	1.10
Mbl1	0.43	0.96	1.87	0.37	ND	0.31	0.80	ND	0.48	1.51	0.73	25.74	0.19	18.29	0.12	1.07
Mbl2	0.96	0.67	0.45	0.07	0.18	ND	0.18	0.74	ND	1.37	4.00	24.86	0.45	22.02	ND	2.80
Mbl3	2.74	11.09	9.19	0.79	ND	0.14	0.84	53.68	0.43	1.59	9.69	1.02	1.77	9.79	ND	0.44
Mbl4	0.81	0.82	1.28	ND	2.43	0.33	1.51	60.58	2.38	4.38	48.70	1.70	0.73	89.79	ND	2.33
Mbl5	19.04	45.91	10.13	2.76	4.16	0.26	3.33	209.50	0.54	24.01	125.87	8.03	5.66	156.58	ND	9.65
Mbl6	2.31	13.05	0.10	5.17	0.08	0.50	0.17	83.62	1.93	18.26	34.08	1.01	1.98	65.84	0.13	13.14
Mbl7	1.27	1.52	3.17	3.47	1.32	19.92	0.16	87.17	2.78	35.85	85.38	0.70	1.47	222.21	0.14	2.96
Mbl8	3.89	0.67	0.90	5.30	0.25	0.11	0.19	7.92	0.12	4.50	16.35	0.30	0.66	18.78	0.03	0.65
Mbl9	3.32	0.89	0.06	4.96	0.01	0.13	0.09	3.23	0.02	0.13	0.87	0.06	0.47	1.32	ND	0.05
Mbl10	2.32	0.69	9.69	3.68	0.02	0.09	0.13	16.01	0.21	3.61	22.48	0.35	1.15	39.42	0.03	0.49
Mbl11	1.05	18.01	3.88	1.44	0.66	4.49	1.13	62.50	0.68	15.25	184.28	4.26	8.23	119.49	0.34	39.97

ND = Non-detectable

B



C

	EphA receptors								EphB receptors					EphrinB ligands		
	EphA1	EphA2	EphA3	EphA4	EphA5	EphA6	EphA7	EphA8	EphB1	EphB2	EphB3	EphB4	EphB6	EfbB1	EfnB2	EfnB3
DAOY	0.21	11.13	1.19	0.31	0.22	ND	0.01	0.02	0.77	4.11	0.42	11.01	0.08	35.53	9.29	13.74
Res-300	0.15	93.10	5.43	0.16	0.32	ND	ND	ND	0.01	3.23	0.43	5.23	ND	75.82	4.46	0.45
Res-256	0.14	50.24	0.01	0.07	0.05	ND	ND	0.01	0.00	0.42	0.04	5.00	0.01	16.40	0.46	0.67
Uw-426	0.11	65.38	6.05	0.21	1.67	ND	ND	ND	0.24	1.62	0.14	7.73	ND	148.65	3.57	0.03
Uw-473	0.10	44.66	0.00	0.13	0.00	ND	ND	0.01	0.01	0.33	0.09	5.92	0.04	29.06	0.36	7.55
Uw-402	0.22	44.97	7.84	0.23	4.79	ND	ND	ND	0.48	2.17	0.04	6.73	ND	186.68	6.82	0.03
AC	0.69	1.82	0.91	2.04	1.34	1.78	0.39	0.53	22.80	0.24	2.59	0.56	0.20	11.01	5.52	1.92
FB	0.27	2.62	16.44	2.34	13.58	1.32	0.24	0.58	13.42	2.14	0.51	2.01	2.19	113.83	25.85	34.92

ND = Non-detectable

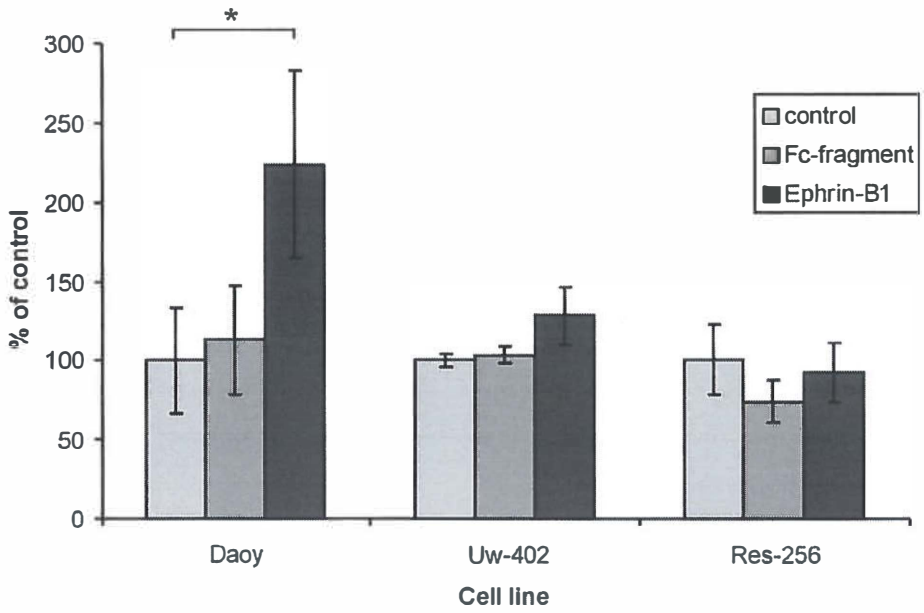


Figure 2. Increased invasion capacity of DAOY medulloblastoma cells upon stimulation with Ephrin-B1. In a trans-well migration assay, stimulation of DAOY medulloblastoma cells with Ephrin-B1 (2 μg/mL) resulted in a marked increase of the invasion capacity of the cells compared to untreated and Fc-fragment treated control cells ($P<0.05$). The cell lines Uw-402 and Res-256 did not show an altered invasion rate.

We selected DAOY, Uw-402 and Res-256 which possess high, intermediate and low EphB2 expression levels, respectively. Stimulation with Ephrin-A1 did not result in an observable phenotype. Ephrin-B1 stimulation however, showed effects on cell adhesion and invasion that correspond with the relative EphB2 mRNA expression levels in the different cell lines ($P<0.05$). Stimulation with Ephrin-B1 markedly increased the invasive capacity of DAOY and, to a lesser extent, Uw-402 in concentrations as low as 0.2 μg/mL whereas no effects were observed on Res-256 (Fig. 2). The effect of Ephrin-B1 on medulloblastoma cell adhesion was assessed on collagen and laminin coated culture dishes. Again, no effects could be observed on

Res-256 while the adhesive capacity of DAOY and Uw-402 decreased substantially (Fig. 3).

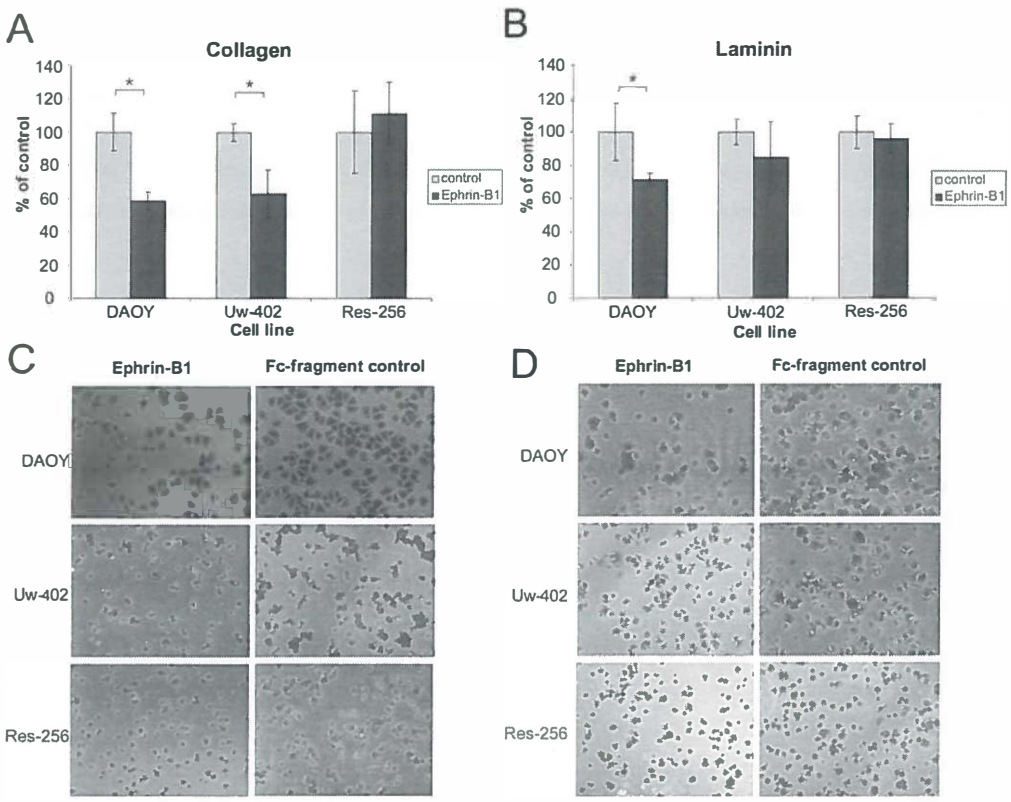


Figure 3. Decreased cell adhesion of the medulloblastoma cell lines DAOY and Uw-402 upon stimulation with Ephrin-B1. Cell adhesion was assessed on a collagen (A, C) and laminin (B, D) matrix. Stimulation of DAOY cells with Ephrin-B1 resulted in a sharp decrease in adhesion compared to Fc-fragment control and untreated cells (data not shown) on both collagen as well as laminin ($P<0.05$). Uw-402 showed the same phenotype on collagen. Res-256, a cell line with low EphB2 expression and phosphorylation, did not show altered cell adhesion upon stimulation with Ephrin-B1.

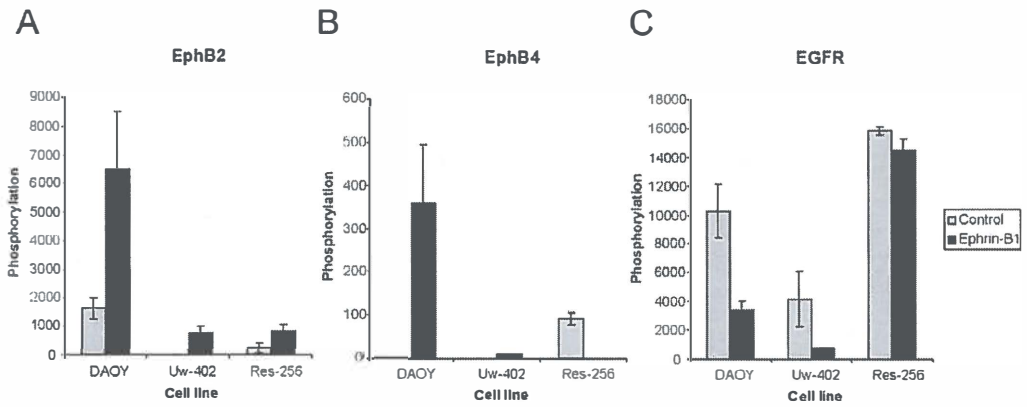


Figure 4. Increased phosphorylation of EphB2 and EphB4 upon stimulation with Ephrin-B1 receptors. Medulloblastoma cell lines were stimulated with Ephrin-B1 or Fc-fragment control for 15 minutes. Subsequently, the phosphorylation of 42 different human receptor tyrosine kinases was assessed applying antibody array screening (Supplementary Table 2). Stimulation resulted in a marked increase in EphB2 (A) and EphB4 (B) phosphorylation in DAOY and Uw-402. No increase in EphB2 and EphB4 phosphorylation could be observed in Res-256, which also possessed only minor mRNA expression of these receptors. The highest basal EphB2 phosphorylation was measured for DAOY, whereas Uw-402 and Res-256 did not show any EphB2 phosphorylation in unstimulated conditions. Strikingly, the change in phosphorylation of EphB2 and EphB4 as observed for DAOY and Uw-402 was accompanied by a marked decrease in EGFR phosphorylation (C). For Res-256, which showed only minor change in Eph receptor phosphorylation upon stimulation with Ephrin-B1, no effects on the EGFR phosphorylation could be observed.

In Bromodeoxyuridine incorporation assays, no direct effect on cell proliferation was measured upon stimulation with either Ephrin-A1 or Ephrin-B1 (data not shown).

Effects of Ephrin-B1 stimulation on receptor tyrosine kinase phosphorylation levels

We assessed the phosphorylation levels of the Eph receptors in the selected cell lines at basal levels and upon stimulation (Fig. 4A and B, Supplementary Table 2). Under basal conditions phosphorylated EphB2 could only be detected in DAOY and in Res-256 at very low amounts. Low levels of phospho-EphB4 could be detected in Res-256. Stimulation of the cells with Ephrin-B1 rendered a substantial increase of

phosphorylated EphB2 and EphB4 in DAOY. To a lesser extent Uw-402 also showed an increased phosphorylation of these receptors, as well as EphA1 and EphA2 upon stimulation. In correspondence with the low mRNA expression of EphB2 and -B4, stimulation of Res-256 did not result in increased phosphorylation of these receptors. Intriguingly, the observed increase in Eph receptor phosphorylation upon stimulation with Ephrin-B1 ligand, as observed for DAOY and Uw-402, was accompanied by a sharp decrease in EGFR phosphorylation (Fig. 4C).

Knockdown of EphB2 abolishes functional effects of Ephrin-B1

To validate the functional relevance of EphB2 in medulloblastoma, DAOY cells were stably transfected with shRNA against EphB2, resulting in a marked knockdown of the mRNA expression of this receptor (Fig. 5A). This knockdown completely abolished the Ephrin-B1 induced effects on cell adhesion and invasion (Fig. 5B, C). Furthermore, the basal adhesive capacity of EphB2 shRNA transfected DAOY cells decreased substantially (data not shown). These results show that activation of EphB2 receptor essentially drives the Ephrin-B1 induced invasive phenotype we observed in the DAOY medulloblastoma cell line.

Effects of Ephrin-B1 stimulation on downstream cell signaling in DAOY and EphB2 shRNA transfected DAOY

By means of phospho-proteome profiler antibody arrays insight in altered downstream signal transduction upon stimulation with Ephrin-B1 (15 minutes) and/or EphB2 knockdown was generated (Fig. 6; Supplementary Table 3). We observed multiple changes in the downstream signaling phospho-proteome, where a decreased activity of Erk, Hck and mTOR and an increased activity of Stat5, Stat2, p38, MSK1/2, Paxillin and p27 are most pronounced. This indicates that multiple prominent cell signaling pathways are affected by altered EphB2 activity. Changes of members of the PI3K-Akt-mTOR pathway as well as the Ras-Raf-MEK-Erk pathway were observed. Furthermore, altered activity of renowned mediators of cell migration like p27 and paxillin is present, providing a cell biological explanation for the observed pro-migratory phenotype of DAOY.

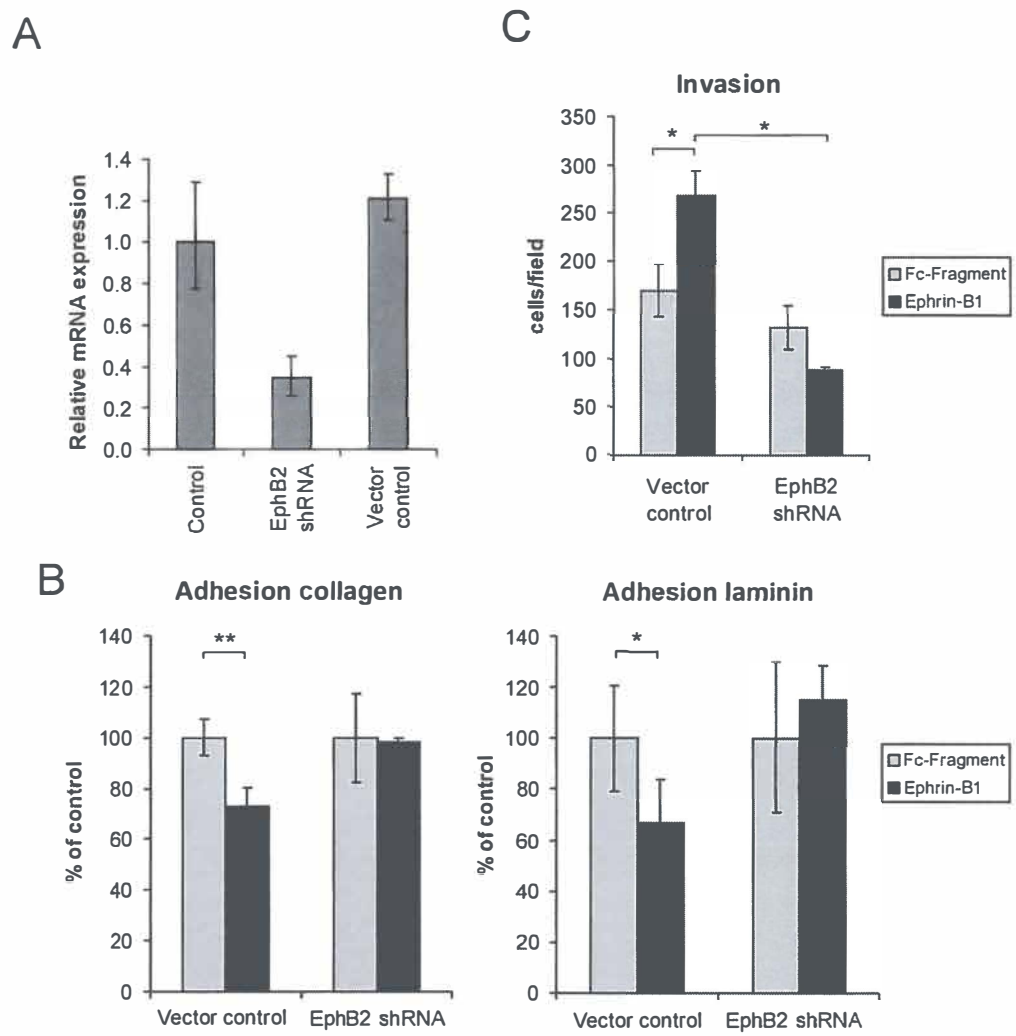


Figure 5. Knockdown of EphB2 expression abolishes the Ephrin-B1 induced effects on DAOY cell adhesion and invasion capacity. DAOY medulloblastoma cells were stably transfected with either shRNA against EphB2 or shRNA empty vector control (A). The decrease in EphB2 expression abolished the Ephrin-B1 induced decrease of cell adhesion on a collagen as well as a laminin matrix whereas the effect of receptor stimulation remained present in cells transfected with the shRNA control vector (B; * $P<0.03$; ** $P<0.01$). The invasive capacity of the medulloblastoma cells was assessed by means of transwell invasion assays (C). Here, the increased invasion upon Ephrin-B1 stimulation could not be observed anymore after EphB2 knockdown (* $P<0.01$).

