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Monoclonal antibodies to murine thrombospondin-1 and thrombospondin-2 reveal differential expression patterns in cancer and low antigen expression in normal tissues

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ABSTRACT

There is a considerable interest for the discovery and characterization of tumor-associated antigens, which may facilitate antibody-based pharmacodelivery strategies. Thrombospondin-1 and thrombospondin-2 are homologous secreted proteins, which have previously been reported to be overexpressed during remodeling typical for wound healing and tumor progression and to possibly play a functional role in cell proliferation, migration and apoptosis. To our knowledge, a complete immunohistochemical characterization of thrombospondins levels in normal rodent tissues has not been reported so far. Using antibody phage technology, we have generated and characterized monoclonal antibodies specific to murine thrombospondin-1 and thrombospondin-2, two antigens which share 62% aminoacid identity. An immunofluorescence analysis revealed that both antigens are virtually undetectable in normal mouse tissues, except for a weak staining of heart tissue by antibodies specific to thrombospondin-1. The analysis also showed that thrombospondin-1 was strongly expressed in 5/7 human tumors xenografted in nude mice, while it was only barely detectable in 3/8 murine tumors grafted in immunocompetent mice. By contrast, a high-affinity antibody to thrombospondin-2 revealed a much lower level of expression of this antigen in cancer specimens. Our analysis resolves ambiguities related to conflicting reports on thrombosponding expression in health and disease. Based on our findings, thrombospondin-1 (and not thrombospondin-2) may be considered as a target for antibody-based pharmacodelivery strategies, in consideration of its low expression in normal tissues and its upregulation in cancer.

INTRODUCTION

Thrombospondin-1 and -2 (TSP1 and TSP2) are 450 kDa adhesive extracellular matrix glycoproteins, which mediate cell-to-cell and cell-to-matrix interactions. TSP1 and 2 share the typical homotrimeric structure, with each subunit consisting of globular N- and C-termini connected by a long stalk **[1]**. Analysis of TSP1 and 2 subunits reveals the presence of an N-terminal domain, which belongs to the laminin G superfamily, of an oligomerization domain (with cysteine residues which may stabilize a disulfide-bonded homotrimeric structure), of a von Willebrand type C domain (VWC) followed by three thrombospondin type 1 repeats (TSR), of three EGF-like domains, of seven calcium binding type 3 repeats and of a globular lectin-like C-terminus **[2, 3, 4][Figure 1]**.

The sequence identity between proteins of the TSP family within a species, as well as between species, increases going from the N-terminus to C-terminus, with the signature domain having the highest identity of 53-82% across the entire family **[2, 5]**. The N-terminal part that ends with TSR region at the C-terminus, which was cloned in this report, has a percentage of sequence identity between mouse and human of 92% for TSP1 and 83% for TSP2.

TSP1- and TSP2-knockout mice have been generated and are viable in the case of single as well as double knockouts **[6]**. TSP1-knockout mice have increased vessel density in retina, skeletal and heart muscle, develop pneumonia from 1 month after birth **[7]**, show reduced levels of active TGF-β1 in lung and pancreas **[8]** as well as increased association of vascular endothelial growth factor (VEGF) with its receptor and higher levels of MMP9 **[9]**. In general, TSP2-knockout phenotype is characterized by fragile skin, lax tendons, twofold increase in bone density, increased vascular density as well as elevated levels of MMP2 and 9 **[10, 11]**.

Thrombospondin 1 and 2 have been suggested to play a functional role in cell proliferation, migration and apoptosis as well as in a variety of physiological and pathological settings such as wound healing, inflammation, angiogenesis and tumor progression **[5]**. TSP1 and 2 interact with a wide range of matrix proteins and cell-surface receptors through specific motifs in their multidomain structure, thus forming complexes that exert (sometimes opposing) effects onto the local environment **[5, 12]**. In cancer, TSP1 and 2 regulate angiogenesis by regulating the activity

and bioavailability of VEGF, as well as by induction of endothelial cell apoptosis, inhibition of endothelial cell migration and suppression of nitric oxide signaling **[13, 14]**. Regional tumor growth is conversely proportional to the expression gradient of TSP1 in the local tumor environment, thus demonstrating the antiangiogenic activity of TSP1 **[15]**. Several studies have further indicated the antiangiogenic and antimetastatic activity of TSP1 and 2 **[16, 17, 18, 19, 20, 21]** and expression of TSP1 and 2 has been associated with increased survival in colon **[19]** and bladder cancer **[17]**. Numerous lines of evidence also support the concept of a TSP1-mediated inhibition of tumor progression in melanoma, hemangioma, squamous cell carcinoma and breast cancer cells **[22, 23]**.

During the early stage of tumor development, the TSP1 level in tumor stroma is high enough to prevent angiogenesis and delay tumor growth. This is corroborated by the fact that expression of TSP1 is increased in dormant tumor cell lines **[24, 25]**. However, prolonged exposure of tumor cells to TSP1 creates a hypoxic environment, leading to an increase in VEGF secretion by tumor cells which then overrides the protective effect of TSP1 thus effectively stimulating angiogenesis **[26, 27, 28, 29, 30]**. It is only after these dormant tumor cells had acquired angiogenic phenotype that TSPs' ability to serve as adhesive proteins as well as their ability to modulate extracellular proteases starts to promote tumor invasion **[31, 32]**.

At the protein level, the expression of thrombospondins in health and disease has mainly been investigated in human specimens, with a specific disease focus. For example, Wong et al. showed absence of TSP1 staining in freshly-frozen healthy breast sections, while reporting strong TSP1 staining in the desmoplastic stroma or at the basement membrane associated with the malignant ductal epithelium of the breast, possibly contributing to an invasive phenotype **[33, 34, 35]**. Rice et al. evidenced immunostaining for TSP1 in the majority of clinical specimens of ductal carcinoma in situ (DCIS). However, it seems that the TSP1 expression is lost in DCIS specimens with more aggressive histological features **[36]**. Additionally, the plasma and tumor level of TSP1 shows correlation not only with microvessel density (MVD) but also with lymph node metastases in both early and advanced breast cancer patients **[37]**. By contrast, Ioachim et al. demonstrated that in around 63% of the patient samples examined in the study, breast cancer cells

and stromas were negative for TSP1 expression, which correlated with the increased risk of disease recurrence **[38]**.

Strong stromal expression of TSP1 was observed in 43% of clinical specimens of vertical growth phase melanoma. This finding was significantly associated with predictors of aggressive tumor behavior, such as increased thickness and level of invasion, high proliferative rate, high MVD, tumor ulceration, vascular invasion as well as decreased survival **[39]**. Additionally, TSP2 is strongly expressed in melanoma metastases while being absent in primary tumors, suggesting a prometastatic role of TSP2 in this setting **[40]**.

