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Basement membrane extract attenuates the more malignant gene expression profile accentuated by fibronectin in prostate cancer cells

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Abstract

Prostate cancer (PCa) has high mortality rates, with most of the deaths resulting from the development of metastasis. Fibronectin (FN) plays key roles in cell adhesion and affects the migratory behavior of cells. In the tumor microenvironment and also in the blood plasma during metastasis, FN displays increased expression, however its role in prostate cancer remains poorly understood. This study aimed to unveil the specific roles of FN as a soluble component, alone or in combination with a complex basement membrane. To investigate the impact of FN in neoplastic prostate cells, we evaluated the gene expression of LNCaP cells by RT-qPCR after exposure to soluble FN (25 µg/mL) either alone or in combination with a basement membrane. When FN was the predominant matrix element, such as in blood plasma, PCa tumor cells increased their expression of genes related to an invasive behavior and resistance to apoptosis, including *CDH2, ITGA5, AKT1*, and *BCL2*. However, the combined presence of FN and a complex basement membrane had the opposite effect on LNCaP cells, in which the expression levels of *CDH2, ITGA5, AKT1*, and *BCL2* were reduced. Hierarchical clustering analysis with LNCaP and RWPE-1 cells showed that LNCaP cells exposed to an enriched extracellular matrix displayed an expression pattern more similar to that shown by RWPE-1 cells, a cell line that illustrates characteristics of the normal prostate epithelium. These findings provide the groundwork for future studies addressing the role of FN in tumor growth, particularly in the context of cancer evolution/progression from a solid primary tumor to a transitory circulating state.

Keywords Fibronectin · Prostate cancer · Integrins · Cadherins · Metastasis · RWPE-1

Introduction

Prostate cancer (PCa) continues to be a leading cause of death among men worldwide [1]. Early diagnostic rates have increased with the onset of the prostate-specific antigen (PSA) test, allowing for great improvements in 5-year survival rates [2]. However, even with these advancements,

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most PCa-related deaths still occur due to the development of metastasis [3].

Metastasis is a multi-step process mediated not only by genetic changes occurring within tumor cells, but also by physical remodeling of the tumor microenvironment [4, 5]. The spread of cells to distant sites requires the involvement of stromal components, along with differential expression of molecules related to cell–cell and cell–matrix interactions [6–8]. To successfully disseminate themselves, tumor cells must be able to complete all of the following steps: (1) detach from the primary tumor mass; (2) degrade the basement membrane; (3) invade the stromal surrounding tissue; (4) intravasate into blood and lymphatic vessel lumens; and, finally, (5) disseminate and colonize other organs, such as lymph nodes, bones, lung, liver, and brain [9–12].

Once tumor cells have reached the blood stream, they activate the coagulation cascade and surround themselves with fibronectin (FN) molecules, forming a thrombus that protects them against the cytotoxic activity of immune cells

[12, 13]. The thrombus becomes a protective shield that stabilizes these circulating tumor cells and provides a favorable microenvironment that sustains cell adherence amongst each other and to the blood vessel wall, which will later be necessary for extravasation to the surrounding tissue [12–14]. Additionally, the FN-rich thrombus microenvironment has been shown to activate important intracellular signaling cascades via surface receptors, such as the AKT pathway, which mediates tumor survival [15].

FN is a multifunctional modular glycoprotein present in numerous tissues throughout the body, being an important component in blood plasma [16], as well as in connective tissue matrices [17]. High FN levels have been documented in diverse processes such as wound healing and arthritis, and many types of cancer [18–20]. With regard to PCa, however, there is limited data on FN expression levels in tissue samples and changes in serum FN concentration in patients [21], which have rather generated contradictory information about the role of FN in this disease.

Identifying and understanding the functions of FN in prostate tumor cells will provide insights into the mechanisms of PCa progression and metastasis. We therefore sought to evaluate the impact of FN on the expression of cadherins, integrins, and important genes involved in cell proliferation and survival (such as AR, AKT1, PTEN, STAT3, MYC, BCL-2 and BAX), using the LNCaP cell line as a model [22]. For this, the cell line was exposed: (1) only to FN, mimicking the interactions between tumor cells and this molecule in the plasma; and (2) to FN in combination with a basement membrane extract, mimicking the interaction in primary tumors. We also investigated whether these exposures would approach or distance the expression of the evaluated genes between the neoplastic cell line (LNCaP) and a non-malignant cell line (RWPE-1).

