Targeting tumor vasculature

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Summary

Targeting tumor vasculature is the common theme for the three separate studies presented in this thesis. The studies comprise the roles of components of three signaling pathways (networks) in several steps of blood vessel formation, and related tumor growth and dissemination. With our first study, we wanted to elucidate the role of selected Wnt signaling members in *in vitro* and *in vivo* angiogenesis. The aim of the second study was to reveal the advantages of simultaneous targeting of all of the constituents of the VEGFR family over the sole inhibition of VEGF and VEGFR2. Finally, in our third study we wanted to delineate the role of ErbB2 signaling mediator Memo in tumorigenesis, vasculogenesis and metastatic abilities of 4T1 mouse mammary carcinoma cells.

Wnt signaling is highly conserved signaling pathway involved in several developmental processes and regulation of adult tissue homeostasis. Accumulating data from mouse knock out and *in vitro* angiogenesis studies indicate its role in blood vessel formation. To decipher the role of Wnt signaling components in angiogenesis, we used human umbilical vein endothelial cells (HUVEC), and well vascularized Lewis lung carcinomas grown in mice, as our *in vitro* and *in vivo* models, respectively. In our *in vitro* studies, we checked for the abilities of recombinant Wnt3a, a member of the family considered to activate only canonical Wnt pathway, and Wnt5a, so called non canonical Wnt, to induce HUVEC proliferation, migration and survival. We found that Wnt3a is a novel proangiogenic factor with the ability to induce HUVEC proliferation and migration, but without an effect on their survival. The effects were mediated through the common Wnt downstream effector protein Dishevelled. Proliferation induced by Wnt3a is VEGFR signaling independent. Wnt5a did not show an effect on any of those processes, but it activated signaling, demonstrated by Dishevelled phosphorylation. To test the role of Fz6, a member of Wnt receptor family repeatedly shown to be expressed on vasculature from different tissues, in *in vivo* angiogenesis, we injected Fz6 knock out mice and their wild type littermates with Lewis lung carcinoma cells. The ablation of Fz6 did not have an effect on the kinetics of tumor growth, quantity nor appearance of tumor vasculature.

While the knowledge about the role of Wnt signaling in angiogenesis is only emerging, the importance of vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) in vessel formation is well documented. In our work, we wanted to test the effects of simultaneous inhibition of VEGFR1, VEGFR2 and VEGFR3 in comparison to inhibition of VEGFR2 or VEGF solely, on tumor growth and spread. We found that targeting all three VEGFRs is more effective in inhibition of functionality of the lymphatics surrounding the primary tumor and subsequent

metastatic spread, than targeting VEGFR2 or VEGF alone. Moreover, we show that B16/BL6 melanoma cancer cells display an *in vitro* autocrine VEGF/VEGFR signaling loop whose inhibition chemosensitizes them to platinum based chemotherapeutics.

ErbB2 receptor tyrosine kinase belongs to the family of epidermal growth factor receptors. It is overexpressed in 20% of human breast cancers, and its expression correlates with highly metastatic disease and poor clinical outcome for patients. In a screen for ErbB2 effectors, Memo, a novel signaling protein that is mediating migration induced through ErbB2 and several other receptor tyrosine kinases, was previoulsy identified in our lab. To test the role of Memo in cancer growth, we made use of 4T1, a highly metastatic mouse mammary carcinoma cell line. Downregulation of Memo via stable transfection of shRNA in these cells decreased *in vivo* primary tumor growth when compared to control cells. The inspection of tumor vasculature revealed that tumors derived from Memo knock down (KD) clones were less vascularized, which could possibly explain their growth delay. To delineate the mechanisms behind decreased vascularization, we measured the concentration of secreted VEGF in the medium of control and Memo KD clones. Our results show that Memo KD clones secrete less VEGF into the medium than control clones, indicating that this might relate to the impairment in vascularization of tumors derived from these cells. To check the role of Memo downregulation in metastasis formation, we injected control and Memo KD clones into the tail vein of BALB/c mice. We found that Memo downregulation decreases the metastatic behavior of these cells. In conclusion, with this study, we describe the effects of Memo downregulation on several aspects of tumorigenesis in mouse mammary carcinoma model. Further studies are needed to decipher the signaling pathway that Memo is a part of, and that is responsible for the described effects on primary tumor, its vascularization and metastasis formation.

T A B L E O F C O N T E N T S

I. INTRODUCTION

Targeting tumor vasculature

The era of anticancer chemotherapy began in the first part of the last century with the use of nitrogen mustards and antifolate drugs in the treatments of lymphoid tumors [1]. Ever since, the strategy to directly target cancer cells was the major chemotherapeutic approach in anticancer treatment.

However, in 1971, Judah Folkman proposed that inhibition of angiogenesis (targeting tumor endothelial cells) could be an effective way to treat human cancer [2]. The growth of tumor tissue is highly dependent on blood supply. Moreover, endothelial cells are not displaying high mutation rates like cancer cells do and they are more reachable to drugs than cancer cells. For these and other arguments, tumor endothelium seemed to be a reasonable target in anticancer treatments. This idea gave rise to a new research field that aimed to identify molecules and pathways responsible for blood vessel formation. Among them, the family of vascular endothelial growth factors (VEGFs) and their receptors (VEGFRs) were recognized as central players in physiological and pathological angiogenesis. An extensive work in the field led to the creation and the recent (2004) FDA (Food and Drug Administration, USA) approval of Avastin (Bevacizumab, Genentech), a humanized monoclonal antibody against VEGF for the first line treatment of patients with colorectal carcinoma in combination with 5-fluorouracil (5-FU) based chemotherapy [3]. Avastin was the first drug on the market designed specifically to target angiogenesis. From then on, the use of Avastin was approved for several other types of human cancer, and two new agents targeting VEGF and VEGFR signaling network are used in clinics in anticancer treatments.

Lymphatic system, although not that significant for the primary tumor growth, was shown to be a major promoter of tumor cell spread from the site of origin. Metastatic spread of tumor cells is the underlying cause of most cancer related deaths, and this is the reason why a lot of effort is being currently made to understand the molecular mechanisms governing formation of tumor associated lymphangiogenesis and cancer cell dissemination.

While the contribution of VEGF and VEGFR family members in the process of vascular formation is indisputable, the research field is focusing on the identification of other pathways and factors that play a role in several steps of vessel formation.

In further chapters, I will present the Wnt signaling and its role in angiogenesis, give a glimpse into the role of VEGF and VEGFR signaling in vessel formation, and finish with the brief overview of ErbB2 signaling and its mediator Memo in breast cancer cell biology. These are meant to be the introductory chapters to the research part presenting the role of components of Wnt signaling in angiogenesis, the efficacy of novel VEGFR targeting kinase inhibitor, and the role of Memo in tumor growth, vascularization and dissemination.

1. Wnt signaling in angiogenesis

1.1. Wnt signaling

1.1.1. Timeline

The initial studies on Wnt signaling originate from separate work in the fields of developmental genetics using fruitfly *Drosophila melanogaster* as a model to trace developmental phenotypes to their genetic cause, and an attempt from cancer research to understand the mechanism by which mouse mammary tumor virus (MMTV) induces tumorigenesis.

In 1973 *Drosophila* '*wingless*' (*Wg*) phenotype (mutant lacking wings) was detected in progeny of flies irradiated by X-rays [4]. Later on, in a separate genetic screen for mutations that are affecting segmentation pattern during *Drosophila* embryonic development, Nüsslein-Volhard and Wieschaus showed that the *Wg* mutations are causing in addition the loss of naked cuticle portion of abdominal segments in *Drosophila* embryo, where a fraction of each segment was deleted and replaced by the mirror image duplicate of the remaining part [5] [6]. Subsequently, a work from the same lab and others identified mutations in *armadillo* (*arm*, β-catenin in vertebrates), *dishevelled* (*dsh*) and *porcupine* (*porc*), causing similar segment polarity defects as seen in Wingless mutants; mutations in *zeste white 3/shaggy* (*zw3*, encodes glycogen synthase kinase 3β) caused opposite phenotypes (reviewed in [7]). Additional studies from the laboratories of Perrimon, Nusse and Wieschaus showed that these segment polarity genes are all components of the same transduction pathway at present known as the canonical Wnt pathway [8]. Moreover, analysis of epistatic relations among *arm*, *dsh*, *porc* and *zw3* from the same studies using the appearance of abdominal segments as readout ('all naked' vs. 'all denticle') revealed the sequence of events in the pathway.

In parallel to work from the field of developmental genetics that dissected relations in the Wnt pathway through loss of function mutations in large-scale genetic screens, an opposite approach (gain of function mutations) led to discovery of the Wnt gene in mammals. In the 1970s and 1980s tumor viruses were studied intensively in the field of cancer research because of their ability to infect and transform non malignant mammalian cells. In 1982 in their attempt to explain the tumorigenic properties of MMTV, Roel Nusse and Harold Varmus found that very frequently mammary tumors caused by this virus contained the insertional site of the virus in the same region of the host genome. The insertion was activating the expression of an unknown gene that they named *Int1* (integration 1) [9]. Five years later the work from Nusse and Lawrence laboratories showed that *Int1* is mammalian homologue of *Drosophila Wg* gene [10] [11]. Hence, the term Wnt was coined as an amalgam of *Wg* and *Int* [12].

Together with studies using *Drosophila* and mice as research models, another significant contribution to our understanding of Wnt signaling comes from the field of *Xenopus leavis*

embryogenesis. In 1989 Randall Moon's laboratory showed that injection of vertebrate Wnt1 mRNA into early *Xenopus* embryos results in duplication of the body axis and twin-headed embryos [13]. Subsequently, axis duplication in frog embryos was used as an assay to characterize components of the Wnt pathway (similar to above mentioned segment polarity assays in *Drosophila*) and to reveal novel biochemical interactions between these components. As an example, the central biochemical event of a canonical Wnt signaling pathway – β-catenin interaction with transcription factors LEF-1 and TCF – was confirmed in studies using injection of LEF-1 and XTcf-3 (maternally expressed *Xenopus* homolog of the mammalian transcription factors Tcf-1 and Lef-1) mRNAs into *Xenopus* embryos [14], [15], [16].

Taken together, listed experiments from *Drosophila* and *Xenopus* embryogenesis, and mammalian viral oncogenesis, show high conservation of components and signaling steps of Wnt canonical pathway among species, and confirm the concept reported elsewhere that the same set of signaling pathways are regulating development and induce cancer when mutated [17], [18].

1.1.2. Ligands

1.1.2.1. Comparative genomics and evolution

Wnts are a highly conserved, metazoan-restricted family of secreted signaling proteins comprising 19 members in humans, 12 in Zebrafish, 7 in *Drosophila* (for a detailed overview of Wnt signaling components across different organisms see [19]).

In vertebrates, Wnt genes are categorized in twelve distinct subfamilies defined by criteria of forming paralogous groups (originating by duplication) that contain orthologous Wnt genes (originating by speciation) [20].

The majority of human *Wnt* genes share about 35% of amino-acid sequence identity which is higher in case of members of the same subfamily (from 58% to 83%). Members of a subfamily are, however, not closely linked within the genome suggesting that they were generated by larger genome rearragements (genome duplications or gene translocations) [21].

In vertebrates, the orthologs in different species are highly similar in sequence. For example, human WNT1 and mouse Wnt1 are 98% identical and human WNT5a and *Xenopus* Wnt5a are 84% identical at the amino-acid level [21]. The sequence identity between orthologous proteins in humans and flies ranges between 21% to 42%, while the evolutionary relationship between human and the five *Caenorhabditis elegans* Wnt genes is less apparent [21].

The finding from phylogenetic analyses on Wnt genes that came as a surprise was that Wnt family diversification happened early on during evolution, before the Cambrian explosion, which was the time of intense divergence within metazoan subkingdom resulting in the appearance of most of the major metazoan taxonomic groups [22]. The unexpected insight

came from the analysis of Wnt genes presence in the sea anemone *Nematostella vectensis* which contains nearly all of the Wnt genes found in vertebrates. *N. vectensis*, a species belonging to cnidarians, was shown to display eleven of the twelve Wnt subfamilies [23] arguing that at least eleven of twelve Wnt gene subfamilies must have already been present as early as before the divergence of cnidarians and bilaterians. The staggered expression along the oral-aboral axis of Wnt genes during *Nematostella* embryogenesis ('the Wnt code', [23]), suggests an ancient and primary role of Wnts in gastrulation and axial differentiation, the function that is taken over by *hox* genes in bilaterians (the *hox* expression cluster or 'the *hox* code', that is present in bilaterian embryogenesis is missing during cnidarian development) [24]. Of a note, besides of the rich Wnt gene repertoire found in *Nematostella*, the studies from the same organism and related cnidarian *Hydra* show that other components of canonical and non canonical Wnt pathways (Wnt/planar cell polarity and Wnt/Calcium pathways, introduced in further chapters of the thesis) were already present in these ancient organisms [24].

In conclusion, the findings from cnidarian genomes show that highly branched Wnt gene family and the plentitude of Wnt pathways already existed in organisms with simpler body plans, much before the emergence of complex bilaterian morphological and anatomical constitution in whose patterning Wnt signaling, together with several other core developmental pathways, plays the major role.

In contrast to many *Wnt* genes present in *Nematostella* genome, there are fewer Wnts both in nematode and a fly (5 Wnt genes in *C. elegans* and 7 in *Drosophila*) than in their common ancestor Urbilateria, which exemplifies the major gene loss that occurred in the protostome lineage of bilaterian evolution [20]. So, although many of the *Wnt* genes already existed in a genome of lower organisms, they were apparently lost in some of their more developed descendants showing that there is no simple correlation between complexity of an organism on the morphological and genetic level.

1.1.2.2. Regulation of Wnt expression

1.1.2.2.1. Regulation at the transcriptional level

The most of the studies on *Wnt* transcription regulation were performed in mice analyzing the interference with Wnt1 effects on central nervous system (CNS) patterning. Expression of Wnt1 is one of the earliest signals associated with CNS patterning, and targeted disruption of this gene in mice causes severe abnormalities in midbrain and cerebellar development [25], [26]. The cis-acting regulatory region of *Wnt1* was shown to be a 5.5 kilobase (kb) enhancer 3' of the coding region [27]. Within this enhancer, an evolutionary conserved 110 base pair (bp) region was found to be sufficient to activate lacZ reporter in the *Wnt1* pattern at neural plate stages [28]. Since then, many efforts have been invested to detect transcription factor(s)

binding to these regulatory sequences and activating the expression of *Wnt1*. Pax-2, a paired domain containing transcription factor, and Cubitus interruptus (Ci), a transcription regulator involved in hedgehog (hh) signaling in *Drosophila*, were suggested as candidates that bind *Wnt1* regulatory region based on expression studies, but until now, there is no direct evidence of interaction between these factors and *Wnt1* gene elements [29].

While the activators of *Wnt1* expression have not yet been identified, there are several proteins known to repress *Wnt1* expression, like Emx2, a homeodomain transcription factor, and Six3, a member of the *Six* family of homeobox genes [29].

1.1.2.2.2. Alternative splicing and post-transcriptional regulation

The majority of human *WNT* genes contain four coding exons that transcribe mRNA that is translated into proteins approximately 350 amino acids long, ranging in molecular weight from 39 kDa (Wnt7a) to 46 kDa (Wnt10a) [21], [30]. All Wnts contain 23 to 25 conserved cysteine residues important for proper protein folding and multimerization (Figure 1a). Several members of the family appear in different splicing variants, an example of which is shown in Figure 1b.

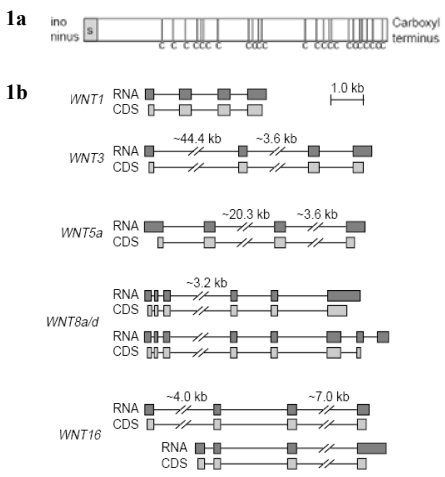


Figure 1a Structural features of the Wnt protein. The amino terminus contains a signal sequence (S) that mediates interactions with Wnt receptors, but carboxyl terminus was shown to be required for activation of these receptors [31]. All Wnts contain 23 to 25 conserved cysteine residues (C) with similar spacing, suggesting that the folding of Wnt proteins depends on the formation of multiple intramolecular disulfide bonds.

Figure 1b Structures of selected members of the human *WNT* gene family. Exons are shown as boxes and introns as lines. For each gene, 'RNA' represents the portion of the gene that is transcribed and 'CDS' represents the portion that encodes protein. *WNT8a/d* is an example of a gene with 3' alternative splicing and *WNT16* is an example of a gene with alternatively used 5' exons. Adapted from [21].

As mentioned before, members of the Wnt gene family play significant roles during development, and therefore tight regulation of their expression in space and time is of a vital importance for an organism. As an example, transient expression of *Xenopus Wnt8* (*Xwnt8*) in ventral and lateral mesoderm during gastrulation is required for muscle development, but its concomitant downregulation following gastrulation is necessary to prevent overcommitment of these cells to a myogenic fate at the expense of scleroteme formation [29]. The negative regulation of the expression of *Xwnt8* is achieved at the level of RNA stability and translation, and is mediated by the 3'-untranslated region (3'-UTR) of *Xwnt8* [32].

1.1.2.2.3. Post-translational modifications

Unlike the many growth factors that were identified and purified as soluble fractions that promote cell growth in cell culture, Wnts were discovered in genetic screens, as described in previous chapters (1.1.1.). The reason behind this is the fact that Wnts are not freely soluble and therefore are difficult to purify [33]. The primary protein structure of Wnts contains a signal sequence for secretion, several highly charged amino-acid residues and many potential glycosylation sites. According to the primary amino-acid sequence, the Wnt proteins were expected to be soluble. In contrast, due to the post-translational modifications, Wnts turned out to be highly hydrophobic. Within the endoplasmic reticulum (ER), immature Wnts undergo *N*-glycosylation whose significance for signaling, targeting or secretion of the protein has not been deciphered yet. More importantly, murine Wnt3A and *Drosophila* Wg were shown to be post-transcriptionally palmitoylated at a highly conserved cysteine residue [33], [34]. While it was shown that this lipid modification is necessary for Wnt function, the role that it plays is still not completely clear. It was suggested that Wnt palmitoylation may protect the modified cysteine from forming a disulfide bond, preventing the abberant folding of the protein [30]. A palmitate group can also act as intracellular signal that allows the proper routing of the protein through, for example, compartments of the Wnt-producing cells where Wnts acquire their signaling potential; or to lipid rafts, the hydrophobic signaling domains within the cell membrane [30], [35]. Furthermore, it was shown that palmitoylation of Wnt proteins is needed for their *N*-glycosylation probably by serving to anchor the proteins to the ER membrane in close proximity to the oligosaccharyl transferase (OST) complex that is glycosylating Wnts. Finally, hydrophobicity due to palmitoylation could be important after secretion for targeting Wnt to membranes of receiving cells, and for their association with the receptor [30], [35].

Of the proteins that might be involved in Wnt palmitoylation (glycosylation as well), the ER membrane-bound *O*-acyltransferase (MBOAT) Porcupine is the most likely candidate [30], [35].

1.1.2.3. Secretion and gradient formation

After being translated, Wnt proteins move through the ER and trans-Golgi network (TGN) within the producing cell to acquire the post-translational modifications described above (chapter 1.1.2.2.3.). There are indications that, following the exit from TGN, Wnt proteins might be secreted through regulated secretion route, contrary to unregulated, 'default' secretory pathway. For example, a fraction of *Drosophila* Wg has been found in the endosomal compartment as well as in recycling vesicles [36]. It is not clear, however, whether this endocytic part of Wg has been retrieved from the surface, or it enters endosomes directly during the secretion process. Another indication that Wnts might be secreted through

alternative secretory route comes from the fact that Wg has been found to associate with lipid rafts, which are, besides serving as signaling platforms within the cell membrane [37], domains that can partition proteins into specialized sorting and secreting routes [38]. Recently, the multipass transmembrane protein Wntless (Wls) located at the Golgi and cell surface membranes, but shown to be present on endosomes as well, has been implicated in Wnt secretion. Lack of Wls results in inhibition of the Wnt transport to the surface of a producing cell, and its concomitant intracellular accumulation [39], [40]. Most probably Wls acts as a cargo receptor assisting Wnt to reach the site from which it gets released. The other Wnt regulator that was discovered to be important for its secretion is retromer, a conserved multiprotein complex composed of members of the Vps family [41], [42]. The current hypothesis based on the recent work from several groups is that retromer serves to retrieve Wls from endosomal compartments to the *trans*-Golgi network after its internalization from the plasma membrane [43], [44], [45], [46], [47], reviewed in [48]. In this way retromer prevents Wls degradation in lysosomes and makes it ready for further rounds of Wnt transport.

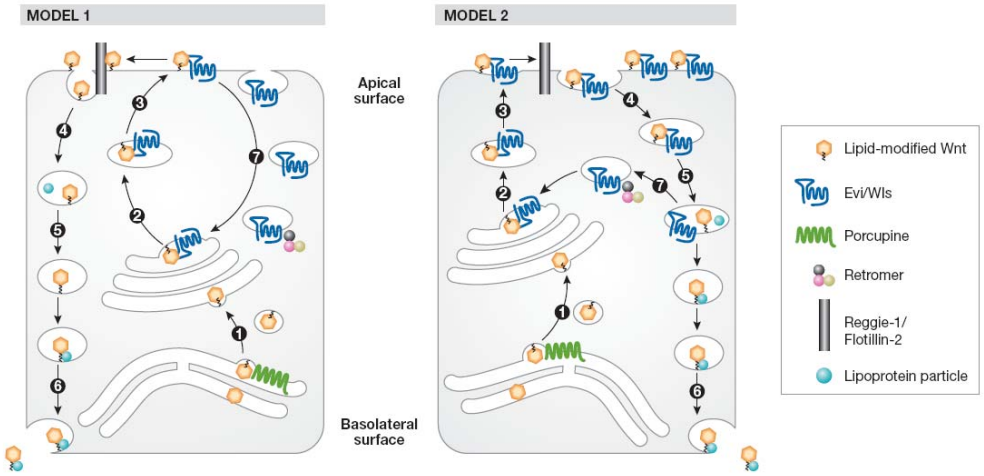


Figure 2. Models of Wls-dependent Wnt secretion. Wnt is lipid-modified in the endoplasmic reticulum by Porc (step 1) and travels to the Golgi where it binds to Wls (step 2), facilitating its delivery to the apical plasma membrane (step 3). In model 1, Wnt and Wls dissociate on the plasma membrane. Wnt associates with Reggie-1/Flotillin-2 containing microdomains, and is internalized (step 4). In endosomal compartments, Wnt is loaded on lipoprotein particles by an unknown mechansim (step 5), and is released from the basolateral surface for long-range signalling (step 6). In model 2, Wnt and Wls are internalized together (step 4), and dissociate in endosomal compartments (step 5). There, Wnt is loaded on lipoprotein particles and released from the basolateral surface (step 6). In both models, Evi is recycled to the Golgi in a retromer-dependent manner (step 7). Note that, in both models, membranebound Wnt signals from the apical membrane to induce short-range targets, whereas the long-range concentration gradient forms on the basal surface. Evi, Evenness interrupted; Porc, Porcupine; Wls,Wntless. Adapted from [48].

A different mechanism has been proposed for Wnt extracellular gradient formation that enables these proteins to bind receptors and signal several cell diameters away from the secreting cell. It has been speculated that loading of Wnts to lipoprotein particles which might serve as vehicles for the movement, as well as their association with protein Reggie-1/Flotillin-2 are important for their distant signaling [30], [49], [48]. The regulation and processes involved in Wnt trafficking are summarized in Figure 2.

1.1.3. Receptors

The first *fz* gene was found in genetic screens for regulators of tissue polarity in *Drosophila* [50], [51]. However, due to the functional redundancy among members of the Fz family in this model, the connection with Wnt signaling was realized only later on, after several other downstream components of the pathway and their relations were already described [52].

Human and mouse genome contain ten, *Drosophila* four and *C. elegans* three *fz* genes. Human *fz* genes code for sequences ranging from 537 to 706 amino acid residues. Analysis of *fz* primary sequence shows high homology to *smoothened* (*smo*), another conserved *Drosophila* gene whose product is involved in transducing Hedgehog signaling [53]. In addition, Fz- and Smo-like receptors have been identified in the social amoeba *Dictyostelium discoideum*, despite the absence of Wnt and Hedgehog genes in its genome, implying an interesting aspect on the evolution of Wnt signaling [54].

Fz are 7 transmembrane spanning (7-TMS) receptors exhibiting extracellular N-terminus with cystein rich domain (CRD) important for ligand binding or accumulation (Figure 3.). The *N*-terminus is also a site of *N*-glycosylations whose biological relevance is unknown. Intracellular domain of human Fz-1, -2, -4, -5, -7, -8 and -10, but not those of Fz-3, -6, and -9, contain the C-terminal motif 'Ser/Thr/-Xxx-Val' which constitutes a binding motif for proteins with a PDZ domain [55].

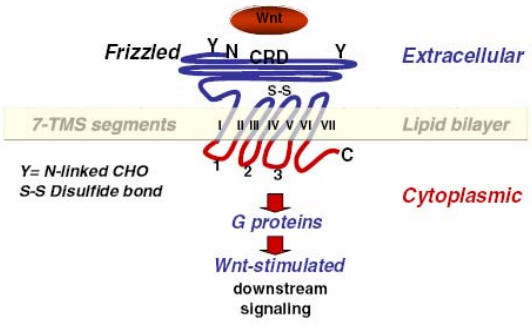


Figure 3. Schematic depiction of Frizzled structure. The three domains of a receptor are the extracellular, the 7 transmembrane spanning segments, and the cytoplasmic domain. The seven transmembrane segments (labeled in Roman numerals I–VII) of the receptor constitute the lipid bilayer domain.
A conserved disulfide bridge (S–S) occurs between two extracellular loops. The intracellular loops (labeled 1, 2, and 3) and the C-terminal tail of a GPCR constitute the cytoplasmic domains that are intimately involved in signal propagation to heterotrimeric G-proteins. Adapted from [55].

Fz receptors belong to the superfamily of G protein-coupled receptors (GPCRs, reviewed in [56]) with which they share many structural similarities, including 7-TMS region and the organization of extra- and intracellular segments. The general functional feature of GPCRs is the activation of trimeric G proteins that consist of the GDP/GTP bound alpha subunit (G_α) and the tightly associated beta and gamma subunits ($G_{\beta\gamma}$). Upon activation of a receptor its intracellular domain serves as guanine nucleotide exchange factor (GEF) that is catalyzing the exchange of G protein bound GDP for GTP, which results in dissociation of a heterotrimeric protein into the activated G_α^{GTP} and $G_{\beta\gamma}$ subunits. Activated subunits from both G_α and $G_{\beta\gamma}$ families are able to transduce signaling to downstream components.

Besides sharing the structural features of GPCR family, accumulating data indicate the involvement of G proteins in some instances of signaling downstream of activated Fz receptors. The first evidence for the requirement of G protein subunits $G_{\alpha 0}$ and $G_{\alpha q}$ for signaling via Fz1 was provided by the lab of Craig Malbon and Randall Moon. The authors used pertussis toxin (ptx) and antisense oligodeoxynucleotides to uncouple specific G protein subunits from the receptor activation in mouse F9 teratocarcinoma stem cells. Upon blockade of $G_{\alpha 0}$ and $G_{\alpha q}$ subunits, the effect of Wnt8 acting through Fz1 on the formation of primitive endoderm by mouse F9 teratocarcinoma cells was suppressed [57]. In another study Katanaev et al expressed ptx or cholera toxin under the eye specific promoter to target G protein subunits in *Drosophila* eye. Blockade of $G_{\alpha 0}$ by ptx suppressed the appearance of the eye phenotype induced by ectopic expression of Fz [58]. Several other strategies like the use of chimeric receptor consisting of portions of the beta adrenergic receptor and C terminus of rat Fz1 [59], or the overexpression of human Fz1 and Fz2 in yeast and the interference with G protein regulated mating pathway [60], all indicate that in some cases G proteins are involved in signaling by Fz receptors [61].

It is considered that G_{α}^{GTP} subunit could potentially act through axin, a scaffold protein involved in Wnt canonical signaling (described in further chapters); or, in the case of other Wnt pathways, the interaction with phospholipase C γ (PLCγ) or the tubulin cytoskeleton are possible [54]. The interaction of $G_{\beta\gamma}$ subunit with Dishevelled protein (Dvl, introduced in further chapters) was shown by Randall Moon lab [62], suggesting that $G_{\beta\gamma}$ subunit, which stays at the plasma membrane after trimer dissociation, could recruit Dvl from the cytoplasm and mediate its activation.

However, the evidence for direct role of Fz receptors as GEF is still lacking. At the moment, there is a consensus in literature that it is probable that both G protein dependant and independent signaling can occur downstream of Fz receptors [54].

1.1.4. Non conventional ligands and receptors in Wnt signaling

Besides Wnt and Fz proteins, several other unrelated molecules are involved in signaling as initiators or transducers of the Wnt signal. The structures of known ligands, inhibitors, receptors and co receptors participating in Wnt signaling are shown in Figure 4.

Norrin is a secreted protein with a cystein-knot motif, unrelated to Wnt, but with the ability to activate the β-catenin pathway through Fz4 and LRP5. Norrin is encoded by the Norrie disease gene and is essential for vascular development in the eye and ear. The Norrie disease is an X-linked human congenital retinal disease caused by mutations in *norrin* gene and manifested as blindness because of the incomplete vascularization of the peripheral retina [63]. More detailed description of Norrin action during eye and ear vascularization is provided in chapter 1.2.3.

R-spondin is protein with a thrombospondin type I repeat domain. It was shown to be required for Wnt dependent myogenesis in *Xenopus* [64], and its role in angiogenesis has been demonstrated recently [65], (chapter 1.2.3.).

Several natural occurring inhibitors of Wnt signaling are known, and their downregulation, as described in chapter 1.1.6., has been detected in some cancers. The five sFRP family members are antagosists that directly bind to Wnts preventing them from interaction with Fzs, or inhibit the signaling by forming nonfunctional complexes with Fzs. It is thought that during development sFRPs control morphogenesis gradients of Wnt activity.

The Dkk family comprises four members all of which inhibit Wnt signaling either by binding to low-density lipoprotein receptor-related protein 5/6 (LRP5/6, see below) thus preventing Fz-LRP complex formation, or by promoting endocytosis and removal of LRP5/6 from the cell surface membrane. Dkk was shown to be essential for *Xenopus* embryos head formation [64].

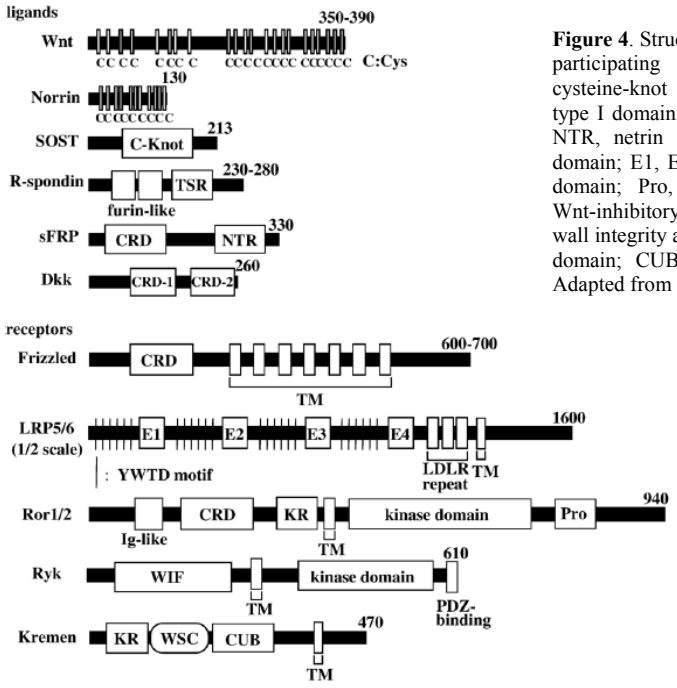


Figure 4. Structures of ligands and receptors participating in Wnt signaling. C-Knot, cysteine-knot motif; TSR, thrombospondin type I domain; CRD, cysteine rich domain; NTR, netrin domain; TM transmembrane domain; E1, EGF-like domain; KR, kringle domain; Pro, proline-rich domain; WIF, Wnt-inhibitory factor domain; WSC, cell wall integrity and stress response component domain; CUB, C1q-Uegf-BMP-1 domain. Adapted from [64].

In addition to Fz proteins described in previous section, receptors from LRP family as well as transmembrane receptors Ror, Ryk and Kremen, are participating in Wnt signal reception.

LRP5, LRP6 and their *Drosophila* homologue Arrow are a type I single-pass transmembrane proteins belonging to a subfamily of the low density lipoprotein (LDL) receptor family, whose members play important roles in metabolism and development (reviewed in [66]). LRP5/6 and Arrow appear to be specifically required as coreceptors for Wnt/β-catenin signaling as discussed in chapter 1.1.5.2.2.

1.1.5. Heterogeneity of signaling pathways initiated by Wnts

The complexity of Wnt intracellular signaling pathways parallels the complexity observed in the heterogeneity of Wnt ligands and receptors. Signaling cascades initiated by Wnts include the Wnt β-catenin pathway, usually referred to as 'Wnt canonical pathway', Wnt planar cell polarity (PCP) and Wnt-calcium pathways. The existence of several other pathways stimulated by Wnts has been reported recently [67].