Yoshida et al. have reported TSP2 overexpression in colorectal cancer specimens, compared to extraneoplastic mucosa, while overexpression of the TSP1 was not found in the same setting **[41]**. However, Miyanaga et al. demonstrated TSP1 expression in tumor stroma of clinical samples of colorectal cancer which correlates with the inhibition of tumor angiogenesis and suppression of tumor growth **[42]**. Sutton et al. in their study of colorectal liver metastases showed TSP1 expression around the blood vessels in 25% and in the stroma in 33% of patients. However, the presence of TSP1 in liver metastases correlated with poor prognosis, while the opposite was true for primary tumors. The antiangiogenic properties of TSP1 may keep the primary tumors in check, while its proangiogenic and proinvasive nature enables metastases to thrive **[43]**. TSP1 levels did not correlate with MVD in ovarian cancer where the immunostaining of cancer tissue was weak **[44]**.

The study of antigen expression is particularly important for the development of antibody-based therapeutic agents. Intense research efforts aim at identifying accessible target proteins which are over-expressed at site of disease, while being undetectable in normal tissues. Such target antigens may be ideally suited for the development of armed antibody therapeutics, such as antibody-drug conjugates **[45]** or antibody-cytokine fusion proteins **[46, 47]**. Indeed, TSP1 and TSP2 over-expression in tumor specimens has emerged from chemical proteomics studies, based on *in vivo* biotinylation procedures, followed by organ homogenization, purification on streptavidin resin, tryptic digestion and mass spectrometry-based peptide quantification **[48, 49]**.

Studies of TSP1 knockout mice revealed a restricted expression pattern in normal adult mouse tissues. While exhibiting a pattern of widespread expression during embryo development, TSP1 expression in adult mouse is restricted to alpha-granules of platelets, activated endothelium, ovary, cornea, lens, healing wounds, skeletal muscles, neointima and atherosclerotic plaque. TSP2, on the other hand, in adult mouse is restricted to only a few places e.g. adrenal cortex, bone marrow and stromal cells **[1]**.

The Protein Atlas Project is the first genome-wide initiative, which provides a comprehensive documentation of patterns of protein expression in human normal tissues and in cancer by means of immunohistochemical analysis **[50]**. The staining of TSP1 is moderate to strong membranous and occasionally cytoplasmic and is evidenced in few normal tissues, mainly secondary lymphoid organs, such as spleen and lymph node, but also smooth and heart muscle, breast, pancreas, gallbladder, duodenum, colon and rectum. Strong cytoplasmic and membranous staining is shown in certain cancer types e.g. colorectal, breast, ovarian, endometrial, pancreatic and testis cancer as well as in lymphoma. However, only one monoclonal antibody has so far been characterized for TSP1 in the Protein Atlas Project, while the reliability of the TSP2 expression findings was classified as “uncertain”.

It is often convenient to study patterns of antigen expression in the mouse, since freshly frozen preparations of healthy organs are readily available and since expression patterns often correlate with findings in humans. For this reason, we generated high affinity monoclonal antibodies specific to murine TSP1 and TSP2 using antibody phage technology **[51, 52]**. We isolated the monoclonal antibodies EB1 and EB2, which recognize the cognate murine TSP1 and TSP2 antigens in ELISA, BIAcore and on frozen tissues. An immunofluorescence analyses of murine cancers as well as human tumor xenografts suggest that thrombospondin-1 may be a better target for antibody-based pharmacodelivery applications.

MATERIALS AND METHODS

Cloning and expression of murine TSP1, TSP2 and human TSP2 fragments

The murine TSP1 and 2 cDNA were obtained from Origene, human TSP2 cDNA was purchased from Thermo Fisher Scientific while human platelet TSP was procured from Athens Research. The N-terminal fragment of TSP1 encompassing laminin G like domain, oligomerization domain, VWC and three TSRs (residues 19-547) was amplified using the forward primer (Operon Eurofins) 5'-TGA TCC TCC TGT TCC TCG TCG CTG TGG CTA CAG GTG TGC ACT CGA ACC GCA TTC CAG AGT CTG GGG GAG-3' and the backward primer 5'-ATA GTT TAG CGG CCG CTT AAT GGT GAT GGT GAT GGT GTG GGC AGT CCT GCT TG-3'. This was followed by another amplification using the forward primer 5'-CCC AAG CTT GTC GAC CAT GGG CTG GAG CCT GAT CCT CCT GTT CCT CGT CGC TGT GGC-3' and the same backward primer as in the previous reaction. This fragment was further inserted into a pCEP4 vector (Invitrogen) using HindIII-NotI restriction enzymes (NEB). In the case of murine TSP2, the fragment (residues 19-549) comprising domains corresponding to those cloned in the case of TSP1 fragment, was amplified using forward primer 5'-CTC CTG TTC CTC GTC GCT GTG GCT ACA GGT GTG CAC TCG GGT GAC CAC GTC AAG GAC ACT TCA TTT G-3' and backward primer 5'-GGT GAT GGT GAG GGC AGC TTC TCT TGT TGC ACA TTT GGT GTT CTG TCA CAT CCC CGA CAC AG-3'. This was followed by another amplification using the forward primer 5'-CGG GGT ACC GTC GAC CAT GGG CTG GAG CCT GAT CCT CCT GTT CCT CGT CGC TGT GGC TAC AGG TGT-3' and the same backward primer as in the previous reaction. This fragment was further inserted into a pCEP4 vector using KpnI-NotI restriction enzymes (NEB). In the case of human TSP2, fragment (residues 19-549) comprising domains corresponding to those cloned in the case of murine TSP2 fragment, was amplified using forward primer 5'-TGA TCC TCC TGT TCC TCG TCG CTG TGG CTA CAG GTG TGC ACT CGG GTC ACC AGG ACA AAG ACA CGA CCT T-3' and backward primer 5'-ATA GTT TAG CGG CCG CTT AAT GGT GAT GGT GAT GGT GGG GGC AGC TCC TCT TGT TGC ACA T-3'. This was followed by another amplification using the forward primer 5'-CCC AAG CTT GTC GAC CAT GGG CTG GAG CCT GAT CCT CCT GTT CCT CGT CGC TGT GGC-3' and the same backward primer as in the previous reaction. This DNA fragment was further inserted into a pCEP4 vector using HindIII-NotI restriction enzymes. The obtained vector was then used to transiently transfect human embryo kidney (HEK 293-EBNA) cells according to the

established protocol **[53]**. Proteins containing 6x His tag at the C-terminus, were purified from the culture supernatant on Day 6 post-transfection, using nickel resin (Qiagen), dialysed against PBS (pH=7.4) and stored at -80°C.

