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BASIC RESEARCH

Ex vivo interaction between blood components and hormone-dependent breast cancer cells induces alterations associated with epithelial-mesenchymal transition and thrombosis

T. N. Augustine[a], K. Pather[a], D. Mak[a], D. Klonaros[a], K. Xulu[a], T. Dix-Peek[b], R. Duarte[b], and W. J. van der Spuy[a]

[a]School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa; [b]Department of Internal Medicine, School of Clinical Medicine, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

ABSTRACT

The prevalence of breast cancer is steadily increasing with metastasis and thromboembolic complications identified as the most common causes of death. The acquisition of an aggressive phenotype by hormone-dependent breast cancers is mediated by Transforming Growth Factor Beta 1 (TGF-β_1) expression and is associated with epithelial-mesenchymal transition (EMT) and, potentially, increased propensity for thrombosis. We investigated this phenomenon by assessing the effect of platelet-rich plasma (PRP) and whole blood (WB) on parameters of EMT and hypercoagulation *in vitro*. MCF-7 breast cancer cells were cultured under standard conditions, followed by co-culture with PRP or WB. Cells were processed for real-time PCR (*TGF-β_1* and *vimentin*), electron microscopy or immunocytochemistry (TGF-β_1). Micrographs were qualitatively assessed, and real-time PCR data analyzed with PAST Statistical Software. The addition of blood components heightened TGF-β_1 immunolocalization and significantly increased corresponding gene expression. Both PRP and WB significantly increased *vimentin* expression and induced a shape change from a typical epithelial phenotype to a spindle-shape morphology, indicative of EMT. Fibrin fiber, network and plaque formation indicated a hypercoagulatory environment. The results thus show that in preparation for hematogenous metastasis, hormone-dependent breast cancer cells assume an aggressive phenotype associated with EMT, simultaneously increasing the propensity for the formation of thrombo-emboli.

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Introduction

Breast cancer is the most commonly diagnosed cancer among females[1], with the majority of deaths linked to metastatic spread.[2] Successful haematogenic metastasis is dependent on the dissociation of cells from the primary tumor, proposed to be strongly associated with platelets which promote the initi steps of the invasive process.[3–5] The cross-talk between platelets and tumor cells, either within the tumor bed or across leaky blood vessels is facilitated by tumor-secreted agonists including tissue factor and thrombin, major players in the coagulation cascade.[6,7] The resulting activation of platelets induces the release of granular contents containing growth factors and matrix metalloproteinases, which enable tumor cell invasion into the vascular system.[7,8]

During subsequent metastasis, platelets physically shield circulating tumor cells from immunosurveillance and shear forces.[9] A resulting effect is the formation of microthrombi, aggregates of platelets and fibrin network formation, which lead to thromboembolic complications – a leading cause of cancer related death.[10,11] It is postulated that these microthrombi allow for sustained cytokine signaling which in turn, may enhance the tumor metastatic profile or permit an immunosuppressive microenvironment within the circulation.[9,12] While this remains essentially unknown[13], such studies are revealing a more complex role for platelets in tumors progression.

Tumor cells secrete a variety of inflammatory cytokines including transforming growth factor (TGF)-β_1 which acts as a tumor suppressor in early stage cancers by causing cell cycle arrest at the G1/S phase, inducing cellular differentiation or promoting apoptosis; conversely in later stage tumors, including breast cancer, TGF-β_1 promotes tumor progression.[14–17] An increase in TGF-β_1 expression is regarded as an essential driver

CONTACT T. N. Augustine tanya.augustine@wits.ac.za School of Anatomical Sciences, Faculty of Health Sciences, University of the Witwatersrand, Room 2P15, 7 York Road, Parktown, Johannesburg 2193, South Africa

