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Differential vascular expression and regulation of oncofetal tenascin-C and fibronectin variants in renal cell carcinoma (RCC): implications for an individualized angiogenesis-related targeted drug delivery

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Abstract The study was aimed at determining the vascular expression of oncofetal fibronectin (oncfFn) and tenascin-C (oncfTn-C) isoforms in renal cell carcinoma (RCC) and its metastases which are well-known targets for antibody-based pharmacodelivery. Furthermore, the influence of tumour cells on endothelial mRNA expression of these molecules was investigated. Evaluation of vascular ED-A$^+$ and ED-B$^+$ Fn as well as A1$^+$ and C$^+$ Tn-C was performed after immunofluorescence double and triple staining using human recombinant antibodies on clear cell, papillary and chromophobe primary RCC and metastases. The influence of hypoxic RCC-conditioned medium on oncfFn and oncfTn-C mRNA expression was examined in human umbilical vein endothelial cells (HUVEC) by real time RT-PCR. There are RCC subtype specific expression profiles of vascular oncfFn and oncfTn-C and corresponding patterns when comparing primary tumours and metastases. Within one tumour, there are different vessel populations with regard to the incorporation of oncfTn-C and oncfFn into the vessel wall. In vitro tumour-derived soluble mediators induce an up regulation of oncfTn-C and oncfFn mRNA in HUVEC which can be blocked by Avastin®. Vascular expression of oncFn and oncTn-C variants depends on RCC subtype and may reflect an individual tumour stroma interaction or different stages of vessel development. Therefore, oncFn or oncTn-C variants can be suggested as molecular targets for individualized antibody based therapy strategies in RCC. Tumour-derived VEGF could be shown to regulate target expression.

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Introduction

Angiogenesis is a crucial step in tumour progression. In tumour vessels, the perivascular extracellular matrix (ECM) contains oncofetal isoforms of tenascin-C (oncfTn-C) and fibronectin (oncfFn) not detectable in quiescent

endothelium (Neri and Bicknell 2005; Eble and Niland 2009). Isoforms of Fn and Tn-C are generated by alternative splicing (Schwarzbauer 1991; Orend and Chiquet-Ehrismann 2006). Large variants containing up to nine extra domains in case of Tn-C or the extra domains A and/or B in case of Fn are expressed during embryogenesis and tissue reorganization processes like wound healing and tumour growth (therefore named as "oncofetal"). The restricted appearance in adults makes them promising structures for antibody-based targeted pharmacodelivery in tumour therapy (Schliemann and Neri 2010). To exploit the oncofFn and oncfTn-C variants in targeted drug delivery, knowledge on their expression pattern in tumours is of substantial clinical interest. Differences in their occurrence and vascular distribution in various tumours and tumour subtypes have been shown recently (Berndt et al. 2010). Humanised small immunoprotein (SIP) format antibodies against several oncofFn and oncfTn-C splice variants already exist. The antibody L19, targeting the extra domain B of fibronectin (ED-B$^+$ Fn), is already subjected to clinical evaluation as radio or immunoconjugate (Carnemolla et al. 2002; Santimaria et al. 2003; Neri and Bicknell 2005; Johannsen et al. 2010). The SIP format antibody F8, targeting the fibronectin extra domain A (ED-A$^+$ Fn), could be shown to accumulate in tumour vessels, too (Villa et al. 2008). The existence of large Tn-C isoforms in a lot of tumours has been known for nearly two decades (Borsi et al. 1992; Hindermann et al. 1999; Berndt et al. 2006). Their preferential vascular expression could be shown more recently (Brack et al. 2006). A recombinant antibody recognizing the splicing domain A1 of Tn-C (F16) conjugated to IL2 in combination with chemotherapeutic agents led to tumour growth reduction in a breast cancer xenotransplant model (Mårlind et al. 2008) and to size reduction in a glioblastoma xenotransplant model (Pedretti et al. 2010). The antibody is currently evaluated in clinical phase Ib trials in combination with doxorubicin or paclitaxel. Furthermore, an antibody against the splicing domain C of Tn-C (G11) binds selectively in tumour vessels and atherosclerotic plaques (Berndt et al. 2006; Silacci et al. 2006; von Lukowicz et al. 2007; Berndt et al. 2010).

Because of the obvious occurrence of oncofFn and oncfTn-C variants in association to vascular structures in renal cell carcinoma (RCC), this tumour entity is a good candidate for targeted pharmacodelivery using the mentioned SIP antibodies (Berndt et al. 2010). Promising results in clinical phase I trials suggests the activity of L19-IL2 in patients with mRCC (Johannsen et al. 2010). Although crucial for planning individual treatment strategies, there is no information on the differential expression and histological distribution of the vascular antigens recognized by the above mentioned antibodies in RCC subtypes and metastases. Therefore, we examined the blood vessel associated expression of A1$^+$ and C$^+$ Tn-C as well as of ED-A$^+$- and ED-B$^+$ Fn isoforms in clear cell, papillary and chromophobe RCC. Furthermore, we compared their distribution pattern in clear cell RCC primaries and metastases in human and in RCC orthotopic xenograft models. To analyse the impact of tumour-derived factors on vascular expression of oncofetal Fn and Tn-C, the mRNA expression of the Tn-C A1 domain and of the Fn EDA and EDB domains was assessed in human umbilical vein endothelial cells (HUVEC) after stimulation with conditioned medium of different RCC cell lines prepared under hypoxic conditions.