Mass spectrometric analyses of TSP1 and 2

Five micrograms of TSP1 or 2 were diluted with MilliQ water, reduced with TCEP (Sigma) and alkylated with iodoacetamide (Thermo Fisher Scientific). The reaction was quenched with cysteine (Fluka), the samples diluted with trypsin digestion buffer (50 mM Tris-HCl, 1mM CaCl2, pH 8.0) and 0.08 µg sequencing grade-modified porcine trypsin (Promega) was added. Peptides were desalted, purified and concentrated with C18 microcolumns (OMIXs tips, Agilent) according to the manufacturer's guidelines, lyophilized and stored at -20°C.

Mass spectrometric analysis was carried out with the 4800 MALDI TOF/TOF Analyzer (AB Sciex, USA). The resulting spectra were processed and analyzed using the Global Protein Server Workstation version 3.6 (GPS Explorer, Applied Biosystems), which uses internal MASCOT version 2.1 (Matrix Sciences, London, UK). The MS/MS data were searched against a database of TSP1 and 2 expression constructs and all human proteins downloaded from the UniProt website. Peptides were considered correct calls when the confidence interval was greater than 95%.

Screening of phage display libraries and selection of antibodies

The recombinant TSP fragments were biotinylated with EZ-link Sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's instructions. The level of biotinylation was estimated by mass sprectrometry. Biotinylated TSP1 fragment was immobilized on Nunc MaxiSorp (Thermo Fisher Scientific) wells at a final concentration of 40 µg/mL for the first two rounds of panning, while the final concentration was 20 µg/mL in the third round. The selection was performed using ETH2-GOLD library according to the established protocol **[54]**.

Fifteen micrograms of the biotinylated TSP2 fragment was captured by 100 mg of streptavidin-coated magnetic beads (Dynabeads, Invitrogen), followed by blocking with 5% milk PBS solution containing 0.1% Tween for 1 hour, at room temperature. After two washing steps, ETH2-GOLD library was added to the TSP2-coated-beads and incubated for 3 hours at room temperature under overhead rotation. Unbound phage particle were washed away with 5 washing steps with PBS-0.1% Tween followed by 2 washings with PBS. Elution of phage particles was performed using 100mM solution of triethylamine. Consequent TG1 E.coli infection and phage

amplification was performed according to the established protocol **[55]**. Bacterial supernatants harboring soluble scFvs, were screened by ELISA and positive clones were further assessed for their ability to bind the immobilized antigen on a high-coating-density chip using surface plasmon resonance (SPR) on the BIAcore3000 instrument (GE Healthcare).

Construction and screening of affinity maturation libraries

Affinity maturation libraries were constructed by introducing sequence variability into CDR1 regions of both heavy and light chain. This randomization of positions 31, 32, 33 in the heavy chain and positions 31, 31a, 32 in the light chain was performed by PCR using partially degenerated primers as described elsewhere **[55]**. This procedure yielded the anti-TSP1 3G3 affinity maturation library of 260 million clones while the affinity maturation library of anti-TSP2-2E4 contained 600 million clones. One round of biopanning was performed with these libraries according to the protocol described above.

Characterisation of scFv antibody fragments

Single chain variable fragments (scFv) were expressed in TG1 E.Coli and purified by affinity chromatography from the culture supernatant using Protein A Sepharose (Sino Biological) as described previously **[51]**. Purified scFvs were characterized by SDS-PAGE and size exclusion chromatography (SEC) on S75 Superdex column (GE Healthcare). Monomeric fraction from the SEC analysis were isolated and used for affinity measurement using BIAcore3000 instrument as described elsewhere **[56]**.

Sequencing of scFv genes

The DNA was amplified by PCR using primers 5'—CAG GAA ACA GCT ATG ACC ATG ATT AC—3' and 5'—GAC GTT AGT AAA TGA ATT TTC TGT ATG AGG—3' (Sigma). Sequencing was performed by GATC Biotech (Germany) according to the standard Sanger method.

Cloning and expression of small immune proteins (SIP)

Single chain variable fragments obtained from library screening were reformatted into a SIP format employing a previously described procedure **[57, 58]**. In brief, the scFv sequence was fused in frame with the sequence of the CH4 domain of the human IgE secretory isoform IgE-S2

using overlap extension PCR. The DNA fragment was inserted into pcDNA3.1 vector using HindIII-NotI restriction enzymes. The obtained plasmid was used to transiently transfect Chinese hamster ovary cells (CHO) as described elsewhere **[59]**. On Day 6 post-transfection SIPs were purified from the culture supernatant by affinity chromatography using Protein A Sepharose.

Immunofluorescence on frozen tissue sections

Healthy tissues were excised from C57BL/6 mice while human tumor xenografts were grown subcutaneously and excised from BALB/c nude mice. Murine tumors were grown and excised from appropriate mouse strains. K1735 was grown in C3H mice, Cloudman S91 in DBA/2J, F9 in Sv129, Renca, WEHI164 and CT26 in BALB/c while B16 and C1498 were grown in C57BL/6. Tumors were embedded in freezing medium (Microm) and stored at -80 °C until sectioned. Tissue sections (10 µm) were fixed for 10 min with ice-cold acetone, rehydrated with PBS and blocked with 20% FCS in PBS. Purified anti-TSP SIPs EB1 and EB2 were applied to slices at a concentration of 2 µg/mL in 3% bovine serum albumin (BSA). The CH4 domain of SIPS was detected with rabbit anti-human-IgE IgG (Dako), which was then detected by Alexa Fluor 594 goat anti-rabbit IgG antibody (Molecular Probes). Blood vessels were detected with mouse anti-CD31 antibody (Invitrogen) followed by donkey Alexa Fluor 488 anti-mouse IgG antibody (Molecular Probes). Nuclei were counterstained with DAPI (Invitrogen). All commercial binding reagents were diluted according to the manufacturer's recommendation in 3% BSA solution. Rinsing with PBS was performed in between all incubation steps. Slides were mounted with Fluorescent mounting medium (Dako) and analyzed with a Zeiss AxioVision 4.7 image analysis software (Carl Zeiss AG).

Flow-cytometric analysis of murine platelets

Flow-cytometric analysis of murine platelets was performed using FITC-labeled SIP fragments (EB1, EB2 and KSF) as described previously [60]. In brief, blood was obtained by cardiac puncture of DBA/1J mouse (Janvier) using sodium-citrate as anticoagulant, and was fixed immediately in 4% paraformaldehyde (4°C, 1:10 dilution) for 15 minutes. In order to detect intercellular thrombospondin, 100 µL of fixed whole blood was incubated for 10 minutes with 400 µL of precooled methanol (-20°C). Following two rounds of washing with PBS and centrifugation for 15 minutes at 1200xg, 5 µL of whole blood was incubated for 20 minutes with 2 µg/mL of FITC-labeled SIP fragments (EB1, EB2 and KSF) in 2% BSA solution. As a platelet-

specific positive control, anti-CD41 coupled to PE (BD Pharmingen) was used, and only CD41-positive cells were gated for the analysis.