of epithelial-mesenchymal transition (EMT), a conversion to a more malignant phenotype with heightened migration and invasive capacity to facilitate metastasis.[14,17,18] TGF-β1 enables EMT by acting at multiple levels – via Smad and non-Smad-dependent signaling pathways, TGF-β1 ligation induces the expression of EMT transcription factors Snail, Slug, Zeb1 and Zeb2 and Twist.[19,20] More recent evidence indicates that TGF-β1 further drives EMT at a post-translational level. by regulating miRNAs, including miR-200, miR-30 and Let-7, identified as key modulators of the EMT transcription factors.[21] EMT is evidenced by phenotypic alterations that include the loss of cell-cell junctional complexes required for adhesion and maintenance of polarity, with the acquisition of mesenchymal markers including N-cadherin, fibronectin and vimentin.[17,18,21] The upregulation of vimentin, an intermediate filament protein facilitates cancer progression, specifically by mediating reorganization of cytoskeletal components and controlling downstream effectors that drive EMT.[22,23] *Vimentin* upregulation has also been identified *in vitro* in MCF-7 cells, which typically contain low amounts of *vimentin* but in response to pro-tumorigenic factors, including exogenous TGF-β_1[24], upregulation serves to mediate processes of EMT and tumor growth.[22,25,26] Additionally, tumor growth can be facilitated by TGF-β_1, is implicated in inhibiting cytotoxic T cell function, thereby limiting the capacity of the immune system to prevent tumor growth.[13] In the metastatic process, TGF-β_1 is further implicated in confounding the hemostatic balance of the coagulation and fibrinolytic processes.[27] Specifically, TGF-β_1 is associated with enhanced platelet aggregation[28] and thus may play a crucial role in the development of thrombi. In circulation, platelets are the main storage site for TGF-β_1 which is released from α-granules during the activation process[9,12]; however, other cell types including tumor cells themselves, lymphocytes and macrophages also secrete TGF-β.$_1$[29,30] This highlights a multifactorial relationship that may promote survival mechanisms or maintain a more aggressive tumor phenotype during haematogenic spread. Understanding tumor cell response *ex vivo* to vascular components would thus allow us to evaluate the outcome of the reciprocal interactions on tumor progression.

The objective of this study was thus to investigate the ability of vascular components i.e. whole blood and platelet-rich plasma, to induce alterations associated with EMT in luminal phenotype breast cancer cells.

Materials and methods

Ethical approval for this study was obtained from the Human Ethics Research Committee at the University of the Witwatersrand, South Africa, with the following codes: #M081036 and #M140155.

Peripheral blood samples were obtained from healthy female volunteers (n = 6) by venipuncture at the Charlotte Maxeke Johannesburg Academic Hospital, Parktown, South Africa. Principal exclusion criteria were pregnancy, autoimmune disease, immunodeficiency, cancer and previous history of cancer, and the use of oral contraceptives. Whole blood (WB), was collected in 4.5 ml Vacuette® tubes (Greiner Bio-One, #A140436N) containing 3.2% sodium citrate as anticoagulant. For generation of platelet-rich plasma (PRP) whole blood was centrifuged at 267 X g for 2 min and the buffy coat isolated.

To investigate the interactions between blood components and tumor cells, MCF-7 cells (Sigma Aldrich, St. Louis, USA, 86012803) were seeded on glass coverslips at an initial density of 1×10^5 cells/well in 24-well cell culture dishes. Cells were plated in triplicate for both qPCR and immunocytochemistry (ICC) assays. Cells were initially incubated at 37°C in 5% CO_2 in 200 µl Dulbecco's Modified Eagles Medium (DMEM; Lonza, Walkersville, MD, USA; #12-604F) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS; Gibco, Life Technologies, Johannesburg, South Africa; #10500), 1% Penicillin/Streptomycin (P/S; Sigma-Aldrich, St. Louis, MO, USA; #P3539) and 0.1% Amphotecerin B (Lonza, Walkersville, MD, USA; 17–836R) for 24 h. The cells were washed in phosphate buffered saline (PBS) prior to hormone deprivation for 48 h in Phenol Red-Free Dulbecco's Modified Eagles Medium (PRF-DMEM supplemented with L-glutamine, 5 ml Dextran Coated Charcoal (DCC)-stripped FBS and 0.1% P/S) to synchronize the cell cycle. Subsequently, cells were washed in PBS followed by a 5 min incubation at room temperature with either 200 µl PRP or 200 µl WB. Hormone-deprived control samples were maintained in PBS for 5 min.

Scanning Electron Microscopy (SEM)

Cells were fixed in 2.5% formaldehyde/glutaraldehyde for 15 min followed by rinsing thrice with 0.1 M PBS, and secondary fixation in 1% osmium tetroxide also followed with PBS rinsing. Cells were thereafter dehydrated through an increasing series of ethanol (30%, 50%, 70%, 90%, and three times absolute ethanol), and dried using hexamethyldisilazane. Samples were then mounted onto aluminum stubs and coated by carbon evaporation. Samples were examined using a ZEISS ULTRA plus FEG Scanning Electron Microscope with acceleration voltage set at 1 kV; at the University of Pretoria, Central Microscopy and Microanalysis Unit. Micrographs were assessed qualitatively.