1.1.5.1. Dishevelled phosphorylation as a common step in Wnt signaling

The first known downstream effect of Wnt binding to its receptor that seems to be shared among all Wnt signaling branches is Dvl phosphorylation and subsequent activation. Dvl is a cytoplasmic modular protein consisting of 500 to 600 amino acids. There are three Dvl homologs in human and mice but only one Dvl has been identified in *Drosophila* [68]. The mechanism by which Dvl gets recruited to receptor upon initiation of signaling, as well as the nature of 'activated' Dvl and the role of phosphorylation in it, are unknown. It was reported that Fz can directly bind to PDZ domain of Dvl, but with weak affinity [69]. Casein kinase-1ε (CK1ε) [70] and -2 (CK2) [71], as well as PAR1 [72] have been implicated in Dvl phosphorylation. Protein kinase C δ (PKCδ) was shown to be important for its localization and activity in a branch of noncanonical Wnt pathway [73].

As mentioned, Dvl phosphorylation was shown to mediate β-catenin and PCP pathways [74], as well as Wnt-calcium pathway [75]. However, the mode of Dvl action is different depending upon which pathway is being initiated because different domains of the protein are involved [68].

1.1.5.2. Wnt β-catenin pathway

1.1.5.2.1. β-catenin 'destruction complex'

In the absence of activated Wnt β-catenin signaling, the transcription co-factor β-catenin is binding to its 'destruction complex' that consists of axin, adenomatous polyposis coli (APC), glycogen synthase kinase-3β (GSK-3β) and casein kinase-1α (CK1α). Axin is a cytoplasmic scaffold protein which provides a platform for binding of β-catenin, APC and kinases. It has been demonstrated in a recent work that assembly of a destruction complex is a highly cooperative event that involves the recruitment of components via indirect interactions with other bound partners, which results in robustness of a process of complex formation [76]. In a complex, CK1α is a 'priming' kinase whose phosphorylation of β-catenin on Serine 45 (Ser45) residue precedes and is required for subsequent GSK-3 phosphorylation of Ser33, Ser37, and Threonine 41 (Thr41) residues, positioned at the amino terminal region of the protien [77]. Phosphorylated β-catenin gets ubiquitinylated by E3 ubiquitin ligase β-TrCP (β-transducin repeat containing protein; or its mouse homolog FWD1), and targeted for

proteasomal degradation [77], [78]. It has been recently shown that the role of APC in a complex is to protect phosphorylated sites of β-catenin from the action of phosphatases, before ubiquitination takes place. In the absence of functional APC, Ser/Thr protein phosphatase 2A (PP2A) cleaves unprotected phosphate groups and inhibits β-catenin degradation [79].

1.1.5.2.2. Dissasembly of 'destruction complex' upon pathway activation

In addition to binding of a Wnt to Fz, for a Wnt β-catenin signaling to take place, the presence of a coreceptor LRP5/6 is required. Binding of Wnt to Fz and LRP5/6 causes the formation of their ternary complex [80].

Two groups have demonstrated that upon ternary complex formation, CK1γ and GSK-3β phosphorylate LRP6 at Thr and Ser residues respectively [81], [82], [83]. For phosphorylation by CK1γ at Thr residue to take place, aggregation of LRP6 in so called signalosomes at the plasma membrane is required. Formation of LRP6 signalosomes was shown to be Dvl dependent [84]. The phosphorylation of LRP6 at Ser residue by GSK-3β is axin dependent event and requires the recruitment of a complex formed by Dvl-Axin-Gsk3β [85]. It was recently shown that formation of phosphatidylinositol 4,5-bisphosphates [PtdIns (4,5)P2] by action of frizzled and disheveled on phosphatidylinositol-4-kinase type IIα and phosphatidylinositol-4-phosphate 5-kinase type I (PIP5KI), is needed, but not sufficient for phosphorylation of LRP at both residues [86]. Phosphorylation of cytoplasmic tail of LRP6 and recruitment of Axin to activated co receptor causes disassembly of β-catenin 'destruction complex'.

The experiments mentioned above indicate the crucial role of Axin in regulation of the Wnt β-catenin signaling, predicted by some earlier reviews [87]. Unlike signaling via receptor tyrosine kinases (RTKs, presented in other sections of the thesis), where signal gets amplified by action of receptor as a catalyst on downstream components, the removal of a negative regulator Axin in Wnt signaling seemes to imply stoichiometric rather than a catalytic mechanism of signal transduction [83]. To test whether amplification step takes place during Wnt β-catenin signaling as well, Baig-Lewis et al used a chimeric Frizzled2-Arrow protein containing the intracellular portion of Arrow (homolog of mammalian LRP) in *Drosophila* that lack endogenous Wg and Arrow. The authors showed that the rescue of Wnt signaling by fusion protein in these flies was only partial indicating that Arrow not only plays an essential role as a Wg coreceptor, but also may function downstream of signal initiation to amplify the initial Wg signal [88]. Indeed, the extracellularly truncated dimerizing version of Arrow strongly amplified Wnt signaling in a corresponding developmental context, but without an ability to initiate it [88].

This argues that amplification of Wnt signal takes place *in vivo*, and that this process is separated (conveyed by different domains of Arrow) from signal initiation (Figure 5) [88], [89]. The amplification function of Arrow/LRP appears to be particularly important for distal signaling, and may reflect a general mechanism by which cells detect low levels of diffusible molecules such as may occur in the distal part of a morphogen gradient [88].

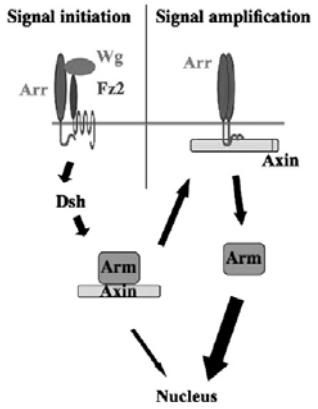


Figure 5. A two-step model for Wg signal transduction. The Wg receptor complex consisting of Arrow and Frizzleds initiates the cytoplasmic cascade in response to Wg binding. Dishevelled transmits this response to the Axin complex and may destabilize the complex sufficiently to transduce a minor signal to the nucleus, via free Armadillo. For maximal signaling, Axin relocates to the cell membrane, where it interacts with clustered Arrow/LRP and becomes inactivated. Armadillo may then be released from Axin and newly synthesized Armadillo is no longer destroyed, resulting in amplified transcriptional activation in the nucleus. Adapted from [88].

Upon disassembly of 'destruction complex', β-catenin is freed, accumulates in cytoplasm and enters the nucleus in a process that requires Rac1 activation. Rac1 is a protein from Rho family of GTPases and its role in β-catenin translocation was shown to be dependent on phosphorylation of β-catenin at S191 and S605, which is mediated by JNK2 kinase [90].

1.1.5.2.3. β-catenin action on transcription

In the nucleus, β-catenin associates with T-cell factor/lymphoid enhancer factor (Tcf/Lef) transcription regulators. There are four *tcf/lef* genes in a vertebrate genome whose target sites on DNA are highly conserved. In the absence of β-catenin, Tcf acts as a repressor of gene transcription by forming a complex with Groucho/Grg/TLE proteins. Upon activation of the signaling, translocated β-catenin physically displaces Groucho from Tcf/Lef and induces transcription from various target genes, including *c-myc*, *cyclinD1*, *fra1*, *c-jun* etc.

In addition to inducing Tcf/Lef and acting as a transcriptional activator on its own, β-catenin was shown to bind coactivators that affect the local chromatin, like the histone acetylase CBP and Brg-1 that are a component of the SWI/SNF chromatin remodeling complex.

Other β-catenin nuclear partner, Legless/Bcl9 and Pygopus were found in *Drosophila* genetic screens. The formation of a trimer between Legless/Bcl9, Pygopus and β-catenin has been implicated in nuclear import/retention of β-catenin and transcription [91], [92], [93], [94].

An interesting view on regulation of transcription by Wnt β-catenin comes from the Martinez Arias laboratory. In their reviews, the authors argue in favor of existence of hierarchy between core developmental pathways Hedgehog (Hh), Bone morphogenetic proteins (BMPs), Wnt, Steroid hormone receptor, Notch and RTKs, in transcription during cell fate

specification. To filter the noise that is inherent to every biological system (and so it is to transcriptional networks that guide cell transitions) the authors hypothesize the existence of initiation and stabilization phases of gene transcription during cell transitions. Because of the ability of Wnt proteins to spread over large cell populations, the interactions of β-catenin with the chromatin remodeling complexes, and the fact that in some cases it was shown that Wnt β-catenin signaling is involved in maintenance rather than initiation of transcription, the Wnt β-catenin would be, they hypothesize, a candidate for developmental transcription noise filtering pathway (together with Notch) [95], [96].

1.1.5.3. Wnt Planar cell polarity pathway

Organization of cells in apical-basolateral and planar planes are of a crucial importance for a tissue to act as a unit. The involvement of Wnt pathway in planar cell polarity (PCP) was realized early on with discovery of *Drosophila fz* and *dvl* mutants. Flies deficient for these genes display disoriented cuticular hair and bristles (Figure 6a-b), as well as disordered ommatidia and photoreceptors in the eye [50], [97], [98], [99]. A similar phenotype of disoriented fur/hair was described in *fz6* deficient mice [100], [101], Figure 6c-d.

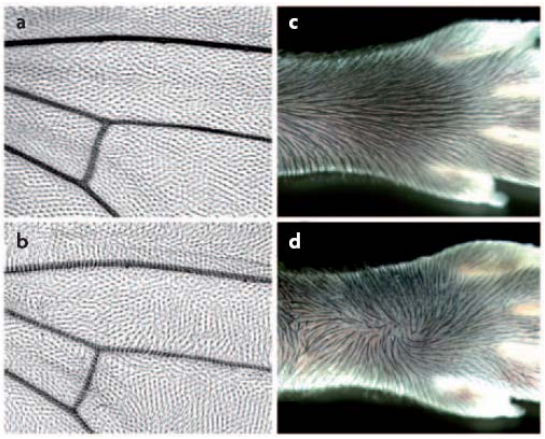


Figure 6. PCP and the organization of tissues. Proximal-distal orientation of hairs on appendages in *Drosophila melanogaster* and mouse. **a** Wing cells of *D. melanogaster* generate an actin hair that points distally in wild-type cells. **b** Mutations in planar cell polarity (PCP) genes disrupt this orientation and instead, wing hairs create swirls and waves (a fz mutant is shown). **c**,**d** The pattern of mammalian fur/hairs, similar to *D. melanogaster* wing hairs, is regulated by frizzled (Fz)/PCP signalling. In fz mutant animals, hairs do not point uniformly distally but appear in swirls and waves (a wild-type and a Fz6 mutant mouse paw are shown in **c** and **d**, respectively). Adapted from [102]

In case of *Drosophila* hair and bristles, planar cell polarity stems from antagonizing distribution of components of Fz/Fmi core PCP proteins within individual hair producing cells. The components of the Fz/Fmi system include Fz and Flamingo (Fmi, also known as Starry night/Stan), Dishevelled (Dsh; Dvl in mammals), Prickle (Pk), Strabismus/Van Gogh (Stbm/Vang) and Diego (Dgo; Diversin and Inversin in vertebrates). In *Drosophila* pupal wing cells the mentioned proteins are asymmetrically distributed, with Fz-Dsh-Dgo complexes enriched at the distal end of each cell, and Stbm-Pk complexes localizing proximally (Figure 7., reviewed in [103]). Fmi, an atypical cadherin thought to provide a homophilic adhesion function, is evenly distributed at both ends of the cell. An actin based hair will always form at a distal side of each cell, where Fz and Dsh are localized. A similar

pattern of PCP components distribution has been recently described in mammalian hair germs [104], [105].

It is thought that coordinated polarization within the plane of cells arises from intercellular communication between Fz, Fmi, and Vang [106].

Besides regulating hair follicle orientation in the skin, the planar cell polarity pathway was shown to govern vertebrate convergent extensions, neural tube closure, eyelid closure, hair bundle orientation in inner ear sensory cells etc.

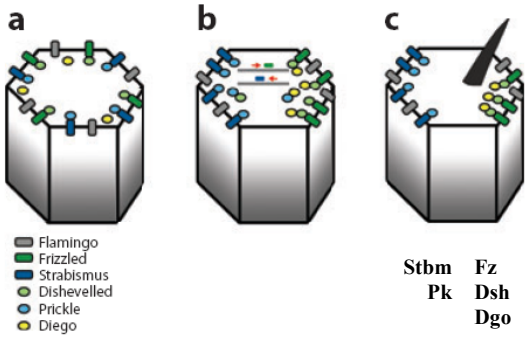


Figure 7. Schematic presentation of the generation of asymmetric core PCP protein localization in *Drosophila* wing cells. In pupal wing cells, the core PCP proteins of the Fz/Fmi cassette become asymmetrically localized to proximal and distal cell membranes. Proximal is left and distal is right in all panels. A single cell at different stages maturing from left to right is shown in the respective panels *a*–*c*. (*a*) Schematic of the localization of the core PCP proteins prior to any asymmetry detection at the onset of their interactions. (*b*) During polarization the Fz-Dsh-Dgo complexes become enriched at the distal end of each cell, whereas the Stbm-Pk complexes concentrate proximally. (*c*) Final stage of polarization with all complexes resolved in either distal or proximal cell ends. An actin-based hair is formed close to the distal vertex of each cell (where Fz-Dsh are localized). Adapted from [103].

The Wnt pathway initiated during planar cell polarity is schematically outlined in Figure 8b. Until now, PCP ligand for Fz has not been found, but in mammals Wnt5a and Wnt11 have been shown to have a permissive role [101], [103]. Upon Fz activation the signal is transduced via Dvl to downstream members of Rho family of GTPases, Rho and Rac whose action through RhoA/Rho kinase (ROCK) and Jun-N-terminal kinase (JNK), respectively, induce reorganization of cytoskeleton.

1.1.5.4. Wnt calcium pathway

Wnt5a and Fz2 are the example of a ligand receptor pair that initiates the Wnt Calcium (Wnt Ca^{2+}) pathway. Upon ligand binding, phospholipase C (PLC) gets activated via $G_{\beta\gamma}$ (see chapter 1.1.3.) and Dvl. PLC is catalyzing the conversion of phosphatidylinositol-4,5-biphosphate (PIP_2) into inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 gets freed from the membrane and binds to IP_3 receptor which releases calcium ions from intracellular stores. Released calcium acts on calcium-sensitive proteins, like protein kinase C (PKC), calcium-calmodulin dependent kinase II (CamKII), and/or calcineurin (CaCN) [107].

Wnt Ca^{2+} signaling was shown to be important in processes of cell adhesion and tissue separation during gastrulation.

The schematic depiction of Wnt Ca^{2+} and other Wnt pathways described in previous chapters are shown in Figure 8. Different Wnt signaling routes are mediated through distinct Dvl domains.

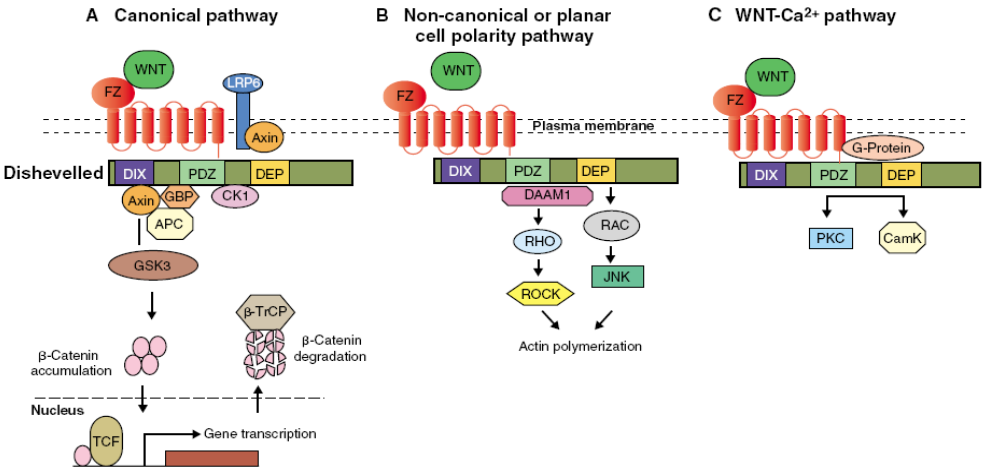


Figure 8. Dishevelled mediates at least three signaling cascades. (A) The canonical Wnt pathway. Wnt signals are received by Fz receptor and LRP coreceptor. The signal is transduced through Dvl and various other proteins, leading to the stabilization of β-catenin, which then together with LEF/TCF controls the transcription of target genes. (B) The non-canonical or planar cell polarity (PCP) cascade. A signal is received by Fz receptor and transduced via Dvl to Rac and Rho, which then activate downstream targets to modulate the actin cytoskeleton. (C) The Wnt Ca2+ pathway. Wnt signaling through the Fz receptors and Dvl and G-proteins leads to the release of intracellular calcium and signaling via PLC, CamKII and PKC. GBP, GSK3 Binding Protein; JNK, Jun kinase; ROCK, Rho-associated coiled-coil forming kinase. See text for other abbreviations. Adapted from [68].

1.1.6. Wnt signaling in cancer

Although mammalian Wnt1 was discovered through genetic screens for insertional site of MMTV at the beginning of eighties (see chapter 1.1.1.), the connection between Wnt signaling and human cancer was established more than a decade after. The laboratories of Polakis, Vogelstein and Kinzler showed in 1993 that known tumor suppressor APC binds to β-catenin [108], [109]. Mutations in APC were previously found to cause human hereditary intestinal cancer syndrome known as Familiar Adenomatous Polyposis (FAP). FAP patients inherit mutations in APC on one allele, and, if the mutation on the second allele is acquired, they develop benign colon adenomas (polyps). Additional mutations in *K-Ras*, *p53* and *Smad4* genes through clonal evolution are required for polyp to progress to malignant colon carcinoma [110]. Besides FAP, acquisition of APC mutations in both alleles was frequently identified in sporadic colorectal cancers (CRC) [110].

Three years after revealing interaction between APC and β-catenin, it was realized that mutations that inactivate APC lead to constitutive stabilization of β-catenin [111]. Inappropriate stabilization of β-catenin, documented in 90% of colorectal cancers [112], [113], increases its action as a transcription cofactor and causes upregulation of Tcf/Lef transcriptional repertoire in intestines [114], [115]. The subsequent work from Hans Clevers group helped to decipher the role that Wnt β-catenin pathway plays in maintenance and pathology of highly proliferative intestinal epithelium. In an intestinal crypt, the Wnt gradient provides proliferating signals for progenitor cells [116] and controls the expression of the EphB/EphrinB countergradient that establishes the crypt-villus boundary [117]. Disruption of

Wnt β-catenin signaling depletes the epithelial stem cell compartment [118], [119], [120], while its upregulation, as mentioned, starts a genetic program that evokes abnormal proliferation of crypt epithelial cells and adenomatous state. In their attempt to reveal the genes that cause transformation of normal epithelium, Clevers lab found that ablation of cMyc rescues APC deficiency in the intestine [121], [122]. Currently, several groups are trying to find other molecules that are involved in either Wnt β-catenin transcription, or regulation of the pathway. Recently, Randall Moon lab, in their RNA interference screen in human DLD1 colon adenocarcinoma cells, found that AGGF1, a chromatin-associated protein is required for β-catenin transcription [123]. In a separate work, two groups discovered that CDK8, a cyclin dependent kinase, is needed for β-catenin binding to cMyc promoter, and that transcription factor E2F1, that is negatively regulated by CDK8 and retinoblastoma tumor suppressor protein (pRb), counteracts the activity of β-catenin [124], [125], [126].

Besides its evident role in colon cancer formation, deregulated Wnt β-catenin signaling has been detected as a primary cause of the human hair follicle tumors [113], [110].

Wilms tumor, a cancer of the kidney whose appearance is closely connected to development, was shown to display mutations in β-catenin in 10% of cases [127]. In a recent study, Rivera et al found that inactivating mutations in another gene, WTX are present in about one third of Wilms tumors [128]. WTX, as shown by Major et al, is a novel component of β-catenin destruction complex that negatively regulates β-catenin and in this way acts as a tumor suppressor [129], [130].

Mutations in β-catenin have not been reported in breast cancer. However, there is evidence, that the expression of negative regulators of pathway, like sFRP1, is suppressed in breast carcinomas [131]. Reintroduction of these regulators in human cancer cell lines, as shown by recent work from our lab, reduces their ability to grow as xenografts [132].

As described above, deregulated Wnt β-catenin pathway is frequently detected in several types of human carcinomas. For this reason, a lot of effort is being made to design therapeutics that would block the aberrant pathway activation. Some already existing substances, like non steroidal antinflammatory drugs (NSAIDs) were shown to reverse polyp growth in patients with FAP most probably by acting on the capability of cells to degrade β-catenin [112].

As the central event in β-catenin action on transcription is its association with TCF/LEF factor, the formation of this complex is of an interest as a pharmaceutical target. High throughput screens of natural compounds revealed three molecules that had the ability to inhibit TCF/β-catenin complex formation that in addition showed efficiency in secondary assays of *Xenopus* axis duplication and inhibition of growth of colon cancer cell lines with constitutively active Wnt β-catenin signaling [133]. However, the essential role of Wnt β-catenin signaling in homeostasis of adult tissues and stem cell compartments [113], [110] as

well as the binding of β-catenin to TCF, E-cadherin and APC through overlapping domains, raise concerns about the negative effects of TCF/β-catenin interaction blockade on healthy tissues [112].

While the causative role of deregulated Wnt β-catenin signaling in different types of human cancer is well documented, the role of non canonical Wnt pathways in tumorigenesis is less evident. Several publications indicate the expression of Wnt5a as a marker of highly aggressive melanomas. In melanoma, Wnt5a was shown to enhance cell migration and invasion acting through Fz5 and PKC [134], [135].

1.2. Angiogenesis

Angiogenesis is a process of new blood vessel formation from preexisting vasculature [136], [137]. During development initial vascular plexus is formed by vasculogenesis, a process that involves direct aggregation of angioblasts, precursors of endothelial cells that share an origin with haematopoietic progenitors. Vasculogenesis is followed by angiogenesis that causes subsequent expansion and refinement of vascular network, as shown in Figure 9.

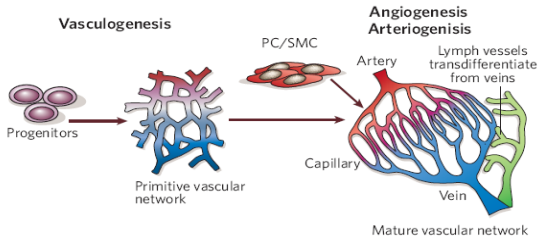


Figure 9. Development of the vascular systems: during vasculogenesis, endothelial progenitors give rise to a primitive vascular labyrinth of arteries and veins. During subsequent angiogenesis, the network expands, pericytes (PCs) and smooth muscle cells (SMCs) cover nascent endothelial channels, and a stereotypically organized vascular network emerges. Lymph vessels develop via transdifferentiation from veins. Adapted from [138].

In addition to wound healing that is characterized by extensive angiogenesis, the only adult tissues in which angiogenesis takes place are cycling ovary and placenta during pregnancy.

Deregulated excessive angiogenesis is the cause or a hallmark of several pathological states, for example cancer, diabetic eye disease, rheumatoid arthritis. In contrast, insufficient angiogenesis is seen in conditions such as the coronary heart disease, stroke and delayed wound healing, to name a few [136].

1.2.1. The beginnings of angiogenesis research field

The foundations to the field of angiogenesis were given by surgeon Judah Folkman at the beginning of 1970s. Although the connection between tumor growth and vascularization was realized by early tumor researches more than a century ago, it was only sporadically investigated in several publications since then on (reviewed in [139], [140]). However, in 1971 Judah Folkman postulated that antiangiogenesis could be an effective strategy in cancer treatment [2]. Subsequently, the Folkman group identified several angiogenesis factors and inhibitors, and improved our understanding of the role of angiogenesis in tumor formation [136], [140]. Many of the angiogenesis assays used nowadays were established by the same

lab. Moreover, like stated by Napoleone Ferrara, the researcher whose group participated in identification of vascular endothelial growth factor (VEGF), 'the hypothesis that targeting angiogenesis could be a strategy to treat cancer and various other diseases has been significant in inspiring many investigators to join this field' [139]. Another aspect of Judah Folkman's work and angiogenesis research that was visionary at the time was that it promoted the concept of targeted therapy for the treatment of cancer.

The subsequent studies in the angiogenesis field resulted in discovery of signaling pathways involved in blood vessel formation and maturation, and more important for cancer research, in creation of an effective anticancer drug that is targeting angiogenesis.

1.2.2. Signaling pathways governing blood vessel formation

Blood vessels consist of an endothelial cell monolayer that is lining the lumen of the vessel, followed by a layer of supportive smooth muscle cells (or pericytes in case of capillaries) embedded in the vascular basement membrane (VBM) that consists of type IV collagen, laminin and proteoglycans.

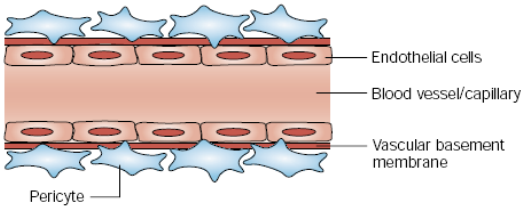


Figure 10. Blood vessel architecture. The vascular basement membrane interacts directly with the pericytes, which are on the outside, and the endothelial cells, which line the inside of the vessel. Adapted from [141].

In majority of healthy adult tissues the balance between angiogenic factors and inhibitors is keeping endothelial cells in quiescent, non proliferative state (Figure 11.). Endothelial cells are among the most quiescent cells of the body with a turnover time usually counted in months. However, during angiogenesis they proliferate rapidly within days. It was suggested that in conditions such as cancer formation or wound healing, the balance of angiogenic activators and inhibitors shifts in favor of activators, and the so-called angiogenic switch, followed by the expansion of vascular bed, takes place.

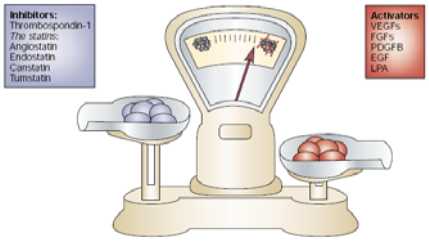


Figure 11. The angiogenic balance. Angiogenesis is orchestrated by a variety of activators and inhibitors. Activators of endothelial-cell proliferation and migration are mainly receptor tyrosine kinase ligands, such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), but can also be of very different origin, such as lysophosphatic acid (LPA). The first described angiogenic inhibitor was thrombospondin-1, which modulates endothelial-cell proliferation and motility. Many inhibitory molecules, such as 'statins', are derived from larger proteins that have no effect on angiogenesis. Adapted from [142].

1.2.2.1. Tumor angiogenesis

Many of the steps of physiological angiogenesis are recapitulated by tumor blood vessel formation, although, because of the access to excessive amounts of growth factors, tumor associated vasculature is more leaky, tortuous, disorgarnized and less functional.

During cancer formation, hypoxia is the most prominent trigger of angiogenic switch. In the situation of low oxygen in tissues that are not adequately perfused with vasculature, the hypoxia inducible factor (HIF) is initiating transcription from the set of angiogenic activators. In the presence of oxygen, a specific proline residue of HIF in the oxygen dependent degradation domain of the protein, gets hydroxylated which facilitates the recognition of HIF by von Hippel Lindau tumor suppressor (VHL) which is the substrate recognition component of the ubiquitin protein ligase complex that catalyzes the transfer of ubiquitin to lysine residue of a substrate. Hydroxylated and polyubiquitinylated HIF is targeted for degradation. In the absence of oxygen, hydroxylation and VHL mediated polyubiquitinylation of HIF are suppressed, so HIF accumulates, enters the nucleus and activates 'angiogenic' gene expression, among which VEGF seems to be the most relevant for the induction of angiogenic response (reviewed in [143]). High levels of VEGF expression alone were shown to be sufficient to evoke angiogenic response in quiescent vasculature [144]. The action of angiogenic growth factors (VEGF; basic fibroblast growth factor, bFGF; platelet derived growth factor, PDGF), chemokines and matrix metaloproteinases (MMPs) causes degradation of the VBM and endothelial cell sprouting. Degradation of the mature VBM promotes endothelial cell proliferation and migration, as well as the liberation of sequestered vascular growth factors and the detachment of pericytes.

The recent work from several groups added to our understanding of the sprouting angiogenesis. Three different cell types can be distinguished in angiogenic sprouts. The tip cells are located at the very end of the growing sprout. These cells are of a migrating and non proliferative phenotype and characterized by the expression of the Notch ligand Delta like ligand 4 (Dll4). The Dll4 acts in paracrine manner on Notch receptors expressed by adjacent highly proliferative stalk cells to downregulate the expression of the vascular endothelial growth factor receptor 2 (VEGFR2, see chapter 3.1.3.) in these cells. The downregulation of VEGFR2 causes the neighboring stalk cells to become less responsive to VEGF proliferative signals and finally acquire the quiescent tube cell phenotype. In this way, Notch signaling limits the action of VEGF on vasculature and protects the nascent blood vessels from excessive proliferation [145], [146], [147], [148], [149], [150]. Recently, it was shown that inhibition of Notch signaling in tip cells causes the upregulation of the vascular endothelial growth factor receptor 3 (VEGFR, chapter 3.1.4.) in these cells [151] and results in sprouting angiogenesis [152], [153]. Consequently, the inhibition of Notch signaling in tumor vasculature causes the increase in the number of vascular sprouts because of the elevated

levels of VEGFR2 and VEGFR3. Paradoxically, this results in slower tumor growth because the overall vascular system, although consisting of more sprouts, is less mature and less functional.

Experiments mentioned above highlight the importance of stabilization and maturation of nascent blood vessels for their proper function. This process requires the recruitment of supporting mural cells (smooth muscle cells or pericytes) and generation of extracellular matrix. Several pathways are known to be involved in this processes, among them platelet derived growth factor (PDGF), sphingosine-1-phosphate (SIP1), angiopoietin1-Tie2, and transforming growth factor β (TGFβ).

In addition to sprouting angiogenesis described above, several other processes contribute to tumor blood vessels formation (Figure 12). Intususspective angiogenesis is a process of remodeling of existing vessels (vessels become thinner or increase in volume) that gives rise to new blood vessel usually placed in the lumen of preexisting vessel. This process does not require proliferation of endothelial cells and is very rapid (within hours or even minutes). It was shown recently that after irradiation or anti angiogenic therapy tumors switch from sprouting angiogenesis to intususspection probably because this is the fastest way to reconstitute more stabe vascular network [154].

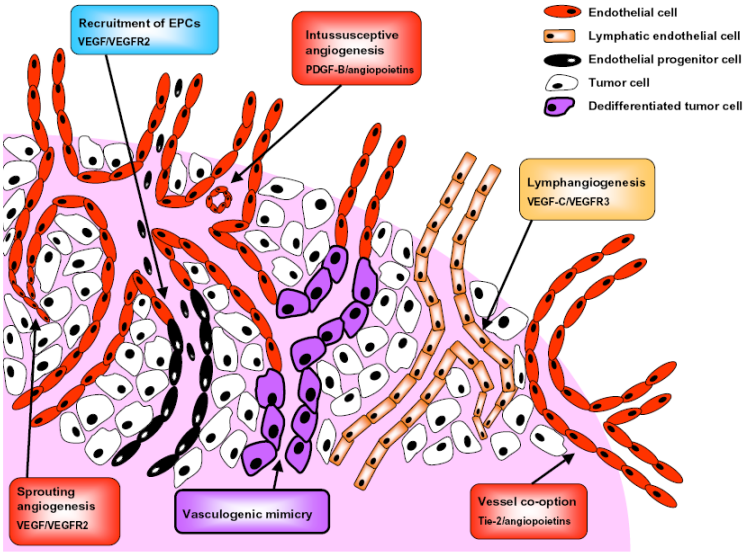


Figure 12. Different mechanisms of tumor vascularisation. Diagram represents the six different types of vascularisation observed in solid tumors, including sprouting angiogenesis, intussusceptive angiogenesis, recruitment of endothelial progenitor cells, vessel co-option, vasculogenic mimicry and lymphangiogenesis. The main key players involved in these processes, if known, are indicated. Adapted from [155].

Vessel cooption is the 'hijack' of host vasculature by avascular tumors seen in rat C6 glioma, murine Lewis lung carcinoma, murine ovarian cancer, human melanoma, and human Kaposi sarcoma.

More than a decade ago it was described that tumor blood vessels can be formed by incorporation of circulating bone marrow derived endothelial progenitor cells (EPC) that are characterized by expression of surface markers CD34, CD31, VEGFR2, Tie2 and CD14. Mobilization of EPC is promoted by several growth factors, chemokines and cytokines.

Another way of tumor vessel formation is by so-called vasculogenic mimicry or formation of circulatory system by cancer cells themselves. This process is characterized by cancer cell dedifferentiation and formation of tube like structures [155].

1.2.3. The role of Wnt pathway in angiogenesis

The evidence for involvement of Wnt signaling in angiogenesis is provided by studies of mice that are deficient for components of the pathway, and by *in vitro* studies using endothelial cells to recapitulate several steps of angiogenesis.

Mice deficient for Wnt2, Wnt4, Wnt7b, Frizzled4 and Frizzled5 display different localized angiogenic phenotypes.

Wnt2 knockout mice suffer from defects in placental vascularization due to reduced capillaries of fetal origin. The placental defects result in reduced weight of newborn pups and their perinatal lethality [156].

The role of Wnt4 during development is to regulate sex specific vasculature and steroid production in mammalian gonads. Wnt4 represses mesonephric endothelial and steroidogenic cell migration in the XX gonad, preventing the formation of a male-specific coelomic blood vessel and the production of steroids. Wnt4 deficient mice develop characteristics of male gonads in XX background [157].

Wnt7b null mice die perinatally because of respiratory failure originating presumably from a defect in major pulmonary vessel [158]. They also fail to regress transient retinal vasculature, the process that is dependent on macrophage derived Wnt7b signals [159].

Fz-5 was shown to be essential for angiogenesis in yolk sac and placenta [160].

