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Tissue Factor associates with survival and regulates tumor progression in osteosarcoma

Chris Tieken^a, Michiel C. Verboom^b, Wolfram Ruf^d, Hans Gelderblom^b, Judith V.M.G. Bovée^c, Pieter H. Reitsma^a, Anne-Marie Cleton-Jansen^c, and Henri H. Versteeg^a

^aEindhoven Laboratory for Experimental Vascular Medicine, Department of Thrombosis and Hemostasis, Leiden University Medical Centre, 2333 ZA Leiden, The Netherlands ^bDepartment of Clinical Oncology, Leiden University Medical Centre, 2333 ZA Leiden, The Netherlands

^cDepartment of Pathology, Leiden University Medical Centre, 2333 ZA Leiden, The Netherlands

^dDepartment of Immunology and Microbiology, The Scripps Research Institute, La Jolla, CA, USA1

Abstract

Osteosarcoma is the most common primary malignant bone tumor. Patients often develop lung metastasis and have a poor prognosis despite extensive chemotherapy and surgical resections. Tissue Factor is associated with poor clinical outcome in a wide range of cancer types, and promotes angiogenesis and metastasis. The role of Tissue Factor in OS tumorigenesis is unknown. 53 osteosarcoma pre-treatment biopsies and four osteosarcoma cell lines were evaluated for Tissue Factor expression, and a possible association with clinical parameters was investigated. Tissue Factor function was inhibited in an osteosarcoma cell line (143B) by shRNA knockdown or specific antibodies, and pro-tumorigenic gene expression, proliferation, matrigel invasion and transwell migration was examined. 143B cells were implanted in mice in the presence of Tissue Factor-blocking antibodies, and tumor volume, micro-vessel density and metastases in the lung were evaluated.

Tissue Factor was highly expressed in 73.6% of osteosarcoma biopsies, and expression associated significantly with disease-free survival. Tissue Factor was expressed in all four investigated cell lines. Tissue Factor was knocked down in 143B cells, which led to reduced expression of *IL-8*, *CXCL-1*, *SNAIL* and *MMP2*, but not *MMP9*. Tissue Factor knockdown or inhibition with antibodies reduced matrigel invasion. Tissue Factor antibodies limited 143B tumor growth *in vivo*, and resulted in decreased intra-tumoral micro-vessel density. Furthermore, lung metastasis from the primary tumor was significantly reduced. Thus, Tissue Factor expression in osteosarcoma reduces metastasis-free survival in patients, and increases pro-tumorigenic behavior both *in vitro* and *in vivo*.

Corresponding author: Henri H. Versteeg; h.h.versteeg@lumc.nl Leiden University Medical Centre, Albinusdreef 2, 2333 ZA, Leiden, The Netherlands. Tel: +31 715263872 Fax: +31 71526755.

Conflict of interest

The authors declare that they have no conflicts of interest in this research.

Keywords

osteosarcoma; coagulation; tumor; migration

Introduction

High-grade conventional osteosarcoma (OS) is the most common primary malignant bone tumor, mostly affecting adolescents. OS is often located in the metaphysis of long bones, usually in the distal femur, proximal tibia and proximal humerus, and less frequently in the pelvis, spine or skull.(1) The introduction of multi-agent chemotherapy has improved three-year survival from 20% to 60–70%, but no further improvements have been made in the last few decades.(2) OS is highly metastatic and 30–40 percent of all patients develop lung metastases mostly within 2–3 years. Resected tumors are analyzed for chemotherapy-induced necrosis,(1,3) which is a predictor of survival in patients.(4) But metastatic disease at diagnosis, the site and size of the tumor also associate with survival.(1) A suitable prognostic marker for disease progression that can be determined in pretreatment biopsies would be more preferable as this allows for tailored treatment at an earlier time point.

Tissue Factor (TF) is a trans-membrane glycoprotein, functioning as a hemostatic envelope for blood vessels and tissues.(5) Upon tissue damage, TF is exposed to the bloodstream and binds to the plasma protein Factor VII (FVII) to initiate coagulation. FVII is activated upon binding, leading to Factor X (FX) activation by the TF:FVIIa complex. This ultimately leads to prothrombin activation, fibrin deposition, platelet activation and clot formation. TF is often up-regulated on the surface of tumor cells. In colorectal cancer, mutation of the *K-RAS* oncogene and the loss of p53 results in TF up-regulation via MAPK and PI3K pathways.(6) In glioma cells, overexpression of the oncogenic epidermal growth factor receptor *EGFRvIII* and loss of PTEN function enhance TF expression, again via MAPK and PI3K pathways.(7) TF expression has been associated with metastasis in pancreatic(8), colorectal(9) and lung(10) cancer.

