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ORIGINAL ARTICLE

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Evaluation of different commercial antibodies for their ability to detect human and mouse tissue factor by western blotting

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Abstract

Background: Western blotting is used to measure protein expression in cells and tissues. Appropriate interpretation of resulting data is contingent upon antibody validation.

Objectives: We assessed several commercial anti-human and anti-mouse tissue factor (TF) antibodies for their ability to detect TF by western blotting.

Material and Methods: We used human pancreatic cancer cell lines expressing different levels of TF and a mouse pancreatic cancer cell line expressing TF with a matched knockout derivative.

Results: Human and mouse TF protein detected by western blotting correlated with levels of TF mRNA in these cell lines. The apparent molecular weight of TF is increased by N-linked glycosylation and, as expected, deglycosylation decreased the size of TF based on western blotting. We found that four commercial anti-human TF antibodies detected TF in a TF-positive cell line HPAF-II whereas no signal was observed in a TF-negative cell line MIA PaCa-2. More variability was observed in detecting mouse TF. Two anti-mouse TF antibodies detected mouse TF in a TF-positive cell line and no signal was observed in a TF knockout cell line. However, a third anti-mouse TF antibody detected a nonspecific protein in both the mouse TF-positive and TF-negative cell lines. Two anti-human TF antibodies that are claimed to cross react with mouse TF either recognized a nonspecific band or did not detect mouse TF.

Discussion: Our results indicate that there is a range in quality of commercial anti-TF antibodies.

Conclusion: We recommend that all commercial antibodies should be validated to ensure that they detect TF.

KEYWORDS

antibody, glycosylation, tissue factor, validation, western blotting

Axel Rosell and Bernhard Moser contributed equally to this work.

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Essentials

- Western blotting is commonly used to detect tissue factor (TF).
- We assessed commercial anti-TF antibodies for their ability to detect either human or mouse TF.
- Four commercial anti-human TF antibodies successfully detected TF in cell extracts from cell lines.
- Detection of mouse TF was more variable with some antibodies failing to recognize mouse TF.

1 | INTRODUCTION

Concerns have been raised about the reproducibility of preclinical research.[1,2] This led the National Institutes of Health to describe the principles and guidelines for reporting preclinical research (https://www.nih.gov/research-training/rigor-reproducibility/principles-guidelines-reporting-preclinical-research). The section entitled "Consider Establishing Best Practice Guidelines" suggests that authors provide a description of biological material "with enough information to uniquely identify the reagent." For antibodies, the source, dilution, and how they were validated should be included. These guidelines have been endorsed by many journals, including *Arteriosclerosis*, *Thrombosis and Vascular Biology*.[3]

Scientific studies use western blotting to measure levels of a given protein in cells and tissues. However, this technique requires use of validated polyclonal or monoclonal antibodies, which can be obtained from investigators or companies. Polyclonal antibodies generally give a stronger signal in western blotting than monoclonal antibodies because they bind to multiple epitopes on the protein, whereas monoclonal antibodies have a higher specificity. In 2016, the International Working Group for Antibody Validation was convened to highlight the need for validating antibodies in common research applications.[4] It provided guidelines aimed to ensure antibody targeting and data reproducibility. For western blotting, several recommendations were made. Among them are (i) eliminating or reducing protein expression through genome editing or RNA interference to alter the levels of the target protein, (ii) performing comparative studies with antibodies that target the same protein at nonoverlapping epitopes, (iii) using an antibody-independent method to analyze the protein, such as mass spectrometry, and (iv) labeling the target protein with an affinity tag to employ an independent method of detection.

Tissue factor (TF) is a transmembrane protein that functions as a high-affinity receptor against factor VII (FVII) and FVIIa.[5] The TF-FVIIa complex is the initiator of the coagulation protease cascade. The mature human TF is composed of 263 amino acids, and the predicted molecular weight without glycosylation is ~36 kDa. However, human TF has 3 N-linked glycosylation sites (Asn 11, Asn 125, and Asn 137) in the extracellular domain that increase the apparent molecular weight to ~50 kDa.[6-10] Similarly, mature mouse TF is composed of 266 amino acids and has 4 N-linked glycosylation sites (Asn 9, Asn 29, Asn 141, and Asn 172) that increase the apparent molecular weight from ~36 kDa to ~50 kDa.[11,12] Deglycosylation of human TF reduces its molecular weight to ~36 kDa.[13]

An array of monoclonal antibodies have been generated against human TF.[14-18] In addition, numerous companies sell anti-human TF monoclonal and polyclonal antibodies, including Novus Biologicals, Cell Signaling Technologies, Bio-Rad Laboratories, Abcam, and BD Biosciences. There are far less well-characterized antibodies against mouse TF. Three rat anti-mouse TF monoclonal antibodies have been reported, including 2 that inhibit mouse TF (1H1 and 21E10).[19,20] Many companies sell anti-mouse TF monoclonal and polyclonal antibodies, including R&D Systems, Abcam, and BioMedica Diagnostics.

In this study, we evaluated several commercial anti-human and anti-mouse TF antibodies for their ability to detect human and mouse TF by western blotting.

