The Intrinsic Pathway does not Contribute to Activation of Coagulation in Mice Bearing Human Pancreatic Tumors Expressing Tissue Factor

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The intrinsic pathway of coagulation has been shown to contribute to thrombosis.[1] Factor (F) XII can be activated by a variety of agents, such as polyphosphate, and promotes coagulation by converting FXI to FXIa.[1] Thrombin can also activate FXI through a positive feedback loop.[1] FXII$^{-/-}$ and FXI$^{-/-}$ mice had reduced thrombosis compared with wild-type mice.[2–4] Similarly, wild-type mice treated with antisense oligonucleotides (ASOs) against either FXII or FXI had reduced thrombosis compared with controls.[5,6] Importantly, reducing FXI expression or inhibiting FXIa activity significantly reduced postoperative venous thromboembolism in patients who underwent total knee arthroplasty without increasing bleeding.[7,8]

There are very few studies on the role of the intrinsic pathway in cancer-associated thrombosis. We showed that extracellular vesicles (EVs) derived from the human prostate cancer cell line PC3 contained polyphosphate.[9] EVs are small membrane vesicles released from a variety of cells, including cancer cells.[10] PC3-derived EVs induced a high rate of death in wild-type mice in a pulmonary embolism model whereas FXII$^{-/-}$ or FXI$^{-/-}$ mice were protected.[9] Similarly, the administration of the anti-FXIIa antibody prevented death of wild-type mice that received PC3-derived EVs.[9] PC3-derived EVs also expressed tissue factor (TF) and inhibition of TF also protected wild-type mice from EV-induced death.[9] One study found that patients with non-metastatic colorectal cancer have significantly lower plasma levels of FXII zymogen but not FXI compared with healthy controls, which suggests activation and consumption of FXII.[11]

Pancreatic cancer is associated with a high incidence of venous thromboembolism (5–26%).[12,13] TF is expressed by pancreatic cancer cell lines and tumors.[14–16] TF-positive EVs are released from cancer cells and circulate in the blood in both patients and mouse models.[14,17,18] We and others showed that EV TF activity is associated with venous thromboembolism in patients with pancreatic cancer.[19,20] Importantly, in mice bearing human pancreatic BxPC-3 tumors TF derived from the tumor enhanced venous thrombosis.[21]

In this study, we investigated the role of FXI in the activation of coagulation in a mouse model of pancreatic cancer. We used BxPC-3 cells modified to express the luciferase reporter.[21] Tumors were grown orthotopically in Crl:NU-*Foxn1^{nu}* male mice and imaged using the IVIS Lumina. Blood was collected from the inferior vena cava into citrate and platelet-poor plasma was prepared by centrifugation at 4,500 × *g* for 15 minutes. Mice were treated with either a

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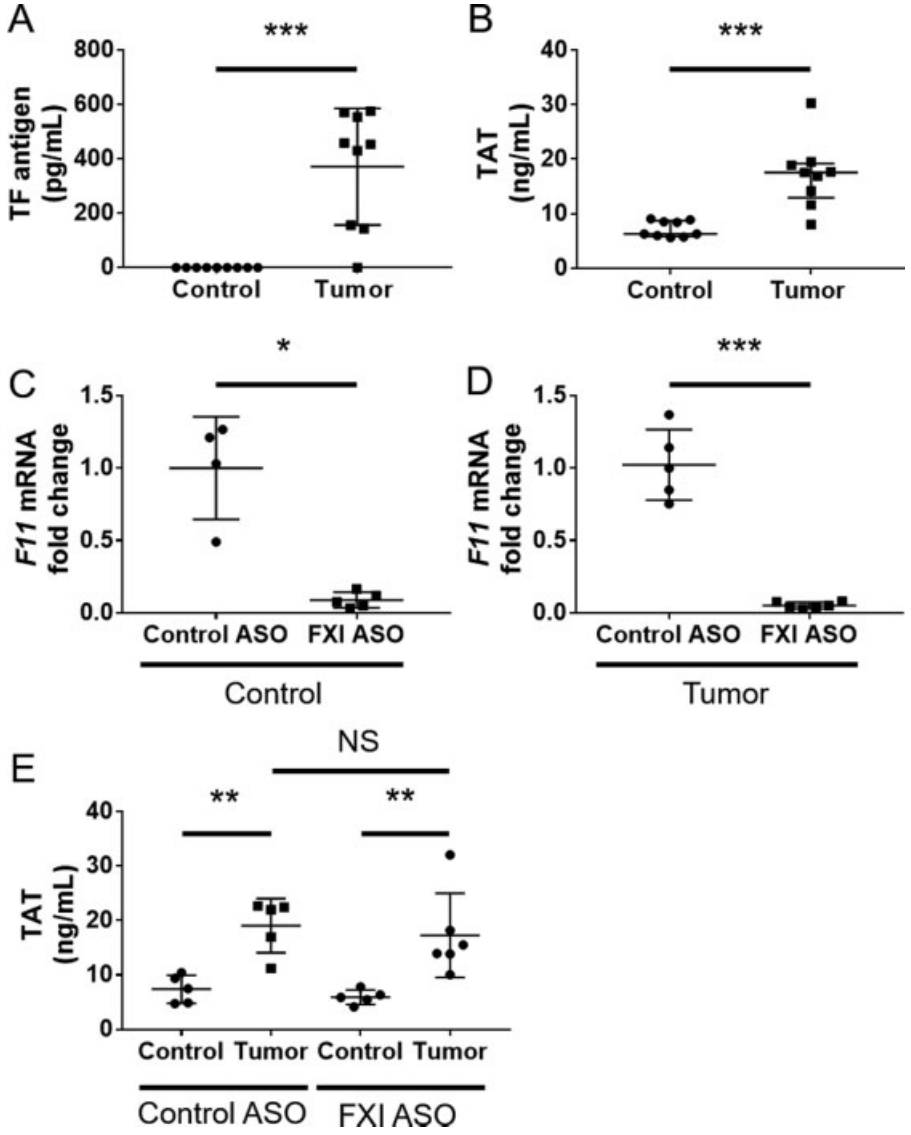

