UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

PG-BGA

RELAÇÃO ENTRE A FIBRONECTINA E O CÂNCER DE PRÓSTATA: ANÁLISE DE GENES E MICRORNAS.

BRUNO MARTINUCCI

Dissertação apresentada ao Programa de Pós-Graduação em Biologia Geral e Aplicada do Instituto de Biociências, Campus de Botucatu, UNESP, como requisito para a obtenção do grau de Mestre, Área de concentração Biologia Celular Estrutural e Funcional.

Profa. Dra. Flávia Karina Delella

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Palavras-chave: Câncer de próstata; Fibronectina; In Vitro; MicroRNAs; Regulação gênica.

A minha família, a minha irmã, e a Brenda...

Dedico

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LISTA DE ABREVIATURAS

ZP: zona periférica;

ZC: zona central;

ZT: zona de transição;

AR: receptor de andrógeno;

PSA: antígeno específico da próstata (*prostate-specific antigen*);

PAP: fosfatase ácida prostática (*prostatic acid phosphatase*);

MEC: matriz extracelular;

FN: fibronectin;

EMT: transição epitelial-mesenquimal (*epitelial-mesenchymal transition*);

CaP: câncer de próstata;

INCA: Instituto Nacional do Câncer;

FC: fatores de crescimento;

miRNAs: microRNAs;

RISC: complexo indutor de silenciamento de RNA;

oncomiRs: microRNAs oncogênicos;

STAT3: sinal tradutor e ativador de transcrição 3;

STATs: proteínas transdutoras e ativadoras de transcrição.

RESUMO

O câncer de próstata (CaP) continua a ser uma das principais causas de morte entre os homens. Mesmo com suas altas taxas de mortalidade e incidência, pouco ainda se sabe sobre os aspectos moleculares desta doença. Entre os diversos fatores envolvidos na carcinogênese prostática, elementos da matriz extracelular (MEC) desempenham papel fundamental. Na próstata, a fibronectina (FN), proteína multimodular que tem sido relacionada ao desenvolvimento de múltiplos tipos de câncer, a migração e invasão celular, tem sua expressão restrita ao compartimento estromal. Contudo, no desenvolvimento tumoral, o padrão de expressão da FN é alterado, com secreção desregulada e falta de organização da matriz. Desta forma, para investigar o impacto da FN no CaP, as células neoplásicas LNCaP foram expostas somente à FN solúvel (25μg/mL) e em combinação com uma membrana basal. Nossos resultados demonstraram que quando a FN é o elemento predominante, as células tumorais desenvolvem um comportamento invasivo e resistência à apoptose. No entanto, na presença de uma membrana basal, a FN diminuiu o potencial maligno e metastático destas células, que neste novo ambiente exibiam perfil de expressão gênica mais semelhante às células RWPE-1, linhagem celular que ilustra as características do epitélio prostático normal. Consequentemente, sabendo que a relação entre as células tumorais e a MEC é regulada em múltiplos níveis, os microRNAs emergiram como importantes moléculas reguladoras. Portanto, também investigamos o impacto da FN na expressão de miRNAs em células LNCaP e PC-3. Nossos resultados mostraram que cinco miRNAs apresentavam expressão diferencial (miR-21, miR-29b, miR-125b, miR-221 e miR-222) após exposição à FN. Para uma análise mais profunda, analisamos a expressão regular de possíveis mRNAs alvos destes miRNAs em dados de RNAseq disponíveis, construímos redes de interação protéica baseadas no banco de dados STRING e realizamos as análises de enriquecimentos de via e de função gênica para avaliarmos de maneira mais abrangente os possíveis efeitos desta proteína. De maneira geral, nosso estudo mostrou que a FN pode estar envolvido na progressão do CaP através da modulação de vias de sinalização, como PI3K/AKT, resposta a drogas e hipóxia. Desta forma, acreditamos que nossos resultados fornecem a base para estudos futuros, abordando o papel da FN no crescimento tumoral, particularmente no contexto de evolução/progressão do câncer de um tumor primário sólido para um estado circulante transitório.

ABSTRACT

Prostate cancer (PCa) continues to be a leading cause of death among men. Even with its high mortality and incidence rates, little is known about the molecular aspects of this disease. Among the various factors involved in the carcinogenesis of the prostate, components of the extracellular matrix (ECM) play key roles. In the prostate, fibronectin (FN), a multimodular protein that has been linked to the development of multiple cancers and involved with cell migration and invasion, has its expression restricted to the stromal compartment. However, in tumor development, the pattern of expression of FN becomes altered, with deregulated secretion and a lack of matrix organization. Thus, in order to investigate the impact of FN on PCa, the neoplastic cells LNCaP were exposed only to soluble FN (25µg/mL) or in combination with a basement membrane. Our results demonstrated that when FN is the predominant element, tumor cells develop an invasive behavior and become resistant to apoptosis. However, in the presence of a basement membrane, FN decreases the malignant and metastatic potential of these cells, which in this new environment displayed a gene expression profile more similar to the RWPE-1 cells, a cell line that illustrates the characteristics of the normal prostate epithelium. Consequently, knowing that the relationship between tumor cells and the ECM is regulated at multiple levels, microRNAs have emerged as important regulatory molecules. Therefore, we also investigated the impact of FN on the expression of miRNAs in LNCaP and PC-3 cells. Our results showed that five miRNAs exhibited differential expression (miR-21, miR-29b, miR-125b, miR-221 and miR-222) after exposure to FN. For a more in-depth analysis, we analyzed the basal expression of mRNAs possibly targeted by these miRNAs in published RNAseq data, constructed protein interactions networks based on the STRING database, and performed pathway enrichment and gene function analyzes to evaluate more comprehensively the possible effects of this protein. Overall, our study showed that FN may be involved in the progression of PCa through the modulation of signaling pathways, such as PI3K/AKT, drug response and hypoxia. Thus, we believe that our results provide the basis for future studies addressing the role of FN in tumor development, particularly in the context of cancer progression from a solid primary tumor to a transient circulating state.

1. INTRODUÇÃO

1.1 *A Glândula Prostática Humana*

A glândula prostática é um órgão sexual masculino encontrado em todos os mamíferos que desempenha importante papel na reprodução. Possui forma de cone truncado, pesa aproximadamente 20g e, conforme ilustrado na Figura 1, situa-se inferiormente à bexiga urinária, anteriormente ao reto, envolvendo a uretra prostática (Wilson, 1987).

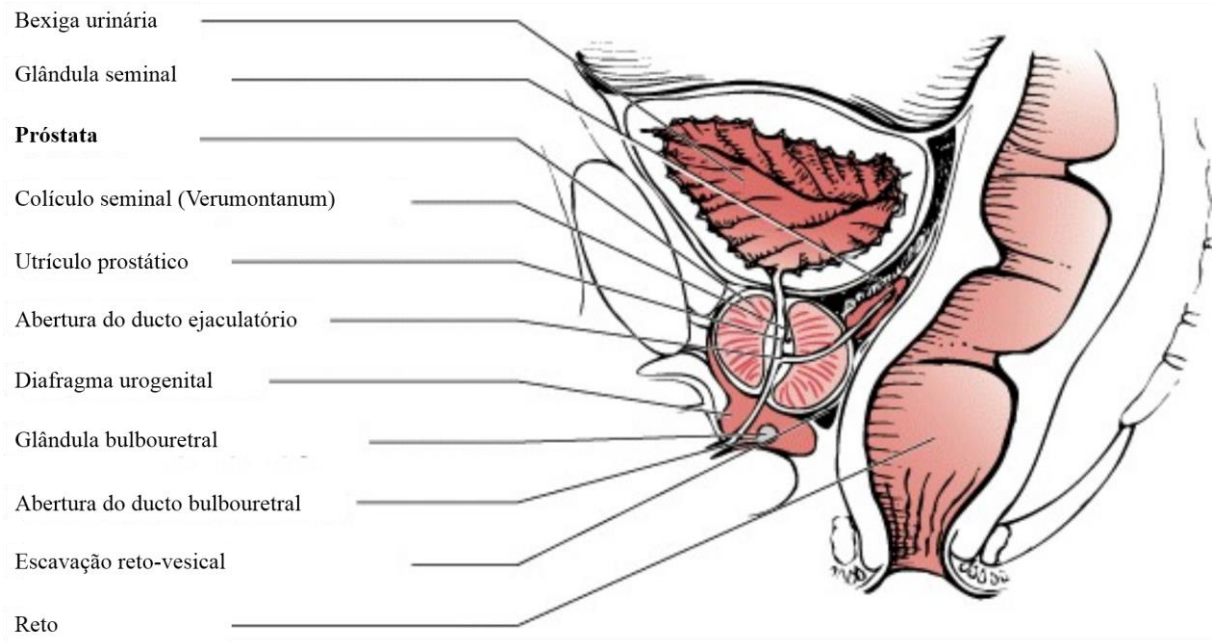

Figura 1. Localização anatômica da próstata (modificado de Smith et al., 2003).

Anatomicamente, a próstata humana pode ser dividida em três zonas glandulares distintas (Figura 2): a zona periférica (ZP) que representa cerca de 70% do órgão, a zona central (ZC) que representa aproximadamente 20% da glândula e a zona de transição (ZT) responsável pelos 10% restantes das regiões glandulares (McNeal, 1988,1981).

Além das zonas glandulares, ainda há o estroma fibromuscular anterior composto pelo esfíncter prostático, músculo detrusor anterior, esfíncter interno, e uma parte do esfíncter estriado uretral (Timms, 2008).

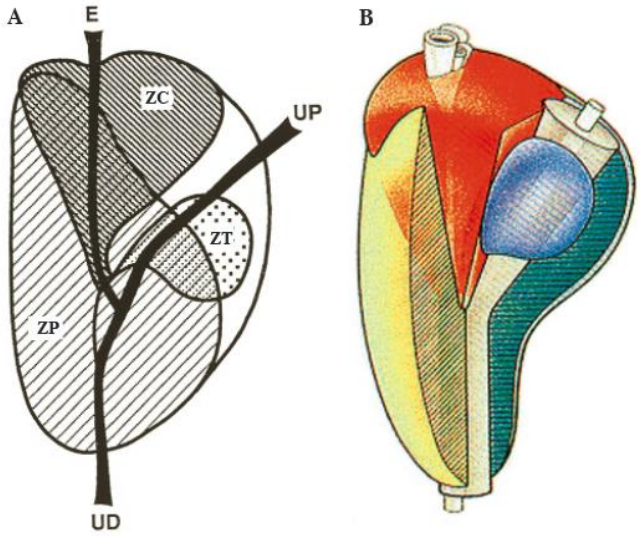

Figura 2. Zonas anatômicas da próstata. (A) Diagrama sagital da próstata humana adulta mostrando as três grandes regiões glandulares: Zona Central (ZC), Zona Periférica (ZP) e Zona de Transição (ZT); e as estruturas relacionadas: Uretra Distal (UD), Uretra Proximal (UP) e Ducto Ejaculatório (E). (B) Representação tridimensional das três zonas glandulares: ZP (amarela), ZC (vermelha), ZT (azul); e do estroma fibromuscular anterior (verde). (Modificado de Timms, 2008).

Morfologicamente, a próstata é composta por elementos glandulares e por elementos estromais (Lee et al., 2011). Os elementos glandulares são revestidos pelo epitélio prostático e se organizam para formar alvéolos e ductos excretores (Vilamaior et al., 2005; Vilamaior et al., 2000; De Carvalho et al., 1997; De Carvalho & Line, 1996).

Este epitélio apresenta elevado nível de organização celular, sendo composto por cinco tipos celulares (Figura 3):

i) células epiteliais basais – células intermediárias com morfologia achatada ou cilíndrica, formam uma única camada sobre a membrana basal, possuem como marcadores clássicos as citoqueratinas 5 e 14, além de expressarem CD44, p63 (proteína homóloga da proteína p53) e a proteína anti-apoptótica Bcl-2 (Bonkhoff & Remberger, 1996; Brawer et al., 1985);

ii) células epiteliais secretoras – revestem os lúmens dos alvéolos prostáticos, são classificadas como cilíndricas altas, expressam citoqueratinas 8 e 18 (Brawer et al., 1985), são dependentes de andrógenos (Collins & Maitland, 2006), e possuem como característica importante a expressão do receptor de andrógeno (AR), a secreção do antígeno específico da próstata (PSA – *prostate-specific antigen*) e da fosfatase ácida prostática (PAP – *prostatic acid phosphatase*) (Leav et al., 1996);

iii) células transitórias amplificadoras – expressam marcadores tanto das células epiteliais basais quanto das células epiteliais secretoras e representam um estado transitório entre a célula basal e secretora (Rizzo et al., 2005; Bonkhoff et al., 1994);

iv) células neuroendócrinas – células diferenciadas presentes em pequena porcentagem no epitélio prostático, não sensíveis ao andrógeno (Bui & Reiter, 1998), com papel biológico pouco conhecido (Oldridge et al., 2012), e co-expressam citoqueratinas 5, 14, 18, assim como o marcador neuroendócrino cromagranina A (Rumpold et al., 2002);

v) células do tipo *stem cells* – representam geralmente uma pequena subpopulação de células quiescentes, expressam CD133 e ao serem estimuladas possuem papel biológico na homeostasia, regeneração e reparo tecidual prostático (Richardson et al., 2004).

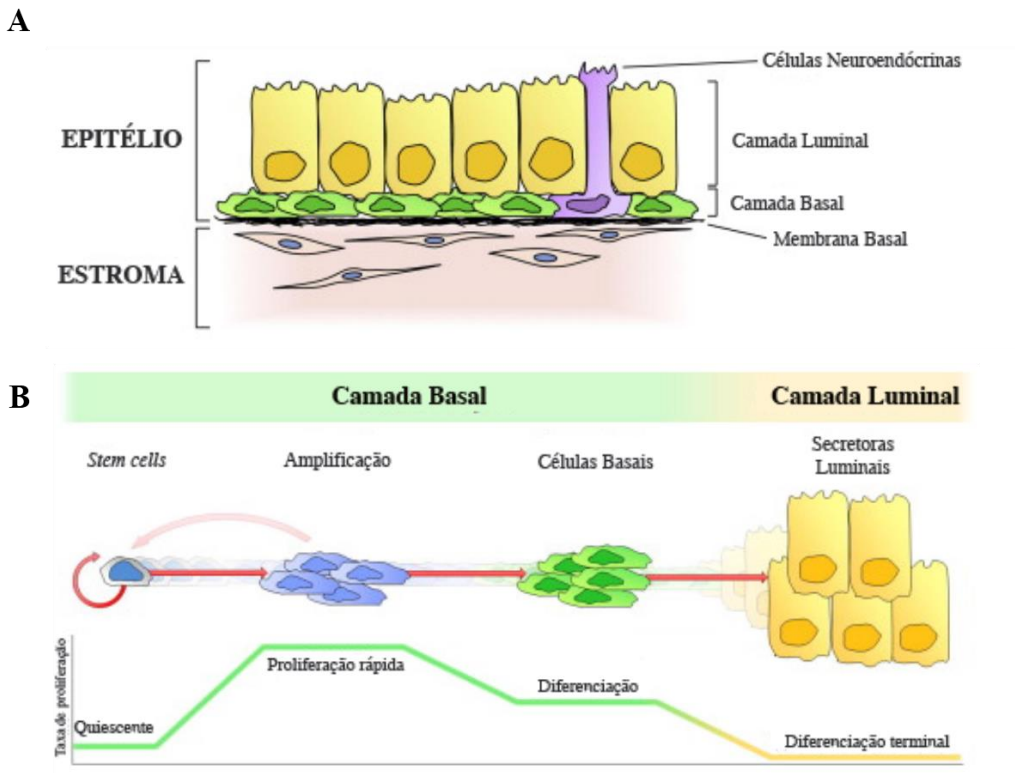

Figura 3. Representação esquemática do epitélio prostático. (A) Arquitetura do epitélio prostático normal. (B) Via hierárquica do epitélio da próstata. (Modificado de Oldridge et al., 2012).

1.2 *O Estroma Prostático*

Antes considerado como uma estrutura inerte de suporte para as células e resistência mecânica para os tecidos e órgãos, o estroma é hoje considerado um ambiente dinâmico de grande importância visto que possui papel fundamental na regulação de inúmeros eventos celulares (Hynes, 2009; Tuxhorn et al., 2001).

Na próstata, o estroma envolve os ductos e alvéolos glandulares (Figura 4), sendo composto pelos tipos celulares: **miofibroblastos** – células encontradas principalmente em locais de remodelação tecidual que expressam pró-colágeno I (Krušlin et al., 2015; Ayala et al., 2003); **fibroblastos** – expressam vimentina e laminina (Micke & Ostman, 2004; De Wever & Mareel, 2003); e **células musculares lisas** – expressam desmina, α-actina, miosina, distrofina, entre outros (Antonioli et al., 2007); além de nervos, vasos sanguíneos e linfáticos (Marker et al., 2003).

A função primária das células estromais é a de orientar o desenvolvimento e diferenciação das células epiteliais através da síntese de diferentes elementos estruturais e reguladores da matriz extracelular (MEC) (Hall et al., 2002; Lin & Bissell, 1993).

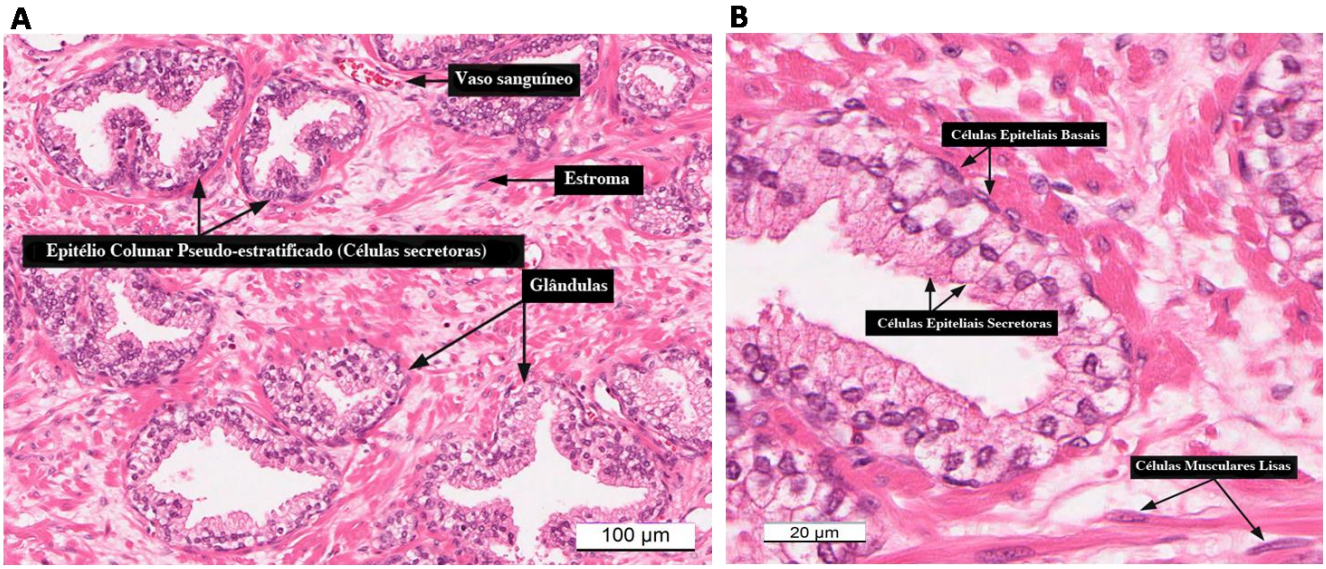

Figura 4. Histologia prostática. (A) Fotomicrografia da próstata corada em H.E em aumento de 20x. (B) Fotomicrografia do epitélio prostático e seu estroma circundante corado em H.E em aumento de 100x (Modificado de www.proteinatlas.org).

