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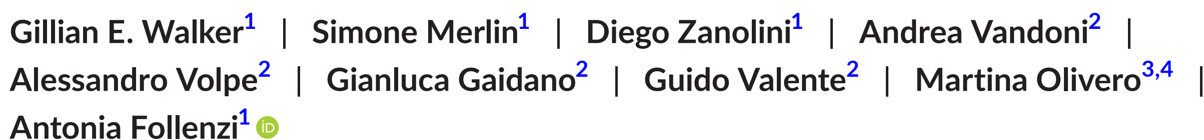
ORIGINAL ARTICLE

Factor VIII as a potential player in cancer pathophysiology



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Abstract

Background: Trousseau sign was the first demonstration of a close relationship between cancer and thrombosis. Currently, venous thromboembolism (VTE) is five to six times more likely to occur in cancer patients, whereas there is a greater risk of cancer diagnoses following thromboses. In considering novel players, factor VIII (FVIII), an essential coagulation cofactor with emerging extracoagulative functions, has been identified as an independent VTE risk factor in cancer; however, the basis of this increase is unknown.

Objective: To investigate the possible direct expression and secretion of FVIII by cancer cells.

Methods: Bladder cancer, with a high VTE risk, and normal bladder tissue and epithelium, were used to investigate FVIII. Factor VIII protein and secretion were examined in bladder cancer cell lines. Expanding to other cancers, the Cancer Cell line Encyclopedia database was used to analyze FVIII, tissue factor, FV, FVII, FIX, FX, and von Willebrand factor (VWF) mRNA in 811 cell lines subdivided according to origin. Factor VIII protein synthesis, secretion, and bioactivity were investigated in a profile of cancer cell lines of differing origins.

Results and conclusions: Although expressed in the normal bladder epithelium, FVIII mRNA and protein were higher in matched bladder neoplasms, with synthesis and secretion of bioactive FVIII evident in bladder cancer cells. This can be extended to other cancer cell lines, with a pattern reflecting the tumor origin, and that is independent of VWF and other relevant players in the coagulation cascade. Here, evidence is provided of a possible independent role for FVIII in cancer-related pathophysiology.

KEYWORDS
cancer, expression, factor VIII, secretion, thrombosis

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1 | INTRODUCTION

In the physiological state, an equilibrium exists between the systems of coagulation and fibrinolysis. With cancer, this equilibrium is disrupted, with patients prone to present with a range of hemostatic disorders.[1,2] As described by Armand Trousseau more than a century ago and more recently others in large population studies, thrombotic diseases with no foundation could also be a clinical marker for occult cancer.[2–4] Whether cancer activates a prothrombic switch and/or coagulation-related players stimulate tumor growth and metastasis,[2,3] the underlying links remain to be clearly defined.

It has been observed that cancer-associated thrombosis is predominant for certain types of malignancies, in particular pancreas, stomach, gastrointestinal, lymphoma, ovarian, and primary brain tumors,[5] with a high venous thromboembolism (VTE) risk also evident for bladder cancer.[5,6] In examining the established mechanisms relating cancer to thrombosis, tumor cells and/or stromal tissue influence the hemostatic pathway and cancer pathophysiology through the release of procoagulant proteins such as tissue factor (TF) and inflammatory cytokines.[7–9] In bladder cancer, increased serum TF levels have been reported[10]; however, recent studies of malignant bladder cancer cell lines demonstrated that, in contrast to TF, tumor cell-derived vascular endothelial growth factor-A (VEGF-A)[11] and interleukin-1[12] were the functional proteins. Both promote the release of von Willebrand factor (VWF) from endothelial cells (ECs),[11,13,14] an important cancer-related biomarker.[15–17] In addition to being a mediator of platelet–endothelial adhesion and platelet aggregation, VWF also functions as a carrier protein of factor VIII (FVIII), an essential cofactor in the coagulation cascade[18] with relevant emerging extracoagulative functions.[19] Several epidemiological studies have reported high levels of circulating FVIII in cancer patients, and that FVIII is independently associated to an increased VTE risk.[20–22] The basis of these elevated levels, however, remains to be understood.

In examining the potential causes of elevated circulating FVIII levels in cancer patients, it should be considered not only the sites of FVIII production, both physiological and cancer-related, but also that a body's response to cancer shares many wound healing and inflammatory proteins. In a physiological state, hepatocytes have long been considered the principal producers of FVIII; however, it is now largely accepted that the main production comes from the liver sinusoidal endothelial cells,[23,24] with FVIII also produced by organ-specific ECs, and hematopoietic cells including monocytes, macrophages, and megakaryocytes.[25] In exclusively damaged ECs *in vitro*, exogenous VEGF has been demonstrated to increase FVIII directly,[26] whereas tumor-associated macrophages attracted by chemokines to solid tumors, are well recognized for producing angiogenic and growth-promoting factors.[27] Although EC damage and inflammatory cell populations such as tumor-associated macrophages in the tumor microenvironment may contribute to the elevated FVIII levels seen in cancer patients, it remains unknown the contribution of the tumor cells themselves, with no direct evidence of FVIII synthesis to date.

Based on these observations, the goal of this investigation was to examine the expression/synthesis of FVIII in cancer, using bladder cancer as the model system. Herein, we demonstrate that FVIII expression is higher in bladder cancer with respect to normal bladder tissue, and it is synthesized and secreted by bladder cancer cells. Further, we demonstrate that this can be extended to cancers of differing origins with a pattern independent of VWF and other relevant players in the coagulation cascade, therefore providing evidence of a possible independent role for FVIII in cancer-related pathophysiology.

ESSENTIALS

- Factor VIII (FVIII) has been identified as an independent vascular thromboembolism (VTE)-risk factor in cancer, however, the basis of this increase is unknown.
- Investigate the direct expression and secretion of FVIII by cancer cells.
- Observation that FVIII is synthesized and secreted in a bioactive form directly by cancer cells in a pattern that reflects the origin and is independent of the expression of other coagulation factors.
- Evidence is provided of a possible independent role for FVIII in cancer-related pathophysiology.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

The human cell lines used are described in Table S1. The human bladder carcinoma cell line ECV-304 was grown and maintained in medium 199 with 10% fetal bovine serum (FBS; EuroClone) and 1% penicillin/streptomycin (P/S; Sigma Aldrich). Dulbecco modified Eagle medium (DMEM; EuroClone) supplemented with 10% FBS and 1% P/S was used for the following human cancer cell lines: bladder cancer line 5637 and all bone, breast, prostate, gastric, colon, and liver cancer lines used in the study (Table S1). Cancer cell lines grown in Roswell Park Memorial Institute medium with L-glutamine (EuroClone) supplemented with 10% FBS and 1% P/S included: thyroid, specifically WRO and 8505C; the hematopoietic cell (HC) lines U-937, Jurkat, and VL51; and all ovarian cancer cell lines (Table S1). The following human cancer cell lines were grown in Roswell Park Memorial Institute medium with L-glutamine and supplemented with 15% FBS and 1% P/S were the HC lines DOHH2, VAL, ROS50, and OCI-Ly8, whereas the thyroid cancer line FTC-133 was grown in DMEM/Ham's F12 (EuroClone) supplemented with 10% FBS and 1% P/S. The neuroblastoma cell lines were grown in Opti-MEM (EuroClone) supplemented with 15% FBS, 1% non-essential amino acids (Sigma), and 1% P/S. Chinese Hamster Ovary (CHO) cells and CHO previously transduced at multiplicity of infection 20 with a lentiviral vector (LV) containing the B domain-deleted form of human FVIII under the control of the ubiquitous PGK promoter (LV-PGK-FVIII), used as negative and positive controls

respectively for the FVIII antigen and activity assays, were maintained in DMEM:F12 supplemented with 10% FBS and 1% P/S. All cell lines were maintained and treated at 37°C in 5% CO_2.