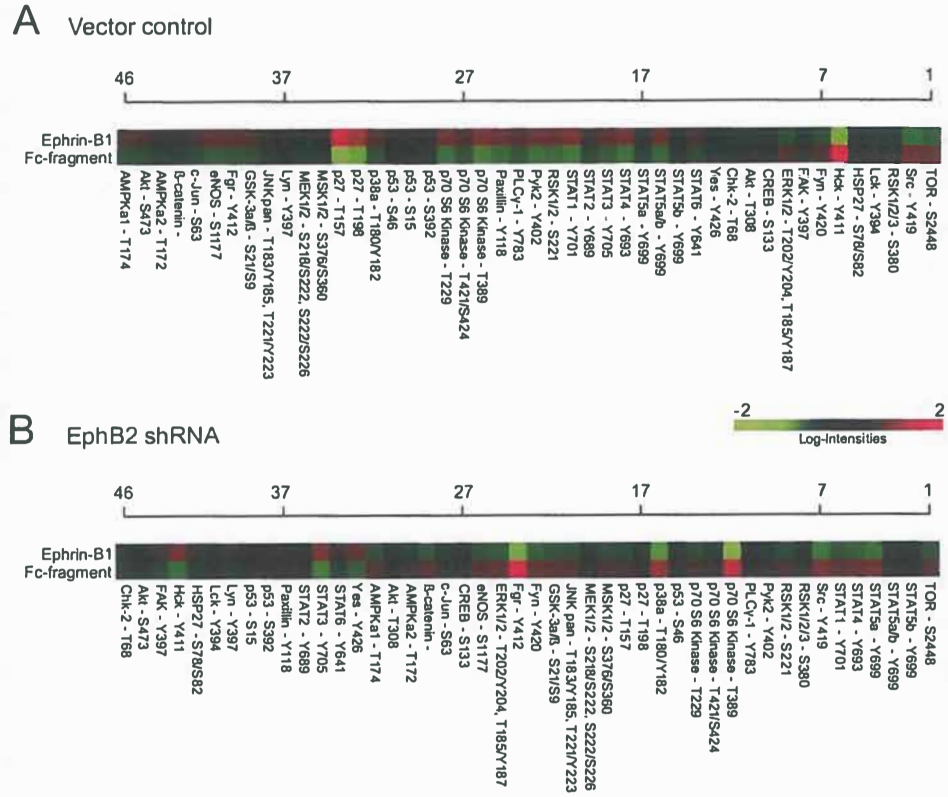


Figure 6. Downstream cell signaling in DAOY medulloblastoma cells in response to Ephrin-B1 stimulation changes upon EphB2 knockdown. DAOY control cells (A) as well as EphB2 shRNA transfected DAOY cells (B) were treated with Ephrin-B1 or Fc-fragment control (15 minutes) upon which the downstream signal transduction activity was assess by means of phospho-proteome profiling (Supplementary Table 4). Changes in phosphorylation levels of multiple cell signaling mediators upon Ephrin-B1 stimulation are abolished or inverted as a result of EphB2 knockdown.

Eph promoter methylation in tumor samples and cell lines

Recently, Kuang *et al.* provided evidence for epigenetic silencing as an important regulatory mechanism in the expression of Eph/Ephrin family genes in acute lymphoblastic leukemia (ALL).[11] Based on the observed Eph/Ephrin expression pattern we hypothesized on a similar regulatory mechanism in medulloblastoma. Hence, medulloblastoma cell lines were cultured in the presence of the

demethylating agent 5-Aza-2'-deoxycytidine. This resulted in an increased expression of all EphB receptors in DAOY with an average fold change of 3.3 (1.9-5.1). (Supplementary Fig. S2). This includes the EphB2 receptor which is already being expressed at high basal levels in DAOY. With the exception of EphB6, all EphB receptors show increased expression in Res-256 upon demethylation treatment. For Uw-402 an increase in receptor expression could only be observed for EphB3 and –B4. These results provide evidence for a potential epigenetic regulation of the Eph receptors in medulloblastoma. We performed methylation analysis of the Eph promoters in a set of primary medulloblastoma samples. This did not indicate a changed promoter methylation of the Eph receptors in medulloblastoma compared with normal cerebellum, suggesting that the 5-Aza-2'-deoxycytidine induced effects are established indirectly (Supplementary Table 4).

Discussion

Increasing evidence points towards a crucial role for Eph/Ephrin signaling in cancer progression by coordinating essential cell migratory and invasive processes. Solid tumors, including medulloblastoma are characterized by a profound tumor cell invasion of the tumor cells into surrounding tissue, thus complicating treatment and resulting in relapse and the development of metastases. We sought to clarify the role of Eph/Ephrin signaling in medulloblastoma progression and identified a key role for EphB2 signaling in tumor cell adhesion and invasion.

Our data point towards a selective overexpression of EphB2 and its corresponding ligand Ephrin-B1 in medulloblastoma. Recently, gene expression data of a larger cohort of medulloblastomas was published by Kool *et al.* showing a significant increase in the expression of EphB2 and Ephrin-B1 compared to normal cerebellum as well, thus confirming the expression changes we found in our cohort.[32] One might speculate that the dramatic effects we observed on *in vitro* cell adhesion and invasion are indicative for a role in local spread of the tumor, thus contributing to the formation of metastases or local relapse. Nakada *et al.* previously reported a role for EphB2 signaling in glioblastoma adhesion and migration as well as proliferation [12]. The fact that we observed no direct effects on medulloblastoma proliferation is supported by recent literature describing that EphB2-induced migration and proliferation are controlled independently by activation of distinct pathways.[22] The

exact role of Eph/Ephrin signaling in local recurrence and formation of metastases should be clarified by means of future *in vivo* experiments.

An increase in the phosphorylation levels of EphA1 and –A2 could be appreciated in the medulloblastoma cell line Uw-402 upon stimulation with Ephrin-B1. Although no phenotype was observed stimulating medulloblastoma cell lines with Ephrin-A1, our results are indicative for a modest activation of the mentioned A-type Eph receptors by Ephrin-B1 in Uw-402. Promiscuity in receptor binding of Ephrin ligands has been observed previously[3] whereas interaction between Ephrin-B1 and A-type Eph receptors has not been described before.

Li *et al.* recently observed a striking decrease of the EGFR phosphorylation levels in glioma cell lines upon treatment with Ephrin-A5.[16] They show that activation of EphA2 results in recruitment of c-Cbl to EGFR, indicative for ubiquitinylation and subsequent degradation of the EGF receptor. In addition, Larsen *et al.* showed that EphA2 and EGFR can be co-immunoprecipitated in multiple cell lines, providing evidence for a direct interaction.[33] In our study we observed a decrease in EGFR phosphorylation levels in DAOY and Uw-402 upon treatment with Ephrin-B1. Possibly the observed Ephrin-B1 phenotype in medulloblastoma is (partly) derived from altered EGFR activity. Curiously, Ephrin-A5 has binding affinity for EphB2 as well so possibly the observed effect of Ephrin-A5 on EGFR activity in glioma is caused by EphB2 activation.[34] In a more recent study a reciprocal effect of EGFR activity on the expression of EphA2 was observed in a number of cancer cell lines including glioma.[35] Furthermore, a decreased EGF-induced cell motility was shown upon EphA2 downregulation. In our opinion this underscores the complexity of this protein interaction, which apparently functions bidirectional.

EphB2 activation in DAOY resulted in a decreased phosphorylation of several members of the Ras-Raf-MEK-Erk as well as the PI3K-Akt-mTOR signal transduction pathways. Pasquale *et al.* previously described a downregulation of Akt-mTOR signaling in response to EphA2 activation in multiple cancer cell types.[36] Possibly this process takes place in response to EphB2 activation in medulloblastoma as well. R-Ras signaling is known to play a key role in medulloblastoma metastasis formation.[37] A direct link between EphB2 and R-Ras has been described previously.[38] In concurrence with these data, we observed a strong decrease in Erk activity upon EphB2 stimulation. As observed previously for EphA4, the Jak/STAT signaling seems

to be activated upon stimulation of EphB2.[39] Interestingly, we also observed decreased activity of members of this pathway in response to Ephrin-B1 stimulation.
We observed an increased phosphorylation of paxillin and p27 upon Ephrin-B1 stimulation, suggesting a role for these kinases in migration and adhesion of medulloblastoma cells upon Eph receptor activation. For p27 a key role in tumor cell motility and migration has been described.[40-42] Paxillin is a well known mediator of cell motility and thus has been implicated in tumor invasion and formation of metastases for a variety of malignancies.[43,44] Furthermore, Vindis *et al.* describe that EphB1-mediated cell migration requires paxillin activation.[45] In correspondence with our data, recently the suggestion was made that p27 as well as paxillin may play a role in medulloblastoma cell motility.[46,47] The exact role of altered signal transduction on the observed phenotypes needs to be clarified.
Expression of Eph receptors as well as Ephrin ligands was observed in primary medulloblastoma tissue. Likely, the medulloblastoma microenvironment will be highly influenced by alternate Eph receptor and Ephrin ligand expression as well. However, targeting the Eph/Ephrin can elicit effects on the tumor cells and the microenvironment that are not necessarily beneficial from a cancer treatment perspective. Upregulation of Ephrin-A1 in endothelial cells and consequent activation of EphA2 has been reported to have an important function in the pro-angiogenic effects of VEGFA and TNFα.[48,49] Enhanced tumor suppressor signaling in response to EphA2 activation is therefore bound to be accompanied by an enhanced tumor angiogenesis.[50,51] More recently, a key role for Ephrin-B2 reverse signaling in VEGFR2 induced angiogenesis and lymphangiogenesis has been reported.[9,10] In an Ephrin-B2 knockout mouse model the intracranial astrocytoma growth was substantially impaired due to decreased angiogenesis. Possibly the characteristically high vascularity of pilocytic astrocytoma and the presence of VEGFR2 kinase activity is indicative for a role of EphB/Ephrin-B signaling as well.[52] Whether the observed overexpression of EphB2 in medulloblastoma results in enhanced Ephrin-B2 mediated VEGFR2 function, thus inducing tumor angiogenesis is an intriguing concept that should be aim of future study. Possibly interplay between B-type Eph receptors and the corresponding Ephrin-B ligands is responsible for a tumor-promoting phenotype through enhanced angiogenesis as well as tumor cell invasion.

Although the Eph/Ephrin signaling mechanism is still largely unknown, the presence of counteracting effects in response to activation of different Eph receptors is indicative for a tightly controlled balance in relative expression of the various receptors that ultimately determines the phenotype. Since the effects of Ephrin-B1 stimulation in DAOY stably transfected with EphB2 shRNA resulted in a further decrease of the invasive capacity instead of an increase as observed in normal DAOY cells one might speculate upon an opposite phenotype due to a perturbed Eph receptor expression balance. Here, we did not observe an indication of direct epigenetic regulation of Eph expression in medulloblastoma. The increase in B-type Eph receptor expression upon 5-Aza-2'-deoxycytidine treatment, however, does suggest indirect epigenetic regulation.

In conclusion, the observed deregulation of the Eph/Ephrin expression in medulloblastoma enhances the invasive phenotype *in vitro*. This suggests a potential role in tumor invasiveness and metastasization. Here, the complexity of the Eph/Ephrin signaling network calls for more detailed tumor-specific insight in the effects of Eph and Ephrin signaling inhibition on the tumor as well as the microenvironment to allow potential application as a target for anticancer therapy.

Acknowledgements

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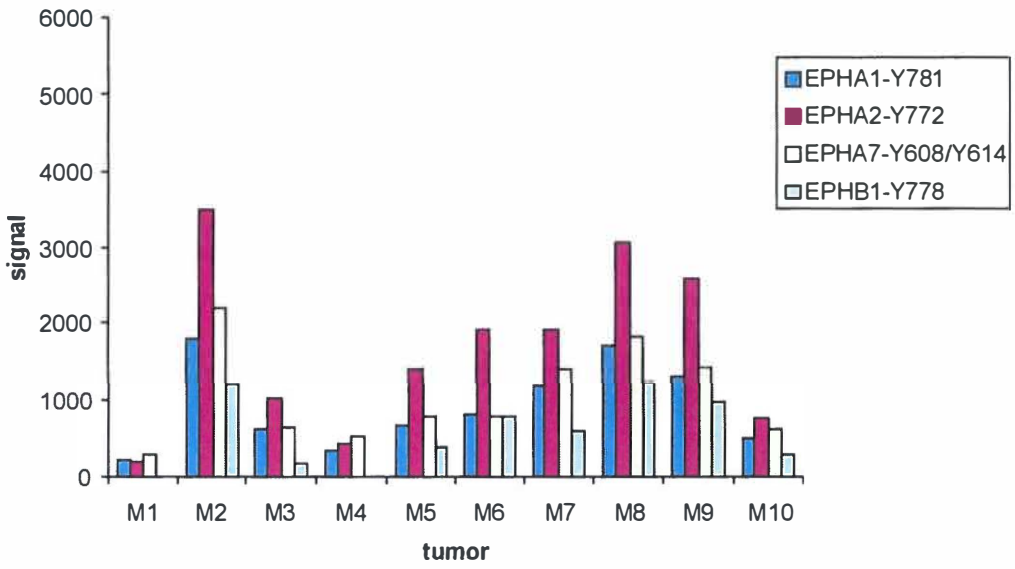
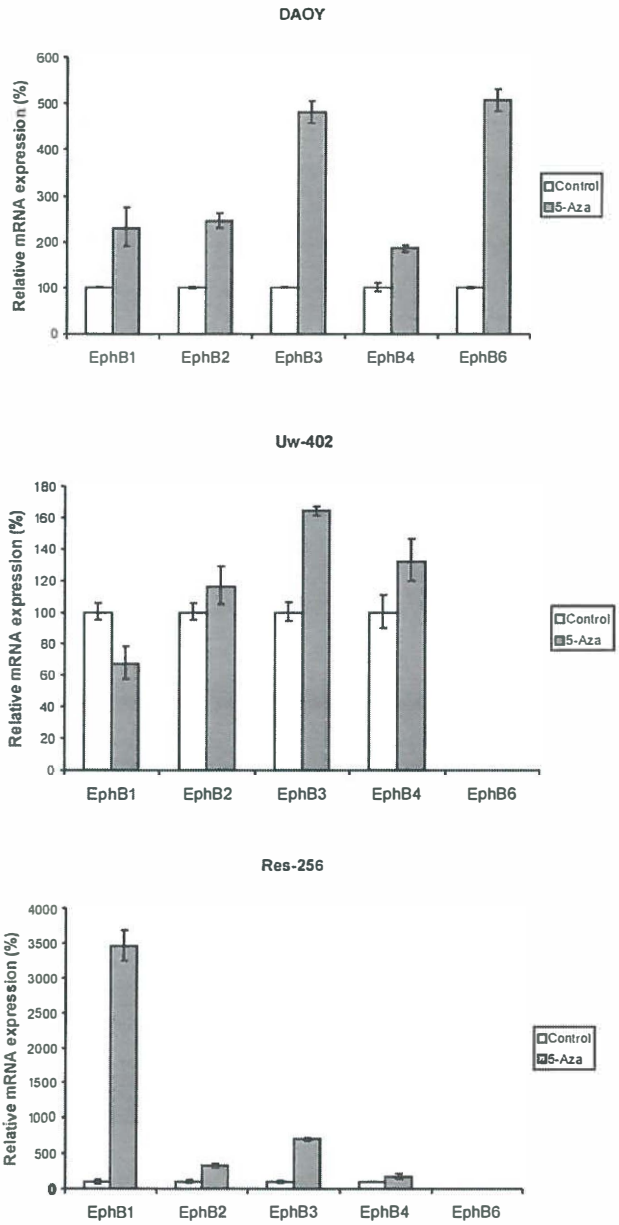
Supplementary Fig. S1. Eph receptor peptide phosphorylation. Histograms showing the phosphorylation levels of all Eph receptor-derived peptides present on the kinase activity profiling array upon application of medulloblastoma tissue. M1 – M10 represent the individual medulloblastoma samples. The protein of origin as well as the tyrosine phosphorylation site have been depicted in the figure.



