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CAIO CÉSAR DAMASCENO MONÇÃO

EFEITOS MOLECULARES E FUNCIONAIS DA INIBIÇÃO DE MELK EM LINHAGENS DE CÂNCER DE PRÓSTATA: ESTUDO *IN VITRO*

MOLECULAR AND FUNCTIONAL EFFECTS OF MELK INHIBITION IN PROSTATE CANCER CELL LINES: IN VITRO STUDY

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Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Mestre em Biologia Celular e Estrutural, na Área de Biologia Celular.

Dissertation presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Master in Cellular and Structural Biology, in the area of Cellular Biology.

Orientador: Sérgio Luis Felisbino

ESTE TRABALHO CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELO ALUNO CAIO CESAR DAMASCENO MONÇÃO E ORIENTADA PELO PROF. DR. SÉRGIO LUÍS FELISBINO.

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Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa de Pós-graduação em Biologia Celular e Estrutural do Instituto de Biologia.

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RESUMO

O câncer de próstata (CaP) é o segundo tipo de câncer mais incidente e o quinto em causa de mortes por câncer em homens no mundo. Mesmo com os avanços das estratégias terapêuticas empregadas no tratamento do CaP, faz-se necessário a busca de novos alvos moleculares que possam oferecer um tratamento mais personalizado e eficaz, principalmente nos casos avançados e metastático. Dentre as diversas proteínas que vem se destacando como possível alvo em tumores, a MELK (do inglês, Maternal Embryonic Leucine Zipper Kinase) apresenta-se super expressa em vários tipos de cânceres, incluindo CaP, e está relacionada com caráter proliferativo, metastático e de resistência aos tratamentos convencionais. Nesse sentido, nossa hipótese é de que MELK tenha papel relevante na agressividade de CaP avançado e que sua inibição possa contribuir para um tratamento mais efetivo. Assim, o presente trabalho teve como objetivo avaliar o efeito do silenciamento de MELK na radioterapia e na quimioterapia com Docetaxel, bem como investigar as novas vias de sinalização celular na qual MELK participa e cujo seu silenciamento afeta. Para isso, foi realizado silenciamento gênico de MELK em células de CaP DU-145, seguido de ensaios funcionais de viabilidade celular e capacidade clonogênica in vitro, com exposição à radioterapia ou ao quimioterápico Docetaxel. Também foram avaliadas as alterações moleculares causadas pelo silenciamento de MELK nas linhagens de CaP DU-145 e LNCaP, através de análise proteômica, seguido de enriquecimento de possíveis vias de sinalização. Foi observado que a inibição da expressão de MELK, sozinha ou associada à radioterapia e à quimioterapia com Docetaxel em células DU-145, diminui significativamente a formação de colônias e a viabilidade celular. A análise proteômica das células LNCaP e DU-145 com MELK silenciada enriqueceram importantes vias relacionadas com controle do ciclo celular, progressão e recidiva tumoral, e metástase, como AKT-mTOR, MYC, AR, AURKA/AURKB e SYK. Até então, a compreensão sobre o papel de MELK na fisiologia celular está ligada, principalmente, à via FOXM1 em tumores neurais, fazendo com que os resultados moleculares obtidos no presente estudo tragam maior clareza da atuação de MELK no CaP. Desta forma, MELK apresenta-se como potencial alvo terapêutico para o CaP, no contexto de terapias alvo-dirigidas.