Fz4 is the most studied member of Wnt signaling network in context of angiogenesis, since its deregulation or mutations in its unconventional ligand Norrin are causing human retinal diseases Familial Exudative Vitreoretinopathy (FEVR) and Norrie disease, respectively. These are retinal vascular diseases characterized by incomplete retinal neovascularization that causes varying degrees of visual impairment. Several evidences (the similarity of vascular phenotype, specificity of binding, activation of downstream canonical pathway etc) indicate that these molecules function like ligand receptor pair that is involved in retinal vascularization [161], [162], [63], [163]. Fz4 and Norrin mutant mice also display defects in female reproductive angiogenesis [159].

Like elaborated in previous chapters, many of the ‘general’ angiogenic factors (VEGFs, angiopoietins), that influence vasculature in several different tissues, have been described. However, little is known about tissue specific regulators of blood vessel formation. The vasculature of brain forms highy specialized structure named blood brain barrier (BBB) that is preventing the invasion of toxins and pathogens from blood to neural tissue. It was presumed that blood vessels in brain form by similar process as throughout body and that they

subsequently acquire this highly specialized feature because of the instructive role of surrounding neural tissue. However, several very recent papers found that brain vasculature is differing from that in other tissues by the presence of activated Wnt β-catenin signaling. The McMahon group showed that dual depletion of Wnt7a and Wnt7b and interruption of downstream Wnt canonical pathway during development causes defects in vasculature of central nervous system [164]. In agreement with this study, the important role of canonical Wnt signaling in induction of proteins that form blood brain barrier was reported by the Dejana group [165]. Barres and coworkers showed that conditional inactivation of β-catenin in endothelial cells specifically disrupts central nervous system vasculature, and renders the rest of the vasculature intact [166]. Taken together, these studies indicate that in stead of displaying systemic effect on vascular biology (like in case of VEGF), canonical Wnt signaling is essential for proper vascular formation locally in central nervous system. However, the studies from Wnt2, Wnt4, Wnt7b, Frizzled4 and Frizzled5 mice show that influence of Wnt signaling network is not limited to angiogenesis coupled with neural tissue. Given the redundancy among family members and multiplicity of signaling pathways initiated by Wnts (see previous chapters) it is not simple to reconcile the angiogenic phenotypes of individual members of the signaling network into a completed picture. However, the essential role of Wnt signaling in angiogenesis in many different contexts is emerging.

The expression of Wnts and Fzs has been documented in various endothelial and smooth muscle cell types [167], [168]. Moreover, Wnts are paracrine factors that could be expressed by variety of cells and activate signaling on neighbouring endothelium. Masckauchan et al detected expression of Fz4, Fz5, Fz6 genes on Human Umbilical Vein Endothelial Cells (HUVEC) [169]. The expression of Fz1, 3, 5, and 7 was reported by other labs [163].

The D'Amore group revealed the versatile role of Wnts in *in vitro* endothelial cell biology. Wnt1 and Wnt5a showed opposing effects on proliferation and tube formation of bovine aortic endothelial cells (Wnt1 activated both processes; Wnt5a slowed them down, and also did not induce the transcription from βcatenin target genes). Wnt2 showed no effect [170].

Endogenous activity of canonical Wnt pathway has been documented in cultured endotheial cells [168]. It was shown by Kitajewski group that ectopically expressed β-catenin, Wnt1 and Wnt5a induce proliferation of human umbilical vein endothelial cells. In this context, Wnt1 activates canonical pathway, while the Wnt5a acts through non canonical Wnt signaling pathway [169], [171]. The permissive role of planar cell polarity pathway in endothelial cell biology has been documented [172].

Recently, involvement of atypical Wnt signaling ligand Rspondin3 (Rspo3) in commitment of progenitor cells towards endothelial cell lineage was confirmed [65]. Rspo3 was shown to be efficient in cell culture experiments, inducing proliferation and angiogenesis of cultured endothelial cells.

In our study, we show that Fz4 and Fz6 are expressed by human umbilical vein endothelial cells (HUVEC) and can convey the Wnt signal to downstream components. We report Wnt3a as a novel proangiogenic factor with the ability to induce HUVEC proliferation and migration, but without an effect on their survival. Proliferation induced by Wnt3a is VEGFR signaling independent. Wnt3a was able to induce canonical Wnt pathway in our model. In contrast to promotive effects of Wnt3a on in vitro angiogenesis, Wnt5a did not induce proliferation or migration. To test the role of expressed Fz6 in *in vivo* angiogenesis, we injected Fz6 deficient mice and their wild type littermates with Lewis lung carcinoma cells. The ablation of Fz6 did not have an effect on quantity or appearance of tumor vasculature.

2. VEGF receptors in cancer

2.1. Vascular endothelial growth factors, discovery and biology

The members of the vascular endothelial growth factor (VEGF) family are secreted dimeric glycoproteins characterized by six conserved cysteine residues that form the so-called cysteine knot motif.

The first member of the family, VEGF or VEGFA, was discovered in 1983 and named tumor vascular permeability factor (VPF) because of its ability to induce vascular leakage in the skin [173]. Later on, in a separate study, Napoleone Ferrara and colleagues described a novel vascular endothelial cell specific growth factor (VEGF) isolated from media conditioned by bovine pituitary follicular cells [174]. In 1989 work from the Ferrara and Connolly groups demonstrated that VEGF and VPF were the same molecule [175], [176], [177].

VEGF is a heparin-binding, homodimeric glycoprotein weighing 45 kDa. The human *VEGF* gene consists of eight exons separated by seven introns. Four different isoforms are generated by alternative splicing, containing 121, 165, 189 and 206 amino acids respectively after signal sequence cleavage. The isoforms are termed $VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, of which $VEGF_{165}$ is the major isoform. The splicing isoforms differ in their ability to bind heparin and consequently range in their availability; from freely diffusible $VEGF_{121}$, to almost completely sequestered $VEGF_{189}$ and $VEGF_{206}$. $VEGF_{165}$ is of intermediate properties, because it is secreted, but with a significant fraction remaining bound to the cell surface and extracellular matrix [178].

Although angiogenesis is a complex process involving several signaling pathways, it is thought that the availability of VEGF is the limiting step in the creation of novel blood vessels. VEGF is a strong mitogen for endothelial cells, inducing their migration, survival and permeability. Inactivation of a single VEGF allele results in embryonic lethality [179], [180], while disruption of the autocrine VEGF signaling loop in endothelial cells causes their progressive degeneration [181].

VEGF is expressed by most cancer cell types and some tumor stromal and cancer infiltrating cells [178]. It was shown in 1993 that inhibition of VEGF suppresses angiogenesis and tumor growth in mice [182]. These results were the first confirmation of an early Judah Folkman hypothesis (see previous chapters) about simultaneous targeting of tumor angiogenesis and growth.

The other VEGF family members include placental growth factor (PlGF), VEGFB, VEGFC and VEGFD. VEGF homologues encoded by Orf viruses are collectively named VEGFE and VEGF like proteins found in snake venoms are usually termed VEGFF. The specificity of binding between VEGFs and their receptors is depicted in Figure 13.

PlGF is mainly expressed in placenta, heart and lungs. PlGF knockout mice do not display any apparent phenotype, but were shown to recover poorly from experimental myocardial

infarction and hind limb ischemia [183]. While PlGF seems to be dispensable for developmental angiogenesis and adult vascular homeostasis, accumulating data suggest that it might play important role in cancer, in particular because of its ability to recruit macrophages and bone marrow progenitor cells. Because of this quality and other proangiogenic effects, targeting PlGF is considered to be a strategy which might improve existing antiangiogenic therapies [184].

VEGFB is one of the least characterized members of the family. Like PlGF, it is not required in angiogenesis during development, as in adult tissues. The role of VEGFB in tumor angiogenesis and the effects of its blockade on tumor growth remain to be determined.

While VEGF, PlGF and VEGFB are considered to be factors involved in regulation of vascular growth and homeostasis, VEGFC and VEGFD have been characterized as lymphangiogenic factors. Unlike other family members that generate different isoforms by alternative splicing, VEGFC and VEGFD undergo proteolytic processing. This type of modification strongly increases their affinity for their receptors, vascular endothelial growth factor receptor 2 and 3 (VEGFR2 and VEGFR3). Hence, the degree of posttranslational processing of these factors may in part determine the rate of (lymph)angiogenesis. The enzymes known to process VEGFC and VEGFD are the serine protease plasmin and members of proprotein convertase family [185].

VEGFC was independently found and cloned in 1996 by the groups of Kari Alitalo and William Wood [186], [187]. During development, VEGFC, in parallel to its receptor VEGFR3, is expressed predominantly in regions where lymphatic vessels develop [188]. In adult mice, VEGFC is expressed highly in lymph nodes and to a lesser extent in the heart, lung and kidney [189]. Overexpression of VEGFC in mouse skin keratinocytes and in chick differentiated chorioallantoic membrane induces lymphangiogenesis without effecting angiogenesis [190], [191], [192]. The lymphangiogenic activity of VEGFC was shown to be mediated through VEGFR3, as modified protein able only to bind this receptor is sufficient to induce lymphangiogenic response [193]. In 2004, the studies on VEGFC knockout mice showed that this factor is required for the development of lymphatic vasculature, but is dispensable for the formation of blood vessels. In VEGFC deficient mice endothelial cells do commit to lymphatic lineage, but fail to sprout and form the lymphatic system. Because of this defect these mice die prenatally. The requirement of VEGFC was shown to be dose dependent as heterozygous mice survived, however they still suffered from cutaneous lymphatic hypoplasia and lymphedema. The lymphangiogenesis observed in VEGFC deficient mice was rescued by introduction of recombinant VEGFC and VEGFD, but not VEGF [194]. Besides of its developmental role in the formation of lymphatics, it was shown that VEGFC mRNA gets upregulated in endothelial cells in response to proinflammatory cytokines [189].

VEGFD was discovered in 1998 [195]. Unlike human VEGFD which binds VEGFR2 and VEGFR3, mouse homolog binds exclusively to VEGFR3. During development, mouse VEGFD is expressed in limb buds, heart, lung and skin. In adult human tissues, VEGFD mRNA is prominently expressed in the heart, lung, skeletal muscle, colon and small intestine [195]. As in the case for VEGFC, overexpression studies have shown that VEGFD strongly induces lymphangiogenesis [193], [196], [197]. In spite of this, ablation of VEGFD did not prevent development of normal lymphatic system in mice as was observed in the VEGFC knockout [198].

The action of VEGFC and VEGFD on lymphatics is mediated through VEGFR3, while the signaling through VEGFR2 was shown to evoke effects on the blood vascular endothelium in some experimental models. These experiments also showed that VEGFD is a stronger angiogenic factor than VEGFC [192].

Several years ago, work from groups of Michael Detmar, Michael Pepper and Marc Achen showed that ectopic expression of VEGFC or VEGFD in breast [199], pancreatic [200], and other mouse tumor models [201], increases lymphangiogenesis and promotes tumor metastasis. Subsequently, other studies confirmed the role of the VEGFC, VEGFD and VEGFR3 axis in tumor lymphangiogenesis and metastatic spread, suggesting that targeting these pathways may suppress cancer progression (reviewed in [185]).

2.2. Vascular endothelial growth factor receptors

The VEGF receptors are comprised of two families, VEGFRs and neuropilins. VEGFRs which are closely related to Fms, Kit and PDGF receptors, are type III receptor tyrosine kinases consisting of seven extracellular immunoglobulin-like domains (six in the case of VEGFR3), a single transmembrane spanning region and an intracellular consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (Figure 13). Upon ligand binding receptor dimerization is induced.

Neuropilins are coreceptors for VEGFs. They consist of a large extracellular domain of about 860 amino acids, followed by single transmembrane spanning domain and intracellular domain that is conserved among family members (reviewed in [202], [203]).

2.2.1. VEGFR1

VEGFR1 (or Fms like tyrosine kinase, Flt1), the first known member of the family, was discovered in 1992 [204]. It is expressed on the surface of vascular endothelial cells, but also on bone marrow cells such as macrophages and monocytes. VEGFR1 binds VEGF, PlGF and VEGFB. Through alternative splicing, VEGFR1 encodes a soluble variant, the precise biological role of which is not known.

Interestingly, this receptor binds VEGF with a much higher affinity than does VEGFR2, however the binding induces only weak tyrosine kinase activity. For this reason it was suggested that VEGFR1 acts as a decoy receptor that regulates angiogenesis by sequestering VEGF [205]. Studies of VEGFR1 knockout mice confirmed this hypothesis, showing that the loss of this receptor causes embryonic lethality due to vascular overgrowth and disorganization [206], [207]. Moreover, mice expressing membrane anchored variant of the receptor lacking the tyrosine kinase domain are not only viable, but also do not exhibit any vascular defects [208].

The ability of VEGFR1 to signal however, seems to be important for the effects observed in bone marrow derived cells, namely monocyte migration and differentiation, and the recruitment of endothelial cell progenitors from bone marrow [209], [210]. VEGFR1 signaling is also crucial for the release of hepatotrophic growth factors by liver sinusoidal endothelial cells, the mechanism shown to be essential for protection of the liver from toxic damage [211]. Another important characteristic of this receptor is that by intracellular crosstalk it can strengthen the signaling induced by VEGFR2. It is thought that in this way VEGFR1 plays an essential role during pathological angiogenesis and in wound healing [205], [212], [213].

The expression of VEGFR1 is elevated in lung, prostate, breast and colon cancer, pulmonary adenocarcinoma, hepatocellular carcinoma, glioblastoma, multiple myeloma and nephroblastoma. In the case of breast cancer and non small cell lung cancer, expression of VEGFR1 is predictive of poor prognosis, metastasis and recurrence. It was shown over the past several years that inhibition of VEGFR1 suppresses tumor growth and metastasis in various tumor models [184].

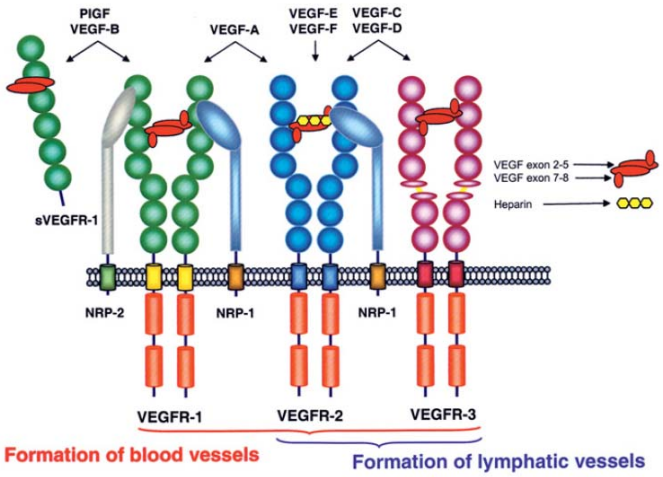


Figure 13. Schematic representation of VEGF family ligands and their receptors. VEGFs show distinct patterns of receptor specificity. VEGF binds to VEGFR1 and -2 and to receptor heterodimers, while VEGFC and -D bind VEGFR2 and -3. Receptor-specific interactions have been described for some VEGF variants: PlGF and VEGF-B exclusively bind VEGFR1 and VEGFE interacts only with VEGFR2. VEGFF variants interact with either VEGFR1 or -2. Adapted from [214].

2.2.2. VEGFR2

VEGFR2 (kinase insert domain receptor, KDR or fetal liver kinase, Flk1) was found to be a receptor for VEGFs in 1992 [215]. It binds VEGF, VEGFC, VEGFD, VEGFE and VEGFF. VEGFR2 is expressed on the surface of endothelial cells (vascular and lymphatic), neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes and hematopoietic stem cells [189]. Its expression is down regulated in the adult blood vascular endothelial cells, and is again upregulated in the endothelium of angiogenic blood vessels [189].

VEGFR2 was shown to be the main transducer of VEGF's proliferative, migratory, survival and permeability effects on endothelial cells. Mice deficient for this receptor die during embryogenesis due to failed vascular development and hematopoiesis [216].

Signaling pathways and proteins activated by phosphorylated VEGFR kinase include phospholipase Cγ, PI3 kinase, Ras GTPase activating protein and the Src family kinases [217], [218]. Activation of Raf-Mek-Erk upon phosphorylation of Tyr1175 and recruitment of PLC γ1 pathway is thought to be essential for induction of proliferation. Signaling through focal adhesion kinase (FAK) was shown to play a role in the migration induced through VEGFR2, while its survival effects are transduced by the action of the PI3kinase/Akt pathway (Figure 14).

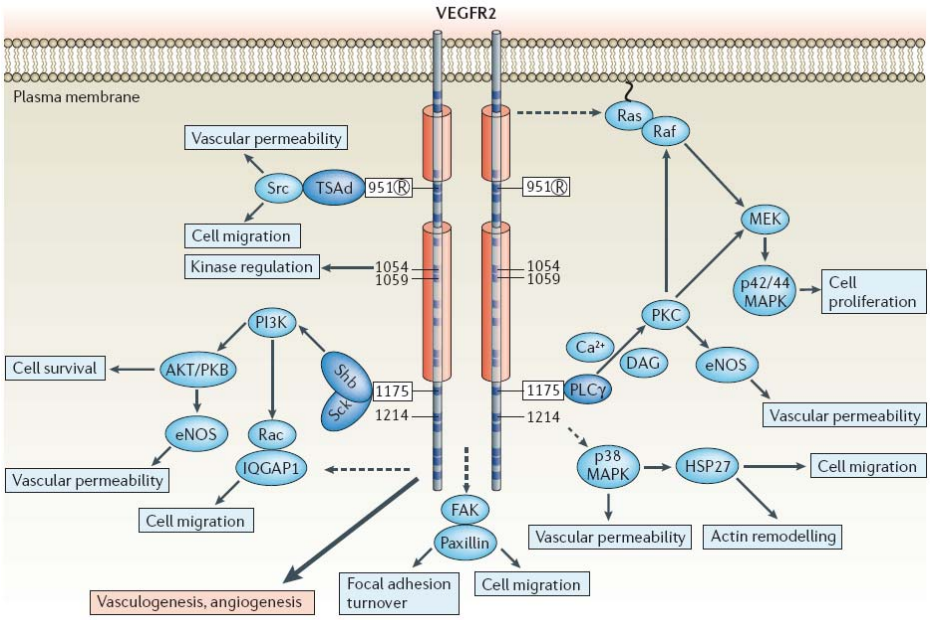


Figure 14. VEGFR2 phosphorylation sites and signal transduction. Intracellular domains of dimerized and activated VEGFR2 is shown with tyrosine-phosphorylation sites that are indicated by numbers. Circled R indicates that use of the phosphorylation site is regulated dependent on the angiogenic state of the endothelial cell. Dark blue squares in the receptor molecules indicate positions of tyrosine residues. Binding of signaling molecules (dark blue ovals) to certain phosphorylation sites (boxed numbers), initiates signalling cascades (light blue ovals), which leads to the establishment of specific biological responses (pale blue boxes). The mode of initiation of certain signalling chains is unclear (dashed arrows). Final biological outcomes that are coupled to the respective receptors are indicated in pink boxes. DAG, diacylglycerol; eNOS, endothelial nitric oxide synthase; FAK, focal adhesion kinase; HSP27, heat-shock protein-27; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase; PI3K, phosphatidylinositol 3′kinase; PKC, protein kinase C; PLCγ, phospholipase Cγ; Shb, SH2 and β-cells; TSAd, T-cell-specific adaptor. Adapted from [217].

Vascular permeability is thought to be induced through Src family kinases, nitric oxide action or the effect on components of adherens and gap junctions (VE cadherin, β-catenin, Occludin) [217].

2.2.3. VEGFR3

VEGFR3, also known as fms like tyrosine kinase 4, Flt4, which binds VEGFC and VEGFD, is expressed on all endothelia during development, but is restricted in adulthood to lymphatic endothelial cells and certain fenestrated blood vasculature [189]. However, it is upregulated in proliferating endothelial cells and in pathologic conditions such as tumors (see chapter 1.2.2.1), [219]. Missense mutations in VEGFR3 in humans have been linked to hereditary lymphedema [189].

VEGFR3 signaling is involved in angiogenesis, lymphangiogenesis and hematopoiesis. The ablation of VEGFR3 causes embryonic lethality due to defective remodeling of the primary vascular plexus and abnormal hematopoiesis [220]. Overexpression of soluble VEGFR3 in mouse skin inhibits fetal lymphangiogenesis, while the other vasculature remains normal [221]. Recently it was shown that simultaneous deletion of the VEGFR3 ligands VEGFC and VEGFD in mice does not phenocopy the vascular phenotype of VEGFR3 deficient mice, but resembles the phenotype observed in the single VEGFC knock out mice [222]. This suggests that either another as yet unknown ligand is binding to this receptor to induce angiogenic signals, or that VEGFR3 is exerting its angiogenic role through heterodimerization with another receptor.

Activation of VEGFR3 induces proliferation, migration and survival of lymphatic endothelial cells [223]. Activated receptor was shown to recruit Shc and Grb2 via phosphorylated Tyr1337. Also, VEGFR3 can form functional heterodimers with VEGFR2 inducing different phosphorylation pattern of the cytoplasmic tail of receptor than when the homodimers are formed [224].

In conclusion, VEGFR3 is considered to be attractive target in anticancer treatments because of its upregulation in some tumors, and of its important and separate roles in angiogenesis versus lymphangiogenesis [153].

2.2.3. Neuropilins

Neuropilins (NRPs) were discovered in 1987 as antigens for antibodies that bind neuropiles and plexiform layers in the *Xenopus* tadpole nervous system. A decade later, it was shown that these molecules are receptors for class III secreted semaphorins, which transmit repulsive signals during axon guidance [203]. In 1998 the group of Michael Klagsbrun found that NRPs are expressed by endothelial cells and are able to bind VEGFs [225]. Neuropilins are VEGFs coreceptors that enhance their signaling. NRP1 binds VEGF, VEGFB and PlGF, while NRP2

binds VEGF, VEGFC and PlGF. In terms of the receptors, VEGFR2 and VEGFR1 interact with NRP1 and NRP2, respectively.

In addition to neuronal abnormalities, overexpression of NRP1 causes vascular defects [226]. NRP1 knockout mice die embryonically due to cardiovascular abnormalities [227]. In contrast to the essential role of NRP1 during development, NRP2 knockout mice are viable into adulthood, but show lower degrees of retinal neovascularization after ischemia [203].

In parallel to the discovery of the role of NRPs in angiogenesis, the role of these receptors in tumor biology became apparent. NRP1 and/or NRP2 expression is correlated with increased tumor angiogenesis and aggressiveness in many different tumor types. Accumulating evidence suggests that neuropilins play a role in the survival of tumor cells [203], [228].

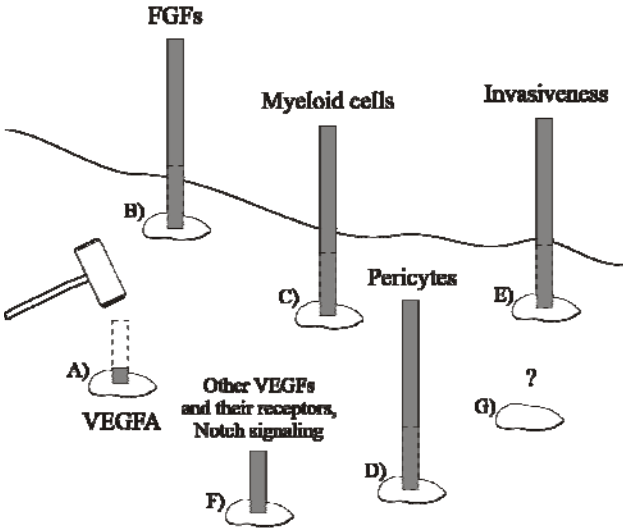
Recently, it was shown that antibody mediated blockade of NRP1 in combination with anti VEGF treatments results in an additive effect on tumor growth inhibition. Vessels from tumors treated with both anti NRP1 and anti VEGF antibodies were less efficient in their ability to associate with pericytes in comparison to tumors treated only with anti VEGF targeted therapies [229].

2.3. Targeting VEGF/VEGFR signaling in cancer

2.3.1. Targeting angiogenesis

Avastin (Bevacizumab, Genentech, [3]) is a humanized VEGF monoclonal antibody that was approved by the Food and Drug Administration (FDA) for anticancer treatment in combination with chemotherapy in 2004. Avastin was the first drug on the market created to specifically target angiogenesis. At present, it is used as a first line treatment in combination with chemotherapy for late stage metastatic colorectal cancer, NSCLC and previously untreated metastatic breast cancer. Due to the primary role of VEGF in angiogenesis (chapter 3.1.), the strategy to block this growth factor in order to reduce tumor vasculature was reasonable. However, emerging data suggest that supression of several other pathways and processes should be taken into consideration in order to accomplish greater effects on tumor growth reduction. The mode of action of Avastin and mechanisms of tumor adaptation to therapy are depicted and discussed in Box 1.

Avastin is not the only cancer therapeutic targeting members of the VEGF family or their receptors. Sorafenib (Nexavar, Bayer/Onyx) and sunitinib (Sutent, Pfizer) are VEGFR tyrosine kinase inhibitors approved for treatment of renal carcinoma (both), gastrointestinal stromal tumors (sunitinib) and hepatocellular carcinomas (sorafenib) as single agents [230]. It is important to emphasize that tyrosine kinase inhibitors, because of their mode of action at the ATP binding pocket, are rather more selective than specific in blockade of their targets. Consequently, it is possible that some of the observed clinical effects are due to non specific inhibition of other tyrosine kinases [231].



Box 1. Figure is schematically showing mechanisms of tumor adaptation or escape from angiogenesis inhibition by an anti VEGF agent.
The adaptation includes the increase in other growth factors (FGFs for example), recruitment of bone marrow derived cells (like myeloid cells), pericyte recruitment and invasiveness [232]. Accumulating data suggest that contribution of other VEGFs (including PlGF) and VEGFRs to tumor angiogenesis should be considered when thinking about targets additional to anti VEGF therapy.
A) Decreased level of active VEGF after Avastin treatment reduces tumor growth and increases OS and PFS in several late stage human cancers when used as a first line treatment in combination with chemotherapy [230]. However, the mode of action of Avastin on vasculature and tumor cells is not apparent at present. It was suggested that in addition to pruning existing vessels and preventing the formation of new ones, the major role of anti VEGF action is to normalize tumor vasculature and consequently increase delivery and efficacy of classical chemotherapeutics [233].
B) Anti VEGF treatment induces hypoxia in tumor tissue that, in return, increases the upregulation of other strong proangiogenic factors, like FGF2. Increased expression of other angiogenic growth factors could be the mode of tumor escape, but it is possible that this quality is acquired by tumors prior to treatment, like in case of late stage breast cancer [232]. Therefore, the inhibiton of several proangiogenic pathways could decrease tumor angiogenesis and growth more efficiently. In addition to VEGFs and FGFs, an attractive candidate for supression would be HIF, factor that induces transcriptional response in hypoxia (see chapter 1.2.2.1.).
C) Hypoxia induces recruitment of various bone marrow derived cells that either serve as building blocks for nascent vessels (endothelial and pericyte progenitors) or local reservoir of growth factors (myeloid cells, tumor associated macrophages, monocytes), [234].
D) In response to vascular regression caused by antiangiogenic therapy endothelial cells increase pericyte or smooth muscle cell recruitment. Coverage of blood vessels by pericytes decreases their sensitivity to anti VEGF treatment. Consequently, combinatorial inhibition of VEGF and PDGF shows improved efficacy on inhibition of angiogenesis and tumor growth [235]. However, the idea of dual inhibition should be considered with precautions because some experiments indicate that loosening blood vessels by smooth muscle cell dissociation increases metastatic dissemination of tumors [232].
E) Another adaptation of tumor cells to angiogenesis inhibition is increase of their ability to metastasize. Interestingly, it was shown that VEGF itself decreases the ability of glioblastoma cells to disseminate both *in vitro* and *in vivo* [236].
F) Preclinical studies suggest that blockade of PlGF, VEGFR1, VEGFR3 and NRPs is also efficient in tumor growth inhibition [237], [184], [153], [229]. In some cases, when combined with anti VEGF therapy, the inhibitors of these growth factors and receptors show additive effect on tumor growth [229]. The efficiency of these inhibitors and the regimen of their use are waiting to be confirmed in clinical trials.
While the angiogenic effects of VEGFC and VEGFD are understudied, their role in tumor lymphangiogenesis is well known [238]. Induction of these VEGFs increases lymphangiogenesis and metastasis in several preclinical tumor models, suggesting that their blockade could be beneficial for cancer patients [239], see the following chapter.
Preclinical studies also indicate that inhibition of Notch pathway could stimulate tumor growth suppression through inhibition of functional angiogenesis [240], see chapter 1.2.2.1.
It is not excluded that in response to anti VEGF therapy the fore mentioned factors and their receptors get upregulated.
G) The effects of Avastin on tumor growth suppression show that targeting angiogenesis, in addition to being an attractive idea, is efficient strategy in cancer treatment. Nevertheless, further studies are needed to elucidate signaling networks that regulate the process. Simultaneous inhibition of several factors and processes will probably increase the effect on tumor blockade.
OS, overall survival, PFS, progression free survival

2.3.2. Targeting lymphangiogenesis and tumor cells

In addition to its essential role in angiogenesis, involvement of VEGF/VEGFR signaling network in dissemination through lymphatic system and its role in tumor cell biology are equally important for cancer treatment.

Metastasis is the main cause of deaths from cancer and many efforts are being invested to dissect signaling pathways involved in this process. Lymphatic system is important route for tumor cell dissemination, and the presence of tumor cells in lymph nodes is used as

prognostic factor in nearly every type of carcinomas. Previously, it was thought that cancer cells use preexisting vessels to disseminate through lymphatic system. However, eight years ago the inductive role of VEGFC/VEGFD/VEGFR3 axis in tumor lymphangiogenesis has been documented (chapter 3.1.). Since then, several reports tried to evaluate the effect of VEGF/VEGFR inhibition in tumor spread through lymphatics.

The groups of Alitalo, Sleeman and Joos delivered soluble VEGFR3 to either human lung cancer xenografts, mammary tumor cells growing in rats, or murine melanoma model. Soluble receptor binds expressed ligands and therefore prevents them from signaling with endogenous signaling partners. Introduction of soluble VEGFR3, they showed, causes reduction in intratumoral lymphangiogenesis and metastasis in regional lymph nodes and lungs [241], [242], [243]. In another study, the same inhibitory effect on lymphangiogenesis and metastasis was achieved by blocking the expression of VEGFC using RNA interfering approach [244]. The recent work from the group of Mihaela Skobe found that inhibition of VEGFR3 with antibody is more efficient in suppression of metastasis than inhibition of VEGFR2. Furthermore, the authors show that combination of antibodies against both receptors has a capability of suppressing metastasis even if the treatment is applied after metastasis formation, the concept that they are further investigating [245]. In addition to these studies, the anti(lymph)angiogenic and antimetastatic properties of VEGFD antibody were described by Stacker and collaborators in a VEGFD dependent mouse tumor model [201].

However, in contrast to reports mentioned above, Wong and colleagues achieved the reduction in tumor lymphangiogenesis by blocking VEGFC by siRNA or usage of soluble VEGFR3 in prostate cancer cells, but this did not correlate with incidence of metastasis to lymph nodes. It is important to note that they report the strong reduction in intratumoral lymphatics (more than 95%), but the surrounding marginal lymphatics at the tumor-stromal interface was not affected. This suggests that these marginal vessels are more important for tumor cell dissemination than intratumoral lymphatics [246].

Clinical data suggest that VEGFC expression in tumor cells correlates with lymph node metastasis in lung, colorectal and prostate cancer and may be a prognostic factor in ovarian and cervical cancer. VEGFD expression is associated with lymphangiogenesis in colorectal cancer and poor prognosis in epithelial ovarian carcinoma.

Accumulating data indicate the role of VEGF/VEGFR signaling in tumor cell biology. VEGFRs and neuropilins have been detected on tumor cells of various types [230] [228], [203]. Functional VEGF/VEGFR autocrine signaling loops have been described in breast cancer, melanoma, and leukemias [247], [248], [249]. Furthermore, it was shown that VEGFRs promote invasion of lung adenocarcinoma cells, and migration of colorectal and pancreatic carcinoma cells [250], [251], [252], [253]. Consequently, the effects of

VEGF/VEGFR inhibitors in certain conditions can partially be attributed to the direct effect on tumor cells.

In our study [254], we wanted to test the effects of pan VEGFR tyrosine kinase inhibitor (PTK787/ZK222584, Vatalanib, Novartis Pharma/Schering [255]), in comparison to those of an anti VEGFR2 blocking antibody (DC101, ImClone Systems, [256]) and anti VEGF antibody (Pab85618 or Avastin, anti mouse and anti human VEGFA antibody, respectively). We compared their ability to inhibit tumor growth, vascularization and metastatic dissemination in a mouse melanoma model. In addition, we tested their effects on *in vitro* tumor cell biology.

PTK787/ZK (PTK/ZK in further text) is tyrosine kinase inhibitor selectively blocking VEGFR1, VEGFR2, VEGFR3, PDGFR-β and c-Kit [257]. It was shown to reduce tumor growth, angiogenesis and metastasis formation in preclinical models [258], [259], [154] and it displayed activity in clinical trials [260], [261], [262]. We used this inhibitor in orthotopic B16/BL6 melanoma model, an aggressive metastatic subline derived from B16 cells [263]. We showed that inhibition of VEGFR1, VEGFR2 and VEGFR3 by PTK/ZK displays comparable effect to VEGF and VEGFR2 inhibitors on primary tumor growth. PTK/ZK and DC101 had similar inhibitory effects on lymphangiogenesis in metastatic sites, but only PTK/ZK reduced the formation of metastasis. Inspection of peritumoral lymphatic vessel functionality performed by checking their ability to uptake injected dye showed that functional lymphatics was reduced in PTK/ZK treated tumors. Using human lymphatic endothelial cells (LECs) as an *in vivo* model, we showed that besides of VEGFA, VEGFC also increases proliferation and survival of these cells, an effect that can be blocked by PTK/ZK, but not Avastin.

In regard to tumor cell biology, we report the existence of autocrine VEGF/VEGFR signaling loop in B16/BL6 melanoma cells whose inhibition by PTK/ZK, but not DC101, reduced the migration of these cells in Boyden chamber. In addition, we show that interruption of PI3K and ERK1/2 survival pathways in B16/BL6 melanoma cells by addition of PTK/ZK or DC101 sensitizes these cells to platinum based chemotherapeutics.