2 | METHODS

2.1 | Antibodies

Table 1 shows the in-house and commercial anti-human and mouse TF antibodies that were used in this study. The anti-human TF mouse monoclonal antibody HTF1 has been described previously.[15] These antibodies were purchased from different companies or provided by colleagues (Dr C. Wu, University of Kentucky [Abcam ab189483] and Dr L. Sommerville, Duke University [BioMedica Diagnostics 4515]). A mouse anti-human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) polyclonal antibody (Santa Cruz Biotechnology, Cat #sc-47724) and a goat anti-mouse GAPDH polyclonal antibody (Santa Cruz Biotechnology, Cat #sc-48167) were used as loading controls.

2.2 | Cell lines

The human pancreatic cancer cell lines BxPC-3, HPAF-II, PANC-1, and MIA PaCa-2 were obtained from American Type Culture Collection (Manassas, VA, USA). AsPC-1 was obtained from Dr Bruce A. Sullenger (Duke University). BxPC-3 and AsPC-1 cells were cultured in RPMI1640 medium (Thermo Fisher Scientific, Waltham, MA, USA; Cat #11875-093). HPAF-II cells were cultured in MEM (Thermo Fisher Scientific, Cat #11095-080). PANC-1 and MIA PaCa-2 cells were cultured in Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific, Cat #11965-092). All cells were grown in a 5% CO_2 incubator. We established a mouse pancreatic cancer cell line called KPC2 from KPC mice ($\underline{Kras}^{G12D/+}$, $\underline{p53}^{R172H/+}$, $Elas^{CreER/+}$).[21] KPC2 cells were cultured in RPMI1640

TABLE 1 Anti-human and anti-mouse tissue factor antibodies that were assessed in the present study

Target species	Host species	Ab type	Description	Company	Cat #	Lot #	Conc.	Application	WB validation
Human	Mouse	Monoclonal	TF9-10H10	In-house	-	-	1.0 mg/mL	IHC, WB	Y-Human
Human	Mouse	Monoclonal	Mouse anti Human CD142 (TF9-10H10)	Bio-Rad	9010-5059	170131	1.0 mg/mL	FCM, IF, IHC, WB	Y-Human
Human	Mouse	Monoclonal	Purified Mouse Anti-Human CD142 (HTF-1)	BD Biosciences	550252	6357515	0.5 mg/mL	FCM, Inh	Y-Human
Human	Rabbit	Polyclonal	Coagulation Factor III/Tissue Factor Antibody	Novus Biologicals	NBP2-15139	42928	0.64 mg/mL	IHC, WB	Y-Human
Human	Rabbit	Polyclonal	Tissue Factor/ CD142 Antibody	Cell Signaling	47769	1	0.055 mg/mL	WB	Y-Human
Mouse	Goat	Polyclonal	Mouse Coagulation Factor III/ Tissue Factor Antibody	R&D Systems	AF3178	WQY0214032	0.2 mg/mL	FCM, IP, WB	Y-Mouse
Mouse	Rabbit	Monoclonal	Recombinant Anti-Tissue Factor antibody (ERP18160-175)	Abcam	ab189483	GR3196777-2	0.576 mg/mL	IP, WB	Y-Mouse
Mouse	Rabbit	Polyclonal	Rabbit anti-mouse Tissue Factor IgG	Biomedica	4515	150408	1.0 mg/mL	FCM, IHC, Inh, WB	N-Mouse
Human[a]	Rabbit	Polyclonal	Anti-Tissue Factor antibody	Abcam	ab104513	GR3224342-1	0.5 mg/mL	IHC, WB	N-Mouse
Human[b]	Rabbit	Monoclonal	Anti-Tissue Factor antibody (EPR8986)	Abcam	ab151748	GR150188-8	0.21 mg/mL	IHC, ICC, IF, WB	N-Mouse

FCM, flow cytometry; ICC, immunocytochemistry; IF, immunofluorescence; IHC, immunohistochemistry; Inh, inhibitory; IP, immunoprecipitation; N, no; WB, western blotting; Y, yes.

[a]Predicted to cross react with mouse/rat.

[b]Cross reacts with mouse/rat.

medium (Thermo Fisher Scientific). All media contained 10% fetal bovine serum (Omega Scientific, Tarzana, CA, USA; Cat #FB-02) and 1% antibiotics-antimycotic (Thermo Fisher Scientific, Cat #15240-062).

2.3 | Generation of a mouse TF knockout cell line

The dimeric clustered regularly interspaced short palindromic repeats (CRISPR) RNA-guided FokI nuclease strategy was applied to knock out the mouse TF gene in KPC2 cells as described.[22] In brief, CRISPR plasmid pSQT1313 (dual guide RNA [gRNA] plasmid) and pSQT1601 (CRISPR-associated protein 9 [Cas9] plasmid) were obtained from Addgene (Watertown, MA, USA; Cat #53370 and 53369). gRNA oligonucleotides were designed and generated to target the ATG start codon of the TF (*F3*) gene (TF gRNA plasmid). For transfection, two 6-cm dishes of KPC2 cells with ~70% confluency were prepared the day before transfection. Two micrograms of Cas9 plasmid, 250 ng of linear puromycin plasmid (Clontech, Mountain View, CA, USA; Cat #631626) and 0.5 μg of TF gRNA plasmid were premixed in serum-free RPMI1640 medium and cotransfected into KPC2 cells using Xtreme Gene 9 (Roche, Basel, Switzerland; Cat #XTG9-RO) to generate TF knockout (KO) cells. Cas9 control cells were generated by only cotransfecting with the Cas9 plasmid and the linear puromycin plasmid. Transfected cells were subsequently cultured in medium containing 2 μg/mL of puromycin for selection. On day 10, 25 emerged cell colonies were selected using standard cloning cylinders (Fisher Scientific, Waltham, MA, USA; Cat #0790710). Standard PCR and cloning techniques were applied for screening positive colonies. Deletion of the TF gene was confirmed by sequencing each of the alleles.