Supplementary Fig. S2. Effect of 5-aza-2'-deoxycitidine on EphB expression. Medulloblastoma cell lines were treated with the demethylating agent 5-Aza-2'-deoxycytidine (5-Aza) during three days. This resulted in a marked increase in the expression of all EphB receptors in DAOY. In Uw-402 only EphB3 and –B4 showed increased expression.



Supplementary Table 1. Primers Eph receptors and ligands for qRT-PCR and primers Eph promoter methylation studies.

Gene	Forward primer	Reverse primer
EphA1	TCTGATGGACACAAGCAAGG	GGTCAGTGTCTCTGCGTCCT
EphA2	AGGGCAAGGAAGTGGTACTG	CTTTGCCATACGGGTGTGT
EphA3	GCTTGTACCCATTGGCAAGT	CTTTGTCTGCCCGGAAGTAA
EphA4	CAGATGGTGAATGGCTGGTA	TCTGAAAAAGCCTCGGTCAC
EphA5	AAATGCCCTTCTGTGGTACG	CAGCTCTGGATGTGAGGTGA
EphA6	ATTCGCCTAGAAGGGGTTGT	CGACCAACTGGATGACTGTG
EphA7	GGATTTCCTCTCCACCCAAT	GTTAGTCCGCAGCCAGTTGT
EphA8	CATCCTCTCTCTCCGCATCT	GTAGAAGCCCAGCTCACAGG
EphB1	CTTTGACCCTCCAGAAGTGG	CTCCACATTGTCGTCACAGC
EphB2	ATGCGGAAGAGGTGGATGTA	CCTTGAAAGTCCCAGATGGA
EphB3	CTGGAAGAGACCCTCATGGA	ATTCATGGCCTCATCGTAGC
EphB4	TTCGAGCACCCCAATATCAT	GAACTGTCCGTCGTTTAGCC
EphB6	ACTTCCTCAAGGGGAGCTGT	CTTCCGCTGGAAGACGAC
EfnB1	TCAACCCCAAGTTCCTGAGT	GCAGATGATGTCCAGCTTGT
EfnB2	GGAATTCCTCGAACTCCAAA	GTCCACTTTGGGGCAAATAA
EfnB3	CCTGGAGCCTGTCTACTGGA	CGATCTGAGGGTACAGCACA
GAPDH	GGTGGTCTCCTCTGACTTCAACA	GTGGTCGTTGAGGGCAATG

Supplementary Table 2. Medulloblastoma cell lines were stimulated with Ephrin-B1 or Fc-fragment control and receptor tyrosine kinase phosphorylation was determined by antibody array screening.

	DAOY		DAOY		Uw-402		Uw-402		Res-256		Res-256	
	EphrinB1		Fc-fragment		EphrinB1		Fc-fragment		EphrinB1		Fc-fragment	
Protein	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD	Average	SD
EGFR	3381.0	622.6	10251.0	1878.7	722.0	0.0	4153.5	1932.6	14472.5	794.9	15853.0	276.6
Axl	881.7	285.9	884.3	121.2	940.3	69.2	0.3	0.3	177.3	183.8	117.0	109.1
c-Ret	1875.7	284.3	2838.3	26.0	4382.8	2249.1	0.0	0.0	437.8	391.3	343.5	274.2
Dtk	1569.2	91.6	1493.8	440.2	519.8	0.0	368.3	0.0	383.3	313.4	319.5	39.7
EphA1	17.5	0.2	2695.5	607.0	1267.0	363.5	0.0	0.0	330.0	258.6	0.0	0.0
EphA2	0.0	0.0	1094.0	17.3	641.0	315.4	0.0	0.0	0.0	0.0	0.0	0.0
EphA3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	18.5	-145.8
EphA4	890.2	152.3	1301.8	170.9	1232.8	128.1	660.8	102.1	124.3	107.4	0.0	0.0
EphA6	0.0	0.0	0.0	0.0	0.0	0.0	131.3	43.5	0.0	0.0	0.0	0.0
EphA7	854.7	368.1	371.8	2.1	38.8	40.5	0.0	0.0	3.8	4.7	0.0	0.0
EphB1	1080.7	973.6	262.8	84.3	577.3	122.3	9.3	13.9	0.0	0.0	0.0	0.0
EphB2	6476.2	2028.7	1643.8	381.2	777.3	213.0	0.0	0.0	845.3	248.4	262.0	178.1
EphB4	358.2	137.4	0.0	0.0	8.8	0.7	0.0	0.0	0.0	0.0	90.5	13.5
EphB6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	104.0	7.8
ErbB2	2469.2	267.9	2848.3	621.2	1040.3	22.8	5.3	1.9	163.3	91.2	1047.0	1463.2
ErbB3	0.0	0.0	757.3	161.7	0.0	0.0	0.0		2441.8	896.5	3178.5	464.6
ErbB4	0.0	0.0	618.3	317.3	2274.3	1026.3	0.0	0.0	9.8	7.0	130.5	55.5
FGFR1	72.2	21.2	0.0	0.0	0.0	0.0	115.8	176.5	234.8	189.2	44.5	140.1
FGFR2a	146.7	12.2	0.0	0.0	251.8	168.1	0.0	0.0	0.0	0.0	192.0	15.0
FGFR3	1842.7	148.0	1223.8	585.9	0.0	0.0	200.8	45.2	210.3	51.5	171.5	101.3
FGFR4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	130.0	259.5	355.5	416.8
Flt-3	0.0	0.0	0.0	0.0	125.3	254.8	0.0		0.0	0.0	0.0	0.0
HGFR	15.2	19.5	19.3	11.1	2290.3	1567.4	0.0	0.0	0.0	0.0	66.5	136.7
IGF-I R	722.7	72.9	1888.8	244.3	321.3	17.2	0.0	0.0	6810.8	542.3	10373.0	397.1
Insulin R	474.7	37.9	937.8	100.2	763.8	93.9	423.8	98.3	1670.3	170.6	4009.0	151.0
M-CSFR	1335.2	204.9	450.3	125.6	141.8	53.4	282.8	103.0	536.8	549.1	120.0	16.8
(Mer)	4602.5	1294.6	2067.5	325.0	104.0	47.3	0.0	0.0	527.5	12.5	84.0	90.1
MSPR	588.2	202.2	2479.3	1319.2	82.3	58.0	17.3	0.6	0.0	0 0	30.0	2248.6
MuSK	223.2	39.4	0.0	0.0	169.8	34.6	0.0		80.8	96.7	216.0	287.5
PDGFRa	0.0	0.0	0.0	0.0	341.8	174.7	0.0	0.0	0.0	0.0	88.5	394.4
PDGFRb	1742.7	173.9	384.3	57.2	0.0	0.0	0.0	0.0	1231.3	147.9	1057.0	164.3
ROR1	1013.2	51.5	2651.3	342.6	934.8	209.6	62.3	20.7	202.8	136.4	418.0	190.0
ROR2	544.2	80.4	1554.8	460.4	301.3	24.7	42.3	107.4	0.0	0.0	0.0	0.0
SCFR	200.2	27.1	0.0	0.0	0.0		161.3		0.0	0.0	214.5	46.6
Tie1	799.7	133.7	734.8	53.0	7.8	5.4	84.3	9.5	0.0	0.0	109.5	29.8
Tie2	1747.2	568.8	451.3	97.0	0.0	0.0	35.3	35.2	83.3	122.9	0.0	0.0
TrkA	911.5	75.2	612.5	317.4	162.0	167.4	0.0	0.0	0.0	0.0	76.5	123.2
TrkB	1728.2	517.9	1473.3	355.9	225.3	346.2	0.0	0.0	0.0	0.0	25.5	-571.8
TrkC	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
VEGFR1	0.0	0.0	0.0	0.0	44.8	23.1	0.0	0.0	0.0	0.0	81.5	36.2
VEGFR2	0.0	0.0	0.0	0.0	0.0	0.0	505.3	421.3	66.8	45.3	193.5	351.8
VEGFR3	688.2	101.6	0.0	0.0	146.8	22.2	133.3	1.6	135.8	94.7	290.5	275.7

Supplementary Table 3. DAOY medulloblastoma cell lines stably transfected with EphB2 shRNA or vector control were stimulated with Ephrin-B1 or Fc-fragment control and downstream kinase phosphorylation was determined by antibody array screening.

This table can be provided upon request.

Supplementary Table 4. The promoter methylation status of the most prominent EphA and EphB receptors was determined in a set of primary medulloblastomas and compared with the situation in normal cerebellum.

PYRO result	EPHA2	EPHA4	EPHA5	EPHA6	EPHA7	EPHA10	EPHB1	EPHB2	EPHB3	EPHB4
	4CpG	3CpG	3CpG	3CpG	2CpG	2CpG	4CpG	4CpG	4CpG	4CpG
medulloblastoma	5.88	3.29	6.3	4.41	2.3	3.48	3.27	3.07	4.51	3.41
medulloblastoma	5.19	3.17		3.87	2.35	3.02	3.06	3.08	4.41	3.18
medulloblastoma	6.29	3.8		5.45	2.5	3.09	2.4	2.82	4.93	3.92
medulloblastoma	5.95	3.4	7.58	4.48	2.59	3.28	3.37	3.04	4.45	4.22
medulloblastoma	5.89	3.49	7.62	5.1	2.25	4.53	2.97	2.49	4.45	3.62
medulloblastoma	6.13	3.63	9.07	5	2.31	2.94	3.08	3.51	5.05	4.95
medulloblastoma	6.18	4.33	18.03	4.94	2.24	3.39	3.3	2.93	4.6	4.48
medulloblastoma	9.08	4.76	8.8	6.08	3.3	4.01	3.68	4.11	5.74	4.28
medulloblastoma	6.45	4.1	8.15	4.83	0.86	3.13	2.77	2.88	5.15	3.81
medulloblastoma	5.86	4.79	8.47	4.9	0.93	4.63	2.73	4.07	5.39	3.61
medulloblastoma	6.34	3.57			2.31		4.06	4.14	5.21	6.02
medulloblastoma	6.11	3.04	6.97	2.38 (1CpG)	1.8		3.65		4.96	5.78
medulloblastoma	8.86	3.94			2.08		6.97	3.51	5.05	6.55
medulloblastoma	6.25	3.66	3.89 (1 Cpg)			2.71	3.92	4.4	4.97	13.65
medulloblastoma	6.43	2.76	8.32		0.57 (1CpG)		3.29		5.04	6.23
normal cerebellum	7.12	4.06	8.83	6.25	2.53	4.89	3.21	3.17	5.08	5.03
normal cerebellum	7.75 (3CpG)	3.6	8.5	5.64	2.36	2.33	3	3.57	5.14	4.77
normal cerebellum	6.93 (3CpG)	4.97	11.16	6.19	2.72	2.62	3.27	4.5	5.65	4.53
normal cerebellum	6.65	4.4	10.44	5.35	2.69	2.81	3.53	2.77	7.01	1.25

Chapter 6

Kinetic Substrate Profiling of the Human Protein Tyrosine Kinome Reveals Functional Dichotomy in Kinase-Substrate Interactions

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Abstract

The molecular determinants driving amino acid composition of kinase substrate motifs remain unresolved. Two opposing schools of thought exist: a first one whose adherents interpret the available data as to suggest that little evolutionary pressure is present determining amino acid sequences in substrates and that specificity of kinases for substrates is mainly regulated at the level of physical interaction of the kinase protein with the targets for its enzymatic action. Here, protein scaffolding makes an important contribution to the selectivity of the kinase-substrate interactions. A second interpretation is that evolutionary pressure forces substrate peptides to acquire high specificity for specific kinase enzymes and low specificity for other kinases, thus coupling the appropriate substrates to the correct enzymatically active kinase. We decided to test a sample of kinases from the human tyrosine kinome (10 % of total) against a library of peptide substrates.

Receptor tyrosine kinases generally phosphorylated their functionally annotated peptide substrates with high initial phosphorylation velocities (V_{ini}). Tyrosine kinases functioning as downstream signaling effectors like Src and ZAP70 phosphorylated their functionally annotated substrates at highly diverse V_{ini} values. Furthermore, the results suggest the presence of a functional dichotomy in substrate phosphorylation where a subset of peptides shows low phosphorylation rates and for phosphorylation likely depend on scaffolding by other protein domains and another subset displaying high phosphorylation rates on specific kinases, possibly allowing their phosphorylation even in the absence of a strong physical kinase-substrate interaction.

Based on our data we propose that variability in substrate motif recognition explains the apparent contradictory interpretations of the forces that drive kinase substrate selection.

Introduction

Phosphorylation of protein substrates by kinases is among the most principal processes governing cellular physiology. Underscoring the functional importance of kinases in mediating signal transduction leading up to a physiological phenotype, protein kinases constitute one of the most abundant classes of enzymes, accounting for approximately 2% of the human genome.[1] The signaling network complexity and the high number of potential protein interactions that can occur greatly rely on a meticulous precision in the control of selectivity in protein interactions.[2]

Two opposing schools of thought with regard to the factors driving protein substrate selection for kinase enzymatic activity exist: a first one whose adherents interpret the available data as to suggest that little evolutionary pressure is present determining amino acid sequences in substrates and that specificity of kinases for substrates is mainly regulated at the level of physical interaction of the kinase protein with the targets for its enzymatic action outside the active site.[3-7] According to this interpretation tyrosine kinases have developed specificity for tyrosine-containing substrates through regulation of kinase activity itself and the recruitment of substrates to the activated kinase domain as the major factors governing the phosphorylation of specific phosphorylation sites. Supporters of this view point towards the large number of SH2 and SH3 domain-containing proteins and the apparent specificity these confer as evidenced by the absence of downstream signal transduction when these domains are deleted or mutated.[8-10] Also the existence of scaffolding proteins and the apparent necessity of such proteins as indicated by genetic mutants lends further credit to this interpretation. Finally, various phosphorylation sites on proteins exist that can act as substrates for a wide range of different kinases in *in vitro* experiments.

A second interpretation is that evolutionary pressure forces the primary amino-acid sequence of substrates to acquire high specificity for specific kinase enzymes and low specificity for other kinases and that thus appropriate protein substrates are coupled to correct kinase enzymatic activity.[4-7,11] In this situation the substrate and kinase have to diffuse towards each other, bind and, as a result, the substrate is phosphorylated. This requires complementary surfaces of substrate and kinase, which have an attraction towards each other. The strong conservation in substrate

motifs throughout eukaryotes underlies this notion as it shows that substantial evolutionary pressure on substrate motif composition exists. Also bioinformatics analyses of substrate motifs for kinases for different protein substrates clearly yield specific substrate consensus sequences (e.g. proline-directed kinases) and various theoretically-predicted super-substrates have thus been confirmed.[12]

The molecular determinants driving amino acid composition of substrate motifs for kinases in proteins remain unresolved. Hence it is fair to say that we do not understand the forces that drive substrate selection in mammalian cells, prompting investigations into this area. Here we employed the potential of peptide array-based kinase activity profiling in the detection of kinase-protein interactions by kinetically measuring phosphorylation in order to characterize substrate motif specificity within the human tyrosine kinome. We hypothesize that functionally annotated kinase-substrate interactions, characterized by a strong interaction domain-dependent physical interaction, can elicit a low initial phosphorylation velocity (V_{ini}) whereas less stringent physical interactions will result in high V_{ini} values due to the retained primary amino-acid specificity.

Methods

Kinome profiling recombinant kinases

Kinase activity profiles were determined using the PamChip tyrosine kinase microarray system (PamGene International B.V.). This microarray consists of 144 unique tyrosine containing peptide sequences covalently coupled to a porous membrane enabling constant flow-through of the reaction mixture (www.PamGene.com). Each peptide represents a 13 amino acid sequence corresponding to a putative endogenous human phosphorylation site, which functions as a tyrosine kinase substrate (annotation:[13]). Phosphorylation is visualized in real time by measurement of the fluorescent signal emitted as a consequence of fluorescein-labeled anti-phosphotyrosine antibody binding (PY20).

The following recombinant tyrosine kinases were included: VEGFR1 (#7784, Cell Signaling), VEGFR2 (#7407, Cell Signaling), VEGFR3 (#7790, Cell Signaling), InsR (0122-0000-1, ProQinase), ALK (14-555, Millipore), Abl (14-529, Millipore), Src (14-326, Millipore), ErbB4 (14-569, Millipore) and ZAP70 (14-404, Millipore). The reaction

mixture (35 uL) consisted of 1x PK buffer (New England Biosciences), 100 µM ATP (Sigma Aldrich), 20 µg/mL FITC labeled PY20 (Exalpha) and 0.5 to 6 µL kinase, correcting for the intrinsic activity. Prior to loading the reaction mixture onto the array a blocking procedure was performed with 0.2% Bovine Serum Albumin (Calbiochem). After loading the kinase containing incubation mixture onto the PamChip arrays (in triplicate), incubation was continued for 60 cycles utilizing the PamStation96. Phosphorylation was measured at multiple time points, i.e. every 5th cycle during the run to study enzyme kinetics.