3. ErbB2 interactome in breast cancer

3.1. ErbB2, an amplifier of EGFR signaling network

ErbB2 (HER2, Neu) is a member of the epidermal growth factor receptor family that comprises four transmembrane spanning receptor tyrosine kinases. Other family members are the founding epidermal growth factor receptor (EGFR or ErbB1), ErbB3 and ErbB4. Structurally, ErbB family members consist of an extracellular EGF related peptide binding region, a transmembrane helix, and an intracellular region comprised of a well conserved tyrosine kinase domain and a less conserved regulatory C terminal tail.

Historically, the *ErbB* oncogene was found through its presence in the genome of avian erythroblastosis virus (*v-ErbB*), a transforming retrovirus that rapidly induces leukemia in red blood cell precursors (an erythroleukemia). Later on, in 1984, it was shown that the coding sequence for epidermal growth factor receptor closely relates to that of the erbB oncogene. This realization was the first connection made between a growth factor receptor and an oncogene. Subsequently, the remaining members of the family, ErbB2, ErbB3, and ErbB4, were discovered. ErbB2, the main focus of several further chapters, was discovered on the basis of its amplification in a human mammary carcinoma cell line [264], [265].

Several epidermal growth factor (EGF) related peptides have been shown to activate the members of this RTK family including EGF, transforming growth factor (TGF) α, amphiregulin, betacellulin, heparin-binding EGF, epiregulin and neuregulins. Interestingly, no ligands have been found that bind to the ErbB2 receptor, and the ErbB3 receptor does not display kinase activity, thus it can only signal when coupled to another member of the family. Ligand binding induces receptor homo or heterodimerization and subsequent activation of their intrinsic RTK activity. ErbB heterodimers induce downstream signaling more strongly than homodimers [266].

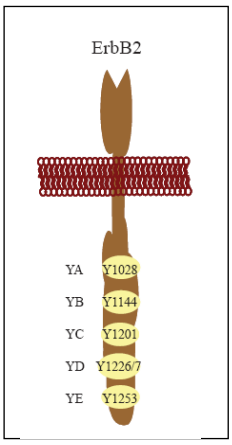
Though the ErbB2 receptor does not bind ligands, it has been shown by Nancy Hynes' group to be the preferential dimerization partner for the other members of the family [267]. For several reasons, ErbB2 is considered to be the amplifier of the ErbB signaling network. First, protein microarray data show that this receptor binds to a much larger subset of phosphotyrosine binding proteins, than the other activated receptors. Second, heterodimers containing ErbB2 are characterized by higher affinity and broader specificity for ligands because of the slow rates of growth factor dissociation. Finally, ErbB2 containing heterodimers undergo slow endocytosis and more frequently recycle back to the cell surface [268]. For these reasons, deregulation of ErbB2 signaling has detrimental effects on cell fate, which will be described in further chapters.

3.2. ErbB2 signaling in breast cancer

In 1987 the groups of William McGuire, Axel Ullrich and Dennis Slamon showed that overexpression of the *erbB2* oncogene in human breast cancers correlates with relapse and decreased survival of patients [269]. Subsequently Nancy Hynes group confirmed these findings and reported a correlation between ErbB2 protein expression and both the nodal status and nuclear grading of breast carcinomas [270]. These findings laid down the foundations for ErbB2 targeted therapy for a subset of breast cancers (for a review on the role of ErbB receptors and their signaling in breast and lung cancers, see [271]). Molecular analyses of gene expression patterns of breast carcinomas and normal breast tissue revealed that ErbB2 overexpressing cancers represent one of six major molecular subtypes of normal and transformed breast tissue [272]. The exact mechanism by which ErbB2 overexpression exerts its oncogenic effects is still poorly understood. However, the poor clinical outcome of patients with tumors bearing the ErbB2 amplicon is connected to its ability to confer resistance to various chemotherapeutic agents and to increase the proliferation, migration, survival and angiogenic capabilities of the cancer cells. For these reasons, the signaling elicited through this receptor is of a central interest in breast cancer research and breast cancer targeted therapy.

3.3. The role of ErB2 binding partners in breast cancer

The extracellular EGF related peptide binding region of the ErbBs consists of four domains. The binding of a ligand to domains I and III induces a conformational change that exposes domain II, which is involved in receptor homo and heterodimerization. The key residues necessary for ligand binding are not conserved in ErbB2's extracellular domain and the structure of the ligand binding domain prevents the binding of EGF related peptides to this site. However, ErbB2 constitutively adopts a conformation in which the dimerization domain II is exposed, making it amenable to binding ligand bound receptors [273]. Still, other structural mechanisms exist to prevent spontaneous dimerization of ErbB2 [266].



Upon ligand binding and receptor dimerization, the phosphorylation of the intracellular domains and recruitment of signaling mediators take place. Five tyrosine phosphorylation sites within the ErbB2 intracellular domain have been described (Figure 15). These sites are refered to as YA, YB, YC, YD and YE, respectively [274], [275]. It was shown by the Muller group that each of the ErbB2 YA-YD sites individually can mediate *in vitro* transformation of normal breast cells via the recruitment of different cytoplasmic mediator proteins to mutated constitutively activated ErbB2 [276].

Figure 15. ErbB2 intracellular phosphorylation sites.

Further analyses were performed by this group to determine the contribution of individual phosphorylation sites to the tumorigenic and metastatic capabilities of the ErbB2 receptor in breast cancer [276]. The authors used mutant, constitutively active rat ErbB2 receptor (Neu) bearing only one autophosphorylation site at a time, expressed under the mammary specific promoter MMTV in transgenic mice. They showed that the biological profiles of tumors originating from signaling through the ErbB2 sites YC, YD, and YE are strikingly similar in pathologic features and gene expression patterns. In contrast, signaling elicited through the YB site induced tumors of different morphology (focal papillary tumors, in contrast to YC, YD and YE which induced multifocal, solid and nodular tumors). Consequently, the number of lung metastasis from these tumors was significantly higher than observed for any other individual ErbB2 phosphorylation site. These findings indicate that different roles exist for each phosphorylation site and the proteins that they recruit, in ErbB2 mediated tumorigenesis and metastasis formation.

The main cytoplasmic signaling mediators of activated ErbB2 and their contribution to the breast tumorigenesis and metastasis are discussed below.

3.3.1. ShcA

The mammalian ShcA gene encodes for a protein with three splice isoforms, namely p46, p52 and p66. All of the isoforms are similar in structure, containing two phosphotyrosine binding motifs (PTB) and a single Src homology 2 (SH2) domain, enabling them to bind docking sites on activated receptor tyrosine kinases. Human ShcA also contains a central collagen homology 1 (CH1) region which contains three tyrosine phosphorylation sites at residues 239/240 and 317 (313 in mice), respectively. When phosphorylated, these sites are capable of inducing Ras/MAPK dependent and independent signaling [277]. In the context of ErbB2 signaling, ShcA is found to associate with phosphorylated YD and YE sites [278].

Clinical data suggest that increased ShcAY317 phosphorylation, in combination with reduced p66ShcA levels correlates with nodal status, disease stage and relapse in breast cancer patients [277]. The contribution of ShcA to ErbB2 mediated angiogenesis, tumorigenesis and metastatic dissemination has been untangled recently by several groups at McGill University, Canada. First, it was shown by the laboratory of Morag Park that ShcA is essential for induction of VEGF expression through the Met/HGF and ErbB2 receptors, and for the initiation of tumor angiogenesis [279]. The contribution of the individual ShcA phosphorylation sites to cancer and metastasis formation downstream of ErbB2 has been analysed by the laboratories of William Muller and Tony Pawson. Using an immortalized mammary epithelial cell line transformed with either activated ErbB2 (Neu), or the variants mutant for each of the phosphorylation sites, they show that all three ShcA tyrosine phosphorylation sites are important for ErbB2 induced mammary tumor outgrowth,

intravasation and angiogenesis. Moreover, they provide evidence to suggest that ShcA expression within the mammary epithelium is essential for ErbB2 induced mammary tumor development [280]. It is worth mentioning, however, that ShcA is necessary for several steps of tumor formation and dissemination not only downstream of ErbB2, but also of Polyoma middle T antigen.

Besides the described roles for ShcA downstream of ErbB2 in VEGF induction, tumor formation and metastasis, its role in mammary carcinoma cell motility and invasion has been confirmed *in vitro*. It was shown by Peter Siegel's group that ShcA is necessary for transforming growth factor (TGF) β induced cell motility and invasion via ErbB2 [281].

Taken together, the experiments described herein reveal an essential role for ShcA in ErbB2 induced VEGF production, tumor angiogenesis, mammary tumor outgrowth, intravasation and TGF-β induced motility.

3.3.2. Grb2

Grb2 (growth factor receptor bound protein 2) is an adaptor protein which binds to several receptor tyrosine kinases, among them ErbB2 via the YB (direct) and YD (through Shc) sites. Two different isoforms of Grb2, obtained through alternative splicing, exist and the protein contains one SH2 and two SH3 domains.

The significance of Grb2 binding to the YB site of activated ErbB2 has been deciphered by the Muller laboratory. The ErbB2 YB site overexpressing mice exhibited a significantly higher number of metastasis in lungs, compared to any of the other ErbB2 tyrosine site models. These results indicate that the recruitment of different signaling mediators induces differential biological responses downstream of activated ErbB2 [274], [276].

3.3.3. Memo

Memo (mediator of ErbB2 driven cell motility) is a protein that was first identified in our lab based on its ability to bind the phospho YD site of the ErbB2 receptor [278]. It is encoded by a single gene, and is found in all branches of life, from bacteria to humans. The crystal structure of Memo revealed that it is homologous to class III nonheme iron dependent dioxygenases. However, no metal binding or enzymatic activity has been detected for Memo [282]. Memo does not contain the typical adaptor protein domains such as PTB or SH2. Initially, it was suggested that ShcA mediates its binding to the YD site of ErbB2, as Memo was detected along with ErbB2 in ShcA immunoprecipitates. Moreover, immunodepletion of Shc from reticulocytes decreased the binding of Memo to the ErbB2 phospho YD site [278]. However, it was shown that the immobilized YD peptide is able to bind specifically to purified Memo, suggesting that at least in a this *in vitro* system Memo and ErbB2 interact directly [282]. Interaction of Memo with cofilin and PLCγ1 was shown to be essential during

ErbB2 induced cell migration [283]. Memo deficient, ErbB2 expressing breast cancer cells form actin fibers and grow lamellipodia in response to ErbB2 activation, but do not organize the microtubule cytoskeleton [284]. Although discovered as an ErbB2 binding partner, we were able to show that Memo has a broader role downstream of receptor tyrosine kinases, as its downregulation also impaired the migratory ability of T47D cells in response to FGF-2 (activator of several FGFRs) and EGF (ErbB1 ligand).

The current studies in our lab are aimed at deciphering the contribution of Memo to tumorigenesis, vasculogenesis, metastasis formation and *in vitro* motility downstream of several receptor tyrosine kinases (RTKs). We are using cell lines and mouse tumor models driven by different RTKs to dissect the role of Memo in their oncogenic sinaling.

In the work presented here, I describe the effects of Memo downregulation on tumor growth, angiogenesis, VEGF release, extravasation and colonization of distant organs of 4T1 mammary carcinoma cells.

II. AIMS OF THE WORK

In our lab we are investigating signaling pathways that are driving breast tumorigenesis and metastasis formation. The main focus of our research is deregulated ErbB2 signaling. However, we are interested in contributions of Wnt pathway to tumorigenesis and tumor angiogenesis, and the role of vascular endothelial growth factor receptors (VEGFRs) in tumor growth and metastatic spread.

The main goals of the first project presented in this thesis were to determine the role of the components of Wnt signaling in endothelial cell biology *in vitro* and *in vivo*. Several lines of evidence from cell culture and mouse knock out studies suggest the involvment of this signaling pathway in angiogenesis (see introductory chapters). We wanted to describe and understand the effects of Wnt3a and Wnt5a ligands on human umbilical vein endothelial cell (HUVEC) proliferation, migration and survival. The other goal was to decipher the contribution of Fz6, Wnt receptor shown to be expressed on surface of endothelial cells derived from variety of tissues, to tumor growth and angiogenesis.

The aim of the second project was to delineate the effects of targeting three VEGFRs on tumor growth, vascularization and metastatic spread. We wanted to compare the efficacy of the agent that targets all known receptors to that of the VEGF and VEGFR2 targeting molecules. Regarding the tumor cell biology, our intention was to delineate effects of targeting VEGF and VEGFR family members on proliferation, migration, and chemosenzitation of cancer cells.

ErbB2 receptor tyrosine kinase is a member of epidermal growth factor receptor family and it is overexpressed in 20% of human breast cancers (chapter 3). For this reason our laboratory is interested in finding the proteins that are interacting with this receptor to convey its signaling. Recently, we identified Memo, a novel protein that is mediating ErbB2 driven cell motility [278]. In the study presented here, we wanted to decipher the role of Memo in tumor formation, vascularization and metastatic spread of 4T1 mouse mammary carcinoma model.

III. RESULTS

1. RESEARCH ARTICLE 1, in preparation for submission

Wnt 3a promotes proliferation and migration of HUVEC

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Abstract

Angiogenesis, the process of new blood vessel formation from preexisting vasculature, is characteristic of several pathophysiological processes including cancer. Consequently, untangling signaling pathways that guide angiogenesis is of a central interest for development of antiangiogenesis based therapies. Here we report the inductive role of Wnt3a in proliferation and migration of cultured human umbilical vein endothelial cells (HUVEC), but without an effect on their survival. In a search for probable downstream mediators of Wnt3a effects on HUVEC biology, we found that upon treatment with Wnt3a, phosphorylation of Dvl and β-catenin stabilization take place. Moreover, we identified upregulation of c-Myc, Tie-2 and Connexin 43 transcripts in same conditions, indicating that these genes might be involved in proangiogenic responses of HUVEC treated with Wnt3a. Wnt5a did not show any effect in same assays, though it did induce Dvl phosphorylation. Our microarray data performed on cultured HUVEC revealed that Wnt receptors Fz4 and Fz6 are expressed at significantly higher levels than other Fzs. The analyses of the role of Fz6 receptor in tumor angiogenesis *in vivo* revealed that ablation of this receptor in mice does not affect the ability of LLC cells to grow, and does not influence their vascularization. Taken together, our data indicate that Wnt3a has the ability to induce the canonical Wnt pathway in cultured HUVEC, and can stimulate their proliferation and migration.

Introduction

Wnts are a family of secreted glycoproteins that bind to transmembrane Frizzled (Fz) receptors and initiate signaling cascades with indispensable roles during development, cell proliferation, migration, and survival [285]. Several pathways downstream of Wnt binding to Frizzled are known, Wnt β-catenin (or Wnt 'canonical' pathway) being the most studied. In the absence of activated Wnt β-catenin signaling, the transcription co-factor β-catenin is targeted for degradation by a destruction complex that consists of axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK-3β). Activation of the canonical Wnt pathway results in recruitment of the cytoplasmic mediator Dishevelled and disassembly of destruction complex causing the stabilization of the β-catenin. Stabilized β-catenin accumulates in the cytoplasm, enters the nucleus, and acts as a co-activator of T-cell factor/lymphoid enhancer factor-1 (TCF/LEF) transcription factors and initiates transcription from their target genes. Among several 'non-canonical' Wnt pathways, Wnt planar cell polarity (PCP) and Wnt calcium pathways are the best understood, for a review see [286]. Both pathways activate Dishevelled, but diverge further down to either GTPases Rho/Rac, or calcium activated protein kinase C (PKC) and calcium/calmodulin dependent-protein kinase II (CamKII), respectively. Although usually described as individual pathways, reported

crosstalk and shared components between downstream Wnt signaling branches suggest the possibility that Wnts act through complex intracellular signaling network [287].

Angiogenesis is a process of new blood vessel formation from pre-existing vasculature [138]. In order to form blood vessels, endothelial cells need to proliferate, migrate and survive. The importance of Wnt signaling in these processes is emphasized by the fact that mice deficient for Fz4 [288], Fz5 [160], or Wnt2 [156] are impaired in blood vessel formation. Mutations within human Fz4 gene cause defective vascularization of the eye and the ear [161], [63].

In this study, we show that Fz4 and Fz6 are expressed by Human Umbilical Vein Endothelial Cells (HUVEC) and can convey the Wnt signal to downstream components. We report that Wnt3a induces HUVEC proliferation and migration, but without an effect on their survival. Proliferation induced by Wnt3a is VEGFR signaling independent.

Results

Human Umbilical Vein Endothelial Cells express Fz-4 and Fz-6

It has already been shown that Frizzled receptors and other components of the Wnt pathway are expressed by HUVEC [168]. To analyze the expression of Frizzled receptors in the HUVEC used in our experiments we performed a microarray analysis. The results indicate that Fz4 and Fz6 are expressed at a much higher level than the other Frizzled receptors (Figure 1a). Fz4 receptor is known to stimulate PKC activation, [289] and β-catenin stabilization [290], [291], [63]. Fz6 induces PKC activation too, [289] and is involved in tissue/planar cell polarity [101]. It was shown that Fz6 can negatively regulate the canonical Wnt β-catenin pathway [292]. The expression of both receptors was confirmed by PCR (Figure 1b).

Wnt3a and Wnt5a induce Dvl3 phosphorylation in HUVEC

Signaling cascades stimulated by Wnts are diverse including the Wnt β-catenin, Wnt planar cell polarity (PCP) and Wnt-calcium pathway. The first known downstream effect of Wnt binding to its receptor Frizzled is Dishevelled phosphorylation and activation. Dvl phosphorylation was shown to mediate β-catenin and PCP pathways [74] as well as Wnt-calcium pathway [75], and is established as a general biochemical assay for Wnt protein function [293].

In order to test the functionality of expressed Fz4 and Fz6 receptors, we treated HUVEC with commercially available Wnt3a and Wnt5a ligands. Historically, Wnt3a was considered as a member of a subclass of Wnt family that activate only the canonical pathway [294]. However, accumulating evidence suggests that the outcome of downstream signaling is dictated by the ligand and receptor context in each system studied. For example, Wnt5a, a typical ‘non-

canonical Wnt' [294], was shown to stabilize β-catenin if co-expressed with Fz4 and LRP5 [291]. In the same manner, it was reported that Wnt3a can act through a non-canonical pathway during osteoblastogenesis [295]. In our experiments we used Wnt3a and Wnt5a to try to activate different Wnt signaling routes through the expressed Fz4 and Fz6. Both Wnts were used at concentration of 200 ng/ml, and Dvl phosphorylation, as demonstrated by the shift in Dvl band in Western blot, was checked 1, 2 and 8 hours after treatment. As shown in the Figure 2a, both Wnts induced a shift in Dvl3 band indicating pathway activation. Wnt3a appeared to induce Dvl phosphorylation more potently than Wnt5a. When used together with Wnt ligands, recombinant Fz4 abrogated the effect on Dvl3 phosphorylation (Figure 2b), which shows that it can compete with the endogenous receptors to bind to Wnts.

Wnt3a induces HUVEC proliferation in a VEGFR signaling independent manner

To analyze the effect of pathway activation on HUVEC proliferation, we used Wnt3a and Wnt5a in a BrdU ELISA incorporation assay. VEGF-A, a factor known to induce HUVEC proliferation [296] was used as a positive control. As shown in Figure 3a, Wnt3a stimulated HUVEC proliferation, but the effect was abolished when Wnt3a was added in a combination with recombinant Fz4. In spite of the ability to induce Dvl3 phosphorylation (Figure 2), Wnt5a did not increase HUVEC proliferation in our assay even at a concentration of 800 ng/ml (Figure 3b). Since activated Wnt signaling can induce VEGF transcription [297], we tested if the effect of Wnt3a on HUVEC proliferation is due to VEGF upregulation, by employing a VEGFR kinase inhibitor PTK787/ZK [255] together with Wnt3a in a proliferation assay. PTK787/ZK did not influence Wnt3a induced proliferation, showing that stimulation of proliferation by Wnt3a in HUVEC is VEGFR signaling independent (Figure 3a).

To check for the ability of Wnt3a to induce HUVEC proliferation in the presence of CamKII inhibitor, we used this growth factor together with increasing concentration of KN93, an inhibitor that prevents the binding of calmodulin to CamKII. As seen from the Figure 3c, KN93 was able to reduce Wnt3a induced proliferation when used at concentration of 0.5 μM, indicating the requirement of CamKII for Wnt3a induced proliferation

Wnt3a induces HUVEC migration, but neither Wnt promotes survival in a condition of medium starvation

The ability of endothelial cells to migrate is required during blood vessel formation. We performed migration assays with HUVEC in Boyden chambers using Wnt3a and Wnt5a as chemoattractants in the lower chamber. As shown in Figure 4, the number of migratory HUVEC in response to Wnt3a was significantly higher compared to control or Wnt5a, indicating that Wnt3a stimulates HUVEC migration.

Next, we tested whether either of the Wnt ligands can rescue HUVECs from starvation induced apoptosis. For this purpose, we took advantage of the YO-PRO reagent, a dye that enters only apoptotic cells. VEGF was used as a positive control. Neither of the Wnts influenced HUVEC survival (Figure 5), while VEGF significantly increased it.

Wnt3a but not Wnt5a induces β-catenin stabilization

To check for the downstream signaling pathway initiated by Wnt3a and Wnt5a, we did immunoblot analysis with antibody detecting β-catenin 2h, 8h and 24h after stimulation. We detected increase in β-catenin band upon treatment with Wnt3a, but not Wnt5a (Figure 6), indicating canonical Wnt pathway activation.

Wnt3a and Wnt5a transcriptional targets in HUVEC

To try to understand the mechanism by which Wnt3a induces biological effects in HUVEC, we checked for the up-regulation of known Wnt target genes that are angiogenesis related. c-Myc is known canonical Wnt transcriptional target gene [298] with documented roles in cell proliferation and migration [299]. Another protein involved in cell-cycle progression downstream of canonical Wnt signaling is CyclinD1 [300]. MMP-1 and Tie-2, have been shown as transcriptional targets of Wnt5a in HUVEC induced by non-canonical Wnt signaling [171], but are not listed as canonical Wnt target genes[19]. Finally, Connexin43, a cellular gap junction protein, has been reported to be a target of Wnt pathway present in vasculature [168]. We isolated RNA from non-treated or Wnt3a and Wnt5a treated HUVEC samples and performed semiquantitative PCR on prepared cDNA, using primers for c-Myc, Tie-2, Connexin 43, MMP-1 and CyclinD1. As depicted in Figure 7, Wnt3a induces the expression of c-Myc, Tie-2 and Connexin 43 strongly, already 5h after treatment, and the effect persists 24h after adding the ligands. In contrast, Wnt5a did not induce cMyc up-regulation at any time point, but we detected an increase in Tie-2 and Connexin 43 transcripts 24h after treatment. Neither Wnt3a nor Wnt5a influenced transcription of MMP-1 and CyclinD1. In conclusion, our data show that the expression of genes known as both canonical (c-Myc), and non-canonical Wnt target genes (Tie-2) are upregulated upon Wnt3a treatment.

In vivo LLC growth in Fz6 deficient mice

Our in vitro data suggest the positive role of Wnt signaling in endothelial cell biology. Activation of the pathway by Wnt3a through either Fz4 or Fz6 receptor induced Dvl3 phosphorylation in HUVEC and enhanced their ability to proliferate and migrate.

To test the importance of Wnt signaling through Frizzled receptors in endothelial cells in an *in vivo* model, we injected Fz6 deficient mice with Lewis Lung Carcinoma cells (LLC) and monitored tumor growth. LLC cells developed well vascularized tumors already within a

week after injection and served as a good model to monitor effect of Fz6 ablation on blood vessel formation. Tumor growth was measured in comparison to wild-type and Fz6+/- littermates. As seen from the Figure 8a, Ablation of Fz6 did not have an effect on primary tumor growth. The staining of tumor sections with CD31 antibody and subsequent inspection of tumor vasculature revealed no differences between the appearance and quantity of vasculature in control and tumors grown in Fz6 deficient mice (Figure 8b). Fz4 deficient mice we could not use in the same experiment, because we were not able to generate these mice in Bl6 background.

2. RESEARCH ARTICLE 2

Inhibition of multiple vascular endothelial growth factor receptors (VEGFR) blocks lymph node metastases but inhibition of VEGFR-2 is sufficient to sensitize tumor cells to platinum-based chemotherapeutics

Author's contribution to the article

1) Delineating the expression of VEGFRs in MDA435, A375 and B16Bl6 cells *in vitro* and on lymph node metastasis derived from B16Bl6 cells by immunochemistry (Figure 3a and 3d in the article, experiment conducted together with Patrizia Sini, PS)

2) Analysis of the expression of VEGFRs in B16Bl6 cells *in vitro* by flow cytometry cell sorting (FACS) method, before and after membrane permeablization (Figure 3b, experiment conducted together with PS)

3) B16/Bl6 *in vitro* migration assay in the presence or absence of increasing concentrations of PTK/ZK (Figure 4b, experiment conducted together with PS)

4) Defining the molecular pathway that confers the ability of PTK/ZK to chemosensitize cancer cells to platinum based chemotherapeutics *in vitro*.

In order to find the molecular pathway that is mediating PTK/ZK induced chemosensitization, I have checked the status of AKT and phospho AKT, survivin, cleaved caspase 3, and cleaved PARP by immunoblotting after treatment with cisplatin alone, or in combination with PTK/ZK, using B16/Bl6, MDA 435, DU145 and A375 cells. I could not detect differences in protein levels of any of the mentioned downstream mediators of apoptosis after combinatiorial treatment with cisplatin and PTK/ZK in comparison to these agents alone. However, PS found that the effect of PTK/ZK on chemosensitation of B16/Bl6 cells is mediated by MAPK pathway (Figure 5).

5) Delineating the expression of VEGFRs on A549 cells *in vitro* by FACS method (Figure 6c)

6) Analysis of combinatorial effects of cisplatin and PTK/ZK on apoptosis in A549 cells (Figure 6c)

All the Figures were prepared for publication by Patrizia Sini.

Inhibition of Multiple Vascular Endothelial Growth Factor Receptors (VEGFR) Blocks Lymph Node Metastases but Inhibition of VEGFR-2 Is Sufficient to Sensitize Tumor Cells to Platinum-Based Chemotherapeutics

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Abstract

Vascular endothelial growth factor receptors (VEGFR) have important roles in cancer, affecting blood and lymphatic vessel functionality as well as tumor cells themselves. We compared the efficacy of a VEGFR tyrosine kinase inhibitor, PTK787/ZK222584 (PTK/ZK), which targets the three VEGFRs, with blocking antibodies directed against VEGFR-2 (DC101) or VEGF-A (Pab85618) in a metastatic melanoma model. Although all inhibitors exerted comparable effects on primary tumor growth, only PTK/ZK significantly reduced lymph node metastasis formation. A comparable decrease in lymphatic vessel density following blockade of VEGFR-2 (DC101) or the three VEGFRs (PTK/ZK) was observed in the metastases. However, the functionality of lymphatics surrounding the primary tumor was more significantly disrupted by PTK/ZK, indicating the importance of multiple VEGFRs in the metastatic process. The antimetastatic properties of PTK/ZK were confirmed in a breast carcinoma model. B16/BL6 tumor cells express VEGF ligands and their receptors. Blockade of a VEGFR-1 autocrine loop with PTK/ZK inhibited tumor cell migration. Furthermore, the tumor cells also showed enhanced sensitivity to platinum-based chemotherapy in combination with PTK/ZK, indicating that autocrine VEGFRs are promoting tumor cell migration and survival. In summary, our results suggest that, in addition to blocking angiogenesis, combined inhibition of the three VEGFRs may more efficiently target other aspects of tumor pathophysiology, including lymphatic vessel functionality, tumor cell dissemination, survival pathways, and response to chemotherapeutic compounds. [Cancer Res 2008;68(5):1581–92]

Introduction

Metastatic spread of tumor cells is the underlying cause of most cancer-related deaths. Clinical and pathologic evidence shows that, for many human tumors, dissemination of cells via lymphatic vessels to local/regional lymph nodes is an early event in metastatic disease. Recently, there has been an increase in the number of mechanistic studies on tumor-associated lymphangiogenesis and lymphatic metastases (1, 2). Under physiologic conditions, lymphatic vessels in most adult tissues are quiescent. Results from various animal models suggest that lymphangiogenesis can be induced by solid tumors and may promote tumor spread (3). The most extensively studied signaling system promoting tumor lymphangiogenesis includes vascular endothelial growth factor (VEGF)-C and VEGF-D, and VEGF receptor-3 (VEGFR-3), their cognate receptor on lymphatic endothelium (4). An increasing number of clinicopathologic studies have shown a direct correlation between expression of VEGF-C or VEGF-D and metastatic tumor spread (5). These ligands, together with others implicated in tumor lymphangiogenesis, such as platelet-derived growth factors (6) and VEGF-A (7, 8), might enhance metastases by increasing the number of lymphatic vessels, thereby increasing contact between the invading cancer cells and the lymphatic endothelium. Moreover, VEGFR-2, the major blood vessel endothelial cell receptor for VEGF-A, has also been detected in collecting lymphatic vessels and capillaries undergoing active lymphangiogenesis (4). Because mature forms of VEGF-C, VEGF-D, and VEGF-A bind and activate VEGFR-2, and induce VEGFR-2/VEGFR-3 heterodimers (9, 10), VEGFR-2 might also influence lymphangiogenesis.

In addition to endothelial cell expression, VEGFRs are also expressed by tumor cells (11–14). Although the functions of VEGFR family members on tumor cells are not completely understood, the concomitant expression of VEGF and VEGFR suggests that these receptors might mediate biological effects in an autocrine fashion.

Various approaches have been taken to interfere with the VEGF/VEGFR system, including antagonistic antibodies (15, 16), dominant-negative VEGFR mutants (17), recombinant soluble VEGFR proteins (18), and small-molecule tyrosine kinase inhibitors (TKI). Bevacizumab (Avastin, Genentech, Inc.), an anti–VEGF-A antagonist antibody, has been approved for use in combination with 5-fluorouracil (5-FU)-based chemotherapy for treatment of patients with metastatic colorectal cancer (19, 20). PTK787/ZK222584 (PTK/ZK; vatalanib, Novartis Pharma/Schering AG; ref. 21), a potent pan-VEGFR TKI, inhibits angiogenesis, tumor growth, and metastasis formation in experimental carcinoma models (22, 23). PTK/ZK has displayed activity in early clinical trials (24, 25).

Here, we have compared the effectiveness of PTK/ZK with that of an anti–VEGFR-2 blocking antibody (DC101, ImClone Systems;

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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ref. 16) and an anti–VEGF-A blocking antibody (Pab85618 or bevacizumab, anti-mouse and anti-human VEGF-A, respectively) in inhibiting tumor metastases. Our results show that blockade of VEGFR-2 signaling affects primary tumor growth but additional inhibition of VEGFR-1 and/or VEGFR-3 signaling is required for a significant reduction of lymphatic vessel functionality and tumor cell metastatic spread. Blockade of VEGFR-1 and VEGFR-2 on tumor cells inhibits migration and enhances sensitivity to cisplatin/oxaliplatin-induced apoptosis, respectively. These findings suggest that blockade of multiple VEGFRs directly affects the biological functions of both endothelial and tumor cells.

Materials and Methods

Cell culture. B16/BL6 mouse melanoma cells (from Dr. J. Fidler, Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX) were cultured in MEM EBS (AMIMED), 5% FCS, 2% vitamin, 1% nonessential amino acids, and 1% sodium pyruvate; A549, A375, and MB-MDA435 (American Type Culture Collection) were cultured in RPMI 1640, 10% FCS, 1% L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Lymphatic endothelial cells (LEC) were maintained on fibronectin-coated culture dishes (50 μg/mL) in microvascular endothelial cell growth medium (PromoCell, BioConcept AG).

Angiogenesis (chamber) assay. The assay was performed as described (23). Mice were treated with PTK/ZK (25, 50, and 100 mg/kg, p.o.), DC101 (5, 10, 21, and 42 mg/kg, i.p.), or Pab85618 (0.5, 1.5, and 5 mg/kg, i.p.) 4 to 6 h before chamber implantation. Dosing was once daily for 3 days for PTK/ZK, once on day 3 for DC101, and on day 2 for Pab85618. Four days after implantation, animals were sacrificed and the chamber was removed and weighed as described (23, 26).

Tumor models. The B16/BL6 murine melanoma model was used (23). Mice were treated with either vehicle PEG300, PBS (5 mL/kg), PTK/ZK (p.o., once daily at 25, 50, and 100 mg/kg), DC101 (i.p., every 3 days at 10, 21, and 42 mg/kg), Pab85618 (i.p., every 2 days at 1.5 and 5 mg/kg), or an IgG isotype control. No significant loss of body weight occurred in treated animals. On day 21, mice were perfused with 1% paraformaldehyde as described (26). Cervical lymph node metastases were weighed and frozen for histologic analysis.

Frozen BN472 tumors were washed in 0.9% NaCl with 100 μg/mL gentamicin and placed in a Petri dish with Ham's F-12 medium (100 μg/mL gentamicin, 10% FCS), and fragments of ~25 mm^3 were transplanted in mammary fat pads of Brown Norwegian rats (Harlan). Treatment with PTK/ZK, given as described above, was initiated 1 day after transplantation and continued for 4 weeks. Lung metastases were determined by counting foci on the surface of lungs fixed in Bouin's solution. Excised axial lymph nodes were weighed. The A549 non–small lung cancer cells (1.5×10^6) were injected s.c. into NMRI *nu/nu* mice. Treatment started with a palpable tumor size of 20 mm^2. PTK/ZK was given p.o. daily at 100 mg/kg, and cisplatin was given i.p. every 3 days at 2 mg/kg. After 41 days, animals were sacrificed and tumors were excised and weighted.