1.3 *A Matriz Extracelular e a Fibronectina*

A MEC é formada por proteínas estruturais, proteoglicanos de ácido hialurônico e uma grande variedade de glicoproteínas que interagindo entre si resultam em um polímero capaz de influenciar nas propriedades biofísicas e bioquímicas dos tecidos (Byron et al., 2013; Hynes, 2009).

Cada célula é capaz de reagir às forças externas (Bershadsky et al., 2003; Geiger & Bershadsky, 2002), reconhecer a topografia (Spatz & Geiger, 2007; Vogel, 2006; Curtis & Riehle, 2001), e rigidez (Engler et al., 2006; Discher et al., 2005), de seu ambiente extracelular adjacente.

Desta forma, a MEC desempenha papel vital na determinação, diferenciação, proliferação, sobrevivência, polaridade e migração das células, visto que alterações em seus componentes individuais ou em sua organização possuirão impacto significativo sobre o fenótipo celular (Lu et al., 2011; Geiger et al., 2009).

Cada tipo de tecido contém um conjunto específico de componentes extracelulares que são organizados de forma a contribuir com sua função (Hubmacher & Apte, 2013). A MEC da próstata é composta principalmente por fibras colágenas, fibras reticulares, fibras do sistema elástico, proteoglicanos e diversas glicoproteínas, tais como a laminina e a fibronectina (FN) (Delella et al., 2012; Delella & Felisbino, 2010; Vilamaior et al., 2000; De Carvalho et al., 1997)**.**

A FN é uma abundante glicoproteína que desempenha função em diversos processos celulares, tais como, adesão, migração, crescimento e diferenciação (Pankov & Yamada, 2002; Hynes, 1986; Mosher, 1984).

Composta por duas subunidades peptídicas similares de 250-280 kDa, unidas por um par de ligações dissulfeto próximas à sua extremidade C-terminal, a molécula de FN forma um dímero (Figura 5 B-C) (Pankov & Yamada, 2002). Cada subunidade possui três diferentes módulos (F1, F2 e F3) que ao se dobrarem em série geram uma estrutura com comprimento de contorno muito longo, cerca de 120-160 nm (Figura 5 A e D) (Erickson et al., 1981). Além disso, distribuído entre os módulos, cada molécula possui sítios de ligação para outros componentes da MEC, tais como as integrinas, fibras colágenas e proteoglicanos (Figura 5 B) (Plow et al., 2000; Hynes, 1986; Mosher, 1984).

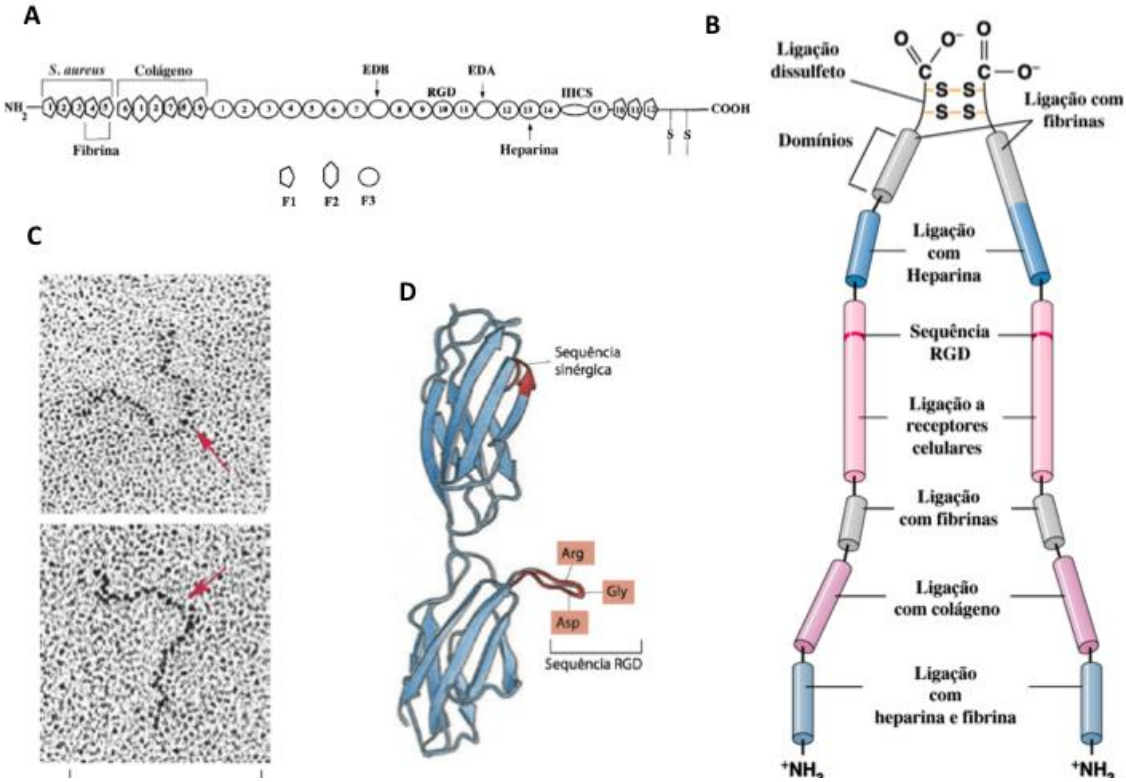

Figura 5. A molécula de fibronectina (FN). (A) Estrutura da FN mostrando a posição dos domínios modulares F1, F2, F3 (Bork et al., 1996); além das regiões de *splicing* alternativo (ED-A. ED-B e III-CS) (Hynes, 1990); (B) Representação simplificada das duas cadeias de FN que formarão um dímero e seus domínios de ligações com outras moléculas (Modificado de Becker et al., 2005); (C) Fotomicrografia eletrônica de moléculas sombreadas por platina; as setas vermelhas marcam a porção c-terminal (Engel et al., 1981); (D) Estrutura tridimensional dos dois tipos de repetições de FN tipo III determinado por cristalografia de raios X (Leahy, 1997).

Embora a FN seja codificada por apenas um gene, por meio de *splicing* alternativo é possível a geração de pelo menos vinte cadeias distintas desta glicoproteína (Kosmehl et al., 1996; Ffrench-Constant, 1995). Nos seres humanos, apenas cinco destas variações podem ser encontradas (Pankov & Yamada, 2002), sendo classificadas como: FN plasmática - isoforma secretada por hepatócitos e que circula na corrente sanguínea em concentração de cerca de 300 ng/mL (Stathakis et al., 1981); ou FN celular – corresponde às demais isoformas, sendo secretada pelas células no local de formação da MEC (Pankov & Yamada, 2002).

Cada forma de FN é responsável em mediar diferentes processos biológicos. Enquanto a FN plasmática é essencial na formação de coágulos durante a fase inicial da cicatrização, a FN celular é responsável em mediar: a fase tardia da cicatrização de feridas, a neovascularização e a angiogênese (To & Midwood, 2011; Grinnell, 1984) . Além disso, a FN celular também está

implicada em outros processos fisiológicos (embriogênese) e patológicos, tais como diabetes, fibrose e câncer (Zhang et al., 2009; Kaspar et al., 2006; Hynes & Yamada, 1982).

1.4 *A Fibronectina e o Câncer*

No câncer, a molécula de FN é maior (Matsuura & Hakomori, 1985), e contêm geralmente as sequências de *splicing*: ED-A e ED-B, acarretando em alterações conformacionais adicionais a molécula.

i) Sequência ED-A: Em tumores com alta remodelação tecidual e com sinalização desregulada, a molécula de FN contendo a sequência ED-A, está relacionada com o desenvolvimento de fibrose (Kelsh et al., 2015; Shinde et al., 2015) e neovascularização tumoral (Rybak et al., 2007). Além disso, esta variante da molécula é associada com o aumento da secreção de VEGF-C através da via de sinalização PI3K/AKT (Xiang et al., 2012), e pode induzir a transição epitelial-mesenquimal (EMT) das células neoplásicas via FAK-SRC por meio da ligação com a integrina α9β1 (Ou et al., 2014).

ii) Sequência ED-B: Durante o desenvolvimento e progressão tumoral, a MEC se torna mais rígida, estando este novo ambiente associado ao aumento da FN ED-B, responsável em propagar o fenótipo tumorigênico (Bordeleau et al., 2015). Assim, a FN ED-B é encontrada principalmente no estroma tumoral (Kaczmarek et al., 1994), tendo relação com o aumento da adesão celular e com a formação de adesões focais, que irão contribuir na maior motilidade das células tumorais (Hashimoto-Uoshima et al., 1997).

Além do *splicing* alternativo, as alterações conformacionais na molécula de FN também podem regular sinergicamente o desenvolvimento e progresso tumoral, visto que são responsáveis em alterar o perfil de expressão de integrinas celulares. A ligação FN-integrina mais abundante é a FN-α5β1, que requer além do sítio de ligação RGD, outros locais da molécula, o que implica em uma ligação forte e organizada entre a célula e a MEC (Li et al., 2003). Tal interação é frequentemente encontrada em tecidos saudáveis, durante o processo de remodelação natural da MEC, como cicatrização de feridas, vascularização e embriogênese (Fig 6A). Durante estes processos, as células dos tecidos se tornam mais contráteis, há um desdobramento natural da molécula de FN, e inicia-se uma forte relação entre a FN e as integrinas α5β1 (Antia et al., 2008), interação esta que irá estimular as células a formarem aderência fibrilares periféricas robustas e reduzir a capacidade de migração de possíveis células invasivas (Jia et al., 2012).

Em contraste, as integrinas αvβ3 requerem apenas o sítio de ligação RGD, o que torna a ligação FN-αvβ3 mais fraca e menos dependente da conformação da molécula de FN (Danen et al., 1995). Este tipo de ligação ocorre em maior frequência nas células tumorais, acarretando em uma reorganização do citoesqueleto no sentido de facilitar a migração celular (Balcioglu et al., 2015) e resistência contra *anoikis* (Zhang et al., 2004) (Fig. 6B).

Sendo assim, a conformação da FN quando alterada durante a tumorigênese, desregula as interações célula-matriz, causando alterações significantes nos processos de sinalização intra-celulares. Desta forma, uma maior compreensão da dinâmica da FN, isto é, da deposição de FN e sua interação com o tumor, é essencial para expandir nosso conhecimento sobre o câncer.

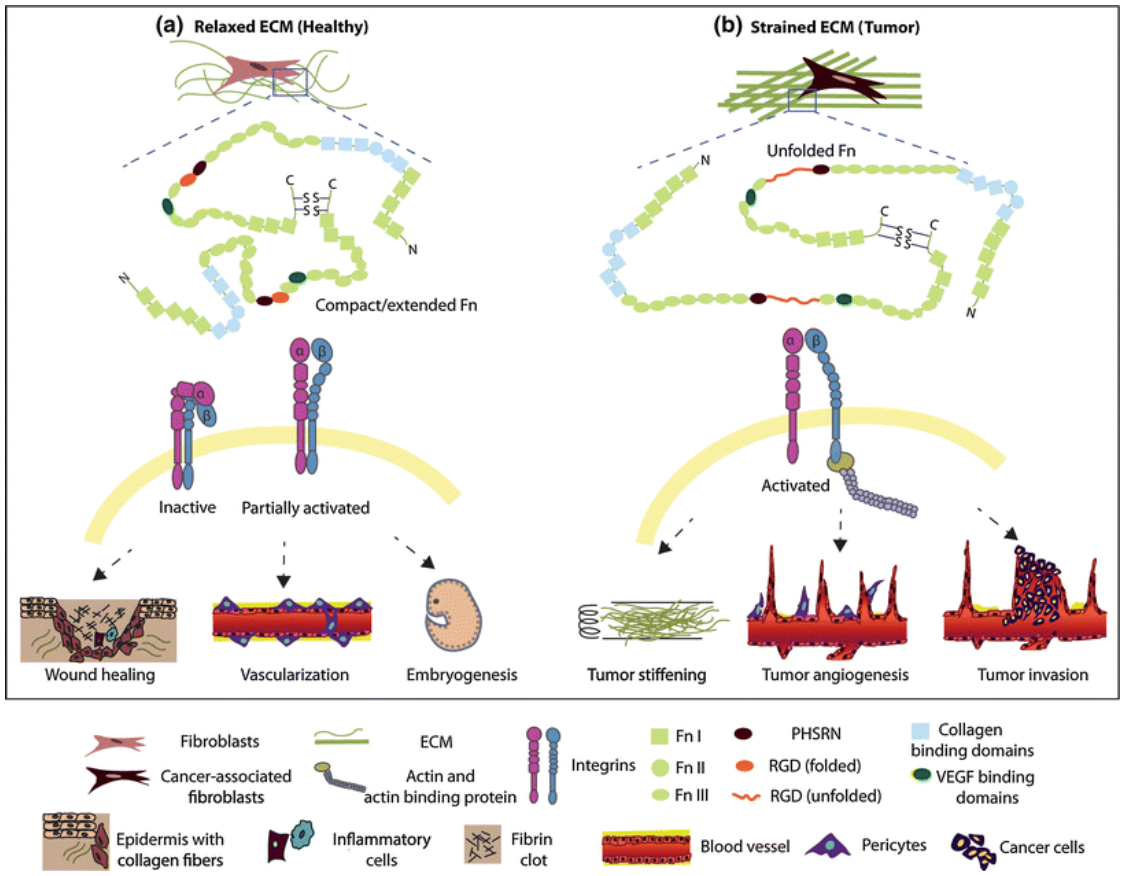

Figura 6. Relação entre a estrutura e a função da FN entre ambientes saudáveis e patológicos. **(A)** Em ambientes saudáveis, a MEC permanece em equilíbrio conformacional para manter a homeostase normal dos tecidos e a FN possui comportamento normal. **(B)** No entanto, a MEC do estroma tumoral perde a sua integridade, o que acarreta na alteração conformacional da FN auxiliando na alteração do fenótipo celular. (Retirado de K. Wang, Seo, Fischbach, & Gourdon, 2015)

1.5 *O Câncer de Próstata*

O câncer de próstata (CaP) continua a ser um dos principais problemas de saúde no envelhecimento masculino, sendo o segundo tipo mais frequente de câncer diagnosticado nos homens em todo o mundo (Torre et al., 2015). Para o ano de 2015, a *American Cancer Society* estimou que cerca de 220 mil homens seriam diagnosticados com CaP, e que aproximadamente 28 mil mortes ocorreriam por este tipo de câncer (Siegel et al., 2015). Já para o Brasil, o Instituto Nacional do Câncer (INCA), estimou que 61.200 casos novos de CaP seriam diagnosticados em 2016 (INCA, Estimativa 2016).

Dentre os diversos fatores que elevam o risco de um homem desenvolver o CaP, podem-se citar como mais importantes:

i) a idade: o aumento da idade é o principal fator de risco para o diagnóstico de CaP. Homens com mais de 50 anos, possuem maior probabilidade de desenvolverem a doença (Stangelberger et al., 2008).

ii) a etnia: os descendentes africanos são os indivíduos que mais desenvolvem o CaP (Grönberg, 2003). Nos Estados Unidos, afro descendentes possuem taxas de incidência e mortalidade notoriamente mais elevadas quando comparadas as taxas das demais etnias (Brawley, 1998). As razões para estas diferenças ainda não são bem elucidadas, mas sabe-se que fatores socioeconômicos, financeiros, culturais e genéticos podem estar envolvidos (Gaston et al., 2003; Liu et al., 2001; Powell et al, 1995; Robbins et al., 2000);

iii) o histórico familiar: homens com casos de CaP na família possuem maior probabilidade de desenvolver a doença, sendo este risco diretamente relacionado com o grau de parentesco e número de parentes afetados (Brandt et al., 2010);

iv) a dieta: homens obesos, com dieta rica em gordura animal apresentam maior pré-disposição a desenvolverem este tipo de câncer (Allott et al., 2013).

No entanto, embora já se conheça os fatores de risco para o desenvolvimento do CaP, a compreensão dos processos moleculares que regulam sua carcinogênese e progressão tumoral estão longe de serem totalmente elucidados.

Em relação a estes processos, o desenvolvimento de uma neoplasia na próstata, como nos demais tipos de câncer, depende do resultado de alterações progressivas e acumulativas em diversos genes, podendo estes serem classificados como: oncogenes ou supressores tumorais (Isaacs & Kainu, 2001).

Na Figura 7 está ilustrado um modelo de como células normais da próstata podem gerar um tumor indiferenciado a partir destas alterações moleculares nestas duas classes de genes (Karayi & Markham, 2004).

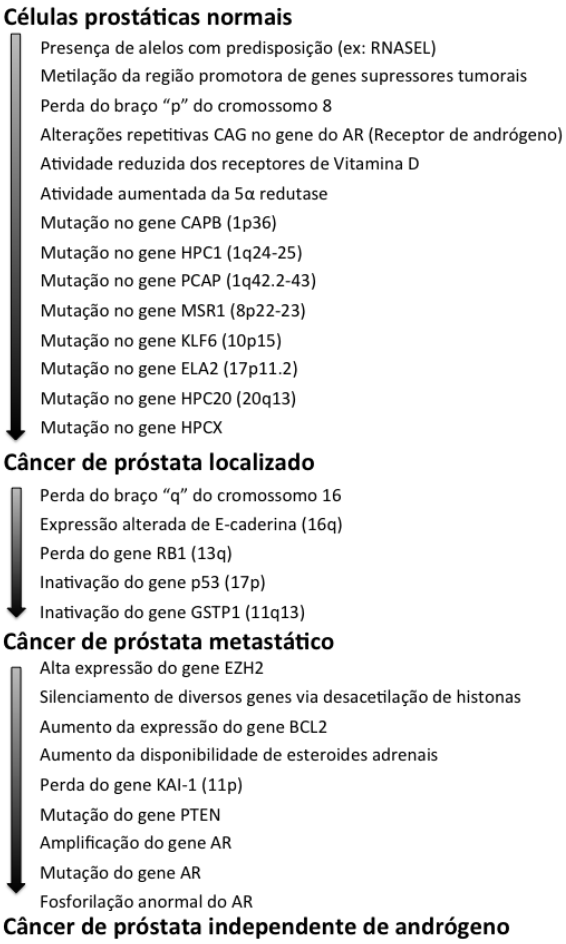

Figura 7. Representação esquemática das alterações moleculares responsáveis pela progressão tumoral no epitélio prostático. (Modificado de Karayl & Markham, 2004).

Os oncogenes auxiliam o desenvolvimento e progressão tumoral (Torry & Cooper, 1991; Varmus, 1989) e derivam de proto-oncogenes, genes que codificam uma variedade de proteínas, tais como: fatores de crescimento (FC), receptores de FC, reguladores de transcrição e replicação, entre outros (Cantley et al., 1991).

Até hoje, nenhum oncogene foi conclusivamente associado como fator essencial responsável para a progressão inicial do CaP (Peehl, 1993). Porém, já se sabe que alguns destes genes são ativados em diferentes fases da progressão tumoral na próstata (Tabela I).

Tabela I. Principais oncogenes alterados no câncer de próstata.

Genes	Localização	Função	Referência
ABL	9q43.1	Proteína Quinase	Parra et al., 2014; Shaul et al., 2005
AR	Xq12	Fator de Transcrição	Balk & Knudsen, 2008
EGR1	5q31.2	Fator de Transcrição	Baron et al., 2006; Parra et al., 2011
SRC	20q12	Proteína Quinase	Fizazi, 2007; Varkaris et al., 2014
C-MYC	8q24.12	Fator de Transcrição	Koh et al., 2010
BCL2	18q21.33	Morte Celular	Chaudhary et al, 1999; Lin et al., 2007
ERBB2	17p11.2	Receptor de FC	Pignon et al, 2009; Savinainen et al, 2002
ERG	3p25	Fator de transcrição	Furusato et al., 2010; Tomlins et al., 2008

Já os genes supressores tumorais podem ser definidos como genes cuja inativação é permissiva para a carcinogênese, sendo esta inativação causada através de deleção ou mutação da sequência de bases do DNA (Knudson, 1971, 1996). Os principais genes supressores tumorais que estão alterados no CaP estão citados na Tabela II

Tabela II. Principais genes supressores tumorais alterados no câncer de próstata.