Data analysis and statistics

Quantification of spot intensities was conducted using Bionavigator software (PamGene International B.V.).

Peptides with phosphorylation intensities comparable to or less than the negative (buffer) control were considered noise. Peptides that did not show a consistent increase in phosphorylation in time were excluded from the data. Furthermore, the threshold signal intensity was determined for each kinase individually by ordering the signals based on signal intensity followed by plotting the second derivative of this dataset. The threshold was set at the point where the trend line deviates from zero.

Fitting

The distribution of the Vini values was studied by binning the signal intensities in bins that were determined by dividing the difference between the lowest and highest signal in 20 equal parts. It was assumed that the binned-distribution of peptide affinities for kinases was either described by:

$$(y) = 1/(\sigma\sqrt{2\pi}) \cdot e^{-(x-x_0)^2/2\sigma^2}$$

where y is the expected positive peptide number at bin number x.[14]

The mean (x_0) is estimated from:

$$\sum_{1 \to x_0} o_n = \tfrac{1}{2}(o_1 + o_2 + \cdots o_\infty)$$

where o_1 is the observed number of positive peptides in bin 1, o_2 the number of positive peptides in bin 2 etc. σ is estimated from the average distance of the observed bin number of all positive peptides to x_0 in this Gaussian, based on the iterative method of the least X^2.

Or by:

$$(y) = 1/(\sigma_\alpha\sqrt{2\pi}) \cdot e^{-(x-x_\alpha)^2/2\sigma_\alpha^2)} + 1/(\sigma_\beta\sqrt{2\pi}) \cdot e^{-(x-x_\beta)^2/2\sigma_\beta^2)}$$

Where σ_α and x_α describe mean and standard deviation of substrates with high V_{ini} values and σ_β and x_β describe mean and standard deviation of substrates with low V_{ini} values, calculated from half distributions based on the iterative methods of the least X^2 as described for the single Gaussian.

Wellness of fit is estimated by calculating the Pearson product moment *R* of the predicted distribution and the observed distribution,[15] whereas for the bimodal distribution the $R^{1\frac{1}{2}}$ is taken to correct for effect that any distribution is better described by two Gaussian distributions than one. The higher of the two numbers was considered to most likely represent the adequate description of the observed distribution.

VEGFR2-APA co-immunoprecipitations

Cells were serum starved for 18 hours followed by stimulation with recombinant VEGF (25 ng/µL) for 5 minutes or inhibition with vandetanib (1 µM) for 10 minutes. After a single wash with phosphate buffered saline (PBS) the cells were scraped and lysed in ice cold Triton lysis buffer (20 mM tris-HCL pH 7.4, 150 mM NaCl, 1.2% Triton X-100, 1 mM EDTA, 1x Complete solution (Roche Diagnostics) and 1 mM Na_3VO_4). The scraped cells were incubated on ice for 20 min followed by centrifugation at 4^oC, 12.000g for 15 minutes. Supernatants were incubated with primary antibodies against either VEGFR2 (55B11 Rabbit mAb, Cell Signaling) or APA (PAB6806 Goat pAb, Abnova) for 2 hours at 4^oC. Protein-G PLUS (sc-2002, Santa Cruz Biotechnology) was added followed by 2 hours incubation. After 3 quick washes with lysis buffer the

samples were boiled in Laemmli's sample buffer for 5 minutes and the supernatant was loaded onto a 7.5% acrylamide gel for SDS-PAGE.

InsR-Syk co-immunoprecipitations

Cells were serum starved over night followed by stimulation with Insulin (200 nM). To study the relevance of Insulin receptor (InsR) activity in generating the Insulin response the cells were pre-treated with the InsR inhibitor BMS-536924 (1 µM) 15 minutes prior to stimulation. Lysis and sample preparation were performed as described above. Supernatants were incubated with primary antibodies against InsR (L55B10, Cell Signaling) over night. Protein-G PLUS (sc-2002, Santa Cruz Biotechnology) was added followed by 2 hours incubation. After 3 washes with lysis buffer the samples were boiled in Laemmli's sample buffer for 5 minutes and the supernatant was loaded onto a 7.5% acrylamide gel for SDS-PAGE.

Immunoblotting

Human umbilical vein endothelial cells (HUVECs) were stimulated with VEGF or treated with vandetanib for 60 minutes. Treatment of HL-60 and U937 with insulin or BMS-536924 was performed for 15 minutes.

To visualize the levels of phosphorylated Syk, a newly developed immunoblotting technique was applied using a phosphate-binding molecule (Phos-Tag AAL-107; NARD Institute).[16] Together with 0.2 mmol/L MnCl2(H2O)4, 0.1 mmol/L Phos-Tag AAL-107 ligand was added to the SDS-PAGE gel (7.5%).

Protein was blotted onto a polyvinylidene fluoride membrane (Millipore). Blots were incubated at 4°C in 5% bovine serum albumin containing a primary antibody against VEGFR2 (55B11 Rabbit mAb, Cell Signaling) or APA (PAB6806 Goat pAb, Abnova) for 4 hours. Incubation with primary antibodies against Syk (sc-1240, Santa Cruz Biotechnology) was performed over night. Antibody staining was visualized by applying horseradish peroxidase secondary antibodies (Dako) followed by enhanced chemiluminescence. b-Actin was probed as a protein loading control (200 µg/mL stock; 1:3000; sc-47778 mouse monoclonal antibody; Santa Cruz Biotechnology).

Results

Variability in phosphorylation rates of functionally annotated substrates: Functional dichotomy in substrate conversion.

Tyrosine kinase activity profiles were successfully generated of nine receptor and non-receptor recombinant tyrosine kinases, thus covering 10% of the human tyrosine kinome. The Pamchip kinase activity profiling allows for measurement of the phosphorylation signal intensities over time providing us with kinetic peptide substrate phosphorylation data (fig. 1). Hence, initial phosphorylation velocities could be determined based on signal intensities at the start of the incubation and after 5-10 min, providing an indication of substrate preference for each kinase (supplementary table 1). As a measure for kinase activity we used these kinetic data to calculate the increase in signal intensity per cycle between cycle 31 and 41, which are the first cycles in which phosphorylation is measured.

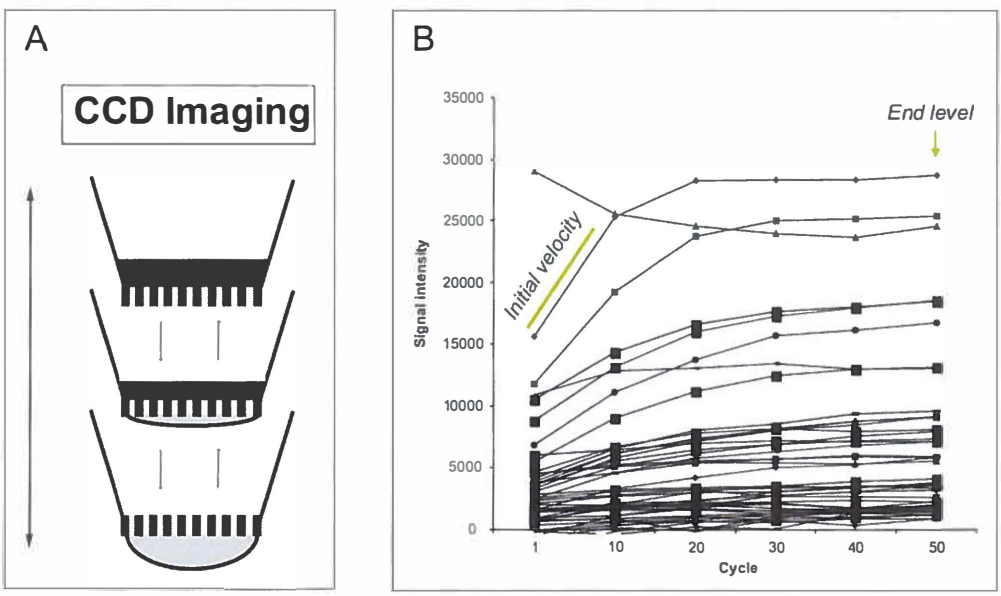


Figure 1: The Pamchip kinase activity profiling technique facilitates kinetic readout of the peptide substrate phosphorylation. In 90 consecutive cycles the sample is pumped up and down through the peptide-spotted membrane (A). Repetitive CCD imaging monitors the phosphorylation of each individual peptide over time (B).

For each kinase a bin plot of the number of peptide substrates with a specific initial phosphorylation velocity was generated (fig. 2). Comparison of the bin plots generated from receptor tyrosine kinases with those generated from tyrosine kinases playing a role in downstream signal transduction suggests a functional difference in the selectivity of substrate phosphorylation. Downstream tyrosine kinases generated peptide phosphorylation in a bimodal distribution, while the receptor tyrosine kinases all show a unimodal distribution (fig. 3).

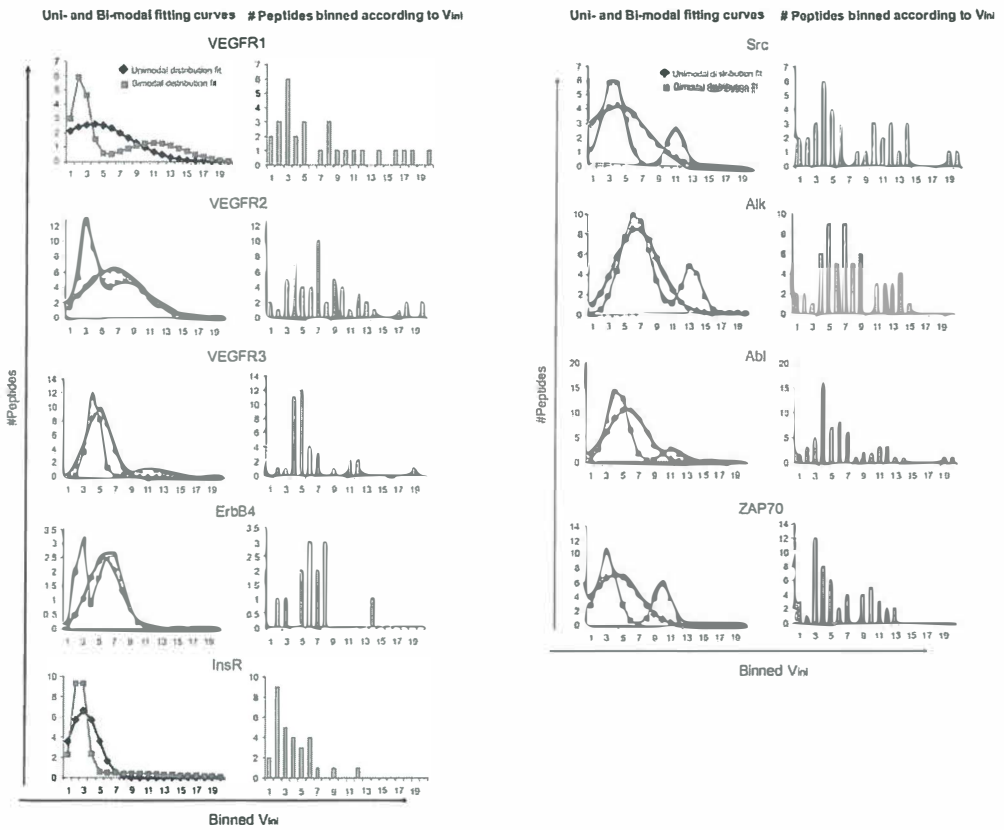
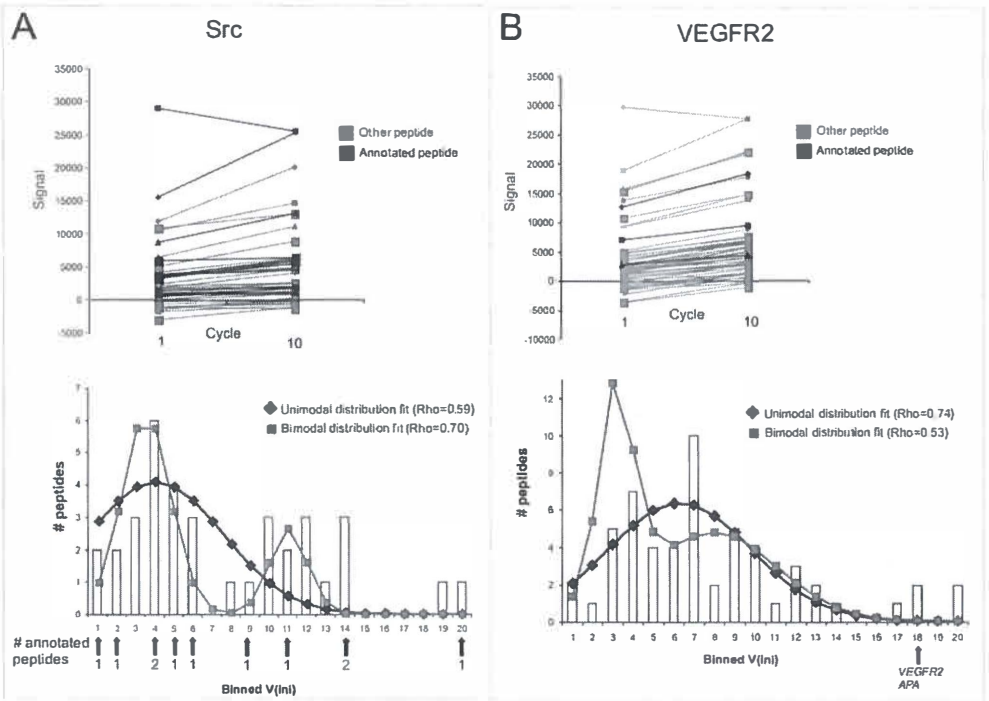


Figure 2: For each kinase the variation in V_{ini} values was visualized in a Bin plot (right plots). Whether the binned distribution of peptide affinities is better reflected by a unimodal or a bimodal distribution has been determined by performing curve fitting, where wellness of fit is estimated by calculating the Pearson product moment of the predicted distribution. The higher of the two numbers (fig. 3) was considered to most likely represent the adequate description of the observed distribution.



C

	rho unimodal distribution	rho bimodal distribution
Src	0.59	0.70
Alk	0.81	0.81
Abl	0.78	0.85
ZAP70	0.69	0.78
InsR	0.87	0.80
Erb	0.78	0.71
VEGR1	0.63	0.60
VEGR2	0.75	0.53
VEGR3	0.88	0.88

To obtain an indication of the functional effects of the observed dichotomous peptide substrate phosphorylation rates we annotated all peptides for potential upstream kinases as collected in the Phospho-ELM database (supplementary table 1).[17] Upon application of recombinant VEGFR2, VGFR2_944_956 was among the peptides with the highest initial velocities, underscoring the functional robustness of this kinase-substrate interaction. Despite the extensive homology in the phosphorylation sites of the various VEGFR family members, the VGFR1_1049_1061 peptide was phosphorylated at a lower rate. The PDYVRKG sequence (central part of VEGFR1_1049_1061) is present in the activation loop of all VEGFR family members.

Figure 3: Kinase activity profiles were generated for 9 recombinant kinases. Initial peptide phosphorylation velocities (V_{ini}) were determined between cycle 1 and 10. Representative examples of a downstream operating tyrosine kinase (A) and receptor tyrosine kinase (B) have been displayed. The upper panels show the increase in phosphorylation signal between cycles 1 and 10 which were used to calculate V_{ini} values. In the lower panels bin-plots provide an indication of the V_{ini} distribution of the various phosphorylated peptides. Curve fitting was performed for either a unimodal or bimodal distribution followed by calculation of the Pearson product moments (C). This indicates that a unimodal distribution provides a better description of the V_{ini} values generated by receptor tyrosine kinases whereas the V_{ini} distribution of downstream tyrosine kinases is bimodal.

The sequence VEGFR_944_956, however, is unique for VEGFR2. Application of recombinant VEGFR1 showed similar results where a high phosphorylation rate was again observed on VGFR2_944_956 while VGFR1_1049_1061 was phosphorylated at a lower V_{ini}. This suggests that the relevance of the primary sequence context differs between these two phosphorylation sites. Recombinant Src evoked phosphorylation of twelve functionally annotated peptide substrates. While many annotated substrates are among the peptides with the highest V_{ini} values, multiple peptides derived from genuine Src substrates (supplementary table 1) show surprisingly low values.

In vivo validation of weak kinase-substrate interactions as observed in the peptide-array data

For each kinase high V_{ini} values were observed on several peptides that have not (yet) been confirmedd as genuine in vivo protein substrates. Figure 4A shows the phosphorylation intensity on an Aminopeptidase A (APA)-derived peptide substrate for each of the applied recombinant kinases, suggesting a highly selective phosphorylation by recombinant VEGFR1 and -2. Furthermore, this peptide is phosphorylated with a high V_{ini} (fig. 3B). The Aminopeptidase A peptide contains the Y12 residue, representing a potential tyrosine phosphorylation site.[18] We speculated on a direct kinase-substrate interaction between VEGFR1/2 and APA. HUVECs were stimulated with VEGF and APA expression and phosphorylation was assessed (fig. 4B).