Lymphatic vessel density quantification. Tumor cryosections were stained for either LYVE-1 or VEGFR-3 as above. Pictures encompassing six representative regions were taken at ×10 magnification (Zeiss Axioplan). Areas of the counted regions were measured using the Openlab 3.1.5 software (Improvision).

Lymphangiography. Mice were anesthetized (Forene, Abbott AG) and 3 to 5 μL of Evans blue (3.5% in saline) were injected in the tumor center. Images were acquired 5 min after injection. The lymphatic network length (μm) connected to the primary tumor was quantified using computer-assisted imaging software (KS-400 Imaging System, Zeiss; ref. 27).

Immunoblot analysis. Treatment of cultures with growth factor was performed after serum starvation and 2 h of incubation with the compound. Immunoprecipitations were performed as described previously (28), and immunocomplexes were resolved by SDS-PAGE and analyzed by immunoblotting. To determine the effect of combination drug treatments on extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT activation, B16/BL6 cells were made quiescent overnight in serum-free medium and pretreated with PTK/ZK (1,000 nmol/L) for 1 h followed by treatment with the indicated concentration of cisplatin (Platinol, Bristol-Myers Squibb GmbH) in growth medium for 6 h at 37°C. Cells were lysed and proteins were analyzed by immunoblotting.

Proliferation and apoptosis assays. Subconfluent LECs (5×10^3 per well) were seeded into 96-well plates for 24 h. For proliferation assays, they were serum starved (basal medium, 1% FCS) and then VEGF-A (20 ng/mL) or VEGF-C (400 ng/mL) was added in the presence or absence of PTK/ZK or bevacizumab. The antiproliferative effect of PTK/ZK and bevacizumab on LECs was evaluated using a bromodeoxyuridine (BrdUrd) ELISA kit, as previously described (28). For apoptosis assays, the medium was replaced with basal medium containing 0.5% FCS and VEGF-A (20 ng/mL) or VEGF-C (400 ng/mL) was added in the presence or absence of PTK/ZK or bevacizumab for 48 h. The percentage of cell death was measured using the YO-PRO assay (29). For the combination studies, cells (B16/BL6, A375, and MB-MDA435) were seeded into 96-well plates and then incubated in the presence or absence of PTK/ZK (1,000 nmol/L) or DC101 (1 μg/mL) combined with increasing concentrations of cisplatin (0–10 μg/mL), oxaliplatin (0–10 μg/mL), or 5-FU (0–10 μmol/L) for 24 h at 37°C. The percentage of cell death and cell number was measured using the YO-PRO assay. Growing A549 cells were incubated with PTK/ZK (1,000 nmol/L) for 24 h followed by PTK/ZK combined with cisplatin (0–25 μg/mL) for an additional 24 h at 37°C. Cells were stained with Annexin V (Vybrant Apoptosis Assay Kit 2, Molecular Probes) and analyzed with a FACSCalibur (Becton Dickinson).

Migration assays. B16/BL6 cells (1×10^5) in MEM EBS plus 0.5% FCS with or without PTK/ZK (0–1,000 nmol/L), IgG (1.25 μg/mL), or DC101 (1.25 μg/mL) were placed on 8.0-μm pore size membrane inserted in 24-well plates (Boyden chambers, Becton Dickinson Labware). MEM EBS plus 0.5% fetal bovine serum (FBS) with or without PTK/ZK (0–1,000 nmol/L), IgG (1.25 μg/mL), or DC101 (1.25 μg/mL) were placed in the bottom wells. After 6 h, cells that did not migrate were removed; cells that had migrated were stained with Hoechst 33342. Membranes were removed and mounted on slides and pictures were taken at ×5 magnification. Migrated cells were counted using the computer-assisted imaging software Imaris (Bitplane AG).

Results

The *in vivo* antitumor and antimetastatic effect of PTK/ZK, DC101, and an anti–VEGF-A antibody. The effective doses and optimal regimens to block VEGF-mediated responses for the multi-VEGFR TKI PTK/ZK, the anti–VEGFR-2 (DC101) and anti–VEGF-A (Pab85618) antibodies were determined as described in Supplementary Fig. S1. A dose-response curve was obtained for primary tumor growth, for weight of cervical lymph nodes, and for a VEGF-driven angiogenesis chamber model (23, 26). The optimal concentration for each compound was chosen based on complete inhibition of angiogenesis in the chamber model and the maximum inhibitory effect on primary tumor growth. DC101, dosed at 42 mg/kg, was more efficient in tumor growth inhibition compared with the 21 mg/kg dose, but at the higher dose, no additional effect was observed on angiogenesis or lymph node metastases weight (Supplementary Fig. S1). The concentrations used in the following experiments were as follows: PTK/ZK, 100 mg/kg; DC101, 42 mg/kg; and Pab85618, 5 mg/kg.

PTK/ZK, DC101, and anti–VEGF-A (Pab85618) were evaluated as antitumor and antimetastatic agents in the syngeneic orthotopic B16/BL6 melanoma model, an aggressive metastatic subline derived from B16 cells (30). When B16/BL6 cells are implanted i.d. in the ears of C57BL6 mice, primary tumors form (Fig. 1*A*). Seven to 10 days after tumor cell implantation, cervical lymph node metastases develop (Fig. 1*B*), with new blood vessel formation becoming evident in the same time frame. The optimal time for antitumor therapy is between 7 days (when there are no significant differences in primary tumor sizes) and 21 days, as subsequently

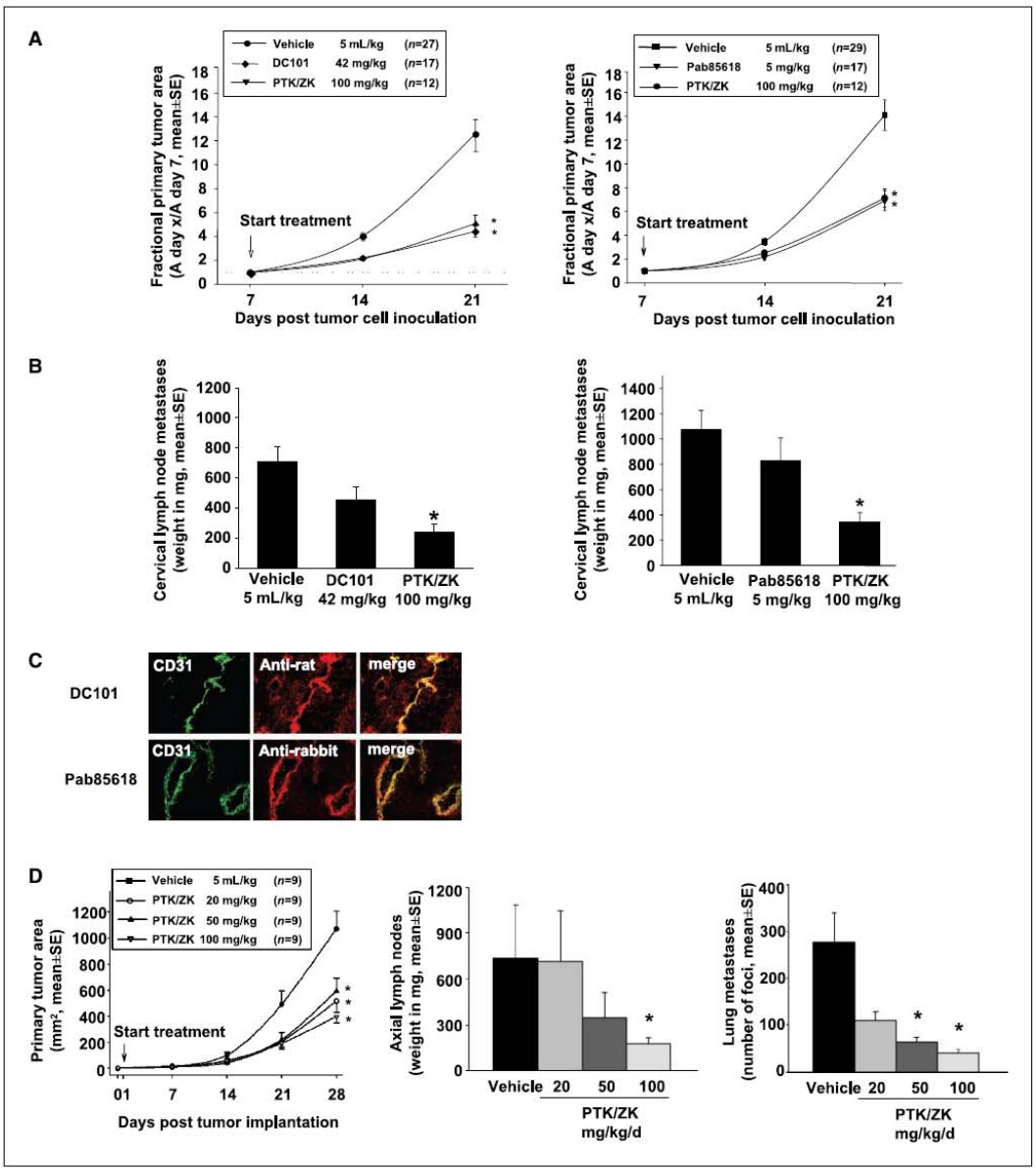


Figure 1. Effect of blockade of VEGFRs on primary tumor and lymph node metastases in the B16/BL6 orthotopic melanoma mouse model and the BN472 orthotopic breast carcinoma model. *A*, B16/BL6 primary tumor growth measured at days 7, 14, and 21 after tumor cell inoculation. *Points*, mean; *bars*, SE. *B*, average weight of cervical lymph node metastases measured on day 21 following treatment between day 7 and day 21. *Columns*, mean; *bars*, SE. *, $P < 0.05$, treatment groups versus control group (one-way ANOVA and post hoc Holm-Sidak test). *C*, immunofluorescent detection of DC101 and Pab85618 in B16/BL6 metastases. Tumor sections from lymph node metastases were stained for CD31 (*green*) and incubated with either anti-rat (DC101 treated; *top*) or anti-rabbit (Pab85618 treated; *bottom*) secondary antibodies (*red*). Images captured for endothelial cells (*CD31*; *green*) were overlapped with images captured for DC101 or Pab85618; colocalization of the two blocking antibodies and CD31 is shown in *yellow* (*merge*). *D, left*, BN472 primary tumor growth measured at days 7, 14, 21, and 28 after transplantation of tumor fragments from donor rats in the mammary fat pads of Brown Norwegian rats. *Points*, mean; *bars*, SE. Average weight of axial lymph node metastases (*middle*) and number of foci of lung metastases (*right*) measured on day 28 following treatment between day 1 and day 28. *Columns*, mean; *bars*, SE. *, $P < 0.05$, treatment groups versus control group (one-way ANOVA and post hoc Holm-Sidak test).

the metastases become necrotic. The agents were given as described in Materials and Methods, and on day 21, the animals were sacrificed and primary tumors and lymph node metastases were analyzed (Fig. 1*A* and *B*). The growth of the primary tumor and metastases in mice treated with either PEG (vehicle control for PTK/ZK) or PBS (vehicle control for DC101 and Pab85618) was not statistically different and the data were pooled in a control group. Similarly, no difference in the effect of IgG versus vehicle control was observed (data not shown). Although statistically significant inhibition of the primary tumor was observed in mice receiving either PTK/ZK (50–63%), DC101 (48%), or Pab85618 (55%; Fig. 1*A*), only PTK/ZK treatment resulted in a significant reduction of the metastases weight (Fig. 1*B*). Furthermore, differences in the frequency of lymph node metastases were also observed, although they did not reach statistical significance. Ninety-five percent (38 of 40) of control mice had metastases, whereas in the PTK/ZK-, DC101-, and Pab85618-treated mice 79% (19 of 24), 88% (21 of 24), and 89% (16 of 18), respectively, had metastases.

To confirm that the antibodies reached the metastatic site, cryosections of lymph node metastases were stained for CD31 and incubated with secondary antibodies against rat (DC101) or rabbit (Pab85618; Fig. 1*C*). Double staining revealed that both DC101 and Pab85618 had diffused into the metastases and were localized around the blood vessels.

The antitumor and antimetastatic properties of PTK/ZK were also assessed in the BN472 orthotopic rat mammary cancer model (Fig. 1*D*). Similar to the B16/BL6 melanoma data, PTK/ZK treatment resulted in a significant dose-dependent reduction in the size of the primary tumor (Fig. 1*D, left*) and in the axial lymph node and lung metastases (Fig. 1*D, middle* and *right*). It was not possible to test the efficacy of DC101 or Pab85618 because these antibodies do not cross-react with rat VEGF-A or VEGFRs.

Taken together, these results suggest that, in the B16/BL6 model, blockade of multiple VEGFRs is more effective in blocking metastasis compared with individual blockade of VEGFR-2 or VEGF-A. Thus, it seems that additional inactivation of VEGFR-1 and/or VEGFR-3 by PTK/ZK may suppress tumor cell dissemination in addition to inhibiting primary tumor cell growth and lymph node metastases. To address whether these observations reflect the blockade of angiogenesis and lymphangiogenesis, we examined the effect of PTK/ZK, DC101, and Pab85618 on blood and lymphatic vessel density in the subsequent experiments.

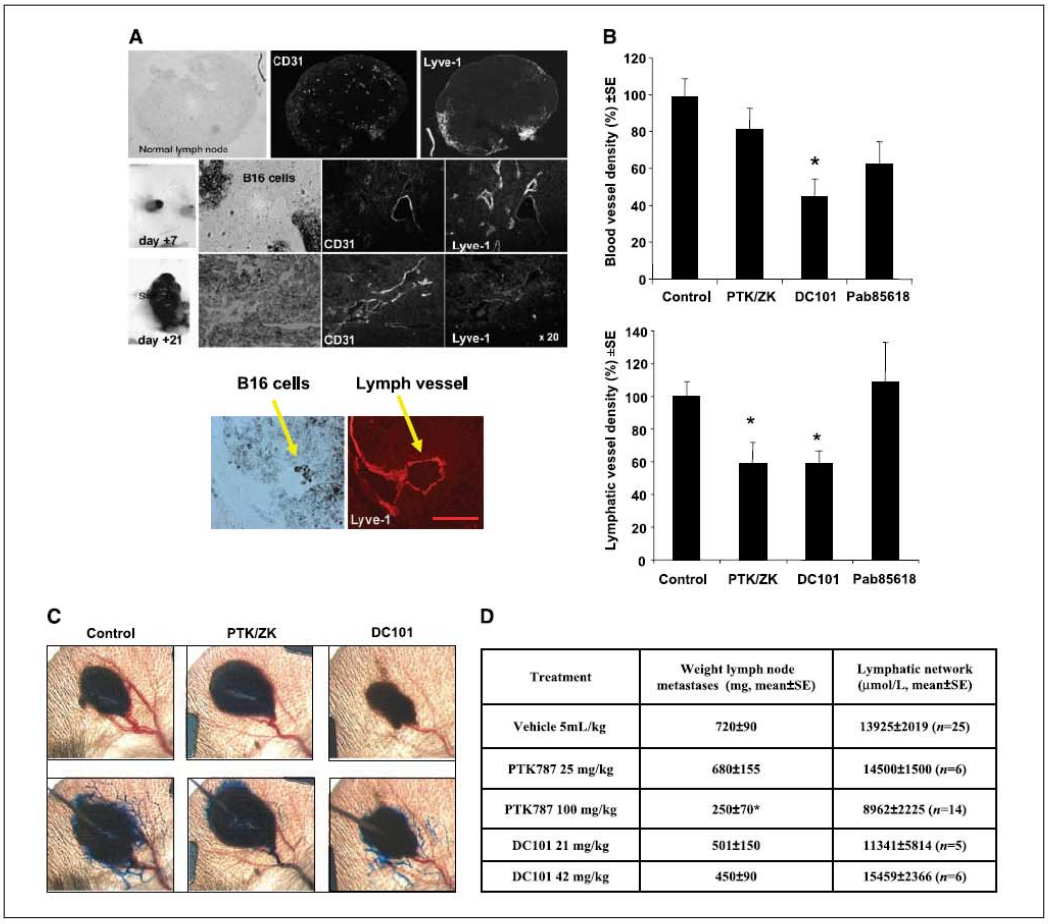
Blockade of intratumoral angiogenesis and lymphangiogenesis is not sufficient to reduce metastatic tumor cell spread. Tumor cells enter the lymphatic system by inducing intratumoral lymphangiogenesis or by coopting preexisting lymphatics in the surrounding tissue (1, 31). We first examined if spread of B16/BL6 tumor cells is accompanied by lymphangiogenesis in the primary tumor and lymph nodes. Blood and lymphatic vessels were visualized 7 and 21 days after tumor cell implantation by immunostaining with antibodies for CD31 and LYVE-1, respectively. In the primary tumor, few lymphatics were observed throughout the entire time course. By day 21, peritumoral lymphatics and, in larger tumors, some intratumoral lymphatics were present (data not shown). In normal lymph nodes, preexisting peripheral lymphatics and blood vessels were observed (Fig. 2*A*) and tumor cell spread was accompanied by an increase in intratumoral lymphangiogenesis (Fig. 2*A*, day 7). Lymphatic vessels with lumen-containing tumor cells were also detected (Fig. 2*A, bottom*). At day 21, lymphatic vessels were present but compressed toward the edge of the tumor (Fig. 2*A*).

Next, we investigated whether the different effects of PTK/ZK, DC101, and Pab85618 on tumor metastases could be attributed to their differential abilities to block tumor angiogenesis and lymphangiogenesis. Cryosections of primary tumors and metastases from treated animals were stained and the blood and lymphatic vessel density was quantified (Fig. 2*B*). Due to the high number of CD31-positive vessels in the ear, we could not distinguish vessels in the skin from vessels in the primary tumor. All three agents reduced the blood vessel density in the metastases (Fig. 2*B, top*). Treatment with either PTK/ZK or DC101 significantly reduced lymphatic vessel density in the metastases (Fig. 2*B*), whereas no effect was observed in the primary tumor (data not shown). Because Pab85618 had no effect on lymphangiogenesis (Fig. 2*B*) or metastatic tumor growth (Fig. 1*B*), it was not further analyzed.

The results show that lymphangiogenesis in the metastases can be significantly reduced to essentially the same extent with DC101 and PTK/ZK treatment (Fig. 2*B*), reflecting that lymphatics formed in the lymph nodes during the metastatic process are equally affected by both. Furthermore, the stronger effect of PTK/ZK compared with DC101 on weight of the metastases is unlikely to be due to its inhibition of angiogenesis (i.e., of nutrient and oxygen supply) because DC101 had even a stronger effects on angiogenesis in the metastatic lymph nodes than did PTK/ZK (Fig. 2*B*). Thus, we were prompted to examine the lymphatics that allow spread from the primary tumor.

Efficient blockade of lymphatic vessel functionality reduces metastatic tumor cell spread. It is well documented that enlarged, hyperplastic lymphatics are present at the periphery of most tumors (32, 33). The increased size of these vessels and the high interstitial fluid pressure within the tumor may facilitate access of tumor cells into the peripheral lymphatics. Therefore, we next examined the effect of PTK/ZK and DC101 on functionality of the lymphatic vessels connected to the primary tumor by measuring their ability to uptake fluid. Fourteen days after tumor cell implantation, a time when the difference in primary tumor size between control and treated mice was not yet significant, Evans blue was injected in the tumor center and images were acquired. The functional lymphatic network connected to the primary tumors was assessed by visual inspection (Fig. 2*C*) and image analysis (Fig. 2*D*). PTK/ZK (100 mg/kg), compared with DC101 (21–42 mg/kg), blocked lymphatic vessel functionality more efficiently, as measured by Evans blue dye uptake (Fig. 2*C* and *D*) and by lymph node metastatic spread (Fig. 1*B*; Supplementary Fig. S1). At 25 mg/kg, PTK/ZK had no significant effect on primary tumor growth and metastases (Supplementary Fig. S1) or on lymphatic vessel functionality (Fig. 2*D*). These data provide evidence that inactivation of VEGFR-2 alone does not impair lymphatic endothelium functionality and metastatic spread of tumor cells as well as blockade of all the VEGFRs. Compared with DC101, PTK/ZK treatment resulted in a strong inhibition of lymphatic functionality, leading to impaired tumor cell spread and a more significant inhibition of metastatic tumor growth in the lymph nodes.

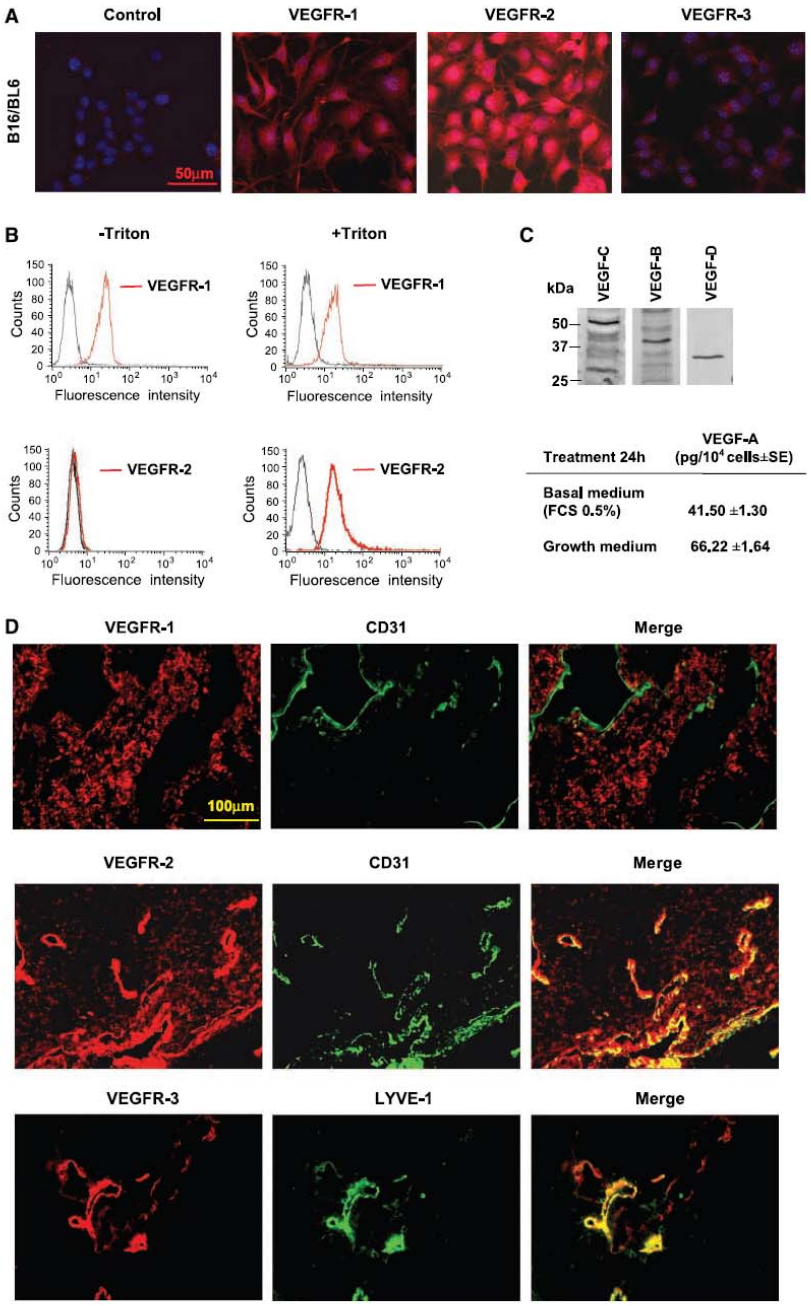
***In vitro* effect of PTK/ZK and bevacizumab on isolated LECs.** To examine potential differences in the role of VEGFR-2 and VEGFR-3 in the lymphatic endothelium, we used human LECs as an *in vitro* model. VEGFR-2, VEGFR-3, and podoplanin, a mucin-like glycoprotein expressed in lymphatic vessels, were detected in the LECs (Supplementary Fig. S2*A*), attesting to the lymphatic nature of the cells. Addition of VEGF-C and, to a lesser extent, VEGF-A increased VEGFR-3 tyrosine phosphorylation (Supplementary



Treatment	Weight lymph node metastases (mg, mean±SE)	Lymphatic network (μmol/L, mean±SE)
Vehicle 5mL/kg	720±90	13925±2019 (*n*=25)
PTK787 25 mg/kg	680±155	14500±1500 (*n*=6)
PTK787 100 mg/kg	250±70*	8962±2225 (*n*=14)
DC101 21 mg/kg	501±150	11341±5814 (*n*=5)
DC101 42 mg/kg	450±90	15459±2366 (*n*=6)

Figure 2. Time course of lymphatic vessel growth and effect of PTK/ZK or DC101 on lymphatic density and functionality in B16/BL6 metastases. *A*, pictures of lymph nodes before implantation and on days 7 and 21 after tumor cell inoculation. Cryosections of normal lymph nodes or metastases were stained for CD31 and the lymphatic vessel marker LYVE-1. *Bottom*, B16/BL6 cells captured inside a lymphatic vessel. *Bar*, 50 μm. *B*, cryosections of B16/BL6 lymph node metastases from mice treated with either vehicle control, Pab85618, DC101, or PTK/ZK were removed 21 d after tumor cell inoculation and stained, and blood and lymphatic vessel density was determined by counting the number of CD31-positive or VEGFR-3–positive vessels per mm^2 in a midsection of a lymph node metastasis, respectively. *Columns*, mean; *bars*, SE. *, $P < 0.05$ versus control (ANOVA with post hoc Holm-Sidak test). *C*, 14 d after tumor cell implantation, the functional lymphatic networks connected to the primary tumors were quantified as described in Materials and Methods. *D*, the length of the functional lymphatic network (μm) and the weight of lymph node metastasis in mice treated with vehicle, PTK/ZK (25 and 100 mg/kg), or DC101 (21 and 42 mg/kg) were quantified as described in Materials and Methods. *, $P < 0.001$ versus control (ANOVA with post hoc Holm-Sidak test).

Fig. S2*B*) and AKT and ERK1/2 activation (Supplementary Fig. S2*C*). Pretreatment with 1,000 nmol/L PTK/ZK blocked VEGF-C–induced and VEGF-A–induced VEGFR-3 phosphorylation (Supplementary Fig. S2*B*), which was accompanied by a blockade of intracellular signaling (Supplementary Fig. S2*C*, *top*). Pretreatment with the anti–VEGF-A blocking antibody bevacizumab resulted in an attenuation of VEGF-A but not VEGF-C–induced AKT and ERK1/2 phosphorylation (Supplementary Fig. S2*C*, *bottom*). We next examined the consequences of VEGFR activity on LEC survival and proliferation. Treatment of LECs with either VEGF-A or VEGF-C led to an increase in BrdUrd incorporation (Supplementary Fig. S2*E*), and coaddition of PTK/ZK (10–1,000 nmol/L) blocked the ability of both VEGF isoforms to stimulate proliferation (Supplementary Fig. S2*E*, *left*). Treatment with bevacizumab (25–100 ng/mL) reduced VEGF-A–stimulated but not VEGF-C–stimulated proliferation (Supplementary Fig. S2*E*, *right*). Furthermore, treatment of serum-starved LECs with either VEGF-A or VEGF-C increased their viability (Supplementary Fig. S2*D*). VEGF-A–induced survival was blocked by both PTK/ZK and bevacizumab in a dose-dependent manner; PTK/ZK addition also resulted in a significant decrease in


A
Control
VEGFR-1
VEGFR-2
VEGFR-3
B16/BL6
50μm
B
-Triton
+Triton
VEGFR-1
VEGFR-2
Counts
Fluorescence intensity
C
kDa
VEGF-C
VEGF-B
VEGF-D
50
37
25
Treatment 24h
VEGF-A (pg/10^4 cells±SE)
Basal medium (FCS 0.5%)
41.50 ±1.30
Growth medium
66.22 ±1.64
D
VEGFR-1
CD31
Merge
100μm
VEGFR-2
CD31
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VEGFR-3
LYVE-1
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VEGF-C–induced cell viability (Supplementary Fig. S2*D*). Interestingly, treatment of the VEGF-C–treated cultures with 1,000 nmol/L PTK/ZK strongly induced apoptosis. Although we can only speculate, the results suggest that LEC cultures became strongly dependent on VEGF-C for their survival during 48 h of VEGF-C treatment. In summary, these results show that both VEGF-A and VEGF-C influence LEC biology via activation of VEGFR-2 and VEGFR-3. Considering that, *in vivo*, tumors are likely to be exposed to different VEGF ligands, blockade of VEGF-A–induced signaling alone might not be sufficient to inhibit activation of the tumor-associated lymphatic endothelium.

Expression of VEGF ligands and receptors in B16/BL6 tumor cells and metastases. VEGFR is also expressed on tumor cells, raising the hypothesis that it can influence tumor cell biology (34). To evaluate if B16/BL6 tumor cells might be targeted by anti-VEGF/VEGFR therapy, we first investigated VEGFRs and ligands in cultured tumor cells. VEGFR-1 and VEGFR-2, but not VEGFR-3, were detected in B16/BL6 cells by immunocytochemistry (Fig. 3*A*). Flow cytometry confirmed the immunocytochemical analysis and showed that VEGFR-2, but not VEGFR-1, was only detectable after a permeabilization step, suggesting an intracellular localization (Fig. 3*B*). These results, together with other reports describing an intracellular redistribution of VEGFR-2 after activation (35), suggest the presence of a VEGF-VEGFR autocrine loop in B16/BL6 cells.

Expression of VEGF ligands was then assessed. The 58-kDa VEGF-C precursor and 29-kDa polypeptide were detected in B16/BL6 cell lysates (Fig. 3*C*); the 21-kDa polypeptide that stimulates both VEGFR-2 and VEGFR-3 was not detectable. However, because it results from processing that occurs after ligand secretion, it might be present. B16/BL6 cells also express VEGF-B and VEGF-D and secrete high levels of VEGF-A (Fig. 3*C*).

We next examined the *in vivo* expression of VEGFRs in sections of B16/BL6 lymph node metastases. CD31 or LYVE-1 staining (Fig. 3*D, green*) was used to highlight the blood or lymphatic vasculature, respectively. Immunofluorescence staining with specific VEGFR antibodies (Fig. 3*D, red*) revealed that vessels (Fig. 3*D, Merge, yellow*) and tumor cells surrounding the vessels (Fig. 3*D, Merge, red*) express VEGFR-1 and VEGFR-2 (Fig. 3*D, top* and *middle*). Expression of VEGFR-3 was restricted to the lymphatic endothelium (Fig. 3*D, bottom, Merge, yellow*) and was not detectable on tumor cells. These data indicate that the B16/BL6 tumor cells also express VEGFR-1 and VEGFR-2 *in vivo* and could respond to autocrine-produced VEGF ligands.

Effect of PTK/ZK and DC101 on B16/BL6 cell migration. We next tested for the presence of an autocrine VEGF/VEGFR loop in B16/BL6 cells. Treatment of cells with PTK/ZK, but not with DC101, caused a decrease in constitutive ERK1/2 phosphorylation with a maximum of inhibition between 15 and 20 min; neither inhibitor had an effect on AKT activation (Fig. 4*A*). To look for a biological effect of VEGFR signaling, we examined proliferation and migration. Neither inhibitor had an effect on cell proliferation as determined by BrdUrd incorporation (data not shown; ref. 26). B16/BL6 cell migration was examined in a Boyden chamber–based assay. Treatment with PTK/ZK blocked basal migration in a dose-dependent manner (Fig. 4*B*), whereas DC101 displayed only a modest, nonsignificant effect (Fig. 4*C*). These results suggest a specific role for VEGFR-1 in ERK1/2 activation and migration of B16/BL6 cells.

PTK/ZK enhances tumor cell response to platinum-based chemotherapy. Based on the reports that VEGF-A protects endothelial cells from chemotherapeutic drug-induced apoptosis (36), we assessed whether the VEGF/VEGFR autocrine loop might also influence B16/BL6 tumor cell chemosensitivity. Treatment of cultures with cisplatin (10 μg/mL), oxaliplatin (10 μg/mL), or 5-FU (10 μmol/L) led to a 30% to 50% decrease in cell number (Fig. 5*A* and *B*; Supplementary Fig. S3*A, right*) and killed 2% to 8% of the tumor cells (Fig. 5*A* and *B*; Supplementary Fig. S3*A, left*). Addition of PTK/ZK to cisplatin (5–10 μg/mL) or oxaliplatin (10 μg/mL), but not to 5-FU (Fig. 5*A* and *B*; Supplementary Fig. S3*A, left*), significantly increased cell death in a dose-dependent manner to 20% to 50%. It should be noted that treatment with PTK/ZK or DC101 alone induced a low level of apoptosis that was variable and dependent on cell density (data not shown). Statistical analyses indicated highly significant interactions between PTK/ZK and cisplatin or oxaliplatin ($P < 0.001$), suggestive of strong combination effects between the TKI and platinum-based chemotherapeutic drugs.

To assess the effect of VEGFR-2 blockade alone in chemosensitization, a similar experiment was performed with DC101. A comparable decrease in B16/BL6 cell number was observed when either PTK/ZK or DC101 was combined with cytotoxic drugs (Fig. 5*A* and *B*). Treatment of B16/BL6 cells with DC101 (1 μg/mL) killed 10% of the cells and addition of oxaliplatin (10 μg/mL) increased cell death to ~30% (Fig. 5*B, bottom*). Statistical analyses indicated significant interactions between the two drugs ($P = 0.045$), suggestive of combination effects. Taken together, these results suggest that B16/BL6 cells are dependent on VEGFR-2 activity for survival and that this autocrine loop protects the tumor cells from platinum-based chemotherapeutics.

To search for the mechanism underlying the chemoprotective effect of VEGFR-2, we examined the combinatorial effect of PTK/ZK and cisplatin on AKT and ERK1/2 activity in B16/BL6 cells. Treatment of tumor cells with cisplatin had no effect on AKT activity and, at low concentrations (up to 6 μg/mL), had no effect on phosphorylated ERK1/2 (P-ERK1/2) level. However, cisplatin at 10 μg/mL lowered ERK1/2 activation (Fig. 5*C*), decreased cell number by 50% (Fig. 5*A, right*), and induced low levels of cell death (5%; Fig. 5*A, left*). Importantly, coaddition of PTK/ZK to cisplatin lowered the threshold for the cisplatin-mediated inhibitory effect on ERK1/2 activity from 10 μg/mL to 4 to 6 μg/mL (Fig. 5*C*), suggesting that ERK1/2 activity may underlie the chemoprotective effect of the autocrine VEGFR-2 loop.