Materials and methods

Tissue material and cells

Frozen tissue samples of 9 clear cell RCC (ccRCC), 10 papillary RCC (pRCC), and 9 chromophobe RCC (chRCC) as well as 9 pairs of RCC primary tumours and metastases (8× ccRCC, 1× pRCC) (from bone, lung and liver) were available for analyses. Diagnosis was performed on the corresponding paraffin-embedded tissue according to the WHO classification criteria of 2004. Ethical approval was obtained from the Local Research Ethic Committee of the Friedrich Schiller University, Jena.

Two pairs of human RCC xenografts and synchronous metastases were obtained by orthotopically implanting 1×10^6 RXF393 or SN12K1 cells in the kidney of 6- to 8-week-old female NCr-nu/nu mice (Harlan Correzzana, Italy) as described (Naito et al. 1986; Frey et al. 2010). Kidney bearing tumour and lung nodules were snap frozen and stored at −80°C. Animal experiments were conducted in conformity with institutional guidelines that are in compliance with national and international laws and policies.

The RCC cell lines A498 and Caki1 were used for the generation of conditioned media. The VHL mutation status was assessed by sequencing all coding exons using primers and method as described previously (Tong et al. 2006). For detection of larger deletions, a MLPA analysis using the P016B kit from MRC Holland (MRC-Holland, Amsterdam, The Netherlands) was done.

Primary human umbilical vein endothelial cells (HUVEC) were isolated as recently described (Heller et al. 2001). Experiments were performed according to the Declaration of Helsinki and were approved by the local research ethics committee. Cells were detached from umbilical veins with 0.01% collagenase and grown in M199 (Biochrom AG, Berlin, Germany) containing 17.5% FCS, 2.5% human serum, 680 μM L-glutamin, 25 μg/ml Heparin, 7.5 μg/ml endothelial mitogen (Hycultec GmbH,

Beutelsbach, Germany) and 100 μM Vitamin C. For experiments, cells of the first and second passage were used.

Immunofluorescence

To visualise oncfFn and oncfTn-C variants, we used recombinant human biotinylated SIP-format antibodies: F8 recognising ED-A$^+$ Fn (mouse and human), L19 targeting ED-B$^+$ Fn (mouse and human), F16 against the A1-domain (human) and G11 recognising the C-domain (mouse and human) of Tn-C. They were detected applying the strep-tavidin and tyramide-Alexa488-based TSA Kit (Invitrogen GmbH, Darmstadt, Germany). Association of the matrix proteins to vascular structures in human tumours and xenografts was analysed by co-staining of the blood vessel specific antigen CD31 as recently described (Berndt et al. 2010).