Apart from coagulation activation, TF can activate cell signaling pathways via TF:FVIIa-dependent protease activated receptor 2 (PAR-2) activation. This leads to MAPK and PI3K activation(11), as well as production of pro-angiogenic factors like *VEGF*, *IL-8*, *CXCL-1* and *CTGF*.(12,13) Both the Akt and MAPK pathways have been implicated in OS cell signaling,(14–16) and mutations in *TP53* were recently shown to occur in the majority of OS.(17)

In 2011, Ichikawa *et al.*(18) reported an osteosarcoma patient with a tumor-associated thrombus, suggesting that OS cells locally activate the coagulation cascade. Interestingly, the primary tumor and tumor cells within the associated thrombus were both positive for TF. The same report showed that the OS cell lines MG63, SAOS-2, TE85 and 143B were able to induce thrombin generation, which is suggestive of functional TF expression. In another study, the OS cell line SAOS-2 was shown to express TF, and the TF:FVIIa complex on these cells induced calcium signaling through PAR-2.(19)

Whether TF is widely expressed in OS and directly influences tumor progression has not been reported. Here we show that TF is expressed in primary OS tumors and associates with metastasis-free survival. Inhibition of TF reduces the aggressive phenotype of OS cells *in vitro*, and diminishes experimental tumor growth and metastasis *in vivo*. These findings indicate that TF is a promising target for adjuvant treatment of OS, and a potential predictor of disease progression.

Methods

Patient Material

Formalin-fixed, paraffin-embedded pre-treatment biopsy tissues were collected from high-grade osteosarcoma patients diagnosed between 1984 and 2003; patients with detectable metastasis at the time of diagnosis were excluded from further analyses. Metastasis-free and overall survival was recorded to determine disease progression over time. All patients were treated with doxorubicin and cisplatin according to a standard protocol in a clinical trial. Human tissue was handled in a coded fashion, according to Dutch national ethical guidelines ("Code for Proper Secondary Use of Human Tissue," Dutch Federation of Medical Scientific Societies). The tissue micro array was constructed as described before.⁽²⁰⁾ TF protein expression was determined in 53 biopsies using a specific TF antibody (4503, American Diagnostica, Stamford, CT, USA) by immunohistochemical staining. In short, slides were deparaffinized, rehydrated, after which endogenous peroxidase activity was blocked with 0.3% H₂O₂. Antigen retrieval was performed using sodium citrate buffer for 10 minutes at 100 °C. Sections were blocked with 5% bovine serum albumin in phosphate-buffered saline and incubated overnight at room temperature with 1:125 diluted primary antibody. Sections were incubated with Envision (Dako, Glostrup, Denmark) for 1 hour, visualized with DAB and counterstained with hematoxylin. This antibody does not recognize the soluble alternatively spliced isoform of TF.⁽²¹⁾ TF staining was assessed by two independent observers (JB and CT); the kappa inter-observer agreement was 0.881. TF protein expression was scored as staining intensity (0 = negative, 1 = weak, 2 = positive, 3 = highly positive) plus percentage of positive tumor cells (1 = 0–25%, 2 = 26–50%, 3 = 51–75%, 4 = 76–100%) resulting in a combined score between 0 and 7. A score below 4 was considered low TF, 4 or higher was considered as high TF.

Chemotherapy induced tumor necrosis of >90% was considered as a good histological response (WHO Classification of Tumors of soft tissue and Bone). Statistical analyses were performed using the statistical software package SPSS (version 21, SPSS Inc., Chicago, IL, USA).

Cell Culture

The cell lines U2OS, SAOS-2, MNNG-HOS, 143B and MCF-7 were purchased from the ATCC (Manassas, VA, USA), the MDA-MB-231mfp cell line was described before⁽²²⁾. Cells were cultured in RPMI1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin. OS Cell line authentication was confirmed by STR profiling using the GenePrint 10 system (Promega,

Madison, WI, USA). TF expression was downregulated in 143B cells by specific TF shRNAs (Sigma Mission library, St. Louis, MO, USA) introduced by lentiviral transduction.