Materials and methods

Cell lines and culture conditions

LNCaP and RWPE-1 cells were purchased from the American Type Culture Collection (ATCC) and cultivated in standard cell culture medium optimal for each cell type. The LNCaP cell line (clone FGC - ATCC® CRL-1740™) represents androgen-sensitive prostate adenocarcinoma, while RWPE-1 cell line (ATCC® CRL-11609™) is commonly used to represent the non-malignant growth. LNCaP cells were cultured in RPMI 1640 medium (Gibco™; 11875093) supplemented with 10% fetal bovine serum (FBS; Gibco™; 16000044) and 1% 100× antibiotic/antimycotic (Gibco™; 15240062). RWPE-1 cells were cultured in Keratynocite Serum Free medium (Gibco™; 37000015) supplemented with 0.05 mg/mL bovine pituitary extract, 5 ng/mL human

recombinant epidermal growth factor, and 1% 100× antibiotic/antimycotic (Gibco™; 15240062). All cell lines were maintained in a humidified incubator with 5% CO_2 at 37 °C.

Coating conditions

Cells were seeded at 4×10^4 cells/cm^2 and all experiments were carried out in triplicate. The LNCaP cells were cultivated for four days under the following conditions: (1) standard medium − Non-exposed group; (2) exposed to 25 μg/ml FN from human plasma (Sigma-Aldrich; F0895), according to an established protocol previously published by our group [23]; (3) plates coated with a basement membrane extract (Geltrex® Reduced Growth Factor Basement Membrane Matrix) (Gibco™; A1569601) plus FN diluted in the medium (25 μg/ml); and (4) plates coated with Geltrex® without FN exposure. To coat the plates, Geltrex® was added, covering the entire well (50 μL/cm^2) of the six-well plates (9.6 cm^2), and the plates were incubated at 37 °C in 5% CO_2 for 30 min to allow polymerization. RWPE-1 cells were cultivated only with standard medium, without coating.

RNA extraction and cDNA synthesis

RNA extraction was performed with the PureLink® kit (Ambion™, catalog number 12183020) according to the manufacturer's instructions. RNA samples were quantified using NanoVue™ Plus spectrophotometer (GE HealthCare), and sample quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies) [24]. Only samples with RNA integrity numbers above 9 were used for further analysis. To remove genomic DNA, we used the TURBO DNA-free™ Kit (Ambion™, AM1907) and 1 μg of RNA was reverse-transcribed into cDNA, using SuperScript® VILO™ Master Mix (Invitrogen™; catalog number 11755500).

Quantitative real-time polymerase chain reaction (RT-qPCR)

For RT-qPCR, the QuantStudio™ 12K Flex system (Applied Biosystems) was used. The cDNA samples to quantify: AKT serine/threonine kinase 1 (AKT1), BCL2 associated X (BAX), BCL2, E-cadherin (CDH1), N-cadherin (CDH2), integrin subunit beta 1 (ITGB1), MYC proto-oncogene (MYC), phosphatase and tensin homolog (PTEN), and signal transducer and activator of transcription 3 (STAT3) mRNAs were amplified using TaqMan® Fast Advanced Master Mix (Applied Biosystems™; 4444557) and TaqMan® Gene Expression Assays (Applied Biosystems™; Supplementary Table 1). For the Androgen Receptor (AR), integrin subunit beta 3 (ITGB3), integrin subunit alpha 5 (ITGA5), and integrin subunit alpha V (ITGAV) mRNAs, the cDNA samples were amplified using Fast SYBR® Green Master

Mix (Applied Biosystems™; 4385617) and primers synthesized by Thermo Fisher Scientific (Supplementary Table 2). Relative gene expression was calculated via the $2-\Delta\Delta C_t$ method [25] and normalized to the *GAPDH* gene expression [C_t means ± SD were as follows: (1) RWPE-1 cells: 27.826 ± 0.07; (2) LNCaP on uncoated plates: 27.178 ± 0.18; (3) LNCaP on Geltrex® coating: 27.509 ± 0.13; and (4) LNCaP on Geltrex® coating plus FN: 27.337 ± 0.09]. For analyses involving only LNCaP cells, gene expression data was normalized using the mean expression values of non-exposed LNCaP cells ($n=3$). For cluster analyses involving RWPE-1 and LNCaP cells, expression data was normalized for each gene, using the mean expression values of all groups ($n=12$).