ABSTRACT

Prostate cancer (PCa) is the second most common type of cancer and the fifth leading cause of cancer deaths in men worldwide. Even with advances in therapeutic strategies used in the treatment of PCa, it is necessary to search for new molecular targets that can offer a more personalized and effective treatment, especially in advanced and metastatic cases. Among the several proteins that have been highlighted as a possible target in tumors, MELK (Maternal Embryonic Leucine Zipper Kinase) is overexpressed in several types of cancers, including PCa, and is related to proliferative, metastatic and resistance to conventional treatments. In this sense, our hypothesis is that MELK plays a relevant role in the aggressiveness of advanced PCa and that its inhibition may contribute to a more effective treatment. Thus, the present work aimed to evaluate the effect of MELK silencing in radiotherapy and chemotherapy with Docetaxel, as well as to investigate new cell signaling pathways in which MELK participates and whose silencing affects. For this, *MELK* gene knockdown was performed in DU-145 PCa cells, followed by functional assays of cell viability and clonogenic capacity *in vitro*, with exposure to radiotherapy or chemotherapy Docetaxel. Molecular changes caused by *MELK* silencing in PCa DU-145 and LNCaP cell lines were also evaluated through proteomic analysis, followed by enrichment of possible signaling pathways. It was observed that the inhibition of *MELK* expression, alone or associated with radiotherapy and chemotherapy with Docetaxel in DU-145 cells, significantly decreases colony formation and cell viability. Proteomic analysis of LNCaP and DU-145 cells with silenced MELK enriched important pathways related to cell cycle control, tumor progression and recurrence, and metastasis, such as AKT-mTOR, MYC, AR, AURKA/AURKB and SYK. Until then, the understanding of the role of MELK in cell physiology is mainly linked to the FOXM1 pathway in neural tumors, making the molecular results obtained in the present study bring greater clarity to the role of MELK in PCa. In this way, MELK presents itself as a potential therapeutic target for PCa, in the context of targeted therapies.

SUMÁRIO

1. INTRODUÇÃO

1.1. Câncer de Próstata

O câncer de próstata (CaP) tem sido altamente relatado pela sua alta incidência em homens, principalmente em países do ocidente. Em nível mundial, o CaP é o segundo tipo de câncer mais frequente[1] e o quinto em causa de mortes por câncer em homens[2], com, aproximadamente, 1,4 milhão de casos e 375 mil mortes em 2020[2]. No Brasil, 65 mil novos casos de CaP foram notificados em 2020, correspondendo a 29.2% do total de casos[3].

O aumento da incidência de CaP em escala mundial tem relação com, dentre outros fatores, o aumento da expectativa de vida, bem como de ações preventivas, de diagnóstico precoce e das práticas de rastreamento, o que reflete a maior incidência de casos em países desenvolvidos[4]. Nesse sentido, a medição da concentração plasmática de PSA (do inglês, *prostate-specific antigen*) é o teste de rastreamento e monitoramento do CaP mais utilizado atualmente. O PSA é uma serina-protease sintetizada pelas células do epitélio prostático e liberado no lúmen glandular em condições anátomo-fisiológicas normais. Todavia, na presença de CaP ou outros distúrbios prostáticos, ocorre remodelação da estrutura glandular, acarretando em aumento de PSA produzido e secretado no sangue, podendo ser detectado em altas concentrações[5].

Largamente utilizado na prática clínica, os primeiros métodos de testagem por PSA apresentam limitações, como frequentes falsos-negativos e falsos-positivos[6], levando a realização de biópsias desnecessárias[7]. Tais incongruências vêm sendo corrigidas com o desenvolvimento de novos biossensores capazes de detectar com maior precisão os níveis de PSA[8]. Em busca de outros biomarcadores não-baseados no PSA, o mais promissor teste indicativo de biópsia é o antígeno de câncer de próstata 3 (PCA3 - do inglês *prostate cancer antigen 3*), um RNA não-codificante longo no qual apresenta-se elevado em >90% dos tecidos de CaP, mas não no tecido normal ou na hiperplasia prostática benigna (HPB)[6,9,10]. Além do PCA3, MALAT-1 (do inglês *metastasis-associated lung adenocarcinoma transcript 1*) é um outro biomarcador promissor no diagnóstico e prognóstico do CaP, já que seus níveis aparentam aumentar com a evolução tumoral[6].