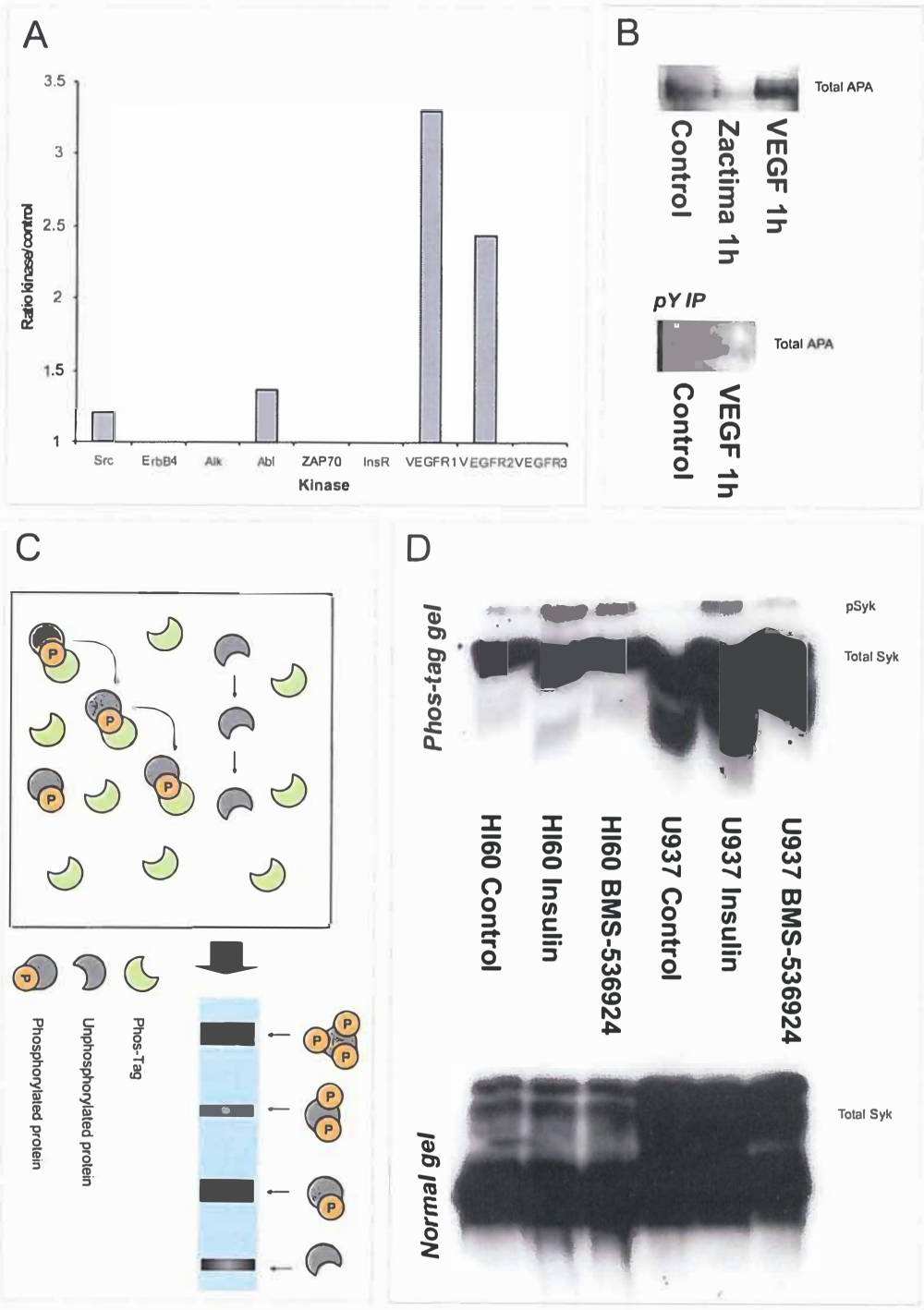

A
Ratio kinase/control
3.5
3
2.5
2
1.5
1
Src
ErbB4
Alk
Abl
ZAP70
InsR
VEGFR1
VEGFR2
VEGFR3
Kinase
B
Total APA
VEGF 1h
Zactima 1h
Control
pY IP
Total APA
VEGF 1h
Control
C
Phosphorylated protein
Unphosphorylated protein
Phos-Tag
D
Phos-tag gel
pSyk
Total Syk
U937 BMS-536924
U937 Insulin
U937 Control
HI60 BMS-536924
HI60 Insulin
HI60 Control
Normal gel
Total Syk

Figure 4: Selectively phosphorylated peptides point towards unidentified weak kinase-substrate interactions. Application of recombinant VEGFR1 and VEGFR2 resulted in marked phosphorylation of an Aminopeptidase A derived substrate whereas other kinases were not able to phosphorylate this peptide (A). This is suggestive for a direct kinase-protein interaction between VEGFR and Aminopeptidase A, which is known to aid in generating pathological angiogenesis in HUVEC upon stimulation with VEGF. Stimulation of HUVEC with VEGF resulted in phosphorylation and overexpression of Aminopeptidase A (B). This effect could be inhibited by adding the combined EGFR/VEGFR2 small molecule inhibitor vandetanib (Zactima).
The effect of InsR stimulation on the phosphorylation status of Syk was assessed by means of Phos-Tag SDS-PAGE (C) Pho-Tag molecules, which are supplemented to the SDS-PAGE gel, retain phosphorylated proteins higher in the gel. Stimulation of InsR (15 min) induced rapid phosphorylation of Syk kinase in HL-60 and U937 AML cells (D). This effect could be abolished by co-treatment with the InsR inhibitor BMS-536924.

An increase in both, expression as well as phosphorylation was observed upon stimulation with VEGF. The combinatorial EGFR/VEGFR2 inhibitor vandetanib was applied to study specificity. APA expression levels dropped dramatically upon this treatment. Co-immunoprecipitation attempts did not show a physical interaction.
Within the Pamchip data we observed substantial phosphorylation of a Syk derived peptide upon application of recombinant InsR-A. A recent paper describes Syk as a key mediator of acute myeloid leukemia (AML) differentiation.[19] Furthermore, an overexpression of InsR is present in 85 percent of all primary AMLs.[20] Our results suggest a possible link between these two observations, i.e. a direct phosphorylation of Syk by InsR kinase activity. To assess this hypothesis we stimulated AML cell lines (HL-60 and U937) with Insulin and determined the effect on Syk phosphorylation (fig. 4C,D). Phos-Tag western blotting provided evidence of an instant increase in phosphorylation of Syk upon this treatment. The specificity of this effect was assessed by co-treatment of the cells with the InsR/IGF-1R inhibitor BMS-536924 (1 µM), which has been shown to reduce AML proliferation in vitro.[21] BMS-536924 abolished the insulin induced Syk phosphorylation, suggesting a link between InsR

activation and the Syk phosphorylation status. In analogy to the VEGFR-APA interaction, co-immunoprecipitation studies did not show evidence of a strong physical interaction between these two proteins.

Discussion

Kinase activity profiles of a panel of purified recombinant tyrosine kinases on a set of 144 peptide substrates representing tyrosine phosphorylation sites in human cell signaling proteins were established. All kinases were promiscuous, for being able to phosphorylate many substrates on the peptide microarray. These experiments show the potential of kinases to phosphorylate particular substrates. Whether they will phosphorylate these substrates in a cellular context depends on subtle regulation mechanisms. In a cellular context substrate specificity may be determined by specific locations of kinase and substrate or by adaptor proteins and kinase domains that recruit specific substrates. The many phosphorylation sites on proteins probably each recruit their specific type of adaptor, thereby selecting the signaling cascade that is put in motion. Understanding the role of each phosphorylation site and the possible combinations of phosphorylations sites will be crucial for detailed dissection of signaling routes and cross talks.

These kinase activity measurements rendered a comprehensive list of phosphorylation signals, representing potential functional kinase-protein interactions. Peptide phosphorylation data of downstream operating tyrosine kinases indicated striking dichotomal substrate specificity. The observed bimodal distribution could not be confirmed applying receptor tyrosine kinases.

Src-induced peptide phosphorylation showed a high diversity in V_{ini} values on functionally annotated substrates. Examples of confirmed Src substrates which are phosphorylated at low V_{ini} rates are PDPK1- and Raf1-derived peptides.[22] This indicates a loss of selectivity on primary protein sequence and ultimate in vivo selection being based on tertiary protein structure and/or protein scaffolding, supporting the concept that downstream operating tyrosine kinases rely on highly diverging means of retaining the necessary selectivity in kinase-protein interactions. Possibly this reflects a subdivision in a class of kinase substrates depending on scaffolding/adapter domains for selectivity and a class of kinase substrates

(additionally) requiring substantial sequence specificity at the phosphorylation site. The dichotomous distribution is not visible for receptor tyrosine kinases.
The selectivity and sensitivity in kinase-substrate interactions varies highly in their dependence on the primary amino-acid sequence relative to their tertiary and quaternary protein structure.[23,24] Furthermore, Eames at al. have discovered that the relative abundance of proteins plays a significant role in the extent of evolutionary pressure in the amino acid sequence.[25] Here, highly abundant proteins possess less evolutionary flexibility due to the higher number of interaction partners.[26] Thus, selectivity is compromised sooner. Possibly, the higher diversity in V_{ini} values, as displayed by downstream tyrosine kinases opposed to the receptor tyrosine kinases, reflects a higher complexity of the surrounding protein environment urging a more extensive control of selectivity in functional protein interactions.

We observed kinase activity on peptides derived from previously identified downstream kinase phosphorylation sites as well as a number of new potential targets. A number of substrates that are known to interact with upstream kinases through scaffolding or adapter domains show relatively low phosphorylation whereas protein substrates that lack a strong physical interaction with the kinase enzyme seem to have been selected for high specificity and affinity. This concept was confirmed for aminopeptidase A (APA), for which an upstream kinase was not yet identified. However, a peptide derived from this protein was selectively phosphorylated by recombinant VEGFR1 and -2 with high velocity, also in intact cells. Strong physical interaction could not be detected however. Correspondingly, we detected specific activity of InsR on a Syk-derived substrate, which could be validated in vitro. Again, conventional validation experiments (i.e. co-immunoprecipitation) rendered no result. Based on these results we speculate on an in vivo interaction for APA-VEGFR and InsR-Syk that is indeed functional but too weak to detect by conventional pull-down methods.
Interestingly, APA appears to be expressed in a highly tumor specific fashion.[27] Furthermore, APA binding amino-acid sequences have been shown to target APA as a tumor-specific endothelial cell marker.[27,28] Possibly this highly specific expression signature explains the apparent focus on the primary amino-acid sequence in determining its kinase specificity.

Two VEGFR-derived peptide substrates showed sensitivity to VEGFR1 and 2 kinase activity. Although the levels of phosphorylation were substantially lower, some activity on these peptides was observed applying some other kinases as well. Despite this overlapping sensitivity to kinases that possess substantial sequence homology, phosphorylation of these substrates is generally considered an indication of VEGFR kinase activity.[29,30] Our data suggest however that the APA-derived substrate is more selectively phosphorylated by VEFGR1 and -2, thus providing a more selective indicator of VEGFR activity.

The direct assessment of aberrant kinase activity in tumor cells has become an increasingly important means of identifying potential targets for therapy. Short amino-acid sequences are generally applied as kinase substrates. High throughput kinase activity measurement approaches such as array based kinome profiling technology make it possible to measure kinase activity on hundreds of different 13-aminoacid peptides at once. From a technological standpoint the kinase activity profiling data interpretation will highly benefit from a non-biased annotation approach. Taking into account all functionally annotated substrates of a kinase of interest has already been shown to improve data interpretation.[17,31] Here we have pointed out, however, that peptides which have not been identified as potential targets of a kinase of interest can provide selective insight in the activity of the kinase as well.

The extent to which peptide phosphorylation is altered when performing kinase activity screening in the presence or absence of specific kinase inhibitors likely will improve the identification of active kinases. This potentially provides a stepping-stone for defining critical targets for treatment of diseases that are characterized by aberrant kinase activity. In our view kinase activity profiling data will gain tremendously in interpretability if approaches will be devised to model the phosphorylation profiles to obtain insight in fundamental changes in the cell signaling network. Here, the kinetic buildup of the phosphorylation signal is a parameter that should not be disregarded. Detailed understanding of the fundamental factors driving selectivity and sensitivity of kinase-substrate interactions is an essential prerequisite. Possibilities to harness the variation in substrate specificity have to be explored to

devise new angles on treatment of disease by means of small molecule inhibitors in the near future.

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Supplementary table 1: Results of the Pamchip kinase activity assays performed with a panel of recombinant tyrosine kinases. The functionally annotated peptides have been highlighted for each kinase.

The full table can be provided upon request.

Example: Src

Peptide	Signal C31	Signal C41	Signal C51	Signal C61	Signal C71	Signal 81	Vini C31-C41
ENOG_37_49	15592.0	25282.5	28268.0	28289.0	28349.5	28702.5	969.1
CD79A_181_193	11790.0	19260.0	23727.5	24977.0	25197.5	25396.5	747.0
EFS_246_258	29028.5	25499.5	24554.0	23955.5	23658.0	24532.0	-352.9
SRC8_CHICK_492_504	8803.0	13181.0	15964.0	17240.5	17978.0	18565.5	437.8
FRK_380_392	10511.0	14310.0	16592.0	17633.5	18045.0	18441.5	379.9
PLCG1_764_776	6817.0	11086.5	13727.0	15682.0	16145.5	16719.0	427.0
RET_1022_1034	5512.5	9014.0	11164.5	12469.0	13011.5	13177.5	350.2
ART_004_EAIYAAPFAKKK	10880.0	12841.5	13047.5	13456.0	12993.5	13088.5	196.2
EPHA1_774_786	4136.5	6554.5	8059.0	8597.5	9366.5	9637.5	241.8
PAXI_24_36	3642.5	6152.0	7274.0	8189.5	8776.5	9172.0	251.0
CDK2_8_20	3921.5	6196.0	7366.0	8066.5	8512.0	9144.0	227.5
SRC8_CHICK_476_488	3340.5	5810.5	7121.5	8061.5	8797.5	9082.5	247.0
FAK2_572_584	4683.0	6684.0	7834.0	8192.5	7961.0	8138.0	200.1
PAXI_111_123	3599.0	5525.5	6445.5	6877.0	7550.5	7927.0	192.7
EPHA7_607_619	3057.0	5123.0	6127.5	6891.0	7194.5	7368.0	206.6
RAF1_332_344	6014.0	6472.0	6978.5	7232.0	7165.5	7156.5	45.8
PGFRB_572_584	2494.0	4579.0	5773.5	6305.0	6804.5	7142.0	208.5
NTRK2_696_708	3842.5	4585.0	5386.5	5689.0	6012.5	5914.0	74.3
FES_706_718	1573.0	3336.0	4191.5	5067.0	5251.0	5868.5	176.3
DCX_109_121	4531.5	5265.0	5575.0	5718.0	5955.5	5837.5	73.4
MET_1227_1239	4503.5	5136.0	5394.0	5387.5	5300.5	5556.0	63.3
P85A_600_612	594.0	1994.0	3081.0	3518.0	3925.0	4138.0	140.0
FER_707_719	871.0	2146.5	2225.0	3063.0	3366.5	3841.5	127.6
PECA1_706_718	2117.5	2695.5	2999.0	3108.5	3514.0	3715.5	57.8
MK10_216_228	2828.0	3217.0	3350.5	3555.5	3461.5	3622.0	38.9
EPHA2_765_777	-536.5	1097.5	1878.0	2789.0	3089.0	3598.5	163.4
RASA1_453_465	2563.5	3101.0	3254.0	3377.0	3524.5	3402.0	53.8
PDPK1_2_14	786.0	1771.0	2372.0	2816.5	3079.5	3247.0	98.5
FAK1_569_581	2423.0	2791.5	3212.5	2724.0	2718.5	2637.0	36.9
TEC_512_524	1465.5	1943.0	1982.0	2154.5	1738.0	2307.0	47.8
JAK2_563_577	1493.0	1811.5	2186.0	2246.5	2367.5	2234.5	31.9
EPHB1_921_933	1684.0	2022.5	2032.5	1730.0	1654.0	2000.5	33.9
EPHB1_771_783	-1881.5	-170.5	631.5	912.0	1654.0	1983.0	171.1
INSR_1348_1360	1990.5	1965.0	1817.5	2087.5	1766.5	1951.5	-2.6
VGFR2_989_1001	-97.0	627.5	787.0	1327.0	1665.0	1780.0	72.5
K2C6B_53_65	810.5	1714.0	2009.0	2204.0	1678.5	1751.0	90.4
VINC_815_827	1445.0	1596.0	1581.0	1542.5	1616.5	1604.5	15.1
PRRX2_202_214	661.0	913.0	1111.0	1388.0	1528.0	1584.0	25.2
EPOR_419_431	-170.5	333.0	965.0	1108.5	1327.0	1554.0	50.4
KSYK_518_530	1488.5	1710.5	1629.0	1284.5	1454.5	1436.5	22.2
ERBB2_870_882	993.0	1110.5	1711.0	1504.5	1342.0	1407.0	11.8
EGFR_1103_1115	-702.5	125.5	-41.0	1090.0	893.5	1305.0	82.8
MK12_178_190	716.0	923.5	-154.5	838.5	1078.5	1209.0	20.8
PGFRB_709_721	-157.5	-353.0	200.5	102.5	1360.5	1176.0	-19.6
MK07_211_223	-192.5	400.5	681.5	587.0	888.0	931.5	59.3
ERBB2_1241_1253	370.0	686.0	1038.0	762.0	400.0	884.5	31.6

Chapter 7

Summary, Discussion & Future Perspectives and Conclusions

Summary

Current pediatric brain tumor treatment protocols comprise surgical resection and when appropriate followed by chemotherapy and radiotherapy. Although the patient survival rates have improved over the recent decades the relative percentage of CNS cancer deaths is rising. This indicates that optimizing current therapeutic options will not suffice in improving treatment outcome. Furthermore, patients often encounter serious treatment related morbidity that can only be overcome by introduction of effective therapeutic alternatives.