PCR

fITF and asTF mRNA expression was determined using a common TF forward primer and specific asTF and fITF reverse primers. Real-time quantitative PCR was performed using SYBR green (Life Technologies, Carlsbad, CA, USA). Primers are described in Supplementary table 1. To determine the presence of human tumor cells in the lungs of mice, mRNA was isolated from snap-frozen lung material and converted to cDNA, a qPCR using mouse *β-actin* and human *GAPDH* housekeeping genes was used to quantify the presence of human mRNA as a measure for lung metastasis.

In Vitro Assays

FXa generation was measured by seeding 3×10^4 cells in a 48-well plate, after attaching overnight, the cells were exposed to 100 nM FX (Kordia, The Netherlands) and 1 nM FVIIa (Novo Nordisk, Malov, Denmark) and FXa levels were quantified using the substrate Xa Spectrozyme (American Diagnostica, Greenwich, CT, USA) at 405 nm. Cell proliferation was determined by a methyl-tetrazolium bromide mitochondrial activity (MTT) assay. Anoikis was determined by seeding 1×10^5 cells in low-adhesion plates and comparing viability on day 0 and 3 using MTT. For cell survival, 1×10^5 cells were seeded overnight in 24-wells tissue culture plates, washed, and placed on serum-free medium. Cell viability was determined on day 3 using MTT. Cells were exposed to either 50 µg/ml 10H10, 5G9 or IgG control antibody, 10 nM FVIIa, or 100 nM FX and 10 nM FVIIa.

Invasion and migration assays were performed using BD Biocoat Matrigel invasion chambers according to the manufacturer's instructions (BD Biosciences, Palo Alto, CA, USA). In short, 3×10^4 cells were added to the upper compartment in the presence of 50 µg/ml antibody and allowed to invade for 18 hours at 37°C. Membranes were stained with crystal violet and invaded cells were quantified. Western blots were performed by lysing cells in sample buffer (Invitrogen, Carlsbad, CA, USA), after which lysates were run on 6–18% gradient gels and transferred to PVDF membranes. Membranes were blocked using 5% milk powder and incubated with the primary antibodies, followed by HRP-conjugated secondary antibodies. Bands were visualized using Western Lightning ECL (PerkinElmer).

In vivo tumor growth assay

1×10^6 143B cells were mixed with 500 µg 10H10, 5G9 (mouse monoclonal antibodies against TF) or a control mouse IgG in PBS and injected subcutaneously (n=6) in NOD-SCID mice (Jackson Laboratories, Westgrove, PA, USA). Tumor growth was measured over time using calipers; tumor volume was calculated as $\text{width}^2 \times \text{length} \times 0.5$. Tumors were harvested after 3 weeks and formalin-fixed, paraffin-embedded for further analysis. Lungs were snap-frozen in liquid nitrogen. Animal experiments were approved by the animal ethical committee of the Leiden University Medical Center. Immunohistochemistry was performed as described above, a vWF antibody (1:5000, DAKO, Glostrup, Denmark) was used to stain microvessels. Ki67 (1:800, DAKO, Glostrup, Denmark) was used to proliferating cells. Ki67 positive nuclei were quantified as described by Tuominen *et al.*(23)

Microvessel density was quantified by counting vWF+ microvessels in at least two fields per tumor at 10× magnification. Means were calculated, a t-test was performed to determine statistical significance between IgG control and 10H10 or 5G9 groups.

Results

TF expression is associated with metastasis free survival in osteosarcoma patients