Fig. 1 Immunofluorescence detection of oncofetal fibronectin and tenascin-C variants in clear cell RCC (**a–d**), papillary RCC (**e–h**), and chromophobe RCC (**i–l**) using the recombinant antibodies F8, L19, F16 and G11 (*green* oncofetal matrix proteins, *red* CD31). Histopathology is demonstrated in H&E stained corresponding frozen sections (**m–o**). (*scale bars* 100 μm)
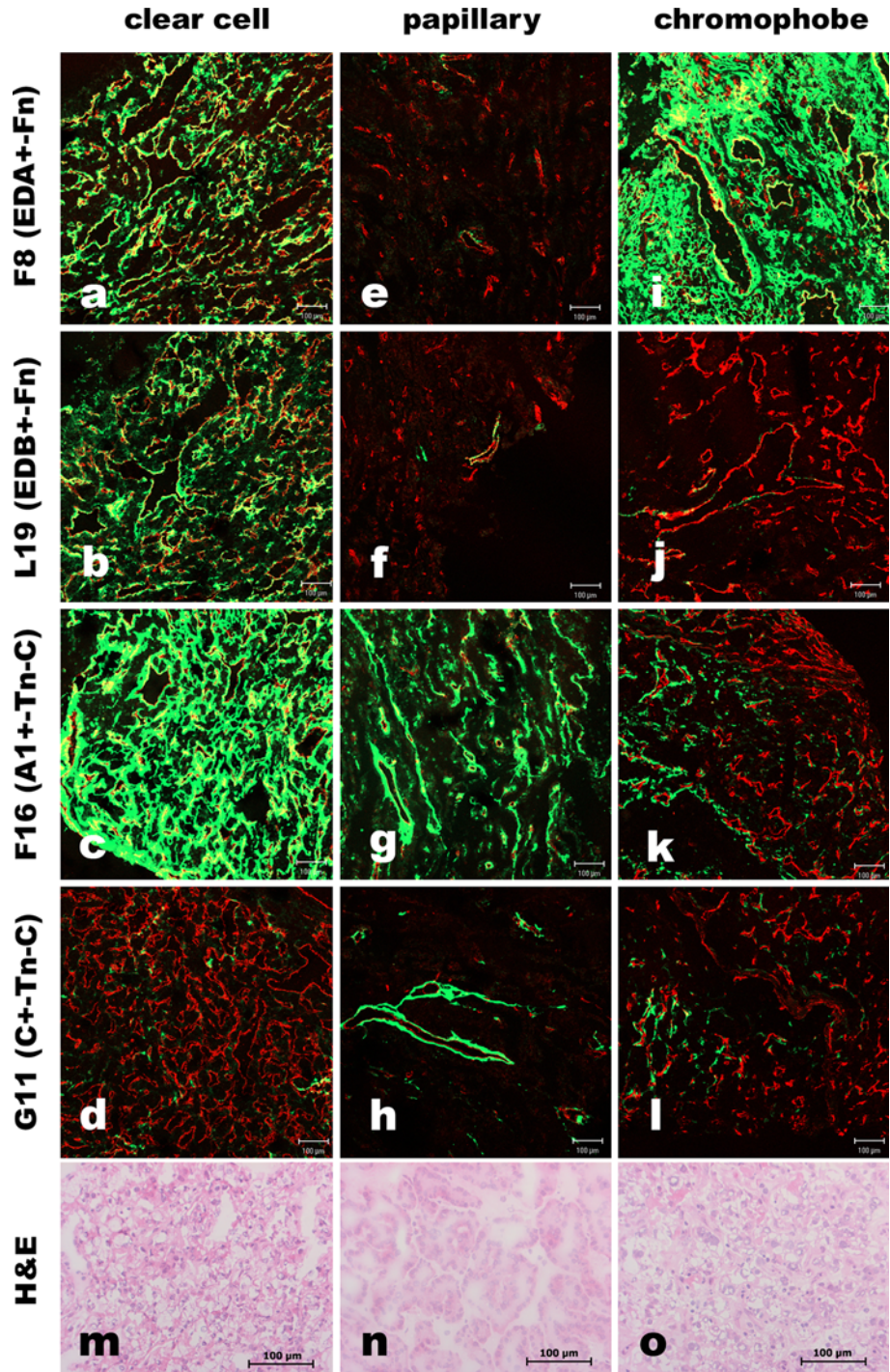
To investigate the vascular deposition of F8 and F16 accessible antigen in relation to one another, an immunofluorescence triple staining was performed. The first SIP was detected by tyramide-Alexa488 signal amplification. The visualization of the second SIP antibody was done by Cy3-coupled streptavidin (Dianova GmbH, Hamburg, Germany). Then, for vessel staining, the CD31 antibody JC70A (DAKO Deutschland GmbH, Hamburg, Germany) and an Alexa633-conjugated goat-anti-mouse IgG (Dianova GmbH, Hamburg, Germany) was applied (detailed protocols are available on request).

For immunfluorescence analysis, the confocal laser scanning microscope LSM 510 (Carl Zeiss Jena GmbH, Jena, Germany) was used. The semiquantitative evaluation of vessels positive for oncfFn and oncfTn-C variants was performed using the following expression score examining the whole section: $0 =$ no staining of CD31 positive vessels, $1 =$ only few CD31 positive vessels are stained, $2 =$ about 50% of CD31 positive vessels are stained or heterogeneous staining pattern with the occurrence of completely detected vessels, and $3 =$ nearly all vessels are positive.

Stimulation experiments

To investigate the impact of RCC derived mediators on ED-A$^+$-, ED-B$^+$ Fn- and A1$^+$ Tn-C mRNA expression in endothelial cells, HUVEC were stimulated with RCC-conditioned media generated under hypoxic conditions. For generation of RCC-conditioned media, cells were cultured 3 days in M199/0.05% HSA (Sigma-Aldrich Chemie GmbH, Munich, Germany) with 1% oxygen in a HERAcell 150i incubator (Thermo Fisher Scientific GmbH, Dreieich, Germany). Medium was collected, centrifuged at 2,000 rpm for 5 min and sterile filtered. Medium was kept at $-20°C$ until use. After starvation of HUVEC in M199/0.05% HSA for 3 h, 500 µl of the hypoxic RCC-conditioned medium was added. To analyse the role of tumour-derived VEGF, 100 ng/ml of Avastin$^®$ (F. Hoffmann-La-Roche AG, Basel, Swizerland) was added. Stimulation was stopped for RNA isolation after 1, 5 or 16 h by adding the RLT buffer from RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). For control, cells were cultivated in starvation medium without additives. Experiments were performed in triplicate and results were given as mean value and standard error of mean.

