The amino-terminal oligomerization domain of Angiopoietin-2 affects vascular remodeling, mammary gland tumor growth, and lung metastasis in mice

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ABSTRACT

Angiopoietin-2 (ANGPT2) is a context-dependent TIE2 agonistic or antagonistic ligand that induces diverse responses in cancer. Blocking ANGPT2 provides a promising strategy for inhibiting tumor growth and metastasis, yet variable effects of targeting ANGPT2 have complicated drug development. ANGPT2₄₄₃ is a naturally occurring, lower-oligomeric protein isoform whose expression is increased in cancer. Here we use a knock-in mouse line (mice expressing Angpt2₄₄₃), a genetic model for breast cancer and metastasis (MMTV-*PyMT*), a syngeneic melanoma lung colonization model (B16F10), and orthotopic injection of E0771 breast cancer cells to show that alternative forms increase the diversity of Angpt2 function. In a mouse retina model of angiogenesis, expression of Angpt2₄₄₃ caused impaired venous development, suggesting enhanced function as a competitive antagonist for Tie2. In mammary gland tumor models, Angpt2₄₄₃ differentially affected primary tumor growth and vascularization; these varying effects were associated with Angpt2 protein localization in the endothelium or in the stromal extracellular matrix as well as the frequency of Tie2-positive tumor blood vessels. In the presence of metastatic cells, Angpt2₄₄₃ promoted destabilization of pulmonary vasculature and lung metastasis. *In vitro*, ANGPT2₄₄₃ was susceptible to proteolytical cleavage, resulting in a monomeric ligand (ANGPT2_{DAP}) that inhibited ANGPT1- or ANGPT4-induced TIE2 activation but did not bind to alternative ANGPT2 receptor $\alpha 5\beta 1$ integrin. Collectively, these data reveal novel roles for the ANGPT2 N-terminal domain in blood vessel remodeling, tumor growth, metastasis, integrin binding, and proteolytic regulation.

STATEMENT OF SIGNIFICANCE

This study identifies the role of the N-terminal oligomerization domain of Angiopoietin-2 in vascular remodeling and lung metastasis and provides new insights into mechanisms underlying the versatile functions of Angiopoietin-2 in cancer.

INTRODUCTION

The angiopoietin (Angpt1, 2 and 4)/Tie receptor tyrosine kinase signaling pathway (*ANGPT*, human; *Angpt*, mouse) regulates vascular remodeling in development and during tumorigenesis (1). Angpt1 is an agonistic Tie2 ligand having a stabilizing and anti-inflammatory role (2), whereas Angpt2 is implicated in vascular remodeling and endothelial cell (EC) activation (3). Angpt4 is required for venous morphogenesis in the retina (4).

In ECs, Angpt2 is stored in and released from EC-specific storage granules, the Weibel-Palade bodies (5). In mouse development, Angpt2 is required for lymphangiogenesis, development of retinal blood vessels and regression of hyaloid vessels in the eye (6). In cancer, Angpt2 promotes vascular sprouting, destabilization, inflammation and metastasis (1). *ANGPT2* expression pattern (low in normal tissue, induced in many cancers) and cancer-promoting effects have identified *ANGPT2* as a potential target molecule for anti-cancer treatments (1), however, treatment responses in patients have been less promising (7, 8) as expected based on preclinical studies. Clearly, thorough characterization of *ANGPT2* is necessary to fully evaluate its potential as a target molecule for anticancer drugs.

Alternative mRNA splicing is a common mechanism for generation of multiple isoforms from a single gene and it represents a major mechanism to increase genetic diversity. In normal development, expression of protein isoforms is carefully regulated, while in cancer significant alterations in the transcriptome are caused by differential or dysregulated mRNA splicing (9). Alternative splicing generates the *ANGPT2*₄₄₃ isoform lacking exon 2 (10) that in full length *ANGPT2* mRNA encodes for 53 amino acids of the coiled-coil domain (CCD) responsible for ligand oligomerization. In cultured human ECs, the relative abundance of *ANGPT2*₄₄₃ is approximately 10% of *ANGPT2* (10, 11) and its expression is also documented in human macrophages (10). *ANGPT2*₄₄₃ is upregulated in tumor cell lines and human tumor biopsies (10, 12). Interestingly, some tumor cells have been shown to express solely *ANGPT2*₄₄₃ (10), and its increased expression has also been detected in human chronic lymphocytic leukemia (12), in hepatitis B -infected liver (13), and in acute lung injury (14). Apart from human malignancies, *ANGPT2*₄₄₃ expression is also reported in canine adrenocortical tumor tissue (15). Our analysis of *ANGPT2* splice variants from The Cancer Genome Atlas (TCGA) mRNA sequencing data (16) revealed that in normal tissue the ratio of *ANGPT2*₄₄₃ to full length *ANGPT2* is less than 20% while in breast cancer patient tissues the ratio is increased (Fig. 1A). Collectively, these data suggest that *ANGPT2*₄₄₃ is expressed in low abundance in unstressed tissues and upregulated in tumorigenesis

and in activated ECs. The *in vivo* functions of ANGPT2₄₄₃ variant and whether they differ from those of ANGPT2 are, however, unknown.

Here, we hypothesized that alternative protein forms increase diversity of the functions of Angpt2 and generated a targeted knock-in mouse allele expressing Angpt2₄₄₃. The *Angpt2*⁴⁴³ mice allowed us to address the physiological importance of a specific domain of Angpt2 in development, in primary tumor growth and in metastasis by using genetic and syngeneic breast cancer and lung metastasis models. We found that alternative protein forms were less oligomerized, potent competitive inhibitors of TIE2 activation affecting venous remodeling, tumor vasculature and metastasis. Our findings identify the *in vivo* importance of the N-terminal domain of ANGPT2 and provide new insights into mechanisms underlying the versatile functions of ANGPT2 in cancer.

MATERIALS AND METHODS

Analysis of *ANGPT2* mRNA variants in human patients

ANGPT2 isoform expression data in human breast cancer was analyzed using The Cancer Genome Atlas cohort data from the TSVdb database (<http://www.tsvdb.com>).

Cell culture

Human umbilical vein endothelial cells (HUVECs, PromoCell) were cultured in Medium 200 (M-200-500, Cascade Biologics) supplemented with growth supplement (LSGS, S00310, Cascade Biologics), penicillin-streptomycin (P/S, Sigma-Aldrich) and 10% heat-inactivated FBS (HI-FBS) (S1810-500, Biowest) in cell culture plates pre-coated with Attachment Factor (123-500, Cell Applications). HUVECs were transfected with human TIE2 (TIE2-WT), ANGPT2, ANGPT2-Flag, ANGPT2-GFP, ANGPT2₄₄₃, ANGPT2₄₄₃-GFP, ANGPT2₄₄₃-His or ANGPT2_{DAP} via retroviral gene transfer (17) (DNA constructs are listed in Supplementary Table SI). An African green monkey kidney fibroblast-like cell line (COS-1, ATCC, RRID:CVCL_0223) was cultured in DMEM (31966-021, Gibco) supplemented with 10% HI-FBS and 1% P/S. The human cervical epidermoid carcinoma cancer cell line (CaSki, ATCC, RRID:CVCL_1100) was maintained in RPMI-1640 medium (30-2001, ATCC) supplemented with 10% HI-FBS and 1% P/S and retrovirally transfected to express H2B-mCherry. C57BL-syngeneic mouse skin melanoma cell line B16F10 (ATCC, RRID:CVCL_0159) was cultured in advanced DMEM F12 (11320033, Gibco) supplemented with

2 mM L-glutamine (G7513, Sigma-Aldrich), 10% HI-FBS and 1% P/S. E0771 mouse breast cancer cell line (CH3 BioSystems) was cultured in RPMI-1640 (ATCC) supplemented with 10% HI-FBS and retrovirally transfected to express ANGPT2, ANGPT2₄₄₃ or ANGPT2_{DAP} when indicated. Cells purchased from ATCC were authenticated by the manufacturer. HUVECs were primary cells from pooled donors used at low passages and authenticated by the expression of EC-specific genes and monolayer morphology. E0771 cells were not authenticated after receiving from the vendor. Non-primary cells were kept in culture for maximum two months, and all cells were tested mycoplasma-negative upon receipt and regularly while performing the experiments with EZ-PCR Mycoplasma test kit (Biological Industries). Details of protein production in COS-1 cells, HUVECs or *E. coli*, ELISA assays, ANGPT2_{DAP} peptide sequence analysis and *in vitro* experiments are described in the Supplementary Materials and Methods.