The expression of TF was investigated in a panel of 53 OS biopsies high-grade OS patients without detectable metastasis at the time of diagnosis. All biopsy specimens were positive for TF by immunohistochemistry (IHC), although immuno-reactivity differed widely between samples. Fourteen patient samples were considered low TF (example shown in Fig. 1A) and 39 samples high TF (Fig.1B), as determined by the IHC score. Age, sex, and median follow up were not statistically different between the TF low and TF high groups, as shown in Table 1. Clinical or biological heterogeneity within the patient group was limited as most patients suffered from conventional OS (supplemental table 2). Patients with high TF more often had a poor histological response to pre-operative chemotherapy compared to patients in the low TF group (odds ratio: 2.21, 95% CI: 0.65–7.52), but this effect did not reach statistical significance. High TF in biopsies significantly associated with reduced metastasis-free survival (Fig. 1C, $p=0.042$). As metastatic spread in OS severely diminishes overall survival in OS, high TF expression also associated with decreased overall survival, although this does not reach statistical significance (Fig. 1D, $p=0.102$). As shown in Table 1, the odds ratio of developing metastatic disease upon high tumor TF expression was 5.70 (95% CI 1.12–28.90), whereas the odds-ratio for overall survival during follow-up was 3.75 (95% CI 0.73–19.14).

TF expression by OS cell lines

In order to establish an *in vitro* cell model to study TF-driven tumor progression expression of TF was investigated in OS cell lines. Full length (fl)TF, alternatively spliced (as)TF and PAR-2 expression was examined by PCR and compared to flTF, asTF and PAR2 levels in two breast cancer cell lines with low and high expression levels of these gene products. The OS cell lines U2OS, SAOS-2, MNNG-HOS and 143B expressed similar levels of full length TF as the breast cancer cell line MDA-MB-231mfp. Expression of asTF, a soluble TF isoform implicated in integrin-dependent tumor cell proliferation, ranged from low to undetectable (Fig. 2A). All OS cell lines expressed PAR-2.

The OS 143B cell line was selected for subsequent experiments because of its aggressive phenotype and ability to metastasize in murine models.(24) Total TF expression could be down regulated in OS 143B cells using an shRNA approach. This resulted in a 70% reduction of TF mRNA compared to a scrambled shRNA (Fig. 2B) and similarly reduced TF protein levels determined by Western blot (Fig. 2C). We subsequently observed a 4-fold decrease in the ability to generate FXa after exposure to FVIIa and FX, showing that TF on OS 143B cells was active, and mRNA reduction led to reduced TF pro-coagulant activity (Fig. 2D).

TF knockdown *in vitro* modulates pro-tumorigenic gene expression but not proliferation

Rapid tumor cell proliferation is a hallmark of cancer progression, and OS 143B is a highly proliferative cell line, both *in vitro* and *in vivo*.⁽²⁵⁾ We found that cell proliferation was not dependent on TF expression levels determined by a MTT proliferation assay (Fig. 3A). Apart from its role in coagulation activation, TF is also able to activate pro-angiogenic pathways by signaling via PAR-2.⁽²⁶⁾ We observed that knockdown of TF significantly reduced the expression of the cytokines *IL-8* and *CXCL-1* (Figure 3B), that are both downstream pro-angiogenic effectors of PAR-2 signaling and well-established readouts of TF signaling.^(13,27)

Expression of the zinc finger transcription factor Snail has recently been linked to OS cell invasion and migration.⁽²⁸⁾ We found that TF knockdown led to a significant decrease in *SNAIL* expression in OS 143B cells (Fig. 3B). An essential step for metastasis to occur is the breakdown by matrix metalloproteinases (MMPs) of the extracellular matrix surrounding the primary tumor, and expression of *MMP2* and *MMP9* was previously shown to regulate OS cell invasion and migration.^(15,29) We observed that knockdown of TF reduces the expression of *MMP2*, but *MMP9* expression remained unchanged (Fig. 3B).

To determine that the effects of TF knockdown were not caused by off-target effects of the TF shRNA, we used two different shRNAs directed against TF. As shown in Fig. 3D, TF shRNAs 2 and 3 reduced TF mRNA levels by 47.1% and 39.4% respectively, which was confirmed by Western blot (Fig. 3C). This led to a similar reduction of *IL-8* expression (Fig. 3D), showing that *IL-8* expression is indeed dependent on TF expression levels.