2.4 | Assessment of TF mRNA

We used the Genevestigator gene expression database (https://genevestigator.com/gv/) for assessment of TF mRNA levels in the human pancreatic cell lines. TF mRNA levels in the KPC2 Cas9 and KPC2 TF KO were measured using RT-PCR and SYBR Green with primers that span the deletion site: forward 5′-cttggacatggcgatcct-3′ and reverse 5′-gttgccactccaaattgtct-3′.

2.5 | Preparation of cell lysates and SDS-PAGE

Cells were washed once with phosphate-buffered saline (PBS), scraped, lysed in RIPA lysis buffer (MilliporeSigma, Billerica, MA, USA; Cat #20-188) containing a protease inhibitor cocktail (Roche, Cat #11697498001) and lysates incubated on ice for 30 minutes. The protein concentration of samples was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific, Cat #23227). Cell lysates were prepared for electrophoresis by addition of SDS sample buffer (Invitrogen, Carlsbad, CA, USA; Cat #NP0007) containing 2.5% β-mercaptoethanol and heated to 95°C for 10 minutes. Forty micrograms of protein were loaded onto 4%-20% gradient Mini-PROTEAN TGX gels (BioRad Laboratories, Hercules, CA, USA; Cat #456-1093). A protein marker (Gel Company, San Francisco, CA, USA; Cat #FPL-008) was used as a standard. Proteins were separated by electrophoresis using Tris/Glycine/SDS buffer (BioRad Laboratories, Cat #161-0732).

2.6 | Western blotting

Proteins were transferred to polyvinylidene difluoride membranes (MilliporeSigma, Cat #IPFL00010) and blocked using protein-free blocking buffer (Thermo Fisher Scientific, Cat #23227). Membranes were probed with various anti-TF antibodies shown in Table 1. All primary antibodies were used at a 1:1000 dilution in blocking buffer. After washing with Tris-buffered saline containing 0.1% Tween 20 (TBS-T), membranes were incubated with either Alexa Fluor 647 chicken anti-mouse IgG (Invitrogen, Cat #A21463), Alexa Fluor 680 rabbit anti-goat IgG (Invitrogen, Cat #A21088), or IR Dye 680 RP goat anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE, USA; Cat #926-68071). All secondary antibodies were used at a 1:10 000 dilution in blocking buffer. Specific antigen-antibody complexes were visualized using an Odyssey imaging system (LI-COR Biosciences). Antibodies were removed with stripping buffer (Thermo Fisher Scientific, Cat #21059), and membranes were reprobed with either anti-human GAPDH or anti-mouse GAPDH antibodies. Both antibodies were diluted 1:1000 in blocking buffer. After washing with TBS-T, membranes were incubated with either IR Dye 800 CW goat anti-mouse IgG (LI-COR Biosciences, Cat #926-33210) or IR Dye 800 CW donkey anti-goat IgG (LI-COR Biosciences, Cat #926-32214). An Odyssey imaging system was used to detect antibody-protein complexes.

2.7 | Deglycosylation

Deglycosylation of human and mouse TF was performed using a commercial enzymatic deglycosylation kit that contains incubation mix, denaturation solution, and detergent solution (Agilent Technologies, Santa Clara, CA, USA; Cat #GK80115). Cell lysates (100 μg) were dissolved in 30 μL of deionized water. Next, 10 μL of 5× incubation mix and 2.5 μL denaturation solution were added. The solution was mixed gently and incubated for 5 minutes at 100˚C. After cooling to room temperature, 2.5 μL of detergent solution was added and mixed. N-Glycanase (2 μL), Sialidase A (3 μL), and O-Glycanase (2 μL) were added to the solution, and samples were incubated at 37˚C for 3 hours.

2.8 | Cellular TF activity assay

Cells were washed with PBS without calcium and magnesium (Thermo Fisher Scientific, Cat #14190-250), reconstituted with Hank's balanced salt solution without calcium and magnesium (Thermo Fisher Scientific, Cat #14170-112), and disrupted by sonication (total of ~24 000 J) using a Q700 sonicator (Qsonica, Newtown, CT, USA). The clotting time was measured using a STart4 coagulation analyzer (Diagnostica Stago, Parsippany, NJ, USA). Briefly, 50 μL of sonicated cellular suspension were added to a mixture of 50 μL of 25 mmol/L $CaCl_2$ solution and 50 μL of human (Pacific Hemostasis Coagulation Control Normal, Level 1, Thermo Fisher Scientific) or mouse plasma. Mouse plasma was prepared from whole blood of C57BL/6J mice by centrifugation at 4500 *g* for 15 minutes. TF activity was calculated using a standard curve generated by Innovin (Siemens, Munich, Germany). TF activity was normalized by protein concentration determined using a BCA assay.