Fig. 1 Measurement of the activation of coagulation in control and tumor-bearing mice with or without suppression of *F11* messenger ribonucleic acid (mRNA) expression. Plasma levels of human tissue factor (TF) protein (**A**) and thrombin-antithrombin (TAT) complexes (**B**) were measured using enzyme-linked immunosorbent assays (ELISAs) in control and BxPC-3 tumor-bearing mice. Control mice (**C**) and BxPC-3 tumor-bearing mice (**D**) were treated with control antisense oligonucleotide (ASO) or FXI ASO and *F11* mRNA levels in the liver were measured. *RPL4* or *HPRT1* mRNA levels were used to correct for variations in input RNA level and reaction efficiency. We measured plasma TAT levels in control mice and BxPC-3 tumor-bearing mice treated with control ASO or FXI ASO (**E**). We used mice with tumors weighing from 1.8 to 3.5 g for these experiments. Four to nine mice were used for each group. Data are shown as mean ± standard deviation (**A, C, D, E**) or median ± interquartile range (**B**) depending on the data distribution. For the two-group comparisons, the unpaired two-tailed Student's *t*-test (**A**), the Mann–Whitney *U* test (**B**), or the unpaired *t*-test with Welch's correction (**C**, **D**) were used depending on the data distribution and variances. For the multigroup comparison, one-way analysis of variance with Tukey's multiple comparisons was used (**E**). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, NS, not significant.

nontargeted (control) ASO or an ASO selectively targeting FXI (Ionis Pharmaceuticals, 50 mg/kg, subcutaneously, single injection). Importantly, the FXI ASO reduced *F11* messenger ribonucleic acid (mRNA) expression in the liver by 98%, and significantly increased the activated partial thromboplastin time (aPTT).[5] Administration of the FXI ASO twice weekly for 3 weeks and then collecting samples 3 days later led to an approximately twofold increase in aPTT, whereas a single administration of the FXI ASO and collecting samples 3 days later led to an approximately 1.3-fold increase in aPTT.[5] We used a single administration of FXI ASOs in control mice or in tumor-bearing mice once the tumors have reached $\geq$ 1.8 g. Livers and blood were collected 3 days after ASO injection. Levels of *F11* mRNA expression were measured as described.[5] Enzyme-linked immunosorbent assays were used to measure plasma levels of human TF protein (Biomedica Diagnostics, Cat#845) and thrombin antithrombin (TAT) complexes (Siemens, Cat#OWMG15).

In a previous study, we showed that BxPC-3 expresses the highest level of TF among human pancreatic cancer cell lines.[15] In addition, BxPC-3 tumor-bearing mice had significantly increased levels of human TF protein and TAT

complexes compared with controls.[21,22] Consistent with our previous studies, we observed increased plasma levels of human TF protein and TAT complexes in tumor-bearing mice compared with controls (►**Fig. 1A**, **B**), which indicated that tumor-bearing mice have an activated coagulation system.

We hypothesized that the intrinsic pathway would contribute to the activation of coagulation in BxPC-3 tumor-bearing mice. Therefore, we examined the effect of suppressing *F11* mRNA expression using ASOs. Similar to a previous study,[5] we observed a 92 and 97% decrease of *F11* mRNA expression in control mice and tumor-bearing mice treated with the FXI ASO when compared with the level of *F11* mRNA expression in control mice and tumor-bearing mice treated with control ASO, respectively (►**Fig. 1C**, **D**). Next, we examined if the reduction of *F11* mRNA expression by treatment with the FXI ASO is associated with a reduction on functional FXI in plasma. Similar to a previous study,[5] we observed a significantly prolonged aPTT in control mice treated with FXI ASO compared with control mice treated with control ASO (Control ASO vs. FXI ASO [mean ± standard deviation] = 27.02 ± 1.40 seconds vs. 31.5 ± 2.63 seconds, $p = 0.01$, unpaired *t*-test). Finally, we measured levels of plasma TAT complexes as a marker of activation of coagulation in control mice and tumor-bearing mice treated with control ASO or FXI ASO. We found that a reduction of *F11* expression did not reduce either the basal level of TAT complexes in control mice or the elevated level of TAT complexes in tumor-bearing mice (►**Fig. 1E**). Therefore, our data indicates that FXI does not contribute to the activation of coagulation in control mice or in tumor-bearing mice. A previous study showed that the extrinsic pathway mediates idling of the coagulation cascade because levels of FIX activation peptide were reduced in individuals with FVII deficiency but not in individuals with FXI deficiency.[23]

The relative contribution of the extrinsic and intrinsic pathways to cancer-associated thrombosis may be different with various cancers. This may depend, in part, on the levels of TF and polyphosphate on the EVs. In pancreatic cancer, the high level of TF expression by EVs may drive the activation of coagulation independently of the intrinsic pathway. In contrast, in prostate cancer, in which there are lower levels of TF expression and higher levels of polyphosphate, the intrinsic pathway may amplify the coagulation cascade and contribute to venous thromboembolism.

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Conflict of Interest
T.K. received a fellowship from SENSHIN Medical Research Foundation during the conduct of the study. A.S.R. and J.R. C. are employees of Ionis Pharmaceuticals. The remaining authors declare no competing financial interests. A.S.R. reports other from Employee and stockholder of Ionis Pharmaceuticals, outside the submitted work.

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