RNA isolation, purification and cDNA synthesis

Total RNA was isolated from the MCF-7 breast cancer cells using the RNeasy Mini kit (Qiagen Inc., Hilden, Germany), per the manufacturer's protocol with the following exceptions. Samples in triplicate were pooled and homogenized with a 21 gauge-needle fitted into an RNase-free syringe in denaturing buffer containing both guanidine isothiocyanate (GITC) and β-mercaptoethanol. RNA was treated with RNase-free DNase I (New England Biolabs, Ipswich, MA) off-column and run through a second column (Qiagen) to clean the RNA. The concentration of RNA aliquots was assessed using a NanoDrop 2000 spectrophotometer (ThermoFischer Scientific, Waltham, USA) per manufacturer instructions. The optical density ratio of ($A_{260\ nm}/A_{280\ nm}$) was evaluated as an indicator of protein contamination. RNA integrity was determined using the RNA 6000 Pico Kit and 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) per the manufacturer's protocol. RNA integrity numbers (RINS) were determined to be 9. cDNA was synthesized from 50ng total RNA in a 20 µl reaction volume using High Capacity cDNA Synthesis kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions, in a MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA) under the following conditions: 25°C for 10 min; 37°C for 120 min; 85°C for 5 sec.

Primer design and identification of putative reference genes for MCF-7 cancer cells

Oligonucleotide primers for the human TGF-β_1 sequence were designed and verified by using the Genbank database Primer-Blast search (https://blast.ncbi.nlm.nih.gov). The three reference genes shown to be most stably expressed in the MCF-7 breast cancer cells used in this study were beta actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein large P0 (RPLP0) (Table 1). These were chosen empirically from a large set of potential reference genes based on the propensity for the qPCR-measured gene expression profiles to remain stable across treatments. All primers were purchased from Integrated DNA Technologies Inc. (IDT, Iowa, US).

Real-time quantitative polymerase chain reaction (qPCR)

Relative gene expression levels were determined in a 20 µl reaction volume using Brilliant II SYBR® Green qPCR Low ROX Master Mix (Agilent Technologies); 0.25 µM of each primer and cDNA to a final concentration of 10 ng/reaction. Real-time PCR was conducted with an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies, CA, USA). Programmable temperature cycling was performed with the following cycle profile:

Table 1. Primer sequences.

Symbol	Name	Primers (5′-3′)	GenBank Accession Number
ACTB	Beta actin	F: GGC CGA GGA CTT TGA TTG CAC R: TTA GGA TGG CAA GGG ACT TCC TGT	NM_001101.3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: TGC ACC ACC AAC TGC TTA GC R: GGC ATG GAC TGT GGT CAT GAG	NM_002046.5
RPLP0	Ribosomal protein, large, P0	F: TGC AGC TGA TCA AGA CTG GAG ACA R: TCC AGG AAG CGA GAA TGC AGA GTT	BC001834.2
TGF-β_1	Transforming Growth Factor Beta 1	F: CTC GCC AGA GTG GTT ATC TT R: AGT GTG TTA TCC CTG CTG TC	NM_000660.5
VIM	Vimentin	F: CCT CTT CCA AAC TTT TCC TCC R: CGT TGA TAA CCT GTC CAT CTC	NM_003380.5

denaturation step at 95°C for 10 min; amplification was performed for 40 cycles of 95°C for 30 sec and elongation 65°C for 1 min; the cycling stage was followed by melt curve analysis at 95°C for 30 sec, 65°C for 1 min, 95°C for 30 sec and 65°C for 15 sec. Two technical replicates were used per sample. Data analysis was performed in concordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[31] Data points that were missing or outliers were imputed.[32]

Relative fold changes in vimentin and TGF-β_1 mRNA expression were then calculated using the $2^{-\Delta\Delta Cq}$ method.[33,34] The mRNA expression of TGF-β_1 and vimentin were normalized using the geometric mean of the three reference genes.[34] The fold change values obtained were thus used for statistical analysis.

Immunocytochemistry (ICC)

Cells were fixed in 4% paraformaldehyde (Saarchem, Muldersdrift, South Africa), rinsed and permeabilized in blocking media consisting of 10% normal goat serum (Dako, Glostrup, Denmark; X0907) in 1% Bovine Serum Albumin (BSA; Sigma Aldrich, A2058)/PBS with 0.1% Tween 20 (Saarchem, MERCK, Johannesburg;) and 0.3 M glycine to penetrate nonspecific binding sites. After removal of the blocking media, cells were incubated overnight at 4° C in rabbit anti-human TGF-β_1 primary antibody (Abcam, Cambridge, England; ab53169) diluted at 1:500 in 1% BSA/PBS. Thereafter cells were rinsed and incubated in the dark for 2 hours in Alexa Fluor 488 goat anti-rabbit antibody (Invitrogen, Life Technologies; A11008) diluted at 1:1,000. Cells were again rinsed, and incubated in 4′,6-damidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA; D1306) diluted at 1:50,000, to stain the cell nuclei. Coverslips were mounted onto slides using Fluoromount (Sigma Aldrich; F4680) and viewed using an Olympus iX51 Inverted Fluorescent Microscope with U-MWIB2 and U-MNU2 filters and captured using CellSens Software Version 11. Three photomicrographs were taken per coverslip. Exposure time was set as followed: 64 ms for bright field and DAPI, and 360 ms for Alexa Fluor 488 at 40X instrument magnification.