RESULTS

Antigen expression and characterization

The N-terminal part of both TSP1 and 2, which comprises laminin G-like domain, oligomerization domain, VWC and ends with three TSRs at the C terminus, was cloned and expressed in mammalian cells [**Figure 1a**]. The resulting TSP fragments were purified on immobilized nickel resins, yielding protein preparations which were pure according to SDS-PAGE and gel filtration [**Figure 1b-d**], but featured a truncation at the N-terminus of a 25 kDa in the case of TSP1 and 20 kDa in the case of TSP2. Mass spectrometry analyses showed that the first N-terminal tryptic peptide detected starts at the position 199 in the case of TSP1 and 308 in the case of TSP2 [**Supplementary Table 1**]. The two protein preparations were biotinylated and used as antigens for antibody selections from phage display libraries.

Antibody isolation and characterization

Monoclonal antibodies specific to TSP1 and TSP2, in single-chain Fv variable (scFv) format **[61]** were isolated from the ETH2-Gold library, a phage display library containing 3 billion human antibody clones **[51]**. After three rounds of panning, antibodies 2E4 and 3G3 were selected for further analysis, because of favorable binding characteristics in BIAcore analysis [**Supplementary Table 2**]. The two antibodies were improved by the construction of affinity-maturation libraries containing 260 and 600 million clones, respectively, based on the combinatorial mutagenesis of residues in the CDR1 regions of variable heavy and light chain, followed by stringent selection. These procedures allowed the isolation of monoclonal antibodies EB1 (specific to TSP1) and EB2 (specific to TSP2), which were used for all subsequent studies [**Supplementary Table 2**]. The two antibodies were expressed in scFv and in SIP format **[57]** and the corresponding protein preparations were characterized by BIAcore analysis using microsensor chips coated with the cognate murine antigen [**Figure 2**] as well as human antigen [**Figure 3**]. Apparent kinetic constants are reported [**Supplementary Table 3**]. Binding studies performed with TSP1 recombinant fragments revealed that EB1 binds to the TSR 2 and 3 domains (data not shown).

Immunofluorescence findings

Figure 4 shows two color immunofluorescence findings with the EB1 or EB2 antibodies (red) and with an anti-CD31 antibody (green), which recognizes vascular structures. Only a faint staining was observed with TSP1 in heart tissue, while the antigen was virtually undetectable in all other normal tissues, including spleen (which has previously been reported to be strongly positive for this antigen in normal human specimens **[50]**).

Freshly frozen tumors, obtained by subcutaneous grafting of seven human cancer cell lines in nude mice or eight murine cell lines in immunocompetent mice, were also stained using the EB1, EB2 and anti-CD31 antibodies [**Figure 5**]. Immunofluorescence microscopic analysis revealed that 5/7 human tumors (MDA-MB-231, SK-RC-52, SKOV3, H460 and SKMEL28) were strongly stained by the EB1 antibody. By contrast, only a weak staining was observed for the EB2 antibody in SK-RC-52, H460 and U87. An immunofluorescence analysis of tumor sections obtained from murine cell lines grafted in immunocompetent mice revealed only weak staining for TSP1 in 3/8 cases (K1735, Renca and Cloudman). Additionally, the EB1 antibody showed strong binding to murine platelets as determined by FACS [**Supplementary Figure 1**]. The EB2 antibody showed TSP2 expression in only 1/8 case (K1735) of murine cancers. As positive control, the F8 antibody was used, which is specific to the alternatively-spliced EDA domain of fibronectin **[55]**. Several immunohistochemical studies had previously reported the ability of F8 to stain the majority of human and murine malignancies **[62, 63]**. We found EDA expression in 7/7 human tumors and in 7/8 murine tumors [**Figure 5**].

DISCUSSION

In this study, monoclonal antibodies specific to murine TSP1 and TSP2 were generated using antibody phage technology and affinity-maturation procedures. These reagents were used to characterize antigen expression in freshly-frozen normal murine tissues and in tumor specimens. Staining of murine healthy organs showed almost complete absence of both TSP1 and 2 in these samples, with an exception of faint staining of the heart for TSP1. An immunofluorescence analysis of tumor sections obtained from human tumor cell lines xenografted in nude mice revealed a strong staining for TSP1 in 5/7 cases, while the staining for TSP2 was only observed in

3/7. By contrast, tumor sections obtained from murine tumor cell lines grafted in immunocompetent mice revealed a much weaker expression of TSP1 in 3/8 cases, while the expression of TSP2 was even scarcer with only 1 positive tissue.

Expression patterns previously reported in the literature for TSP1 and TSP2 in adult murine organs originate from immunohistochemical studies as well as from Northern blotting. Immunostaining for TSP1 in healthy murine organs has been reported in lung, liver, bone and brain while no staining was detected in the heart **[25, 64, 65, 66]**. Literature reports of immunostaining in healthy murine organs for TSP2 are ambiguous regarding the expression in lung and heart. One report reveals the expression of TSP2 in brain, kidney, heart and lung **[67]**, while another report did not detect any staining for TSP2 in heart and lung **[65]**. However, majority of these immunohistochemical investigations relied on polyclonal antibodies. Our immunofluorescence study of healthy murine organs revealed absence of TSP1 and 2 from all tested tissues (heart, spleen, lung, intestine, liver, pancreas, brain and kidney) with the exception of weak staining of heart for TSP1. Northern blot analysis of adult mouse tissues had previously reported TSP1 and 2 expression in kidney, lung and heart, but not in brain and liver **[68]**.

Tumor xenograft specimens stained with anti-TSP1 and -TSP2 antibodies in general revealed abundant depositions of TSP1 in SK-RC-52, MDA-MB-231, SKOV3, H460 and SKMEL-28 cancers. A highly diffuse TSP1 expression characterized by a strong vascular and stromal positivity that was observed in MDA-MB231 tumor outmatches the TSP1 expression level reported in the Protein Atlas as well as the expression of EDA of fibronectin in the same tissue. SK-RC-52, H460 and SKOV3 are characterized by abundant deposition of EB1-accessible TSP1 antigen in tumor vessels as well as in the stroma, which in the case of SKOV3 and SK-RC-52 exceeded the expression of EDA of fibronectin. By contrast, Protein Atlas describes only weak immunostaining for TSP1 in 3/12 patients in renal cancer patient samples. Human melanoma xenograft, SKMEL-28, showed a highly diffuse TSP1 deposition which replicated that observed in the patient samples, as reported by the Protein Atlas. Using freshly-frozen tumor specimens and monoclonal antibodies produced in our laboratory, we have previously reported discrepancies in the Protein Atlas also for other antigens (e.g., MMP1, MMP2 and MMP3) **[69]**. It may thus be important to use additional antibodies for the documentation of antigen expression in the Protein Atlas Project.