Western blot analysis

Whole cell lysates, conditioned media or immunoprecipitated cell or tissue samples were separated under reducing or non-reducing conditions with SDS-PAGE and analyzed by Western blotting as described in (4). Purified human recombinant ANGPT2 protein (R&D Systems) was included as a control when indicated. All antibodies are listed in Supplementary Table SII. See Supplementary Materials and Methods for detailed immunoprecipitation protocols.

Generation of mouse lines

Experiments involving mice were performed under permission from the Finnish Animal Experiment

Board following the regulations of EU and Finnish legislation. A mouse allele expressing solely Angpt2₄₄₃, named as *Angpt2*⁴⁴³, was generated using a conventional BAC recombineering technology. The targeting construct lacked the 156 base pair (bp) long exon 2 and intronic consensus sequences for mRNA splicing sites 834 bp upstream and 1620 bp downstream from exon 2. In the targeting construct, deleted region was replaced with a *neo* cassette including PGK/EM7 promoter and a polyA sequence for guiding neomycin expression as an independent transcript. Final targeting vector contained 9.5 and 1.8 kb genomic arms and a 3 kb *neo* cassette. The SalI-linearized targeting construct was electroporated into B6 ES cells and selected with G418. Genomic DNA isolated from resistant colonies was screened by PCR. Cells from correctly targeted clones were

injected into C57BL blastocysts, and two separate mouse lines were generated by standard methods. For *Angpt2*⁴⁴³ allele expression analysis, PCR from lung cDNA was performed with primers spanning from exon 1 to exon 4 (Supplementary Table SIII) allowing detection of *Angpt2* isoforms. PCR products were separated on a 2% agarose gel and verified by sequencing. *Angpt2*^{443/443} mice were kept in C57BL/6N genetic background. *Angpt4*^{-/-} mouse line has been described before (4), and *Angpt2*^{-/-} mouse line is described in the Supplementary Materials and Methods.

Generation of polyomavirus middle T antigen mouse breast cancer model FVB/N-Tg(MMTV-PyVT)634Mul (Jackson Laboratory, hereafter *PyMT*) has been described elsewhere (18). Male FVB *PyMT* mice were crossed with heterozygous female C57BL/6N *Angpt2*^{+/-443} mice, resulting in four different genotypes in F1 generation (*Angpt2*^{+/+}, *Angpt2*^{+/-443}, *PyMT*; *Angpt2*^{+/+}, *PyMT*; *Angpt2*^{+/-443}) all in the same FVB-C57BL/6N hybrid genetic background. The tumor-bearing female mice were sacrificed at 13 weeks and 18 weeks of age and non-tumor control mice were sacrificed at 19 weeks. Genotyping PCR primers for the *neo* gene -cassette, *PyMT* transgene and *Angpt2* are presented in Supplementary Table SIII.

Histological methods

All histological tissue staining methods, tissue imaging and analysis are described in the Supplementary Materials and Methods. All antibodies are listed in Supplementary Table SII.

Fluorosphere clearance from retinal veins

Fluorescent beads were administrated into carotid artery of 5 months old mice as previously published (4) except that Alexa Fluor 594-conjugated WGA-lectin (Thermo Fisher) was injected 5 min before the beads to visualize blood vessels.

In vivo vascular permeability assay

MMTV-*PyMT* mice were injected with Evans Blue (EB), after 30 min they were euthanized, vasculature was flushed from excess EB and tumor EB content was measured. More detailed protocol is presented in the Supplementary Materials and Methods.

Quantitative RT-PCR

Quantitative RT-PCR was performed as described before (4) using GAPDH and β -actin as housekeeping genes. qPCR primers are presented in Supplementary Table SIII.

B16F10 melanoma cell tail vein injections

B16F10 melanoma cells were trypsinized, washed twice with serum-containing medium and twice with ice-cold HBSS, strained through cell strainer and cell number was adjusted to 1×10^6 cells/ml in HBSS. Adult mice were anesthetized with isoflurane and 1.5×10^5 cells (150 μ l)/mouse was injected into tail vein. After 14 days, the lungs were collected, and metastases analyzed with optical projection tomography as described in the Supplementary Materials and Methods.

Wet/dry lung weight ratio

Lungs from one year old mice were collected and immediately weighed, dried at 65 °C for three days and weighed again to establish wet/dry lung weight ratio (19).

E0771 breast cancer cell injections

Trypsinized E0771 cells were washed twice with serum-containing medium and twice with HBSS, and 1×10^7 cells/ml were suspended in RPMI-1640 medium. 5×10^5 cells (50 μ l) were injected into four mammary fat pads (right and left thoracic and inguinal)/12-weeks-old female transgenic or C57BL/6N mouse. Tissues were collected after 29 days.

Statistical analysis

Mann-Whitney U -test was used in Figures 1A and 4M-N. Otherwise, comparisons between two groups were performed using two-tailed unpaired Student's t-test (in Figures 1E, 1K, 4 and 5) and between multiple groups with one-way ANOVA followed by Bonferroni (in Figure 1H) or Tukey (in Figures 2, 3, 6 and 7) post-hoc tests using OriginPro software. Statistical significances are marked into figures with an asterisk: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Box plots show median as a vertical line, average as a small box, 25-75% interquartile range as a box, 5th and 95th percentile as whiskers and outliers as dots. Dot plots show measurement points and a bar as a mean. In column charts, data is presented as mean $\pm$ SD.