Data analysis

Statistical analyses were performed using PAST Statistical software, version 3.26 software. The non-parametric Kruskal Wallis test was conducted, following which a Dunn's post-hoc test was used to measure the significance ($p < .05$) of differential expression of the target genes or TGF-β1 protein expression across WB- and PRP- co-cultured and control groups.

Results

Ultrastructural analysis

Hormone-deprived MCF-7 breast cancer cells in the control group exhibited a phenotype that is typically associated with transformed epithelial cells. These cells have a polyhedral squamous cell shape with short microvilli (Figure 1a). Bleb-like structures were noted primarily in the control group, being reduced in co-cultured groups. Co-culture with blood components induced morphology consistent with that of highly invasive breast cancer cells, including elongation of the cell to a spindle shape with a protruding nucleus. Cells and cellular processes were noted to overlap (Figure 1b and c). In addition to these cellular characteristics, in co-culture pericellular fibrin fibers were evident highlighting a pro-thrombotic environment. Cells co-cultured with WB displayed denser fibrin network formation than cells co-cultured with PRP, and were often obscured by fibrin plaques, possibly indicative of a higher rate of fibrin formation than in samples where cells were co-cultured with PRP. A representative platelet prior to WB – or PRP–co-culture with MCF-7 cells shows morphology characteristic of early activation (Figure 1d).

Immunolocalization of TGF-β_1

Negative controls indicated the efficacy of the ICC technique, showing no nonspecific binding of the antibodies. Qualitative assessment showed that hormone-deprived (control) MCF-7 breast cancer cells presented TGF-β_1 expression concentrated primarily in the perinuclear cytoplasmic region (Figure 2a). Following co-culture with WB and PRP, higher intensity perinuclear expression was noted (Figure 2b and c), with an increase in diffuse