3 | RESULTS

3.1 | Levels of TF mRNA, protein, and activity in various human and mouse pancreatic cancer cell lines

We selected 5 human pancreatic cancer cell lines that express high (BxPC-3, HPAF-II, AsPC-1), very low (PANC-1), and no (MIA PaCa-2) TF. We used an anti-human TF polyclonal antibody from Cell Signaling Technologies (Danvers, MA, USA) for western blotting. In general, levels of TF mRNA, protein and activity correlated with each other in the different lines (Figure 1). The only discrepancy was the relative levels of TF protein in the TF expressing lines vs their corresponding TF activity, which indicates a different TF protein:TF activity ratio in these lines. BxPC-3, HPAF-II, and AsPC-1 expressed a ~50 kDa band on western blotting, which is consistent with the expected size of glycosylated TF (Figure 1B).[14] Several weaker nonspecific, higher-molecular-weight bands were observed in all the cell lines (Figure 1B).

The mouse pancreatic cancer cell line KPC2 expresses a high level of TF.[21] For this study, 2 new derivative lines were generated for analysis. These include a KPC2 Cas9 control line and a KPC2 TF KO line. We used an anti-mouse TF monoclonal antibody from Abcam (Cambridge, MA, USA; ab189483) for western blotting. Similar to the parental line KPC2 (not shown), KPC2 Cas9 cells expressed a high level of TF mRNA, protein, and activity, whereas no TF expression was detected in the KPC2 TF KO line (Figure 2). KPC2 Cas9 cells expressed an ~50-kDa band on western blotting, which is consistent with the expected size of glycosylated mouse TF (Figure 2B). Two

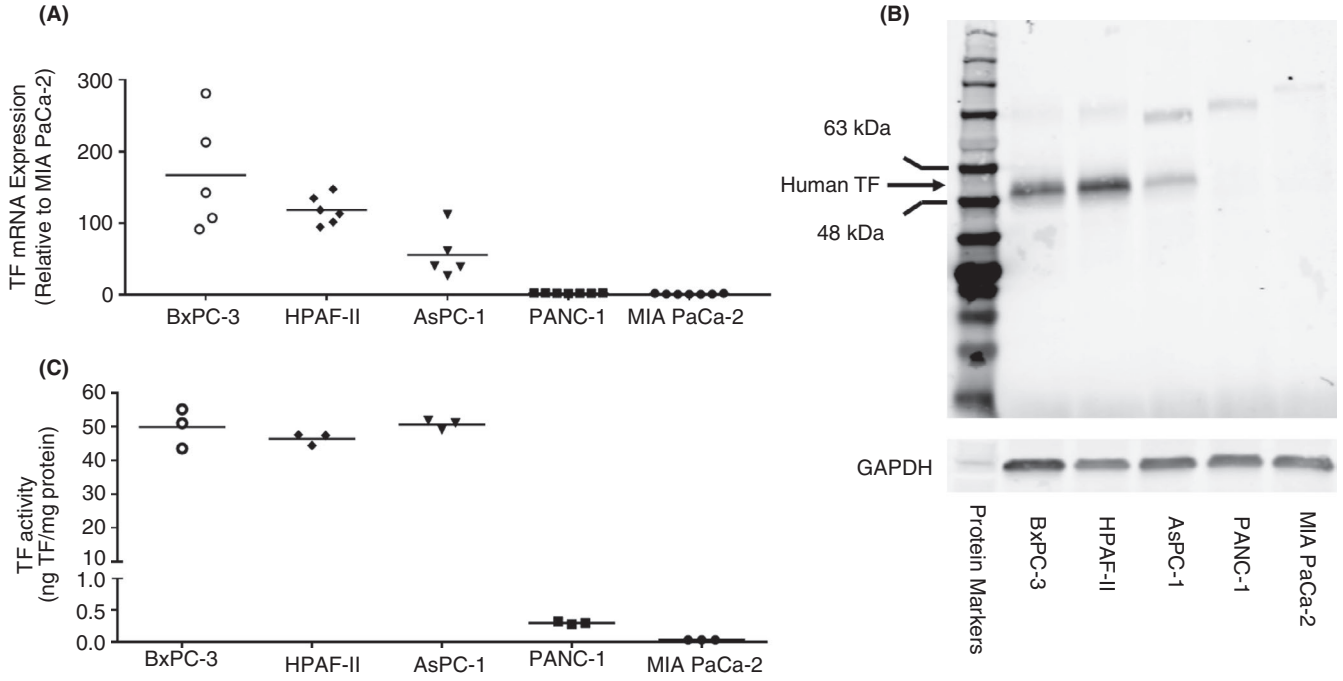

FIGURE 1 Tissue factor expression in human pancreatic cancer cell lines. We used five human pancreatic cancer cell lines that express different levels of tissue factor (TF). A, The level of TF mRNA in the different cell lines was from the Genevestigator gene expression database. B, The level of TF protein in the different cell lines was determined by western blotting using an anti-human TF polyclonal antibody from Cell Signaling Technologies 47769 (1:1000 dilution, final concentration 0.055 μg/mL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using an anti-human GAPDH antibody (1:1000 dilution, final concentration 0.2 μg/mL). Secondary antibodies were used at a 1:10 000 dilution in blocking buffer. A protein marker is shown on the left of the panel. C, The level of TF activity in the different cell lines was determined using a one stage clotting assay. Mean levels of TF mRNA and activity are shown