RT-PCR

Total RNA was isolated from HUVEC using the RNeasy Mini Kit and oncfFn and oncfTn-C rtRT-PCR was performed using the QuantiTect SYBR$^®$ green one step rtRT-PCR Kit (both Qiagen GmbH, Hilden, Germany) on a

iQ^{TM}5 cycler (Bio-Rad Laboratories GmbH, Munich, Germany). GAPDH served as housekeeping gene. Primers used were: ED-A$^+$ Fn forward: 5′-GGAGAGAGTCAGCC TCTGGTTCAG-3′, ED-A$^+$ Fn reverse: 5′-TCTGCAG TGTCTTCTTCACC-3′ (Mighell et al. 1997), ED-B$^+$ Fn forward: 5′-TACCGCATCACAGTAGTTGC-3′, ED-B$^+$ Fn reverse: 5′-CAGGTGACACGCATGGTGTCTGGA-3′ (Mighell et al. 1997), A1$^+$ Tn-C forward: 5′-AGCTT CCAAGAAACACCACTTC-3′, A1$^+$ Tn-C reverse: 5′-CC ATCCCAGCCAACCTCA-3′, GAPDH forward: 5′-GGA GTCAACGGATTTGGT-3′, GAPDH reverse: 5′-GTGAT GGGATTTCCATTGAT-3′ (Giannelli et al. 2005). Cycling conditions were: reverse transcription at 50°C for 30 min, followed by the initial PCR activating step at 95°C for 15 min, and 40 cycles of 94°C for 15 s, annealing for 30 s, 72°C for 40 s. Results were expressed as fold change of the non-stimulated controls ($2^{-\Delta\Delta Ct}/\Delta\Delta Ct$ data analysis
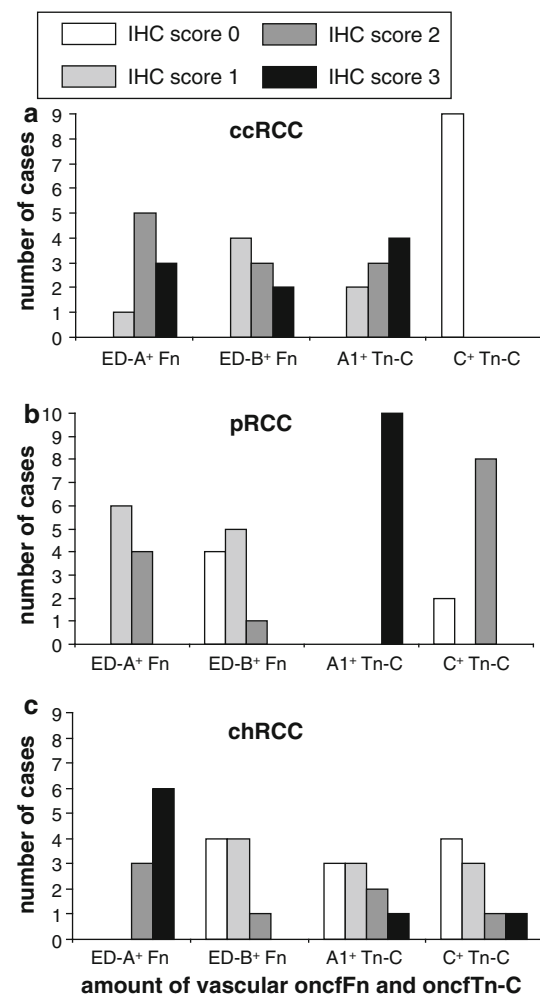

Fig. 2 Results of the semiquantitative evaluation of the vascular deposition of oncofetal fibronectin and tenascin-C variants in RCC subtypes **a** ccRCC (nine cases), **b** pRCC (ten cases), **c** chRCC (nine cases). Evaluation of the immunoreactive scores is explained in "Materials and methods". *IHC* Immunohistochemistry

method). Experiments were performed in triplicate and results were given as mean value and standard error of mean.

Results

Vascular expression of oncfFn and oncfTn-C variants in RCC subtypes

As shown by double immunofluorescence, there were remarkable differences in vessel associated oncfFn and oncfTn-C deposition between RCC subtypes. Within a large number of tumours of one subtype, the expression patterns are highly constant. The most consistent expression pattern could be revealed in ccRCC. ED-A$^+$ and ED-B$^+$ Fn as well as A1$^+$ Tn-C were detectable in all specimens at high abundance around vascular structures. Vascular C$^+$ Tn-C could never be shown (Figs. 1a–d, 2a). Figure 3a, b demonstrates ED-A$^+$ Fn and A1$^+$ Tn-c deposition in ccRCC on vascular level at higher magnification. In pRCC, ED-B$^+$ Fn and ED-A$^+$ Fn are only rarely expressed in blood vessels. In contrast, Tn-C isoforms are vascularly expressed at high level (Figs. 1e–h, 2b). In chRCC, only vascular ED-A$^+$ Fn was regularly detectable at high abundance while ED-B$^+$ Fn is present only

rarely. Tn-C splice variants usually exist, but differ in quantity (Figs. 1i–l, 2c).

As demonstrated by JC70A/F8/F16 triple fluorescence staining, different vessel populations with respect to antigen expression are distinguishable (Fig. 3c): (1) vessels negative for both, (2) vessels showing only ED-A$^+$ Fn or A1$^+$ Tn-C, and (3) vessels showing both. Vessel walls positive for both oncfFn and oncfTn-C splice variants show a stratified patterning with F8 accessible antigen deposited in association to the endothelium and F16 accessible antigen being perivascularly organized (Fig. 3d).