Statistical analysis

Statistical analyses were performed by a *t* test or a parametric one-way ANOVA test with an *a posteriori* Tukey test. Differences were considered statistically significant at $p < 0.05$.

Results

Fibronectin enhances the expression of N-cadherin and integrins αvβ3 and α5β1

The dissemination of tumor cells to distant organs begins with a transformation in phenotype, in which the cells shift from a benign to a malignant phenotype. In this multi-step process, epithelial cells that are normally cohesive, have to acquire migratory and invasive characteristics mediated by cell-adhesion molecules, such as cadherins and integrins [26]. Cadherins are important cell-surface glycoproteins that mediate cell–cell interactions and, studies have shown that a key event in tumor cell migration is the suppression of E-cadherin, and upregulation of N-cadherin [27]. In the other hand, integrins are surface receptors that mediate cell adhesion to the extracellular matrix (ECM), translating mechanical properties into intracellular biochemical signals [28]. Therefore, considering that integrins αvβ3 and α5β1 are FN receptors and that E-cadherins and N-cadherins are crucial for tumor invasion, we sought to evaluate the ability of FN to affect the expression of these genes. For that, LNCaP cells were exposed to soluble FN in the concentration of 25 μg/ml. The exposure to soluble FN decreased the expression levels of *CDH1* (0.62 fold) and increased the levels of *CDH2* (1.98 fold), suggesting that soluble FN may support an invasive behavior. Additionally, we observed a significant increase in the expression of *ITGA5* (2.70 fold), *ITGAV* (2.81 fold), and *ITGB3* (2.71 fold) after exposure to FN when compared with non-exposed cells (Fig. 1).
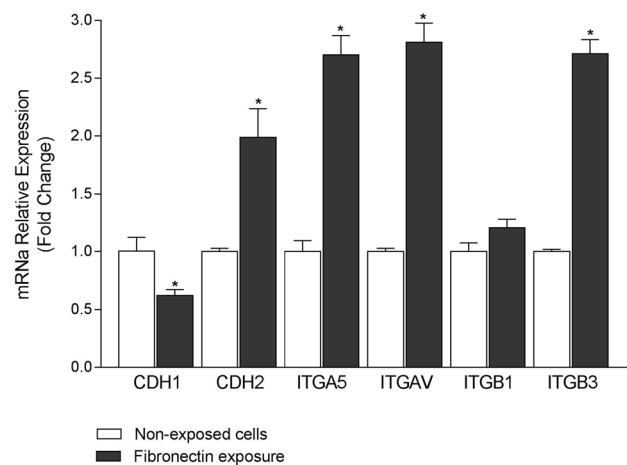

Fig. 1 Relative expression of cell surface receptors genes after FN exposure (fold change versusnon-exposed LNCaP cells). mRNA levels of cadherins and integrins in LNCaP cells: not exposed (white bars) or exposed (black bars). Values are expressed as mean ± SEM; *$p < 0.05$

Fibronectin provides a pro-proliferative intracellular signaling stimulus

The increase in cell proliferation rates and survival are also a crucial event for tumor progression. Cell–matrix interactions mediated by integrins have been shown to modulate these cellular processes by triggering alterations in intracellular signaling pathways [29]. Fibronectin has been shown to support cell survival by increasing Bcl-2 expression through interaction with integrin α5β1 in colon carcinoma and neuronal cells [30]. Therefore, to explore whether FN exposure also supports cell survival and proliferation in PCa, we examined the expression of genes involved in cell signaling, proliferation and cell death pathways. Table 1 presents detailed Gene Ontology (GO) annotations and the major function of each evaluated gene.