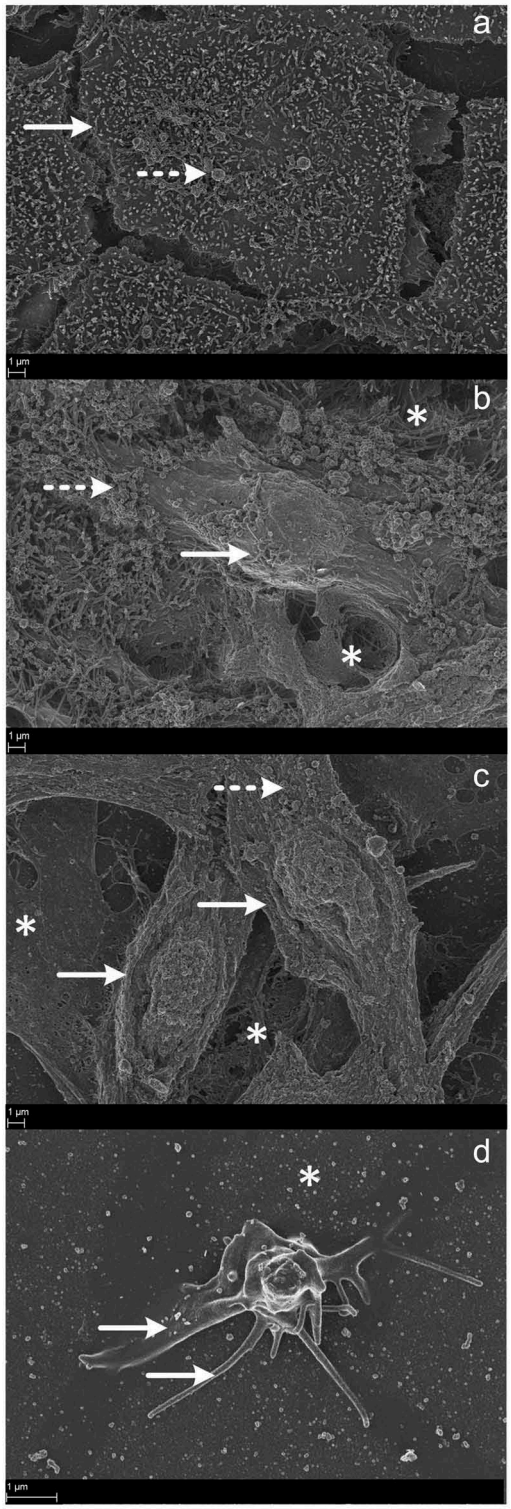

Figure 1. Electron Micrographs showing: A: Control MCF-7 cells, B: MCF-7 cells co-cultured with platelet-rich plasma (PRP), C: MCF-7 cells co-cultured with whole-blood (WB). D) Activated platelet adhered to the coverslip prior to co-incubation with MCF-7 cells. Scale bar: 1µm
A: Control MCF-7 cell (solid arrow) showing typical polyhedral, squamous morphology. The cytoplasm is covered in short microvilli, and bleb-like structures (dashed arrow), with definitive connectives to other cells. B: MCF-7 cell with prominent nucleus (solid arrow) co-cultured with PRP. The cell is spindle-shaped, with thick cytoplasmic processes and an evident reduction in microvilli. Numerous globular aggregates are noted (dashed arrow), interspersed by bleb-like structures associated directly with the cell plasma membrane. Cells are surrounded by fibrin fibers forming dense networks (asterisk). C: MCF-7 cells with prominent nuclei (solid arrows) co-cultured with WB. Cells are spindle-shaped, showing thick cytoplasmic processes. A reduction in cytoplasmic microvilli is evident, although blebbing is noted (dashed arrow). Surrounding fibrin plaques are dense (asterisks). D: Platelet representative of both WB and PRP prior to co-culture with MCF-7 cells, showing early activation with pseudopodia extending out from the body (solid arrows). Microparticle secretion is evident (asterisk).

cytoplasmic TGF-β_1 expression (Figure 3) compared with the control.

TGF-β_1 and Vimentin gene expression in MCF-7 breast cancer cells

In comparison with the control, WB- and PRP- co-culture groups gained significantly higher expression of *TGF-β_1* and *vimentin* (Figure 3a and b). In co-cultured samples, *TGF-β_1* in MCF-7 cells incubated with WB was significantly higher than those incubated with PRP (mean ± SD, 89.22 ± 127.23) (Figure 3a); however, *TGF-β1* expression in both WB and PRP-treated cultures were upregulated when compared with the control samples ($p < .05$). The expression of *vimentin* in co-cultured cells incubated with PRP-exposed cells was significantly higher, albeit more variable, than that in WB-exposed cells, suggesting that the MCF-7 cells were engaged in an active EMT. This finding is further supported by the acquisition of a more spindle shape in treated MCF7 cells as observed morphologically (Figures 1(b,c), 2(b,c)).

Discussion

Metastasis, a prominent feature of cancer-associated treatment failures and death[1], is dependent on the acquisition of a more invasive phenotype and intravasation into the circulation.[4] Reciprocal interactions between cell types within the surrounding microenvironment are essential to inducing tumor cell phenotypic alterations, with participation from cytokines effectively mediating tumor progression.[35] The cytokine TGF-β is a pleiotropic molecule, which has a tumor progression-dependent role – in early tumors, TGFβ action is suppressive and in later stages, facilitative.[36,37] As a driver of tumor progression, TGF-β1 overexpression is associated with inhibition of immunosurveillance[36–38], promotion of platelet aggregation[28,39] and metastasis by enhancing EMT[12,37,40,41] (Figure 4).

Our results show that on exposure to blood components, whether whole blood or platelet -rich plasma, breast cancer cells undergo EMT with associated alterations in TGF-β1 expression. In control samples, following serum deprivation, TGF-β_1 mRNA expression was severely reduced due to hormonal regulation of this growth factor.[42,43] Notably, even with serum-deprivation and reduced TGF-β_1 mRNA expression, definitive protein expression was noted primarily in the perinuclear region. This alludes to activation of the TGF-β signaling pathway or transportation of the protein for release into the extracellular microenvironment.[20,24,44] Multiple cell types in the tumor microenvironment secrete TGF-β_1, with platelet-derived TGF-β_1 providing a large source of this growth factor for tumor progression.[12] However, studies indicate that autocrine expression of TGF-β_1 by tumor cells themselves and subsequent sustained signaling is essential for EMT.[37,40,41] In this study we show that exposure to blood components, be it platelet-rich plasma or whole blood, is a trigger for the induction of TGF-β_1 expression (mRNA and protein) in breast cancer cells. This is a clear indication of the upregulation of TGF-β_1 biosynthesis, a prominent feature of breast tumor cells to promote autocrine regulation of EMT as a factor of tumor progression for invasion, metastasis and ultimately, the establishment of secondary sites.[17,18,20,44,45] Moreover, significantly heightened *vimentin* expression following exposure to blood components offered further evidence of EMT. *Vimentin* is a marker of EMT and mediates organization of the cytoskeleton[22,25], thereby promoting the acquisition of spindle-shaped morphology, as identified in our results, and reflecting the transition to a more invasive phenotype.[4,24,26,40] Further research is warranted regarding the presentation of other EMT-associated genes and invasive assays, which would serve to enhance our understanding of this process. Ultrastructural assessment also revealed extracellular vesicles (blebs) on MCF-7 cells, albeit some obscured by platelet aggregates in samples co-cultured with blood components. Molecules previously thought to have been solely secreted, may indeed be contained within tumor-cell derived extracellular vesicles (including microvesicles and exosomes).[45,46] The release of such vesicles into the tumor microenvironment and circulation is implicated in facilitating tumor progression, with such vesicles found in higher proportions in cancer patient circulation.[45,46] Microparticles from tumor cells undergoing EMT also have been found to contain tissue factor and thrombin, potent agonists of the coagulation cascade.[46,47] In this study, the ability of breast cancer cells to produce a pro-coagulant microenvironment is further highlighted by the induction of fibrin networks following exposure to blood components. While both co-culture systems resulted in