Extracellular matrix components can be attractive targets for antibody-based pharmacodelivery strategies **[70, 71]**. However, to the best of our knowledge, there are no literature reports describing systematically the expression patterns of TSP1 or 2 in murine cancers, which are frequently used to test antibody therapeutics. It had previously been reported that CT26 and Renca cell lines do not secrete TSP1 into their culture medium **[72]**. In our study however, we found weak staining of Renca tumors for TSP1 while the staining for TSP2 was not detected. The findings with CT26 tumor match those reported in the literature, as we could not detect any staining for neither TSP1 nor TSP2. It is at the moment unclear why the expression of TSP1 and 2 in murine cancers is weaker than the one observed in human xenograft.

Conflicting results on the localization of TSP may depend on the precise epitope which is recognized by different antibodies as thrombospondins are known to exist in tissues as intact protein and as proteolytic fragments **[73, 74, 75]**. Also, some studies have suggested that TSP1 promotes angiogenesis and tumor progression, depending on which TSP1 domains are present in the tumor environment **[76, 77, 78]**. In addition, thrombospondins co-exist both in the intracellular and extracellular space as recent studies indicated their intracellular localization and activity **[79]**.

In this study, we demonstrated strong staining for TSP1 and a weak TSP2 expression in MDA-MB-231 tumor xenografts, while previous reports had shown low levels of TSP1 protein and mRNA in the same model **[80, 81]**. We found strong expression of TSP1 in SK-RC-52 renal cell carcinomas (RCC), in agreement with previous reports of elevated TSP1 levels in RCC patient tumor interstitial fluid serum, compared to non-cancer kidney tissue **[82]**. Previous reports of TSP1 expression in SKOV3 cells coincide with our observation of strong TSP1 staining in this tumor **[83]**. The pattern of strong TSP1 and weak TSP2 staining in H460 human lung carcinoma corresponds to previously published gene expression patterns in NSCLC patient samples **[84]**. Similarly, an intense TSP1 staining was observed in SKMEL-28 melanomas, while TSP2 expression was substantially weaker. It has been previously been reported that TSP2 is strongly expressed in human metastatic melanoma, while there are no reports regarding the expression of TSP1 in the same setting **[40]**. Hsu et al. had indicated no detectable TSP1 levels in U87 human glioblastoma cells, while Aghi et al. had reported a strong TSP1 expression and a much stronger

expression of TSP2 in the same model **[85, 86]**. We did not detect any TSP1 immunostaining in U87 human glioblastoma tumor xenografts, while a weak expression of TSP2 could be observed.

We used the clinical-stage F8 antibody as positive control in our immunofluorescence studies, as this reagent has previously been reported to strongly react with the majority of murine and human tumors tested **[55, 62, 63, 87, 88]**. The F8 antibody is specific to the alternatively-spliced extra domain-A of fibronectin, an extracellular matrix component which is virtually undetectable in normal adult tissues (exception made for placenta, uterus and some vessels in the ovaries; **[89]**). In keeping with previous studies, we found EDA expression in 7/7 human and 7/8 murine tumors. In all murine tumors that were positive for EDA, staining intensity was stronger than the one observed for TSP1 and 2. Furthermore, in the majority of human tumors EDA expression was stronger than the expression of both TSP1 and 2. However, EB1 staining intensity in MDA-MB-231, SK-RC-52 and SKOV3 was stronger than the one observed for the F8 antibody, suggesting that anti-TSP1 antibodies may be considered for pharmacodelivery applications in those indications, for which immunohistochemical studies reveal a strong antigen expression (e.g., melanoma **[39]**, esophageal squamous cell carcinoma **[90],** breast **[37]** and colorectal cancer **[42]**).

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FIGURE CAPTIONS

Figure 1: **A.** Schematic representation of the thrombospondin monomer which consists of N-terminal laminin G like domain (1), followed by oligomerization domain (2), VWC domain (3) and three TSR domains (4) that are connecting the N-terminus with the signature domain. The signature domain comprises three EGF-like domains (5), followed by seven calcium binding type 3 repeats (6), and ending with a globular lectin-like C-terminus (7). **B.** SDS-PAGE profile of recombinant murine TSP1 and 2 fragments. M-marker; 1-TSP1 under non-reducing conditions; 2-TSP1 under reducing conditions; 3-TSP2 under non-reducing conditions; 4-TSP2 under reducing conditions. **C. - D.** Size exclusion chromatography of the expressed thrombospondin fragments on Superdex S75 column.

Figure 2: BIAcore profiles of anti-thrombospondin scFv antibodies (left) and SIP antibodies (right) against murine recombinant TSP2 fragment (EB2), as well as against both murine recombinant TSP1 and 2 (EB1).

Figure 3: BIAcore profiles of anti-thrombospondin SIP antibodies against human recombinant TSP2 fragment (EB2), as well as against human recombinant TSP2 and platelet derived human TSP1 (EB1).

Figure 4: Immunofluorescence staining of murine healthy tissues using SIP (KSF), SIP (EB1), SIP (EB2), SIP (F8) and anti-CD31 antibody. SIP antibodies are shown in red as they were detected using anti-human-IgE IgG antibody, which was further detected by Alexa Fluor 594 goat anti-rabbit IgG antibody. Vessels are shown in green, as the anti-CD31 antibody was detected by donkey Alexa Fluor 488 anti-mouse IgG antibody.

Figure 5: Immunofluorescence staining of **(A)** tumors xenografted in nude mice and **(B)** murine tumors grafted in immunocompetent mice using SIP (KSF), SIP (EB1), SIP (EB2), SIP (F8) and anti-CD31 antibody. SIP antibodies are shown in red as they were detected using anti-human-IgE IgG antibody, which was then detected by Alexa Fluor 594 goat anti-rabbit IgG antibody. Vessels are shown in green, as the anti-CD31 antibody was detected by donkey Alexa Fluor 488 anti-mouse IgG antibody.

Supplementary Table 1: List of peptides obtained by tryptic digestion of TSP1 and 2 fragments.

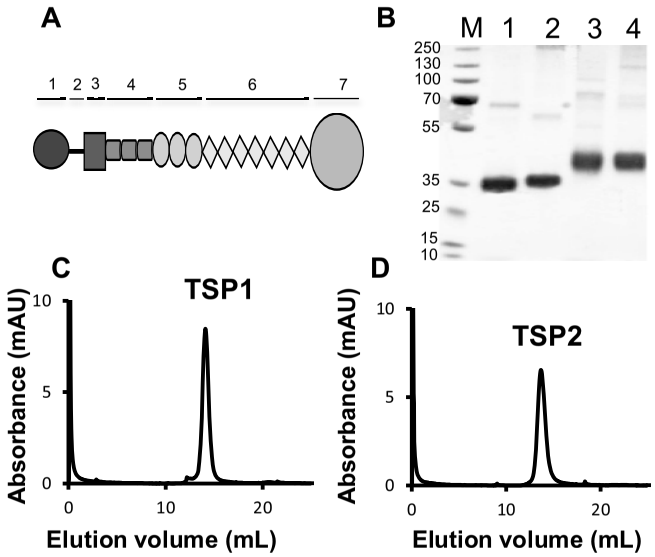
Supplementary Table 2: Aminoacid sequences of 2E4, 3G3, EB1 and EB2 scFvs.