Figure 3. Expression of VEGFRs and VEGF ligands in B16/BL6 melanoma cells. *A*, immunofluorescent staining of VEGFRs in B16/Bl6 cells. The specificity of immunostaining was shown by the lack of signal in the absence of the primary antibodies (*Control*). *Bar*, 50 μm. *B*, B16/BL6 cells were stained with either an anti–VEGFR-1 antibody (*red*), a phycoerythrin-conjugated anti–VEGFR-2 antibody (*red*), or an isotype-specific control (*black*) before (*left*) and after (*right*) a permeabilization step as described in Materials and Methods of the Supplementary Data and analyzed by flow cytometry. *C*, total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti–VEGF-C, anti–VEGF-B, and anti–VEGF-D antisera. Conditioned medium was collected from B16/BL6 cells plated in either basal medium or growth medium, and VEGF-A secretion was measured using an ELISA kit as described in Materials and Methods. *D*, immunofluorescent detection of VEGFRs in B16/BL6 lymph node metastases. Tumor sections were double stained for VEGFR-1 (*red, top*) and CD31 (*green, top*), VEGFR-2 (*red, middle*) and CD31 (*green, middle*), or VEGFR-3 (*red, bottom*) and LYVE-1 (*green, bottom*). The images captured for endothelial cells (CD31 and LYVE-1; *green*) were overlapped with images captured for VEGFR-1, VEGFR-2, and VEGFR-3 (*Merge, yellow*).

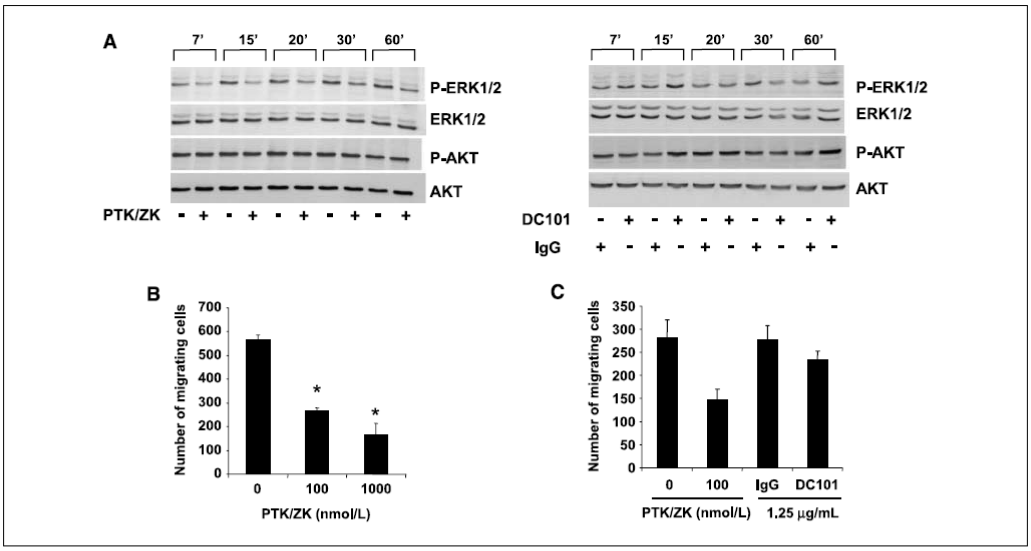

Figure 4. Effect of PTK/ZK and DC101 on *in vitro*–cultured B16/BL6 melanoma cells. *A*, growing B16/BL6 cells were incubated in the presence or absence of PTK/ZK (1,000 nmol/L), DC101 (1.5 μg/mL), or IgG (1.5 μg/mL) for the indicated time. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti–phoshorylated AKT (*P-AKT*), P-ERK, AKT, and ERK antibodies. *B* and *C*, B16/BL6 cells in MEM EBS plus 0.5% FCS with the indicated concentration of PTK/ZK, DC101, or IgG were placed on a membrane with an 8.0-μm pore size inserted in 24-well plates. MEM EBS plus 0.5% FBS with the indicated concentration of PTK/ZK, DC101, or IgG were placed in the bottom wells. A migration assay was performed as described in Materials and Methods. *Columns*, mean; *bars*, SE. *, $P < 0.05$ versus 0 nmol/L PTK/ZK.

To provide evidence supporting a role for VEGFR-2 in chemosensitization, we assessed the interaction of PTK/ZK with chemotherapeutics in additional human tumor cell lines: the VEGFR-2–positive and VEGFR-3–positive MB-MDA435 mammary carcinoma cells (Fig. 6*A*) and A549 non–small lung carcinoma cells (Fig. 6*C*) and the VEGFR-3–expressing A375 melanoma cells (Fig. 6*B*). These tumor cell lines express multiple VEGF isoforms (Supplementary Fig. S4; ref. 37), making it likely that an autocrine VEGF/VEGFR loop is present in each. Treatment of MB-MDA435 and A375 cell lines with cisplatin (10 μg/mL; Fig. 6*A* and *B, right*) or 5-FU (10 μmol/L; Supplementary Fig. S3*B* and *C, right*) reduced cell number by 30% to 60%. Cisplatin killed 10% to 30% of the tumor cells [Fig. 6*A* and *B* (*middle*) and *C* (*right*)]. As observed for B16/BL6 cells, 5-FU had no effect on viability of MB-MDA435 and A375 tumor cell lines (Supplementary Fig. S3*B* and *C, left*); its activity was not examined in the A549 cells. Addition of PTK/ZK to cisplatin (10 μg/mL) increased MB-MDA435 cell death from 30% to 45% (Fig. 6*A, middle*) and A549 cell death from 13% to 16% (Fig. 6*C, right*), whereas coaddition of PTK/ZK did not further increase the effect of cisplatin on viability of the VEGFR-2–negative A375 tumor cells (Fig. 6*B, middle*). Statistical analyses indicated significant interactions between PTK/ZK and cisplatin ($P = 0.005$ and 0.013, respectively) in MB-MDA435 and A549 cells, suggestive of combination effects between the two drugs. In summary, addition of PTK/ZK to platinum-based drugs resulted in increased tumor cell death in the VEGFR-2–positive B16/BL6, MB-MDA435, and A549 cells, but not in the VEGFR-3–positive but VEGFR-2–negative A375 cells, suggesting that the autocrine activation of VEGFR-2, but not VEGFR-1 or VEGFR-3, has a role in tumor cell chemoresistance.

The *in vivo* effects of treatment with PTK/ZK and cisplatin were also examined in the A549 human lung cancer model. Importantly, there was a significant inhibition in tumor area and weight in animals treated with the combination of PTK/ZK and cisplatin compared with either drug alone (Fig. 6*D*).

Discussion

VEGFRs have important roles in cancer, affecting blood and lymphatic vessel functionality as well as tumor cells themselves. The present study was undertaken to assess the effect of selective VEGFR-2 and VEGF-A inhibitors (DC101 and Pab85618) and PTK/ZK, a multiple VEGFR inhibitor, on the growth of primary tumor and lymph node metastases in the B16/BL6 melanoma model. We show that combined inhibition of multiple VEGFRs efficiently targets various aspects of tumor pathophysiology, including lymphatic vessel functionality and tumor cell dissemination. We also address the role of VEGFRs on tumor cells and provide evidence that autocrine activity of VEGFR-1 and VEGFR-2 has a role in tumor cell migration and chemoresistance, respectively.

Our results show that selective blockade of VEGFR-2 or VEGF-A is sufficient to abrogate primary tumor growth, but additional inhibition of VEGFR-1 and VEGFR-3 with PTK/ZK is required for a significant reduction in the metastatic spread of the melanoma cells. PTK/ZK was also very efficient in blocking metastases in the BN472 rat mammary carcinoma model (shown here) and in the RENCA renal cell carcinoma model (22). Moreover, it has previously been shown that, in the VEGF-C ectopically expressing MB-MDA435 breast tumor

model, combination of anti–VEGFR-2 and anti–VEGFR-3 antibodies blocked metastases more efficiently than either antibody alone (38).

Lymphatic vessel endothelium expresses not only VEGFR-3 but also VEGFR-2 (39), raising the possibility that this receptor might also stimulate LEC proliferation and induce lymphangiogenesis. Indeed, VEGFR-2 activation seems to be sufficient to promote tumor lymphangiogenesis in some tumor models (2). Isolated LECs express both VEGFR-2 and VEGFR-3 and, in agreement with other studies (40), show a proliferative and survival response to VEGF-A and VEGF-C. Furthermore, the effects of VEGF-A on cultured LECs were inhibited by the VEGF-A blocking antibody. *In vivo*, however, this antibody was unable to block lymphangiogenesis or metastatic spread, suggesting that activation of the lymphatic VEGFRs occurs in response to VEGF-C and/or VEGF-D.

Importantly, our studies show that simultaneous inhibition of all VEGFRs impaired functionality of the lymphatic vessels connected to the primary tumor and this effect positively correlated with a decrease in lymph node metastases weight. VEGFR-2 blockade was able to lower lymphangiogenesis in the lymph nodes, but this was not sufficient to block metastasis, suggesting that preexisting

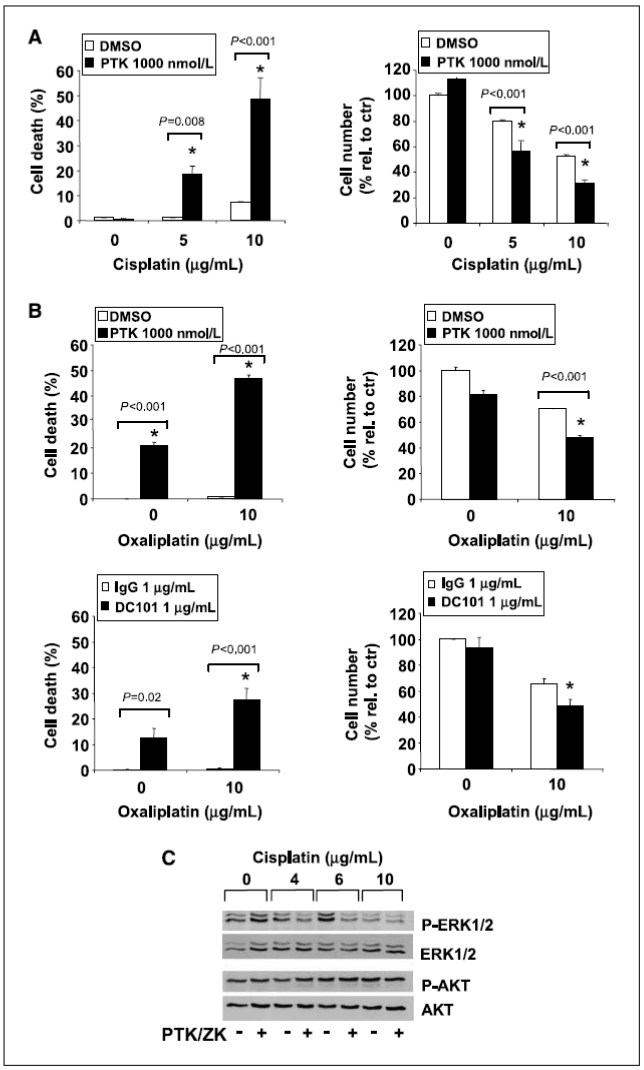

Figure 5. PTK/ZK sensitizes B16/BL6 to platinum-based chemotherapy. *A*, growing B16/BL6 cells (5,000 per well) were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) combined with increasing concentrations of cisplatin (0–10 μg/mL) for 24 h at 37°C. Cell death and cell number were measured using the YO-PRO assay as described in Materials and Methods. *Columns*, mean; *bars*, SE. *, $P < 0.01$ versus 0 μg/mL cisplatin (two-way ANOVA and Holm-Sidak test). *B*, growing B16/BL6 cells (10,000 per well) were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) or DC101 (1 μg/mL) combined with oxaliplatin (10 μg/mL) for 24 h at 37°C. Cell death and cell number were measured using the YO-PRO assay. *Columns*, mean; *bars*, SE. *, $P < 0.01$ versus 0 μg/mL oxaliplatin (one-way/two-way ANOVA and Holm-Sidak test). *C*, B16/BL6 cells were pretreated with PTK/ZK for 1 h followed by 6-h treatment with the indicated concentrations of cisplatin in growth medium. Total cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti–P-AKT, P-ERK, AKT, and ERK antibodies.

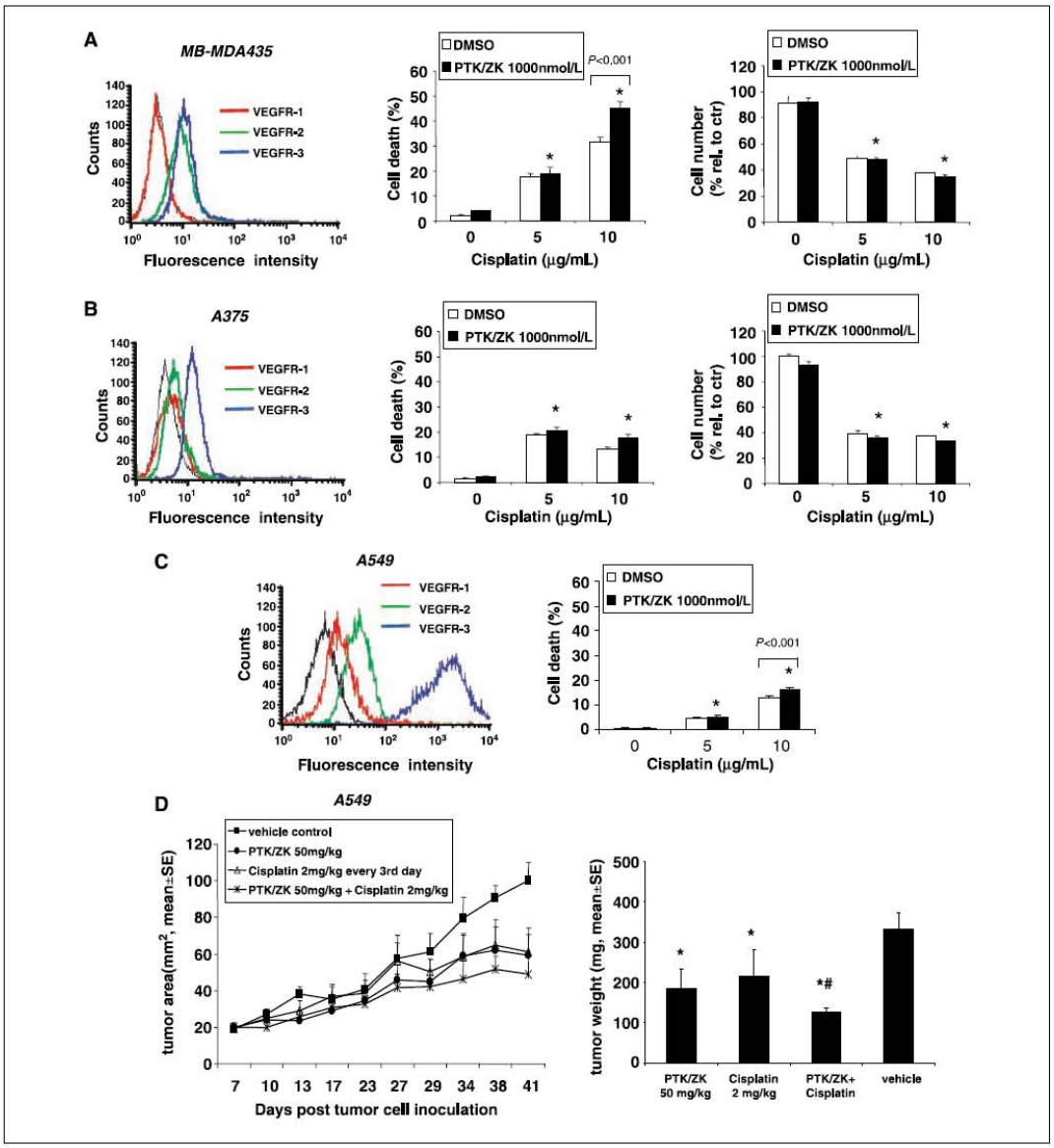

Figure 6. PTK/ZK sensitizes VEGFR-2–positive tumor cells to cisplatin treatment *in vitro* and *in vivo*. *A* and *B*, *left*, MB-MDA435 and A375 cells were stained with an anti–VEGFR-1 antibody (*red*), anti–VEGFR-2 antibody (*green*), anti–VEGFR-3 antibody (*blue*), or an isotype-specific control (*black*) as described in Materials and Methods of the Supplementary Data and analyzed by flow cytometry; *right*, growing MB-MDA435 or A375 cells were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) combined with increasing concentrations of cisplatin (0–10 μg/mL) for 24 h at 37°C. Cell death and cell number were measured using the YO-PRO assay. *Columns*, mean; *bars*, SE. *, $P < 0.001$ versus 0 μg/mL cisplatin (one-way/two-way ANOVA and Holm-Sidak test). *C*, *left*, A549 cells were stained with an anti–VEGFR-1 antibody (*red*), anti–VEGFR-2 antibody (*green*), anti–VEGFR-3 antibody (*blue*), or an isotype-specific control (*black*) after a permeabilization step (0.3% Triton) as described in Materials and Methods of the Supplementary Data and analyzed by flow cytometry; *right*, growing A549 cells were incubated in the presence or absence of PTK/ZK (1,000 nmol/L) for 24 h followed by PTK/ZK combined with increasing concentrations of cisplatin (0–10 μg/mL) for an additional 24 h at 37°C. Cell death was measured using the Vybrant Apoptosis Assay Kit 2. *Columns*, mean; *bars*, SE. *, $P < 0.001$ versus 0 μg/mL cisplatin (one-way/two-way ANOVA and Holm-Sidak test). *D*, *left*, A549 primary tumor area measured every 3 d between day 7 and day 41 after tumor cell inoculation. *Points*, mean; *bars*, SE. *Right*, average of tumor weight measured on day 41 following treatment between day 7 and day 41. *Columns*, mean; *bars*, SE. *, $P < 0.05$, treatment groups versus control group; #, $P < 0.05$, treatment PTK/ZK + cisplatin versus treatment with each agent alone (one-way ANOVA and post hoc Holm-Sidak test).

lymphatics surrounding the tumor are used by the B16/BL6 tumor cells to reach the lymph nodes. Furthermore, in contrast to results with PTK/ZK, blockade of VEGFR-2 alone had little effect on the functionality of lymphatic vessels connected to the primary tumor and this correlated with only a partial blockade of tumor cell spread to the lymph nodes. Taken together, our results suggest that inhibition of tumor angiogenesis, mainly via blockade of VEGFR-2, underlies the essentially equivalent *in vivo* efficacy of PTK/ZK, DC101, and Pab85618 on primary tumor growth. Importantly, blockade of the functionality of peripheral lymphatics surrounding the primary tumor, presumably via inhibition of the other VEGFRs, may underlie the efficient inhibition of metastatic spread observed with PTK/ZK treatment.

In addition to the important activity of VEGFs on endothelial cells, these growth factors might also stimulate the VEGFRs that have been detected on many tumor types (34). Indeed, in breast cancer, melanoma, and some leukemias, VEGF/VEGFR biological activities for autocrine signaling loops have been described (13, 41, 42). Furthermore, VEGFR-3 and VEGFR-1 have important roles in promoting lung adenocarcinoma cell invasion, and colorectal and pancreatic carcinoma cell migration, respectively (14, 43–45). Here, we show that B16/BL6 cells express multiple VEGF ligands as well as VEGFR-1 and VEGFR-2. *In vitro* treatment with PTK/ZK, but not DC101, decreased ERK1/2 activity and strongly reduced B16/BL6 cell migration. These data suggest that an autocrine VEGFR-1 activation loop promotes B16/BL6 cell migration, agreeing with a recent report showing a specific role for VEGFR-1 in placenta growth factor (PlGF)-induced metastatic melanoma spread (46).

Finally, our work shows that autocrine VEGFR activity present in tumor cells mediates chemoprotection and survival. *In vivo*, combined treatment with PTK/ZK and cisplatin had a significant beneficial effect on tumor growth inhibition when compared with either agent alone. *In vitro*, combined treatment with platinum-based cytotoxic drugs and PTK/ZK was more effective in inducing death of the VEGFR-2–positive B16/BL6, A549, and MB-MDA435 tumor cells than either therapy alone. Activation of both phosphatidylinositol 3-kinase and ERK1/2 can protect tumor cells from cisplatin-induced apoptosis (47, 48), and pharmacologic interruption of these survival pathways lowers the threshold for cisplatin-mediated lethality (49, 50). Our results show that treatment of B16/BL6 tumor cells with PTK/ZK alone or in combination with cisplatin resulted in a decrease of ERK1/2 but not AKT activity, suggesting that the autocrine VEGFR activity–mediated chemoprotective effect occurs via activation of ERK1/2 signaling. DC101 also showed interactive effects with platinum-based chemotherapy in the B16/BL6 melanoma cells. Interestingly, A375 melanoma cells, with low or undetectable levels of VEGFR-1 and VEGFR-2, were not sensitized by PTK/ZK to cisplatin-induced death, supporting the concept that VEGFR-2 has a specific role in the chemosensitization effect. Moreover, no increased cell death was observed when PTK/ZK was combined with 5-FU in either B16/BL6 or MB-MDA435 tumor cell lines, suggesting that the sensitization to platinum-based chemotherapeutic drugs occurs via a specific mechanism.

In conclusion, an anti–VEGF-A-targeted antibody can block the VEGF-A contribution to angiogenic and lymphangiogenic signaling, and to tumor cell survival and spread, but is unable to restrict lymphatic spread promoted by PlGF and other members of the VEGF family. PTK/ZK, by blocking all three VEGFRs, inhibits angiogenesis, targets lymphatic vessel functionality and tumor cell dissemination, and, specifically via VEGFR-2 inhibition, enhances the tumor cell response to platinum-based chemotherapeutic drugs. Furthermore, combination of chemotherapeutic drugs and angiogenesis inhibitors has the potential to target both endothelial and tumor cells. Clinical trials addressing the ability of tumor VEGFR expression pattern to serve as a predictive factor for the outcome of combinatorial chemotherapeutics and VEGFR inhibitor therapy may be important in the future.

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3. Memo downregulation impairs 4T1 mammary tumor growth and metastasis formation

Introduction

ErbB2 is a member of the epidermal growth factor receptor family, and is overexpressed in 20% of breast cancers, primarily due to gene amplification. Its overexpression correlates with aggressive disease and poor clinical outcome [271]. Analyses of the ErbB2 cytoplasmic domain revealed five tyrosine phosphorylation sites (YA-YE), four of which (YA-YD) are capable of mediating transformation of normal breast cell lines *in vitro* downstream of mutant, constitutively active ErbB2 [274]. In a screen for ErbB2 binding partners, our lab identified a novel protein named Memo that binds to YD site of activated ErbB2 and mediates motility induced through this receptor [278]. Besides its coupling to heregulin induced ErbB2 signaling, Memo was shown to mediate migration initiated by FGF2 and EGF, implying that it has a broader role downstream of several receptor tyrosine kinases (RTKs). As it is known that deregulation of signaling through RTKs is a trigger for several steps of tumorigenesis and metastasis formation, we wanted to delineate the contribution of Memo to each of these steps. In the work presented here we show the effects of Memo downregulation on primary tumor growth, angiogenesis, VEGF production and the metastatic properties of the highly aggressive mouse mammary carcinoma cell line 4T1.

Results

Memo knock down 4T1 cells form tumors with delayed kinetics

To analyze the role of Memo in tumor growth we made use of 4T1 and 67NR cell lines, both of which originate from a spontaneously arising mouse mammary tumor [301]. The 4T1 cell line is highly metastatic, while the 67NR is a nonmetastatic cell line.

We first stably infected 4T1 cells with a control or *memo*-targeted shRNA construct. These control and Memo knock down (KD) clones (Figure 1a) were then orthotopically injected into the mammary fat pads of BALB/c female mice. As shown in Figure 1b and 1c, growth of the control clones (LacZ.2 and LacZ.M) was more rapid in comparison to the Memo KD clones (7i and 7L). Statistical analysis carried out on individual (Figure 1b) and pooled (Figure 1c) data sets from control and Memo KD clones revealed a significant delay in Memo KD clone tumor growth kinetics.

In contrast, downregulation of Memo in 67NR cells by two different shRNAs (shRNA series 7, clones 7-10.4C, and 7-10.11F; and shRNA series 10, clones 10-10X.1F, and 10-6.10B, Figure 1d) did not affect their ability to grow in mice (Figure 1e). Spontaneous tumor regression was observed in some of the mice injected with both control and Memo KD clones.

Tumors derived from Memo KD clones are less vascularized

The ability of tumor cells to release vascular growth factors and form vasculature is correlated with their ability to grow. In order to grow beyond 1 mm in diameter tumor tissue needs a blood supply. To analyze if the delay in growth of tumors derived from Memo KD clones reflects their reduced vascularization, we examined the vasculature of tumors from Memo KD and control cells. Quantification of the area of tumor sections stained by the endothelial cell specific PECAM antibody at days 4, 7 and 9 after injection, revealed reduced vascularization in Memo KD derived tumors. The difference was most prominent at days 4 and 7, and less striking at day 9 (Figures 2a, b). These results indicate an initial delay in vascularization of Memo KD derived tumors.

Memo KD 4T1 clones release less VEGF in comparison to control clones

VEGF is a strong proangiogenic factor and considered to be the main inducer of angiogenesis in hypoxic tumor tissue (introductory chapter 2). To explain the reduction in vascularization observed in Memo KD derived tumors we performed an ELISA assay to test the level of VEGF secreted in the medium of control and Memo KD cells. We found that Memo KD clones secreted less VEGF into the cell culture medium than control clones (Figure 3), indicating that this might be the cause of their impaired ability to induce tumor angiogenesis *in vivo*.

Memo KD clones are impaired in their ability to extravasate and colonize lungs

Memo was discovered in context of tumor cell migration [278]. For this reason we wanted to examine the ability of Memo KD clones to colonize distant tissues when present in bloodstream. We injected control and Memo KD clones into the tail vein of BALB/c female mice and examined the lungs for the presence of metastasis 12 days after injection. Macroscopic examination of the lungs indicated a difference in the presence of metastases in lungs of the mice injected with control and Memo KD clones. The lungs from the control injected mice were larger and their surface was completely covered with metastases (Figure 4a, b, c). In contrast, mice injected with Memo KD clones displayed less metastases, and the normal lung tissue was clearly visible (Figure 4a). Histological examination of lung tissue sections stained with hematoxylin and eosin, followed by quantification of the surface area covered with metastases revealed a reduced presence of cancer cells in lungs derived from the mice injected with Memo KD cells (Figure 4c). In line with this, the total surface area of lung sections from control injected mice was larger than those in Memo KD injected mice, as they were more densly filled with metastases. These experiments indicate an important role for Memo in tumor cell dissemination, specifically in steps of extravasation from blood vessels and/or colonization of the lungs.

Effects of Memo downregulation on cell survival in conditions of $CoCl_2$ induced oxidative stress

Hypoxia is a main inducer of VEGF expression in tumor tissue and Memo KD cells, as mentioned previously, secrete less VEGF into the medium. To check if Memo has a broader role in the response to oxidative stress, we treated 4T1 control and Memo KD clones (two and three different clones, respectively) with cobalt chloride ($CoCl_2$), a known hypoxia mimicking agent, and measured cell survival. We detected a high variability in the survival rates of different 4T1 clones in $CoCl_2$ induced stress conditions. However, the pooled data display the trend most prominent at concentration of 300 μM $CoCl_2$ that Memo KD clones are more susceptible to oxidative stress than the control clones (Figure 5), which might partially account for their impaired ability to survive in conditions of oxidative stress in tumor tissue.

IV. DISCUSSION

Cancer is a disease characterized by the deregulation of several molecular pathways that in turn orchestrate inappropriate execution of several processes in affected body cells such as proliferation, survival, migration, growth factor release. This results in the formation of malignant tissue that disrupts body's homeostasis. Initial strategies to treat cancer with chemotherapeutics developed in the first part of the previous century took advantage of the fact that in contrast to majority of normal, nontransformed body cells, cancer cells undergo rapid division. The first anticancer drugs (antifolate drugs and DNA alkylating agents) thus exhibited their activity by either inhibiting DNA synthesis in cancer cells, or damaging its structure. However, in the majority of cases, cancer cells develop resistance to these cytotoxic agents, thus yielding only limited success, by delaying disease progression without curing it. Moreover, these treatments often caused unwanted side effects in rapidly dividing healthy tissues. For these reasons, the development of cytotoxic DNA targeting therapeutics reached its efficacy plateau during the nineteen eighties, without fulfilling the final goal of cancer eradication.

At this time, the strategy of targeted therapy began to emerge. Knowledge about the signaling pathways involved in proliferation, survival, migration and molecular cross talk between cancer cells and the surrounding and distal tissues was rapidly increasing. Soon it became clear that deregulation of the signaling pathways orchestrating developmental processes and adult tissue homeostasis are the main cause of malignant transformation. Those pathways include the receptor tyrosine kinases (RTKs), Wnt, Notch, Hedgehog, bone morphogenetic proteins (BMPs) and steroid hormone receptors. Among these, the contribution of the large family of RTKs to cancer formation is currently the best exploited in anticancer treatments [302].

Both DNA targeting cytotoxic agents and RTK inhibitors are tailored against targets within cancer cell itself. However, it is becoming increasingly evident that, although cancer originates from deregulation at the cellular level, the progression to a clinically relevant tumor mass includes recruitment of surrounding nontransformed tissue and the build up of its own vascular supply. For this reason, the cross talk between tumor cells and the surrounding tissue is studied in detail. The same holds true for the process of tumor angiogenesis.

The idea of targeting tumor angiogenesis has been continuously investigated since 1971. In 1971, Judah Folkman suggested that tumor vasculature might be a valuable target in anticancer treatments. In contrast to cancer cells, endothelial cells are not susceptible to high mutation rate and they are easily accessible to drugs. Together with the fact that the cancer tissue is highly dependent on blood supply, these were strong arguments in favor of targeting angiogenesis. The undeniable proof of concept came with the recent clinical trials showing

efficacy of Avastin, a drug targeting VEGF, in certain types of cancer in combination with chemotherapy [3].

Taken together, the clinical data show the indisputable efficacy of targeted therapy. However, like in the case of classical chemotherapy, the benefits for patients in the majority of cases are not absolute, meaning the disease is not cured. An important difference between targeted therapies and chemotherapy however, is that we are currently only at the beginning of exploration of this new paradigm. The knowledge about signaling networks involved in tumor growth, vascularization, stroma and bone marrow cell recruitment, and metastasis formation is only partial. Further studies are needed to untangle the complete signaling networks driving these processes in order for researchers to be able to validate the final reach of targeted therapy, and give rise to novel paradigms in cancer treatment.

The work presented in this thesis is directed towards better understanding the signaling pathways involved in tumor vascularization and related tumor growth and dissemination. We show the involvement of Wnt3a in several steps of *in vitro* angiogenesis, the effects of targeting VEGFRs on tumor growth and dissemination in a preclinical model, and finally, the effects of downregulation of the ErbB2 mediator Memo on tumor growth, vascularization and dissemination in 4T1 mouse mammary carcinoma cells.

Wnts are a family of secreted glycoproteins that bind to the transmembrane Frizzled receptors and initiate signaling cascades with indispensable roles during development, cell proliferation, migration, and survival [285]. Several pathways downstream of Wnt/Frizzled are known, Wnt β-catenin (or the 'canonical' Wnt pathway) being the most studied. Among the 'non-canonical' Wnt pathways, the Wnt planar cell polarity (PCP) and Wnt calcium pathways are the best understood. Although usually described as individual pathways, reported crosstalk and shared components between downstream Wnt signaling branches suggest that Wnts exert their effects through complex intracellular signaling network [287]. Excessive activation of Wnt signaling was confirmed to have causative role in colon cancer formation, and deregulation of its negative regulators has been detected in some cases of breast cancer. For these reasons, targeting Wnt signaling holds great therapeutic promise.

Angiogenesis is the process of new blood vessel formation from preexisting vasculature. In order to grow beyond 1 mm in diameter, tumor tissue needs a blood suply. At present, several anticancer drugs whose primary mode of action is to target angiogenesis are being used in the clinic. However, these agents have been shown to cause resistance that emergs several months after the beginning of the treatment regimen, thus rendering the drug ineffective in future treatments rounds [232], [230]. To improve the efficacy of antiangiogenesis therapy, more detailed knowledge about tumor blood vessel formation is needed. In this context, it is becoming evident that signaling networks other than VEGF/VEGFR play important roles in

tumor angiogenesis. Accumulating data from cell culture and mouse knock out studies suggest a role for the Wnt pathway in angiogenesis (see previous chapters). While known growth factors like the VEGFs and FGFs exhibit their roles on vasculature from many different sites, the current data suggest that the influence of components of Wnt signaling is limited to certain tissue types. For example, conditional inactivation of β-catenin in endothelial cells specifically disrupts central nervous system vasculature, while the rest of the vasculature remains intact [166]. In a similar way, humans with mutations in *Fz4* gene suffer from familial exudative vitreorethinopathy (FEVR), a disease that is characterized by incomplete retinal vascularization.

In the search for novel regulators of angiogenesis, we wanted to test the roles of the Wnt3a and Wnt5a ligands and the Fz6 receptor in different steps of *in vivo* and *in vitro* angiogenesis. Among the numerous Wnt family members, we have chosen to work with Wnt3a and Wnt5a because they are representatives of the so called canonical and noncanonical Wnt ligands, respectively. However, it should be mentioned that the classification of Wnt family members to canonical and non canonical is only conditional, meaning that pathway activation is context dependent [303].