To examine the secretion profile and activity of FVIII in the cancer cell lines described here, each were grown to 75%–80% confluency in maintenance medium. Following serum clearance, the cells were treated with the appropriate serum-free medium (SFM) for 24 h. At completion, the conditioned medium (CM) was centrifuged twice, first at 300*g* 4°C for 10 min to pellet suspension cells, and then a second centrifugation at 2400*g* 4°C for 10 min to remove debris, with the CM stored at −80°C before sample processing. In parallel, the cells were processed for whole cell lysates.

2.2 | Bladder epithelial cell isolation and culture

Human bladder epithelial cells were isolated from cystectomy specimens obtained from the University Hospital "AOU Maggiore della Carità" di Novara, according to previous methods.[28] In brief, from approximately 1 cm^2 of tissue cut from bladder lumen, the epithelium was gently detached from the stroma by scraping into DMEM supplemented with 10% FBS, 2 mM glutamine, penicillin 100 U/ml (2X), and streptomycin 100 μg/ml (2X; Sigma/Merck). The clusters of cells collected were washed twice in 1X PBS and resuspended in serum-free EpiLife medium containing 2 mM glutamine, penicillin 50 U/ml, and streptomycin 50 μg/ml (Thermo Fisher) supplemented with 1X human keratinocyte growth supplement (Thermo Fisher). The cell suspensions were seeded onto 35-mm tissue culture dishes and incubated at 37°C 5% CO_2. After 1 week, colonies of epithelial cells were evident. Cells were passaged 1:3 every 4–5 days and were maintained for up to eight passages (P).

2.3 | RNA isolation and RT-PCR

For total RNA isolation, snap-frozen matched healthy and tumor bladder tissue samples obtained from cystectomies (pathology laboratory, University Hospital "AOU Maggiore della Carità" di Novara, Italy), ECV-304 bladder cancer cells, and isolated primary bladder epithelial cells grown *in vitro*, were mechanically disrupted in Isol-RNA Lysis Reagent (Thermo Fisher). Total RNA concentrations and quality were assessed by spectrophotometry and gel electrophoresis, respectively. Complementary DNA was obtained from 1 μg of total RNA using Maxima H Minus First Strand cDNA Synthesis Kits (Thermo Fisher). All PCR reactions were performed with GoTaq Flexi DNA polymerase (Promega).

The PCR protocols used were as follows: denaturation at 94°C for 30 s, annealing at 56°C for 25 cycles (β-Actin) or 60°C for 30 cycles (hFVIII) and a final extension at 72°C for 7 min. The PCR reactions were performed with the following primers: hFVIII forward- TGTCCACTGAAATGAATAGAATGGAT; FVIII reverse-CAA TGGCTACATAATGGATACACTA CCT; hβ-actin forward: GAGAAAATCT GGCACCACACC, hβ-actin reverse: CGACGTAGCACAGCTT CTC. The PCR products were resolved on 2% agarose gels.

2.4 | Protein sample preparation and western immunoblot

Where described, CLs from normal/neoplastic paired bladder tissue samples and cancer cell lines were mechanically disrupted in RIPA buffer (50 mM Tris pH7.5, 150 mM NaCl, 1% NP40, 1% DOC, 0.1% SDS, 1X complete EDTA free protease inhibitor [1XPI] cocktail [Merck Life Science Srl]), with concentrations determined using the Pierce BCA Protein Assay (Thermo Fisher). Sample quality was assessed by running 1 μg on a 10% PAGE stained with Sypro Ruby Protein Stain (BioRad). Image capture was performed using ChemiDoc Touch Imaging System (BioRad).

The CM samples stored at −80°C at the time of collection were dialyzed using benzoylated dialysis tubing (cutoff 3 kDa; Merck) against water for 24 h at 4°C, lyophilized (LaboGene, Scanvac CoolSafe), and resuspended in 300 μl in 1X PBS with a 1X PI to create concentrated CM (cCM) samples. Sample quality of the cCMs were assessed by running 5 μl against albumin (1 μg) on a 10% PAGE stained with Sypro Ruby.

Where described in the study, all samples and recombinant FL-hFVIII control (Kovaltry, Bayer), were size fractionated on 8% PAGE under reducing conditions and electro-transferred to immuno-blot polyvinylidene difluoride membrane (BioRad). Membranes were incubated either with polyclonal human anti-FVIII (Booster Biological Technology), monoclonal human anti-FVIII GMA-012 (Green Mountain, Burlington, VT), monoclonal human anti-FVIII GMA-8025 (Green Mountain), monoclonal human anti-albumin (Santa Cruz Biotechnology Inc), and detected with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Merck). Total protein was assessed by Sypro Ruby or anti-βActin-HRP conjugated (1:20000; Merck). Immunoreactive proteins were detected using Clarity enhanced chemiluminescence (BioRad) with image capture performed using ChemiDoc Touch Imaging System.

2.5 | Immunostaining

Paraffin-embedded normal and neoplastic tissues were analyzed by immunohistochemistry (IHC) and immunofluorescence (IF). A previously well-characterized in-house polyclonal anti-FVIII antibody that recognizes full-length human FVIII with a sensitivity of 0.6–1 ng/ml, was used.[25] Preimmune rabbit serum collected before FVIII immunization was used as a control. The IHC and IF analyses were performed according to standard procedures. In brief, immunostaining of 5–6 μM sections were done using the in-house polyclonal anti-FVIII (1:1000) with Dako EnVision, which uses diaminobenzidine as the chromogen (Dako). Counterstaining was done with Mayer hematoxylin Diapath (Diapath SpA). All sections were analyzed, and images captured using a Nikon Eclipse E600 optical microscope (Nikon Instruments Europe BV).

Immunofluorescence analyses were performed on normal and neoplastic bladder samples, and isolated bladder epithelial cells. In brief, 5–6 μM paraffin-embedded tissue sections were treated in boiling 50 mM EDTA pH8 for antigen retrieval and blocked for 1 h (5% normal

goat serum, 1% BSA, 0.1 % Triton-X-100) at room temperature. *In vitro* bladder epithelial cells were fixed in 4% PFA for 10 min at 4°C. The cells were permeabilized with ice-cold 0.5% Triton-X-100 in PBS for 10 min and blocked as described previously. Factor VIII was detected the in-house polyclonal anti-FVIII (1:200) in combination with monoclonal anti-cytokeratin 7 (CK7; OV-TL 12/30, Dako, 1:500), in blocking buffer for 90 min at room temperature. Following washes in PBS, secondary goat anti-rabbit ALEXA Flour 488 and goat anti-mouse Flour 546 antibodies (1:500; Molecular Probes, Thermo Fisher Scientific) were added in 1% BSA, 0.1% Triton-X-100 in PBS for 45–60 min. Nuclei were stained with DAPI-Antifade reagent (Molecular Probes, Thermo Fisher Scientific). All samples were visualized and images captured using a Leica DM 2500 florescent microscope (Leica Instruments Europe BV) and analyzed with LASX software (Leica Application Suite, Leica Instruments).

2.6 | Factor VIII antigen and activity assays

An antigen assay for the quantification of FVIII in cancer cell CM was performed using a commercially available kit (Affinity Biologicals Inc), as previously described.[29] The FVIII activity was assessed by two methods: (1) a 1-stage APTT assay using a Coatron M4 coagulometer (TECO Medical Instruments) with a Hemosil Synthasil kit (Instrumentation Laboratory), as described previously[29]; and (2) a two-stage fluorometric FVIII assay using the Factor VIIIa Activity Assay Kit (BioVision Inc) according to the manufacturer's instructions. Standard curves for both FVIII antigen (0–200 ng) and activity assays (0–100 ng) were generated by serially diluting FVIII in culture medium. Results are expressed as ng/ml ± SD with samples analyzed in duplicate from $n = 3$ samples per cell line.

2.7 | Statistical analysis

Data are expressed as mean ± SD, where indicated. A repeated measured ANOVA with Tukey multiple comparison was used to determine differences in the mRNA expression of FVIII, TF, FV, FVII, FIX, FX, and vWF. Correlations between FVIII and TF mRNA was done using Pearson correlation coefficients. Statistical significance was assumed for $p < .05$. The statistical analyses were performed with GraphPad Prism version 8.0 (GraphPad Software).