In recent years the realization has grown that altered activation of signal transduction pathways drives the malignant process. The individual building blocks of the intracellular cell signaling network are the protein kinases, relaying signals by means of phosphorylating downstream targets. During or after translation modification of proteins can occur on multiple levels such as glycosylation, acetylation, lipid attachment, cleavage of protein segments and phosphorylation. These modifications are ultimately decisive for the nature of downstream events. Particularly the phosphorylation status of cell signaling proteins, which forms the basis of rapid and specific response to extracellular stimuli, is commonly considered decisive in the final common pathway controlling the malignant phenotype. The identification of disease-related signal transduction effectors culminated into the acknowledgement of protein kinases as one of the most important classes of potential drug targets to date. The first clinical successes have been demonstrated approximately a decade ago in the treatment of chronic myeloid leukemia using BCR-Abl inhibitors and, more recently, in cancer resulting from mutations in the canonical nutrient signaling pathway using mTOR inhibitors.[1,2] Despite the successful application of relatively broad spectrum kinase inhibitors for the treatment of specific malignancies, insufficient ways to predict the efficacy of inhibiting specific kinase activity gives rise to a discrepancy between the evaluation of aberrant cell signaling and the currently available ensemble of highly specific targeted treatment strategies. Here, more insight in altered activity of cell signaling mediators that are critical to cell survival and tumor progression is warranted. Hence, techniques that robustly characterize kinase activity in clinical cancer samples greatly appeal. **Chapter 2** provides a comprehensive overview of these techniques and discusses their advantages and

disadvantages for systems biology approaches to identify kinase targets in oncological disease. Recent advances in the development and application of array-based peptide-substrate kinase activity screens show great promise in overcoming the discrepancy between the evaluation of aberrant cell signaling in specific malignancies or even individual patients and the currently available ensemble of highly specific targeted treatment strategies. These developments have the potential to result in a more effective selection of kinase inhibitors and thus optimize mechanism-based patient-specific therapeutic strategies. We conclude from the current status of research on the tumor kinome that generating network views on aberrant tumor cell signaling is critical to meet this challenge. Here, a shift in focus can be observed from the measurement of the phosphorylation status of signal transduction mediators as a surrogate for kinase activation to direct quantitative measurement of kinase activity.

Peptide-based kinase activity profiling has the potential to overcome the heterogeneity in patient response upon kinase-targeted therapies by targeting cell signaling pathways that play a decisive role in tumor progression. The addition of proteomics approaches like reverse phase protein arrays will aid in completing the network view on tumor cell signaling.[3]

To obtain insight in the cell signaling profile of pediatric brain tumors we optimized a peptide-based flow-through microarray approach for application of pediatric brain tumor tissue. The tumor types medulloblastoma, low-grade astrocytoma of the cerebellum (primarily pilocytic astrocytoma) and ependymoma were selected for kinase activity profiling as they represent the most frequently occurring childhood brain tumors. As described in **chapter 3** comprehensive tyrosine kinase activity profiles of pediatric brain tumors were generated using the PamChip kinase activity profiling system. Reproducible kinase activity profiles could be generated that were stable irrespective of the time until cryopreservation of the tumor tissue after surgical resection (< 1 hour). The kinase activity profiles of the different brain tumors showed considerable overlap. A set of 30 of 144 substrates proved to be phosphorylated in >90% of all brain tumor lysates. In addition, tumor specific cell signaling activity could be appreciated. Previously reported activity of epidermal growth factor receptor, c-Met, and vascular endothelial growth factor receptor

(VEGFR) in pediatric brain tumors could be observed in our array results, underscoring the validity of the array results. Peptides corresponding with phosphorylation consensus sequences for Src family kinases showed remarkably high levels of phosphorylation. Src activity was confirmed with Phos-Tag SDS-PAGE. Furthermore, the Src family kinase inhibitors PP1 and Dasatinib induced substantial tumor cell death in nine pediatric brain tumor cell lines and not in control cell lines. Thus, this study describes a new high-throughput technique to generate clinically relevant tyrosine kinase activity profiles as we have shown here for pediatric brain tumors. Furthermore, our data provide the first evidence that Src signaling is an interesting potential target for pediatric brain tumor treatment.

As discussed in chapter 3 we evaluated tyrosine kinase activity in pediatric brain tumor tissue lysates using a peptide microarray. When applied to pediatric pilocytic astrocytoma tissue, this analysis revealed extensive phosphorylation of VEGF receptor-derived peptides. Vascular endothelial growth factor receptor (VEGFR) signaling plays a major part in tumor angiogenesis. Tumor cell survival and progression is substantially enhanced in response to improved angiogenesis. Although total protein expression of VEGF and VEGFRs has been described previously for pilocytic astrocytoma, the presence and localization of active VEGFR signaling activity in these highly vascular tumors still remains subject of much debate. In **chapter 4** we aimed to validate this result and determine the presence of VEGFR-2 activity in pediatric pilocytic astrocytoma as the main VEGF receptor in terms of mitogenic signaling. In addition, the localization of VEGFR1-3 mRNA expression was assessed.

VEGFR-2 phosphorylation was determined adopting a proximity ligation assay approach. Proximity ligation assays (PLA) on tumor cryosections showed the presence of phosphorylation of VEGFR-2, which primarily localized to vascular endothelium.

Enrichment of endothelial markers and VEGF receptors in tumor endothelium was determined by qPCR analysis of laser microdissected blood vessels. This analysis showed a 13.6-fold average enrichment of VEGFR-2 expression in the laser microdissected endothelium compared to whole tumor. Also the expression of VEGFR-1 and -3 was highly enriched in the endothelium fraction with an average fold-enrichment of 16.5 and 50.8 respectively.

In conclusion these data provide evidence for an active VEGFR signaling in pediatric pilocytic astrocytoma, selectively taking place in the tumor endothelium. This suggests a crucial role for VEGF/VEGFR-induced angiogenesis in the progression and maintenance of these tumors.

One of the many intriguing potential targets that appeared in our kinase activity screen was EphR signaling. Activity on Eph receptor derived substrates was observed for each of the three tumor types but was most pronounced applying medulloblastoma tissue lysate. Eph/Ephrin signaling has been implicated in various types of cancer enhancing key processes like migration, proliferation and angiogenesis. Both forward signaling downstream of the receptor as well as reverse signaling downstream of the ligands have been implicated in each of these processes essential to tumor growth. In medulloblastoma invading tumor cells characteristically lead to early recurrence and a decreased prognosis. Based on the kinase activity profiling data described in chapter 3 we hypothesized on a key function of the Eph/Ephrin signaling system in medulloblastoma invasion.

In **chapter 5** the functional relevance of Eph forward signaling in medulloblastoma invasion was assessed. Expression of all Eph receptors and Ephrin-B ligands was observed. Compared to normal cerebellum controls significantly higher expression of the B-type Eph receptor EphB2 and, one of its corresponding ligands, Ephrin-B1 was observed. The A–type Eph receptors EphA3 and -A8 were significantly higher expressed in medulloblastoma as well. Interestingly, in correspondence with the expression data of the medulloblastoma tissue, a number of cell lines demonstrate a consistent overexpression of EphB2. Stimulation of medulloblastoma cells with Ephrin-B1 resulted in a marked decrease in *in vitro* cell adhesion and an increase in the invasion capacity of cells expressing high levels of EphB2. Stimulation with Ephrin-A1 did not result in an observable phenotype. The cell lines which showed an Ephrin-B1 induced phenotype possessed increased levels of phosphorylated EphB2 and, to a lesser extent, EphB4 after stimulation. Interestingly, the activation of EphB2 and –B4 is accompanied by a substantial decrease in EGFR phosphorylation.

Knockdown of EphB2 expression by shRNA completely abolished Ephrin ligand induced effects on adhesion and migration. Analysis of downstream signal transduction identified Akt, Erk, STAT5, Paxillin and p27 as downstream signaling

mediators potentially inducing the Ephrin-B1 phenotype. With the exception of p27, each of these signaling proteins has been implicated in Eph receptor-derived signal transduction previously.[4-7]

The fundamental factors driving selectivity and sensitivity of kinase-substrate interactions still remain largely unresolved. Two opposing schools of thought exist: a first one whose adherents interpret the available data as to suggest that little evolutionary pressure is present determining amino acid sequences in substrates and that specificity of kinases for substrates is mainly regulated at the level of physical interaction of the kinase protein with the targets for its enzymatic action. Here, protein scaffolding makes an important contribution to the selectivity of the kinase-substrate interactions. A second interpretation is that evolutionary pressure forces substrate peptides to acquire high specificity for specific kinase enzymes and low specificity for other kinases, thus coupling the appropriate substrates to the correct enzymatically active kinase.

As described in **chapter 6** we employed the potential of peptide array-based kinase activity profiling in the detection of kinase-protein interactions by kinetically measuring phosphorylation in order to characterize substrate motif specificity within the human tyrosine kinome. We decided to test a sample of kinases from the human tyrosine kinome (10 % of total) against the library of substrate peptides. Receptor tyrosine kinases generally phosphorylated their functionally annotated substrates with high initial phosphorylation velocities (V_{ini}). Tyrosine kinases functioning as downstream signaling effectors like Src and ZAP70 phosphorylated their functionally annotated substrates at highly diverse V_{ini} values. Furthermore, peptide phosphorylation data of downstream operating tyrosine kinases indicated a bimodal distribution in substrate specificity, which could not be confirmed applying receptor tyrosine kinases. These results suggest the presence of a functional dichotomy in substrate phosphorylation where a subset of peptides shows low affinity for tyrosine kinases and for phosphorylation likely depend on scaffolding by other protein domains and another subset displaying high affinity for specific kinases, possibly allowing their phosphorylation even in the absence of a strong physical kinase-substrate interaction.

For two kinase-substrate interactions which presented high initial phosphorylation signal buildup, VEGFR2-APA and InsR-Syk, an in vivo interaction could be appreciated whereas the physical interaction proved to be too weak to detect in a co-immunoprecipitation approach. This indicates that these interactions depend on strict protein recognition of the primary amino-acid sequence around the phosphorylation site.

Based on these data we propose that variability in substrate motif recognition explains the apparent contradictory interpretations of the forces that drive substrate selection. We hypothesize that functionally annotated kinase-substrate interactions, characterized by a strong interaction domain-dependent physical interaction, can elicit a low initial phosphorylation velocity (V_{ini}) on peptide substrates whereas less stringent physical interactions will result in high V_{ini} values due to the retained primary amino-acid specificity.

Discussion and future perspectives

Kinase activity profiling

One of the characteristic features of neoplastic growth is an altered cell signaling, favoring many tumor-promoting processes. Changes in expression and activity of key mediators of signal transduction highly influence the coordination of processes that are essential to tumor development, like proliferation, migration and cell death. These effects result from altered signaling within the tumor cells or through indirect effects on the microenvironment by means of growth factor excretion. Since alterations in cell signaling are ultimately responsible for the tumor phenotype, increasing effort is put in devising strategies to normalize aberrant cell signaling in order to stop the neoplastic growth or force the cells into differentiation.

So far the most successful drugs targeting the tumor cell signaling have been small molecule inhibitors with a relatively broad spectrum of binding targets, thus affecting a variety of cell signaling pathways.[8,9] Although effective, targeting non-essential cell signaling processes is accompanied by adverse effects that result in increased patient morbidity. Thus, specific targeting of those signaling routes that are essential to the growth and progression of the specific tumor can be considered the holy grail of cancer treatment.

Increasing numbers of kinase-targeted small molecules with high specificity are becoming available as we speak. However, insufficient ways to predict the efficacy of inhibiting specific kinase activity gives rise to a divergence between the evaluation of aberrant cell signaling and the currently available ensemble of highly specific targeted treatment strategies. Bringing together these two pillars in novel cancer drug development has escalated into one of the most important challenges in cancer research.

The aim of this thesis was to generate comprehensive insight in the activity of cell signaling mediators in pediatric brain tumors by means of high-throughput screening of kinase activity. The applicability of peptide substrate-based kinase activity screening in an array format to generate comprehensive insight in pediatric brain tumor cell signaling was put to the test. Although many kinase-substrate interaction control mechanisms are lost when applying short amino-acid sequences for kinase substrates, reproducible kinase activity profiles could be obtained. Previously described activity of many critical cell signaling kinases could be confirmed, underscoring the validity of the data. Furthermore, we identified activity of a number of potentially druggable cell signaling mediators.

The most prominent challenge in kinase activity profiling data interpretation proves to be annotating the individual peptides to the upstream kinases potentially responsible for the observed phosphorylation. We decided to run each peptide through the rapidly expanding Phopho-ELM database containing all the currently known and validated kinase-substrate interactions, rendering all currently identified potential upstream kinases of a specific substrate (www.phospho.elm.eu.org). In addition to taking into account all the substrates annotated to a single kinase of interest tremendously boosted the interpretability of kinase activity profiling data. Furthermore we adopted the minimal kinome approach, first described by Diks et al., to select those substrates that were constitutively phosphorylated by >90% of all samples included in the study, thus generating a minimal pediatric brain tumor kinome.[10] These new views on interpreting kinase activity profiling data resulted in the selection of a number of new potential targets for pediatric brain tumor therapy. However, the protein-substrate interactions are controlled by many factors that each contribute to the sensitivity and selectivity of a kinase for its substrates. As described in chapter 6 we observed varying activity on substrates derived from kinases

previously reported to engage in a physical protein interaction with the applied kinase that is strong enough to allow co-immunoprecipitation. This variety indicates that for each individual kinase-substrate interaction the extent in which the primary amino-acid sequence and the tertiary protein structure contributes to the selectivity of the interaction can be tremendously diverging. Possibilities to harness this variability in substrate specificity have to be explored to provide a new angle on treatment of disease. Interestingly, the phosphorylation profiles of downstream operating tyrosine kinases such as Src, ZAP70 and Abl displayed a bimodal distribution whether for receptor tyrosine kinases a unimodal fitting provided a better description. This suggests a fundamental difference in the strategies ensuring sufficient selectivity and sensitivity of the kinase-substrate interactions. Whether this functional dichotomy in peptide substrate phosphorylation holds true for the entire tyrosine kinase pool has yet to be confirmed.

Serine/threonine kinases generally do not employ modular recognition or targeting domains to select their substrates. This suggests that the primary amino acid sequence is more important in these proteins to maintain interaction selectivity.[11,12] Recently the Pamchip platform has been adapted to screen kinase activity on serine and threonine substrates. It would be highly interesting to find out whether the varying affinity for tyrosine kinase motifs is present in these other kinase classes as well.[13]

In our studies, taking into account all substrates representing previously identified phosphorylation sites of the kinase of interest has been shown to improve data interpretation.[14,15] However, obtaining a non-biased annotation for each individual peptide substrate will substantially aid the interpretability of peptide substrate-based kinase activity profiles in future experiments. Generating kinase activity profiles of a larger panel of purified or recombinant kinases promises to provide more detailed insight in which kinases are responsible for specific phosphorylation events. In addition the Pamchip profiling platform allows kinetic readout of the phosphorylation buildup. Chapter 6 underscored the relevance of taking into account the initial reaction velocity in the data interpretation. Detailed understanding of the fundamental factors driving selectivity and sensitivity of protein-protein interactions is an essential prerequisite. Moreover, the effects of specific kinase inhibitors on

peptide phosphorylation in complex protein samples will likely shed more light on peptide specificity.

Therapeutic potential of targeting Src signaling

We decided to further study a number of the potential targets as attained from the kinase activity screen and assess their role in the progression of the various pediatric brain tumor types. Src kinase activity proved to be essential to pediatric brain tumor survival as we observed in cell survival assays upon Src inhibition. Pediatric brain tumor cell survival was compromised at LC50 levels that rival that of many other solid tumor tumors.[16-18] This underscores that targeting Src activity could become a valuable addition to pediatric brain tumor treatment. In vivo validation experiments using pediatric brain tumor mouse models such as the model we established for low-grade astrocytoma, is the next step in assessing the clinical applicability of Src inhibition in general and that of Dasatinib in particular. The intracranial orthotopic low-grade astrocytoma mouse model we recently established provides an ideal opportunity to study the role of Src, as well as the efficacy of Dasatinib in low-grade astrocytoma in more detail. Here, an important factor is obtaining sufficient intracranial concentrations of Dasatinib.

The blood-brain barrier separating the circulating blood and the brain extracellular fluid turns out to be a barrier hard to surpass for many small molecule inhibitors. First phase 1 studies for treatment of intracranial CML, however, indicated that sufficient intracranial concentrations of Dasatinib can be reached without unacceptable adverse effects.[19] Furthermore, inhibitors of P-glycoprotein (P-gp; ABCB1) and Breast Cancer Resistance protein (ABCG2) have shown to be able to enhance the intracranial concentrations of Dasatinib tremendously.[20,21] Possibly co-treatment of Dasatinib with these inhibitors of which Elacridar is currently the most promising, could contribute to reaching intracranial concentrations of Dasatinib that are sufficient for a more effective treatment of pediatric brain tumors and Src sensitive brain tumor in general.[22,23] Besides Dasatinib, application of other inhibitors for intracranial therapy might benefit from ABCB1/ABCG2 inhibition in reaching therapeutic concentrations as well.