RESULTS

Cell-secreted ANGPT2₄₄₃ includes two different-size monomers regulated by furin-dependent cleavage of the alternative N-terminus

Analysis of cell lysates from retrovirally transduced HUVECs using polyclonal ANGPT2 antibody showed higher and lower molecular weight (MW) protein forms for ANGPT2 and ANGPT2₄₄₃ under reduced conditions in Western blot (WB) analysis. Analysis of conditioned media (CM) from the same cells under reducing conditions indicated that HUVEC-secreted ANGPT2 is mainly a single MW protein, whereas ANGPT2₄₄₃ showed two distinct bands (Fig. 1B). Under non-reducing conditions, both isoforms run mainly as dimers in the HUVECs' CM, however, in ANGPT2₄₄₃ an increase in monomeric ~50 kDa protein form was detected (Fig. 1C). In addition, when compared to ANGPT2, ANGPT2₄₄₃ was less multimerized in accordance with a previously proposed role of the N-terminus in ligand oligomerization and multimerization (20). To investigate in detail the ANGPT2₄₄₃-derived low MW protein form, His-tagged proteins were immunoprecipitated for peptide analysis. Mass spectroscopy -analysis of trypsin-digested samples identified the first peptide for the uncleaved ANGPT2 and ANGPT2₄₄₃ starting from glutamine 33 (Q33). In contrast, in ANGPT2₄₄₃-derived low MW protein the first peptide identified started from aspartic acid 68 (D68) (Fig. 1D). This cleavage site (...VQR/DAP...) was confirmed by N-terminal peptide sequencing. The resulting ANGPT2₄₄₃-derived ~50 kDa protein was named as ANGPT2_{DAP} based on the first three N-terminal amino acids. Analysis of potential protease cleavage site (<http://prosperous.erc.monash.edu/>) revealed consensus sequence for proprotein convertase 1 and furin-like protein convertase (AVQR/DA). In line with the *in silico* analysis, furin inhibitor decreased the extent of ANGPT2_{DAP} cleavage (Fig. 1E). ANGPT2_{DAP} lacked the entire superclustering domain (Supplementary Fig. S1) needed for formation of higher order multimers, consistent with the enrichment of monomeric ANGPT2 proteins. Similarly to HUVECs, CM from the ANGPT2₄₄₃-transfected COS-1 cells showed less higher-oligomerized/multimerized forms and two distinct monomeric forms while ANGPT2_{DAP} run as a non-disulfide linked monomer in SDS-PAGE (Fig. 1F and G). In solid-phase binding assay, ANGPT2₄₄₃ and ANGPT2_{DAP} bound to TIE2 but with lower binding affinity (Fig. 1H). This was in line with previous results indicating that designed low-oligomerized forms of ANGPT1 bind less to TIE2 (21). In addition to TIE2, ANGPT2 can bind to integrins to enhance sprouting angiogenesis (22) and endothelial

destabilization (23). Our special interest was to investigate ANGPT2 binding to $\alpha 5 \beta 1$ integrin, which is involved in breast cancer metastasis (24), abnormal vascular remodeling (25) and leakage (26). C-terminal ANGPT2 fragment (K275 to F469, common to all variants) bound to $\alpha 5 \beta 1$ at a relatively high concentration but the N-terminal (E72 to A274) fragment did not (Fig. 1I). Binding of full length ANGPT2 showed significantly higher affinity to $\alpha 5 \beta 1$ than the C-terminal fragment, whereas affinity of ANGPT2₄₄₃ was somewhat lower and ANGPT2_{DAP} did not bind to $\alpha 5 \beta 1$ at 2 nM concentration (Fig. 1J). To test if oligomerization of ANGPT2 is necessary for binding to $\alpha 5 \beta 1$, disulfide bonds in the oligomeric full length ANGPT2 were reduced using dithiothreitol (DTT). DTT treatment efficiently reduced $\alpha 5 \beta 1$ binding (Fig. 1K).

Lower-oligomeric Angiopoietin-2 forms have differential effects on endothelial cell functions and are potent competitive inhibitors of Angiopoietin-1 and -4

To test whether ANGPT2 forms mediate differential biological effects, ligand isoforms were first compared in TIE2 activation assays as single ligands or in the presence of ANGPT1 or ANGPT4. Similarly to ANGPT2 (27), ANGPT2₄₄₃ acted as a weak TIE2 agonist (about 20% of tyrosine phosphorylation of ANGPT1-stimulated TIE2) while ANGPT2_{DAP} did not activate TIE2 (Fig. 2A, left). ANGPT2₄₄₃ has been shown to be more potent competitive inhibitor of ANGPT1 than native ANGPT2 (10). In our experimental setting, ANGPT2₄₄₃ was approximately twice as effective as ANGPT2 to reduce ANGPT1-induced TIE2 phosphorylation, while ANGPT2_{DAP} was the most potent competitive inhibitor (Fig. 2A, right). In EC-EC junctions, ANGPT2 inhibits ANGPT1-induced TIE2 activation (17), and in this subcellular compartment ANGPT2₄₄₃ and ANGPT2_{DAP} were more potent to do so (Fig. 2B). Similarly to ANGPT1 blocking, low oligomeric ANGPT2 forms were effective to reduce ANGPT4-induced TIE2 activation in EC-EC junctions (Supplementary Fig. S2). WB analysis (Fig 2A) suggested that ANGPT2 and ANGPT2₄₄₃ can activate TIE2 at low level when present as single ligands. In the retracting cell edges, a cell compartment that does not contain VE-PTP (17) downregulating the agonistic role of ANGPT2 (28), ANGPT2 and ANGPT2₄₄₃ induced some TIE2 activation but ANGPT2_{DAP} did not (Supplementary Fig. S3).

We next compared ANGPT2 forms in functional EC assays. In cultured HUVECs (that express ANGPT2 but no other angiopoietins at significant levels) endogenous ANGPT2 increases cell viability (29, 30). *ANGPT2* mRNA silencing reduced HUVEC cell number, and viability was improved when these cells were cultured in the presence of ANGPT2 or ANGPT2₄₄₃ but not with ANGPT2_{DAP} (Fig. 2C). In fibrin gel model of angiogenesis, native ANGPT2 promotes

tube formation (27). Similarly, in our sprouting assay in fibrin gel ANGPT2 elongated sprouts while other forms did not (Fig. 2D). In contrast, lower oligomeric ANGPT2₄₄₃ and ANGPT2_{DAP} increased the number of sprouts, suggesting that full length and lower oligomeric ANGPT2 forms may have complementary functions. We have previously shown that substrate-bound native ANGPT2 weakly supports HUVEC adhesion and spreading (31). This was also true for lower oligomeric ligand forms (Fig. 2E), indicating that extracellular matrix (ECM) -bound (negative cellular effect) and soluble ANGPT2 (weak agonist) have differential cellular effects.

As indicated in Fig. 1A, *ANGPT2* and *ANGPT2*⁴⁴³ mRNAs are simultaneously expressed in tumor tissue. To test if the presence of N-terminally variable ANPGT2 proteins may negatively affect their assembly into oligomeric forms (dominant-negative effect), COS-1 cells were retrovirally transduced either with single ANGPT2-Flag or ANGPT2₄₄₃-His or both (Fig. 2F). Comparison of CMs collected from single and double-transduced cells revealed that simultaneous expression of ANGPT2-Flag and ANGPT2₄₄₃-His did not affect their mobility in non-reducing SDS-PAGE analysis (Fig. 2G) indicating that these N-terminally different proteins do not form intermolecular oligomers.

***Angpt2*⁴⁴³ interferes with the venous development in the mouse retina model of angiogenesis**

To investigate the *in vivo* importance of *Angpt2*₄₄₃ variant, a gene-targeted mouse allele was generated, in which alternative exon 2 was replaced with a *neo* gene -cassette (Supplementary Fig. S4A) thus expressing solely *Angpt2*⁴⁴³. RT-PCR analysis confirmed that the sequence encoded by exon 2 was lacking in homozygous *Angpt2*^{443/443} mice mRNA (Supplementary Fig. S4B-D).