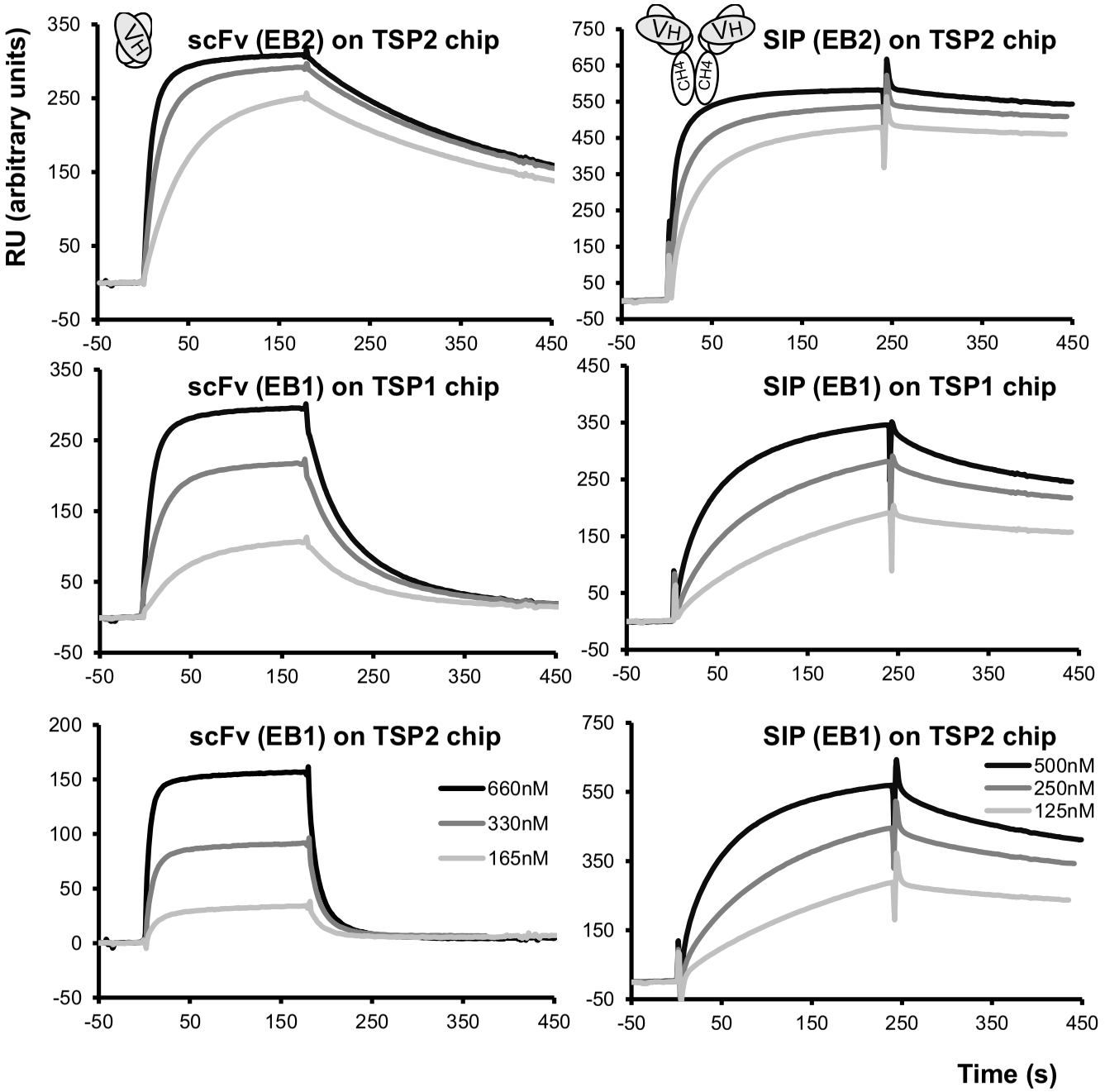
Supplementary Figure 1: Flow-cytometry of murine platelets after membrane permeabilization with methanol using FITC-labeled KSF-SIP (shaded), EB2-SIP (dotted line) and EB1-SIP (full line).

Supplementary Table 3: Apparent dissociation constant (Kd), apparent kinetic association (Kon) and apparent kinetic dissociation (Koff) constants for EB1- and EB2-SIP at the concentration of 125 nM as determined by BIAcore analysis on a chip coated with murine recombinant TSP1, human platelet TSP1 as well as murine and human recombinant TSP2.

High affinity monoclonal antibodies to murine and human TSP1 and 2 were raised
Both antigens are virtually undetectable in normal mouse tissues
Strong positivity of human tumor xenografts for TSP1 was detected
Study revealed much lower level of TSP2 expression in cancer specimens
TSP1 (and not TSP2) may be considered as a target for antibody-based pharmacodelivery