Vascular expression of oncfFn and oncfTn-C variants in RCC primary tumours and corresponding metastases

In human RCC, there is an obvious correspondence in the vascular expression patterns when comparing primary tumours and metastases. As expected for ccRCC, F8, L19, and F16 accessible antigens are expressed in large amounts in primary tumours (Figs. 4a–d, 5) and, to a larger extend, also in metastases (Figs. 4e–h, 5).

In the murine RCC, xenograft models a comparable vascular expression of the extra domains A and B of Fn and A1 of Tn-C could be shown using the antibodies F8, L19 and F16. Because the antibody F16 only detects human A1$^+$ Tn-C, at least this Tn-C isoform must be derived from

Fig. 3 a, b Immunofluorescence double staining for CD31 (antibody JC70A, *red*) and for EDA$^+$ Fn (antibody F8, *green*) (**a**) or for CD31 (antibody JC70A, red) and A1$^+$ Tn-C (antibody F16, *green*) (**b**) in a representative ccRCC at higher magnification (nuclei stained with DAPI in *blue*, bars 100 μm). **c, d** Immunofluorescence triple staining for CD31 (*blue*), A1$^+$ Tn-C (*green*) and EDA$^+$ Fn (*red*) in ccRCC demonstrating a heterogeneous incorporation of EDA$^+$ Fn and A1$^+$ Tn-C in the vascular ECM (**c**) and vessel wall stratification with regard to these proteins (EDA$^+$ Fn colocalized with endothelial cells, A1$^+$ Tn-C deposited in the perivascular rim) (**d**) [*scale bars* 50 μm (**c**) and 20 μm (**d**)]
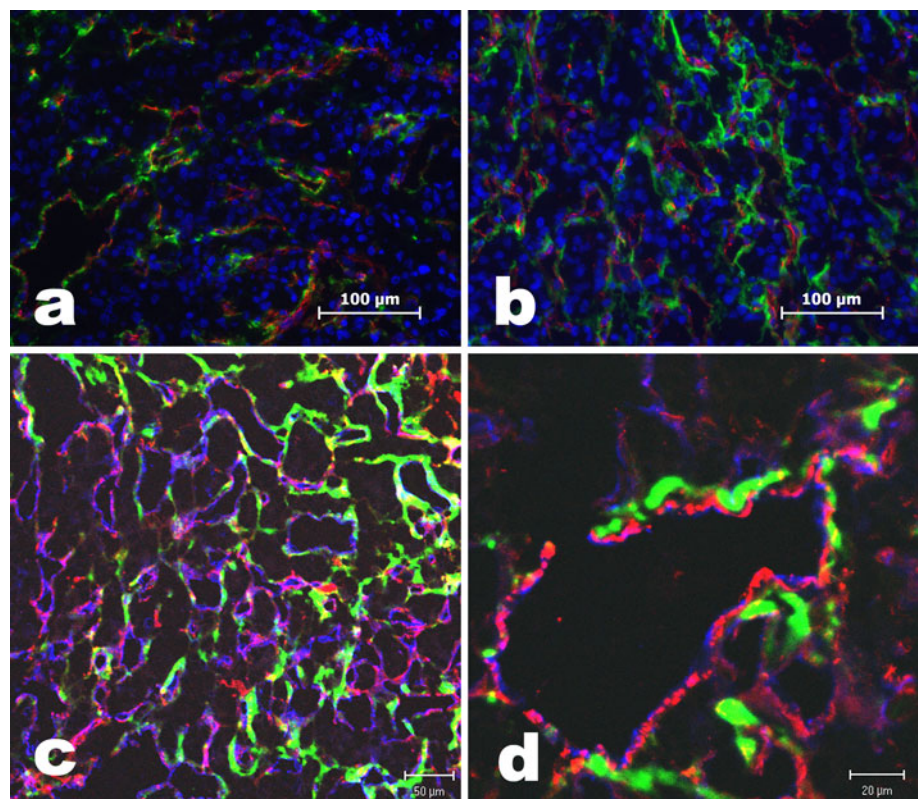
the carcinoma cells themselves. Also here, vascular expression patterns are the same in both primary tumours (Fig. 4i–l) and metastases with only quantitative differences (Fig. 4m–p).

Regulation of oncfFn and oncfTn-C mRNA expression in HUVEC by RCC-derived factors

To examine the relevance of RCC-derived factors for ED-A$^+$-, ED-B$^+$ Fn- and A1$^+$-Tn-C mRNA expression in endothelial cells, stimulation of HUVEC with A498- and Caki1-conditioned media generated under hypoxic

conditions was performed. Mutation analysis revealed the loss of one copy of the VHL gene in A498. Furthermore, a homozygous deletion of 4 bp (TGAC) in exon 2 was present which leads to a frame shift and therefore to a truncated VHL protein version with loss of function (confirmation of the results presented by Shinojima et al. 2007). In Caki1, only a homozygous substitution A > T in the 3′UTR was found and predicted to be a polymorphism (http://www.MutationTaster.org) with no alteration of protein function.