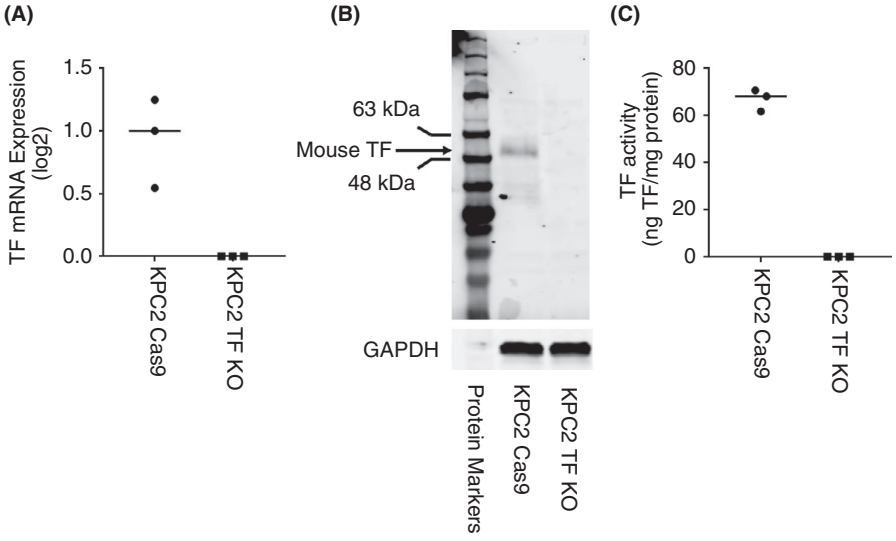

FIGURE 2 Tissue factor expression in mouse pancreatic cancer cell lines. We used a mouse pancreatic cancer cell line that expresses tissue factor (TF) (KPC2 Cas9) and a TF knockout line (KPC2 TF KO). A, The level of TF mRNA in the 2 cell lines was determined by RT-PCR. B, The level of TF protein in the 2 cell lines was determined by western blotting using an anti-mouse TF monoclonal antibody from Abcam ab189483 (1:1000 dilution, final concentration 0.576 μg/mL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using an anti-mouse GAPDH antibody (1:1000 dilution, final concentration 0.2 μg/mL). Secondary antibodies were used at a 1:10 000 dilution in blocking buffer. A protein marker is shown on the left of the panel. C, The level of TF activity in the 2 cell lines was determined using a 1-stage clotting assay. Mean levels of TF mRNA and activity are shown

weaker and lower-molecular-weight bands (~35 and 30 kDa) were also observed in KPC2 Cas9 cell lysate. These may represent nonglycosylated forms of mouse TF. These data indicate that these human and mouse pancreatic cancer cell lines can be used to assess the ability of different commercial anti-TF antibodies to detect TF by western blotting.

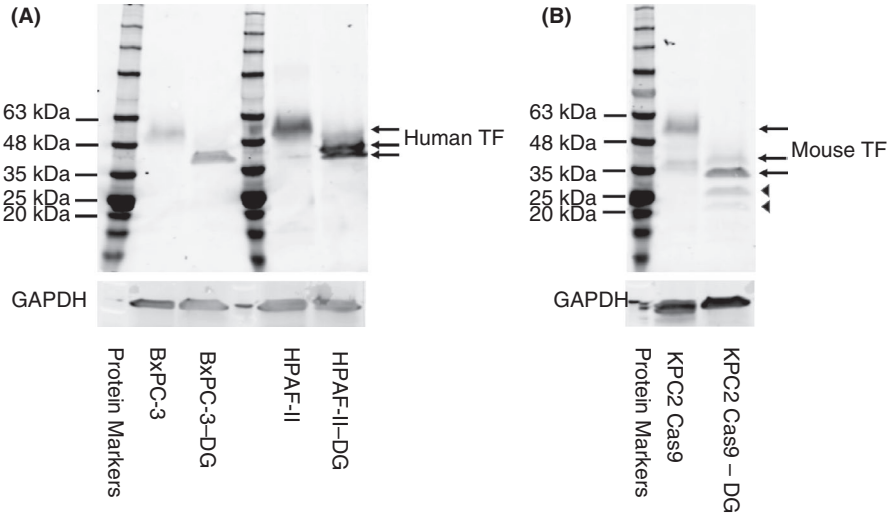

FIGURE 3 Deglycosylation of human and mouse tissue factor. We enzymatically deglycosylated cell lysates from human and mouse pancreatic cancer cell lines. We then determined the molecular weight of nondeglycosylated and deglycosylated human and mouse tissue factor (TF). The various forms of human and mouse TF are indicated by the arrows. Arrowheads indicate possible degradation products. Human and mouse TF were detected using Cell Signaling Technologies 49969 antibody (1:1000 dilution, final concentration 0.055 μg/mL) and Abcam ab189483 (1:1000 dilution, final concentration 0.576 μg/mL), respectively. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using an anti-human or anti-mouse GAPDH antibody (1:1000 dilution, final concentration 0.2 μg/mL). Secondary antibodies were used at a 1:10 000 dilution in blocking buffer. A protein marker is shown on the left of each panel. DG, deglycosylation