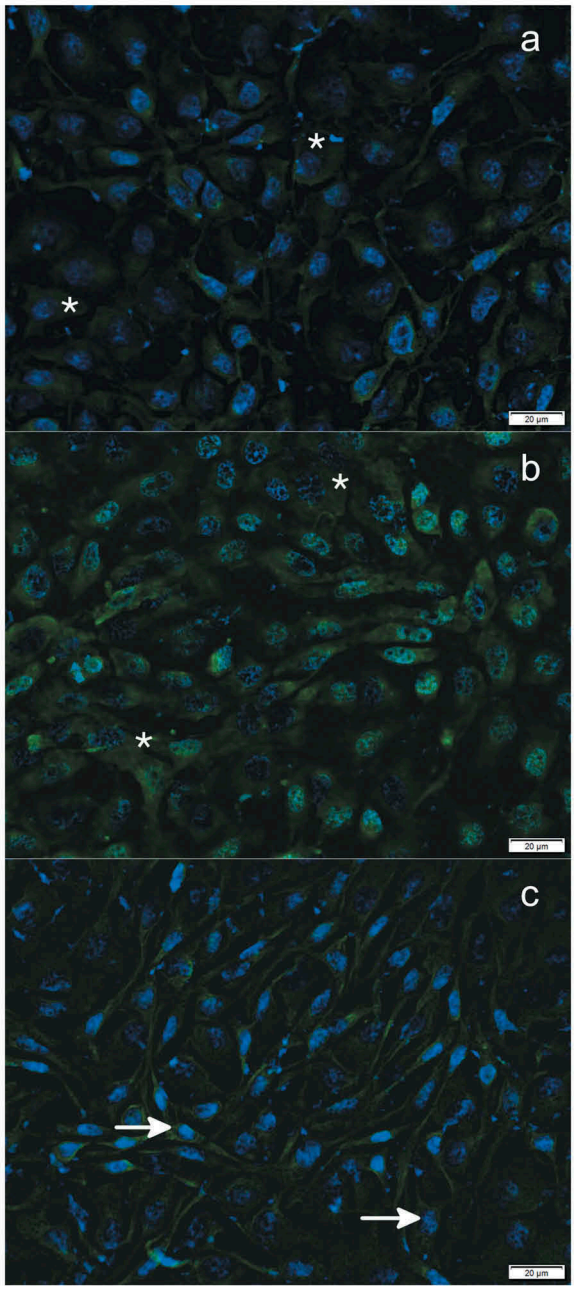

Figure 2. Immunofluorescence of TGF-β1 in: A: Control MCF-7 cells, B: MCF-7 cells co-cultured with whole-blood (WB), C: MCF-7 cells co-cultured with platelet-rich plasma (PRP). Scale bar: 20µm.
Qualitative assessment of TGF-β1 expression in control MCF-7 cells. A: TGF-β1 was localized primarily to the perinuclear region, with cells having a typical polyhedral, squamous appearance (asterisk). B: Incubation with WB induced an increase in TGF-β1 expression and intensity in both the perinuclear (arrow) and cytoplasmic regions (asterisk). C: The addition of PRP induced higher intensity TGF-β1 expression in the perinuclear region, with more diffuse cytoplasmic staining (arrow). In both co-culture groups, heterogeneous expression is noted, with TGF-β1 expression low or absent in some cells. Additionally, cells that appear more spindle-shaped or mesenchymal, show higher intensity TGF-β1 expression.