3 | RESULTS

3.1 | FVIII protein is expressed in the epithelial cells of normal urothelium

Factor VIII was detected in the epithelial cells of normal bladder tissue by IF using a previously characterized in-house polyclonal anti-FVIII antibody costained with CK7, an intermediate filament protein present in epithelial cells (EpC; Figure 1A). Factor VIII positivity was observed in a repeatable manner in EpCs of the urothelium, shown by CK7 costaining, in all samples analyzed ($n = 3$; Figure 1A), whereas controls with preimmune serum were negative (Figure S1). Although only qualitative, a stronger positivity was observed in the EpCs facing the lumen of the transitional epithelium, whereas FVIII positivity independent of CK7 was also observed randomly in cells bordering the basal cell layer.

To confirm the EpC synthesis of FVIII, bladder EpCs (BEpCs) from normal bladder tissue ($n = 10$) were isolated and cultured. Epithelial cells were successfully isolated from five samples. In the most successful isolation, the BEpCs were maintained in culture for >1 month and up to P8 before senescence (Figure 1B). Factor VIII mRNA in BEpCs (P1–P5) and ECV-304, a bladder carcinoma cell line with endothelial-like characteristics, was detected and as early as P1 for BEpCs, with levels sustained in later passages (Figure 1C). Further, FVIII and CK7 costaining by IF confirmed the positive expression of FVIII protein in BEpCs, with FVIII localization perinuclear with a punctate pattern (Figure 1D). Preimmune serum was negative (Figure S2).

3.2 | Factor VIII protein is present in human bladder carcinomas predominantly in neoplastic cells

In a total of nine urothelial carcinomas, including papillary, *in situ*, and invasive carcinomas, FVIII protein was analyzed by IHC. Interestingly, almost all urothelial neoplasms (7/9) were positive for FVIII in the cytoplasm of neoplastic cells, regardless of the type of carcinoma, with the intensity of expression increasing from bladder dysplasia, putative precursors of bladder cancer, through to invasive carcinomas (Figure 2). The strongest expression was observed in the invasive bladder carcinomas, whereas two cases with diffuse squamous metaplasia were negative (data not shown). The normal urothelium was slightly positive.

3.3 | Factor VIII in human bladder carcinomas is synthesized and secreted by the neoplastic cells

To establish if bladder tumors express different levels of FVIII with respect to normal bladder tissue, sample sets of normal bladder mucosa and tumor tissue from 14 subjects who underwent a radical cystectomy, were collected. In RT-PCR experiments performed on the first seven sample sets (patients 1–7), FVIII mRNA was detected in all samples. Although only qualitative, we observed in five of the seven subjects analyzed a higher expression of FVIII mRNA in the tumor tissues, when compared with adjacent apparently healthy tissue (Figure 3A). This observation was further supported by intracellular FVIII protein expression in a second set of samples ($n = 7$; patients 8–14; Figure 3B). In six of the seven subjects analyzed, a higher expression of FL-FVIII protein was detected by western immunoblot (WIB) in the tumor tissue (Figure 3B).

To determine whether the FVIII detected in the bladder carcinomas is synthesized by the neoplastic BEpCs, costaining with FVIII and CK7 was performed ($n = 5$). As demonstrated, FVIII is synthesized by the neoplastic BEpCs, with an intense signal observed at the

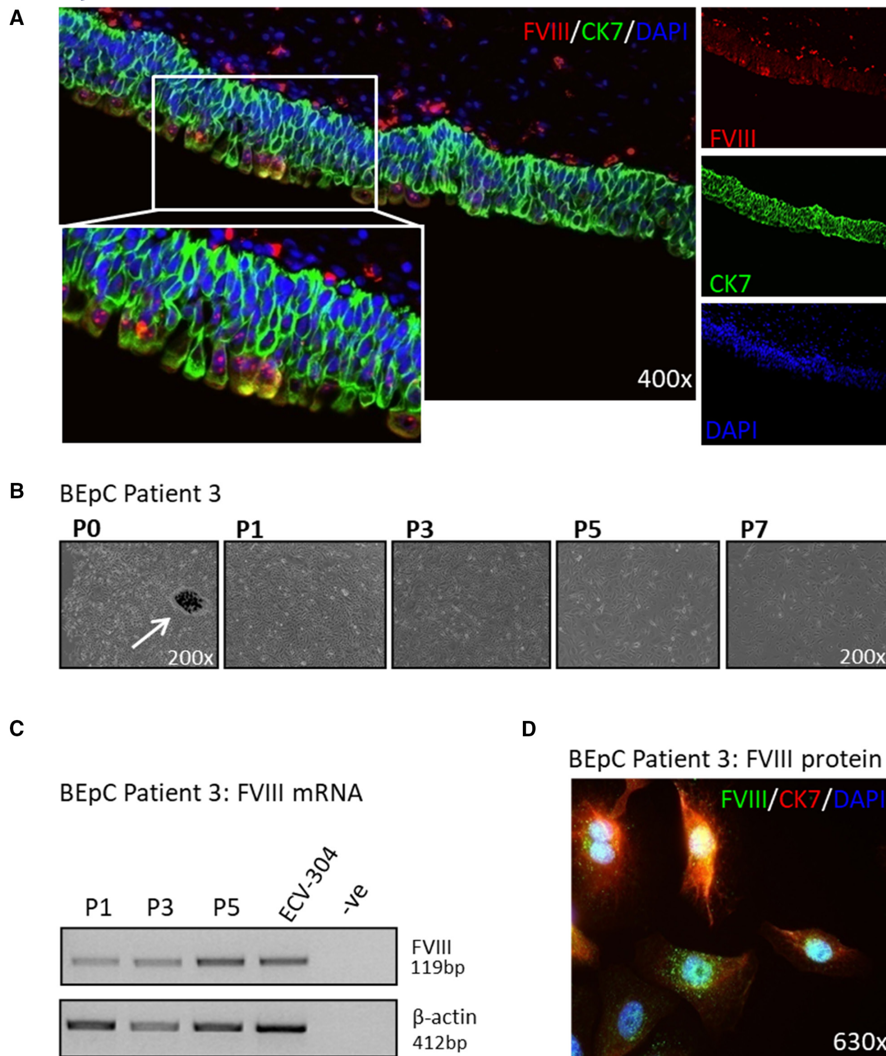

FIGURE 1 Factor VIII is expressed in the normal human urothelium epithelium. (A) Representative image of immunofluorescence performed on sections of normal bladder tissue ($n = 3$) using anti-FVIII antibody (1:1000), costained with the epithelial marker CK7 (1:500). Magnification 400×. (B) Primary culture of normal human BEpC isolated from patient 3 (bladder epithelial cells [BEpC3]) shown from passage (P)0 to P7 ($n = 5$ patients). White arrow indicates one of the colonies of isolated EpCs. Magnification 200×. (C) Factor VIII and the internal control β-actin mRNA analyzed by RT-PCR in BEpC3 with increasing passages (P1, P3, P5) and the bladder cancer cell line ECV-304. (D) Representative immunofluorescence image of FVIII protein costained with CK7 in the isolated bladder epithelial cells at P4 from BEpC3. Magnification 630×

advancing tumor border (Figure 3C), whereas staining with preimmune serum was negative (Figure S3).

Because the bioactive form of FVIII is a secreted protein, we wanted to establish whether FVIII, when synthesized, is secreted by BEpCs. Using two human bladder cancer cell lines, ECV-304, with endothelial-like characteristics, and 5637, a grade II bladder carcinoma, we evaluated by WIB the synthesis and secretion of FVIII in SFM after 24 h (**Figure 3D**). Both cell lines were healthy after 24 h in SFM and expressed intracellularly similar levels of FL-FVIII protein. In contrast, the secretion profile for FVIII from both cell lines was quite distinct (Figure 3D), with 5637 cells secreting higher levels of FL-FVIII and processed forms, when compared with ECV-304 cells.