TF inhibition reduces cell migration and invasion

Although metastasis is a multi-faceted process that is difficult to study *in vitro*, the ability of tumor cells to migrate and invade artificial extra-cellular matrices is an established readout for metastatic behavior. Matrigel invasion was 2.3-fold reduced after TF knockdown in OS 143B cells, trans-well migration was 4.1-fold reduced (Fig. 4A). Next TF function in OS was targeted using antibody-mediated blockade specific to TF. The monoclonal TF antibodies 10H10 and 5G9 are specific inhibitors of distinct processes in TF biology; 10H10 inhibits TF:FVIIa dependent PAR-2 activation while 5G9 inhibits FX activation via the TF:FVIIa complex.⁽³⁰⁾ The TF:FVIIa:FXa complex can also activate PAR-2 as well as trigger subsequent coagulation activation. Matrigel invasion was significantly reduced in OS 143B cells in the presence of the TF antibodies 10H10 or 5G9 compared to a non-specific mouse IgG (Fig. 4B). This inhibiting effect was not explained by 10H10 and 5G9-dependent reduction in proliferation or cell survival, as these antibodies did not substantially influence proliferation or loss-of-anchorage-induced apoptosis (anoikis) (Sup. Fig. 1A and B) in serum-containing media, although addition of high amounts of FVIIa, alone or in combination with FX, could inhibit serum starvation-induced apoptosis (Sup. Fig. 1C). To exclude that this effect was specific for OS 143B cells, we also determined matrigel invasion in the OS cell lines U2OS, SAOS-2 and MNNG-HOS, and observed that the TF antibodies 10H10 and 5G9 both significantly reduced matrigel invasion (Fig. 4C).

Inhibition of TF by specific antibodies reduces tumor growth and lung metastasis

We next evaluated whether TF inhibition can attenuate OS tumor development *in vivo*. OS 143B cells were injected in the presence of the TF antibodies 5G9, 10H10 or an isotype-matched control IgG1 in NOD-SCID mice. The TF antibodies 5G9 and 10H10 antibodies significantly reduced tumor volume over time (Fig. 5A). TF does not regulate OS cell proliferation *in vitro*, thus we reasoned that tumor growth is likely dependent on TF-driven angiogenesis. Tumor sections stained for the proliferation marker Ki67 did not show a significant difference in the percentage of proliferating tumor cells when tumors were treated with the TF antibodies 10H10, 5G9 or control IgG (Fig. 5B and 5C). In contrast, micro-vessel density in tumors treated with TF antibodies 5G9 and 10H10 was significantly decreased (Figure 5D and E). Thus, inhibition of TF reduces tumor growth by attenuating angiogenesis. The OS 143B cell line is capable of metastasizing from the primary tumor to distant sites in murine models.(24) By comparing human to mouse housekeeping-gene expression in the lungs we quantified the presence of tumor cells, and observed a more than 200-fold reduction in mice treated with TF antibodies (Fig 5F).

Discussion

This study shows that TF is expressed in primary osteosarcoma, and associated with metastasis-free survival based on pre-treatment biopsy material. Prognosis of disease progression is important for both the patient and treating physician to determine the course of treatment. Treatment usually consists of pre-operative chemotherapy after the diagnosis has been established based on histological examination of a biopsy. Several studies have shown that a good histological response to pre-operative chemotherapy associates favorably with survival.(4,20) This also allows patients with a poor response to chemotherapy to receive alternative chemotherapy regimens, which may improve survival.(31)

Recently, more efforts have been put into identifying adequate predictors of OS progression based on biopsies. A meta-analysis showed that *VEGF* associated with overall and disease-free survival(32). It is interesting to note that *VEGF* expression has been associated with TF expression in lung(33), colorectal(34) and prostate(35) cancer. Increased microRNA-9 expression associated with survival(36), while low expression of microRNAs 183(37) and 223(38) combined with upregulation of Ezrin or Ect2 respectively associated with both metastasis and poor response. The loss of expression of the tumor suppressor p16 associated with decreased survival in patients.(20) *HER2* expression and *TP53* mutations associate with disease progression in multiple cancer types, but they were not associated with OS progression.(17,39) We have shown here that high expression of TF in OS biopsies associates with decreased metastasis-free survival. It should be noted that the confidence interval was wide (1.12–28.90) despite the fact that most of the patients suffered from conventional OS (Sup. Table 2). Rather, as osteosarcoma is a rare tumor type, we believe the limited number of included patients explains the wide confidence interval. Despite this, we believe that the association between TF and metastasis-free survival provides a rationale for optimized treatment decisions based on TF expression in biopsy material, which is considerably earlier collected than resected material after neo-adjuvant chemotherapy and surgery.