Development of mouse retinal vasculature is particularly sensitive for changes in Tie/Angiopoietin signaling pathway (32) and was used as a model to analyze possible vascular phenotypes in *Angpt2*⁴⁴³ mice. As *Angpt2* is required for formation of the retinal capillary plexus (6, 33), postnatal and adult retinas were analyzed (Fig. 3A-C). At postnatal day (P) 6 we found no delay in sprouting angiogenesis, however, *Angpt2*^{443/443} retinas showed impaired lumen formation in the veins, which is dependent on *Angpt4* (4) and *Tie2* (34) (Fig. 3D-E). In normal mouse retina, two or occasionally three main veins extend to the retinal periphery and branch to form an annular structure (Supplementary Fig. S4E). Interestingly, in *Angpt2*^{443/443} as well as in *Angpt4*^{-/-} mice, the number of veins extending retinal periphery was elevated (Fig. 3F), indicating that *Angpt2*⁴⁴³ inhibits *Angpt4*-dependent vascular development. Also, in the heterozygous *Angpt2*^{+/-443} mice vein diameter was diminished and number of veins extending to periphery elevated (Fig. 3B-C and E-F), suggesting a dose-dependent effect of *Angpt2*₄₄₃. To rule out the possibility that defects in

Angpt2^{+/-443} and *Angpt2*^{443/443} mice are caused by differences in Angpt2 protein levels or location, P4 retinas were stained with Angpt2 antibody. Similar levels of staining in retinal tip cells and neuronal layer were detected in all genotypes (Supplementary Fig. S4F). These data suggested that exon 2 deletion does not have a loss-of-function effect, which we further investigated by comparing retinal phenotype to Angpt2-deficient mice. In contrast to *Angpt2*^{443/443} mice, *Angpt2*^{-/-} retinal vasculature lacked hierarchical organization of arterial and venous trees (Supplementary Fig. S4G). Furthermore, retinal vasculature in *Angpt2*^{-/-} mice did not extend to the periphery resulting in avascular areas that were not observed in other genotypes (Supplementary Fig. S4H). Instead, exon 2 deletion more closely resembled the phenotype observed in *Angpt4*^{-/-} mice and may rather interfere with Tie2 signaling that promotes vessel enlargement and vein morphogenesis (2, 4, 34, 35). This was confirmed by retina whole mount staining of phosphorylated Tie2 (pTie2) and total Tie2 which showed decreased pTie2/Tie2 ratio in *Angpt2*^{443/443} retinal veins at P8 (Fig. 3G-H) when vein morphogenesis occurs.

Developmental defect in *Angpt4*^{-/-} veins also includes maturation abnormality of SMCs (4). Reduced α SMA staining was indeed seen in some *Angpt2*^{443/443} P12 retinal veins (Fig. 3I-J) but this was less severe than reported in *Angpt4*^{-/-} veins. To investigate if venous drainage function is reduced in *Angpt2*^{443/443} mice, fluorescent beads were injected into carotid artery and their distribution in retinal veins was investigated shortly after injection. Some veins in *Angpt2*^{+/-443} and *Angpt2*^{443/443} mice had increased accumulation of beads (Fig. 3K), however, decrease in drainage was not as prominent than observed in *Angpt4*^{-/-} mice, in which venous narrowing is also more severe (4).

Mammary gland tumor growth and lung metastasis are reduced in *PyMT*; *Angpt2*^{+/-443} mice

Primary tumor growth in the MMTV-*PyMT* model has previously been shown to be dependent on Angpt2 (36). To test whether Angpt2₄₄₃ has a differential effect, as one can expect based on its proteolytic processing, lower integrin binding and increased potency to interfere TIE2 signaling, we generated *PyMT*; *Angpt2*^{+/-443} mice by crossing transgenic *PyMT* (18) males (in pure FVB/N background) with *Angpt2*^{443/443} females (in pure C57Bl/6N background). This allowed us to investigate tumor growth in a homogenous F1-hybrid genetic background. As both full length *ANGPT2* and *ANGPT2*⁴⁴³ are simultaneously expressed in human tumor tissues, heterozygous state (*Angpt2*^{+/-443}) in mice should well represent the ANGPT2 isoform balance in cancer (Fig. 1A). Two cohorts of tumor-bearing mice were sacrificed at 13 and 18 weeks of age and the mammary glands

and tumors were harvested for gravimetric (Fig. 4A) and carmine alum (Fig. 4B) analysis. Mammary gland weights were equal in non-tumor *Angpt2*^{+/+} and *Angpt2*^{+/-443} controls and showed no alterations in their vasculature. A trend for decreased tumor weight was observed in *PyMT*; *Angpt2*^{+/-443} already at 13 weeks of age. In the older cohort, the total primary tumor burden in the *PyMT*; *Angpt2*^{+/-443} mice was smaller than in the *PyMT*; *Angpt2*^{+/+} mice (Fig. 4A).

Angpt2 has various effects on tumor vasculature including angiogenesis (36), vessel lumen size (37), pericyte (PC) coverage (37, 38) and leakage (39). Immunofluorescent microscopy revealed decreased blood vessel (CD31) count (Fig. 4C-D) and reduced number of lumen structures in the tumor vessels (Fig. 4E) in *PyMT*; *Angpt2*^{+/-443} mice. Those vessels where lumens were identified, however, showed no differences in the lumen size when compared to the *PyMT*; *Angpt2*^{+/+} mice (Fig. 4F) suggesting that not all tumor vessels express or respond to *Angpt2*⁴⁴³ equally. There was a trend for increased number of PC-deficient vessels in the *PyMT*; *Angpt2*^{+/-443} whole tumor sections stained for CD140b and CD31 (Fig. 4G). Based on transmission electron microscopy (TEM), in which stromal and tumor compartments can be reliably distinguished in high resolution, PC abnormalities were primarily found in *PyMT*; *Angpt2*^{+/-443} stromal blood vessels, while PC coverage was not different in vessels that were located within the tumor cell mass without defined perivascular ECM (Fig. 4H-J). In addition, vascular permeability was increased in the *PyMT*; *Angpt2*^{+/-443} mammary tumors (Fig. 4K).

To rule out that slower tumor growth is not caused by lack of *Angpt2*⁴⁴³ allele expression, we investigated mRNA expression using *Angpt2*⁴⁴³-specific qPCR primers spanning from exon 1 to exon 3 (forward) and exon 4 (reverse) (Supplementary Table SIII). *Angpt2*⁴⁴³ mRNA was detected in *PyMT*; *Angpt2*^{+/-443} but not in *PyMT*; *Angpt2*^{+/+} tumors (Supplementary Fig. S5A). Based on qPCR primers from exons 8 and 9, common to both variants, total *Angpt2* expression was at the same level (Supplementary Fig. 5B). *Angpt1* and *Vegfa* are well-known factors that can modulate destabilizing and angiogenic effects of *Angpt2* on vasculature (1). *Angpt1* mRNA levels remained unchanged, while *Vegfa* levels were increased in *PyMT*; *Angpt2*^{+/-443} mice (Supplementary Fig. S5B). Expression levels of *Calx*, *Eno1*, *Glut1*, *Ldha* and *Pgk1*, which upregulation would indicate hypoxic conditions in mouse breast cancer tumors (40, 41), were found to be unaltered (Supplementary Fig. S5B). Number of apoptotic ECs, PCs and tumor cells as well as necrotic areas in tumors were not changed (Supplementary Fig. S5C-F).

Metastases were annotated from HE-stained lung sections (Fig. 4L). *PyMT*; *Angpt2*^{+/+} mice showed larger metastatic area (Fig. 4M) and a trend for increased metastasis count (Fig. 4N, *P*=0.057) when compared to *PyMT*; *Angpt2*^{+/-443} mice. Notably, 67% of the *PyMT*; *Angpt2*^{+/-443} mice

had less than 0.5% metastasis versus the lung area, whereas 100% of the *PyMT; Angpt2^{+/+}* lungs had more than 0.5% metastasis (Fig. 4M).