After FN-only exposure, LNCaP cells showed significantly increased expression of pro-proliferative genes *AKT1* and *MYC*; while that of *PTEN* was reduced compared with that in the non-exposed cells. With respect to genes related to cell death, *BCL2* expression was highly increased, whereas that of *BAX* showed a two-fold decrease in expression when compared with that in the non-exposed cells (Fig. 2). As briefly mentioned, the genes *AKT1* and *MYC* support cell proliferation and survival, while PTEN has the opposite role, being a negative regulator of the PI3K-AKT/PKB signaling pathway [31]. Similarly, the *BCL2* gene suppresses apoptosis, while *BAX* has pro-apoptotic functions [32]. Therefore, our results indicate that FN supports cell survival and proliferation in PCa, corroborating with its roles in colon carcinoma and neuronal cells.

Table 1 Gene ontology (GO) annotation and function of evaluated genes

| Gene	Related GO annotation[a]	Function
AR	Transcription factor activity and DNA binding	Cell signaling
AKT1	Protein binding and protein kinase activity	Cell signaling
PTEN	Protein kinase binding and magnesium ion binding	Cell signaling
STAT3	Transcription factor activity and DNA binding	Proliferation
MYC	Transcription factor activity and DNA binding	Proliferation
BCL2	Protein homodimerization activity and protein binding	Death
BAX	Protein homodimerization and heterodimerization activity	Death

[a]Only the two main GO annotations are mentioned
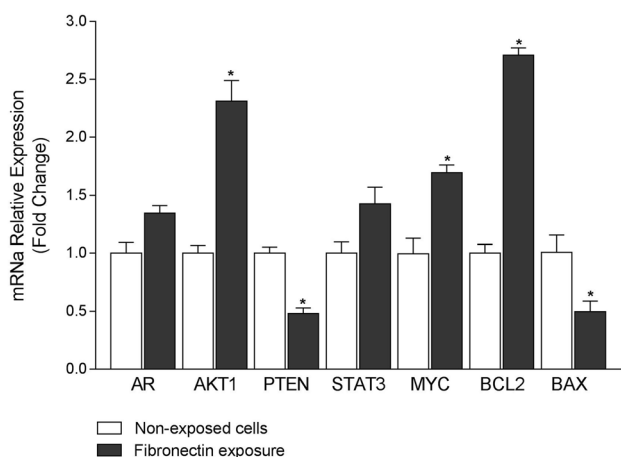
Fig. 2 Relative expression of genes related to pathways that transfer signals from the extracellular to the intracellular compartment (fold change versus non-exposed LNCaP cells). mRNA levels of genes related to cell signaling, proliferation, and survival were evaluated in LNCaP cells: non-exposed (white bars) or FN-exposed (black bars). Values are expressed as mean ± SEM; *$p < 0.05$

Fibronectin inversely alters gene expression when combined with a basement membrane extract

The ECM is a complex and highly dynamic structure that undergoes constant remodeling to precisely maintain its composition [33]. In the tumor setting, activated fibroblasts have been shown to alter the normal dynamic and composition of the ECM, supporting tumor progression [34]; while the exposure of tumor cells to a normal and balanced ECM is capable of restraining tumor progression [35]. Therefore, considering that each component of the ECM surrounding tumor cells play important roles in determining the cell's phenotype and behavior, we sought to evaluate if FN would have the same impact in LNCaP cells when present in an ECM-enriched environment, like those found in primary tumor sites. For that, we cultivated LNCaP cells on top of a basement membrane (Geltrex®) with soluble FN diluted in the medium.

As seen in Fig. 3a, the combination of Geltrex® and FN led to a gene expression pattern opposite to that of the one
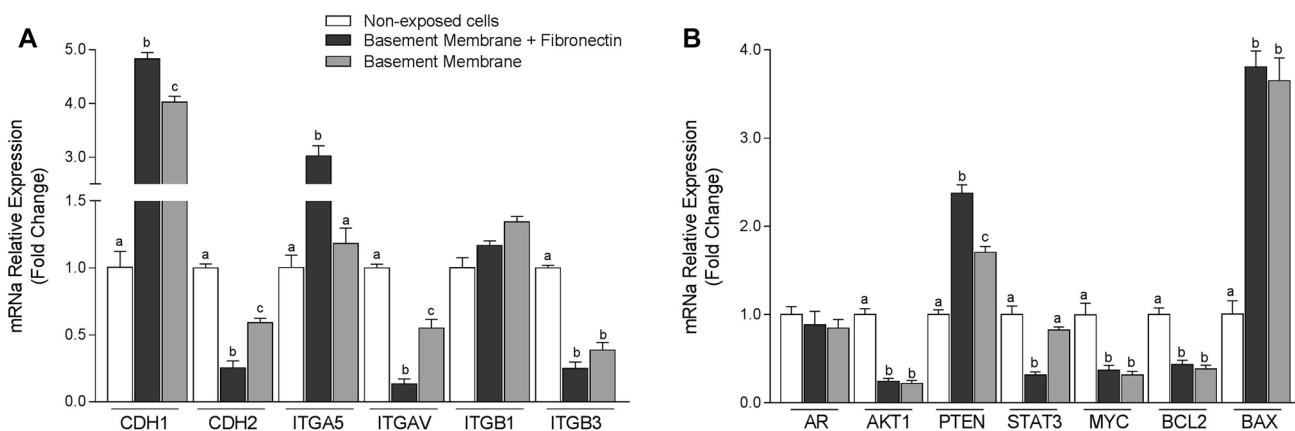