3.4 | Human FVIII is expressed in human cancers

Establishing that bladder cancer cells can synthesize and secrete FVIII, we wanted to determine whether this could be extended to other types of human cancers. We investigated FVIII protein expression by IHC in a series of human tumors of differing types and origins ($n = 90$; Table S2).

Considering the results collectively, a strong positive expression was observed in the majority of the tumors, where FVIII was observed as a diffuse staining in the cytoplasm or as small perinuclear aggregates in neoplastic cells (Figure 4). With respect to FVIII positivity, a 100% positivity was seen in most tumor categories, excluding endometrial and prostate carcinomas where no expression was detected, whereas a partial positivity was seen in thyroid, urothelial, and ovarian carcinomas (Table S2). As expected, the endothelial cells of the blood vessels were selectively positive (Figure 4); low-pressure vessels were consistently positive (veins), whereas high-pressure vessels were negative or faintly positive (arteries). Controls with preimmune serum were negative.

Although the sample sizes are small, from the IHC results for FVIII in all the human tumors, we observed three possible categories based on qualitative levels and distribution (Figure 4; Table S2). In the first category, FVIII expression showed a medium–strong positivity in all neoplastic cells and all samples. These included neoplasia of the gastrointestinal tract, pancreatic adenocarcinomas (data not shown), and lymphoma. Among liver tumors, hepatocellular carcinomas were positive. A proportion of the renal cell carcinomas were positive; all clear cell carcinomas ($n = 5$) were positive, whereas for

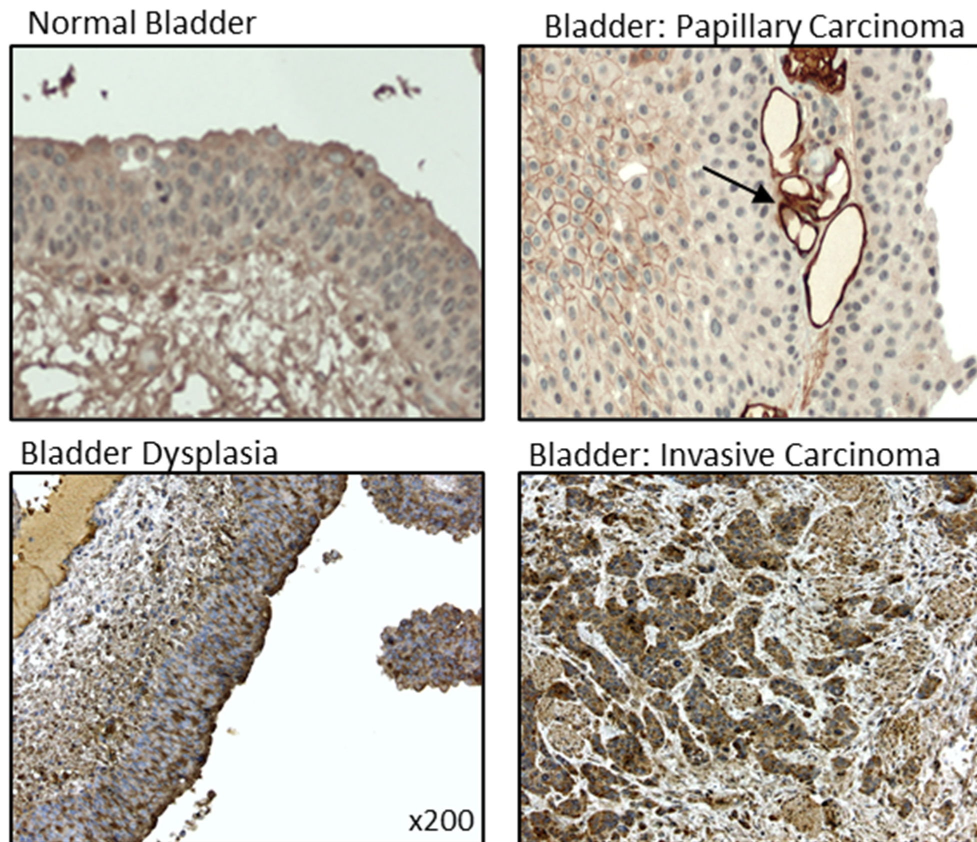

FIGURE 2 Factor VIII protein is present in human bladder carcinomas. Representative photomicrographs of human FVIII antigen using an in-house polyclonal anti-FVIII in normal bladder tissue, papillary carcinoma, bladder dysplasia, and invasive bladder carcinoma. Positive immunoreactivity shown by the dark brown stain. Magnification 200×

the renal papillary carcinomas (n = 4) only type 2 (n = 2), the more aggressive tumors, were FVIII-positive. All cases of breast infiltrating ductal carcinomas and lung adenocarcinomas were positive; however, the staining showed a lower intensity. All urothelial neoplasms, as described previously, fall into this category.

In the second category of tumors, FVIII positivity was only present in a percentage of the neoplasms investigated, and in some cases, this was dependent on the histological type/stage. For example, three of five ovarian serous carcinomas were positive and had expression specific to neoplastic cells. For the thyroid papillary carcinomas (n = 7), FVIII localized exclusively to the tall cells, which are an indicator of clinically more aggressive tumors.

Finally, in a third category, including endometrial and prostatic adenocarcinomas, and thyroid follicular carcinomas (n = 3), FVIII expression was very low or negative. In prostatic adenocarcinoma, FVIII positivity was evident only in the stromal smooth muscle cells.

3.5 | Factor VIII expression with respect to other key factors of the coagulation cascade in human cancer cell lines

Factor VIII expression was examined across a representative range of established cell lines from several tissues/fluids to determine if there is an origin-related expression, and then with respect to other players in the coagulation cascade. Using the Cancer Cell line Encyclopedia database from the Broad Institute (https://portals.broadinstitute.org/ccle), which contains an expression profile of 41 496 genes in 934 human cancer cell lines of different origins, FVIII mRNA expression was investigated. In the Cancer Cell line Encyclopedia database, mRNA expression levels were obtained using Affymetrix U133 plus 2.0 arrays with RMA normalization, with the final data expressed as the log2 gene expression signal (log2 signal).[30] Of the 934 cell lines, FVIII expression was available for 811, which in turn were subdivided according to their cancer origin (16 groups). According to the average FVIII expression, the groups were assigned from the highest expressing group, thyroid (7.24 ± 1.14 log2 signal), to the lowest, neuroblastoma (5.15 ± 0.58 log2 signal) with a significant difference in expression seen between all groups ($p < .0001$; Figure 5A). Among the top five expressing groups (thyroid, lung, HCs, bone, pancreas), similar levels of FVIII were seen from lung (6.79 ± 1.22 log2 signal) to pancreas (6.75 ± 1.15 log2 signal).

Because of the tight relationship between VWF and FVIII, we examined VWF expression in the same panel. In contrast to FVIII expression, very low levels of VWF mRNA were detected (Figure 5B). A significant difference, however, was seen in VWF mRNA between groups, explained by significant differences between HCs and the lung ($p < .001$), stomach ($p < .05$), colon ($p < .05$), and liver groups ($p < .05$).