3.2 | Deglycosylation of human and mouse TF

Both human and mouse TF are glycosylated. Therefore, one would expect that the size of both proteins would be decreased by deglycosylation. We used an anti-human TF polyclonal antibody from Cell Signaling Technologies and an anti-mouse TF polyclonal antibody from Abcam (ab189483) for western blotting. Deglycosylation of human TF expressed by BxPC-3 and HPAF-II reduced the molecular weight from ~50 kDa to ~40 kDa (Figure 3A). An additional band was observed in the deglycosylated HPAF-II sample that likely represents partially deglycosylated protein. Deglycosylation of mouse TF expressed by KPC2 Cas9 cells decreased the size to ~35 kDa (Figure 3B). A higher-molecular-weight band (~38 kDa) may be a partially deglycosylated product. In addition, 2 lower-molecular-weight bands were observed that might represent degradation products (Figure 3B). These results indicate that the ~50-kDa band observed in TF expressing human and mouse pancreatic cancer cell lines is a glycosylated form of TF because its molecular weight is decreased by deglycosylation.

3.3 | Detection of human TF by western blotting using commercial anti-human TF antibodies

Four commercially available anti-human TF antibodies (2 monoclonal and 2 polyclonal antibodies) that are claimed to detect human TF by western blotting were randomly selected for validation. We used a TF-positive cell line (HPAF-II) and a TF-negative cell line were used (MIA PaCa-2). An "in-house" anti-human TF monoclonal antibody TF9-10H10 served as a positive control.[14,15] All the commercial anti-human TF antibodies detected a single band at ~50 kDa, which is the expected size for glycosylated TF (Figure 4).

3.4 | Detection of mouse TF by western blotting using commercial anti-mouse TF and anti-human TF antibodies

We used 3 commercial anti-mouse TF antibodies and 2 anti-human TF antibodies to detected mouse TF by western blotting. The mouse pancreatic cancer cell lines KPC2 Cas9 and KPC2 TF KO were used as positive and negative controls, respectively. The R&D Systems goat polyclonal antibody AF3178 and the Abcam ab189483 monoclonal antibody detected at band at ~50 kDa in KPC2 Cas9 cells, which is the expected size of glycosylated TF, but nothing was detected with KPC2 TF KO (Figure 5). The anti-mouse TF polyclonal antibody from BioMedica Diagnostics detected a nonspecific band at ~48 kDa that was present in both the KPC2 Cas9 and KPC2 TF KO lines (Figure 5). Similarly, the anti-human TF polyclonal antibody Abcam ab151748 detected a nonspecific band at ~50 kDa that was present in both the KPC2 Cas9 and KPC2 TF KO lines (Figure 5). Finally, the anti-human TF polyclonal antibody ab104513 from Abcam failed to detect mouse TF (Figure 5). These data indicate that in our limited survey, 2 of 3 commercial anti-mouse antibodies detected mouse TF by western blotting whereas the third anti-mouse TF antibody recognized a nonspecific protein. Both anti-human TF antibodies failed to detect mouse TF.

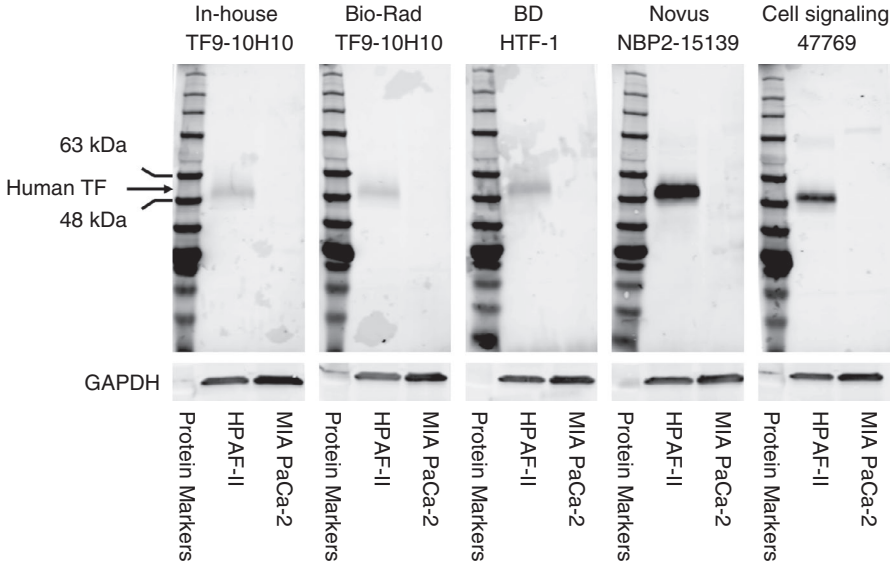

FIGURE 4 Detection of human tissue factor by western blotting using commercial antibodies. We used a tissue factor (TF)-positive cell line (HPAF-II) and a TF-negative cell line (MIA PaCa-2). Each primary antibody was diluted 1:1000. The final concentration of each antibody was as follows: Novus Biologicals (final concentration 0.64 μg/mL); Cell Signaling Technologies (final concentration 0.055 μg/mL), HTF-1 (final concentration 0.55 μg/mL), in-house TF9-10H10 (final concentration 1.0 μg/mL), Bio-Rad Laboratories TF9-10H10 (final concentration 0.055 μg/mL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using an anti-human GAPDH antibody (1:1000 dilution, final concentration 0.2 μg/mL). Secondary antibodies were used at a 1:10 000 dilution in blocking buffer. A protein marker is shown on the left of each panel