Mesmo com o avanço dos métodos de detecção e vigilância, o desenvolvimento e progressão do CaP ocorre, na maioria dos casos, de forma lenta, localizado no interior do órgão e assintomático, dificultando o diagnóstico precoce[6]. Após a realização de exames não invasivos, como PSA, junto com toque retal ou exame retal digital e confirmada a suspeita de CaP, são realizadas biópsias, seguida de análise histopatológica para o estadiamento do tumor de acordo com o padrão de Gleason[1]. O sistema de Gleason classifica o estadiamento histopatológico do tumor de 1 (bem diferenciado) a 5 (mal diferenciado), onde os dois padrões mais frequentes observados nessa escala são somados, estabelecendo o *score* de Gleason, variando de 2-10 [1,11]. Os *Scores* de Gleason 7, 8, 9 e 10 estão associados com a doença mais agressiva e com pior prognóstico de cura, enquanto os *Scores* de Gleason $\leqslant$ 6 são considerados de melhor prognóstico[1,12].

A progressão entre o CaP pré-clínico até a doença clínica ocorre através de uma sequência de estágios (figura 1), tendo início como uma neoplasia intraepitelial prostática (do inglês, *prostatic intraepithelial neoplasia*)[13], evoluíndo para adenocarcinoma micro-invasivo, adenocarcinoma invasivo e, posteriormente, para CaP metastático[13,14]. Na progressão do CaP, existe ainda dois sub-estágios da doença entre a doença localizada e a metastática, e são descritos como: CSPC (do inglês, *castration-sensitive prostate cancer*) e CRPC (do inglês, *castration-resistant prostate cancer*)[15]. No estágio inicial de adenocarcinoma, o CaP é classificado como CSPC por apresentar vias de AR (do inglês, *androgen receptor*) ativas e sensibilidade aos tratamentos antiandrogênicos, característica molecular que é perdida conforme a doença avança em direção ao estágio metastático, onde o CaP passa a ser classificado como CRPC[1,15].

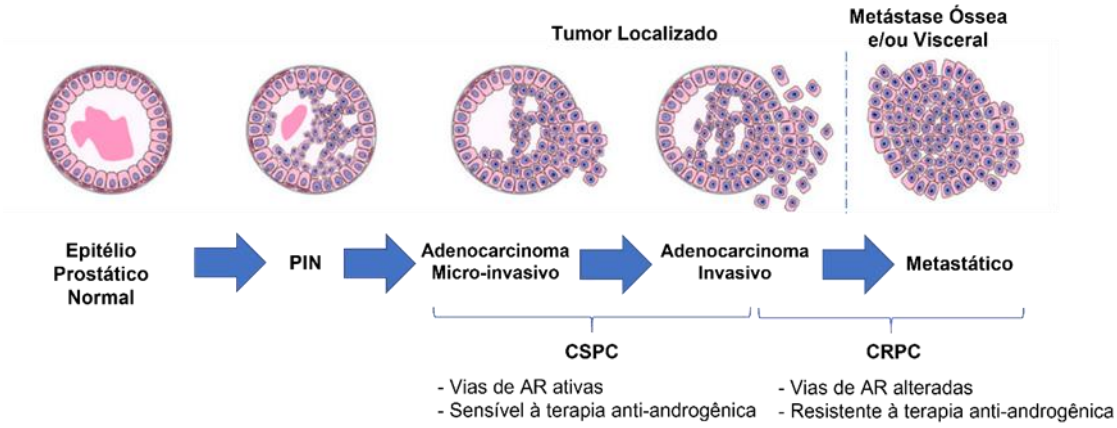

Figura 1: Desenvolvimento do CaP. Esquema representativo dos estágios de iniciação e progressão tumoral no CaP, bem como os subestágios CSPC e CRPC e suas características clínicas. Adaptado de Sekhoacha et al[14]

Hormônios androgênicos, fundamentais no desenvolvimento normal da próstata, atuam também na iniciação do CaP ao se ligarem com AR citoplasmático[16,17], provocando sua dimerização e posterior translocação para o núcleo, onde regula a transcrição de genes alvos[17]. Atualmente, prostatectomia e radioterapia são os tratamentos padrões nos estágios iniciais do CaP, e a terapia de privação androgênica é utilizada a partir dos estágios intermediários (grau III). Entretanto, a privação androgênica está normalmente associada com resistência a castração e recidiva do CaP, devido a mutações genômicas de AR[14].