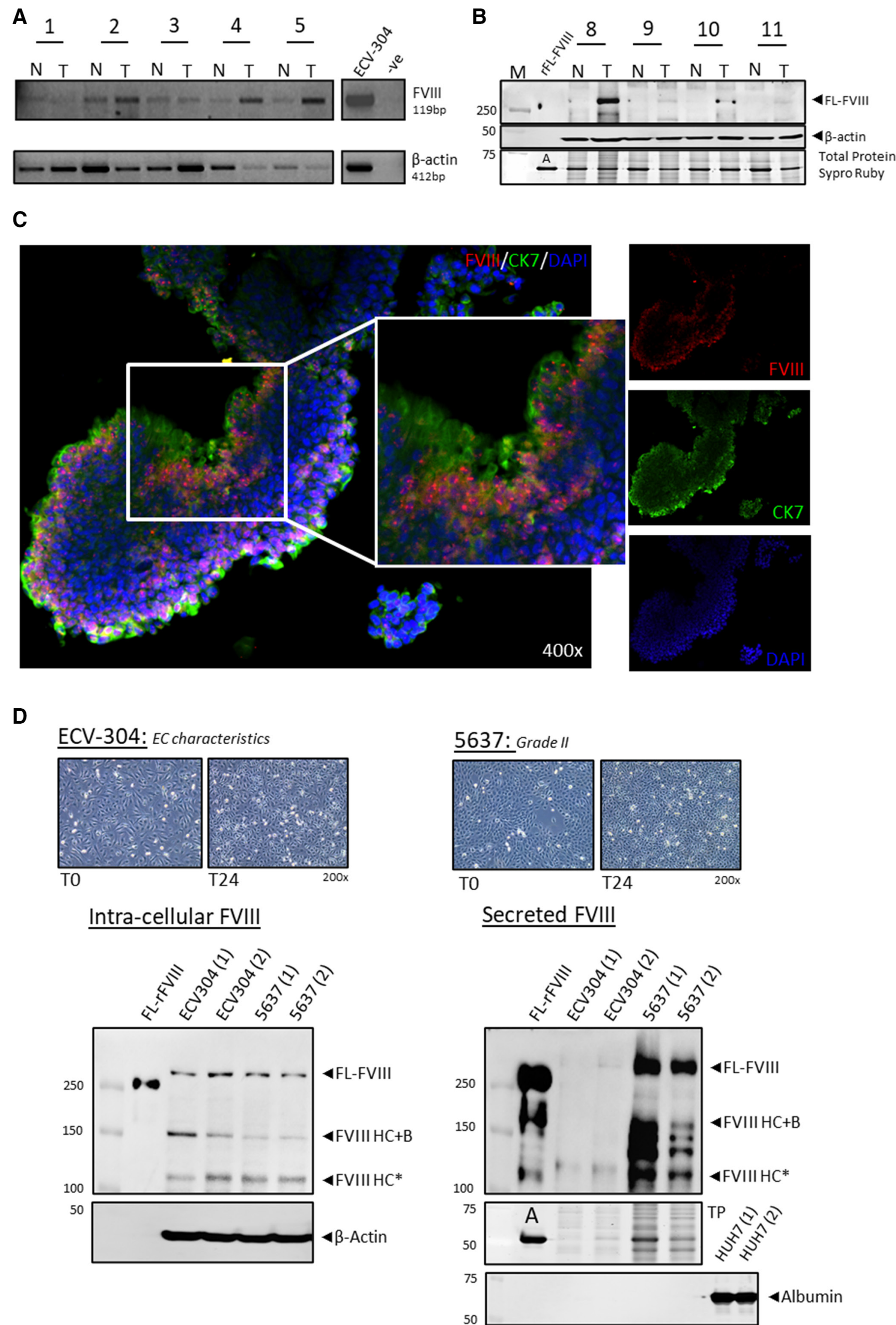

FIGURE 3 Factor VIII protein is synthesized and secreted by bladder carcinoma cells. (A) Factor VIII and the internal control β-actin mRNA analyzed by RT-PCR in bladder cancer tissue (T) and matched healthy tissue (N) from five (patients 1–5) of the seven patients analyzed. The bladder cancer cell line ECV-304 was used in this case as a positive control. (B) Representative cropped western immunoblot (WIB) of FVIII protein showing FL-FVIII in bladder cancer tissue (T) and matched healthy tissue (N) from four (patients 8–11) of the seven patients analyzed using the monoclonal human anti-FVIII GMA-012 antibody (1:10000). Protein loaded is demonstrated by β-Actin and Sypro Ruby total protein stain of 1 ug of protein. A recombinant human FL-FVIII Kovaltry (rFL-FVIII) was used as a positive control. The experiment was repeated with three independent FVIII antibodies. (C) Representative image of immunofluorescence performed on sections of an invasive bladder carcinoma using anti-FVIII antibody (1:1000), costained with the epithelial marker CK7 (1:500; $n = 3$). Magnification 400×. (D) Representative cropped WIBs of FVIII protein in duplicate both intracellular (20 μg) and in the conditioned medium (20 μl; secreted FVIII) in ECV-304 and 5637 bladder carcinoma cell lines after 24 h in serum-free medium. In the representative WIB, monoclonal human anti-FVIII GMA-012 antibody (1:10000) was used, with β-Actin-HRP (1:20000) or Sypro Ruby total protein serving as loading controls. Albumin was investigated on the FVIII WIB for FBS contaminants. Cell experiments were performed in triplicate with duplicate samples shown for each cell line and three independent FVIII antibodies used. A, albumin control for total protein gel; FL-FVIII, Kovaltry; FVIII-HC+B, heavy chain +B domain; FVIII-HC, heavy chain