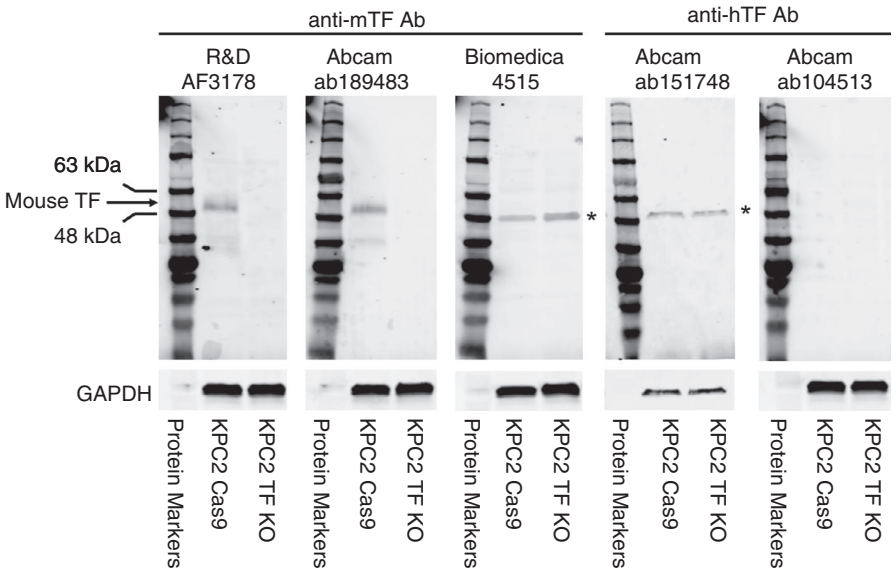

FIGURE 5 Detection of mouse tissue factor by western blotting using commercial antibodies. We used a tissue factor (TF)-positive cell line (KPC2 Cas9) and a TF-negative cell line (KPC2 TF KO). Each primary antibody was diluted 1:1000. The final concentration of each antibody was as follows: R&D Systems (final concentration 0.2 μg/mL), Abcam ab189483 (final concentration 0.576 μg/mL), BioMedica Diagnostics (final concentration 1.0 μg/mL), Abcam ab151748 (final concentration 0.212 μg/mL), and Abcam ab104513 (final concentration 0.55 μg/mL). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was detected using an anti-mouse GAPDH antibody (1:1000 dilution, final concentration 0.2 μg/mL). Secondary antibodies were used at a 1:10 000 dilution in blocking buffer. A protein marker is shown on the left of each panel. The asterisk marks a nonspecific band. All data was generated with blots run in parallel and probed in parallel except the data for ab151748 was obtained on a different day using different samples

4 | DISCUSSION

In this study, we evaluated different commercial anti-human TF and anti-mouse TF antibodies for their ability to detect human and mouse TF by western blotting. In our limited survey, we found that all the commercial anti-human polyclonal and monoclonal antibodies examined detected TF in human pancreatic cancer cell lines by western blotting. In contrast, only 2 of 3 commercial anti-mouse TF

antibodies detected mouse TF, and the other recognized a nonspecific band. Two anti-human TF polyclonal antibodies that claimed to either cross react or predicted to cross react with mouse TF did not detect mouse TF by western blotting.

The cost of antibodies can be substantial with the price of TF antibodies ranging from approximately $130 to $450. Thus, investigators rely on company technical support services or other online resources when selecting an antibody. However, the quality of technical support provided by different companies can vary widely. A "best-case scenario" was experienced by our research team with the technical service department of Cell Signaling Technologies. This group readily provided western blot data for their antibody using several cell lines, including BxPC-3 and HPAF-II. In addition, Cell Signaling Technologies has detailed information on their website (

https://www.cellsignal.com/products/primary-antibodies/tissue factor-cd142-antibody/47769). In contrast, the technical services department for a separate company told us that "we couldn't get a QC [quality control] image from the antibody originator for you," and we were not provided information about the antibody originator.

Another source available for selecting an antibody is the Human Protein Atlas (HPA; https://www.proteinatlas.org), which, among its various purposes, is designed to serve as a free, independent source for antibody validation. However, in our experience, this source must be reviewed with caution for information on anti-TF antibodies. Data on the HPA website indicates that the TF antibody HPA049292 from MilliporeSigma recognized an ~50-kDa band in a confirmed TF-positive cell line U-251 MG, and no signal was observed with known TF-negative samples. This antibody should have received a "supported" score. Surprisingly, it was given an "uncertain" grade in the HPA. In contrast, a second antibody HPA069132 from MilliporeSigma that recognized a nonspecific protein at ~30 kDa in TF-negative samples was given a "supported" grade. We expressed our concerns to the HPA organization and were told by a representative that "the initial scoring of western blots is based on the theoretical molecular weight of the unmodified proteins related to each gene," which in the case of TF would be ~36 kDa. It is clear that HPA's scoring criteria for anti-TF antibodies does not account for posttranslational modifications, such as glycosylation, and does not consider nonspecific binding of antibodies to TF-negative cell lines and samples. Therefore, such sources of antibody validation should be carefully evaluated prior to use.