Fig. 3 Relative expression of cell surface receptors and genes involved in signal transduction from the extracellular to the intracellular compartment (fold change versus non-exposed LNCaP cells). mRNAs levels of cell surface receptors and genes related to cell signaling, proliferation, and survival were evaluated in LNCaP cells: non-exposed (white bars), exposed to FN in combination with a basement membrane (black bars), and exposed only to basement membrane (grey bars). Values are expressed as mean ± SEM. Bars with different letters are significantly different ($p < 0.05$)

observed with only FN. In this case, *CDH1* expression level showed an increase of 4.83-fold compared with that in the non-exposed cells. *CDH2* that had previously displayed a markedly increase in its expression levels had a four-fold decrease in expression. For the integrins, both subunits of integrin αvβ3 (ITGAV and ITGB3) had significantly decreased expression, while the expression of *ITGA5* was increased. The integrin subunit β1 had the same level of relative expression among all groups (Figs. 1, 3a).

Likewise, regarding the expression of genes related to cellular behavior, the presence of a more complex basement membrane shifted the gene expression observed following FN-only exposure (Fig. 3b). In this case, *AKT, STAT3, MYC*, and *BCL2* had significantly decreased expression by more than two-fold, when compared with that in the non-exposed cells; while *PTEN* and *BAX* showed significantly increased expression. All fold change values are presented in Supplementary Table 3. Therefore, as expected, our results demonstrate that although the FN itself is capable of supporting a more invasive phenotype in tumor cells, when the cells are surrounded by a more balanced microenvironment this phenotype is restrained.

Fibronectin exposure distances gene expression pattern between LNCaP and RWPE-1 cells

In this study, we sought to assess how FN would affect the expression levels of genes associated with cell–cell/cell–matrix interactions, cell proliferation and survival. More specifically, we aimed to evaluate if those expression changes would occur in a manner that resemble a more invasive and aggressive phenotype, or a more benign phenotype. For this, we compared the expression patterns of LNCaP cells cultivated under three different conditions: (i) non-exposed, (ii) exposed to FN, and (iii) exposed to FN in combination with a basement membrane, to the expression patterns of RWPE-1 cell lines (non-malignant cells) cultivated in a standard culture.

Hierarchical clustering analysis (Fig. 4) indicated a clear distinction among the groups, in which LNCaP cells exposed only to FN acquired a very distinct expression pattern when compared to RWPE-1 cells; while LNCaP cells exposed to the combination of FN with a more complex basement membrane showed an expression profile similar to the expression pattern of RWPE-1 cells.

Discussion

FN is one of the most abundant adhesion proteins in blood plasma [36], and has been shown to support stabilization and survival of circulating tumor cells in the blood stream during metastatic progression [12]. The role of FN in cancer is not yet fully understood; whereas some studies suggest that the adhesive activity of FN suppresses tumor cell invasion [18, 37, 38], others indicate that FN may assist in the invasion and metastasis processes [12, 39–41].