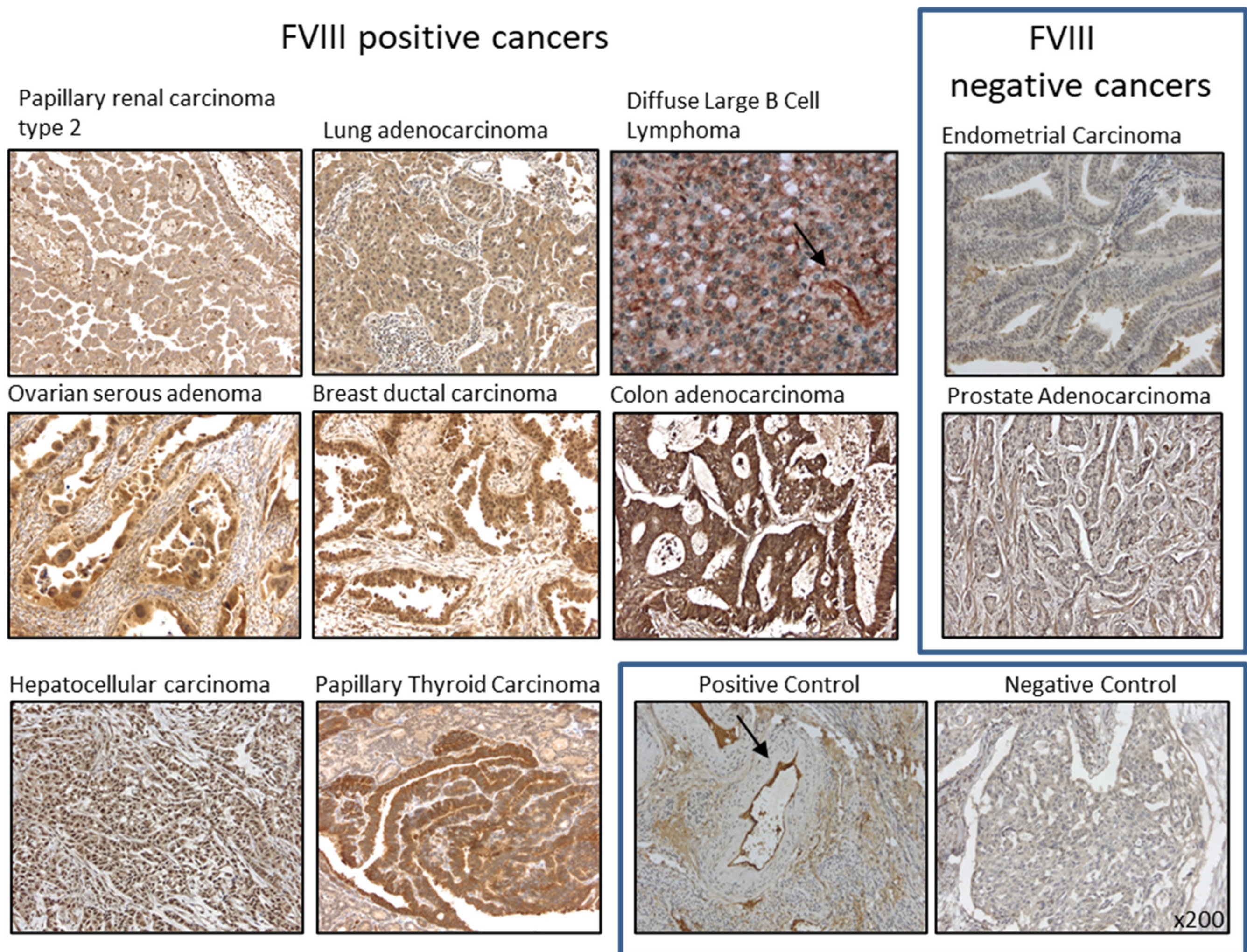

FIGURE 4 Factor VIII protein is present in a range of human cancers. Representative photomicrographs of human FVIII antigen in a range of human cancers using an in-house polyclonal anti-FVIII (1:1000). Positive immunoreactivity shown by the dark brown stain. The samples are divided into FVIII-positive and FVIII-negative cancers. The black arrows indicate endothelial cells of blood vessels. Negative control is preimmune rabbit serum (PRS). Magnification 200×

With a demonstrated role for TF in cancer and cancer-associated thrombosis, we investigated its expression according to the same groups (Figure 5C). As for FVIII, a significant difference in TF expression was seen between groups ($p < .0001$). On average, the TF expression levels were higher with respect to FVIII, excluding however, HCs, bone, skin, and neuroblastoma. In the case of bone and skin, there was no significant difference between TF (bone: 7.2 ± 1.96 log2 signal; skin: 6.81 ± 2.02 log2 signal) and FVIII (bone: 6.76 ± 1.18 log2 signal; skin: 6.73 ± 1.36 log2 signal). Likewise, for neuroblastoma, both TF (5.26 ± 1.2 log2 signal) and FVIII (5.15 ± 0.58 log2 signal) expression levels were similar but the lowest overall. Of interest were the results of the HC group (n = 190), which although having one of the highest average FVIII expression levels (6.78 ± 1.17 log2 signal), had the lowest level of TF (4.89 ± 1.09 log2 signal). No correlation was seen between the two factors in HCs (FVIII vs. TF, r = .083, ns).

In a similar fashion, we investigated other key players in the coagulation cascade; FV, FVII, FIX, and FX (Figure S4). For FVII, mRNA levels were evidently lower than those seen for FVIII and TF across the groups. In the case of FIX, no significant differences were seen among groups, whereas for FV ($p < .0001$), FVII ($p < .0001$), and FX ($p < .0001$), differences in expression were observed. In multiple comparison tests, the significant differences for FV were seen for liver (n = 28) and stomach (n = 39), whereas for FVII, liver and breast (n = 59). For FX, exclusively liver.

3.6 | Factor VIII protein synthesis and secretion shows origin-dependent expression patterns

Because of the limited correlation between mRNA and protein synthesis, we evaluated FVIII protein synthesis and secretion from n = 27 cell lines subdivided according to their origins (Table S1). Of the 16 groups investigated at the mRNA level, we were able to evaluate 11 of these, excluding the lung, pancreatic, skin, kidney, and endometrial groups. Although only representative and not quantitative,

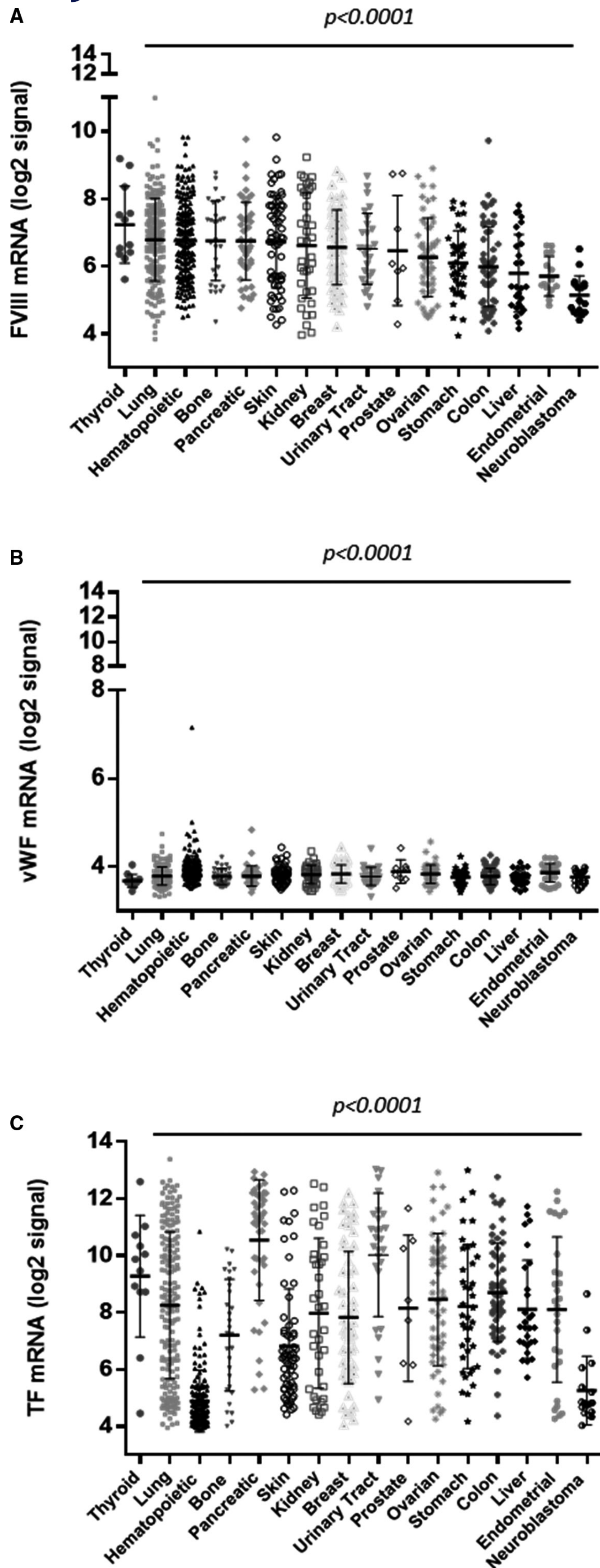

FIGURE 5 Factor VIII mRNA expression with respect to von Willebrand factor (VWF) and tissue factor (TF) in human cancer cell lines. The mRNA levels are expressed as the mean of log2 signal ± SD and are subdivided according to tumor origin into 16 groups. The groups are organized from the highest expressing FVIII group (thyroid) to the lowest (neuroblastoma) with this organization maintained for all coagulation factors. (A) FVIII expression. (B) VWF expression. (C) TF expression

it was observed with three independent FVIII antibodies that all cell lines examined, expressed to differing degrees, FL-FVIII and/or processed forms (Figure 6A–D; Figure S5A–D). Thyroid cancer cell lines, which demonstrated the highest average expression of FVIII mRNA among all groups examined, each expressed intracellular FVIII including FL-FVIII. Of these, however, only one, WRO, secreted detectable levels of FL-FVIII after 24 h, despite the low levels of total protein detected in the cCM (Figure 6A). Expression of intracellular FVIII, but undetectable levels of the secreted protein, was also observed for one breast cell line (MCF7), prostate, a colon line (Caco2), and both neuroblastoma cell lines (Figure S5B,C). For all other cell lines, FVIII was detected in the CM after 24 h, with the majority secreting FL-FVIII and no other evident processed forms. Evidence of processed forms of FVIII was only observed for hepatocellular carcinoma (Huh7, HepG2), the high-grade breast cancer cell line, MDA-MB-231, all bone cancer cell lines, one colon line (SW480), and the gastric line (GTL-16). The presence of contaminating serum factors was excluded by the absence of albumin, which was only expected in the liver cancer secretomes (Figure 6A,D). Another interesting observation was seen for the WRO thyroid cancer cell line, where a high molecular weight complex of FVIII (FVIIIc) was detected in addition to FL-FVIII (Figure 6A). This FVIIIc was seen only for one other group, the HCs. Despite the low levels of protein detected in the cCM, all cell lines examined ($n = 6$), regardless as to whether they were lymphoma or leukemia, expressed exclusively the FVIIIc form in the secretome (Figure 6B; Figure S5A).