To determine which Fz receptors are expressed by endothelial cells, microarray analysis was performed on HUVEC, a primary endothelial cell line isolated from human umbilical vein. We found that Fz4 and Fz6 are expressed at significantly higher levels by HUVEC than the other receptors of the same family. Our results are in agreement with those obtained by Masckauchan *et al*, with the difference that we did not detect the Fz5 transcript [169]. Differences in the Frizzled expression from tissues of different individuals are well known, and are probably due to functional redundancy among the family members [168]. Interestingly, when Daneman *et al* analysed expression of the Frizzled receptors in endothelial cells isolated from the murine central nervous system they found that Fz4, Fz6 and Fz8 are expressed in these cells, indicating the significance of these receptors for *in vivo* endothelial cell biology [166].

In order to activate the Fz4 and Fz6 receptors expressed by HUVEC, we treated the cells with recombinant Wnt3a and Wnt5a ligands. As readout of pathway activation we monitored the electrophoretic band shift of the protein Dishevelled (Dvl), which indicates its phosphorylation status. As current data suggest that Dvl is downstream of all known Wnt pathways, Dvl phosphorylation is considered to be a general biochemical assay for Wnt protein function [293]. Treatment with either ligand resulted in phosphorylation of the Dvl protein, indicating functional activation of the Fz4 and/or Fz6 receptors.

Angiogenesis is a tightly regulated, multistep biological process. In order to form blood vessels, endothelial cells need to proliferate, migrate and survive. Using our *in vitro* system we wanted to test the role of Wnt3a and Wnt5a on HUVEC proliferation, migration and

survival. Using the BrdU incorporation assay, we were able to show that Wnt3a induces HUVEC proliferation and migration, but has no effect on their survival. Contrary to what was presumed in the literature [163], proliferation induced by Wnt3a is VEGFR signaling independent as it was not affected by a VEGFR inhibitor. This result suggests that Wnt3a is a direct, VEGF independent, proangiogenic factor.

In contrast to Masckauchan *et al*, we did not detect any proliferative effects of Wnt5a on HUVEC, which may be explained by the difference in receptor expression as previously mentioned.

To discern which receptor conveys Wnt3a induced proliferation, we made use of purified Fz4-cysteine rich domain (CRD). When administered to HUVEC together with Wnt3a, the Fz4-CRD protein inhibited the proliferative effects of Wnt3a, suggesting that this receptor might transduce the Wnt3a proliferative signals.

In a search for downstream pathway activation, we checked the status of β-catenin after Wnt3a stimulation, and detected its stabilization, indicating activation of canonical Wnt pathway.

In order to explain the mechanism by which Wnt3a induces its biological effects in HUVEC, we treated the cells with the ligand and looked for up regulation of known angiogenesis related Wnt target genes by semi-quantitative PCR. c-Myc is a known β-catenin transcriptional target gene [298] with documented roles in cell proliferation and migration [299]. Another cell cycle regulator downstream of canonical Wnt signaling is CyclinD1 [300]. Several proangiogenic genes, among them MMP-1 and Tie-2, have been shown to be transcriptional targets of Wnt5a and non-canonical Wnt signaling in HUVEC [171], but are not listed as canonical Wnt target genes. Finally, Connexin43, a cellular gap junction protein, has been reported to be a target of the Wnt pathway in the vasculature [168]. We were able to show that Wnt3a induces the expression of c-Myc, Tie-2 and Connexin 43 transcripts, while it did not influence the transcription of MMP-1 or CyclinD1. In conclusion, our data show that the expression of genes known as both canonical (c-Myc) and non-canonical (Tie-2) Wnt target genes is upregulated upon Wnt3a treatment. Some of these genes (c-Myc for example) could be potential mediators of Wnt3a effect on HUVEC proliferation and migration.

Downstream targets activated by the Wnt Calcium (Wnt Ca^{2+}) pathway include calcium-sensitive protein calcium-calmodulin dependent kinase II (CamKII) [107]. To check if Wnt3a is exhibiting its effect on HUVEC proliferation via the Wnt Ca^{2+} pathway and CamKII, we administered Wnt3a together with increasing concentrations of KN93, a selective CamKII inhibitor which binds to the calmodulin binding site of the enzyme. KN93 was able to reduce Wnt3a induced proliferation when used at concentration of 0.5 μM, indicating the requirement of CamKII for Wnt3a induced proliferation.

Taken together, β-catenin stabilization and upregulation of known canonical Wnt target gene c-Myc indicate activation of canonical Wnt pathway upon Wnt3a treatment. However, as mentioned, we also detected upregulation of Tie2 transcript, a gene that was shown to be downstream of non canonical Wnt signaling in HUVEC. We have also shown that CamKII inhibitor that is blocking a branch of Wnt calcium pathway decreases proliferation induced by Wnt3a. These results suggest the possibility that Wnt3a induces both canonical and non canonical Wnt signaling in our model.

It was stated previously that expression of Fz6 has been repeatedly confirmed in several independent analyses of endothelial cells. For this reason, we wanted to test the role of this receptor in *in vivo* angiogenesis. We chose to investigate the role of Fz6 ablation using the Lewis lung carcinoma tumor model as it is well established that several of the steps of normal, physiological angiogenesis are recapitulated by tumors when forming blood vessels. Another reason to choose a tumor model is because of its relevance to cancer research studies. We compared the growth of tumors in mice deficient for Fz6 and their wild-type littermates. We did not detect significant differences in primary tumor growth, nor vascularization of tumors derived from Fz6 deficient mice in comparison to their wild-type littermates. However, it should be kept in mind that the tumor cells, surrounding activated stroma and recruited bone marrow derived cells are potential source of different growth factors that could interfere with the effects of Fz6 ablation.

Taken together, our data implicate Wnt3a as a novel proangiogenic factor that plays a role in the induction of HUVEC proliferation and migration, but without an effect on their survival. Ablation of Fz6 in mice did not appear to affect either tumor growth or vascularization. However more appropriate models are needed to draw conclusions about the requirements of the pathways downstream of this receptor for tumor growth and angiogenesis.

While the role of Wnt family members in angiogenesis is only emerging, the role of VEGF/VEGFR signaling network is very well documented. However, questions remain about the contribution of individual components to tumor growth, vascularization, dissemination and susceptibility to standard chemotherapeutics. To determine the effects of simultaneous inhibition of three VEGFRs in comparison to inhibition of either VEGFR2 or VEGF alone, we used the pan-VEGFR inhibitor PTK787/ZK (PTK/ZK, Vatalanib, Novartis Pharma/Schering), an anti VEGFR2 blocking antibody (DC101, ImClone Systems, [256]) and anti VEGF antibodies (Pab85618 and Avastin, anti mouse and anti human VEGFA antibodies, respectively). We show in an *in vitro* tumor model that simultaneous inhibition of all three VEGFRs by PTK/ZK significantly reduces functionality of the lymphatics surrounding tumors as well as the metastatic spread as compared to the individual administration of the other agents. *In vitro*, we show that VEGFA and VEGFC can induce

lymphatic endothelial cell (LEC) proliferation and survival. PTK/ZK was able to suppress the effects of both of these VEGFs, while Avastin was only successful in inhibiting the effects of VEGFA on LEC proliferation and survival. Increasing evidence suggest that anti VEGF/VEGFR treatment effects not only endothelial, but also tumor cell biology (chapter 2.3.2.). We show here that PTK/ZK reduces the *in vitro* migration of B16/BL6 tumor cells, probably by primarily inhibiting VEGFR1. Interruption of PI3K and ERK1/2 survival pathways in B16/BL6 melanoma cells by PTK/ZK or DC101 also sensitized these cells to platinum based chemotherapeutics. Taken together, our results dissect individual contributions of several members of the VEGF/VEGFR signaling network to tumor growth, vascularization, lymphatic functionality, metastatic spread and *in vitro* tumor cell biology.

Two decades ago, the Hynes group was among those who reported the discovery of amplification of the ErbB2 oncogene and the overexpression of its protein in a subset of breast cancers [269], [270]. Overexpression of the ErbB2 receptor tyrosine kinase is detected in 20% of cases of human breast cancer, and correlates with highly aggressive disease and poor clinical outcome for patients. Since these initial discoveries, oncogenic ErbB2 signaling has remained among the top research focuses in the field of breast cancer research.

Recently, our group reported the finding of a novel protein that mediats microtubule outgrowth and cancer cell migration downstream of activated ErbB2. The protein was named Memo, or mediator of ErbB2 driven cell motility. Memo binds to the phospho YD site of the ErbB2 receptor (Tyr1226/7) [278]. Interaction of Memo with cofilin and PLCγ1 downstream of activated ErbB2 was shown to be essential for maintaining the directionality of cell migration induced through this receptor [283]. Although discovered in a screen for ErbB2 binding partners, we were able to show that Memo has a broader role downstream of RTKs, as its downregulation impaired not only the migratory ability of T47D cells in response to heregulin (ErbB2 activator), but also in response to FGF2 (activator of several FGFRs) and EGF (ErbB1 ligand) stimulation. Being aware of the importance of signaling through RTKs in tumor formation, angiogenesis, growth factor release, bone marrow cell recruitment and several steps of tumor dissemination, we wanted to test the role of Memo in the *in vivo* growth and dissemination of the 4T1 cell line which was isolated from a mammary tumor that spontaneously arose in a BALB/cfC3H mouse.

Memo was downregulated in 4T1 cells by the stable transfection of a Memo shRNA construct. To determine the effects of Memo downregulation on primary tumor growth, we injected control and Memo downregulated 4T1 clones into the mammary fat pads of mammary glands of BALB/c female mice. We observed a significant delay in primary tumor growth upon Memo downregulation. To explain this effect, we inspected the vasculature of tumors derived from control and Memo knock down clones. The staining for the endothelial

cell specific surface molecule PECAM (platelet endothelial cell adhesion molecule, also known as CD31) revealed that tumors derived from Memo knock down (KD) clones were less vascularized in comparison to control tumors of similar size. In order to explain the impairment in angiogenesis observed in Memo downregulated tumors, we investigated the ability of Memo knock down cells to secrete vascular endothelial growth factor (VEGF), a strong angiogenic growth factor. Using an in *vitro* VEGF ELISA assay we found that Memo knock down cells secrete less VEGF into the medium than control cells, which may account for the observed *in vivo* effects on tumor vascularization.

As previously mentioned, metastasis formation is the main cause of cancer related deaths. The process of metastasis formation includes several steps which together are reffered to as the 'invasion metastasis cascade' [304]. These include: localized invasion, which enables *in situ* carcinoma cells to breach the basement membrane; intravasation into either lymphatic or blood microvessels; transport through the circulation; settlement in microvessels of various organs; extravasation; micrometastasis formation; and finally colonization, or macrometastasis formation. The likelihood of a single cancer cell successfully completing all these steps is low, however, the magnitude of the cancer cells present in a tumor and their high mutation rates explain why metastsis related deaths are one of the main healthcare issues of our time. For this reason, signaling pathways that are driving individual steps of 'invasion metastasis cascade' are of a central interest in cancer research.

To test the role of Memo in metastasis formation *in vivo*, we again made use of the 4T1 cell model, which has been shown to be highly metastatic. To check the ability of Memo knock down 4T1 cells to extravasate and colonize distant organs, we performed tail vein injections and quantified the numbers of metastatic foci that were present in the lungs after twelve days. We were able to show that downregulation of Memo in 4T1 cells reduces the number of lung metastases after tail vein injection, indicating a role for Memo in the invasion-metastasis cascade.

We know from data generated in our lab that 4T1 cells display autocrine FGFR signaling loop whose inhibition slows down primary tumor growth and reduces number of lung metastasis [305]. As Memo was also shown to be required for migration downstream of FGF, we wanted to determine if the effects of Memo KD are mediated through the FGFR pathway. We first checked the status of FGFR signaling in Memo KD cells. Our initial data show that the FGF1 and FGFR1 transcripts are decreased in Memo KD cells, suggesting that the Memo downregulation does affect FGFR signaling, potentially explaining the effects observed on 4T1 tumor cell biology. To confirm the involvement of Memo in FGFR mediated signaling, further characterization of the status of the components of this signaling network after Memo downregulation is needed. Stable reintroduction of key component (eg FGF1, FGFR1) into Memo KD cells would further our understanding of where Memo is acting in this pathway.

With our future studies we would also like to decipher the signaling pathway that influences VEGF production in 4T1 cells which, as our data suggest, is disrupted upon Memo downregulation. In this context, the interaction of Memo with signaling molecule ShcA, which is known to be required for VEGF induction downstream of some RTKs (introductory chapter 3.3.1.), could be interesting to explore in more detail.

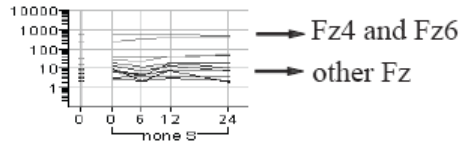
Taken together, our data show that Memo knock down reduces 4T1 *in vivo* growth, vascularization, dissemination and *in vitro* VEGF secretion. Further studies are needed to decipher the functional link between Memo and the signaling pathways driving these processes in 4T1 cells.

V. FIGURES

Wnt 3a promotes proliferation and migration of HUVEC

Figure 1.

a)



b)

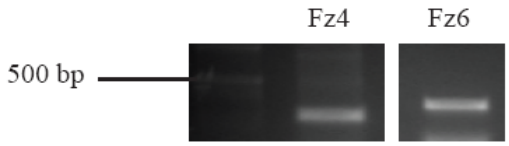
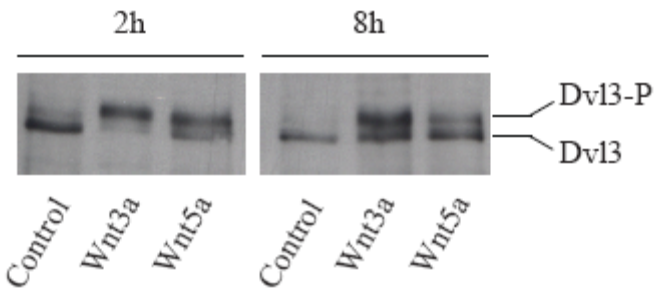


Figure 2.

a)


2h
8h
Dvl3-P
Dvl3
Control
Wnt3a
Wnt5a
Control
Wnt3a
Wnt5a

b)

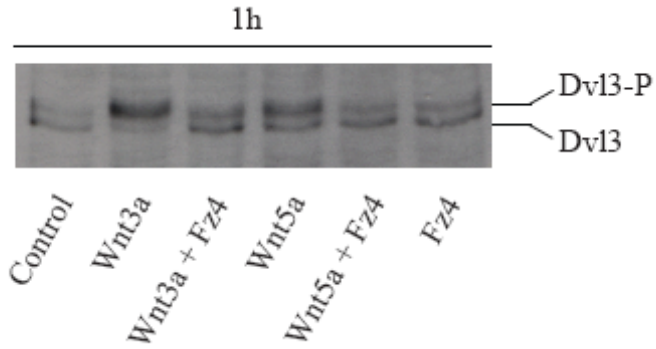
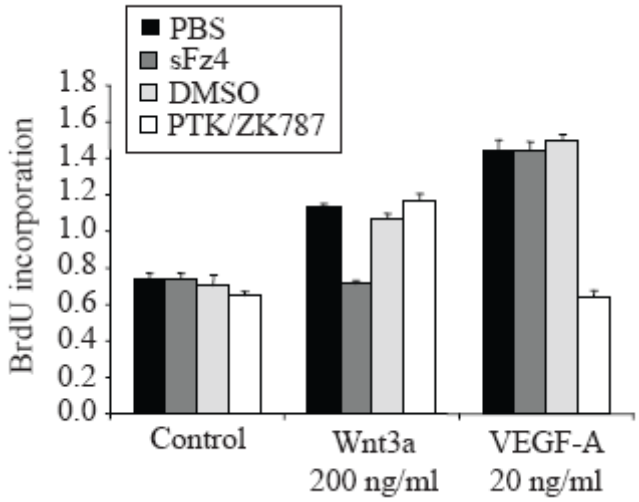
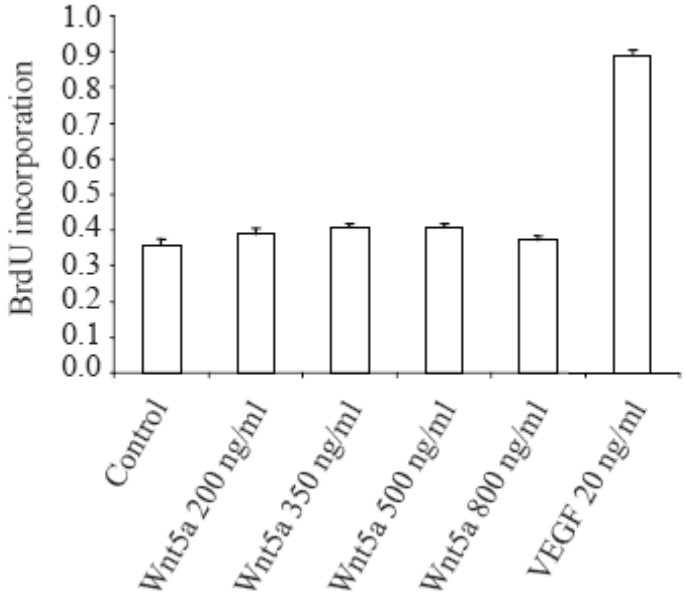

1h
Dvl3-P
Dvl3
Control
Wnt3a
Wnt3a + Fz4
Wnt5a
Wnt5a + Fz4
Fz4

Figure 3.

a)


PBS
sFz4
DMSO
PTK/ZK787
BrdU incorporation
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Wnt3a
200 ng/ml
VEGF-A
20 ng/ml

b)


BrdU incorporation
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
Wnt5a 200 ng/ml
Wnt5a 350 ng/ml
Wnt5a 500 ng/ml
Wnt5a 800 ng/ml
VEGF 20 ng/ml

c)

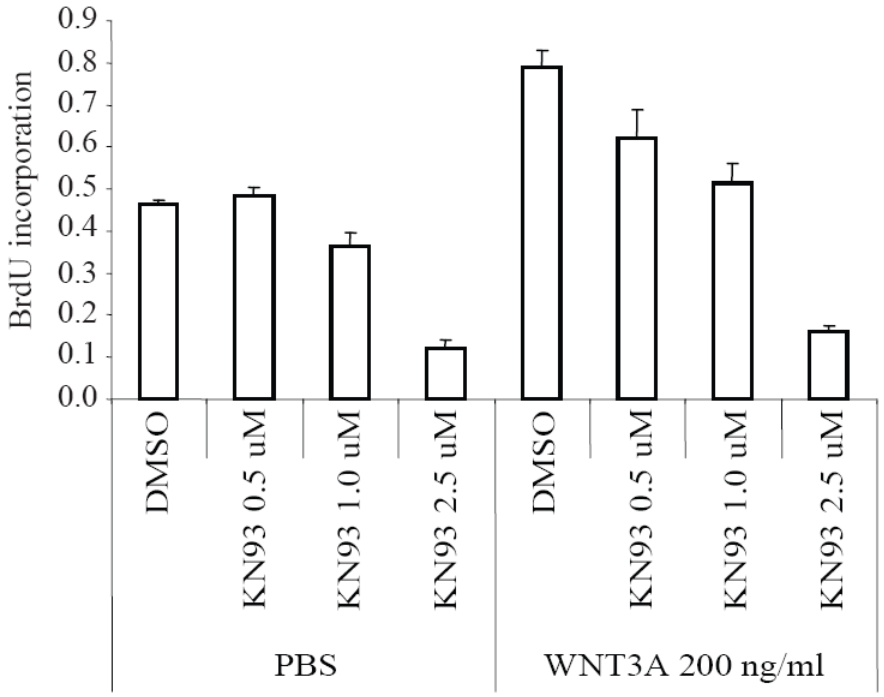
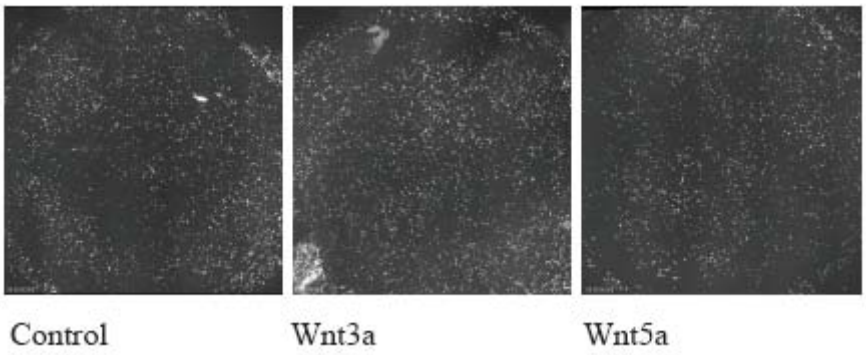
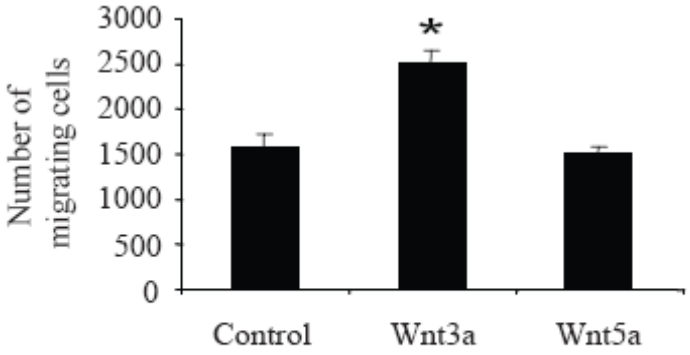

BrdU incorporation
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
DMSO
KN93 0.5 uM
KN93 1.0 uM
KN93 2.5 uM
DMSO
KN93 0.5 uM
KN93 1.0 uM
KN93 2.5 uM
PBS
WNT3A 200 ng/ml

Figure 4.

a)



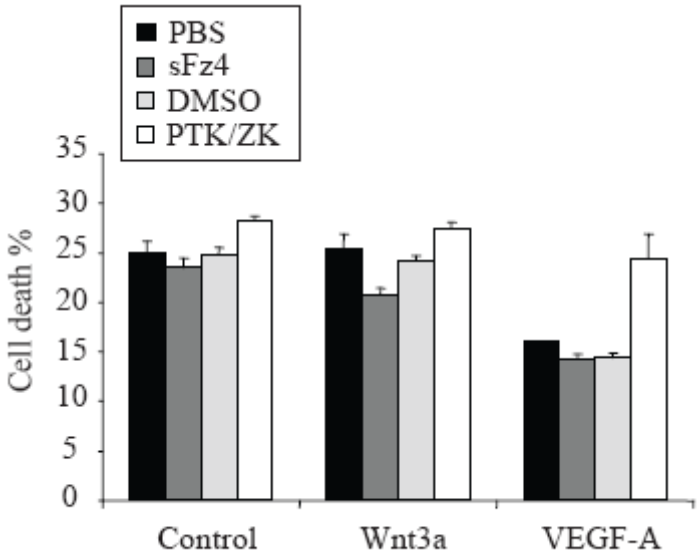
b)



*SE $p<0.05$ versus control (ANOVA)

Figure 5.

a)


PBS
sFz4
DMSO
PTK/ZK
Cell death %
35
30
25
20
15
10
5
0
Control
Wnt3a
VEGF-A

b)

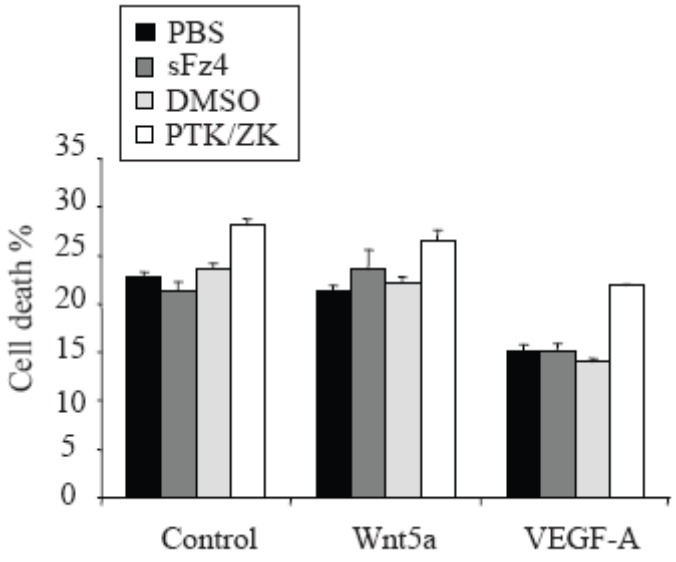
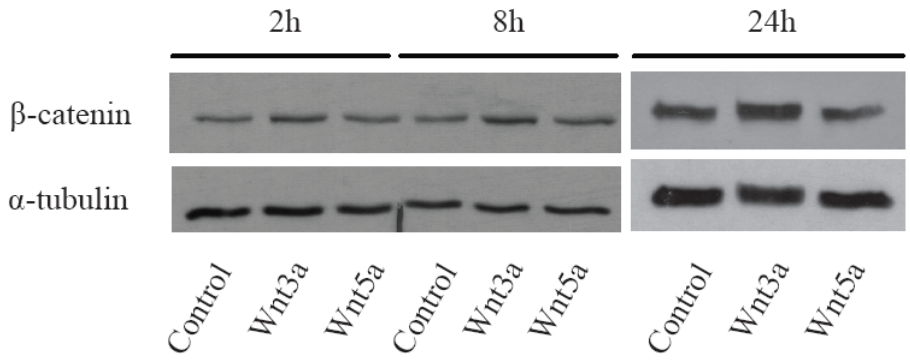

PBS
sFz4
DMSO
PTK/ZK
Cell death %
35
30
25
20
15
10
5
0
Control
Wnt5a
VEGF-A

Figure 6.

a)


2h
8h
24h
β-catenin
α-tubulin
Control
Wnt3a
Wnt5a
Control
Wnt3a
Wnt5a
Control
Wnt3a
Wnt5a

b)

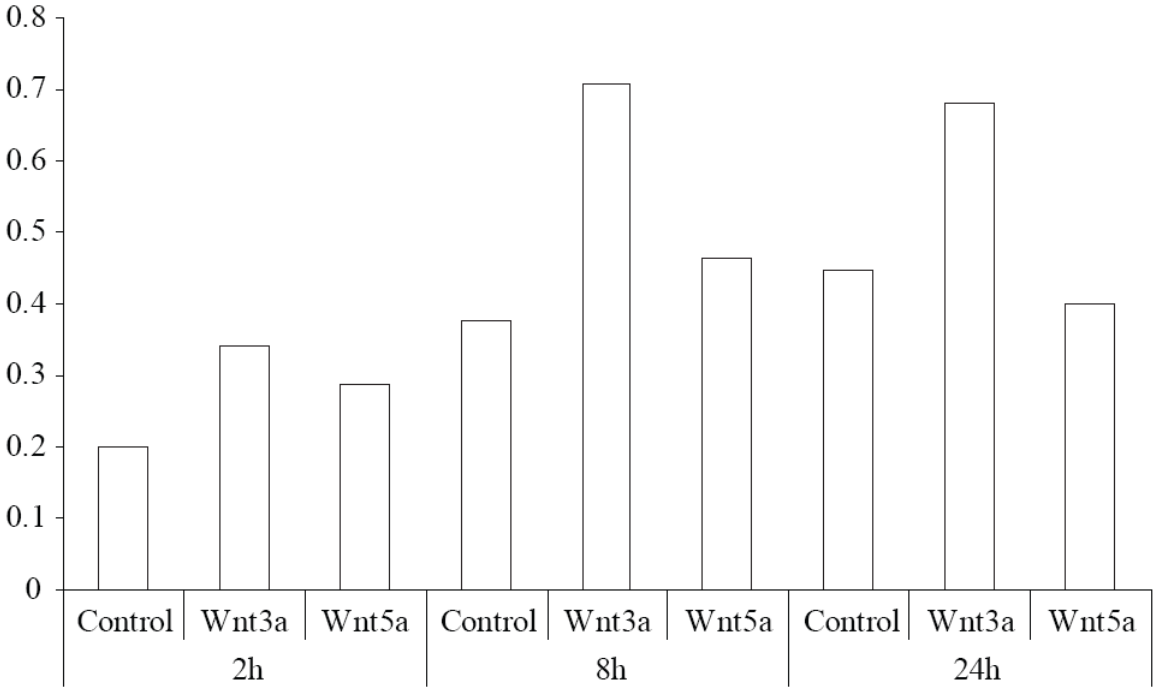

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Control
Wnt3a
Wnt5a
Control
Wnt3a
Wnt5a
Control
Wnt3a
Wnt5a
2h
8h
24h

Figure 7.

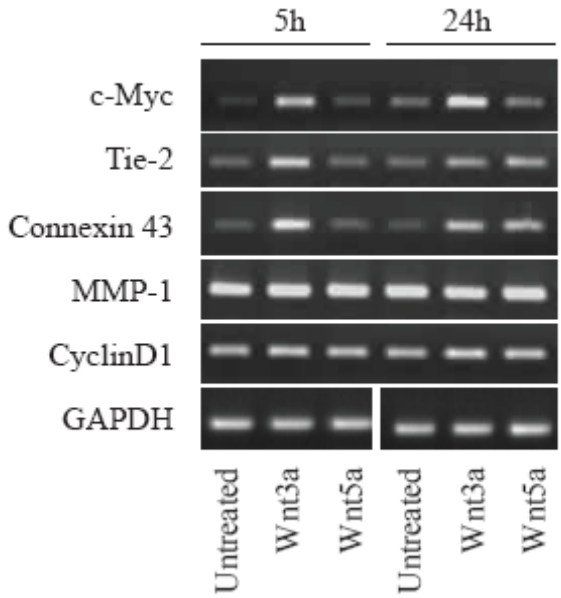
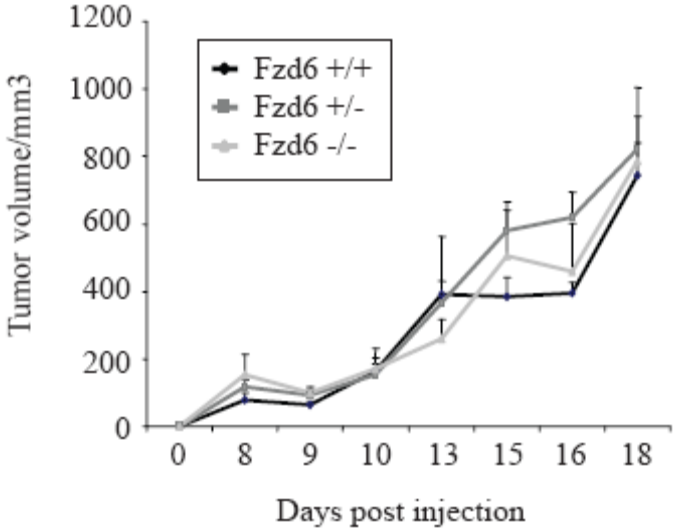

5h
24h
c-Myc
Tie-2
Connexin 43
MMP-1
CyclinD1
GAPDH
Untreated
Wnt3a
Wnt5a
Untreated
Wnt3a
Wnt5a

Figure 8.

a)


1200
1000
800
600
400
200
0
Tumor volume/mm3
Fzd6 +/+
Fzd6 +/-
Fzd6 -/-
0
8
9
10
13
15
16
18
Days post injection

b)

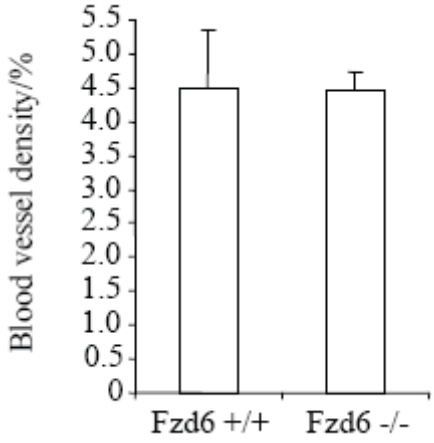

5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Blood vessel density/%
Fzd6 +/+
Fzd6 -/-

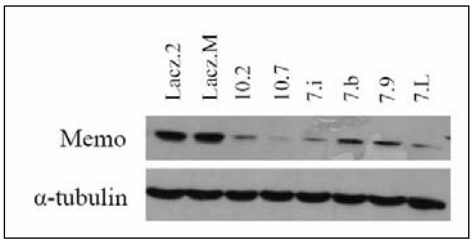
Table 1.

Primer		Sequence 5'-3'	Size/bp
Connexin43	Forw	AACTGCTGGAGGGAAGGTGTGG	333
	Rev	CATGAGCCAGGTACAAGAGTGTGG	
c-Myc	Forw	TGCTCCATGAGGAGACACCG	200
	Rev	ATGTGTGGAGACGTGGCACC	
cyclinD1	Forw	GTCCATGCGGAAGATCGTCG	185
	Rev	TCTCCTTCATCTTAGAGGCCACG	
MMP-1	Forw	GCCTGATGTGGCTCAGTTTGTCC	194
	Rev	TGTCTGCTTGACCCTCAGAGACC	
Tie-2	Forw	TTATTTCTGTGAAGGGCGAGTTCG	223
	Rev	AATATCAGGTACTTCATGCCGGG	
Fz4	Forw	GGAAATGGTTGGGTGAAGCCTG	215
	Rev	TTTTTGATGCTGGGGTCGGG	
Fz6	Forw	TCTCTGCTGTCTTCTGGGTTGG	346
	Rev	TCCGCTTTCACCTCTCTCATTG	

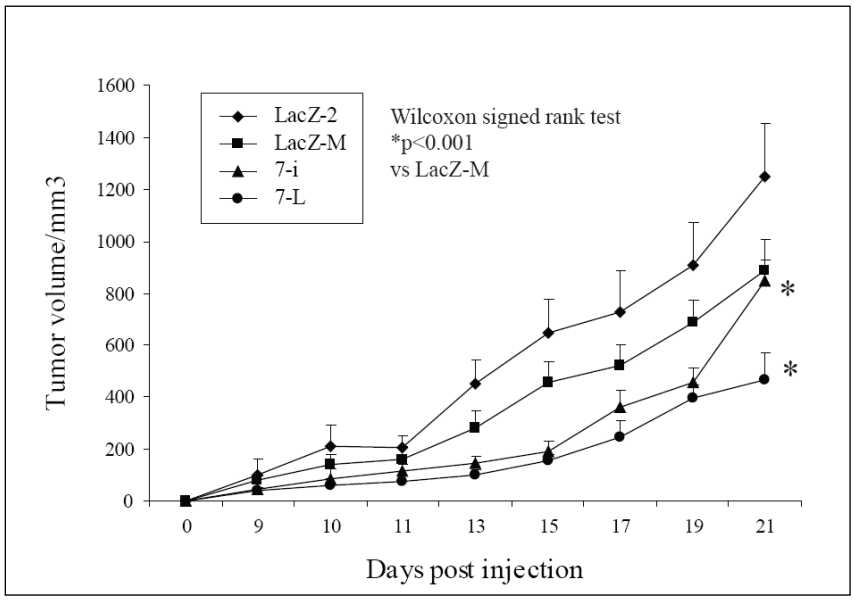
Memo downregulation impairs 4T1 mammary tumor growth and metastasis formation

Figure 1.