Figure 1 and 2





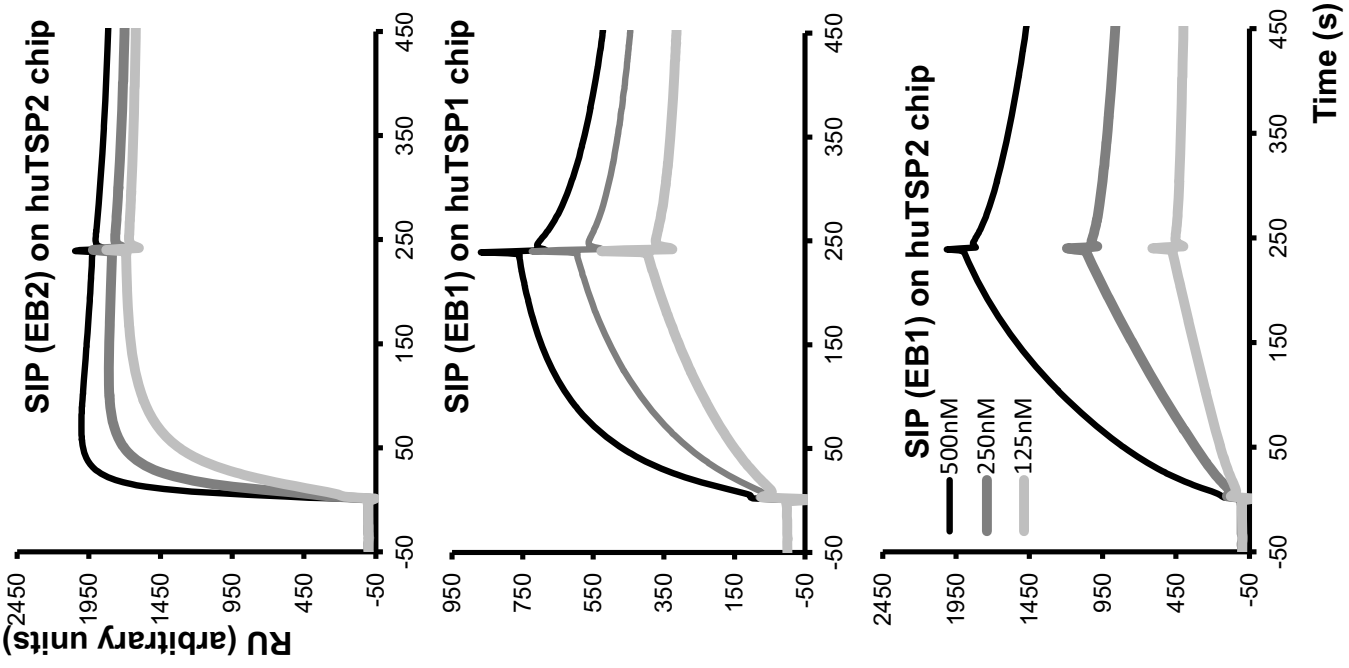


Figure 3

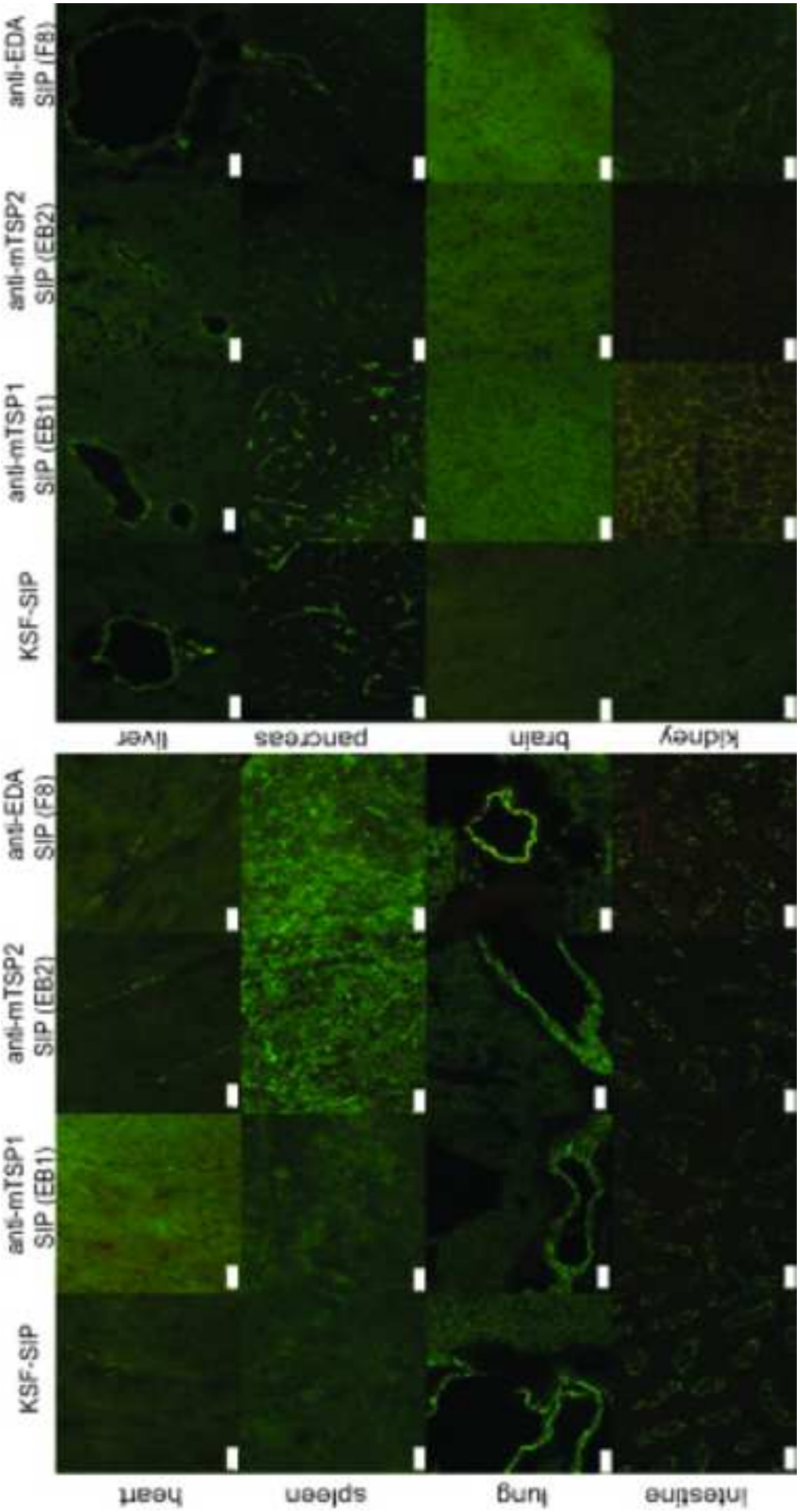

anti-EDA SIP (F8)
anti-mTSP2 SIP (EB2)
anti-mTSP1 SIP (EB1)
KSF-SIP
kidney
brain
pancreas
liver
anti-EDA SIP (F8)
anti-mTSP2 SIP (EB2)
anti-mTSP1 SIP (EB1)
KSF-SIP
intestine
lung
spleen
heart