Angpt2 is expressed in some endothelial cells in tumor vasculature and sequestered in the tumor stroma

To investigate further the observed heterogeneity in lumen-forming and PC-deficient vessels in MMTV-*PyMT* tumor vasculature, we analyzed *Angpt2* mRNA and protein localization using *Angpt2 in situ* hybridization (4) and an Angpt2-specific antibody (25). Notably only a subset of ECs showed *Angpt2* expression (Fig. 5A–D) as has previously been shown also in the vasculature of human glioma (42). Interestingly, a large proportion of Angpt2 immunoreactivity was located in the perivascular matrix (Fig. 5E–H) in both *PyMT; Angpt2^{+/+}* and *PyMT; Angpt2^{+/443}* tumors (Fig. 5H–I).

As Angpt2 immunostaining was surprisingly more intense in the subendothelial ECM than in the ECs where it was produced, we next investigated ECM secretion of ANGPT2 further using ANGPT2-GFP HUVECs. Additional motivation to investigate ECM secretion mechanism was the observation that ECM-bound and soluble ANGPT2 showed differential effects (Fig. 2) and that how paracrine, EC-produced ANGPT2 can affect PCs that locate within the perivascular ECM. In stable adherent HUVECs, both ANGPT2-GFP and ANGPT2₄₄₃-GFP were located in matured Weibel-Palade bodies (WPBs) containing also von Willebrand Factor (vWF) (Supplementary Fig. S6A). To analyze ECM secretion, we used total internal fluorescence (TIRF) microscopy to visualize ANGPT2-GFP with high axial resolution in freshly plated, actively spreading HUVECs where ECM constituents are secreted and EC-ECM contacts are formed and remodeled (Supplementary Movies 1-2). Interestingly, ANGPT2-GFP was detected on the basal cell membrane first as vesicles that emerged to the TIRF field and were then fast immobilized when they became in contact with the ECM (Supplementary Movie 2). In fixed and vWF-stained ANGPT2-GFP HUVECs, TIRF imaging revealed co-localization of ANGPT2-GFP and vWF in the subendothelial ECM (Supplementary Fig. S6B–C). A previous study suggested that ANGPT2 and vWF are secreted from ECs by exosomes (43). However, our analysis of the ultrastructure of the EC-ECM interphase revealed ANGPT2 immunostaining at the basal cell membrane and ECM-interphase but not within the membrane-covered extracellular vesicles (Supplementary Fig. S6D–H).

***Angpt2₄₄₃* enhances lung colonization of tail vein injected B16F10 melanoma cells**

Angpt2 blocking has previously been shown to decrease lung metastasis in an B16F10 metastasis model (44) so we next investigated lung colonization of syngeneic B16F10 melanoma cells in

Angpt2^{+/+}, *Angpt2*^{+/⁴⁴³} and *Angpt2*^{443/443} mice using optical projection tomography of whole lung lobes (Fig. 6A-B). In *Angpt2*^{+/+} mice, extensive B16F10 colonization (> 3% of total lung volume) was observed in 7.1% of lung samples, in 44.4% of *Angpt2*^{+/⁴⁴³}, and in 60% of *Angpt2*^{443/443} lungs. Increased volume of melanoma cell colonies was observed in *Angpt2*^{443/443} mice when compared to *Angpt2*^{+/+} mice ($P=0.008$ in two-tailed t-test). In the *Angpt2*^{+/⁴⁴³} mice, the average volume of melanoma colonies was roughly in between *Angpt2*^{443/443} and WT mice, however, due to notably high variation in *Angpt2*^{+/⁴⁴³} mice, comparisons of all three groups in ANOVA resulted in a P -value of 0.052 (Fig. 6B).

In a previous study, *Angpt2* overexpression increased while *Angpt2*-blocking antibodies alleviated vascular destabilization in the B16F10 model (44). To investigate if *Angpt2*⁴⁴³ delivers stronger destabilizing effects than native *Angpt2*, ultrastructure of the lung vasculature was investigated using TEM. As illustrated in Fig. 6C-D, EC-EC adherent junction area (Fig. 6E) and the length of EC-EC contacts (Fig. 6F) were decreased in both *Angpt2*^{+/⁴⁴³} and *Angpt2*^{443/443} host vessels close to the metastasis foci. EC degenerative changes (cell organelle -free cytoplasm, pyknosis, EC-ECM detachment and vacuolization) were most frequently observed in B16F10-injected *Angpt2*^{443/443} mice (Fig. 6G). In non-tumor cell-loaded lungs we found no changes in pulmonary vasculature (Tie2 phosphorylation, markers for permeability, lung wet/dry weight ratio indicative of edema) in either *Angpt2*^{+/+}, *Angpt2*^{+/⁴⁴³} or *Angpt2*^{443/443} mice (Supplementary Fig. S7A-C). This is in line with previous data indicating that in the normal lung ECs *Angpt2* is not significantly expressed (45), but it is induced in the host vessel ECs in response to tumor cell injection (46). Accordingly, *Angpt2* protein was localized in some ECs at the margin of B16F10 colonies (Supplementary Fig. S7D). In comparison to MMTV-*PyMT* mammary tumors, B16F10 cells were closely associated with host pulmonary vessels, induced only little intratumoral angiogenesis, did not form tumor stroma and the pulmonary ECM did not show *Angpt2* immunoreactivity. *Vegfa*, which at low levels enhances *Angpt2*-induced vascular regression (47), was slightly downregulated in B16F10-colonized lungs of *Angpt2*^{+/⁴⁴³} and *Angpt2*^{443/443} mice, while levels of *Angpt1* were not significantly changed (Supplementary Fig. S7E). As in *PyMT* tumors, total *Angpt2* mRNA levels were equal in all genotypes (Supplementary Fig. S7E), while the *Angpt2*⁴⁴³ variant-specific primer pair showed *Angpt2*⁴⁴³ allele expression only in the *Angpt2*^{+/⁴⁴³} and *Angpt2*^{443/443} mice but none in the WT mice (Supplementary Fig. S7F). Our observation that WT mice do not naturally express *Angpt2*⁴⁴³ goes along with the GenBank database (<https://www.ncbi.nlm.nih.gov/genbank/>) and indicates that *ANGPT2*⁴⁴³ is not expressed in all mammalian species and that the splicing process generating *Angpt2*⁴⁴³ is not completely conserved.

To test if vascular destabilization by ANGPT2₄₄₃ could also be observed in human cell-based assay, we performed *in vitro* EC transmigration assay (48) using CaSki cancer cell line with non-transduced and ANGPT2 or ANGPT2₄₄₃-expressing HUVECs. In this assay, ANGPT2₄₄₃ but not ANGPT2 increased tumor cell transmigration (Fig. 6H).