VEGFR and angiogenesis

Within our research group Sie et al. described the variation in vascular patterning in glioblastoma, pilocytic astrocytoma and ependymoma. Here, the extensive vascularization that was observed in pilocytic astrocytoma was highly similar to that in high grade gliomas.[24,25] The irregular vessel structure, vessel dilatation, abnormal spacing, decreased/abnormal pericyte coverage and irregular basement membrane structures suggest extensive vessel immaturity and instability. We provided evidence for a selectively vascular expression and activity of VEGFR2 in pilocytic astrocytoma. This highly distinguishes this tumor type from its high-grade counterparts the anaplastic astrocytoma and glioblastoma multiforme. In the latter types, the applicability of anti-angiogenic therapies in general and VEGF-targeted therapy in specific has been subject of intense (pre-)clinical study. The rationale behind targeting VEGF is to establish a normalization of blood-vessel structuring and consequently blood flow, resulting in an increased delivery of concomitantly administered chemotherapeutic agents.[26] Increased intratumoral delivery of chemotherapeutics in response to anti-angiogenic treatment has, however, been disputed.[27,28] Possibly the partly restoration of the blood-brain barrier plays an important role in impairing the delivery of chemotherapeutics in these studies. To complicate the matter even further, recent studies showed that antibody-based anti-VEGF therapy can lead to increased vascular damage instead of vessel normalization eliciting brain hemorrhage.[29-31] The importance of angiogenesis in progression of peditatric pilocytic astrocytoma, however, is becoming evermore clear.[24,25]

Clinical trials assessing the therapeutic value of anti-VEGF therapy in colorectal cancer using the monoclonal antibody Bevacizumab have shown beneficial functional changes.[32] In glioblastoma, anti-VEGF therapy results in vessel normalization, thus decreasing cerebral edema and normalize the functional and structural aspects of the tumor vasculature.[33] Although with a high inter-patient variability, these effects only last several months upon which the abnormal vessel characteristics return. This is suggestive for an altered pro-angiogenic cell signaling mechanism that is independent of VEGFR activation.[34] Maintaining the angiogenic phenotype irrespective of decreased VEGFR signaling activity suggests signaling redundancy. Synergistic maintenance of the angiogenic and neovascularization profile independent of VEGFR signaling has recently been described for FGF and PDGF-BB.[35] Furthermore, inhibition of VEGFR2 signaling has been shown to elicit an increase in expression of

angiopoetins and ephrins.[36] This implicates that multiple pro-angiogenic factors play a part in taking over the angiogenic phenotype after VEGFR inhibition. Combinatorial targeting of multiple pro-angiogenic approaches deserves further research in its ability to overcome resistance to anti-angiogenic therapy. Furthermore, low-dose metronomic chemotherapy in combination with anti-angiogenic agents promises to be a more effective approach in counteracting the proliferation of endothelial cells.[37]

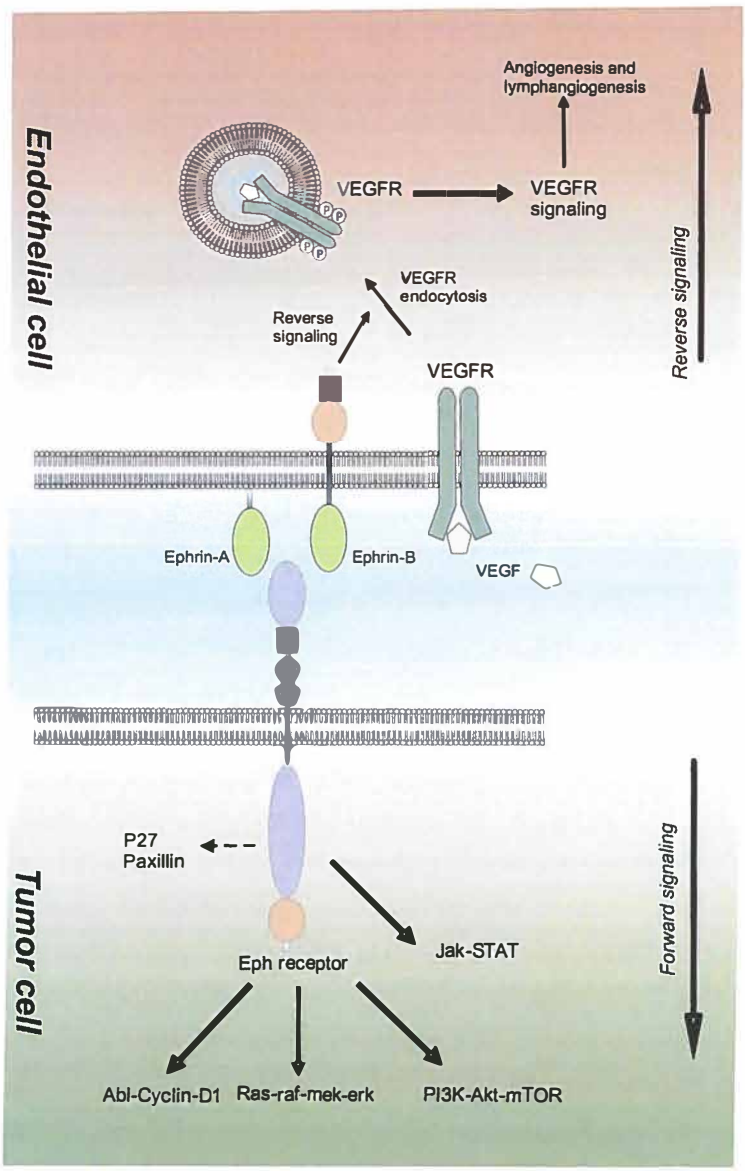

Figure 1: Interplay between Ephrin reverse signaling and VEGF/VEGFR induced (lymph)angiogenesis. Eph-Ephrin signaling can be bi-directional where downstream signaling can be initiated downstream from the receptor, as well as the Ephrin-B ligands. Not until last year the reverse (Ephrin-B) signaling has been implicated in mediating the effects of VEGFR activation. In this thesis an aberrant expression profile of Eph receptors and ephrin ligands implicates involvement of this signaling-family in coordinating the pro-migratory and invasive character of medulloblastoma. Based on the recently established role for Ephrin-B2 in angiogenesis we speculate upon a role for Eph/Ephrin signaling in medulloblastoma angiogenesis as well.

The selectively vascular expression of VEGFR2 we observed in pilocytic astrocytoma illustrates a fundamental difference in the role of VEGF/VEGFR in the onset and progression of tumor angiogenesis. Screening of the vascular expression of angiogenic factors in different tumor types might provide insight in how these differences manifest themselves in a specific vessel signature and how they can be exploited in a therapeutic setting. By means of proximity ligation assays we were able to validate VEGFR2 activity. Laser microdissection allowed us to quantitatively assess the local differences in VEGFR expression. As already posed by Langenkamp et al., assessment of local expression profiles of signal transduction mediators critical for tumor angiogenesis has hitherto not been possible, but now has come within reach.[38] In addition, this approach facilitates generating a better insight in the molecular interactions between tumor and endothelium. Newly developed low-density gene-expression array technology allows simultaneous measurement of mRNA expression of multiple genes of interest, for instance factors involved in coordination angiogenesis in various laser microdissected cell populations. Thus we suggest expanding the laser microdissection study that we employed to study VEGFR expression differences and compare different tumor areas as well as different vessel types with each other.

Laser-microdissection followed by phospho-kinase measurement has recently been successfully executed by means of reverse phase protein arrays.[39] However, laser microdissection technology does not yet allow sufficient preservation of kinase activity during the dissection procedure. Since, not the phosphorylation state but the ultimate activity of cell signaling kinases is decisive in the eventual phenotype we

strongly advocate further development of laser-microdissection technology to facilitate subsequent kinase activity profiling. This will increase our understanding of the molecular basis of tumor vascular heterogeneity as well as of tumor endothelial responsiveness to anti-angiogenic therapies.

Eph/Ephrin signaling in medulloblastoma

Successful tumor growth inhibition by either inhibiting the Eph receptor signaling or stimulating receptors with agonists has been reported previously. This suggests that activation of different Eph receptors can not only result in complementing effects but in opposing effects as well. Literature supports this claim by presenting evidence for tumor-suppressing signaling in response to activation of multiple Eph receptors. The observed deregulation of the Eph/Ephrin expression we observed in medulloblastoma enhances the invasive phenotype, suggesting a potential role in local tumor cell invasion and the formation of metastases. Here, we focused on the role of EphB forward signaling and identified a key role for EphB2. One of the complicating characteristics of Eph/Ephrin signaling is the bi-directional signaling downstream from either the receptor (forward signaling) or the ligand (reverse signaling) (fig. 1). Consequently, studying the relevance of EphB2 signaling on the phenotype of the tumor as a whole should be the next step in assessing the clinical applicability of anti-EphB2 therapy.

Since the Eph receptor family is the largest RTK family described so far, the consequence of tempering with the Eph/Ephrin signaling network is hard to predict. Most likely the Eph receptor resulting in selective forward signaling which is not involved in any tumor-suppressing processes will be the most plausible successful target for therapeutic approaches. Targeting the Eph/Ephrin can, however, elicit effects on the tumor cells and the microenvironment that are not necessarily beneficial from a cancer treatment perspective. Although overexpression of EphA2 is associated with a poor prognosis, increased metastasis and decreased survival, stimulation with Ephrin-A1 has been shown to result in a tumor growth suppressing effect.[40-45] Whether this effect is caused by an increased signaling downstream of EphA2 or it is due to the concomitant decrease in EphA2 is unknown so far. In addition, upregulation of Ephrin-A1 in endothelial cells and consequent activation of EphA2 has been reported to have an important function in the pro-angiogenic effects

of VEGFA and TNFα.[46,47] Thus, the profit that might be obtained from increased tumor suppressor function is bound to be lost as a consequence of enhanced tumor angiogenesis. A similar dichotomy has been reported for EphB2, where an overexpression in gastrointestinal and liver tumors and a functional role in stimulating the invasion of glioblastoma multiforme are conflicting with its tumor-suppressing role that has been suggested in colorectal and prostate cancer.[7,48-53] EphB2 seems to enhance glioblastoma multiforme invasiveness through R-Ras and mobilization of integrins.[7,53,54] Although contradictory results have published, EphB2/EphB3 and EphB4 appear to have contrasting roles in the intestine.[48-50,55-57] Here, tumor-suppressing roles have been described for EphB2 and -B3 while EphB4 signaling enhances tumor growth. Thus, promoting EphB kinase activity, although more challenging, could instead be useful for regenerative medicine. Whether this holds up in medulloblastoma as well is an intriguing concept that should be subject of investigation, preferably in suitable (orthotopic) in vivo models.

A number of gene expression studies identified a distinct molecular subclassification in medulloblastoma.[58,59] In this publically available medulloblastoma microarray data we assessed the Eph receptor expression levels in the individual subclasses and indentified EphB2 overexpression in each subclass. Interestingly, the subclass identified by characteristic Wnt-signaling aberrations has an additional overexpression of EphB4, -A3, -A4 and -A7 that is not detected in the other subclasses. In colon cancer the expression of B-type Eph receptors has already been identified being under control of the Wnt-signaling pathway, thus influencing cell positioning and proliferation.[60] Whether the Eph receptor gene expression differences result in a distinct EphR/Ephrin phenotype in individual medulloblastoma subclasses should be subject of further study.

The small molecule inhibitor Dasatinib previously mentioned as a promising inhibitor of Src also potently inhibits Eph receptors and could therefore be potentially effective in the treatment of tumors with a tumor-promoting Eph/Ephrin phenotype. A beneficial effect of Eph receptor inhibition by Dasatinib has been reported previously for pancreatic cancer.[61] Also the beneficial effect of Dasatinib in the treatment of colorectal cancer has been suggested to derive, at least in part, from Eph receptor inhibition.

Increasing evidence emerges that the various downstream signaling effects, both adverse as well as beneficial, of Eph receptor activation result from selective activation of key signal transduction pathways. Focusing on EphB2, Genander et al. showed that EphB2 regulates migration through PI3K and proliferation through Abl-Cyclin D1 in a strictly selective manner.[62] Although not studied in detail, a proliferative effect of EphB stimulation or EphB2 knockout could not be observed in our studies. This indicates that in medulloblastoma the activation of EphB2 result in selective activation of migration/invasion pathways whereas signaling resulting in cell proliferation is not affected. Amongst others, the PI3K-Akt signaling activity is modulated by altered EphB2 activity in our study as well. Recently, co-immunoprecipitation of EphA2 and EGFR has been observed in multiple cell lines.[63] In our study the stimulation of medulloblastoma cells with Ephrin-B1 resulted in a substantial decrease in EGFR activity. Possibly the observed Ephrin-B1 phenotype we observed in medulloblastoma is (partly) derived from altered EGFR activity. Co-immunoprecipitation studies should clarify whether EphB2 and EGFR engage in a physical interaction or whether these effects are established indirectly.

Culturing of medulloblastoma cell lines in the presence of demethylating agents resulted in an increased EphB receptor expression. Promoter methylation levels of EphB receptors, however, did not show an increased Eph promoter methylation, indicating that epigenetics does not play a direct role in the regulation of Eph expression. In a pilot experiment we observed that the expression of various EphRs is more prominently increased upon inhibition of histone acetylation *in vitro*. Whether this properly reflects the situation in patient material remains to be addressed.

Interplay between Eph/Ephrin and VEGFR signaling

The function of Ephrin ligands we identified in mediating medulloblastoma cell motility has been reported for the motility of endothelial cells as well.[64] As for VEGF, HIF-1a has been implicated in regulating the expression.[65] Despite these clear indications that VEGF and Ephrin induced cell signaling are intertwined in regulating cell migration as well as angiogenesis, clear links between these pathways had yet to be discovered. Recently, Ephrin-B2 reverse signaling has been implicated in VEGFR-induced angiogenesis and lymphangiogenesis, for the first time presenting evidence

for direct interplay between Eph/Ephrin and VEGF/VEGFR signaling (fig. 1).[66,67] Sawamiphak et al. present evidence for a role of Ephrin-B2 reverse signaling in VEGF-induced VEGFR2 activation while Wang et al. describe the same phenomenon for VEGFR3. The suggestion was made that the effects of Ephrin-B2 on VEGFR2 signaling are mediated by a PDZ-binding protein.[66] Since we reported an overexpression of one of the complementary receptors EphB2 in medulloblastoma this might indicate concomitant stimulation of the angiogenic phenotype in medulloblastoma as well. Medulloblastomas are generally highly vascular CNS tumors. Whether the extent of vascularization is a prognostic factor for outcome is subject of debate, as illustrated by conflicting results.[68,69] Nonetheless, a recent publication does suggest that anti-angiogenic therapy improves survival in recurrent/refractory medulloblastoma.[54] The pro-migratory phenotype that results from EphB2 activation could therefore potentially be accompanied by effects on angiogenesis.

As mentioned previously, the beneficial effects of anti-VEGF therapy are often of only transient nature and may result in drug resistance and increased cell migration.[70] Thus, the identification of other key signaling proteins mediating these processes might provide alternatives in the development of anti-angiogenic strategies. Ultimately, the complexity of the angiogenic signaling network calls for more detailed tumor-specific insight in the effects of Eph and Ephrin on the tumor as well as the microenvironment to allow potential application as a target for anticancer therapy. Here, in vivo loss of function studies have to be performed. The Ephrin-B2 mouse model recently applied in dissecting the role of Ephrin-B2 in astrocytoma angiogenesis has high potential in serving as a pragmatic continuation of studying Eph/Ephrin function in tumor progression.[66]

Conclusions

The selection of cell signaling mediators playing a decisive role in carcinogenesis and maintenance of malignancy has been identified as an essential prerequisite in overcoming the heterogeneity in patient response to tumor- and patient-specific kinase-targeted therapeutic strategies. This thesis describes the optimization of the kinase activity profiling protocol and data processing to generate comprehensive insight in pediatric brain tumor kinase activity and cell signaling network. Based on the obtained kinase activity profiles a number of potentially druggable targets for

therapeutic options were selected. Subsequent in vitro studies provided valuable insights in their roles in pediatric brain tumor progression. Here, active Src signaling was found to be essential to pediatric brain tumor survival. Selectively vascular expression and activity of VEGFR2 was observed in pilocytic astrocytoma. Aberrant EphR expression and activity proved to be of critical importance in mediating medulloblastoma invasion. In vivo validation of these new insights in aberrant pediatric brain tumor signal transduction should be pursued to establish the therapeutic applicability of these targets.

The kinetic readout of peptide substrate phosphorylation provided new insights in the basic factors determining the selectivity of kinase-substrate interactions within the cell. Here, a high diversity in the relevance of the primary amino-acid sequence in determining substrate specificity urges more study of the fundamental factors driving selectivity and sensitivity of protein-protein interactions in order to establish effective and specific kinase targeting.