Besides associations with clinical parameters it was also shown that specific inhibition of TF reduces the pro-tumorigenic behavior of OS cells in both *in vitro* and *in vivo* experimental models. Experimental data by others shows that TF drives pro-tumorigenic cellular processes, although this is mostly shown in tumor models of epithelial origin.(26) In this paper we have shown that TF inhibition also reduces angiogenesis and metastatic behavior in tumor cells of mesenchymal origin.

We show here that knockdown of TF expression by shRNAs reduces the expression of pro-angiogenic factors *IL-8* and *CXCL-1* *in vitro*, and TF antibodies reduced micro-vessel density in OS tumors *in vivo*. Although angiogenesis is a crucial process for tumor development, a higher micro-vessel density in OS biopsies was associated with more favorable survival rates and a good response to chemotherapy.(40) This paradox is likely caused by better access of chemotherapeutic agents to the tumor cells via the more densely organized intra-tumoral vascular network. We also note that despite reduced micro-vessel density as a consequence of co-injection of tumor cells with TF antibodies did not reduce the number of proliferating cells. Although this appears contradicting, coagulation factor FVII, alone or in combination with FX, inhibits osteosarcoma tumor cell apoptosis. Thus, 10H10 or 5G9 treatment may inhibit this cell survival *in vivo*, and Ki67 positivity is the resultant of equilibrium between proliferation and cell death. In support, a reduction in vascular density while maintaining Ki67 positivity *in vivo* that was comparable to control, was also demonstrated after knockout of tumor cell PAR2, the functional TF/FVII receptor.(41) In this study it also remained unclear whether TF induces tumor growth through enhanced vascular density, or whether TF induces tumor growth resulting in more vessel growth. However, as TF is not associated with a proliferative advantage *in vitro*, we deem the latter scenario unlikely.

Both TF knockdown and inhibition by antibodies reduced the invasiveness of OS cells *in vitro*, and lung metastasis was reduced after TF antibody administration *in vivo*. At present, we believe that the inhibitory action of our TF inhibitory antibodies may be ascribed to TF's role in invasion, rather than its role in fibrin/platelet plug formation on the surface of cancer cells which protects the metastatic cell from the immune system and shear stress.(42) The reason for this is that 10H10 primarily interferes with PAR2 activation rather than TF coagulant function and clot formation (30). We also note that 10H10 could be an interesting approach to targeting TF-driven metastasis in OS. Indeed, clinical use of TF antibodies could be complicated by possible bleeding complications caused by their anticoagulant effects. A clinical trial using a monoclonal TF antibody reported no major bleeding, although dose-dependent minor bleeding was observed.(43)

While this study directly proves that TF dictates OS cell behavior *in vitro* and in an *in vivo* model of OS, it also has a number of limitations. Although it is known that tumor cell grafting in an orthotopic setting results in tumor growth and dissemination that is different from that observed after subcutaneous grafting, we decided to use the subcutaneous model, based on three considerations; i) subcutaneous growth allows more accurate tumor volume measurements in time that grafting in bone, ii) orthotopic graftment, but not subcutaneous grafting, often leads to onset of humane endpoints before detection of wide-spread tumor cell dissemination,(44) hampering measurement of metastasis, and iii) xenografts from

subcutaneously and orthotopically grown OS tumor cells show similar patterns of protein expression and similar histology reminiscent of primary human osteosarcoma.(24)

A second limitation is the limited availability of spontaneously metastasizing OS cell lines(24) which precluded us from confirming the role of TF in OS progression *in vivo* using other cell lines. Nevertheless, as TF antibodies incubated with three other OS cell lines consistently inhibited invasion, a prerequisite for metastasis, we believe that TF's role in invasion is essential for early metastasis.

Finally, our work did not show how TF drives OS progression. It is known that serum contains sufficient levels of FVII to activate PAR2,(45) and a role for 10H10 in inhibiting TF-dependent PAR2 activation is consistent with this view.(30) However, 10H10 also disrupts TF-integrin $\beta 1$ complexes,(30) and interplay between TF and integrins was previously shown to regulate cell migration.(46) Furthermore, we believe that MMP2 and Snail are not solely responsible for the effects of TF on migration. In the future, knockdown approaches should enable us to decipher whether TF-dependent migration, invasion and metastasis is dependent on PAR2 activation or TF-integrin $\beta 1$ complexation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TF in Osteosarcoma biopsies reduces metastasis-free and overall survival. The presence of TF protein was determined by IHC in 69 biopsies. **A** low TF expression and **B** high TF expression in OS biopsies. Bars represent 50 μm . **C** Metastasis free survival is significantly reduced in patients with high TF (p : 0.042, log-rank test). **D** High TF in biopsies reduced overall survival in trend (p : 0.102, log-rank test).