Genes	Localização	Função	Referência
RB1	13q14.1-14.2	Divisão celular	Bookstein et al., 1990
TP53	17p13.1	Divisão celular	Al-Maghrabi et al, 2001; Navone et al., 199
PTEN	10q23.3	Divisão celular	Cairns et al., 1997; Dong, 2001
CDH1	16q22.1	Adesão celular	Umbas et al., 1992, 1994
BRCA1	17q21	Crescimento celular	Anderson et al, 1993; Gao et al, 1995
BRCA2	13q12.3	Proliferação	Anderson & Badzioch, 1993
RNASEL	1q25	Ativ. dos *interferons*	Rökman et al., 2002; Wang et al., 2002
MSR	8p22-23	Receptor de Macrófago	Xu et al., 2001
GSTP1	11q13	Detoxificação	Harden et al., 2003
KLF6	10p15	Divisão celular	Chen et al., 2003; Narla et al., 2001

Contudo, além dos mecanismos genéticos aos quais estas duas classes gênicas são submetidas, tais como: mutação do DNA, translocação ou deleção cromossômica; é importante destacar também o papel dos mecanismos epigenéticos no desenvolvimento do CaP.

Os mecanismos epigenéticos são em sua maioria reversíveis e definidos como mudanças hereditárias responsáveis em alterar a expressão gênica sem alterar a sequência do gene ou estrutura cromossômica (Reik & Walter, 2001). Tais mecanismos envolvem modificações

bioquímicas das proteínas histona, modificações no DNA em si (ex. taxa de metilação), e a expressão de RNAs não codificantes, entre eles os microRNAs (miRNAs) (Calin & Croce, 2007; Jaenisch & Bird, 2003; Jenuwein & Allis, 2001).

1.6 *MicroRNAs e Câncer*

Os miRNAs são RNAs não-codificadores de tamanho variando entre 17 a 25 nucleotídeos, que apresentam importante papel na regulação de diversos processos biológicos por modulação da expressão gênica (Ambros, 2004).

Evolutivamente conservados e amplamente distribuídos entre as espécies, os miRNAs reprimem pós-transcricionalmente a expressão gênica pelo reconhecimento de locais complementares na região 3' não traduzida (3' UTR) de seus RNAs mensageiros alvos (Tie & Fan, 2011; Ambros et al., 2003). Atualmente, mais de 35828 miRNAs maduros de cerca de 220 espécies, sendo 2588 miRNAs maduros humanos, estão registrados no banco de dados *miRBase* (versão 21, 2014; www.mirbase.org).

A biogênese de um miRNA (Figura 8) inicia-se pelo processamento de um longo transcrito primário conhecido como pri-miRNA. Uma única molécula de pri-miRNA possui precursores que darão origem a miRNAs (pré-miRNAs), sendo preferencialmente transcritos pela RNA polimerase II, a partir de genes independentes, íntros, ou até mesmo éxons de genes codificadores de proteínas, mantendo assim características tais como estrutura de cap na sua região 5' e cauda de poli (A) na sua região 3'(Kim, 2005; Lee et al., 2004).

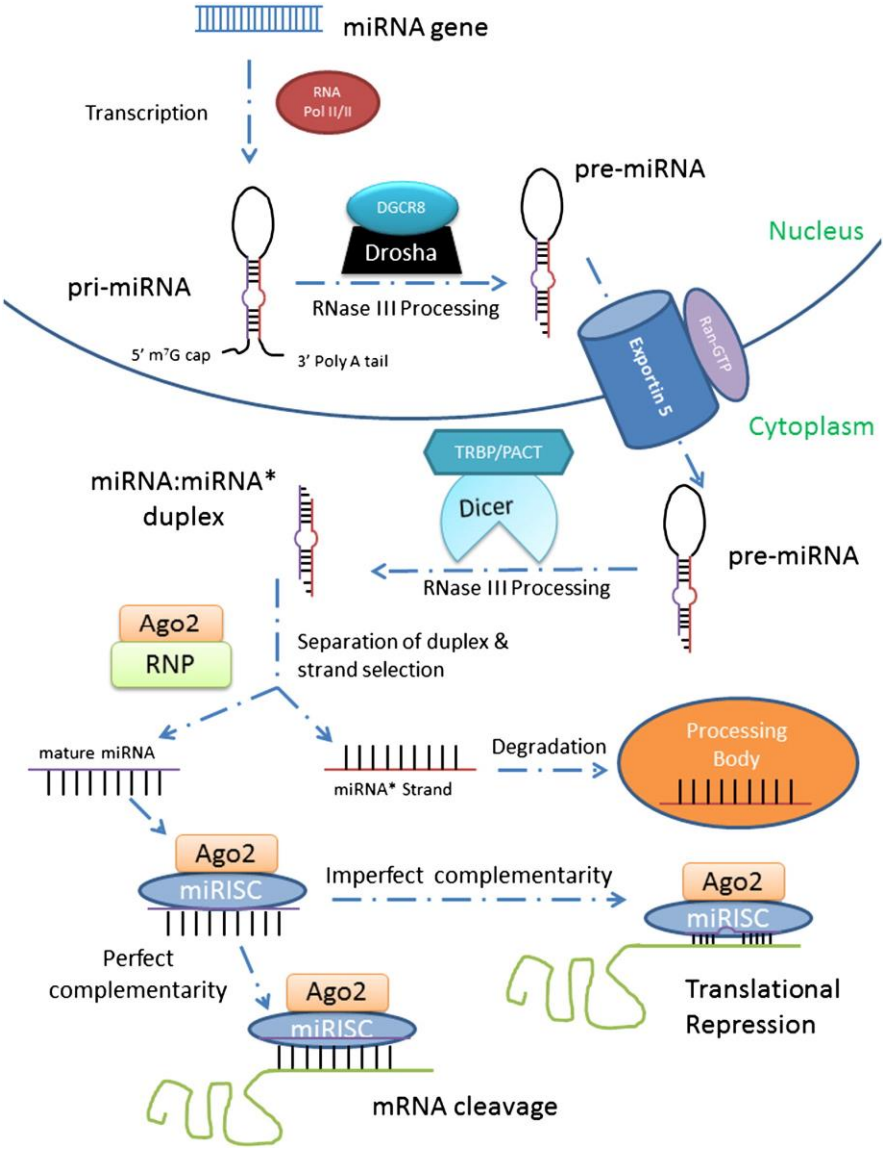

Figura 8. Biogênese dos miRNAs (Retirado de Rutnam *et al.*, 2013).

No núcleo, a enzima RNAse III (conhecida como Drosha) processa o pri-miRNA em um pré-miRNA – estrutura de ~ 70 nucleotídeos com forma de "*hairpin*" – "grampo de cabelo" (Zeng & Cullen, 2005). Formas ativas da enzima Drosha requerem a formação de complexos, a partir da ligação com cofatores, como a proteína DGCR8 (*Di George Syndrome critical region gene* 8) em humanos (Denli et al., 2004; Gregory et al., 2004). O complexo de ligação proteína-RNA gerado a partir da junção entre a DGCR8 e a Drosha é chamado de "microprocessador", que em humanos possui ~ 650 kDa (Gregory et al., 2004).

Após o processamento nuclear, cada pré-miRNA é exportado para o citoplasma pela proteína exportina-5 (EXP5), membro da família de receptores de transporte nuclear sendo

convertido para miRNAs maduro e funcional pela Dicer – enzima pertencente à família das RNAs polimerases III (Yi et al., 2003).

Ao ser clivado pela Dicer, a molécula de RNA é separada em duas fitas (miRNA:miRNA*), sendo que uma das fitas de aproximadamente 22 nucleotídeos (fita guia ou miRNA), associa-se à proteína Argonauta – proteínas semelhantes a RNAses endonucleares – para formar o complexo indutor de silenciamento de RNA (RISC), enquanto a outra fita (fita passageira ou miRNA*) é geralmente degradada (Kim et al., 2009; Rand et al., 2005).

O complexo miRNA-RISC irá interagir com sítios ligantes da região 3' UTR do RNA mensageiro alvo, devido à complementaridade total ou parcial de uma sequência de 5-7 nucleotídeos (região *seed*) inibindo sua tradução ou o degradando (Lee & Dutta, 2009).

Previsões apontam que os miRNAs podem regular a expressão de um número elevado de genes (Bartel, 2009). Este fato ocorre devido a possibilidade de um único miRNA poder ter diversos mRNAs alvo, e da mesma forma, um único mRNA poder ser regulado por diferentes miRNAs.

Desta forma, os miRNAs promovem uma regulação orquestrada de seus alvos, controlando vias de sinalização e funções biológicas complexas (Goto et al., 2015; Van Rooij et al., 2008).

No câncer, a expressão de miRNAs é característica para cada tipo de tecido, estágio do tumor e variações clínicas (Lee & Dutta, 2009). De maneira geral, os miRNAs podem estar super ou pouco expressos em células tumorais (Volinia et al., 2006). A super expressão ocorrerá, geralmente, devido a processos moleculares de amplificação, desmetilação nas regiões promotoras dos miRNAs ou desregulação de um fator de transcrição, enquanto a baixa expressão poderá ocorrer devido à deleções, silenciamento epigenético ou perda da expressão de fatores de transcrição (Croce, 2008).

Os miRNAs podem atuar como oncogenes ou supressores tumorais, conforme ilutrado na Figura 9 (Esquela-Kerscher & Slack, 2006). Sendo classificados como microRNAs oncogênicos (oncomiRs) quando possuem a habilidade de reprimir genes supressores tumorais ou relacionados à apoptose, e miRNAs supressores tumorais quando suprimem a expressão de oncogenes ou de genes relacionados à proliferação celular (Shi et al., 2008).

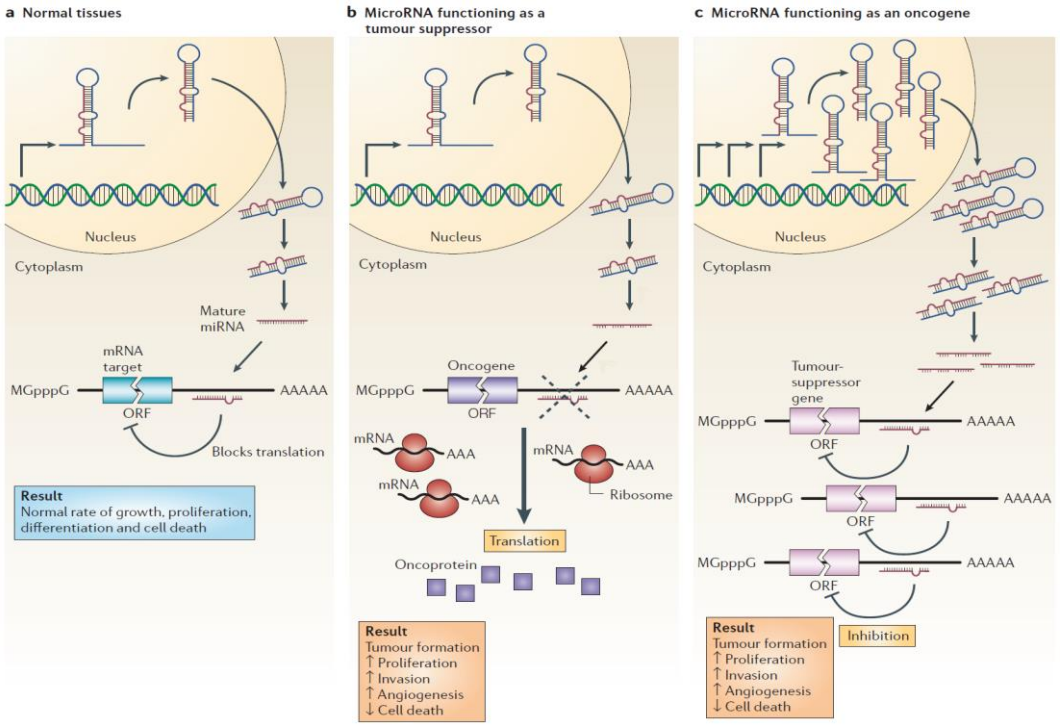

Figura 9. miRNAs podem funcionar como supressores tumorais ou oncogenes. a) Função dos miRNAs em tecidos normais. b) Redução ou eliminação de um miRNA que funciona como supressor tumoral. c) Amplificação ou super expressão de um miRNA que possui papel oncogênico. (Esquema proposto por Esquela-Kescher & Slack, 2006).

No CaP, a maioria dos miRNAs supressores tumorais encontram-se em baixa concentração e expressão, sendo um exemplo o miR-145. Este miRNA encontra-se bastante presente em tecidos germinativos e derivados da mesoderme, possuindo importante papel regulador no microambiente celular ao silenciar o gene c-MYC, inibindo a proliferação e crescimento celular (Sachdeva & Mo, 2010; Sachdeva et al., 2009). Acredita-se que a baixa expressão do miR-145 em células tumorais prostáticas pode estar envolvida com a maior expressão do protoncogene ERG, gene pertencente a família de fatores de transcrição ETS presente em 50-70% dos tumores de próstata, causando assim maior avanço na progressão tumoral (Hart et al., 2013; Furusato et al., 2010).

O miR-21 no CaP, como na maioria dos demais tipo de câncer, encontra-se altamente expresso, sendo seu papel nos processos de invasão e metástase bem definido (Lee & Dutta, 2009). Tal miRNA é responsável em promover a mobilidade e invasão celular ao inibir a expressão do gene PTEN, um supressor tumoral que inibi a invasão celular por meio do

bloqueio da expressão de diversas MMPs (Meng et al., 2007). Importante oncomiR, o mir-21 é ativado pelo STAT3 (sinal tradutor e ativador de transcrição 3) e pela via de sinalização induzida por IL-6 (Löffler et al., 2007).

O STAT3 pertence a uma família de sete proteínas transdutoras e ativadoras de transcrição (STATs) que são ativadas em resposta a proteínas de sinalização extracelulares (Rawlings et al., 2004). Através da modulação da transcrição de genes responsáveis na regulação de uma grande variedade de processos biológicos (diferenciação celular, proliferação, apoptose, metástase, e resposta imune), o STAT3 impulsiona a progressão do câncer em diversos tipos de tecidos, estando associado com mau prognóstico em diversos tipos de câncer (Cao et al., 2013; Ren et al., 2010; Chen et al., 2007).

Estudos recentes têm demonstrado que os miRNAs podem modular diretamente a via de sinalização STAT3 (Cao et al., 2013). O miR-125b, trata-se de um miRNA de grande interesse na área de pesquisa do câncer por estar desregulado em uma ampla variedade de tumores (Xie et al., 2013). Sabe-se que este miRNA é capaz de regular negativamente o STAT3 suprimindo a proliferação e migração de alguns tipos de células tumorais (Liu et al., 2011).

Tais evidências permitem concluir que os miRNAs possuem importante papel na pesquisa sobre o câncer. Pois, embora, muitos estudos envolvendo a expressão de miRNAs no processo tumoral já existam, muito ainda há para se saber, dada a complexidade dos fatores envolvidos.

Dentre a enorme diversidade de interações que ocorrem no processo de carcinogênese, a relação da MEC com os miRNAs tem se tornado um campo de destaque.

Atualmente, pesquisas estão sendo realizadas no intuito de relacionar a alteração da FN pela expressão anormal dos miRNAs. Sabe-se que, alguns miRNAs como o miR-143, miR-146a e miR-199a, relacionados a regulação normal sobre a FN, quando alterados podem estar relacionados com a aparição de processos patológicos no indivíduo (Lee & Dutta, 2009; Zhang et al., 2009).

Contudo, poucos estudos são realizados para se compreender a relação inversa, ou seja, a FN sendo responsável na alteração dos miRNAs e consequentemente na alteração da expressão gênica.

Desta forma, torna-se importante a realização de estudos mais profundos para se entender como a MEC pode regular a expressão de miRNAs, visto que a maioria das pesquisas mostram apenas o papel dos miRNAs na regulação da expressão de moléculas da MEC.

1.7 *Estudos in vitro e as linhagens celulares prostáticas*

O estudo da expressão gênica e dos miRNAs mostra-se, desta forma, uma importante ferramenta para a melhor compreensão do CaP.

Entretanto, a expressão destas moléculas são características para cada tipo de tecido, estágio do tumor e variações clínicas (Hu et al., 2013; Barrett et al., 2011; Lee & Dutta, 2009), o que torna a obtenção de biópsias de diferentes fases do CaP que acompanhem tais mudanças genômicas e transcriptômicas uma tarefa de extrema dificuldade.

Como alternativa surgem os estudos *in vitro* envolvendo linhagens celulares, que possibilitam uma análise do comportamento celular em condições controladas e uniformes (Sobel & Sadar, 2005). Dentre as diversas linhagens prostáticas existentes, ocupando papel de destaque está a linhagem de carcinoma prostático LNCaP e a linhagem que exibe características do epitélio normal prostático, a RWPE-1.

Células LNCaP

A linhagem celular LNCaP, dependente de andrógeno, foi isolada a partir de lesão metastática do linfonodo supracravicular esquerdo de um homem caucasiano de 50 anos com diagnóstico de CaP metastático (Horoszewicz et al., 1980). Estas células produzem PAP e PSA, além de expressarem AR, receptor de estrógeno, EGF e TGF α e β (Horoszewicz et al., 1983). Entretanto, o AR contém uma mutação T877A que resulta em resposta indiscriminada para diversos esteroides (Veldscholte et al., 1990).

Outra característica é que são aneuploides, possuindo 84 cromossomos em 22% das células, podendo ter 86 (20%) até 87 cromossomos (18%) (Gibas et al., 1984), expressam citoqueratina 8 e 18 e possuem o gene TP53 em seu estado selvagem (Van Bokhoven et al., 2003).

Morfologicamente, as células LNCaP são fusiformes e alongadas, possuindo grandes núcleos e cromatina condensada (Figura 10). Devido a característica de crescerem aglomeradas (*clusters* em inglês), as células LNCaP se aderem fracamente ao substrato e são facilmente retiradas por agitação ou pipetagem (Horoszewicz et al., 1983).

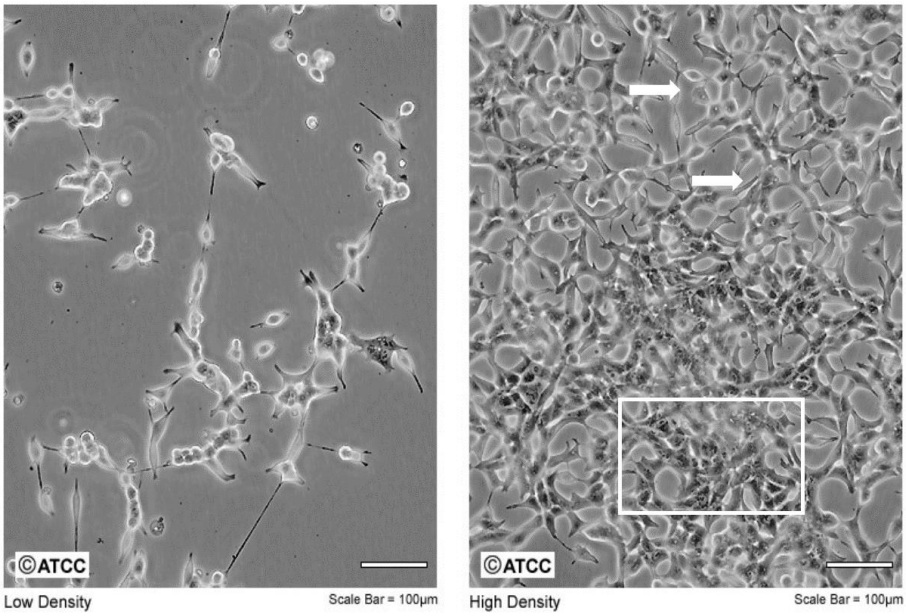

Figura 10. Fotomicrografia de células LNCaP. Visão geral da morfologia das células LNCaP em baixa confluência (esquerda) e alta confluência (direita). As setas representam o crescimento em monocamada e em detalhe no quadrado o crescimento aglomerado (*clusters*) (Retirado de http://www.atcc.org).