During the metastatic process, tumor cells can interact with FN in two different environments: (1) in the primary tumor site, in which the cells are in contact with the FN present in the surrounding stromal microenvironment; or, (2) in blood plasma, where FN is an abundant soluble component [41, 42]. The examination of samples from patients with PCa correlates high levels of FN and increased mortality, promoting FN as a prognostic biomarker for this cancer type [41]. In this study, we showed that exposure to only FN, mimicking the plasma environment, results in increased N-cadherin (*CDH2*) expression and decreased E-cadherin (*CDH1*) expression, suggesting that plasma FN supports the acquisition of an invasive phenotype. Indeed, studies have demonstrated that N-cadherin overexpression is associated with higher invasion rates and the development of an androgen-independent state in prostate tumor cell lines [43]. Changes in integrin expression can also be associated with such invasive behavior. Upregulation of integrins α5β1 and αvβ3 has been associated with invasiveness in several cancers, as lung cancer and bone metastasis [44, 45].

Likewise, our findings showed increased expression of integrin subunits α5, αv, and β3 in FN-exposed LNCaP cells. Similar results found in lung cancer presented the activation of integrins αvβ3 by plasma FN, promoting premetastatic condition to cancer cells [12]. Together, our data indicate and reinforces using another cancer cell model that soluble FN may support the adhesion of tumor cells mediated by N-cadherin (CDH2) and integrins αvβ3 and α5β1.

Because intracellular signals downstream of cell surface receptors are crucial for the processes of metastasis and anchorage-independence, we also evaluated the expression status of several genes important for regulating cell growth, proliferation, and survival. After FN exposure, LNCaP cells showed increased *AKT1* expression. *AKT* is required for cell survival and studies have shown that FN can activate the AKT pathway, which could in turn contribute to the development of resistance to apoptosis in PCa cells [40]. Likewise, we detected a significant increase in *BCL2* expression, an anti-apoptotic gene, and a significant decrease in *BAX* expression, an apoptotic inducer gene. Functional studies have demonstrated that FN induces *BCL2* expression with a consequent decrease in apoptosis rates [46–48].

Furthermore, LNCaP cells exposed only to FN displayed higher expression levels of *MYC*, upregulation of which is related to early stages of PCa, required for AR-dependent growth and related to the development of metastatic progression in PCa [49, 50]. Thus, we propose that when FN is the predominant element, tumor cells can develop invasive behavior and resistance to apoptosis.