3.7 | Secreted factor VIII protein from cancer cell lines is bioactive

To investigate whether the FVIII secreted by cancer cells is bioactive and could potentially contribute to the thrombotic risk seen in cancer patients, the CM from cancer cell lines selected from four different groups (liver, bone, HC, bladder), in addition to the control cells CHO and CHO transduced with LV-PGK-FVIII (CHO-FVIII), were analyzed at the same time for FVIII antigen and FVIII activity by two independent methods. The CM from cells was used as opposed to cCM because of the inactivity of FVIII seen in concentrated samples when performing the activity assays (data not shown). The FVIII antigen assay confirmed the presence of FVIII in CM in the cell lines investigated, with the exception the two osteosarcoma cell lines (Figure 7A). Further, the assessment of FVIII activity by both the aPTT and fluorogenic assays, demonstrated that where cancer-derived FVIII could be detected, it was also bioactive (Figure 7B). A very low level of activity in MG63 osteosarcoma cells (0.2 ± 0.1 ng/ml) was detected exclusively by the fluorogenic assay.

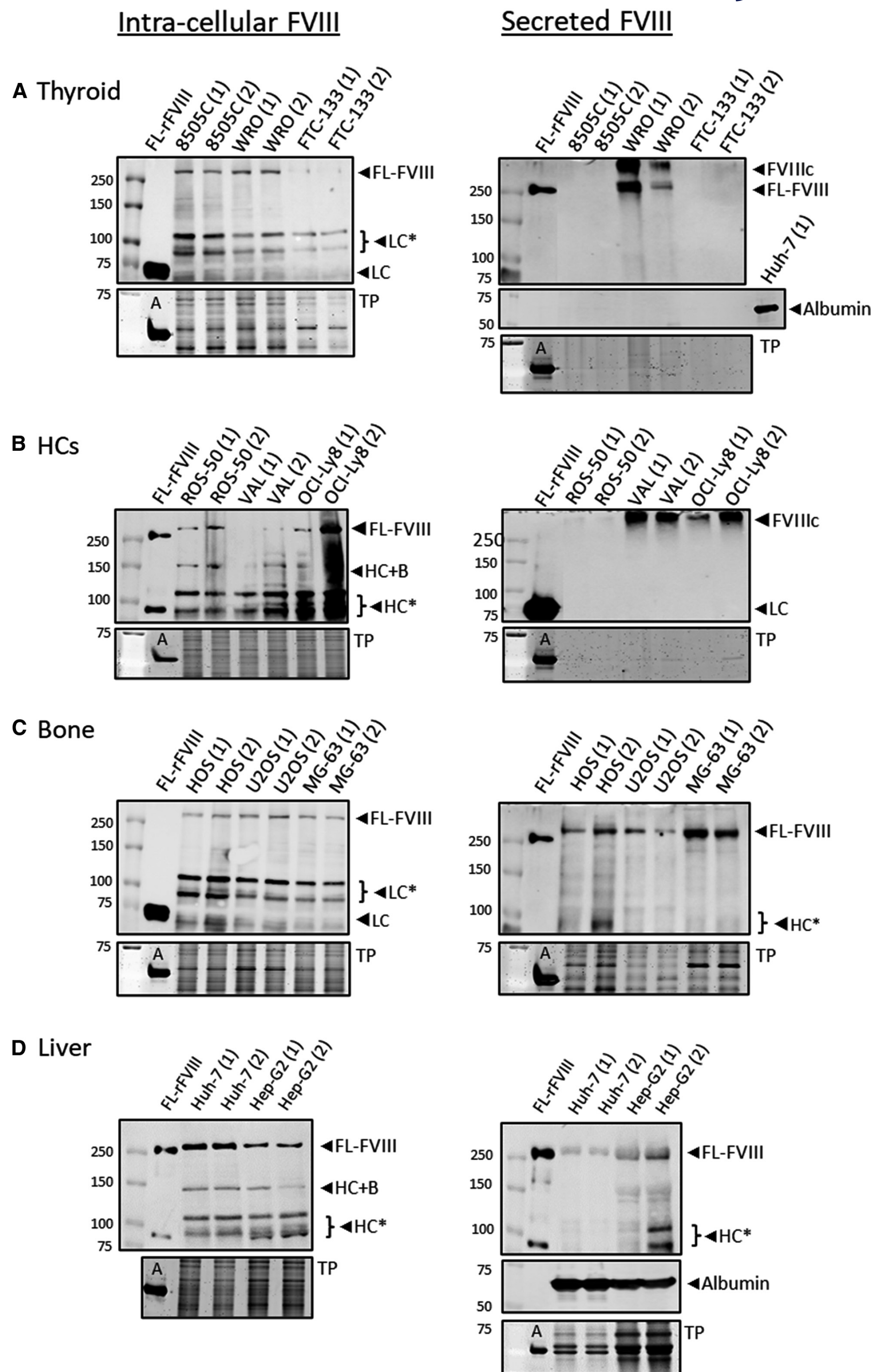

FIGURE 6 Factor VIII is synthesized and secreted by human cancer cell lines. Representative cropped western immunoblots (WIBs) of FVIII protein in duplicate both intracellularly (20 µg) and in the conditioned medium (CM) (20 µl; secreted FVIII) in (A) thyroid, (B) hematopoietic cells (HCs), (C) bone, and (D) liver human cancer cell lines after 24 h in serum-free medium using three independent FVIII antibodies. In the representative WIBs, one of either monoclonal human anti-FVIII GMA-012 antibody (1:10000; (B) CL; (D) CL+CM), monoclonal human anti-FVIII GMA-8025 (1:10000; (A) CL; (B) CM; (C) CL) or polyclonal anti-FVIII (1:5000; (A) CM; (C) CM) is shown, with Sypro Ruby total protein serving as loading controls. Albumin was investigated on the FVIII WIB for FBS contaminants in all CM with results shown only for the thyroid group. Cell experiments were performed in triplicate with duplicate samples shown for each cell line. A, albumin control for total protein gel; FL-FVIII, Kovaltry; FVIIIc, FVIII complex; FVIII-HC+B, heavy chain + B domain; HC, heavy chain; HC*, processed forms of the heavy chain; LC, light chain; LC*, processed forms of the light chain; TP, total protein

4 | DISCUSSION

In the present study, bladder cancer was chosen as the model system to investigate a potential novel tumor-derived coagulative protein, FVIII. Factor VIII was investigated not only because of an independent association to an increased risk of VTE in cancer patients,[20–22] but also because of relevant extracoagulative functions that have recently emerged.[19] Bladder cancer was selected as the model system because of its high VTE incidence rate,[5,6] which correlates with elevated plasma levels of early coagulative markers.[31] In addition, to our knowledge, there are no studies to date related to FVIII production in bladder cancer cells. Here, we show for the first time that FVIII is expressed at a higher level in bladder cancer with respect to matched normal bladder tissue. Further, *in vitro* we demonstrate that FVIII is synthesized and secreted directly by bladder cancer cells, an observation extended to other cancers/cell lines of different origins and histology, with cancer type-specific patterns of expression. Interestingly, when compared with VWF and other coagulation factors integral to the coagulation cascade, FVIII mRNA expression was consistently the highest, except with respect to TF in some but not all groups subdivided according to tumor origin. Finally, in the CM of a group of selected cancer cell lines, FVIII activity could