A linhagem PC-3 (Figura 11) foi isolada a partir de uma metástase óssea de um adenocarcinoma prostático de grau IV de um homem caucasiano de 62 anos de idade (Kaighn et al., 1979). Estas células expressam citoqueratina 8 e 18, não secretam PSA (Kaighn et al., 1979), e embora sejam independentes e não sensíveis a andrógeno, possuem marcação fraca de AR em seu núcleo (Katsuoka et al., 1986). Além disso, esta linhagem expressa níveis elevados de de TGF-a e EGF-R que podem contribuir para o seu crescimento autônomo (Carruba et al., 1994; Ching et al., 1993).

Morfologicamente, as células PC-3 não são uniformes, variando entre aspecto fusiformes e alongado a grandes células arredondas e multinucleares (Figura 11).

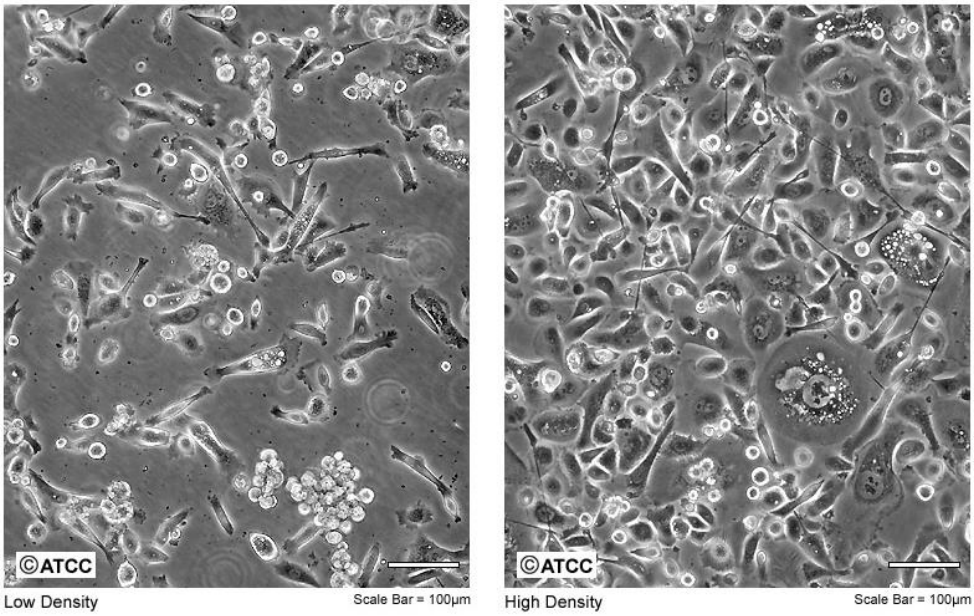

FIGURA 11. Fotomicrografia de células PC-3. Visão geral da morfologia das células PC-3 em baixa confluência (esquerda) e alta confluência (direita). (Retirado de http://www.atcc.org).

Células RWPE-1

A linhagem RWPE-1 (Figura 12) foi isolada da ZP da próstata de homem caucasiano de 54 anos sem CaP e foi transfectada com papiloma vírus humano 18 (HPV-18) para a imortalização das células (Bello et al., 1997).

Essas células expressam AR, citoqueratina 8 e 18 e os genes TP53 e RB1 (Bello et al., 1997); quando colocadas em cultura 3D, organizam-se em ácinos e secretam PSA (Bello-Deocampo et al., 2001).

Utilizar linhagens celulares que exibam características de células normais da próstata, tais como a células RWPE-1, permite um conhecimento mais profundo sobre a etiologia, evolução e prevenção de neoplasias prostáticas ao se identificar alterações celulares e moleculares relevantes no processo de carcinogênese (Achanzar et al., 2001; Quader et al., 2001; Webber et al., 1997).

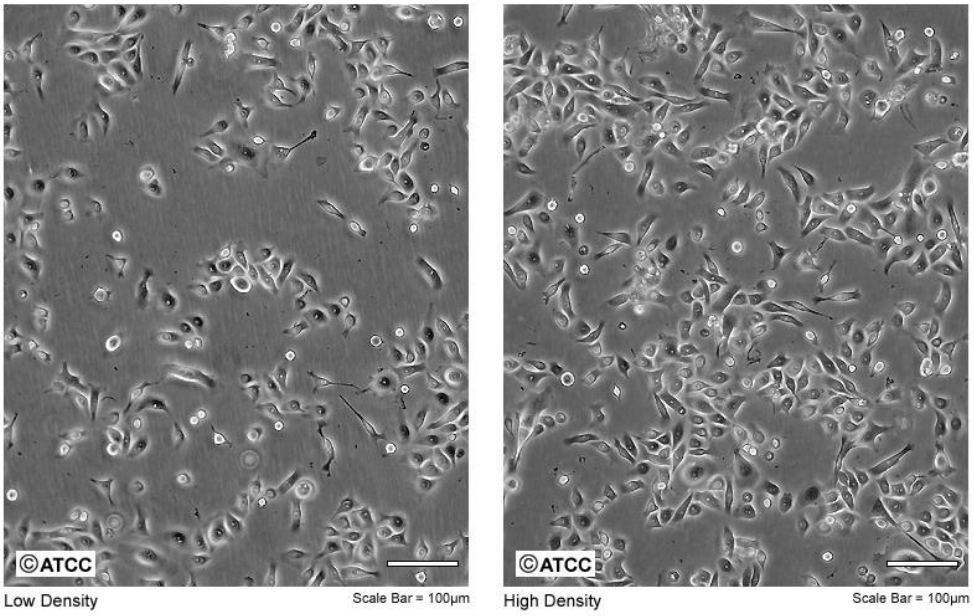

Figura 12. Fotomicrografia de células RWPE-1. Visão geral da morfologia das células RWPE-1 em baixa confluência (esquerda) e alta confluência (direita). (Retirado de http://www.atcc.org).

JUSTIFICATIVA

2. JUSTIFICATIVA

O CaP continua a ser um dos principais problemas de saúde no envelhecimento masculino. Mesmo com suas altas taxas de mortalidade e incidência, pouco se sabe sobre a biologia molecular deste tipo de câncer.

Dentre os diversos fatores que estão envolvidos com a carcinogênese na próstata, a MEC desempenha papel vital, uma vez que sua composição e organização possuem impacto significativo em numerosos eventos biológicos. Na próstata, esta matriz é composta principalmente por fibras colágenas, fibras reticulares, fibras do sistema elástico, proteoglicanos e diversas glicoproteínas, tais como a laminina e FN**.**

A FN é uma abundante glicoproteína que desempenha função em processos celulares, tais como, adesão e migração. Na glândula prostática normal, a expressão da FN se encontra restrita ao compartimento estromal, enquanto que a integrina α5β1 é predominantemente expressa por células epiteliais. Já durante o desenvolviment tumoral, o padrão de expressão da FN é significativamente alterado, sendo desigual e reduzido, sugerindo uma regulação negativa da secreção de FN e falta de organização de uma matriz.

Desta forma, uma maior compreensão da dinâmica entre a FN e as células tumorais, se torna essencial para expandir nosso conhecimento sobre este tipo de câncer, visto que, até os dias atuais ainda não existe um conceito central sobre o papel da FN na biologia molecular do câncer.

HIPÓTESE E OBJETIVOS

3. HIPÓTESE

Considerando as informações descritas, nossa hipótese é de que a FN possua impacto significativo na regulação da expressão de mRNAs e microRNAs relacionados ao câncer nas linhagens tumorais prostáticas, sendo esta regulação favorável a progressão da doença.

4. OBJETIVOS

Avaliar a influência da FN na expressão de mRNAs e miRNAs nas células epiteliais tumorais prostáticas, LNCaP e PC-3.

Objetivos específicos:

- ✓ Avaliar o impacto da FN na expressão de caderinas (*CDH1* e *CDH2*), integrinas (subunidades α e β) e genes relacionados com importantes vias de sinalização celular, tais como: *STAT3, AKT* e *PTEN* em células LNCaP em ambiente de cultura, no qual a FN é elemento predominante, e em conjunto com uma membrana basal;
- ✓ Avaliar se as alterações na expressão gênica influenciadas pela FN encontradas nas células LNCaP aproximam ou distanciam da expressão regular das células RWPE-1;
- ✓ Avaliar o impacto da FN na expressão dos miRNAs: miR-21, miR-29b, miR-34a, miR-125b, miR-145, miR-221 e miR-222, nas células LNCaP e PC-3;
- ✓ Identificar os possíveis mRNAs alvos dos miRNAs diferencialmente expressos após exposição à FN, assim como, avaliar os possíveis efeitos desta exposição por meio da análise de enriquecimento de via, ontologia gênica e interações proteicas.

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CAPÍTULO 1

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Exploring fibronectin exposure on LNCaP cells gene expression related to tumor progression and dissemination

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ABSTRACT

Prostate cancer still has high mortality rates, with most of the deaths resulting from the development of metastases. During the metastatic process, tumor cells interact with fibronectin in two different moments: in the primary tumor site and in blood plasma. Fibronectin is known to regulate important cellular functions, however the major role of this molecule in prostate cancer remains poorly understood. Thus, to investigate the impact of fibronectin in the gene expression of neoplastic prostate cells, we exposed LNCaP cells to fibronectin (25µg/mL) soluble in the media and in combination with a basement membrane. Using RT-qPCR, we demonstrated that when fibronectin is the predominant element, such as in blood plasma, tumor cells develop an invasive behavior and apoptosis resistance. However, together with a basement membrane, fibronectin seems to decrease the malignant and metastatic potential of LNCaP cells. To elucidate this idea, we performed a hierarchical clustering analysis with the LNCaP cells and RWPE-1 cells. Our results demonstrate that LNCaP cells exposed to a more enriched-ECM display a gene expression profile more similar to RWPE-1 cells, a cell line that illustrates the characteristics of the normal prostate epithelium. In conclusion, our findings provide the groundwork for future studies, addressing the role of fibronectin 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; LNCaP; Integrins; Cadherins; Circulating Tumor Cells; 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 [2]. However, even with therapeutic advances and earlier diagnosis, most PCa related deaths still occur due to the development of metastases [3].

Currently, it is known that the metastatic potential is not only inherent from genetic changes that happen within tumor cells, but also relies on changes in the tumor microenvironment [4,5]. Several studies indicate that the preparation of a "fertile soil" for metastasis requires the involvement of stromal components, along with a differential expression of molecules related to cell-cell and cell-matrix interaction in the tumor cells [6–8]. In this scenario, during a successful dissemination, tumor cells should be able to: i) detach from the primary tumor mass; ii) degrade the basement membrane; iii) invade the stromal surrounding tissue; iv) intravasate into blood and lymphatic vessels lumen; and, v) disseminate and colonize other organs, such as lymph nodes, bones, lung, liver and brain [9–11].

Studies demonstrate that circulating tumor cells (CTCs) activate the coagulation cascade and surround themselves with fibronectin (FN) molecules, in order to form a thrombus that protects them against the cytotoxic activity of immune cells[12,13]. Furthermore, the clotted plasma also provides the CTCs a favorable microenvironment that sustains adherence among the cells and to the blood vessel wall [12,14], and activates important intracellular signaling cascades, such as the AKT pathway, via surface receptors [15].

FN is a multifunctional modular molecule that is ubiquitously distributed in the body, being an important component both in blood plasma [16], as in matrices of connective tissues [17]. High levels of this molecule have been documented in processes such as wound healing, arthritis and many types of cancer [18–20]. However, in PCa, only a limited number of experimental data evaluates the expression levels of FN in tissue samples, or changes in the serum concentration of this molecule in patients [21], generating controversial information about the role of FN in this disease.

The elucidation of the functions of FN in prostate tumor cells can provide insights into the mechanisms of PCa progression and metastasis. Thus, using the LNCaP cell line [22], a metastatic androgen-sensitive prostate adenocarcinoma cell line as a model, we sought to evaluate the impact of FN in the expression of cadherins, integrins and genes related to important intracellular signaling pathways. For this, the cell line was exposed: i) only to FN,

mimicking the interactions between cells and this molecule in the plasma; and ii) to FN in combination with a basement membrane, mimicking the interaction in primary tumors. We also investigated if those 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).

MATERIAL AND METHODS

Cell Lines and Culture Conditions

LNCaP and RWPE-1 cells were purchased from American Type Culture Collection (ATCC). 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% 100X 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, 5ng/mL human recombinant epidermal growth factor and 1% 100X antibiotic/antimycotic (Gibco™; 15240062). All cell lines were maintained in a humidified incubator with 5% CO_2 at 37ºC.

Coating conditions

Cells were seeded at 4×10^4 cells/cm^2 and all experiments were carried out three times in triplicates. The LNCaP cells were cultivated for four days in the following conditions: i) in standard medium – Control group; ii) exposed to FN from human plasma (Sigma-Aldrich; F0895) in the concentration of 25 µg/ml, according to an established protocol previously published by our group [23]; iii) in plates coated with Geltrex® (Gibco™; A1569601) plus FN diluted in the medium (25 µg/ml); and iv) in plates coated with Geltrex® without FN exposure. To coat the plates, Geltrex® was added, covering the entire well (50 µL/cm^2), and the plates were incubated at 37ºC in CO_2 for 30 min. RWPE-1 cells were cultivated only in uncoated plates with standard medium.

RNA extraction and cDNA synthesis

RNA extraction was performed with PureLink® kit (Ambion™, 12183020) according to the manufacturer's instructions and the RNA was quantified using NanoVue™ Plus spectrophotometer (GE HealthCare). The quality of the samples was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) [24] and only samples with RNA integrity numbers above 9 were used in the analysis. In order to remove genomic DNA we used TURBO DNA-free™ Kit (Ambion™, AM1907) and 1µg of RNA was reverse transcribed into cDNA using SuperScript® VILO™ Master Mix (Invitrogen™; 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 AKT1, BAX, BCL2, CDH1, CDH2, ITGB1, MYC, PTEN and 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 AR, ITGB3, ITGA5 and ITGAV mRNAs, the cDNA samples were amplified using Fast SYBR® Green Master Mix (Applied Biosystems™; 4385617) and primers synthetized by Thermo Fisher Scientific (Supplementary Table 2). Relative gene expression was calculated via a $2^{-\Delta\Delta Ct}$ method [25] and levels normalized to the GAPDH gene [Ct means ± SD, i) RWPE-1 cells: 27.826 ± 0.07; ii) LNCaP on uncoated plates: 27.178 ± 0.18; iii) LNCaP on Geltrex® coating: 27.509 ± 0.13; and iv) LNCaP on Geltrex® coating plus FN: 27.337 ± 0.09]. For analysis involving only LNCaP cells, gene expression data was normalized using the mean expression values of non-exposed LNCaP cells (n=3). For cluster analysis involving RWPE-1 and LNCaP cells, gene expression data was normalized for each gene using the mean expression values of all groups (n=12).

Statistical Analysis

Statistical analyses were performed using a parametric one-way ANOVA test with "a posteriori" Tuckey-Kramer test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Fibronectin alone or in combination with a basement membrane alters Cadherins and Integrins expression in opposite ways

FN is one of the most abundant adhesion proteins in blood plasma [26], and have been shown to be associated with CTCs in metastatic progression [12]. This way, LNCaP cells were exposed to soluble FN to evaluate the capacity of this molecule to alter the gene expression of surface receptors, such as cadherins and integrins.

Our results showed a decrease in CDH1 mRNA expression levels (0.62 fold), while CDH2 was increased (1.98 fold), when compared to control (non-exposed cells) (Fig. 1). For integrins, we observed a significantly increased expression of: ITGA5 (2.70 fold), ITGAV (2.81 fold) and ITGB3 (2.71 fold), compared to control. These data indicate that soluble FN may support an increased adhesion of tumor cells mediated by integrins αvβ3 and α5β1.

Additionally, to examine whether FN could induce similar expression changes in tumor cells at a more complex environment, as found in the primary tumor site, we cultivated LNCaP cells on top of a basement membrane (Geltrex®) with soluble FN dissolved in the media. LNCaP cells cultivated on top of Geltrex® without soluble FN were used as control for this experiment.

As seen in Figure 1, the combination of Geltrex® and FN lead to an opposite gene expression pattern. In this case, CDH1 mRNA expression level showed an increase of 7.79 fold compared to the control group. Additionally, CDH2 that had previously displayed a markedly increase in expression levels, this time had a 7.92 fold decrease in expression. For the integrins, both subunits of the integrin αvβ3 had significantly decreased expression, while the expression of ITGA5 was maintained in similar levels as the cells only exposed to FN. The integrin subunit β1 had the same level of relative expression among all groups (Fig. 1). All fold change values are presented in Supplementary Table 3.

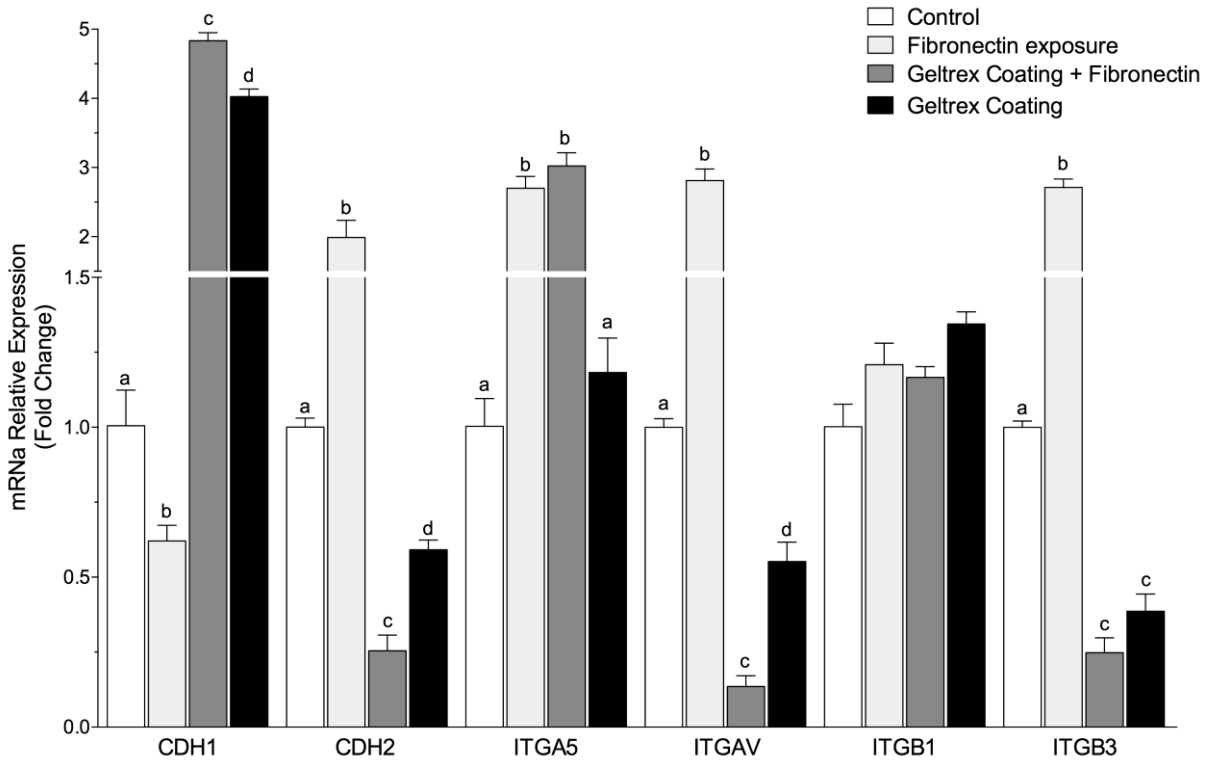

Figure 1: Relative expression of surface receptors after fibronectin exposure (Fold Change from uncoated LNCaP cells). mRNAs levels of Cadherins and Integrins in LNCaP cells: not exposed – control group (white bars); exposed only to fibronectin (light grey bars); exposed to fibronectin in combination with Geltrex® (dark grey bars); and exposed only to Geltrex® (black bars). Values are expressed by mean ± SEM. Bars with different letters are significantly different ($p<0.05$).