ANGPT2₄₄₃ expression increases lung metastasis in orthotopic E0771 breast cancer cell transplantation model

Results from MMTV-*PyMT* and B16F10 models were inconsistent with respect to the lung metastasis potential of *Angpt2*₄₄₃. To test all three genotypes in a model that allowed analysis of both mammary gland primary tumors and lung metastases we next utilized orthotopic injection of E0771 breast cancer cells into mouse mammary fat pad. In the first experimental setting, we injected parental E0771 cells into *Angpt2*^{+/+}, *Angpt2*^{+/-} and *Angpt2*^{443/443} mice. As E0771 model is not strongly metastatic and to test the ANGPT2_{DAP} variant we also generated E0771 cells stably expressing ANGPT2 protein forms and transplanted the resulting E0771^{ANGPT2}, E0771⁴⁴³ and E0771^{DAP} cells into wild-type C57BL mice. In all cohorts, we found no changes in the primary tumor weight (Fig. 7A). In addition, tumor blood vessel count and lung metastasis area were not changed in *Angpt2*^{+/-} and *Angpt2*^{443/443} mice (Fig. 7B-E). In contrast to genetic MMTV-*PyMT* model, E0771 transplantation tumors contained only little ECM-rich stroma and *Angpt2* protein was localized in CD31-positive vascular structures (Fig. 7C). Furthermore, frequency of Tie2-positive blood vessels was low in E0771 tumors when compared to MMTV-*PyMT* tumors (Supplementary Fig. S8A-B), providing likely explanation for differential tumor growth and vasculature in the presence of *Angpt2*₄₄₃.

E0771^{ANGPT2}, E0771⁴⁴³ and E0771^{DAP} cell lines produced ANGPT2 proteins in similar amounts based on WB analysis and ELISA from concentrated CM (Supplementary Fig. S8C-D) before their transplantation. In contrast to ECs, where ANGPT2 is stored in WPBs, E0771 cells immediately secreted ANGPT2 forms into CM as cell lysates were negative for ANGPT2. In E0771^{ANGPT2}, E0771⁴⁴³ or E0771^{DAP} mice, ANGPT2 was present in serum as measured by human ANGPT2 ELISA (Supplementary Fig. S8E). ANGPT2 and ANGPT2₄₄₃ serum concentrations were lower, possibly due to increased solubility of ANGPT2_{DAP}. Primary tumor blood vessel count and lung metastasis area were increased in E0771⁴⁴³ *Angpt2*^{+/+} mice (Fig. 7B-E). As lung metastasis was more enhanced in mice transplanted with ANGPT2 variant -expressing E0771 cells, this cohort was investigated in more detail. As in B16F10-colonized lungs, ultrastructural analysis showed reduced

cortical actin (Fig. 7F) and length of EC-EC junctions (Fig. 7G), and increased EC degeneration adjacent to lung metastasis (Fig. 7H). The junctional alterations were most prominent in E0771⁴⁴³ *Angpt2*^{+/+} mice, and increased EC degeneration was present in E0771⁴⁴³ *Angpt2*^{+/+} and E0771^{DAP} *Angpt2*^{+/+} mice (Fig. 7H).

DISCUSSION

The major obstacle for development of effective cancer treatments is biological diversity in cancer. Alternative mRNA splicing is a main mechanism to generate protein isoforms (9). Additional confusing factors are those isoforms that occur in human cancer but not in normal development or in the pre-clinical animal models. In this study, we characterized one such alternative isoform, ANGPT2₄₄₃, and investigated its relevance for mammary gland tumors and lung metastasis in mice. Our comparison to full length ANGPT2 revealed that lower oligomeric ANGPT2 protein forms have different effects on TIE2 activation and integrin binding, the two key mechanisms how ANGPT2 regulates vascular remodeling. Metastasis is the major cause of cancer morbidity and mortality, currently providing one of the most important challenges in clinical oncology. Vascular normalization, which can be achieved by Tie2 activation and Angpt2 inhibition (44, 49), offers one possible strategy to generate less malignant tumor microenvironment and reduce metastases. ANGPT2 is identified as a molecular constituent of the metastatic microenvironment, and our study directly indicates the metastatic-promoting role of monomeric and lower oligomeric ANGPT2 protein forms in the lungs.

In mammary gland tumor and lung metastasis models the extent of lung metastasis was associated with differential Tie2 expression in ECs and Angpt2 localization in the stromal ECM. In MMTV-*PyMT* tumor vasculature, Tie2 was expressed relatively frequently in ECs, and in this model Angpt2₄₄₃ negatively affected blood vessel count and number of lumen-forming vessels, leading to reduced primary tumor growth. Reduced tumor vasculature and lower primary tumor burden likely decreased seeding of metastatic cells into the lungs in *PyMT*; *Angpt2*^{+/443} mice. In contrast to MMTV-*PyMT*, in E0771 tumor vasculature Tie2-positive vessels were less frequent, stromal ECM compartment was poorly developed, Angpt2 staining was not detected outside of ECs and no alterations in the tumor vasculature in E0771 *Angpt2*^{+/443} or E0771 *Angpt2*^{443/443} mice were found. Interestingly, when ANGPT2₄₄₃-expressing E0771 cells (E0771⁴⁴³) were transplanted into WT mice (E0771⁴⁴³ *Angpt2*^{+/+} mice) blood vessel count and lung metastasis were increased suggesting a complementary effect of Angpt2 and ANGPT2₄₄₃. A probable contributing factor

resulting in differential phenotypes in E0771⁴⁴³ *Angpt2*^{+/+} and E0771 *Angpt2*^{+/-443} mice is that in E0771⁴⁴³ cells ANGPT2₄₄₃ is constitutively expressed under viral promoter and on top of that homozygous *Angpt2*^{+/+} allele expression results in double the amount of endogenous Angpt2 than in *Angpt2*^{+/-443} mice.

Based on published RNAseq data, Tie2 (in ECs) and Angpt1 (in stromal cells) are constitutively expressed in the matured healthy lungs while Angpt2 (in ECs) levels are very low (45). Without seeding of metastatic cells, *Angpt2*⁴⁴³ mice showed no deleterious effect in lung vasculature likely due to insignificant amount of Angpt2 expression. In the B16F10 model, Angpt2 protein was localized in host vessel ECs around the B16F10 cell colonies and ultrastructural analysis revealed EC destabilization in *Angpt2*^{+/-443} and *Angpt2*^{443/443} mice. Ultrastructural changes suggestive for enhanced EC destabilization were also observed in the pulmonary vasculature in E0771⁴⁴³ *Angpt2*^{+/+} lungs when metastatic cells were present. In E0771 model, ANGPT2_{DAP} did not affect primary tumor vasculature; as a large proportion of blood vessels were Tie2-negative and ANGPT2_{DAP} does not bind to ubiquitously expressed integrins, ANGPT2_{DAP} probably lacked a mechanism to enhance metastatic spread in primary tumors. In the pulmonary venules adjacent to lung metastasis, EC-EC junctional changes were less prominent in E0771^{DAP} than in E0771⁴⁴³ mice that may reflect their differential integrin binding shown to have a role in EC junctional destabilization (23). Although EC-EC junctions were less affected, E0771^{DAP} lungs with metastatic foci showed cellular changes suggestive for EC degeneration that may be caused by blocking of Angpt1/Tie2-induced pro-survival signaling. Collectively, these data indicated that the tumor- and lung metastasis-promoting or suppressing roles of ANGPT2₄₄₃ depend on the tumor microenvironment and that simultaneous expression of both ANGPT2 and ANGPT2₄₄₃ may have complementary effects on Tie2 inhibition and integrin binding. In addition, EC-destabilizing effect in ultrastructural studies was evident in those lungs where metastatic cell colonies were present, suggesting metastasis-promoting rather than metastasis-priming effect for Angpt2₄₄₃.