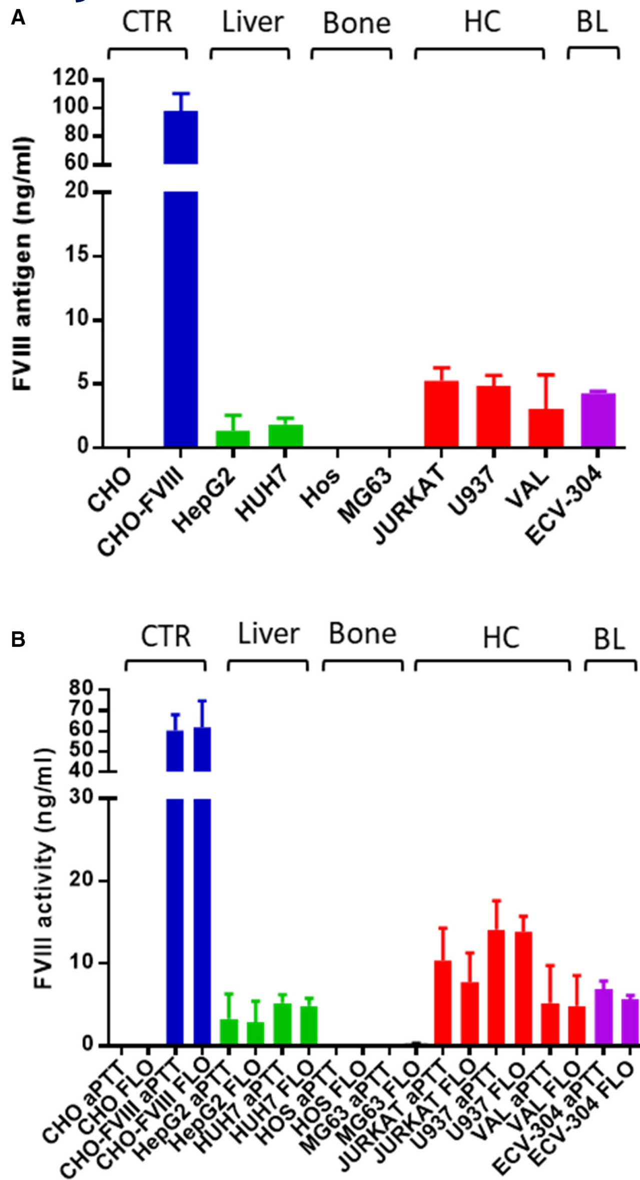

FIGURE 7 Secreted factor VIII protein from cancer cell lines is bioactive. Graphical representation of the measurement of (A) FVIII antigen and (B) FVIII activity by two independent methods (APTT and fluorogenic assay), in the conditioned medium from cancer cell lines selected from four different groups with Chinese Hamster Ovary (CHO) and CHO transduced with LV-PGK-FVIII (CHO-FVIII) serving as negative and positive controls, respectively. Results are given as ng/ml ± SD from triplicate experiments. BL, bladder cancer, CTR, controls

be detected in correspondence to FVIII. Collectively, our observations highlight a potential and possibly independent role for FVIII in cancer pathophysiology.

In considering how FVIII may be differentially regulated in cancer cells, investigations into the regulatory mechanisms in tumor cells that control the production of the hemostatic proteins have largely focused on TF. In several cancer model systems, it has been observed that numerous oncogene and repressor gene mutations involved in neoplastic transformation upregulate TF.[32–35] To date, very little is known with respect to FVIII gene regulation in a physiological setting. An early investigation identified hepatocyte nuclear factor 1α (HNF1α) to be essential for activating transcription of a FVIII minimal promoter.[36] Interestingly, significantly reduced levels of HNF1α have been reported in hepatocellular carcinomas, hepatocellular adenomas, and pancreatic tumors, suggesting HNF1α may play a possible tumor suppressor role.[37,38] Because of the importance of FVIII production by ECs, more recently our laboratory, in the search for EC-specific transcription factors regulating FVIII promoter (pF8) activity, identified that the ETS transcription factors, Ets-1 or an Ets-1/Est-2 combination, can significantly up-regulate FVIII minimal promoters in ECV-304 bladder carcinoma and HEK293T cells.[39] Although ETS transcription factors are involved in normal growth and development, an aberrant dysregulation in cancer is well recognized, with both oncogenic and tumor suppressive roles described for both Ets-1 and Ets-2.[40] With FVIII gene regulation in the physiological state in its infancy, the foundation of the aberrant regulation of FVIII in cancer remains to be established.

There are very few related studies in the literature supporting the concept of a functional role for FVIII in cancer. The most important demonstration of the importance of dysregulated FVIII production/activity, is highlighted by hemophilia A (HA), a congenital bleeding disorder caused by the decrease or absence of FVIII activity.[41] For the moment, the exclusive treatment regimen for HA patients is the repeated and lifelong administration of FVIII.[42] Although for HA patients there is a systemic improvement with FVIII prophylaxis, the opposite has also been observed, with numerous clinical studies showing an association between high circulating FVIII levels and thrombosis.[43–45] Further, FVIII has been shown to enhance metastasis of B16F10 melanoma cells in an HA mouse model,[46] whereas FVIII gene mutations have been shown to be associated to a good prognosis in patients with cutaneous melanoma.[47] Although the functional significance of our findings remains to be investigated, our demonstration that FVIII is directly synthesized and secreted in an origin-dependent fashion and is expressed at higher levels in comparison to other key coagulation factors provides supporting evidence of a potential role of FVIII in cancer pathophysiology.

In evaluating and treating HA patients with FVIII, researchers are beginning to understand that FVIII, beyond its established role, has extracoagulative functions.[19] Although the limitations of the present study are its descriptive nature as opposed to quantitative and low sample numbers, the differential pattern of expression and secretion of FVIII according to tumor origin seen within this study, supports the hypothesis of possible multifaceted functions for FVIII in cancer. Although the mechanisms, to date, are not known, direct FVIII-related effects have been seen at the cardiologic,[48] angiogenic,[29,49] and bone level.[50] Only for bone has a direct mechanism been established, where FVIII has been shown to regulate osteoblast development by the receptor activator of nuclear factor kappa-B ligand, a key regulator of bone remodeling.[51–53] Whether FVIII can in fact play a direct role in tumor growth and metastasis remains to be demonstrated.

From the observation of Armand Trousseau until today, cancer patients have an increased likelihood of hemostatic disorders. Whether

cancer activates a prothrombic switch and/or coagulation stimulates tumor growth and metastasis, the underlying mechanistic link/s are complex and intricately linked. Between the processes of hemostasis, oncogenesis, angiogenesis, and inflammation, numerous proteins, receptor, and signaling pathways are shared. Factor VIII has a demonstrated role in hemostasis and is a recognized proangiogenic factor, whereas we are beginning to touch on its involvement in inflammation with impaired clotting in HA leading to an increase in inflammation and dysregulation of macrophage differentiation.[54] Although FVIII has been identified as an independent risk factor for cancer-related VTE, and here we demonstrate the synthesis and secretion of bioactive FVIII by cancer/cancer cells in an origin-dependent fashion, a clear functional role for FVIII in oncogenesis remains to be established. Factor VIII may provide another piece to the complex puzzle.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Gillian E. Walker, Simone Merlin, Diego Zanolini, and Antonia Follenzi, with the clinical contribution of Guido Valente, Alessandro Volpe, and Gianluca Gaidano, conceived the study and designed the experiments. Gillian E. Walker, Simone Merlin, Diego Zanolini, and Antonia Follenzi performed the experiments and analyzed the data. Andrea Vandoni and Guido Valente performed all immunohistochemistry experiments and analyzed the results. Martina Olivero performed the bioinformatic analyses of the mRNA data from cell lines. Gianluca Gaidano provided valuable cell lines. Antonia Follenzi generated the funding to support the study. Gillian E. Walker and Antonia Follenzi wrote the paper. All authors critically revised the paper and approved the version to be published.

INFORMED CONSENT

On behalf of all the authors of the publication "Factor VIII as a potential player in cancer pathophysiology", I declare that all the authors have read, critically reviewed the paper, and agree to its publication.

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