Fibronectin alone or in combination with a basement membrane supports a differential intracellular signaling stimulus

To explore whether FN alone or in combination with a basement membrane could support also a differential expression of genes related to cellular behavior, we evaluated the expression of genes involved in pathways responsible for signal transduction between the extracellular and intracellular compartments. Table 1 presents the two main Gene Ontology (GO) annotations and the major function of each evaluated gene.

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

Gene	Related GO annotation*	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

*Olny the two main GO annotations are mentioned

After exposure to only soluble FN, LNCaP cells showed a significant increased expression of pro-proliferative mRNAs, such as AKT1 and MYC; while PTEN mRNA had a reduced expression, compared to non-exposed cells. For genes related to cell death, BCL2 expression was highly increased, whereas the expression of BAX showed a 2-fold decrease in expression when compared to control (Fig. 2)

In LNCaP cells exposed to FN in combination with Geltrex®, gene expression analysis showed an opposite pattern. In this case, AKT, STAT3, MYC and BCL2 mRNAs had significantly decreased expression in more than 2-fold, when compared to non-exposed cells; while PTEN and BAX had a significantly increase in expression (Fig. 2).

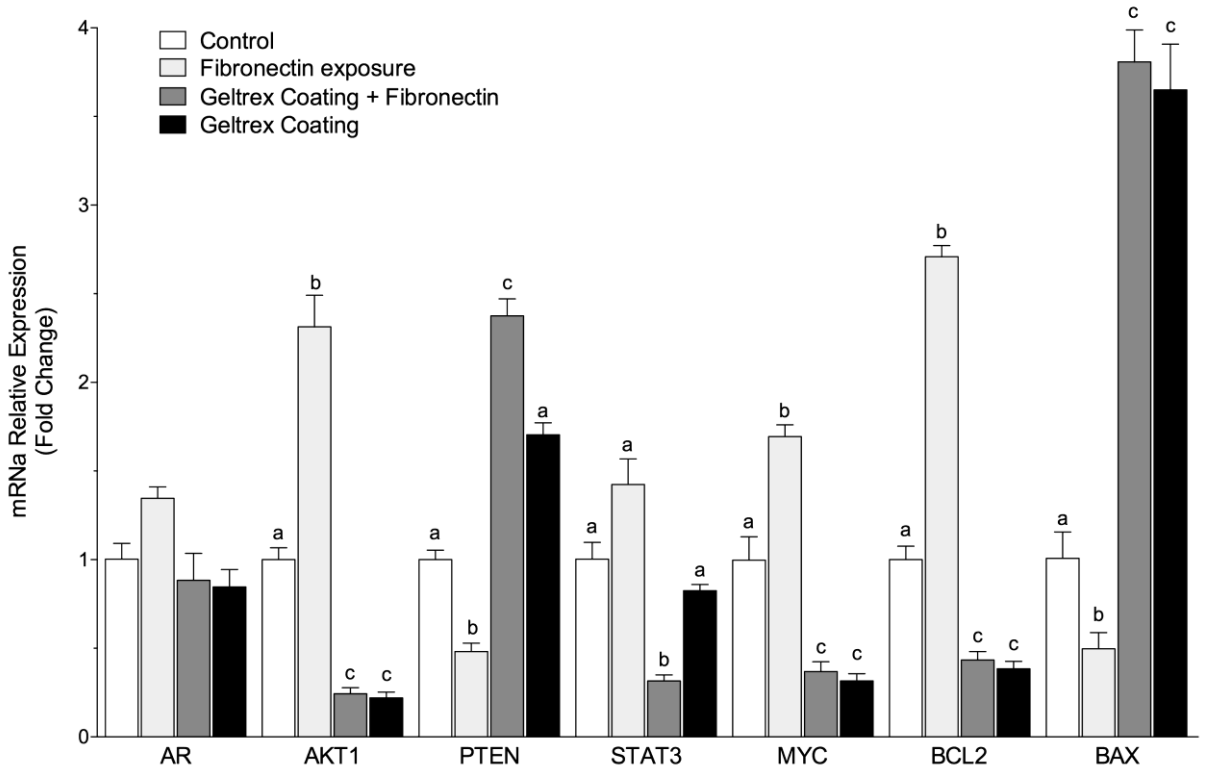

Figure 2: Relative expression of genes related to pathways that transfer signals from the extracellular to the intracellular environment (Fold Change from uncoated LNCaP cells). mRNAs levels of genes related to cell signaling, proliferation and survival; were evaluated in LNCaP cells: not exposed – control group (white bars); exposed only to fibronectin (light grey bars); exposed to fibronectin in combination with Geltrex® (dark grey bars); and exposed only to Geltrex® (black bars). Values are expressed by mean ± SEM. Bars with different letters are significantly different ($p<0.05$).

Fibronectin exposure could approach or distance gene expression pattern between LNCaP and RWPE-1 cells

With our gene expression results, we sought to assess whether FN would approach or distance the gene expression patterns between the tumor and the normal epithelial cell lines. In this sense, we performed a hierarchical clustering to analyze the association among gene expression data of the RWPE-1 cell line and the LNCaP cells i) non-exposed (Control); ii) exposed to FN; and iii) exposed to FN in combination of Geltrex® coating.

The clustering analysis (Fig. 3) indicated a clear distinction among the groups, in which LNCaP cells exposed only to FN acquired a very distinct pattern compared to RWPE-1 cells, which could indicate an acquisition of a more aggressive phenotype. On the other hand, RWPE-1 and LNCaP cells exposed to the combination of the two elements, showed similar expression.

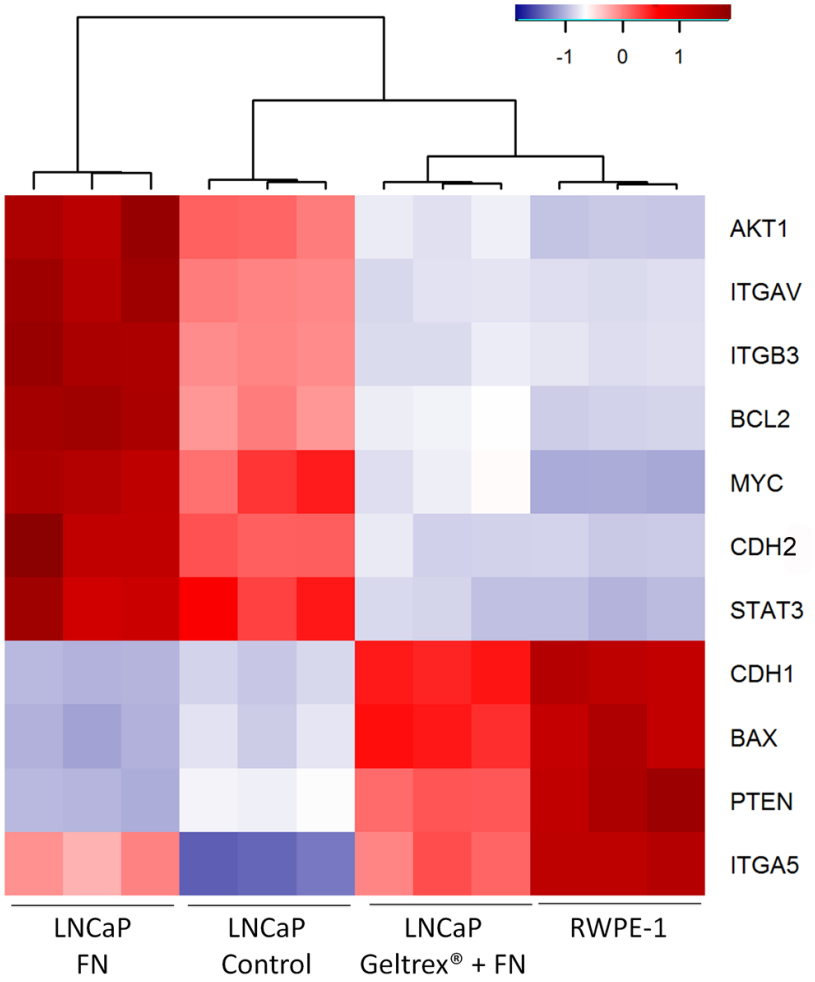

Figure 3: Hierarchical clustering of gene expression data from samples of RWPE-1 cells and LNCaP cells (n=12). The clustering analysis was carried out using DESeq package (R/bioconductor) based on Euclidian distance. Expression data are normalized for each gene using the mean expression of all samples and scaled to Z-scores of the rows, once we wanted to show differences among samples for each gene. Blue boxes indicate lower levels and red boxes, higher levels.

DISCUSSION

Until now, FN appears to have an ambivalent role in cancer. While some authors suggest that FN can suppress the invasion of tumor cells due to its adhesive activity [18,27,28], other research groups have shown that the same element could assist in the processes of invasion and metastasis [12,29,30]. In this study, our findings suggest that both thoughts may be correct.

During the metastatic process, tumor cells interact with FN mainly in two different moments: 1) in the primary tumor site, in which the cells are in contact with the surrounding basement membrane; and 2) in blood plasma, where FN is one of the major components [31,32]. Here, we showed that exposure to FN alone, mimicking the plasma environment, leads to an increased expression of CDH2 mRNA and a decrease in CDH1 expression. This suggests a more invasive phenotype, in line with previous studies that demonstrated that *N-cadherin* overexpression was associated with higher invasion rates in prostate tumor cell lines, and related also to the development of an androgen-independent stage [33].

Such invasive behavior, can also be seen in the expression of integrins. According to several studies, integrins α5β1 and αvβ3 are associated with invasiveness of cancer cells [34,35], and in our study, we showed an increased expression of the subunits: α5, αv and β3 in LNCaP cells exposed to FN.

In addition, knowing that intracellular signal related to cell surface receptors is crucial for the processes of metastasis and anchorage-independence, we evaluate some important genes related to cell growth, proliferation and survival. After FN exposure, LNCaP cells showed an increased expression of AKT1, in line with previous studies that demonstrate that FN induces the AKT pathway, contributing to the development of apoptosis resistance in PCa cells [30]. In addition, we detected also a significant increase in BCL2 expression, consistent with studies that showed higher expression of BCL2 related to an increase in the expression of integrins α5β1 and αvβ3 [36,37].

Furthermore, LNCaP cells exposed only to FN displayed higher expression levels of: i) MYC, gene related to early stages of PCa, required for AR-dependent growth [38,39]; and ii) STAT3, related to the development of metastatic progression in PCa [40]. Thus, we propose that when FN is the predominant element, tumor cells develop an invasive behavior and apoptosis resistance.

However, as we know, in primary tumor sites, tumor cells not only interact with FN, but also with others elements of the extracellular matrix (ECM). Based on this fact, we also

verified the impact of FN exposure combined to a basement membrane (Geltrex®). We supplemented the medium of cells seeded on Geltrex® coating with soluble FN, and showed that LNCaP cells displayed a differential expression pattern of *Cadherins*, with a decreased expression of CDH2 and an increased expression of CDH1, protein related to the degree of cell differentiation and invasion [41,42]. Furthermore, the expression of ITGAV and ITGB3 genes showed now significant lower levels compared not only to the cells exposed to FN alone, but also to cells not exposed.

Accordingly to this new gene expression pattern of cell surface receptors, genes related to cell survival and invasiveness displayed also altered expression in an opposite way. LNCaP cells in the presence of a basement membrane, when exposed to FN showed a decreased expression of AKT1, STAT3, MYC and BCL2, while the expression of BAX, a pro-apoptotic gene, and PTEN, a gene that could sensitize LNCaP cells to an apoptotic stimuli [43], were significantly elevated. In this way, FN exposure in combination with a basement membrane seems to have decreased the malignant and metastatic potential of LNCaP cells.

Thus, we demonstrated that the role of FN depends on the interactions between tumor cells and their substrates, corroborating with Ramos et al. (2016), which reported that changes in the environment could cause a differential effect of fibronectin on cell migration[44].

To further elucidate this idea, we performed a hierarchical clustering analysis with the LNCaP in the two different exposure conditions studied and with the RWPE-1 cells. With our results we showed that LNCaP cells exposed to a more enriched-ECM displayed a gene expression profile more similar to RWPE-1 cells, a cell line that illustrates the characteristics of a normal prostate epithelium [45], while LNCaP cells exposed only to FN acquired a more aggressive behavior.

This is especially important in the context of CTCs biology, since it becomes evident that FN may play a role not only in angiogenesis stimulation [31], but may also sustain survival and proliferation of tumor cells within the blood vessels.

In conclusion, this study provides 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.

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SUPPLEMENTARY DATA

Supplementary Table 1. TaqMan® Assays used in this study.

Gene Name	Gene Symbol	Assay ID
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	Hs99999905_m1
V-akt murine thymoma viral oncogene homolog 1	AKT1	Hs00178289_m1
BCL2-associated X protein	BAX	Hs00180269_m1
B-cell CLL/lymphoma 2	BCL2	Hs00236808_s1
Cadherin 1, type 1, E-cadherin 1	CDH1	Hs01023894_m1
Cadherin 2, type 1 (N-cadherin)	CDH12	Hs00983056_m1
Integrin, beta 1 (fibronectin receptor)	ITGB1	Hs00559595_m1
V-myc avian myelocytomatosis viral oncogene homolog	MYC	Hs00153408_m1
Phosphatase and tensin homolog	PTEN	Hs02621230_s1
Signal transducer and activator of transcription 3	STAT3	Hs00374280_m1

Supplementary Table 2. Primers used in this study

Gene	Forward Primer (5’ to 3’)	Reverse Primer (3’ to 5’)
GAPDH	GAATGGGCAGCCGTTAGGAA	ATCACCCGGAGGAGAAATCG
AR	TGGATAGCTACTCCGGACCT	CCACAGATCAGGCAGGTCTT
ITGB3	CATCACCATCCACGACCGAA	TTGGTGAAGGTAGACGTGGC
ITGA5	GATGCCGTACCGAATCCTGC	GCCTTCTGCCTTGGTCCATT
ITGAV	TCGGGACTCCTGCTACCTCT	AGCTCCCACGAGAAGAAACAT

Supplementary Table 3. Fold change values from non-exposed LNCaP cells.

Gene symbol	FN exposure	Geltrex + FN	Geltrex
CDH1	0.62 ± 0.05	4.83 ± 0.11	4.02 ± 0.11
CDH2	1.98 ± 0.25	0.25 ± 0.05	0.59 ± 0.03
ITGA5	2.70 ± 0.16	3.02 ± 0.19	1.18 ± 0.11
ITGAV	2.81 ± 0.16	0.13 ± 0.03	0.55 ± 0.06
ITGB1	1.20 ± 0.07	1.16 ± 0.03	1.34 ± 0.04
ITGB3	2.71 ± 0.12	0.24 ± 0.04	0.38 ± 0.05
AR	1.34 ± 0.06	0.88 ± 0.15	0.84 ± 0.09
AKT1	2.31 ± 0.17	0.24 ± 0.03	0.21 ± 0.03
PTEN	0.48 ± 0.04	2.37 ± 0.09	1.70 ± 0.06
STAT3	1.42 ± 0.14	0.31 ± 0.03	0.82 ± 0.03
MYC	1.69 ± 0.06	0.36 ± 0.05	0.31 ± 0.04
BCL2	2.70 ± 0.06	0.43 ± 0.04	0.38 ± 0.04
BAX	0.49 ± 0.09	3.80 ± 0.17	3.65 ± 0.25

Values are expressed by mean ± SEM.

CAPÍTULO 2

CAPÍTULO 2

Artigo a ser revisado e submetido para o periódico científico "*Bioscince Reports*".

Modulation of cellular micrornas through fibronectin activity in prostate cancer cells

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ABSTRACT

Prostate cancer remains a major cause of cancer-related deaths among men. Even with its high mortality and incidence rates, little is known about the molecular biology of this type of cancer. Among the many factors that are involved in the carcinogenesis of the prostate, the extracellular matrix plays a pivotal role. In the prostate gland, this matrix is composed mainly of collagen fibers, reticular fibers, elastic fibers, proteoglycans and glycoproteins, such as fibronectin. This multimodular glycoprotein has been implicated in the development of multiple types of cancer, and it has been associated with cell migration and invasion in several metastatic models. Our research group showed that LNCaP cells displayed altered gene expression when exposed to fibronectin. Consequently, knowing that the relationship between tumor cells and the extracellular matrix is regulated at multiple levels, microRNAs emerged as key regulator molecules. In this study, we investigated the impact of fibronectin exposure over the expression of microRNAs in LNCaP and PC-3 cells. Here, five differentially expressed microRNAs were found by qPCR (miR-21, miR-29b, miR-125b, miR-221 and miR-222) after fibronectin exposure in the prostate cell lines LNCaP and PC-3. Further, we analyzed the regular expression pattern of the target mRNAs of these microRNAs in published RNAseq data, annotated them into protein interactions networks based on STRING database, and performed function enrichment analysis for these genes. In summary, we showed that fibronectin impact on microRNAs expression might be involved in the progression of prostate cancer through the modulation of signaling pathways, such as PI3K/AKT, response to drugs and hypoxia. Thus, we believe that a better understanding of the relationship between fibronectin and the expression of microRNAs could improve the comprehension of PCa progression.

Keywords: Fibronectin, LNCaP, PC-3, RNAseq; PI3K/AKT pathway; extacellular matrix;

INTRODUCTION

Prostate cancer (PCa) remains the most common malignancy and a major cause of cancer-related deaths among men [1]. During the development of this disorder, several paracrine stimuli occur between acinar epithelial cells and the surrounding environment, which ultimately modulates the cell behavior and the expression of stromal extracellular matrix (ECM) molecules [2,3].

Among the ECM components, fibronectin (FN) holds a prominent role, regulating a wide spectrum of cellular events [4,5]. This multimodular glycoprotein has been implicated in the development of multiple types of cancer [6–8], and it has been associated with cell migration and invasion in several metastatic models [9,10]. Our research group showed that LNCaP cells, a neoplastic prostate cell line, displayed altered expression of genes involved with cell adhesion, survival, and proliferation, when exposed to FN [11 – data not published]. Consequently, knowing that the relationship between tumor cells and the ECM is regulated at multiple levels, microRNAs (miRNAs) emerged as key regulator molecules.

miRNAs are small non-coding RNAs with about 17 to 25 nucleotides that play important regulatory roles through translational repression [12,13]. Due to the ability to target several mRNAs, miRNAs can coordinate or fine-tune the expression of many proteins. In mammals, predictions indicate that miRNAs may control the activity of ~50% of all protein-coding genes [14], promoting an orchestrated regulation of controlling signaling pathways and complex biological functions [15,16].

However, while it has been well established that miRNAs can regulate the expression of ECM molecules, it is just beginning to become apparent that the expression and function of miRNAs can be influenced by the ECM [17,18]. This way, we believe that a better understanding of the relationship between the ECM and miRNAs will facilitate the discovery of molecular mechanisms underlying PCa progression, which may unveil potential therapeutic opportunities.