Fig. 4 Hierarchical clustering of gene expression data from RWPE-1 cells and LNCaP cells ($n = 12$). The clustering analysis was carried out using DESeq package (R/bioconductor) based on Euclidian distance. Expression data are normalized for each gene, using the mean expression of all samples, and scaled to Z-scores of the rows in order to show differences among samples for each gene. Blue boxes indicate lower levels; red boxes indicate higher levels. (Color figure online)
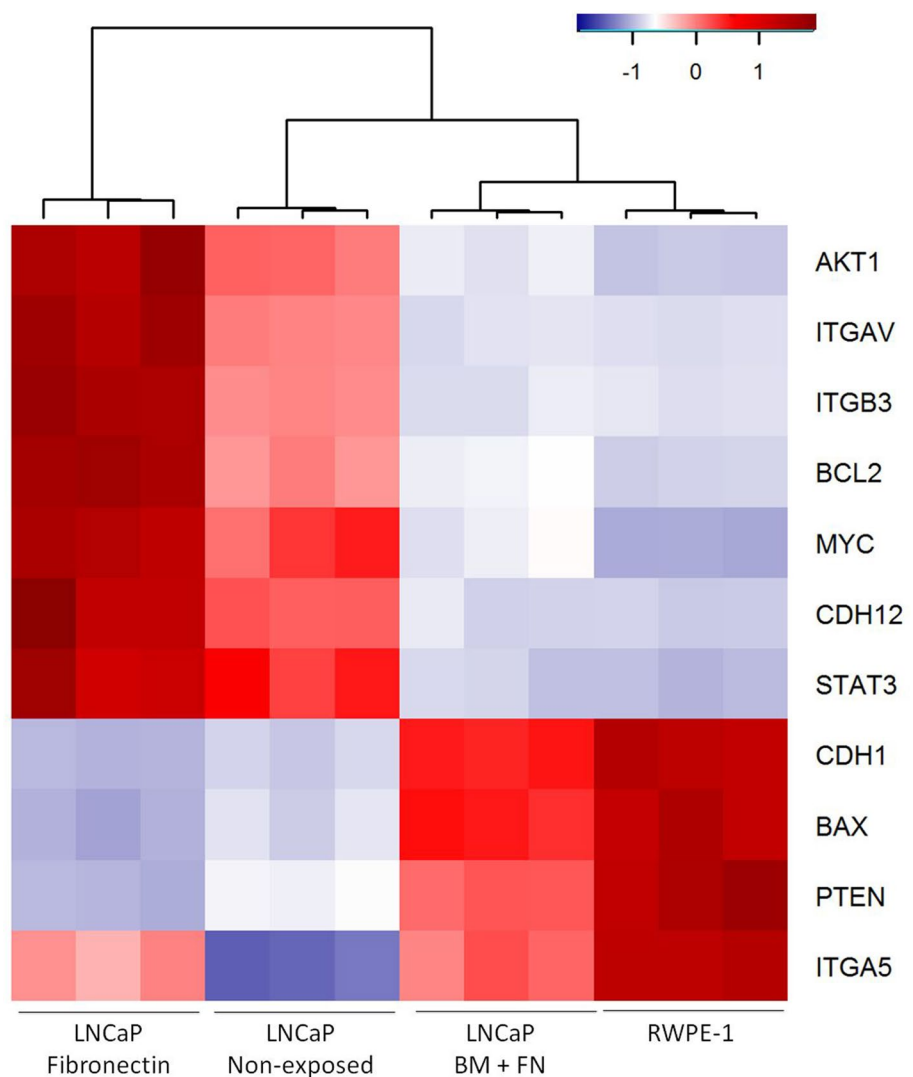
However, in primary tumor sites, tumor cells not only interact with FN, but also with other ECM elements. We therefore examined the impact of exposing our LNCaP cell model to the combination of FN and a basement membrane extract. In this environment, LNCaP cells displayed a differential expression pattern of cadherins, with decreased *N-cadherin* expression and increased *E-cadherin* expression [51, 52]. Furthermore, *ITGAV* and *ITGB3* expression levels were significantly lower than those in FN-only exposed cells as well as in non-exposed cells, revealing basement membrane extract attenuating role in the primary effects of FN on LNCaP cells gene expression.

In accordance with this new expression pattern of cell surface receptors, genes related to cell survival and invasiveness also displayed altered expression, but in an opposite way when compared to those in cells exposed only to FN. LNCaP cells in the presence of a basement membrane extract and FN showed decreased expression of *AKT1, STAT3, MYC*, and *BCL2*; while expression of *BAX*, a pro-apoptotic gene,

and *PTEN*, a gene that could sensitize LNCaP cells to an apoptotic stimuli [53], were significantly elevated. This way, the presence of the basement membrane components (Geltrex® content: laminin, type IV collagen, heparin/heparin sulfate proteoglycan, and entactin/nidogen, with reduced growth factor) seems to restrain the malignant and metastatic potential of LNCaP previously highlighted with exposure to FN. In these sense, our findings reinforce the results already found for breast cancer [35, 54–56] and contribute to emphasize these results also for prostate cancer in showing that a more complex ECM created a rich microenvironment favoring intercellular connections and the correction of malignant behavior. Thus, our findings show that the role of FN depends on the interactions between tumor cells and their substrates, corroborating the findings of Ramos et al. [57], who reported that changes in the microenvironment could cause a differential effect of FN on cell migration.

To further elucidate this idea, we performed a hierarchical clustering analysis with the LNCaP under the

two different exposure conditions and the non-malignant RWPE-1 cells. Interestingly, results showed that the gene expression profile of LNCaP cells exposed to a more enriched ECM was more similar to that of RWPE-1 cells [58], while LNCaP cells exposed only to FN seemed to have acquired a more aggressive behavior, underlining that FN favors tumor growth, survival, and metastatic progression. This is especially important in the context of circulating tumor cells' biology, since it becomes evident that FN may play a role not only in angiogenesis stimulation [41], but may also sustain survival and proliferation of tumor cells within the blood vessels.

In conclusion, this study shows that the role of FN in prostate cancer is a function of the composition of the surrounding microenvironment. Our results provide the groundwork for future research addressing the role of FN signaling in tumor growth, particularly in the context of cancer evolution/progression from a solid primary tumor to a transitory circulating state.

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest.

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