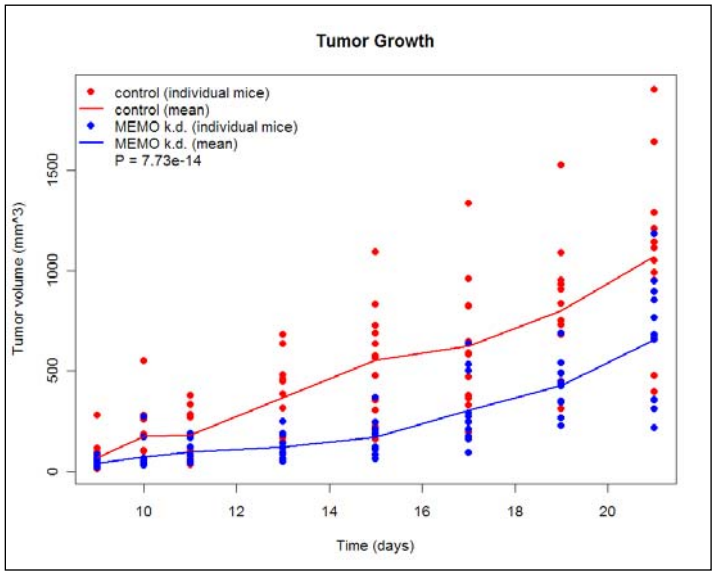
a)



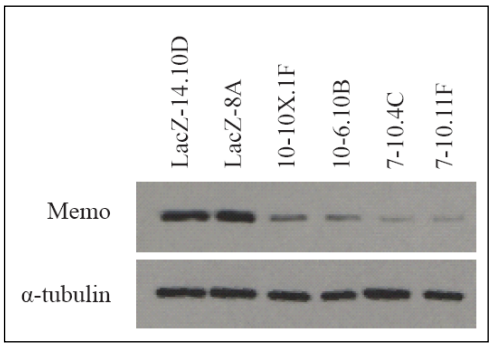
b)



c)



d)


LacZ-14.10D
LacZ-8A
10-10X.1F
10-6.10B
7-10.4C
7-10.11F
Memo
α-tubulin

e)

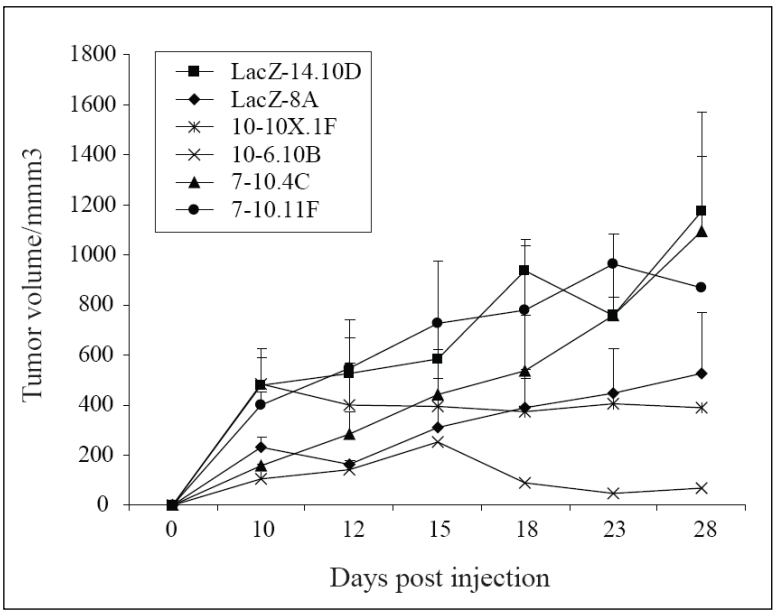
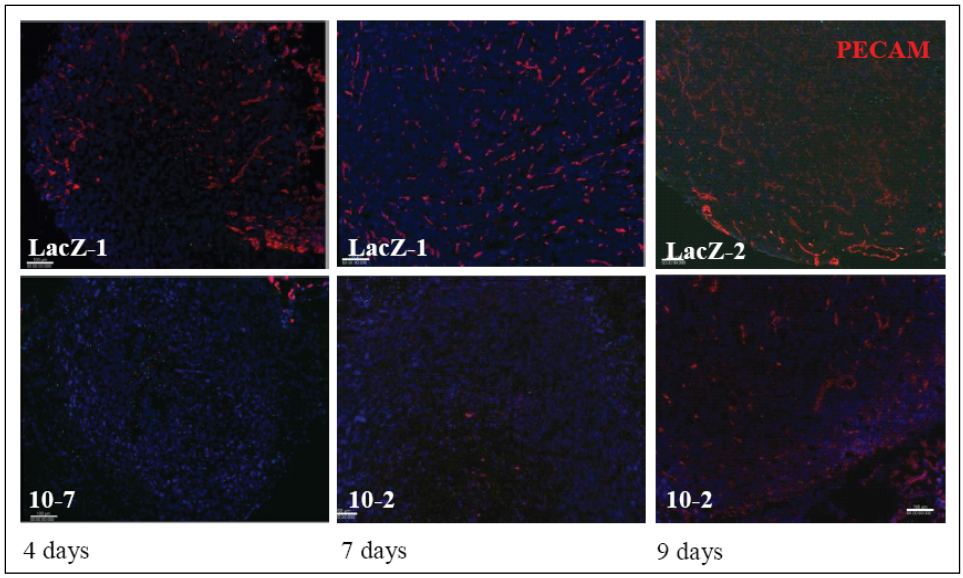

LacZ-14.10D
LacZ-8A
10-10X.1F
10-6.10B
7-10.4C
7-10.11F
Tumor volume/mmm3
1800
1600
1400
1200
1000
800
600
400
200
0
0
10
12
15
18
23
28
Days post injection

Figure 2.

a)


PECAM
LacZ-1
LacZ-1
LacZ-2
10-7
10-2
10-2
4 days
7 days
9 days

b)

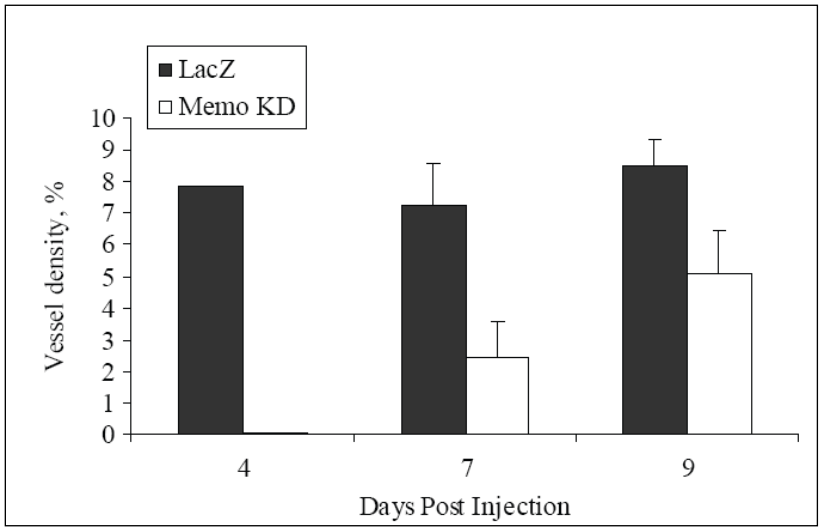

LacZ
Memo KD
Vessel density, %
0
1
2
3
4
5
6
7
8
9
10
4
7
9
Days Post Injection

Figure 3.

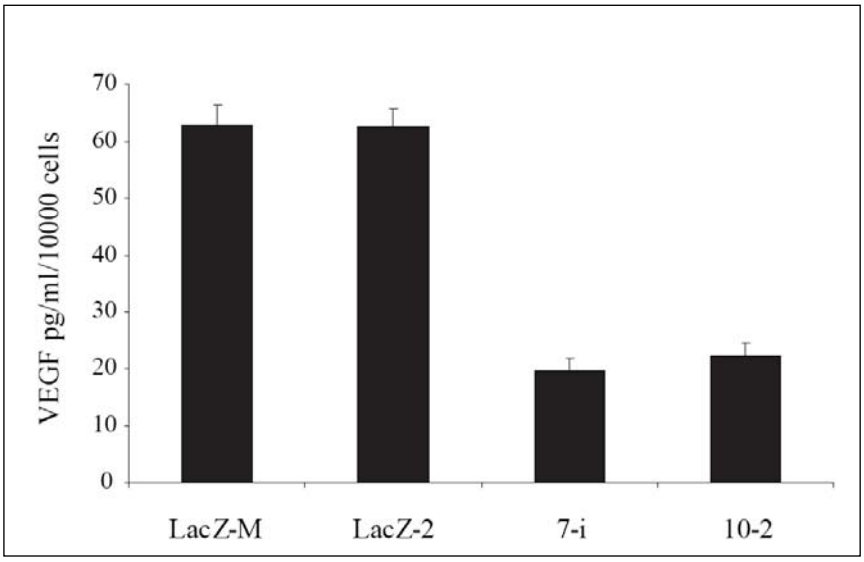
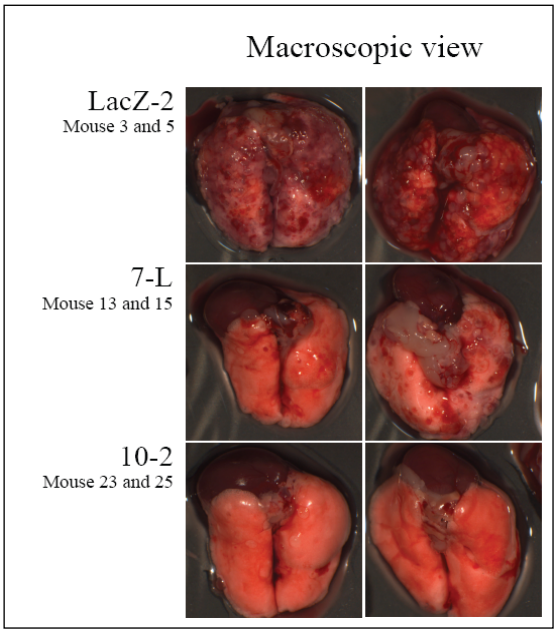
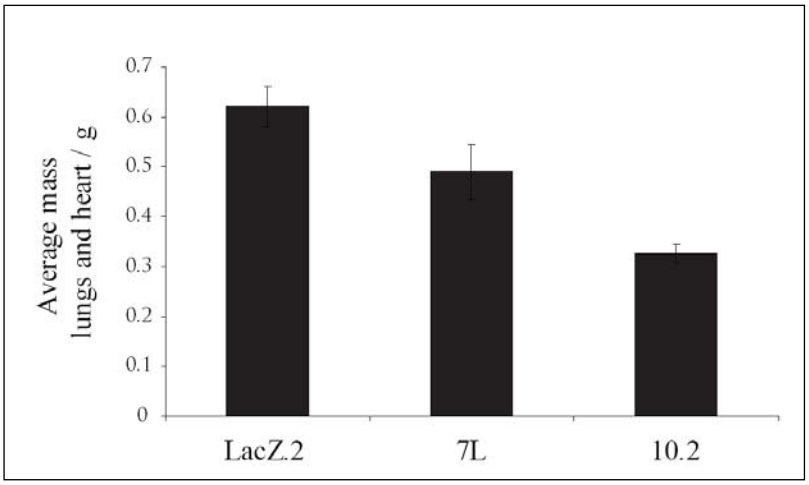


Figure 4.

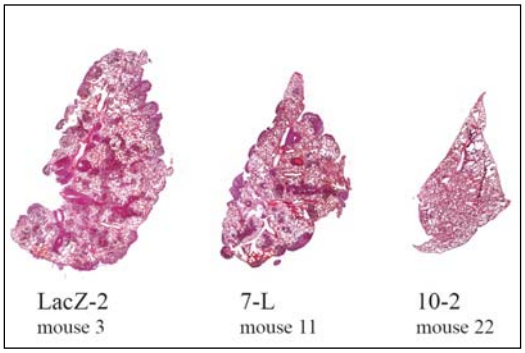
a)


Macroscopic view
LacZ-2
Mouse 3 and 5
7-L
Mouse 13 and 15
10-2
Mouse 23 and 25

b)


Average mass lungs and heart / g
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
LacZ.2
7L
10.2

c)


LacZ-2
mouse 3
7-L
mouse 11
10-2
mouse 22

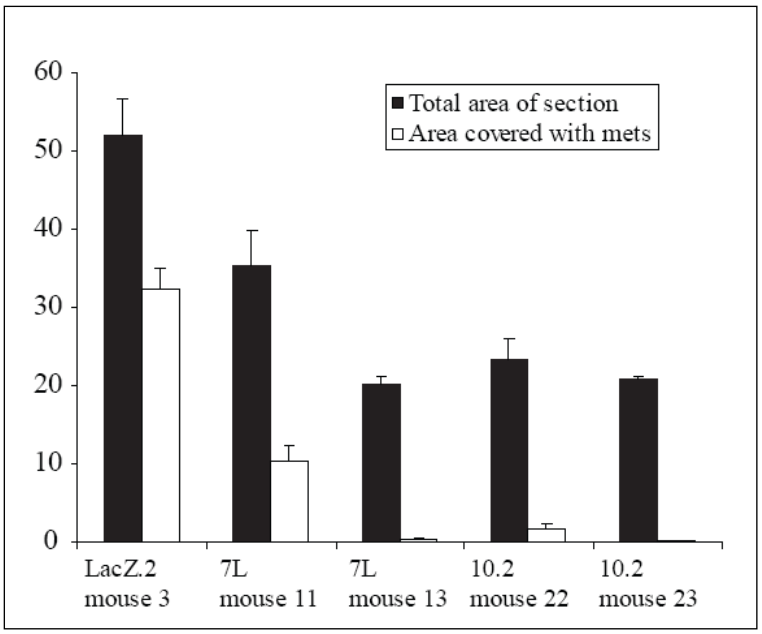
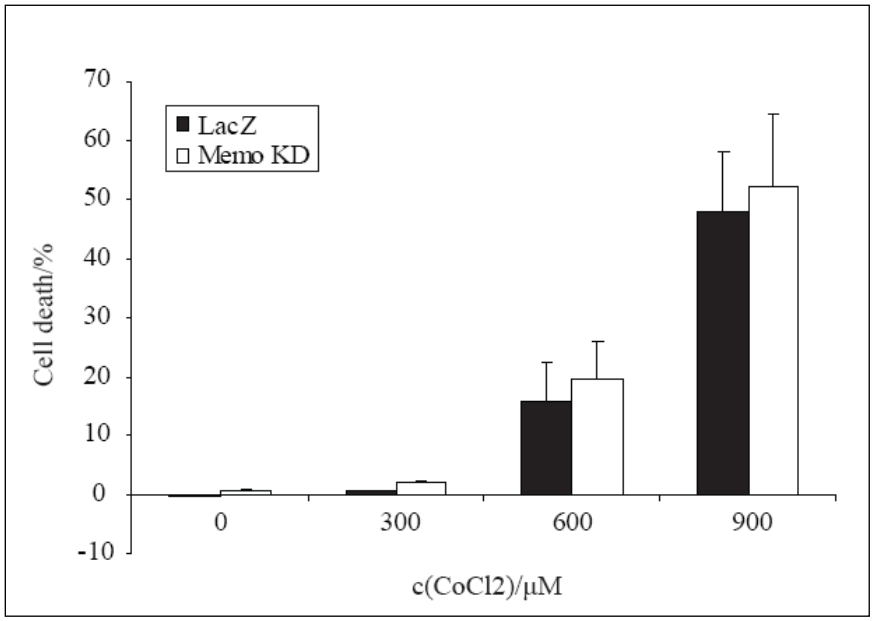

60
50
40
30
20
10
0
Total area of section
Area covered with mets
LacZ.2
mouse 3
7L
mouse 11
7L
mouse 13
10.2
mouse 22
10.2
mouse 23

Figure 5.


70
60
50
40
30
20
10
0
-10
Cell death/%
LacZ
Memo KD
0
300
600
900
c(CoCl2)/μM

VI. FIGURE LEGENDS

Wnt 3a promotes proliferation and migration of HUVEC

Figure 1. HUVEC express Fz4 and Fz6 receptors.

a) Microarray analysis was performed on HUVEC to determine the Fz receptors expressed by these cells. The experiment was performed in Novartis Pharma AG.

b) The expression of Fz4 and Fz6 in HUVEC was confirmed by semi quantitative PCR.

Figure 2. Wnt3a and Wnt5a induce Dvl3 phosphorylation in HUVEC; recombinant Fz4 competes for both Wnts.

a) HUVEC were stimulated with either Wnt3a (200 ng/ml) or Wnt5a (200 ng/ml) for 2h and 8h. Dvl3 was analyzed by immunoblotting with specific antiserum. The slower migrating band represents P-Dvl3.

b) HUVEC were treated with Wnt3a (200 ng/ml), Wnt5a (200 ng/ml) in the presence or no sFz-4 (5 μg/ml) for 1h. Dvl3 was analyzed by immunoblotting with specific antiserum. The slower migrating band represents P-Dvl3.

Figure 3. Wnt3a induces HUVEC proliferation in VEGFR signaling independent manner. HUVEC were incubated with indicated concentrations of a) Wnt3a or VEGF-A in the presence or absence of sFz-4 (5 μg/ml), PTK/ZK (1μM), b) increasing concentrations of Wnt5a, c) Wnt3a and increasing concentrations of CaMKII inhibitor KN93 for 48 hours at 37ºC. Proliferaton was assessed using an in vitro cell proliferation BrdU ELISA kit.

Figure 4. Wnt3a induces HUVEC migration.

a) Wnt3a (200 ng/ml) and Wnt5a (200 ng/ml) were used as chemoattractants in migration assay that was performed in a Boyden Chamber. The cells resuspended in Endothelial Cell Basal Medium (Promo Cell, C-90210) supplemented with 1% FBS were allowed to migrate for 6 hours at 37ºC.

b) The nuclei of migrated cells were stained with Hoechst33342 and the number of migrated cells was counted. Columns, mean; bars, SE. $p<0.05$ versus control (ANOVA).

Figure 5. Wnt3a and Wnt5a are not survival factors for HUVEC.

HUVEC resuspended in Endothelial Cell Basal Medium supplemented with 0.5% FBS were incubated with a) Wnt3a (200 ng/ml) or b) Wnt5a (200 ng/ml) or VEGF-A (20ng/ml), sFz-4 (5 μg/ml) or PTK/ZK (1μM, VEGFR inhibitor, Novartis Pharma/Schering AG) for 48 hours at 37ºC. Cell death was measured using the YO-PRO assay.

Figure 6. Wnt3a and Wnt5a induce β-catenin stabilization

a) HUVEC were stimulated with Wnt3a (200 ng/ml) or Wnt5a (200 ng/ml), in the presence or no sFz4 (5 μg/ml) for 24h. Total β-catenin and α-tubulin were analyzed by immunoblotting with specific antiserum.

b) Quantifications of Western blots were carried out using the ImageQuant software.

Figure 7. Wnt3a and Wnt5a transcriptional targets in HUVEC.

HUVECs were treated for 5 and 24 hours in the presence or absence of Wnt3a (200 ng/ml), or Wnt5a (200 ng/ml). RNA was isolated, transcribed to cDNA and semiquantitative PCR was performed with primers for c-Myc, Tie-2, Connexin 43, MMP-1, Cyclin D1 and GAPDH.

Figure 8. LLC growth *in vivo* in Fz6 knock out mice.

a) Fzd6 +/+, Fzd6 +/-, Fzd6 -/- male and female mice from five littermates aged between 1 and 5 months were injected with 0.5 million Lewis Lung Carcinoma (LLC) cells in 100 μl of PBS, subcutaneously into mid-dorsal region. Tumor growth was measured using a vernier caliper. Graph is presenting average tumor growth. N (Fzd6 +/+ mice) = 9, N (Fzd6 +/- mice) = 10, N (Fzd6 -/- mice) = 8.

b) Tumors were taken at day 18 after injection. Fixed frozen sections were stained for CD31 and the surface positive for staining was quantified using ImagePro software. N (Fzd6 +/+ mice) = 2, N (Fzd6 -/- mice) = 3.

Memo downregulation impairs 4T1 mammary tumor growth and metastasis formation

Figure 1.

Memo knock down 4T1 mouse mammary carcinoma cells form tumors with delayed kinetics.

a) The level of Memo expression in control and Memo knock down (KD) 4T1 clones was determined by immunoblotting. α-Tubulin expression is shown as a loading control.

b) Control and Memo KD 4T1 clones were injected into the mammary gland of female BALB/c mice (0.5 million cells in 100 µl PBS). Tumor growth was followed for 21 days. Data represents the average tumor volume in 6 mice over time.

c) Statistical analysis (Wilcoxon signed rank test) was done on pooled data from control and Memo KD clones to evaluate the effect of Memo downregulation on 4T1 tumor cell growth.

e) The level of Memo expression in control and Memo knock down (KD) 67NR clones was determined by immunoblotting. α-Tubulin expression is shown as a loading control.

f) Control and Memo KD 67NR clones were injected into the mammary gland of female BALB/c mice (0.5 million cells in 100 µl PBS). Tumor growth was followed for 28 days. Data represents the average tumor volume in 3 or more mice over time.

Figure 2.

Tumors derived from Memo KD clones are less vascularized.

a) Sections from tumors derived from control and Memo KD clones were taken at days 4, 7 and 9 post injection and immunostained for the endothelial marker PECAM (shown in red). The tissue is counterstained with the nuclear dye Hoechst (blue).

b) To determine vessel density, the area positive for PECAM was calculated and divided by the total surface area of the analysed section. The graph presents the percentage of the total area covered with vessels.

Figure 3.

Memo KD 4T1 clones secrete less VEGF in comparison to control clones.

The medium from control and Memo knock down cells was analysed for the presence of secreted VEGF by the method of enzyme linked immunosorbent assay (ELISA). Supernatants from three wells of growing cells were combined then assayed in triplicates in a way that standard errors on a graph refer to technical replicates. The graph represents the amount of VEGF per ml of supernatant normalized to 10000 cells.

Figure 4.

Memo KD clones are impaired in their ability to extravasate and colonize lungs.

a) 4T1 control and Memo KD cells were injected into the tail vein of BALB/c female mice (6 mice per group). Mice were sacrificed 12 days after injection. Images depict lungs that were immediately taken out.
b) Graph is presenting average weight of dissected lungs and heart from mice injected either with control or Memo KD clones.
c) H&E staining was done on fixed frozen sections, and images of consecutive sections (12 μM thick) were taken with automatized slidescanner. The graph is presenting total area of sections and area covered with metastasis, that were both measured using Imaris software. Note that, besides of the area covered with metastasis, total area of section is larger too in case of mice injected with control cells.

Figure 5.

Effects of Memo downregulation on survival of 4T1 clones in conditions of oxidative stress induced by $CoCl_2$.

4T1 cells resuspended in growth medium were plated at density 5000 cells per well and allowed to seed overnight. The next day medium was exchanged and the cells were incubated with increasing concentrations of $CoCl_2$ (300 μM, 600 μM, and 900 μM), or equal volume of PBS (control). Cell death was measured using the YO-PRO assay. Graph is showing the percentage of cells undergoing apoptosis.

VII. MATERIALS AND METHODS

Wnt 3a promotes proliferation and migration of HUVEC

Cell culture and reagents

Human umbilical vein endothelial cells (HUVECs) were obtained from Promo Cell and grown on plates coated with 1.5% gelatin, either in Endothelial Cell Growth Medium (Promo Cell, C-22010) supplemented with 2.5% Fetal Bovine Serum (FBS, Sigma, St Louis, MO, USA) or when indicated, in Endothelial Cell Basal Medium (Promo Cell, C-90210) supplemented with 0.5% or 1% FBS. For experiments cells not more than passage 6, were used. Lewis lung carcinoma (LLC) cells were maintained in RPMI1640, 10% FCS, 1% L-glutamine, 100 IU/ml penicillin, 100 µl/ml streptomycin. Recombinant Wnt3a, Wnt5a and Fz4 were purchased from R&D Systems, Minneapolis, MN, USA. Purified VEGF and PTK 787/ZK were provided by Dr. Amanda Littlewood Evans (Novartis Pharma AG, Basel, Switzerland). In this study we used antibodies against β-catenin (BD Transduction Laboratories, Franklin Lakes, NJ, USA), Dishevelled-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), α-tubulin (Lab Vision Corporation, Fremont, CA, USA), CD31 (BD Biosciences Pharmingen, San Jose, CA, USA), and HRP-conjugated anti-mouse secondary antibody (GE healthcare, Little Chalfont, Buckinghamshire, UK).

Detection of Fz4 and Fz6 by PCR

RNA from HUVECs cultured in 6 well plates was isolated using the RNeasy MiniKit (Qiagen) following the instructions from the manufacturer. cDNA was obtained using Ready-to-Go You-Prime First-strand beads (Amersham Biosciences). Semiquantitative PCR was done using primers for Fz4 and Fz6 (sequences and amplified bands' size are given in Table1). Conditions for PCR were: 94ºC for 5 minutes, followed by 29 cycles of 94ºC for 1 minute, 55ºC for 1 minute and 72ºC for 1 minute and 20 seconds.

Western blot analysis

HUVECs grown in Endothelial Cell Growth Medium with 2.5% FBS were treated with Wnt3a (200 ng/ml), Wnt5a (200 ng/ml), recombinant Fz4 (5 µg/ml), or PBS (control). Cells were harvested at different time points in NP-40 protein extraction buffer (1% Nonident P-40, 50mM Tris pH7.5, 120 mMNaCl, 5 mM EDTA [ethylenediaminetetraacetic acid], 1 mM EGTA [ethylene glycol tetraacetic acid], 2 mM sodium vanadate, 20 mM β-glycerophosphate, 10 µg/mL aprotinin, 10 µg/mL leupeptin, 0.5 mM PMSF [phenylmethylsulphonyl fluoride], 50 mM NaF, and 1 mM dithiothreitol).

Equal amounts of protein samples were denatured for 10 minutes at 95°C in sample buffer and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was done using antibodies against β-catenin, Dishevelled-3 and α-tubulin in 10% horse serum in TBS-T buffer (0.2M NaCl, 25 mM tris pH 7.5, 0.5 ml/l Tween-20), followed by incubation with α-mouse-HRP secondary antibody. The signal detection was performed with ECL (enhanced chemiluminiscence, GE Healthcare) according to manufacturer's instructions. Quantifications of Western blots were carried out using the ImageQuant TL version 2005 software package from Amersham Biosciences (now part of GE Healthcare).

BrdU incorporation assay

HUVECs were plated on a 96 well plate at a density of 5000 cells/well and incubated over night in a complete growth medium. The next day, medium was exchanged to Endothelial Cell Basal Medium supplemented with 1% FBS. Twenty-four hours later cells were treated with Wnt3a (200 ng/ml), Wnt5a (200 ng/ml), VEGF (20 ng/ml) or an equal volume of PBS in the presence or absence of recombinant Fz4 (5 μg/ml), PTK/ZK787 (1μM) or equal volumes of PBS and DMSO as a control. The treatments were done in Endothelial Cell Basal Medium supplemented with 1% FBS. BrdU was added to cells 12 hours prior to the assay that was performed 2 days after treatment with ligands. BrdU detection was done using the Cell Proliferation Biotrak ELISA System (GE Healthcare) following the instructions of the manufacturer.

Migration assay

The cell migration assay was performed in a 24-well Boyden Chamber. HUVECs were resuspended in Endothelial Cell Basal Medium supplemented with 1% FBS and added to the upper chamber of the well, and either Wnt3a (200 ng/ml) or Wnt5a (200 ng/ml) were used as chemo-attractants in the lower chamber. The cells were allowed to migrate for 6 hours at 37ºC, and then the cells were removed from the upper surface by scraping with a cotton swab. Cells that had migrated across the membrane were fixed in 4% PFA/PBS and the nuclei stained with Hoechst 33342. The number of cells was counted. Each condition was done in triplicate.

Survival assay

HUVECs were plated on 96 well plate at density 5000 cells per well, and grown over night in complete growth medium. To measure the effects on survival, cells were then grown in Endothelial Cell Basal Medium supplemented with 0.5% FBS. The cells were treated with Wnt3a (200 ng/ml), Wnt5a (200 ng/ml), VEGF (20 ng/ml) or an equal volume of PBS in the presence or absence of recombinant Fz4 (5 μg/ml), PTK 787/ZK (1μM) or equal volumes of

PBS and DMSO as control. Cells were incubated with ligand for 48 hours at 37ºC. Survival was measured using the YO-PRO assay. YO-PRO is a fluorescent dye that enters the nuclei of apoptotic cells, but not those that are viable. To determine the cells undergoing apoptosis and total cell number, respectively, we measured the fluorescence after the addition of the dye to the cells in culture, and subsequently after the cell lysis.

Wnt pathway target gene detection

HUVECs were treated with Wnt3a (200 ng/ml), Wnt5a (200 ng/ml) or an equal volume of PBS as a control for 5h or 24h. RNA was isolated using the RNeasy MiniKit (Qiagen) following the instructions from the manufacturer. cDNA was obtained using the Ready-to-Go You-Prime First-strand beads (Amersham Biosciences). Semiquantitative PCR was done using primers for c-Myc, Tie-2, Connexin 43, MMP-1 and CyclinD1. Primers (see Table 1 for sequences and amplified bands' size) were designed using Vector NTI. Conditions for PCR were: 95ºC for 4 minutes followed by 10 cycles of 95ºC, 60ºC, 72ºC for 30 seconds and 25 cycles of 95ºC, 55ºC, 72ºC for 30 seconds.

***In vivo* tumor growth**

Wild-type, heterozygous and Fz6 knock-out mice [100] backcrossed into the Bl6 background were injected with 0.5 million LLC cells in 100 µl of PBS. The injection was done subcutaneously into the mid-dorsal region and tumor growth was measured using a vernier caliper. Tumor volume was calculated using formula for the volume (V) of ellipsoid, $V=(4/3)\pi abc$, with the assumption that the height of the tumor equals its width (b=c).

Fixed frozen sections were stained for CD31 and the surface positive for staining was quantified using ImagePro software (Media Cybernetics, Bethesda, MD, USA).

Memo downregulation impairs 4T1 mammary tumor growth and metastasis formation

Cell culture and reagents

The mouse mammary tumor cell lines 4T1 and 67NR, obtained from Dr. R. Weinberg, Whitehead Institute for Biomedical Research, USA, were grown in DMEM supplemented with 10% heat inactivated FCS (Amimed, Allschwil, Switzerland) and Penicillin and Streptomycin.

PECAM antibody was obtained from BD Biosciences Pharmingen, San Jose, CA, USA, and α-tubulin from Lab Vision Corporation, Fremont, CA, USA.

Injection of tumor cells into mammary gland

Half a milion 4T1 and 67NR cells were resuspended in PBS and injected into the fourth, inguinal mammary fat pad of 8-12 week old female BALB/c mice. Tumor growth was measured with a vernier caliper, and tumor volume calculated using the formula for the volume of ellipsoid, $V=(4/3)\pi abc$, in case of 4T1, and cylinder, $V=\pi r^2 h$ in case of 67NR clones.

Vessel quantification

PECAM immunostaining on tumor sections, taken 4, 7 and 9 days after injection, was performed to visualize vasculature (sectioning, staining and images acquisition, were done by Dr Regis Masson)**.** Area positive for PECAM was calculated using Image Pro (image processing software, Media Cybernetics) and divided with total surface of selected area of interest to get the percentage of vessel coverage.

VEGF ELISA

Control and Memo knock down cells were plated at density of 8000 cells per well in 96 well plate in a growing medium. The medium was collected after 24 h and the concentration of secreted VEGF assessed using ELISA kit (Quantikine Immunoassay mouse VEGF, R&D Systems) following the instructions from manufacturer. Supernatants from three wells of growing cells were joined together and divided into three wells on an ELISA plate in a way that standard errors refer as technical replicates.

Tail vein injection

BALB/c female mice were injected into the tail veins with 7.5x105 4T1 mammary carcinoma cells (6 mice per group, injection perfomed by Julien Dey). Mice were sacrificed 12 days after injection, lungs and heart were taken out and weighed. Subsequently, lungs were imaged and prepared for histology. H&E staining was done on fixed frozen sections (12 um thick), and

images of consecutive sections were taken with Mirax slidescanner. Area covered with metastasis was measured using Imaris software.

Survival assay

4T1 control and Memo knock down clones (two and three different clones, respectively) were plated in 96 well plate at density 5000 cells per well, and grown over night in complete growth medium. To measure the effects of Memo downregulation on 4T1 cell survival in conditions of hypoxia, the medium was exchanged the next day and the cells were incubated with increasing concentrations of $CoCl_2$ (300 μM, 600 μM, and 900 μM), or equal volume of PBS (control). Cell death was measured using the YO-PRO assay. YO-PRO is a fluorescent dye entering the nuclei of apoptotic cells, but not those that are viable. To determine the cells undergoing apoptosis and total cell number, respectively, we measured the fluorescence after the employment of the dye to the cells in culture, and subsequently after the cell lysis. Graph is showing the percentage of cells undergoing apoptosis.

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