For this, we identified miRNAs differentially expressed under FN exposure in the prostate cell lines: i) LNCaP – androgen-sensitive [19]; and ii) PC-3 – androgen independent [20]; using both TarBase and miRTarBase databases to determine the validated mRNA targets of the identified miRNAs. Further, we analyzed the expression pattern of these mRNAs in the two prostate cell lines, annotated them into protein interactions networks based on STRING database, and performed function enrichment analysis for these genes.

MATERIAL AND METHODS

Cell Lines and Culture Conditions

The prostate cancer cell lines LNCaP (clone FGC - ATCC® CRL-1740™) and PC-3 (ATCC® CRL-1435™) were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS - Gibco BRL, Grand Island, NY) and 1% 100X Antibiotic/Antimycotic (Gibco BRL, Grand Island, NY). The cells were maintained in a humidified incubator with 5% CO_2 at 37ºC.

Fibronectin exposure

The cells were seeded at 4×10^4 cells/cm^2 and exposed to FN derived from human plasma (Sigma-Aldrich; F0895) in the concentration of 25 μg/ml, according to an established protocol previously published by our group [11,21]. The experiments were carried out three times in triplicates.

RNA extraction

RNA was isolated from LNCaP and PC-3 cells using the PureLink® kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quantification and quality of the RNA was checked by NanoVue™ Plus spectrophotometer (GE HealthCare, Little Chalfont, UK), and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) [22], respectively. Only samples with RNA integrity numbers above 9 were used in further analysis. In order to remove genomic DNA we used the DNAse I, Amplification Grade (Invitrogen, Carlsbad, CA).

miRNAs expression detection

In a previous study, our group demonstrated that FN exposure promoted differential expression of several mRNAs (11), because of this, we decided to evaluate if FN would also affect the expression of miRNAs. Finally, we chose 7 miRNAs known to be associated with PCa (Table I).

Table I. microRNAs evaluated

miRBase ID	Assay ID*	Reference
hsa-miR-21-5p	000397	[23]
hsa-miR-29b-3p	000413	[24]
hsa-miR-34a-5p	000425	[25]
hsa-miR-125b-5p	000449	[26]
hsa-miR-145-5p	002278	[27]
hsa-miR-221-3p	000524	[28]
hsa-miR-222-3p	002276	[15]

* Taqman® Assay ID

For these miRNAs, 10 ng of RNA was reverse transcribed using TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific primers (Applied Biosystems, Foster City, CA). The reaction conditions were used as follows: 16 °C for 30 minutes, 42 °C for 30 minutes and 85 °C for 5 minutes. The qPCR reactions were performed on a QuantStudio™ 12K Flex system (Applied Biosystems, Foster City, CA) using TaqMan® Fast Advanced Master Mix and sequence-specific TaqMan MicroRNA Assays (Table I /Applied Biosystems, Foster City, CA). Relative gene expression was calculated via a $2^{-\Delta\Delta Ct}$ method [29], and levels were normalized using the endogenous reference RNAs: RNU43 and RNU48. For statistical analyses, we performed a parametric one-way ANOVA test with "a posteriori" Tuckey-Kramer test. Differences were considered statistically significant at $p < 0.05$.

Identification of target genes from the differentially expressed miRNAs

Each miRNA has a plurality of target genes. In order to identify these targets genes, we used the miRTarBase v.6.0 [30], and TarBase v.7.0 [31] databases. These two databases use algorithms to produce a collection with experimentally validated miRNA targets, and we only used interactions that were proved by the following techniques: i) reporter assay; ii) western blot; and iii) q-PCR.

Gene expression profiles

To evaluate the expression of the identified validated target mRNAs in LNCaP and PC-3 cells, we downloaded the mRNA expression profiles of these cell lines from The Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo/). The accession number of

LNCaP mRNA expression profile is GSE29155 [32], containing 7 samples from LNCaP cells and 4 samples of prostate epithelial cells (PrEC), which were used as control in this study. The mRNA profiles were detected using the platforms GPL9052- Illumina Genome Analyzer and GPL9115 – Illumina Genome Analyzer II. The accession numbers of PC-3 mRNA expression profiles are GSE64025 [33], and GSE68645 [34], both containing 2 samples from PC-3 cells. The platform used was GPL10999-Illumina Genome Analyzer IIx in GSE64025, and Illumina Hiseq 2500 in GSE68645. From all studies, we obtained the raw expression data. All the samples were downloaded using a custom bash script in a GNU/Linux machine. After downloaded, the data was firstly analyzed using FastQC software (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) in order to obtain quality information. Adaptor trimming and quality filtering were both carried out using Trimmomatic software version 0.36 [35], based on the following parameters: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 and MINLEN:22. For the quantification and comparison procedures, the remaining reads were aligned against the Homo sapiens genome, version hg19, using RSEM version 1.2.12 [36]. Read count data from all the transcripts were extracted from the alignment files using HTSeq software [37]. Such read count information was then inserted in an R/Bioconductor environment in order to obtain differential expression data by using EdgeR package.

Expression pattern of the target mRNAs

Differential mRNA expression analysis was carried out using the edgeR Bioconductor package [38]. We used the human primary prostate epithelial cells (PrEC - Lonza) as a normal control and edgeR was employed to find the differentially expressed genes between these cells and the neoplastic prostate cell lines, LNCaP and PC-3. To visualize and generate the heatmap of the differential mRNAs discovered, we used the ComplexHeatmap Bioconductor package [39]. The hierarchical clustering of genes was based on Euclidian distance and expression data are scaled to Z-scores of the rows.

Interaction networks construction of the miRNA targets

To verify the synergy of one gene with others and the interactions of its proteins, the online software STRING v.10 [40] was used to evaluate these interactions between the differentially expressed genes (http://string-db.org).

Gene Ontology

A functional classification of the differentially expressed genes predicted to be targeted by the miRNAs evaluated was performed using Gene Ontology (GO) enrichment analysis applying the DAVID Tool [41]; and searched for over-represented GO terms in three categories, namely biological process, molecular function and cellular component.

Gene Set Enrichment Analysis

The gene set enrichment analysis (GSEA) considers functionally similar or function-related genes as a whole. In this strategy, we can assesses the biological functions or biological properties by calculating the overall significance of gene expression changes in a defined set of genes [42]. The fold change between the raw counts from RNA-seq NGS data of LNCaP and PC-3 *versus* PrEC was calculated, and target genes were ranked by the order of expression in each cell line. These lists were uploaded as a pre-ranked gene list to GSEA v.2.0 (Broad Institute, Cambridge, MA), and the analysis was performed using a weighted enrichment statistic and default normalization mode.

RESULTS

Impact of fibronectin exposure on miRNA expression

In the last study of our group we showed that FN was capable of altering gene expression of LNCaP cells [11]. To further evaluate the impact of FN on PCa, we analyzed 7 miRNAs known to be involved in this disease and that were known to be involved in the regulation of these genes previously evaluated by our group. The qRT-PCR analyses for LNCaP and PC-3 cells is given in Figure 1, showing the miRNAs' relative expression. In LNCaP cells exposed to FN it was found that: hsa-miR-21-5p (1.55 fold); hsa-miR-29b-3p (2.61 fold); hsa-miR-125b-5p (2.20 fold); hsa-miR-221-3p (2.42 fold) and hsa-miR-222-3p (1.64 fold) displayed higher expression when compared to the same cells, when non-exposed. Additionally, FN exposure effects was similar in PC-3 cells, but in a lesser extent. In the androgen-insensitive cell line, the numbers of miRNAs affected were lower than in the LNCaP. Only the miRNAs: hsa-miR-21-5p (1.66); hsa-miR-29b-3p (1.83 fold); hsa-miR-221-3p (1.71) and hsa-miR-222-3p (1.36 fold) showed a higher expression, with the differences between the relative expression

value of the unexposed and exposed cells being smaller. The hsa-miR-34a-5p and hsa-miR-145-5p had the same level of relative expression in both culture conditions in the two cell lines.

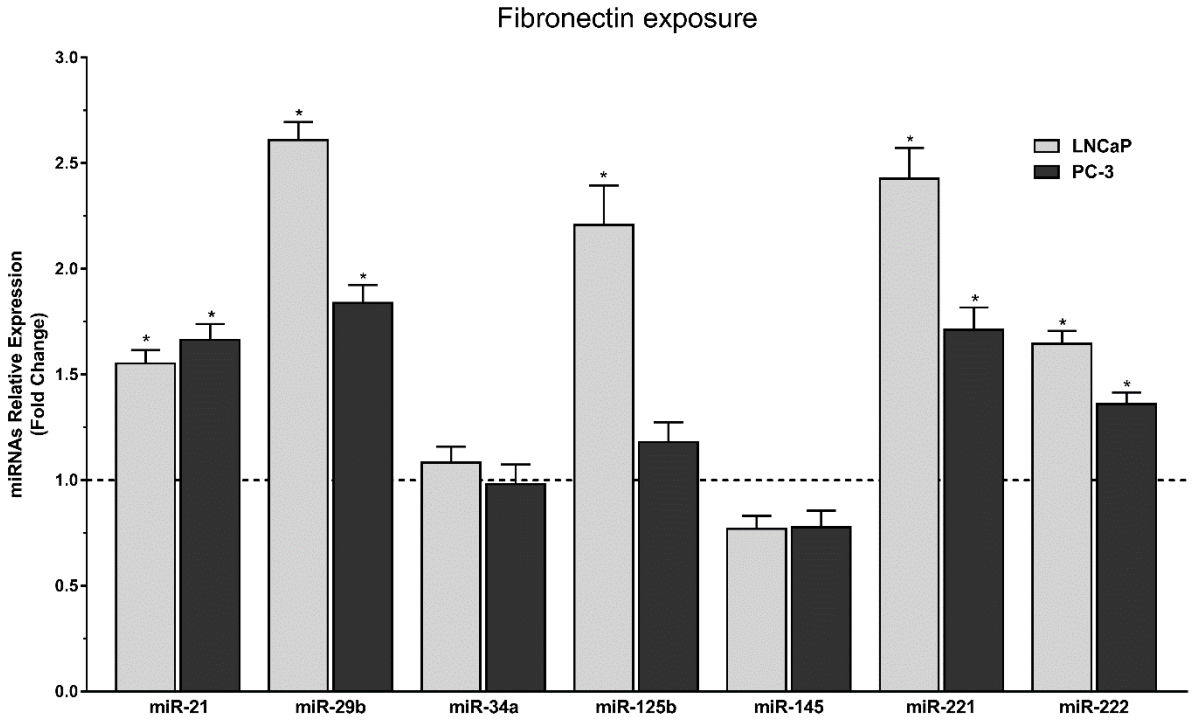

Figure 1: Relative expression of miRNAs after FN exposure in LNCaP and PC-3 cells (Fold Change from unexposed cells from each cell line). Light grey bars represent the miRNAs levels of LNCaP cells exposed to FN, and dark grey bars the PC-3 cells. The line represents the value of LNCaP and PC-3 cells non-exposed. Values are expressed by mean ± SEM (Standard error of the mean * $p<0.05$ between cells exposed and non-exposed in the same cell line).

Target Genes of the Differentially Expressed miRNAs

To explore the biological functions of the differentially expressed miRNAs identified after FN exposure in both cell lines, the validated mRNA targets were downloaded from two databases: TarBase v6.0 and miRTarBase v4.5. As shown in Table II, the targets of the differentially expressed miRNAs: i) hsa-miR-21-5p; ii) hsa-miR-29b-3p; iii) hsa-miR-125b-5p; iv) hsa-miR-221-3p; and v) hsa-miR-222-3p, were defined as target genes, proven by several test methods.

Table II. Target genes of differentially expressed miRNAs

	hsa-miR-21-5p	hsa-miR-29b-3p	hsa-miR-125b-5p
miRTarBase v.6.0 and TarBase v.7.0	AKT2; ANKRD46; ANP32A; APAF1; BASP1; BCL2; BCL6; BMPR2; BTG2; CCL20; CCR1; CDC25A; CDK2AP1; CLU; COL4A1; DAXX; DERL1; DOCK4; DOCK5; DOCK7; DUSP10; E2F1; EGFR; EIF4A2; ERBB2; FASLG; FMOD; GAS5; GDF5; HNRNPK; HPGD; ICAM1; IGF1R; IL1B; IRAK1; ISCU; JAG1; JMY; LRRFIP1; MAP2K3; MARCKS; MEF2C; MSH2; MSH6; MTAP; MYD88; NCAPG; NCOA3; NFIA; NFIB; NTF3; PCBP1; PDCD4; PIAS3; PLAT; PLOD3; PPARA; PPIF; PTEN; PTX3; RASA1; RASGRP1; RECK; REST; RHO; RHOB; RPS7; RTN4; SATB1; SERPINB5; SERPINI1; SETD2; SIRT2; SMAD7; SMARCA4; SMN1; SOD3; SOX5; SP1; SPRY2; STAT3; TCF21; TGFBI; TGFBR2; TGFBR3; TGIF1; TIAM1; TIMP3; TM9SF3; TNFAIP3; TNFRSF10B; TOPORS; TP53BP2; TP63; TPM1; VEGFA; VHL; WWP1; YOD1	ADAM12; BACE1; BBC3; BCL2; BMP1; CDC42; CDK6; CLDN1; COL1A1; COL3A1; COL4A1; COL4A2; COL5A3; CTNNBIP1; DNAJB11; DNMT1; DNMT3A; DNMT3B; DUSP2; ESR1; FAM193A; FGA; FGB; FGG; FOS; GRN; GSK3B; HDAC4; HMGA2; IFNG; IFRD1; IMPDH1; MAPKBP1; MCL1; MMP15; MMP2; MMP24; MYCN; NASP; NCOA3; NID1; NKIRAS2; PIK3CG; PIK3R1; PPP1R13B; PTEN; RAX; S100B; SCAF8; SFPQ; SP1; TBX21; TCL1A; TET1; TGFB1; TGFB2; TGFB3; VEGFA	ABTB1; AHRR; AKT1; ARID3A; ARID3B; BAK1; BBC3; BCL2; BCL2L2; BCL3; BMF; BMPR1B; CBFB; CCNJ; CDH5; CDKN2A; CSNK2A1; CYP24A1; DGAT1; DUSP6; E2F2; E2F3; EIF4EBP1; EIF5A2; ENPEP; EPO; EPOR; ERBB2; ERBB3; ETS1; FGFR2; GLI1; GRIN2A; GSS; HK2; HMGA1; HMGA2; ICAM2; IGF2; IKZF2; IKZF3; IKZF4; IL6R; IRF4; KLF13; LIN28A; LIN28B; LIPA; MAN1B1; MAPK14; MCL1; MEGF9; MMP13; MUC1; MXD1; NCOR2; NES; NKIRAS2; NTRK3; PCTP; PIAS3; PIGF; PIK3CD; PPP1CA; PRDM1; PRKRA; RAF1; RPS6KA1; SCNN1A; SGPL1; SIRT7; SMAD4; SMO; STARD13; TBC1D1; TET2; TNFAIP3; TP53; TP53INP1; VDR; VPS4B; VPS51

	hsa-miR-221-3p	hsa-miR-222-3p
miRTarBase v.6.0 and TarBase v.7.0	ADAM1A; ANXA1; APAF1; ARIH2; ARNT; ASZ1; BBC3; BMF; BNIP3; BNIP3L; BRAP; CDKN1B; CDKN1C; CERS2; CORO1A; CREBZF; CTCF; DDIT4; DICER1; DIRAS3; DKK2; DVL2; ESR1; ETS1; FMR1; FOS; FOXO3; GJA1; HECTD2; HMGXB4; HOXB5; ICAM1; KIT; MDM2; MEOX2; MGMT; MMP2; MYBL1; NAIP; PAK1; PIK3R1; PTEN; RAB1A; RAD51; RB1; RECK; RUNX1; SELE; SIRT1; SOCS1; SOCS3; SSX2IP; STAT5A; TBK1; TCEAL1; TICAM1; TIMP3; TMED7; TNFSF10; TP53; TRPS1; USP18; WEE1; ZEB2	ADAM1A; BBC3; CDKN1B; CDKN1C; CERS2; CORO1A; DICER1; DIRAS3; DKK2; ESR1; ETS1; FOS; FOXO3; GJA1; ICAM1; KIT; MGMT; MMP1; PPP2R2A; PTEN; RECK; SELE; SOD2; SSX2IP; STAT5A; TCEAL1; TIMP3; TMED7; TNFSF10; TP53; TRPS1; VGLL4

Gene Expression Profile of Target Genes

Raw gene expression data of the RNA-seq datasets were preprocessed and normalized, as show in Supplementary Figure 1. After normalization, we performed differential analysis between the neoplastic prostate cancer and the PrEC cell lines using the R statistical analysis package, edgeR. All the information about the expression analysis, such as: mean-variance and relationship among samples, can be seen in Supplementary Figure 2. In this study, only the genes that are validated targets of the differentially expressed miRNAs and that displayed: false discovery rate (FDR) and p-value lower than 0.05 and absolute log fold changes (base 2) greater than 1.0, were considered as differentially expressed genes (DEGs) in the LNCaP and PC-3 cells.

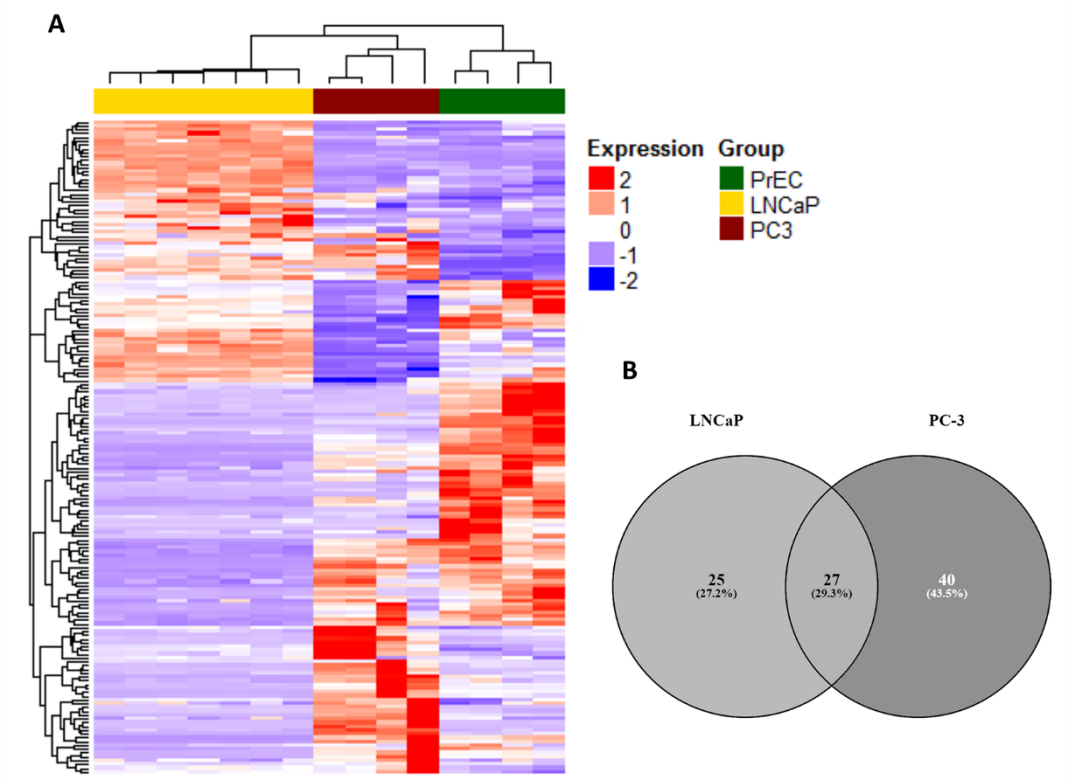

Figure 2: Transcriptional profile of target genes **(A)** Hierarchical clustering analysis of the target genes of differentially expressed miRNAs after FN exposure in PrEC (green), LNCaP (yellow) and PC-3 (red) cells. The clustering analysis was carried out using ComplexHeatmap package (R/bioconductor) with the entire list of the target genes presented in Table II to see their regular profile in the two neoplastic prostate cell lines. Shades of red and blue are used to illustrate whether the expression values is above (red) or below (blue) the mean expression value across all samples (each row in the data was normalized from -2 to +2). **(B)** Overlap of the upregulated target genes observed by transcriptome sequencing in LNCaP and PC-3 cells. The complete list of these genes is presented in Supplementary Table I.