the formation of fibrin networks, closely associated with the breast cancer cells, whole blood alone caused fibrin plaque formation, indicative of hyperactivation of the coagulation system.[48] Inflammatory conditions are capable of altering fibrin network architecture, which ultimately affect the efficacy of fibrinolysis and contributes to thrombosis.[48,49] The kinetics of clot formation is largely dependent on thrombin concentrations (note: "normal", not physiologically low as with bleeding disorders) and are associated with the

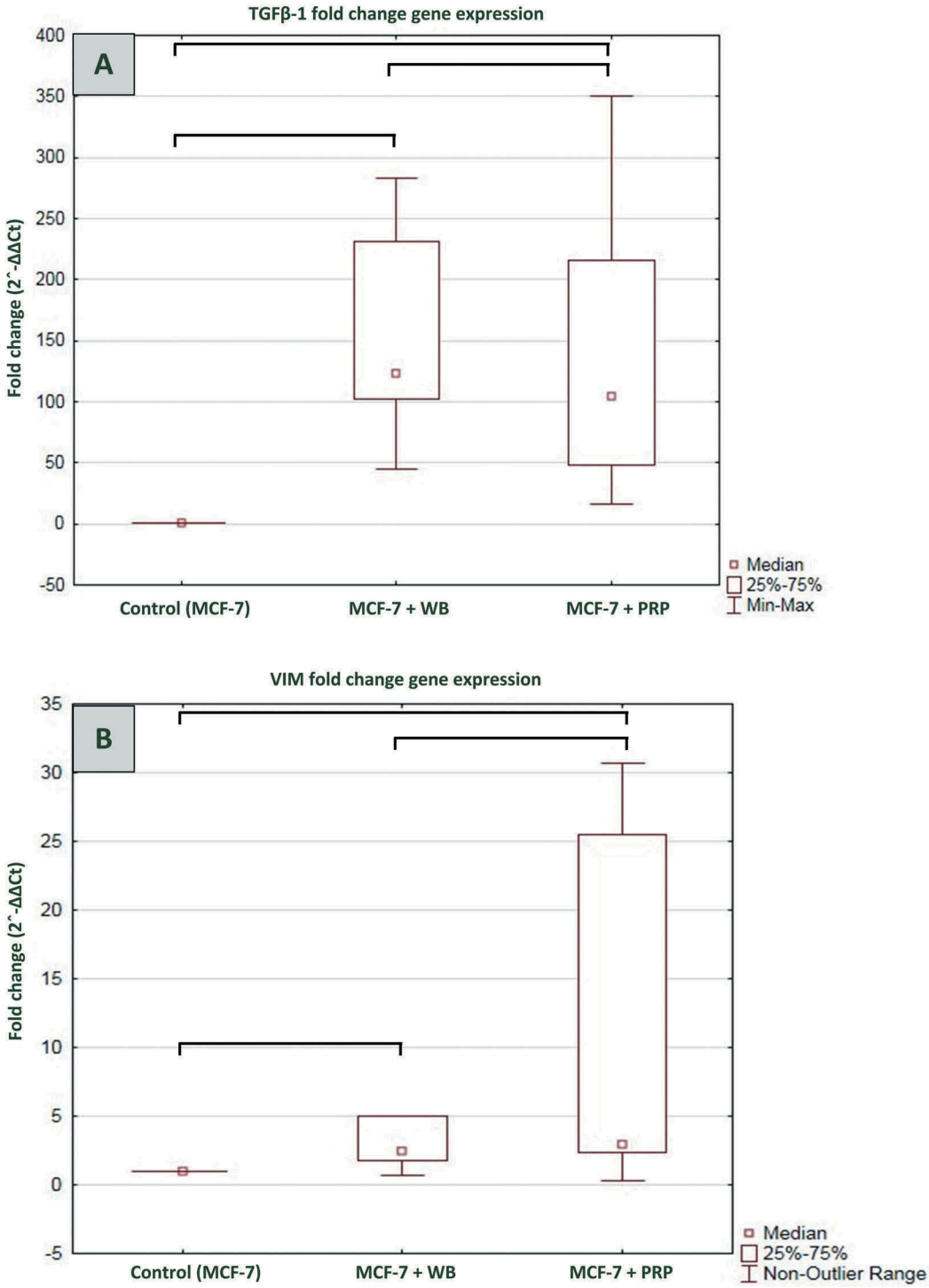

Figure 3. Box and Whisker plots comparing fold change expression in (A) TGF-β1 and (B) vimentin following whole blood (WB) and platelet-rich plasma (PRP) co-culture with MCF-7 breast cancer cells

MCF-7 control cells were used as a baseline for TGF-β1 and vimentin expression and the fold change in gene expression was calculated in relation to the control. TGF-β1 was upregulated in comparison with the control (pβ0.05), with that from WB co-cultures significantly higher than in PRP co-cultures. Vimentin was upregulated in comparison with the control (pβ0.05) and was significantly higher in PRP co-cultures than in WB co-cultures.