Figure 4

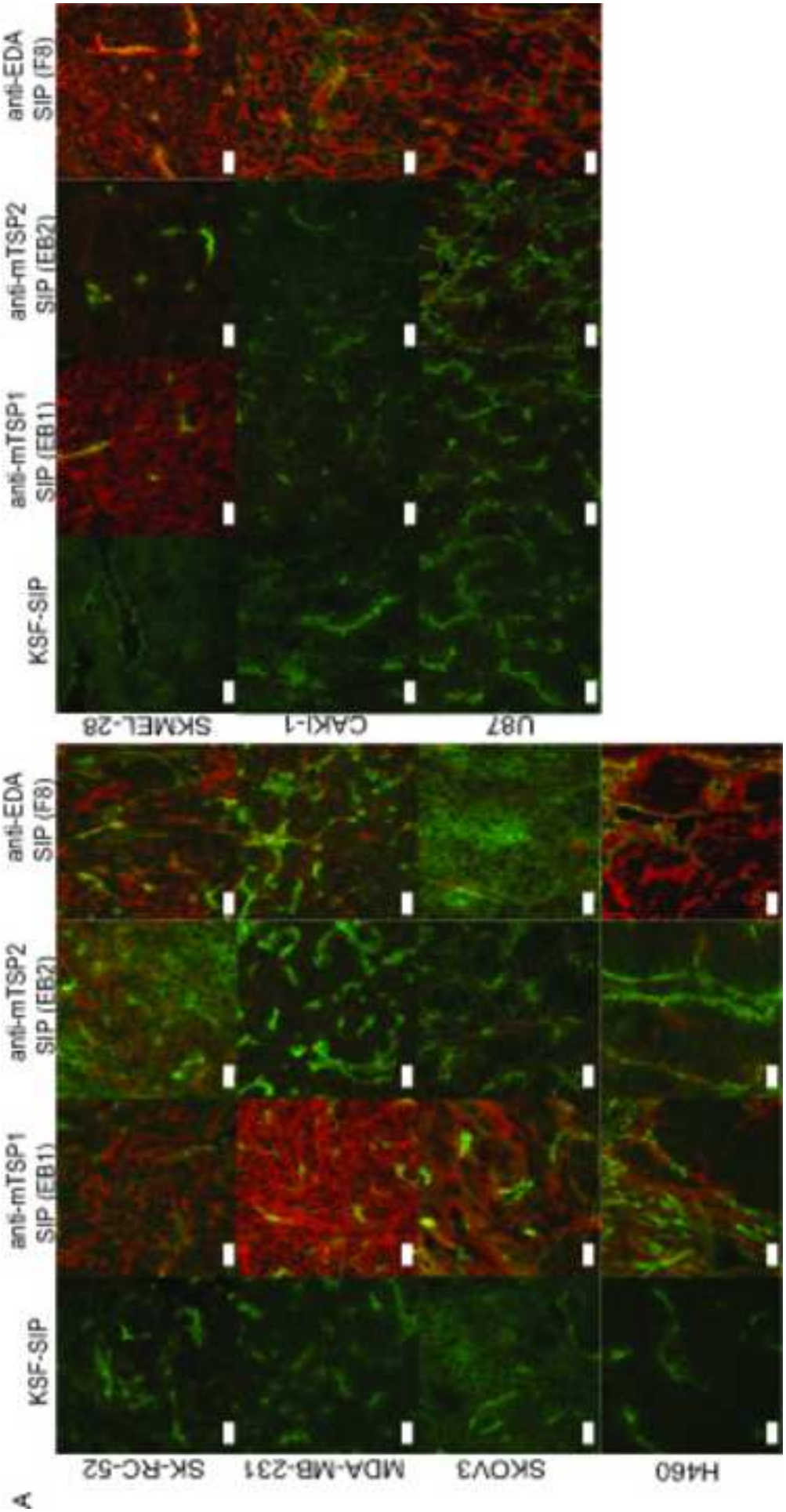


Figure 5a

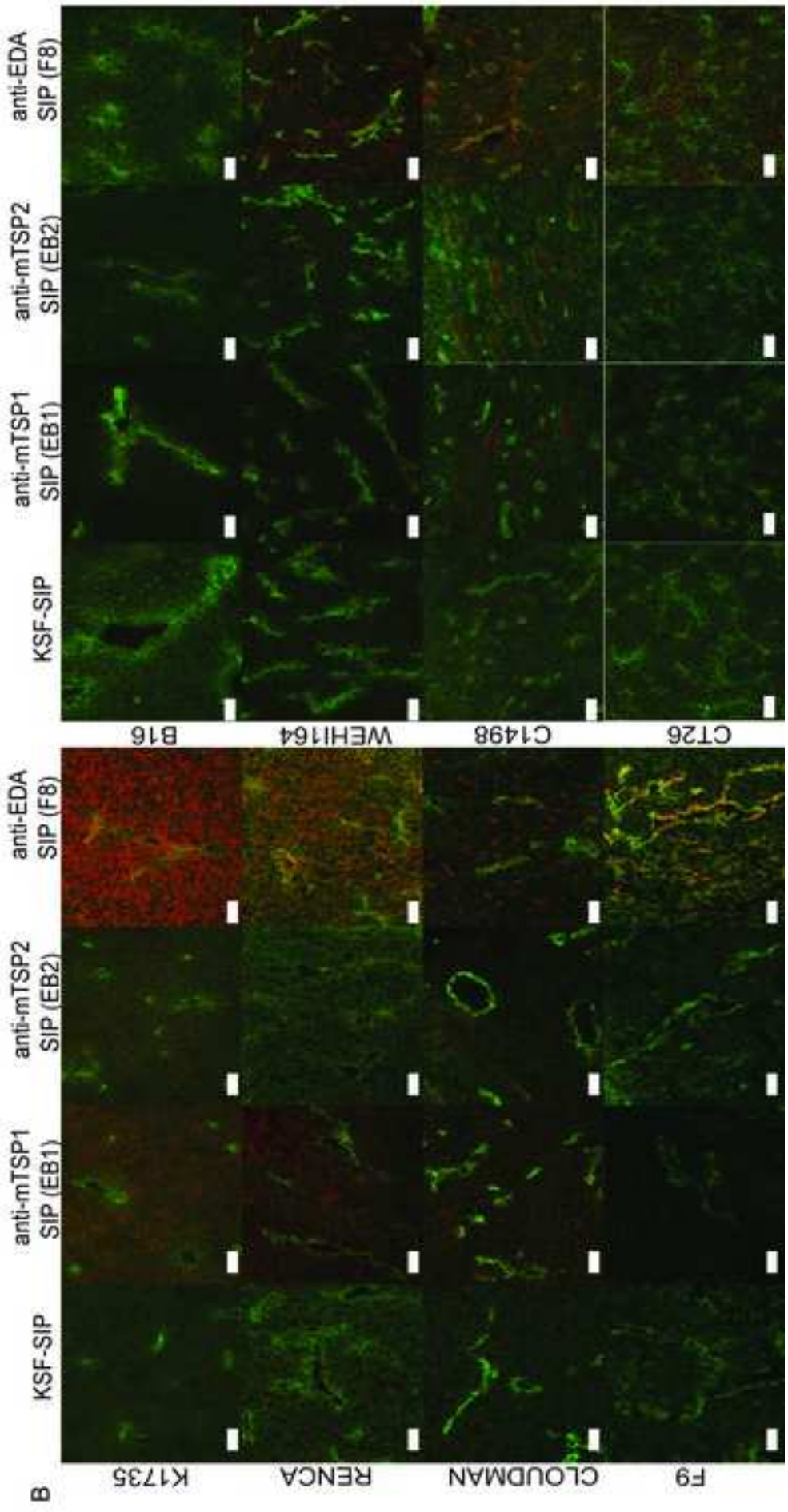


Figure 5b