There is a general increase in ED-A$^+$-, ED-B$^+$ Fn- and A1$^+$ Tn-C mRNA expression in HUVEC with the highest
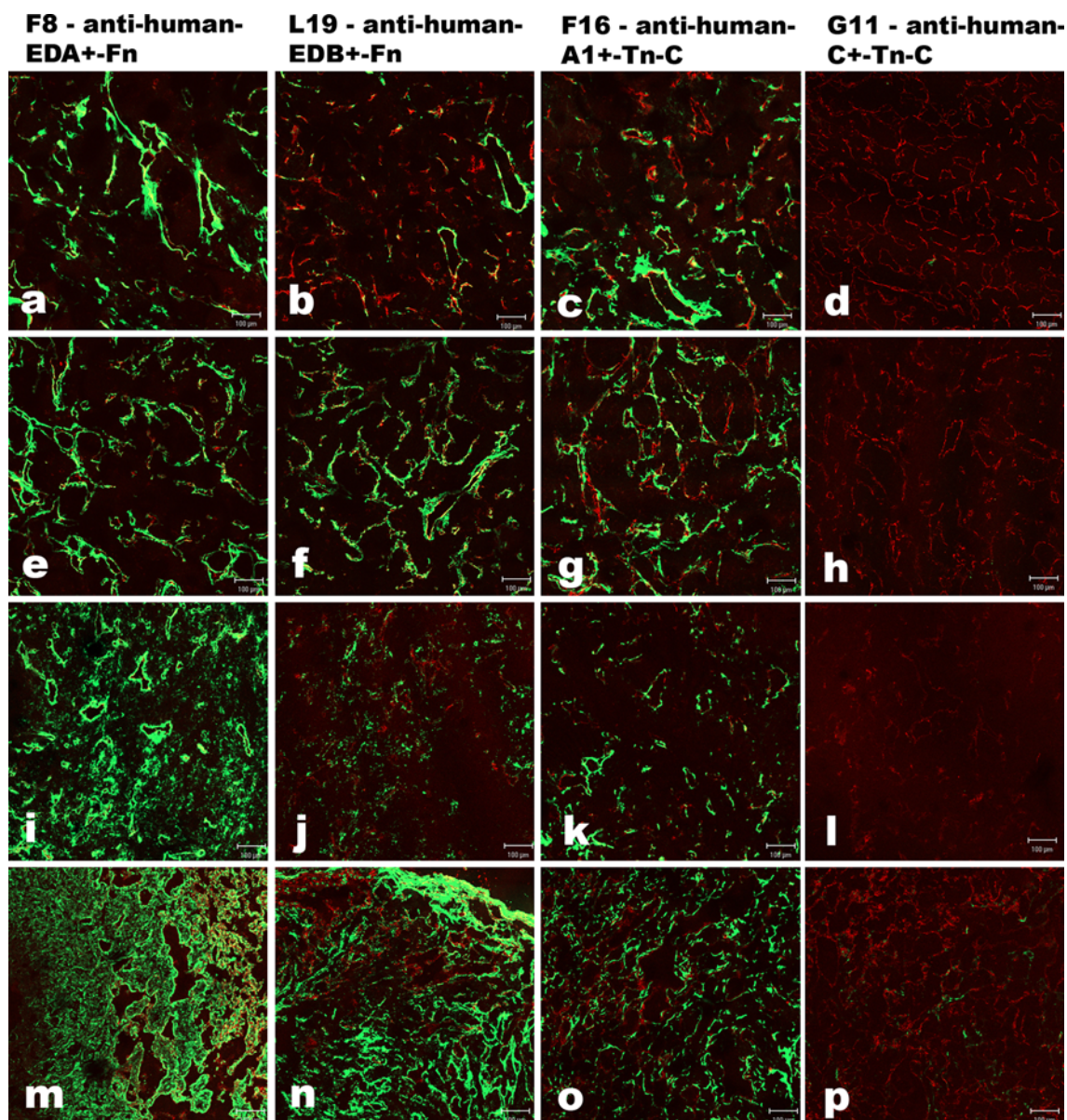