O CaP recidivante é denominado CaP “resistente à castração”, pois o tumor permanece dependente da função do AR por vias de sinalização não canônicas[17,18]. As vias não-canônicas de sinalização de andrógeno são classificadas em dois tipos: via não genômica e via ativada sem andrógenos, sendo que ambas resultam em aumento da proliferação e sobrevivência celular, da migração e inibição da apoptose [14,17]. Na via ativada sem andrógenos, ocorre participação de AR ativado por fatores de crescimento, como EGFR, PI3K/AKT e PKA, e citocinas, como receptores de interleucina-6 (IL-6)[17].

Na evolução da doença, em cerca de 30% dos casos que apresentam recorrência após prostatectomia e terapia antiandrogênica, é comum a presença de células com fenótipo neuroendócrino no tecido tumoral[19–21], sendo a variante mais prevalente nos CRPCs e nos tumores prostáticos metastáticos[22]. Essa alteração

fenotípica é evidenciada pela perda de características de adenocarcinoma típico e presença de marcadores neuroendócrinos, como cromogranina A, sinaptofisina e CD56[21], bem como a frequente perda da expressão de AR nestas células, evadindo as pressões terapêuticas impostas pelos tratamentos convencionais[22].

Fundamentado nas características fisiopatológicas, hormonais e genéticas, o CaP é de grande complexidade, principalmente no que diz respeito à heterogeneidade histológica e à diversidade de fatores envolvidos no desenvolvimento inicial e na progressão tumoral[23,24]. Esse contexto fica evidenciado pelo fato de, em um caso de CaP ou até dentro de um mesmo tumor, ser frequente a observação de diferentes alterações genéticas e epigenéticas, o que dificulta a determinação de marcadores de prognóstico e tratamento adequado[25].

Apesar dos avanços de detecção e terapêuticos, é desafiador detectar pacientes em estágios iniciais de CaP, bem como estabelecer terapias mais efetivas, principalmente para quadros avançados, como no CRPC [26], onde os tratamentos não são curativos e prolongam a vida dos pacientes por 30 meses em média[27]. Neste sentido, a identificação de novos marcadores e alvos relacionados à progressão do CaP é fundamental, possibilitando melhor direcionamento das abordagens terapêuticas para os pacientes, bem como o desenvolvimento de novas estratégias de tratamento, trazendo respostas clínicas superiores dentro da medicina personalizada. Assim, diversas proteínas de interesse estão sendo investigadas, dentre elas MELK.

1.2. MELK e o câncer de próstata

MELK (do inglês, *maternal embryonic leucine zipper kinase*) é uma proteína serina/treonina quinase pertencente à família snf1/AMPK, altamente conservada entre diversas espécies. Apresenta importante papel na proliferação e na regulação do ciclo celular, na apoptose, na proliferação de células-tronco e na organogênese[28–30]. Do ponto de vista molecular, em condição tecidual normal, MELK é ativada através da ligação com a proteína ZPR9 (do inglês, *zinc-finger-like protein*) após estímulos de estresse, acarretando na ativação de vias apoptóticas e de bloqueio do ciclo celular[31]

De forma contrastante, no contexto do câncer, MELK está relacionada com a iniciação e a progressão de diversos tumores, estando o aumento da sua expressão associado ao pior prognóstico de pacientes com câncer de mama, cólon, pâncreas, ovário, cérebro, próstata, carcinoma endometrial, neuroblastoma, linfoma não-Hodgkin, mieloma e leucemia[30,32–37]. Além disso, o aumento da expressão de MELK também está envolvido na transformação maligna de células-tronco normais em células-tronco tumorais[29], no aumento da capacidade de invasão celular e de metástase[38], bem como a sua relevância no CRPC e seu potencial como alvo terapêutico para esse tipo de tumor[39].