Figure 2.

TF is expressed and active on OS cell lines. **A** mRNA expression of flTF, asTF and PAR-2 in OS cancer cell lines. MCF7 and MDA-MB-231mfp were included as a negative and positive control respectively. **B** qPCR and **C** Western blot showing that TF expression is reduced in 143B cells using a TF shRNA. A scrambled shRNA does not alter TF expression. **D** The ability to generate FXa by 143B after exposure to 1 nM FVIIa and 100 nM FX is attenuated because of TF expression knockdown. n.s.: not significant, *: $p < 0.05$, $p < 0.01$ *** $p < 0.001$. All values expressed as mean $\pm$ SD, Students t-test was used.

Figure 3.

TF knockdown reduces pro-tumorigenic gene expression but not cell proliferation. **A** TF knockdown did not alter 143B cell proliferation by MTT assay. **B** TF knockdown significantly reduced expression of pro-angiogenic factors *IL-8* and *CXCL-1*, the EMT marker *SNAIL*, and *MMP2* but not *MMP9*. **C** TF expression was knocked down in 143B using two alternative TF shRNA construct. **D** TF shRNAs 2 and 3 significantly reduced TF expression, resulting in a dose-dependent IL-8 downregulation * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. All values expressed as mean $\pm$ SD, Students t-test was used.

Figure 4.

TF inhibition reduces migration and invasion *in vitro*. **A** TF knockdown reduces matrigel invasion and transwell migration. **B** TF antibodies 5G9 and 10H10 significantly reduce matrigel invasion. **C** Matrigel invasion is reduced by both TF antibodies 5G9 and 10H10 in U2OS, SAOS-2 and MNNG-HOS. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$. All values expressed as mean $\pm$ SD, Students t-test was used.

Figure 5.

TF antibodies inhibit OS tumor growth and metastasis *in vivo*. **A** 143B Cells mixed with 5G9, 10H10 or a nonspecific IgG control were subcutaneously injected in NOD-SCID mice. Both 10H10 and 5G9 significantly reduced tumor volume. **B** Representative Ki67 staining of tumor sections **C** The percentage of Ki67 positive nuclei were quantified per tumor field **D** Tumors were analyzed by IHC to assess vascular density (vWF), inset shows a high magnification of a vWF⁺ micro-vessel. **E** Quantification of micro-vessels per field. **F** qPCR on lung cDNA comparing human GAPDH to mouse β -actin to quantify the metastatic load. Control represents lung cDNA from a mouse not exposed to tumor cells. Error bar = SEM. n.s.: not significant *p < 0.05 **p < 0.01

Table 1

Association of Tissue Factor with OS patient and tumor characteristics

Characteristic	TF low N (%)	TF high N (%)	Total N (%)	p-value	Odds Ratio (95% CI)
Total	14 (100)	39 (100)	53 (100)		
Age at diagnosis, y (SD)	15.5 (6.7)	15.3 (6.6)	15.5 (6.6)	0.37	
Sex, female n (%)	8 (57.1)	18 (46.2)	26 (49.1)	0.49	
Median follow-up, mo (range)	208 (33–348)	142 (6–303)	172 (6–348)	0.09	
Histological response to chemotherapy					
Good (>90% necrosis)	7 (50.0)	11 (28.2)	18 (34.0)		2.21 (0.65–7.52)
Poor (≤ 90% necrosis)	6 (42.9)	27 (69.2)	33 (62.2)		
Undetermined ^a	1 (7.1)	1 (2.6)	2 (3.8)		
Metastasis during follow-up					
Yes	2 (14.3)	20 (51.3)	21 (39.6)		5.70 (1.12–28.90)
No	12 (85.7)	19 (48.7)	32 (60.4)		
Overall Survival					
Deceased	2 (14.3)	15 (35.9)	17 (32.1)		3.75 (0.73–19.14)
Alive	12 (85.7)	24 (64.1)	36 (67.9)		

^aTwo patients did not receive pre-operative chemotherapy.