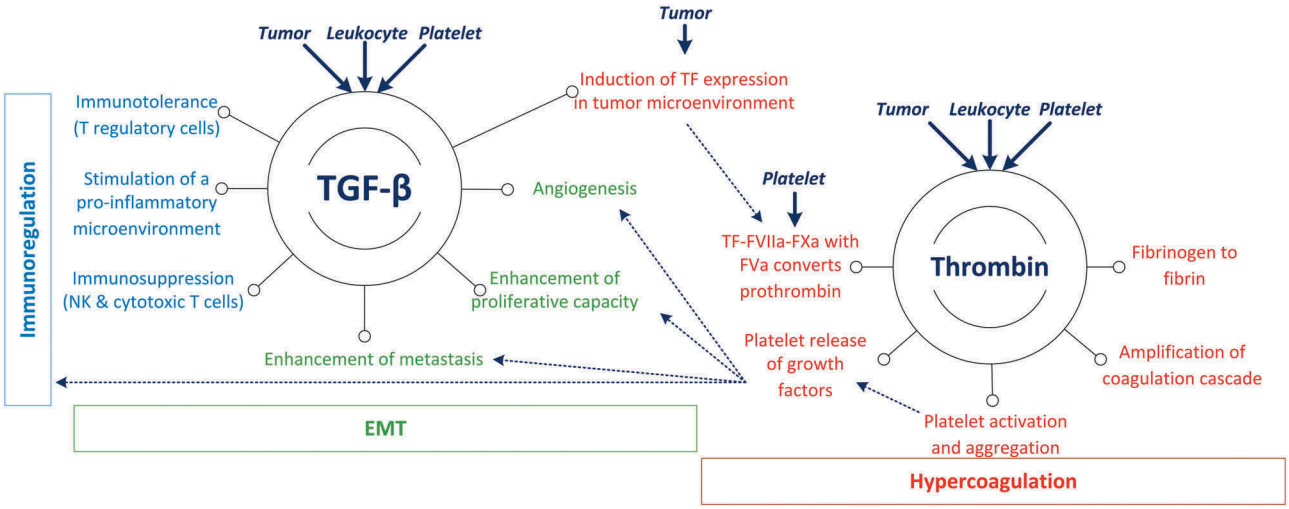

Figure 4. TGF-β in late stage cancers is a powerful mediator linking the processes of hypercoagulation, immunoregulation and epithelial-mesenchymal transition (EMT) to facilitate tumor progression.

construction of sturdy, thick/major fibers, loosely contained within a network and more susceptible to fibrinolysis than the tightly packed, thin/minor fibers formed in the presence of higher thrombin concentrations.[49] The formation of fibrin plaques without the experimental addition of thrombin, as observed in our results, indicates an inflammatory cell culture state. Such plaque formation has been observed in patients with inflammatory disorders, autoimmune disease and cancer.[48,50,51]

Additionally, tumor cells, including the MCF-7 cell line, are themselves capable of producing a peak thrombin concentration of 271 nM, with time to peak approximately 11.9 min, highlighting a pro-thrombotic phenotype that accounts for the heightened risk of thromboembolic complications in cancer patients.[52] In our laboratory, platelet-rich plasma exposed to MCF-7 cells for only 5 min displayed ultrastructural characteristics of platelet activation and induction of thin fibrin networks[53], with time-dependent assays highlighting enhanced pro-thrombotic activity, and consistent with ongoing pro-coagulant secretion by MCF-7 cells with longer duration co-culture. The ultrastructural findings in the present study supports the production of thrombin by MCF-7 cells, as evidenced by the formation of fibrin networks and plaques in the culture environment when blood components were incubated with breast cancer cells.

Our study affirmed that upon exposure to blood components, as a proxy for the interaction between tumor cells and the vascular system, hormone-dependent breast cancer cells undergo epithelial-mesenchymal transition that is coupled with the induction of a pro-thrombotic microenvironment, potentially facilitated by sustained signaling from TGF-β_1. In this sense, TGF-β plays a dominant role, thereby linking EMT processes, coagulopathy and immunoregulation[24], to deconstruct the complexity of the microenvironment and facilitate an.[47] *Ex vivo* assessments of the interaction between tumor cells and components of the circulation are necessary understanding of factors involved in tumor progression, which may in turn affect other co-morbidities.

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Declaration of interest

The authors have no conflicts of interest to declare.

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