To investigate the mechanisms underlying differential effects of ANGPT2 forms, their molecular and cellular characteristics were compared in biochemical and EC assays and in mouse retina model of angiogenesis. Collectively these data indicated that the main Tie2-dependent mechanism how Angpt2₄₄₃ mediates its effects is via competitive inhibition of Tie2 activation by stronger agonistic ligands Angpt1 and Angpt4. Tie2-signaling interfering functions on the integrity of lung endothelium by Angpt2₄₄₃ is well in line with the effect of *Angpt1* and *Tie2* deletions resulting in increased colonization of melanoma cells as previously published (50). In addition, manipulations that artificially increase the oligomerization state of native ANGPT2 have been shown to increase TIE2 activation and vascular protection (51). In conditions where TIE2

expression is low (such as in tip cells in vascular sprouts and in some tumor ECs) ANGPT2 can also bind to integrins to promote sprouting and vascular destabilization (22, 23). Our data confirmed earlier results that ANGPT2 binding to $\alpha 5\beta 1$ occurs via C-terminal peptide (52) and further indicated that N-terminal domain-mediated oligomerization increases ANGPT2 valency to $\alpha 5\beta 1$ binding. In the mouse retina model, however, we found no sprouting phenotype suggestive for impaired ANGPT2/integrin signaling implying that affinity of Angpt2₄₄₃ is still sufficient to support physiological angiogenesis in mouse retina.

Based on literature, a change in protein structure and folding may result in an increased susceptibility to proteolytic cleavage (53), which can be caused by alternative splicing (54) and involve coiled-coil domains (55, 56). We propose that lack of exon 2 -encoded part of the coiled-coil domain in ANGPT2₄₄₃ results in a conformational change that may unmask protease recognition sequences and/or remove the protective effect of native protein structure against proteolysis. Outstanding remaining question to completely understand the importance of ANGPT2₄₄₃ in cancer would be the investigation of native ANGPT2 protein forms in malignant tissues. Despite a vast number of different experiments, our analysis failed to reliably show Angpt2 and its fragments in WB from mouse tissue lysates. Based on ELISA, in our experimental settings, Angpt2 concentrations were lower than typical WB detection range. Nevertheless, it is interesting that Angpt2 fragments have been previously identified in mouse tumors (57) and that angiopoietin-like proteins are known to be cleaved by furin *in vivo* (58) in their N-terminuses.

Currently, Trebananib (AMG386) is the most extensively evaluated ANGPT2 inhibitor in clinical cancer trials. In combination with doxorubicin, Trebananib improved the objective response rate (ORR) and duration of response, but not the progression-free survival (PFS) (8). In combination with Paclitaxel, Trebananib improved PSF and ORR (59), however, in combination with Paclitaxel and Carboplatin, Trebananib showed no effect in a phase 3 clinical trial for ovarian cancer (7). As Trebananib prevents the interaction of both ANGPT1 and ANGPT2 with TIE2 (60) it can interfere with ANGPT1-induced blood vessel stabilization and therefore not result in a best possible outcome. As binding to TIE2 occurs via ANGPT2 C-terminal domain (common to all ANGPT2 forms investigated) it is most likely that the pharmaceuticals that prevent ANGPT2 binding to TIE2 do not separate between different ANGPT2 forms. Our finding that less oligomerized Angpt2₄₄₃ promotes lung metastasis and vascular destabilization further justifies the development of novel drugs targeting ANGPT2, such as ABTAA antibody that clusters ANGPT2 for TIE2 activation and more effectively prevents lung metastasis than Angpt2 blocking alone (49).

All in all, we observed marked heterogeneity between the tumor models. Among them, MMTV-*PyMT* best recapitulated the multistep processes of tumorigenesis and metastasis,

relevant stromal microenvironment, Tie2-positive ECs and PC coverage. Common to all primary tumor models, expression of *Angpt2*₄₄₃ did not increase mammary gland tumor growth; and common to all lung metastasis models, *Angpt2*₄₄₃ decreased vascular integrity of endothelial cell-cell junctions adjacent to metastatic cell foci. From a translational perspective, TCGA provided the most comprehensive cancer patient data available which takes into account mRNA splice variants. This included 1081 primary tumor samples, however, number of individuals in advanced/metastatic stage was low (n=7) thus lacking statistical power to reliably compare to our mouse data. Nevertheless, our study provides a rationale for further investigation of *ANGPT2*⁴⁴³ as a risk factor for the progression of metastasis in patients.

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FIGURE LEGENDS

Figure 1. *ANGPT2*⁴⁴³ mRNA expression in human breast cancer tumors and characterization of ANGPT2 protein isoforms. **A**, Relative abundance of *ANGPT2*⁴⁴³ mRNA in normal (n=111) and in primary tumor (n=1081) tissues based on TCGA RNA-seq data (pooled data from stages I-IV). **B**, Cell lysates and conditioned medium (CM) of non-transduced HUVECs and HUVECs retrovirally overexpressing ANGPT2 or ANGPT2₄₄₃ analyzed in reduced Western blot (WB) with ANGPT2 antibody. **C**, WB in non-reducing conditions. Gray arrowhead, disulfide-linked dimers; white arrowhead, monomers; black arrowhead, oligomeric/multimeric forms. **D**, Analysis of the 50 kDa protein derived from ANGPT2₄₄₃ CM. Black boxes indicate N-terminal amino acids identified in mass spectroscopy and arrow the first amino acid in N-terminal peptide sequencing. Bolded, consensus sequence for furin/furin-like protein convertase (...AVQR/DA...). **E**, WB analysis of HUVEC CM in the absence and presence of furin inhibitor, quantification of cleaved (DAP)/non-cleaved (443) forms on the right. Arrowhead, ~50 kDa ANGPT2_{DAP} (n=3 experiments). **F-G**, WB analysis of reduced and non-reduced CM from COS-1 cells transfected with the ANGPT2 forms indicated. White arrowhead, monomers. **H**, Binding affinity of ANGPT2 variants to TIE2 analyzed by ELISA. ANGPT2 binding to TIE2 (250 ng/ml) was set to one at ANGPT2 concentration 500 ng/ml (n=3 experiments). ***P<0.001, *P<0.05 vs. control; †††P<0.001, †P<0.05 vs. ANGPT2; ###P<0.001, ##P<0.01, #P<0.05 vs. ANGPT2₄₄₃. **I-K**, Binding affinities of N- and C-terminal ANGPT2 fragments (I) and full length ANGPT2 isoforms (J) to integrin $\alpha 5\beta 1$ and the effect of reduction of disulfide-linked oligomers by DTT (K) (n=3 experiments). Mean $\pm$ SD.

Figure 2. TIE2 activation and cellular effects of ANGPT2 isoforms. **A**, Left, immunoprecipitation (IP) and WB analysis of TIE2 tyrosine phosphorylation following stimulation with ANGPT1 (A1), full length ANGPT2 (A2_FL) and isoforms ANGPT2₄₄₃ (A2_443) and ANGPT2_{DAP} (A2_DAP). Right, competitive inhibition of ANGPT1/TIE2 phosphorylation by ANGPT2 isoforms. HUVECs were first pre-treated with ANGPT2 forms indicated (60 mins, 500 ng/ml) and then stimulated with ANGPT1 (30 mins, 250 ng/ml) (n=3 experiments). **B**, TIE2 activation in HUVEC cell-cell junctions (n=17 to 26 40x confocal microscopic fields, each consisting of 12 to 26 P-TIE2/TIE2 measurements). Quantification of analysis below (n=3 independent experiments). Mean±SD. **C**, Rescue of *ANGPT2*-silenced HUVECs by supplemental ANGPT2 protein forms. HUVECs stained with TRITC-phalloidin (left), box blot (right) indicates cell numbers in 10x microscopy field relative to negative control (conditioned media, CM) (n=24 microscopy fields analyzed from 3 different shRNA treatments). **D**, HUVEC sprouting in fibrin gel, TRITC-phalloidin staining (left) and quantification of data (right). Data from two experiments, sprouts quantified from 5 to 8 ANGPT2, ANGPT2₄₄₃ and ANGPT2_{DAP} and non-transduced (NT) HUVEC spheroids. ns, statistically not significant. **E**, HUVEC adhesion on substrate-linked angiopoietins, ANGPT1 (A1) and BSA were used as positive and negative controls, respectively. Two independent experiments done in triplicate. **F-G**, COS-1 cells were transduced with ANGPT2-Flag or ANGPT2₄₄₃-His or both and resulting protein forms were analyzed from CM.