In the heatmap (Figure 2A), it is shown that normalized values from the three cell lines for all the DEGs targets clusters each cell line in a distinct clade. Also, shown in Figure 2, are the numbers of DEGs exclusive to LNCaP (n = 25), PC-3 (n=40) and mutually differentially expressed in both cell lines (n= 27) (Figure 2B). The list of these genes is presented in Supplementary Table I.

Therefore, with the increase in the relative expression of some miRNAs by FN exposure, and knowing that the major function of the miRNAs is the translational repression of target mRNAs, we decided to use only the target genes that were upregulated, and could be repressed. The list of these genes can be seen in Table III.

Table III. Up-regulated genes in LNCaP and PC-3 cells

LNCaP				PC-3			
Genes	**LogFC**	**P-value**	**FDR**	**Genes**	**LogFC**	**P-value**	**FDR**
BMF	6.548	1.78E-12	1.43E-11	ZEB2	8.014	5.18E-09	4.51E-08
TGFBR3	6.295	3.14E-19	4.04E-18	PTX3	7.916	3.58E-41	2.69E-39
E2F1	5.398	1.12E-43	4.23E-42	NES	7.653	1.05E-51	1.25E-49
SOD3	5.262	4.70E-13	3.96E-12	TGFBR3	6.717	7.41E-23	2.21E-21
SELENBP1	5.249	5.04E-50	2.31E-48	BMF	6.636	5.55E-12	6.83E-11
BMPR1B	4.965	3.11E-37	9.37E-36	MEF2C	6.537	6.59E-27	2.51E-25
ARNT2	4.405	2.02E-08	1.06E-07	ADAM12	6.285	2.43E-19	5.80E-18
ARIH2OS	4.077	2.08E-09	1.23E-08	E2F1	5.413	9.12E-44	7.70E-42
IGF2BP2-AS1	4.053	2.71E-03	6.29E-03	SOD3	4.781	1.13E-11	1.34E-10
CCR10	3.624	4.33E-03	9.57E-03	MYBL1	4.489	1.78E-09	1.66E-08
E2F2	3.585	1.07E-13	9.47E-13	MMP13	4.425	8.25E-06	4.30E-05
PRDM12	3.257	1.32E-02	2.57E-02	RTN4RL2	3.864	1.59E-06	9.47E-06
ERBB3	3.252	1.17E-31	2.86E-30	E2F2	3.705	8.58E-16	1.53E-14
MAN1B1-AS1	2.923	1.19E-12	9.66E-12	IGF2BP2-AS1	3.704	7.03E-03	1.82E-02
BCL2	2.878	1.75E-03	4.24E-03	PRDM12	3.332	1.17E-02	2.81E-02
IKZF3	2.871	7.95E-06	2.95E-05	CDKN2A	3.312	8.75E-07	5.47E-06
PDCD4-AS1	2.736	5.75E-05	1.87E-04	SOCS3	3.166	1.23E-13	1.80E-12
MARCKSL1	2.666	4.02E-16	4.27E-15	PPIF	3.054	1.99E-15	3.43E-14
LIN28A	2.640	1.02E-02	2.05E-02	CCR10	3.036	1.22E-02	2.93E-02
PIK3CD	2.640	1.11E-27	2.21E-26	DNMT1	2.868	5.92E-29	2.52E-27
MMP15	2.599	2.36E-19	3.06E-18	ICAM1	2.754	4.95E-13	6.75E-12
BBC3	2.528	1.11E-07	5.38E-07	DNMT3B	2.624	2.96E-08	2.32E-07
RASGRP1	2.482	2.65E-03	6.17E-03	LIN28A	2.564	8.84E-03	2.22E-02

Table III. Continued

LNCaP				PC-3			
Genes	**LogFC**	**P-value**	**FDR**	**Genes**	**LogFC**	**P-value**	**FDR**
DNMT3B	2.464	3.21E-07	1.47E-06	FOSL1	2.540	3.43E-13	4.74E-12
DUSP2	2.360	6.13E-05	1.98E-04	NCAPG2	2.484	5.58E-08	4.18E-07
EPOR	2.142	3.47E-07	1.57E-06	HOXB5	2.472	1.76E-03	5.37E-03
EPOR.1	2.142	3.47E-07	1.57E-06	CDC25A	2.441	4.13E-13	5.67E-12
BCL6	1.992	1.67E-10	1.10E-09	MSH2	2.380	5.40E-08	4.06E-07
USP18	1.975	6.60E-05	2.12E-04	RAD51	2.320	3.35E-05	1.55E-04
DNMT1	1.955	2.50E-16	2.68E-15	EPOR	2.297	4.52E-08	3.44E-07
VPS51	1.950	3.65E-11	2.57E-10	EPOR.1	2.297	4.52E-08	3.44E-07
JMY	1.896	4.79E-05	1.58E-04	YOD1	2.285	9.97E-13	1.33E-11
ERBB2	1.811	3.14E-15	3.12E-14	SIRT1	2.182	9.67E-11	1.04E-09
TCEAL1	1.805	8.96E-06	3.30E-05	DOCK4	2.152	6.43E-17	1.26E-15
TET1	1.793	1.34E-03	3.34E-03	NASP	2.142	3.03E-17	6.11E-16
DNMT3A	1.731	1.49E-12	1.20E-11	IL1B	1.967	1.35E-05	6.75E-05
NCOR2	1.705	3.47E-06	1.36E-05	SPRY2	1.942	2.49E-06	1.43E-05
ADAM12	1.685	2.47E-02	4.46E-02	BMPR1B	1.933	5.80E-06	3.10E-05
TP53INP1	1.661	1.05E-14	1.00E-13	TGFB3	1.907	1.67E-03	5.15E-03
ANP32A-IT1	1.654	4.67E-03	1.02E-02	CDKN1C	1.905	9.15E-03	2.29E-02
CDKN1B	1.631	3.56E-04	9.99E-04	AHRR	1.899	3.15E-04	1.17E-03
NASP	1.528	1.51E-10	1.00E-09	TET1	1.836	1.05E-03	3.43E-03
DVL2	1.464	1.01E-08	5.53E-08	HDAC4	1.770	2.90E-08	2.27E-07
NCAPG2	1.405	9.70E-04	2.48E-03	DGAT1	1.768	1.34E-06	8.13E-06
TP53	1.268	5.21E-09	2.96E-08	IKZF3	1.606	1.64E-02	3.77E-02
ARID3A	1.259	1.31E-03	3.26E-03	SMOX	1.599	1.28E-04	5.23E-04
MMP24	1.257	6.54E-03	1.38E-02	SOD2	1.538	2.85E-05	1.34E-04
CLDN15	1.15	2.03E-03	4.84E-03	PLAT	1.536	1.99E-05	9.61E-05
SMO	1.129	7.16E-03	1.50E-02	MARCKSL1	1.510	4.62E-06	2.53E-05
AKT1	1.046	2.32E-06	9.36E-06	HMGA1	1.500	2.44E-06	1.41E-05
HDAC4	1.032	6.25E-04	1.67E-03	SMARCA4	1.483	1.28E-05	6.42E-05

Table III. Continued

LNCaP				PC-3			
Genes	**LogFC**	**P-value**	**FDR**	**Genes**	**LogFC**	**P-value**	**FDR**
				SELENBP1	1.468	5.15E-05	2.29E-04
				PDCD4-AS1	1.448	2.26E-02	4.94E-02
				USP18	1.415	5.51E-03	1.47E-02
				SSX2IP	1.415	2.25E-04	8.61E-04
				EIF5A2	1.396	4.49E-05	2.02E-04
				COL4A2	1.371	1.04E-03	3.40E-03
				COL4A1	1.357	2.40E-03	7.09E-03
				NCOR2	1.291	8.83E-04	2.93E-03
				TCEAL1	1.253	4.27E-03	1.18E-02
				CDC42BPA	1.243	1.12E-07	8.07E-07
				PAK1IP1	1.241	1.50E-06	8.97E-06
				RHOBTB3	1.228	1.03E-04	4.31E-04
				COL1A1	1.222	1.64E-02	3.77E-02
				RECK	1.200	1.48E-02	3.45E-02
				TGFB2	1.081	3.09E-03	8.89E-03

LogFC: Log Fold Change

Protein interactions networks for target DEGs

The online software STRING was utilized to predict interactions between the products of the up-regulated target genes. The interactions networks of DEGs in LNCaP and PC-3 cells are presented in Figure 3. Combined scores that weigh the degree of confidence for each interaction are illustrated in Supplementary Table II.

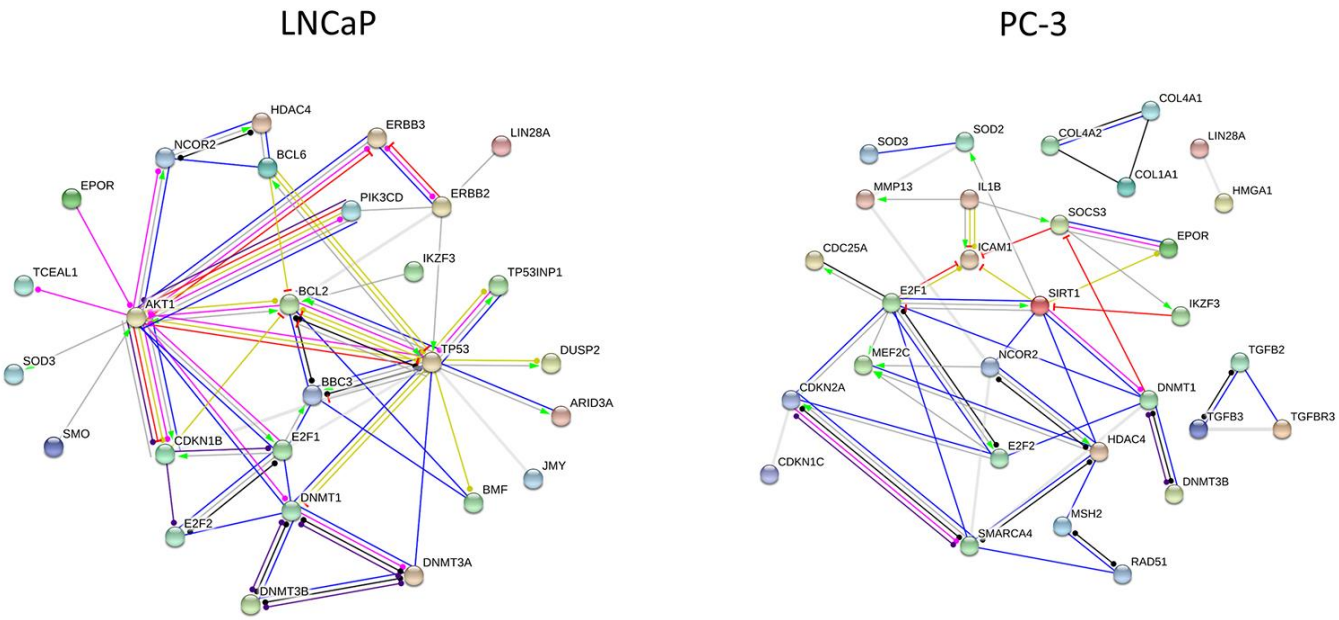

Figure 3: Protein-protein interactions networks generated using STRING. The network is made up to LNCaP cells with 52 upregulated genes, and for PC-3 cells with 67 upregulated genes. In LNCaP cells are presented the importance of the AKT1, BCL2 and TP53 nodes, and in PC-3 cells, the network center on the SIRT1 and the presence of collagens and proteins related to extracellular matrix. Activation (*green*), inhibition (*red*), binding (*blue*), post-translational modification (*fuchsia*), reaction (*black line*). Directionality is indicated by the *arrow*

Function Enrichment Analysis

Next, the target DEGs were used in the functional classification toll provided by DAVID and mapped to identify their biological process, cell location and molecular function. The results for LNCaP and PC-3 cells showed that most of the miRNAs targets were significantly located in the nucleus, highlighting the ECM in the case of PC-3 cells (Figure 4). Moreover, most of these genes tended to be involved with the regulation of the transcription of RNA polymerase II, the response to hypoxia, and, interestingly, response to drugs.

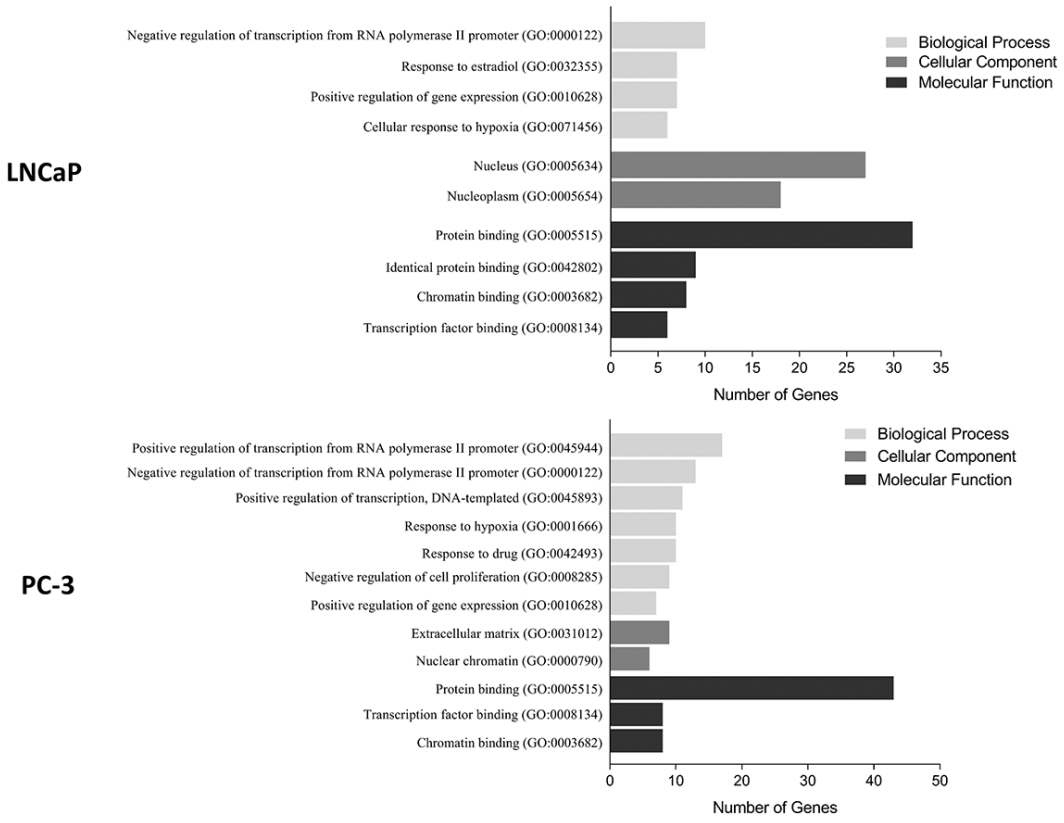

Figure 4: Functional classification of upregulated genes that are target of miRNAs evaluated in LNCaP and PC-3 cells according to DAVID tool. We searched for over-represented GO terms in the three categories, namely biological process, molecular function and cellular component.

Additionally, to further elucidate the possible role of FN exposure in PCa, the same genes were used in the GSEA software. In this analysis (Table IV), we obtained 9 enriched pathways for LNCaP and 4 for PC-3 cells. The most significant pathways were cancer-related, including microRNAs in cancer (hsa05206), prostate cancer (hsa05215), and PI3K/AKT signaling pathway (hsa04151). Moreover, the ECM-receptor interactions (hsa04512) were significantly enriched, demonstrating the importance of the ECM and FN. Thus, the above insights provide some clues for the role of FN in PCa.

Tabela IV. The KEGG Term of Differentially Expressed Target Genes of miRNAs

KEGG paths	*P-value*	*miRNAs*
LNCaP		
Viral carcinogenesis (hsa05203)	1.53E-14	miR-29b, miR-125b, miR-221, miR-222
MicroRNAs in cancer (hsa05206)	9.94E-13	miR-21, miR-29b, miR-125b, miR-221, miR-222
ErbB signaling pathway (hsa04012)	3.20E-09	miR-21, miR-125b, miR-221
Central carbon metabolism in cancer (hsa05230)	2.37E-08	miR-29b, miR-125b, miR-221, miR-222
Prostate cancer (hsa05215)	1.19E-07	miR-21, miR-125b, miR-221, miR-222
Pathways in cancer (hsa05200)	5.96E-05	miR-21, miR-29b, miR-125b, miR-222
Apoptosis (hsa04210)	2.22E-04	miR-21, miR-29b, miR-125b
PI3K-Akt signaling pathway (hsa04151)	1.93E-02	miR-125b
Transcriptional misregulation in cancer (hsa05202)	3.16E-02	miR-221, miR-222
PC-3		
ECM-receptor interaction (hsa04512)	7.36E-18	miR-21, miR-29b
MicroRNAs in cancer (hsa05206)	1.44E-05	miR-21, miR-29b, miR-221, miR-222
Amoebiasis (hsa05146)	9.81E-05	miR-21, miR-29b
Mismatch repair (hsa03430)	2.19E-02	miR-21

DISCUSSION

In the present study, we analyzed the impact of FN over the expression of miRNAs in the neoplastic prostate cell lines LNCaP and PC-3, which are widely employed in PCa research. When choosing the miRNAs that would be evaluated, we decided to analyze 7 miRNAs that are regulators of the genes found to be altered by FN in a previous study [11].

FN stimulates a differential expression of important PCa-related miRNAs

Our results have shown that FN stimulates a differential expression of some miRNAs in both cell lines. LNCaP and PC-3 cells demonstrated a higher expression of miR-21, miR-29b, miR-221 and miR-222. In addition, LNCaP cells also had an increase in the expression of miR-125b. miR-21 has been shown to be upregulated in PCa [43]. This miRNA is related to the development of many types of human cancers, functioning as an oncogene by targeting tumor suppressor genes, such as PTEN, in cancer cells [44].

miR-29b is found in very low expression values in prostatic tumor tissues [45], and when upregulated, inhibits Mcl-1, matrix metallopeptidase-2 (MMP-2) and collagen. Interestingly, previous findings of our group demonstrate that LNCaP cells express higher MMP-2 activity when in contact with FN [21]; suggesting that the increase of this miRNA may be a response to this event.

Among the most important miRNA families, the miR-125 family has been reported to be implicated in a variety of carcinomas [46]. miR-125b targets multiple genes involved in the regulation of apoptosis, including BAK1 and STAT3, but depending on the cell type, miR-125b can either contribute to oncogenesis or tumor suppression [47,48]. In PCa, this miRNA was up-regulated and could be responsible for stimulating the androgen-independent growth [47], which may be the cause of the increased expression of miR-125b after FN exposure only in LNCaP cells, once PC-3 cells are already insensitive to androgen.

Overexpression of the miR-221 and miR-222 promotes growth, metastasis, and invasion of a variety of malignant tumors, including PCa [49]. It was observed that these two miRNAs could affect the proliferative potential of human prostate carcinoma cells [50].

Protein interactions networks and pathway analysis

In view of the importance of these miRNAs in PCa, we sought to understand how the targets of these microRNAs would interact in the evaluated cell lines. For this, we integrated miRNA target information from TarBase and miRTarBase, used a regular mRNA expression profile of LNCaP and PC-3 cells, and considered the gene ontology, pathway enrichment and protein interaction analysis of these candidate genes to better determine the possible effects of FN regulation in PCa. Among the several targets of these miRNAs provided by the databases, 52 and 67 genes were highly expressed in the LNCaP and PC-3 cell lines, respectively, which were chosen for further analysis.