Fig. 4 Immunofluorescence detection of oncofetal fibronectin and tenascin-C variants in a human primary ccRCC (**a–d**) and its corresponding bone metastasis (**e–h**) as well as in a RXF393 orthotopic xenograft on nude mice (**i–l**) and its corresponding lung metastasis (**m–p**) (*green* oncofetal matrix proteins, *red* CD31; *scale bars* 100 μm)
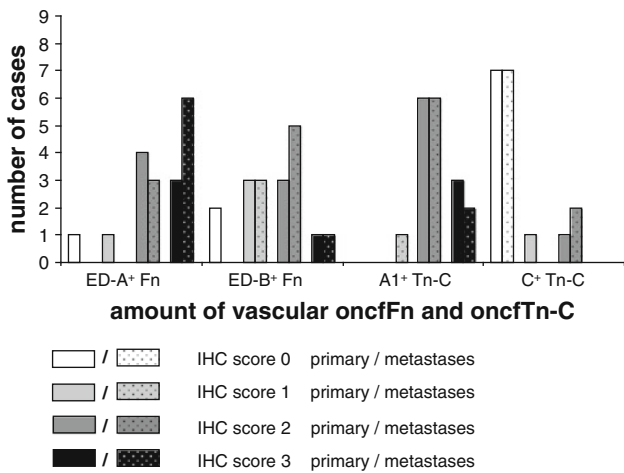
Fig. 5 Results of the semiquantitative evaluation of the vascular deposition of oncofetal fibronectin and tenascin-C variants in primary RCC and their metastases. The graph presents the number of cases assigned to the different IHC scores. Evaluation of the immunoreactive scores is explained in "Materials and methods". In general, there are comparable expression patterns in primary tumours and corresponding metastases. *IHC* immunohistochemistry

up regulation after 16 h independent of the medium used for stimulation. The up regulation after 16 h is up to twofold higher for A498 in comparison to Caki1-conditioned medium with a pronounced effect on $A1^+$ Tn-C (Fig. 6a, c). The addition of the anti-VEGF antibody Avastin® to A498 medium completely blocked the stimulatory effect (Fig. 6b), while in case of Caki1 medium there

was only a partial inhibition of oncfFn and oncfTn-C mRNA expression (Fig. 6d).

Discussion

Angiogenesis-related vascular matrix proteins are well established target structures for antibody based targeted pharmacodelivery (Neri and Bicknell 2005). As recently published, 15/18 (83%) of mRCC patients showed stable disease after two cycles of the antibody conjugate L19-IL2 targeting $ED-B^+$ Fn with a median progression-free survival of 8 months (Johannsen et al. 2010). Furthermore, it could be shown that the immunocytokine F8-IL2 improves the therapeutic efficacy of Sunitinib in a RCC xenograft model (Frey et al. 2010). Therefore, knowledge on the differential vascular expression of the target proteins in RCC subtypes and metastases and their regulation could help stratify patients for targeted delivery therapy and is therefore of high clinical relevance.

Here we are able to demonstrate that the oncofFn and oncfTn-C vascular expression pattern differs in between different RCC subtypes, but profiles are highly consistent within a great number of tumours of the same subtype and when comparing primary tumours and the corresponding metastases. This finding could also be evidenced in mRCC xenograft models used for treatment experimental. The findings are very important for treatment strategy: systemic therapy for RCC patients is used in metastatic situation and
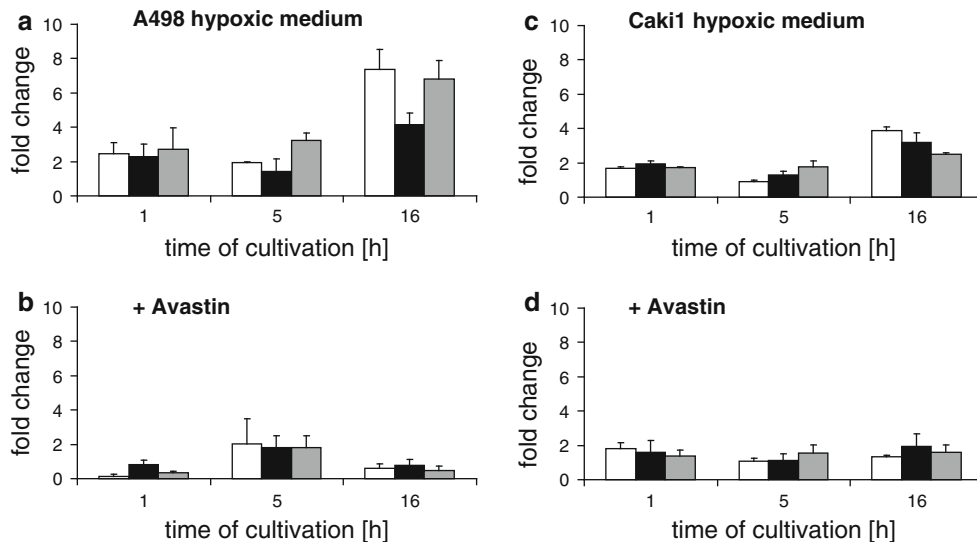

Fig. 6 Changes in oncofetal fibronectin and tenascin-C mRNA expression in HUVEC due to 1, 5, and 16 h stimulation with A498 or Caki1-conditioned medium harvested under hypoxic conditions (**a**, **c**). Results are given as fold changes compared to unstimulated control. There is a general increase in mRNA expression in HUVEC after 16 h incubation with conditioned medium but showing

quantitative and qualitative differences. The mRNA induction after application of A498 hypoxic medium is completely blocked by Avastin® (**b**) while there is only a partial reduction in case of Caki1 hypoxic medium (**d**). (experiments were performed in triplicate; *white* EDA^+ Fn, *grey* EDB^+ Fn, *black* $A1^+$ Tn-C; *error bars* indicate standard error of the mean)

is mainly directed against metastasis because nephrectomy is still standard of care. Metastases show the same vascular ECM structures, that is why vascular based targeted drug delivery in metastatic lesions might be individually adapted on the basis of the expression pattern in primary tumours.