Altogether, array-based kinase activity profiling provided invaluable and comprehensive insight in the pediatric brain tumor signal transduction network. Although substantial challenges in optimizing peptide substrate annotation and data interpretation remain, further implementation of kinase activity-based tumor characterization studies should become a focal point in the optimization of mechanism-based anticancer therapeutic strategies.

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Chapter 8

Nederlandse samenvatting

Nederlandse Samenvatting

In de afgelopen decennia is er aanzienlijke vooruitgang geboekt in de 5-jaars overleving van patiënten met een tumor aan het centraal zenuwstelsel. Desondanks steeg het aandeel patiënten dat overlijdt aan een hersentumor ten opzichte van het totaal aan overlijdensgevallen ten gevolge van kanker bij kinderen. Dit wijst erop dat optimalisatie van de huidige behandelstrategieën niet afdoende is in het verbeteren van het behandelingsresultaat. Daarnaast krijgen patiënten vaak te kampen met aanzienlijke morbiditeit ten gevolgde van de behandeling, wat enkel opgelost kan worden door het introduceren van effectievere alternatieven. Nieuw inzicht in ziekte-gerelateerde signaal transductie activiteit hebben geresulteerd in het erkennen van veranderde activering van signaaltransductieroutes als drijvende kracht achter het ziekteproces. De afzonderlijke bouwstenen van het intracellulaire signaal transductie netwerk zijn de eiwit kinases, die signalen doorgeven door middel van het fosforyleren van downstream eiwitten.
Ongeveer een decennium geleden zijn de eerste klinische successen met kinase remmers behaald in de behandeling van chronische myeloïde leukemie door gebruik te maken van BCR-Abl remmers en, meer recent, in kanker ten gevolge van mutaties in de canonieke nutriënt signaal transductie route in de vorm van mTOR remmers. Ondanks de succesvolle toepassing van kinase remmers met een relatief breed spectrum in de behandeling van specifieke vormen van kanker zijn er onvoldoende middelen om de te verwachten doeltreffendheid van het inhiberen van specifieke kinase activiteit te bepalen. Dit leidt tot een discrepantie tussen het vaststellen van afwijkende signaal transductie en het groeiende ensemble van specifieke kinase remmers en vereist meer inzicht in veranderende activiteit van signaal transductie mediatoren van essentieel belang voor cel overleving en tumor progressie. Technieken die op een robuuste manier kinase activiteit in klinische kanker weefselmonsters kunnen karakteriseren hebben daarom een grote aantrekkingskracht.

Doel van het onderzoek
Onderzoek naar nieuwe therapeutische aangrijpingspunten voor het tegengaan van tumor progressie verschuift van het bepalen van absolute expressieniveaus naar

directe meting van eiwit kinase activiteit. De doelstelling van het onderzoek beschreven in dit proefschrift was het genereren van inzicht in de activiteit van eiwitten betrokken bij de cel signalering in kinderhersentumoren. Hierbij is gebruik gemaakt van een high-throughput analysemethode voor het bepalen van kinase activiteit. Validatie experimenten zijn vervolgens uitgevoerd om de functionele relevantie van een aantal potentiële therapeutische aangrijpingspunten te bepalen. Ons uiteindelijke doel is het identificeren van eiwitten betrokken bij de cel signalering die van essentieel belang zijn voor kinderhersentumor progressie.

Samenvatting

Hoofdstuk 2 geeft een overzicht van technieken die in staat zijn kinase activiteit te kunnen karakteriseren en bediscussieert hun voor- en nadelen voor toepassing als strategie voor het identificeren van kinase aangrijpingspunten in de oncologie. Recente voortgang in de ontwikkeling en toepassing van array gebaseerde peptide-substraat kinase activiteits screening methoden lijken veelbelovend in het overbruggen van de discrepantie tussen het vaststellen van afwijkende signaal transductie in specifieke maligniteiten of zelfs individuele patiënten en de momenteel beschikbare hoog-specifieke gerichte behandelstrategieën. Deze ontwikkelingen resulteren potentieel in een meer effectieve selectie van kinase remmers om zo de patiënt-specifieke en op het mechanisme gebaseerde behandelstrategieën te kunnen optimaliseren. Een verschuiving in de focus van het meten van de fosforylatiestatus van signaal transductie mediatoren als maat voor kinase activering naar rechtstreekse kwantitatieve meting van kinase activiteit kan worden waargenomen. Centraal staat hierbij het in ogenschouw nemen van het signaal transductie netwerk als geheel.

Om inzicht te verwerven in het signaal transductie profiel van kinderhersentumoren hebben we een flow-through peptide-miroarray techniek geoptimaliseerd voor toepassing op kinderherentumor weefsel. De tumor typen medulloblastoma, laaggradig astrocytoma van het cerebellum (voornamelijk pilocytair) en ependymoma werden geselecteerd voor de kinase activiteits profilering aangezien ze de meest frequent voorkomende hersentumortypen bij kinderen vertegenwoordigen. Zoals beschreven in **hoofstuk 3** konden interpreteerbare tyrosine kinase activiteits profielen van kinderhersentumoren worden gegenereerd door gebruik te maken van

het PamChip kinase activiteits profilering systeem. De kinase activiteitsprofielen van de verschillende hersentumoren lieten aanzienlijke overlap zien. Een set van 30 uit het totaal van 144 substraten werd gefosforyleerd door >90% van alle hersentumor lysaten. Bovendien kon tumor-specifieke signaal transductie activiteit worden waargenomen. Eerder in kinderhersentumoren aangetoonde activiteit van de epidermale groeifactor receptor (EGFR), c-Met en de vasculaire endotheliale groeifactor receptor (VEGFR) kon worden bevestigd. Peptides die corresponderen met fosforylatie consensus sequenties voor Src-familie kinases lieten hoge fosforylatiewaarden zien. Src kinase activiteit kon worden bevestigd door middel van Phos-Tag SDS-PAGE. Bovendien induceerden de Src familie kinase inhibitoren PP1 en Dasatinib aanzienlijke tumor celdood in negen kinderhersentumor cellijnen.
Wanneer toegepast op pilocytair astrocytoma weefsel onthult de high-throughput PamChip kinase activiteits profilering techniek een sterke fosforylatie van VEGFR-afkomstige peptiden. Vasculaire endotheliale groeifactor receptor signalering speelt een grote rol in tumor angiogenese. Tumor cel overleving en tumor progressie wordt substantieel versterkt ten gevolge van verbeterde angiogenese. Hoewel totaal eiwit expressie van VEGF en VEGFRs eerder is beschreven voor pilocytaire astrocytomen wordt er nog sterk gedebatteerd over de aanwezigheid en lokalisatie van VEGFR signaal transductie activiteit. In **hoofdstuk 4** is met behulp van proximity ligation assays aangetoond dat VEGFR-2 activiteit aanwezig in pilocytaire astrocytomen. Door de bloedvaten en tumorcellen van elkaar te scheiden met behulp van laser microdissectie kon door middel van qPCR worden aangetoond dat de VEGFR2 expressie vrijwel volledig toe te schrijven is aan de endotheel cellen waaruit de bloedvaten in de tumor zijn opgebouwd. Deze resultaten doen vermoeden dat een actieve VEGF/VEGFR signaal transductie een cruciale rol speelt in de angiogenese binnen deze tumoren maar niet, zoals bij hooggradige astrocytomen, in autocriene stimulatie van tumorgroei.

Hoofstuk 5 beschrijft een studie naar de functionele relevantie van signaal transductie downstream van Eph de receptor in medulloblastoma invasie. Expressie van alle Eph receptoren en Ephrin-B liganden kon worden waargenomen in een cohort van primaire medullobastomen. Vergeleken met normaal cerebellum brachten de tumoren de B-type receptor EphB2 en z'n corresponderende ligand

Ephrin-B1 significant hoger tot expressie. Ook de A-type receptoren EphA3 en –A8 kwamen significant hoger tot expressie. Stimulatie van medulloblastoma cellen met Ephrin-B1 resulteerde in een afname in *in vitro* cel adhesie en een toename in het vermogen tot migreren bij cellen die EphB2 hoog tot expressie brengen. Stimulatie met Ephrin-A1 resulteerde niet in een veranderend fenotype. De cellijnen met een Ephrin-B1 induceerbaar fenotype bezaten verhoogde aanwezigheid van gefosforyleerd EphB2 en, in mindere mate, EphB4 na stimulatie. De activering van EphB2 en –B4 ging gepaard met een afname in EGFR fosforylatie. Het terugschroeven van de EphB2 expressie middels shRNA resulteerde in het voorkomen van de Ephrin geïnduceerde effecten op adhesie en migratie. Analyse van de downstream signaal transductie wijst op mogelijke betrokkenheid van Akt, Erk , STAT5, Paxilline en p27 bij het induceren van het Ephrin-B1 fenotype. Met uitzondering van p27 is elk van deze eiwitten eerder in verband gebracht met Eph receptor-afkomstige signaal transductie.

De factoren die ten grondslag liggen aan de selectiviteit en sensitiviteit van kinase-substraat interacties zijn grotendeels nog niet bekend. Er bestaan twee tegengestelde theorieën. Een eerste wiens aanhangers de beschikbare data interpreteren als een suggestie dat selectiedruk slechts in beperkte mate de aminozuurvolgorde in kinase substraten bepaald en dat kinase specificiteit voor substraten wordt gereguleerd op het niveau van fysieke interactie tussen de kinase en het substraat eiwit. Eiwit scaffolding levert hier een belangrijke bijdrage aan de selectiviteit van de kinase-substraat interactie. Een tweede interpretatie suggereert dat selectiedruk de substraat peptiden dwingt tot een hoge mate van specificiteit voor upstream kinases en lage specificiteit voor andere signaal transductie eiwitten om op die manier de koppeling tussen kinase en substraat correct tot stand te brengen.

Zoals beschreven in **hoofdstuk 6** hebben we het potentieel van de peptide array-gebaseerde kinase activiteits profilering gebruikt om helderheid te verschaffen in kinase-substraat interacties door de fosforylatiesnelheid van peptide substraten te meten om zo de specificiteit in substraat-motief te karakteriseren. We hebben besloten om een aantal kinases (10% van totaal) te testen tegen een bibliotheek van 144 substraat peptiden. Over het algemeen fosforyleerden receptor tyrosine kinases hun functioneel geannoteerde substraten met een hoge initiële fosforylatiesnelheid

(V_{ini}). Downstream opererende kinases zoals Src en ZAP70 fosforyleerden hun functioneel geannoteerde substraten met sterk variabele V_{ini} waarden.
Peptide fosforylatie data van downstream functionerende kinases gaf tevens een indicatie voor een bimodale verdeling in substraat specificiteit. Dit kon niet worden geconcludeerd voor receptor tyrosine kinases. Deze resultaten suggereren een functionele tweedeling in substraat fosforylatie waarbij een subset van peptiden een lage affiniteit voor tyrosine kinases laten zien en voor fosforylatie waarschijnlijk afhankelijk zijn van ondersteuning door andere eiwit domeinen en een andere subset die juist een hoge affiniteit voor specifieke kinases laat zien, wat fosforylatie mogelijkerwijs zelfs mogelijk maakt in afwezigheid van een sterke fysieke interactie tussen kinase en substraat.
Voor twee kinase-substraat interacties die zich presenteerden met een hoge initiële toename van fosforylatie, VEGFR2-APA en InsR-Syk, kon een in vivo interactie worden aangetoond terwijl deze interactie te zwak is om te detecteren in een co-immunoprecipitatie experiment. Dit is een aanwijzing dat deze eiwitinteractie in sterke mate afhankelijk is van herkenning op basis van de primaire aminozuur volgorde in de nabijheid van het te fosforyleren aminozuur.
Gebaseerd op deze resultaten doen we de suggestie dat variatie in substraat motief herkenning de schijnbaar tegengestelde interpretaties van de krachten die substraat selectie bepalen verklaart. We poneren de hypothese dat functioneel geannoteerde kinase-substraat interacties, gekarakteriseerd door een sterke interactiedomein-afhankelijke fysieke interactie, een lage initiële fosforylatiesnelheid op peptide substraten kunnen bewerkstelligen terwijl minder stringente fysieke interacties zullen resulteren in hoge V_{ini} waarden ten gevolge van een behouden primaire aminozuur specificiteit.

Conclusies

De selectie van signaal transductie eiwitten van doorslaggevende betekenis in de carcinogenese en het onderhouden van maligniteit is een essentiële voorwaarde om de heterogeniteit in respons op tumor- en patientspecifieke therapeutische strategieën gericht tegen eiwit kinases het hoofd te kunnen bieden. Dit proefschrift beschrijft de optimalisatie van het kinase activiteitsprofilering protocol en data verwerking teneinde begrijpelijk inzicht te krijgen in kinderhersentumor kinase

activiteit en het signaal transductie netwerk. Gebaseerd op de verkregen kinase activiteitsprofielen konden een aantal potentiële therapeutische targets worden geselecteerd. *In vitro* studies gaven vervolgens meer inzicht in hun specifieke rol in kinderhersentumor progressie. Actieve Src signaal transductie bleek essentieel voor kinderhersentumorcel overleving. Selectieve vasculaire expressie en activiteit van VEGFR werd waargenomen in pilocytaire astrycytomen. Afwijkende EphR expressie en activiteit bleek van doorslaggevend belang in medulloblastoma invasie. *In vivo* validatie van deze nieuwe inzichten in afwijkende kinderhersentumor signaal transductie zal moeten volgen om vast te stellen of deze targets geschikt zijn voor therapeutische toepassing. Het uitlezen van de snelheid van peptide substraat fosforylatie heeft nieuw inzicht verschaft in de factoren die ten grondslag liggen aan het bepalen van de selectiviteit van kinase-substraat interacties in de cel. De hoge mate van diversiteit in de relevantie van de primaire aminozuursequentie in het bepalen van de substraat specificiteit is aanleiding voor meer onderzoek naar de factoren die bepalend zijn voor selectiviteit en gevoeligheid van eiwit-eiwit interacties om zo een effectieve en specifieke kinase targeting te bewerkstelligen.

Samenvattend heeft array-gebaseerde kinase activiteits profilering waardevolle inzichten verschaft in het signaal transductie netwerk van kinderhersentumoren. Hoewel er nog aanzienlijke uitdagingen blijven in het optimaliseren van de peptide substraat annotatie en data interpretatie zou verdere implementatie van tumor karakterisering op basis van kinase activiteit de spil kunnen vormen in het optimaliseren van op mechanisme gebaseerde therapeutische strategieën voor de behandeling van kanker.

Appendix

Abbreviations
Contributing authors
Publications
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Abbreviations

AA	Amino acid
ABCB	ATP-binding cassette sub-family B
ABCG	ATP-binding cassette sub-family G
Abl	c-abl kinase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AMPK	5' adenosine monophosphate-activated protein kinase
APA	Aminopeptidase A
ATP	Adenosine triphosphate
BRCA	Breast cancer 1
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDK	Cyclin-dependent kinase
CLU	Clusterin
CML	Chronic myelogenous leukemia
CNS	Central nervous system
c-Src	Cellular Src kinase
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EGL	External granular layer
Eph	Ephrin receptor
ErbB	Epidermal growth factor receptor family
Erk	Epidermal growth factor receptor (EGFR) family kinases
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FCS	Fetal calf serum
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDP	Guanosine diphosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPI	Glycosylphosphatidylinositol
GTP	Guanosine triphosphate
Her2	Human epidermal growth factor receptor 2
HGF	Hepatocyte growth factor

Abbreviations

HUVEC	Human umbilical vein endothelial cells
IGF	Insulin growth factor
InsR	Insulin receptor
Jak	Janus kinase
KAYAK	Kinase activity assay for kinome profiling
LC50	Lethal concentration, 50%
LMD	Laser microdissection
MAPK	Mitogen-activated protein kinases
MMP	Matrix metalloproteinase
M-PER	Mammalian protein extraction reagent
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mTOR	Mammalian target of rapamycin
NaOH	Sodium hydroxide
P-gp	P-glycoprotein
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PET	Positron emission tomography
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLA	Proximity ligation assay
PLC-γ	Phospholipase C gamma
PNET	Primitive neuroectodermal tumor
PP1	4-amino-5-(4-methylphenyl)-7-(tbutyl)pyrazolo[3,4-d]pyrimidine
PSAP	Prosaposin
PTEN	Phosphatase and tensin homolog
P-Tyr	Phospho-tyrosine
PVDF	Polyvinylidene fluoride
pY	Phospho-tyrosine
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RAF1	RAF proto-oncogene serine/threonine-protein kinase
RCA	Rolling circle amplification
RET	RET proto-oncogene
Rheb	Ras homolog enriched in brain
RIPA buffer	Radioimmunoprecipitation assay buffer
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute medium
RPPA	Reverse phase protein array
RSE	Relative standard error of mean

RT	Room temperature
RTK	Receptor tyrosine kinase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate – polyacrylamide gel electrophoresis
SF	Scatter factor
Shh	Sonic hedgehog
shRNA	Short hairpin ribonucleic acid
Smo	Smoothened
STAT	Signal transducers and activators of transcription
Syk	Spleen tyrosine kinase
TNFa	Tumor necrosis factor alpha
TSAd	T-cell specific adapter protein
Tsc	Tuberous sclerosis complex
UEA1	Ulex europaeus agglutinin-1
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
V_{ini}	Initial phosphorylation velocity
WHO	World health organization

Appendix

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