Gene ontology analysis showed the importance of the nuclear compartment, and highlighted the biological processes responsible for the regulation of the transcription of RNA polymerase II, and the response to hypoxia. Prostate tumors have been reported to possess extensive regions of hypoxia compared to the corresponding normal tissue [51,52]. This hypoxic microenvironment correlates with increased tumor invasiveness, metastasis and resistance to radio and chemotherapy [53,54], being an independent poorer prognostic indicator for patients with PCa. In this way, FN exposure could clarify the existing crosstalk between different cells in the hypoxic prostate tumor environment, contributing to the development of new therapies.

To further comprehend the molecular functions, the protein products of these genes were submitted to the STRING platform. Based on the protein-protein network analysis presented here we can highlight the importance of the AKT1, BCL2 and TP53 genes for the LNCaP cell line.

In fact, it was found in the enrichment pathway analysis that apoptosis and the PI3K/AKT signaling pathways are both significantly enriched in LNCaP cells. Activation of the PI3K/AKT pathway clearly plays a major role in the aggressive nature of many prostate cancers [55]. Studies demonstrate that an improved understanding of the biology of this pathway in PCa will be important to better comprehend the benefits from PI3K/AKT inhibitors and which point in the disease course these inhibitors should be given [56]. Thus, we believe that FN could give us some clues, and also be a key factor in the regulation of this pathway in the development of the resistant form of PCa.

Regarding PC-3 cells, the most enriched pathway was the ECM-receptor interaction. In addition, our results showed important protein interactions related to cell migration and invasion. Docheva et al. [57], demonstrated that PC-3 cells, which originated from bone metastasis, are preconditioned *in vivo* to bind to type I collagen (COL1), having high potential to spread and invade areas containing this type of collagen. Here, it was found that FN could alter the expression of three different types of collagen that interact with each other (COL4A2, COL4A1, and COL1A1), suggesting that FN can act as a regulatory molecule in the progression and occurrence of bone metastasis in PCa.

Another finding that establishes FN as a key molecule in the migration and invasion of PC-3 cells is the fact that this glycoprotein regulates genes such as ICAM1, MMP13 and SIRT1. The SIRT1 gene participates in a wide variety of cellular processes, including proliferation, apoptosis and invasion, via non-histone deacetylation of molecules such as the DNA repair protein P300, P73, NF-κB, FOXO transcriptional factor, and P53 [58,59]. In fact, Cui *et al.* [60] demonstrated that inhibition of SIRT1 gene expression in PC-3 cells can decrease the invasive and migratory ability of this cell line, which may be associated with deregulation of the expression levels of mesenchymal markers such as Vimentin and N-cadherin, and upregulation of the expression of epithelial markers such as E-cadherin.

In summary, few studies have investigated how the ECM can regulate the expression of miRNAs, and here in this study, we investigated the impact of the FN exposure on miRNA expression in LNCaP and PC-3 cells. Five differentially expressed miRNAs were found, which might be involved in the progression of PCa through the modulation of signaling pathways, such as PI3K/AKT, and response to drugs, hypoxia and others. Thus, a better understanding of the relationship between FN and the expression of miRNAs could improve the comprehension of PCa progression. Additionally, these miRNAs may also represent effective targets to improve treatment efficacy in future PCa therapy.

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SUPPLEMENTARY DATA

Supp. Table I. DEGs in LNCaP, PC-3 and in both cells

LNCaP	PC-3	LNCaP and PC-3
AKT1	AHRR	ADAM12
ANP32A-IT1	CDC25A	ARIH2OS
ARID3A	CDC42BPA	BMF
ARNT2	CDKN1C	BMPR1B
BBC3	CDKN2A	CCR10
BCL2	COL1A1	DNMT1
BCL6	COL4A1	DNMT3B
CDKN1B	COL4A2	E2F1
CLDN15	DGAT1	E2F2
DNMT3A	DOCK4	EPOR
DUSP2	EIF5A2	EPOR.1
DVL2	FOSL1	HDAC4
ERBB2	HMGA1	IGF2BP2-AS1
ERBB3	HOXB5	IKZF3
FAM193A	ICAM1	LIN28A
JMY	IL1B	MARCKSL1
MAN1B1-AS1	MEF2C	NASP
MMP15	MMP13	NCAPG2
MMP24	MSH2	NCOR2
PIK3CD	MYBL1	PDCD4-AS1
RASGRP1	NES	PRDM12
SMO	PAK1IP1	SELENBP1
TP53	PLAT	SOD3
TP53INP1	PPIF	TCEAL1
VPS51	PTX3	TET1

Supp. Table I. Continued

LNCaP	PC-3	LNCaP and PC-3
	RAD51	TGFBR3
	RECK	USP18
	RHOBTB3	
	RTN4RL2	
	SIRT1	
	SMARCA4	
	SMOX	
	SOCS3	
	SOD2	
	SPRY2	
	SSX2IP	
	TGFB2	
	TGFB3	
	YOD1	
	ZEB2	

Supp. Table II. STRING Interactions in LNCaP and PC-3 cells

LNCaP			*PC-3*		
Node1	**Node2**	**Combined Score**	**Node1**	**Node2**	**Combined Score**
ERBB2	ERBB3	0.999	DNMT1	DNMT3B	0.999
DNMT1	DNMT3A	0.999	MEF2C	HDAC4	0.999
DNMT1	DNMT3B	0.999	COL4A1	COL4A2	0.997
NCOR2	BCL6	0.999	NCOR2	HDAC4	0.997
AKT1	CDKN1B	0.999	HDAC4	SIRT1	0.997
BCL2	TP53	0.999	CDKN2A	E2F1	0.993
AKT1	ERBB3	0.999	E2F1	SIRT1	0.992
BCL2	AKT1	0.998	SOCS3	EPOR	0.990
NCOR2	HDAC4	0.997	TGFB2	TGFBR3	0.990
TP53INP1	TP53	0.997	SOD3	SOD2	0.983
BMF	BCL2	0.996	SOD2	SIRT1	0.980
BBC3	BCL2	0.996	E2F2	E2F1	0.975
DNMT3B	DNMT3A	0.996	RAD51	MSH2	0.961
TP53	ARID3A	0.996	ICAM1	IL1B	0.960
NCOR2	AKT1	0.993	DNMT1	E2F1	0.959
TP53	BCL6	0.992	TGFB3	TGFBR3	0.957
DNMT1	TP53	0.985	TGFB2	TGFB3	0.956
PIK3CD	AKT1	0.984	DNMT1	SIRT1	0.954
IKZF3	BCL2	0.983	CDKN2A	E2F2	0.948
E2F2	E2F1	0.975	E2F1	CDC25A	0.939
AKT1	TP53	0.975	COL4A1	COL1A1	0.928
BBC3	TP53	0.975	NCOR2	MEF2C	0.928
ERBB2	TP53	0.974	COL4A2	COL1A1	0.919
BCL2	BCL6	0.961	E2F2	DNMT1	0.910
DNMT1	E2F1	0.959	IL1B	MMP13	0.909
DNMT1	AKT1	0.950	E2F2	MEF2C	0.902
SOD3	AKT1	0.947	E2F1	MEF2C	0.901
BCL2	CDKN1B	0.942	IKZF3	SOCS3	0.900
BBC3	BMF	0.942	SOCS3	IL1B	0.892

Supp. Table II. Continued

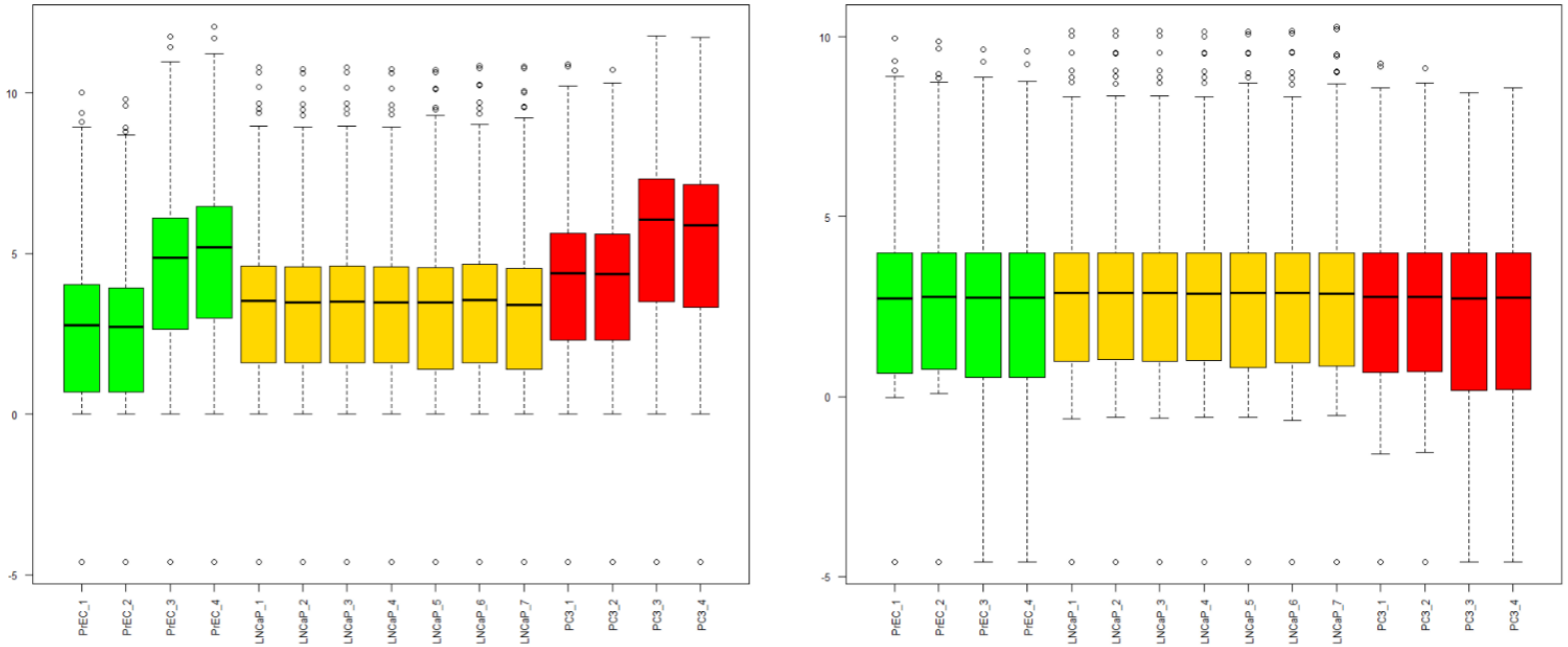
LNCaP			*PC-3*		
Node1	**Node2**	**Combined Score**	**Node1**	**Node2**	**Combined Score**
E2F2	CDKN1B	0.936	SOCS3	ICAM1	0.892
E2F1	AKT1	0.926	NCOR2	SIRT1	0.877
E2F1	CDKN1B	0.924	SMARCA4	E2F1	0.871
AKT1	SMO	0.922	ICAM1	SIRT1	0.854
AKT1	EPOR	0.912	DNMT1	SOCS3	0.842
PIK3CD	ERBB2	0.911	RAD51	SMARCA4	0.822
E2F2	DNMT1	0.910	IKZF3	SIRT1	0.816
BBC3	E2F1	0.900	EPOR	SIRT1	0.810
TP53	DNMT3A	0.897	E2F1	ICAM1	0.804
BMF	TP53	0.863	DNMT1	SMARCA4	0.795
HDAC4	BCL6	0.854	SOD2	MMP13	0.791
DUSP2	TP53	0.838	NCOR2	MMP13	0.788
TP53	CDKN1B	0.832	SMARCA4	HDAC4	0.786
ERBB2	LIN28A	0.831	CDKN2A	SMARCA4	0.761
TCEAL1	AKT1	0.804	NCOR2	SMARCA4	0.759
BCL2	ERBB2	0.778	CDKN1C	CDKN2A	0.756
E2F1	TP53	0.731	HMGA1	LIN28A	0.708
JMY	TP53	0.729	HDAC4	MSH2	0.701
IKZF3	ARNT2	0.691	MYBL1	E2F2	0.687
TP53	ERBB3	0.658	CDKN2A	DNMT1	0.658
ERBB2	CDKN1B	0.638	MEF2C	SIRT1	0.640
IKZF3	HDAC4	0.613	IKZF3	HDAC4	0.613
E2F2	AKT1	0.593	ZEB2	HDAC4	0.604
DNMT1	BMPR1B	0.576	CDKN2A	CDC25A	0.598
E2F1	ARID3A	0.576	E2F2	CDC25A	0.594
BCL2	ERBB3	0.574	MEF2C	BMPR1B	0.587
E2F2	ARID3A	0.570	AHRR	HDAC4	0.585
BCL2	HDAC4	0.569	CDKN2A	MSH2	0.582
PIK3CD	ERBB3	0.562	DNMT1	BMPR1B	0.576

Supp. Table II. Continued

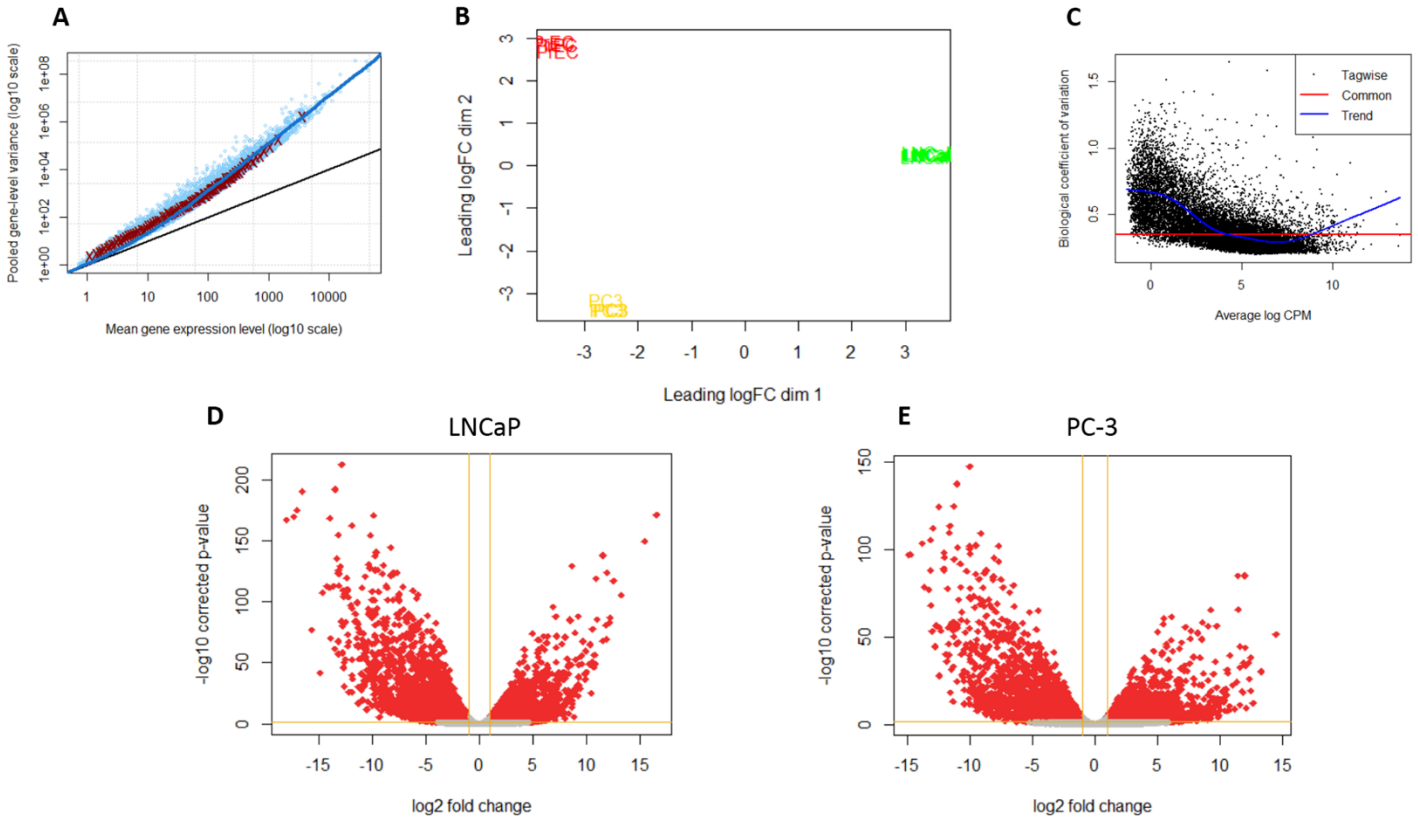
LNCaP			*PC-3*		
Node1	**Node2**	**Combined Score**	**Node1**	**Node2**	**Combined Score**
DNMT3B	LIN28A	0.550	NCOR2	HMGA1	0.574
E2F2	TP53	0.540	TGFB2	BMPR1B	0.569
DNMT3B	TP53	0.537	CDKN2A	DNMT3B	0.568
TP53	HDAC4	0.532	RAD51	SMOX	0.568
PIK3CD	BCL2	0.530	BMPR1B	TGFB3	0.554
AKT1	BCL6	0.517	DNMT3B	LIN28A	0.550
SMO	DVL2	0.517	MMP13	COL1A1	0.546
ERBB3	CDKN1B	0.511	PPIF	SIRT1	0.541
BCL2	EPOR	0.504	MYBL1	SMARCA4	0.533
RASGRP1	AKT1	0.499	MYBL1	NCOR2	0.514
TGFBR3	DVL2	0.495	CDKN2A	SIRT1	0.483
NCOR2	TP53	0.484	SOD2	PPIF	0.474
DNMT1	BCL2	0.472	TGFB2	IL1B	0.472
TP53	EPOR	0.455	SMARCA4	DNMT3B	0.469
TET1	DNMT3A	0.450	TGFB3	COL1A1	0.465
BCL6	CDKN1B	0.418	TGFB2	COL1A1	0.459
TP53	LIN28A	0.418	SMARCA4	SIRT1	0.452
AKT1	ERBB2	0.402	DNMT1	MSH2	0.444
TET1	DNMT1	0.400	NASP	MSH2	0.440
			CDKN1C	CDC25A	0.437
			MYBL1	E2F1	0.429
			DNMT3B	SIRT1	0.421
			COL4A2	PLAT	0.420
			CDKN1C	SMARCA4	0.420
			CDKN1C	DNMT1	0.412
			SMOX	MSH2	0.407
			NES	LIN28A	0.407
			MYBL1	CDC25A	0.405
			CDC25A	MSH2	0.403

Supp. Table II. Continued

LNCaP			*PC-3*		
Node1	**Node2**	**Combined Score**	**Node1**	**Node2**	**Combined Score**
			MYBL1	FOSL1	0.402
			TET1	DNMT1	0.400



Supp. Figure 1. Boxplot of normalized expression profiles. Original gene expression profiles are shown in the left figure. Green, yellow and red boxes represent the PrEC, LNCaP and PC-3 cells, respectively. Upper quartile normalized gene expression profiles by the three cell lines are shown in the right figure. The black line in the box represents the median of gene expression and can indicate the level of data standardization. The black lines of all samples almost at the same level, indicate that the data are well normalized.



(a) Scatter plot demonstrating the variance estimation among samples showing the gene-level variance plotted against the mean gene expression level. The black line show the variance implied by the Poisson distribution. The blue line is the variance estimate used by edgeR package. (b) The MDS plot of the data set. Samples are separated by the cell lineage (PrEC, LNCaP and PC-3). (c) Scatterplot of the biological coefficient of variation against the average abundance of each gene. The plot shows the square-root estimates of the common, trended and tagwise negative binomial dispersions. (d) and (e) Volcano plot of the differentially expressed genes between PrEC and LNCaP, and PrEC and PC-3, respectively. For more information, please see (Chen et al. 2016).