Figure 3. Effects of *Angpt2*₄₄₃ isoform on the retinal vasculature. **A**, Retina whole mounts stained with isolectin B4 (IB4) at P6. Vascular front, discontinuous line; front length from the optic nerve head, arrows. **B-C**, Collagen IV (ColIV) stained adult retinas. Veins, arrows; arteries, arrowheads; veins extending retinal periphery, asterisks. **D-F**, Quantification of image data from A-B. n=27/52/13 mice/genotype in D; n=4 mice/genotype, where 12/24/30 veins/genotype were measured in E; n=21/8/18/8 mice/genotype in F (*A4*^{-/-}, *Angpt4*^{-/-} mice). **G**, P8 retinas stained with total Tie2 or phosphorylated Tie2 (pTie2). Arrows mark areas in developing vein positive for pTie2 signal. **H**, Quantification of image data from G. n=11/4/7 mice/genotype, where 22/8/28 veins/genotype were measured. **I**, P12 retinas stained with αSMA. Veins, arrows; arteries, arrowheads. **J**, Quantification of image data from I. n=3 mice/genotype, where 5/3/6 veins/genotype were measured. Mean±SD. **K**, Fluorescent beads were injected into carotid artery, mice were sacrificed after 30 s and bead clearance from retinal veins was quantified from whole mount images. n=3/3/4 mice/genotype, where 10/18/30 veins/genotype were analyzed.

Figure 4. Mammary gland tumor growth and lung metastasis in MMTV-*PyMT* model. **A**, Weight of mammary glands in controls and MMTV-*PyMT* tumors at 13 and 18 weeks of age. Controls without *PyMT*, n=14/10; 13 weeks MMTV-*PyMT*, n=5/7; 18 weeks MMTV-*PyMT*, n=15/9 mice/genotype. Dot, total mammary gland or tumor weight in an individual mouse; bar, mean. **B**, Whole mammary carcinomas stained with Carmine Alum. **C**, Tumor blood vessels stained for endothelial cells (CD31, green) and pericytes/perivascular mesenchymal cells (CD140b, red). Lumens, white circles. Framed areas magnified on right, pericyte (PC)-deficient vessels indicated by arrows. **D-G**, Quantification of image data from C. n=7/8 mice/genotype, 1-2 tumors were analyzed/mouse in D; n=4/5 mice/genotype, 107-490 veins were analyzed/mouse in E-F; n=4/3 mice per genotype, 1-4 tumors were analyzed/mouse in G. **H**, TEM analysis of stromal blood vessels. Framed area magnified on the right; arrowheads indicate PCs. **I**, Quantification of image data from H. n=37/61 stromal blood vessels, data collected from 115/118 TEM micrographs from 3 mice/genotype. **J**, Quantification of tumor cell mass PCs from TEM micrographs. n=46/93 vessels/genotype from 5 mice/genotype. **K**, Evans blue extravasation in *PyMT* mammary tumors. n=8/3 mouse per genotype, 8 tumors/mouse were analyzed. **L-N**, HE-stained MMTV-*PyMT* lungs, metastases marked with black circles (L) and quantified as percentage of total metastasis area from lung area (M) and number of metastasis (N). n=15/9 mice/genotype.

Figure 5. Angpt2 mRNA and protein localization in the MMTV-*PyMT* mammary gland tumors. **A-D**, *In situ* hybridization and immunostaining detects *Angpt2* mRNA and protein in endothelial cells of some tumor vessels (arrowheads; vessels negative for *Angpt2*, arrows). **E**, Masson trichrome staining of MMTV-*PyMT* mammary gland. Stromal ECM stains as light blue surrounded by tumor cells (black line). **F**, Angpt2 (green) staining in the ECM. Stromal ECM is surrounded by white line and DAPI shows tumor cell mass. **G**, Both Angpt2 (green) and mesenchymal cell marker CD140b (red) IF staining is enriched in the stromal compartment. **H**, Angpt2 (red) protein localization in comparison to stromal collagen (SHG, second harmonic generation). **I**, Quantification of image data from H. n= 3/5 mice/genotype, 1-4 ROIs were analyzed/mouse. Mean±SD.

Figure 6. B16F10 mouse melanoma lung colonization and peritumoral blood vessel destabilization. **A-B**, B16F10 lung colonies imaged by OPT (A). Volumes of melanoma cell colonies were quantified (B). n=14/9/5 mice/genotype. **C-D**, TEM micrographs from lung venules adjacent to melanoma cell foci indicating ultrastructural phenotypes analyzed. Framed areas magnified below (EC basal cell membrane, white arrowheads; BM, black arrows; junctional electron-dense cortical actin, opposite arrows; EC degeneration, asterisk). **E-G**, Quantification of TEM image data. n=5/3/5

mice/genotype; total 73/111/76 EC-EC junctions/genotype and 87/281/76 ECs/genotype analyzed. **H**, Excess ANGPT2₄₄₃ promoted CaSki transmigration through EC monolayer (n=4 experiments). ns, statistically not significant; Mean±SD.

Figure 7. E0771 mouse mammary gland adenocarcinoma transplantation. **A**, Weight of mammary gland tumors. Parental E0771 tumor cells were transplanted in four fat pads of *Angpt2*^{+/+} (controls for all groups), *Angpt2*^{+/-} and *Angpt2*^{443/443} host mice (n=12/7/5 mice/genotype; non-transduced, NT). Alternatively, E0771 cells were retrovirally transduced to express ANGPT2 forms indicated (A2, full length ANGPT2) and transplanted in *Angpt2*^{+/+} mice (n=4/4/5 mice/E0771 cell line). Dot, total tumor weight in an individual mouse; bar, mean. **B**, Quantification of endothelial cell content (CD31-positive area) in tumors. n=11/6/5/4/4/5 mice/group, 1-2 tumors were analyzed/mouse. **C**, Representative images of tumors stained with Angpt2 (green) and CD31 (red). Some blood vessels are positive for Angpt2 (arrowheads); blood vessels negative for Angpt2, arrows. **D**, Lung metastases quantified as percentage of metastatic area from total lung section area. n=12/7/5/4/4/5 mice/group. **E**, Representative images of HE-stained lung sections, metastases marked with black line. **F-H**, Quantification of EC junctional cortical actin area, EC-EC junction length and frequency of EC degenerative changes from TEM image data. n=6/4/3/4 mice/genotype; total 204/76/96/126 junctions/genotype analyzed in F and G, 356/133/204/208 cells/genotype in H.

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Figure 5

Figure 6

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The amino-terminal oligomerization domain of Angiopoietin-2 affects vascular remodeling, mammary gland tumor growth, and lung metastasis in mice

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