All three subtypes show an abundant vascular deposition of ED-A$^+$ Fn and A1$^+$ Tn-C. In contrast ED-B$^+$ Fn is detectable always in vessels supplying ccRCC tissue, sometimes in high amounts but less or even not at all in pRCC and chRCC vasculature. C$^+$ Tn-C is only marginally expressed around RCC vessels, so targeting this isoform with the antibody G11 would not be effective in RCC therapy. These differences may be caused by tumour specific liberation of pro and/or anti angiogenic factors (Eble and Niland 2009; Saharinen et al. 2010). Indeed, RCC subtypes differ in their genetic background affecting the VEGF signalling pathway. A mutation affecting the VHL gene in a large portion of ccRCC and a resulting up regulation of HIF1 target genes is well-known (Brauch et al. 2000). In contrast, in pRCC there is an increase of onco-neuronal cerebella degeneration-related antigen 2 (Cdr2) that leads to the suppression of HIF1 transcription activation (Balamurugan et al. 2009). With respect to this situation, it is necessary to carefully select patients for an antibody-based pharmacodelivery using the antibodies described here. Individual quantitative and qualitative differences in vascular ED-B$^+$ Fn may also be a reason why only 15 out of 18 of the L19-IL12-treated patients with advanced RCC in the clinical trial reported by Johannsen et al. (2010) responded with a stabilisation of disease.

By fluorescence triple staining applying the clinically relevant antibodies F8 (ED-A$^+$ Fn) and F16 (A1$^+$ Tn-C), we are able to demonstrate an intratumoural heterogeneity of vasculature concerning oncfFn and oncfTn-C composition in ccRCC. This might reflect different states of vessel maturation or may be the result of a heterogenic distribution of growth factors and/or hypoxic areas. In line with this, a sequential occurrence of oncofFn isoforms, the generation of special fibronectin fragments as well as the fibronectin fibrillogenesis are important for the regulation of endothelial cell proliferation, recruitment of pericytes as well as vascular smooth muscle cells and thereby for the establishment of a functional vascular system during embryogenesis or wound healing processes (Astrof and Hynes 2009; Zhou et al. 2008). For oncTn-C variants, a migration promoting role could be shown for endothelial cells during processes of tissue remodelling (Zagzag et al. 2002; Ballard et al. 2006; Orend and Chiquet-Ehrismann 2006). In that context, the process of alternative splicing of Tn-C is of functional importance since different splicing domains could be shown to induce different effects: a angiogenesis promoting role for the fibrinogen-homologue globular domain and a angiogenesis inhibiting role for the A2 domain (Schenk et al. 1999; Saito et al. 2008).

Stable expression pattern of vascular oncfFn and oncfTn-C on one hand and the occurrence of different vessel subpopulations on the other hand lead to the suggestion that a combination of oncfFn and oncfTn-C targeting antibodies may improve the therapeutic efficacy. Furthermore, as already demonstrated by a combination of a recombinant SIP antibody and a monoclonal antibody (Berndt et al. 2010), a stratified organization of the vascular ECM concerning oncfFn and oncfTn-C targetable antigens could also be evidenced by use of two "therapeutic" SIP format antibodies. Thus, it might be possible to specifically attack endothelial cells via intraluminal targeting of oncFn and perivascular cells such as pericytes and myofibroblasts by targeting of oncTnC alone or in combination.

In vitro, we are able to show that indeed oncfFn and oncfTn-C mRNA expression in endothelial cells are up-regulated by RCC-conditioned medium. This effect is more pronounced using conditioned medium of A498 cells with a VHL loss of function mutation in comparison to Caki1-conditioned medium. This difference could be explained by the known overexpression of VEGF in A498 cells. The prominent role of VEGF is also supported by the fact that mRNA expression is completely blocked by Avastin$^®$ when added to A498-conditioned medium. In contrast, in case of Caki1-conditioned medium there is only a partial inhibition of mRNA synthesis by Avastin$^®$ indicating an additional role of further angiogenesis related cytokines in ECM protein regulation which must be the object of further studies. These differences in the cytokine spectrum of both cell lines may also be the reason for varying mRNA expression patterns supporting the in vivo results of different but stable immunohistochemical expression pattern in RCC subtypes. A comparable impact of the VEGF/VEGFR system on the expression of ED-B$^+$ Fn expression could recently be evidenced in a human endometrial adenocarcinoma xenograft model (Coltrini et al. 2009). In any case, the relevance of VEGF in the up regulation of potential target structures should be taken into consideration if anti-VEGF therapy and targeted drug delivery on the basis of vascular matrix proteins are combined.

Conflict of interest Dario Neri is a co-founder and shareholder of Philogen, the company which owns the rights over the F8, L19, F16 and G11 antibodies.

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