We found a high degree of variability in the detection of mouse TF by western blotting using commercial antibodies. The R&D Systems goat anti-mouse TF polyclonal antibody AF3178 and the Abcam ab189483 rabbit anti-mouse monoclonal antibody detected TF by western blotting. Indeed, the R&D Systems antibody has been used by our laboratory and others and has the advantage of being antigen affinity purified to increase its specificity.[23-27] Human and mouse TF are highly homologous.[28] However, this does not mean that polyclonal antibodies raised against one species will cross react with TF from another species. For example, the anti-human TF polyclonal antibody ab104513 from Abcam is marketed as predicted to cross react with mouse TF. We contacted Abcam and were told by the technical service department that this claim was based on a paper that used the antibody to detect mouse TF by immunohistochemistry.[29] The Abcam website (https://www.abcam.com/tissue-factor-antibody-ab104513-references.html) lists 13 publications that used this antibody to detect TF from a variety of species, including human, rat, mouse, and pig. Importantly, 2 papers used the antibody to measure levels of mouse TF protein by western blotting.[30,31] We found that this antibody did not recognize mouse TF by western blotting. Similarly, in 2014, Dr Moshe Shashar reported that ab104513 does not recognize mouse TF.[32] This example highlights the need for individual investigators and laboratories to perform in-house validation of antibodies prior to publishing findings.

A major complicating factor in the use of antibodies for western blotting protocols to detect TF is the fact that some anti-mouse TF polyclonal antibodies do not recognize mouse TF but rather bind to a nonspecific protein in western blots. In our study, we found that a rabbit anti-mouse polyclonal antibody from BioMedica Diagnostics 4515 recognized a nonspecific protein in both TF-positive and TF-negative cell lines by western blotting. We could not find any papers that used the BioMedica Diagnostics antibody. Another antibody of concern is Abcam ab151748. This rabbit anti-human monoclonal antibody was raised against a peptide from human TF. Abcam claimed that this antibody detects human, rat, and mouse TF by western blotting. However, the datasheet gave a predicted molecular weight of TF as 33 kDa rather than ~50 kDa for the glycosylated form. We found 29 papers that used this antibody to measure human, mouse, rabbit and canine TF by a variety of methods, including 15 that used the antibody for western blotting (Table S1).[33-61] Importantly, Abcam evaluated the ability of the antibody to detect human TF in wild-type and KO cell lines by western blotting.[62] Based on the data they obtained, they recently withdrew ab151748 and stated that "after evaluation, using a HAP1 knockout cell line, ab151748 was not shown to react specifically with the target protein and has therefore been discontinued." We confirmed that this antibody does not detect mouse TF by western blotting but rather a nonspecific band. This highlights the need for appropriate quality control to fully characterize antibodies. An important downstream implication of this revelation is that published western blot data obtained using ab151748 should be reevaluated.

A final consideration is that even for a given antibody, there may be variation based on the commercial provider. It is interesting to note that at least 15 companies sell TF9-10H10, including Creative Biolabs, MilliporeSigma, Thermo Fisher Scientific, and Novus Biologicals. It would be good to know if these antibodies are all indeed equivalent. Sources of potential variation between vendors include different purification techniques, storage conditions, or buffers and carrier proteins. MilliporeSigma technical services reported that they obtain the antibody from Calbiochem, whereas Bio-Rad, Novus Biologicals, and Thermo Fisher Scientific technical services told us that the source was proprietary.

The study has some limitations. We used human and mouse pancreatic cell lines as positive and negative controls for human and

mouse TF expression. We would expect similar results using other cell lines, activated monocytes and TF-expressing tissues but this was not tested. Our study focused on the ability of commercial antibodies to detect human and mouse TF by western and did not determine the ability of these antibodies to detect TF by other methods, such as immunohistochemistry. Interestingly, the rat anti-mouse TF monoclonal antibody 1H1 detects mouse TF by immunohistochemistry but failed to detect mouse TF by western blotting (data not shown).[19] Therefore, our western blot data cannot be extrapolated to our techniques or vice versa.

The lessons from these examples indicate that full information should be available for commercial antibodies and that it is important to know TF is a glycosylated protein that is differentially expressed by various cell lines and tissues.[12,63,64] In the era of rigor and reproducibility, companies must do a better job validating their antibodies using a variety of methods. Investigators should provide the antibody source, including lot number, and independently validate commercial antibodies as recommended by the National Institutes of Health.

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RELATIONSHIP DISCLOSURE

The authors declare nothing to report.

AUTHOR CONTRIBUTIONS

NM designed the study. AR, BM, YH, RC, GL, and YY performed the experiments. All authors analyzed data and wrote and approved the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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