Na natureza dos tumores, a elevada expressão de MELK é descrita pela sinalização de vias de progressão tumoral e de bloqueio da morte celular [31]. Além da participação da proteína ZPR9, a regulação da atividade de MELK ocorre pela interação com proteínas JNKs (do inglês, *c-Jun NH(2)-terminal Kinases*), pertencentes a família MAPK (do inglês, *Mitogen Activated Protein Kinases*), bem como por mecanismos de autofosforilação[28,29].

Devido a estas características, tal proteína tem se tornado um interessante alvo terapêutico para a compreensão da tumorigênese e da formulação de novas drogas anti-tumorais que sejam capazes de inibir sua atividade[38]. Entretanto, a via de MELK está mais bem descrita em tumores de origem neural, um pouco em tumores mamários e limitada em tumores prostáticos. Em estudos recentes, foi observado que MELK atua na fosforilação e ativação do fator de transcrição FOXM1 (do inglês, *Forkhead box M1*) através da formação de um complexo proteico[40,41]. Por sua vez, o trabalho de Gouazé-Andersson et al.[42] aponta a via FGFR1/FOXM1/MELK como crucial na regulação da radioresistência em células-tronco de glioblastoma. Além disso, a atividade de FOXM1 associada a atividade de MELK mostra-se como mediador central na regulação positiva de fatores envolvidos na plasticidade celular em gliomas, glioblastomas e meduloblastomas[28,43–45]. Atualmente, a plasticidade celular vem sendo cada vez mais descrita em diferentes tipos de tumores sólidos e associada à resistência destes às terapias convencionais, incluindo no CRPC, onde é descrita por estabelecer características neuroendócricas nas células tumorais[22].

2. JUSTIFICATIVA

Mesmo com os avanços terapêuticos, o CaP é uma das principais causas de morte dentre os homens, com destaque para os casos avançados da doença, com a presença do câncer resistente a castração com ou sem metástase. Nesse contexto, é de grande relevância a busca por novos alvos terapêuticos que possam elucidar os mecanismos de resistência aos tratamentos convencionais, bem como contribuir para o desenvolvimento de novas estratégias de tratamentos primários e de terapias adjuvantes. O papel de MELK na progressão e transformação tumoral é destacado em diversos trabalhos, através da diminuição da apoptose, migração e metástase, plasticidade celular e radio- e químioresistência em diferentes tipos de cânceres, incluindo o CaP resistente a castração. Deste modo, a hipótese deste estudo é que MELK tem função relevante na progressão do CaP resistente a castração e que sua inibição pode contribuir, como terapia adjuvante, para os tratamentos dos estágios avançados da doença. Para isso, é fundamental elucidar as principais vias de sinalização onde MELK atua no CaP e caracterizar sua relação com outros fatores importantes na biologia do câncer.

3. OBJETIVOS DO ESTUDO

3.1. Objetivo Geral

O objetivo deste projeto é avaliar os efeitos da inibição de MELK na eficácia de radio- e quimioterapia convencionais utilizadas no CaP, bem como caracterizar vias de sinalização com participação de MELK e sua relação com fatores envolvidos no desenvolvimento tumoral.

3.2. Objetivos Específicos

- Analisar o efeito do silenciamento de *MELK* na viabilidade celular e na capacidade clonogênica da linhagem celular de CaP DU-145, quando exposta à quimioterapia.
- Avaliar o efeito do silenciamento de *MELK* na capacidade clonogênica da linhagem celular de CaP DU-145, quando exposta à radioterapia.
- Descrever as proteínas com expressão alterada após silenciamento de *MELK* nas linhagens celulares de CaP LNCaP e DU-145.

- Elucidar as principais vias e/ou fatores relacionados com MELK nas linhagens de CaP LNCaP e DU-145.

4. RESULTADOS E DISCUSSÃO

Os resultados obtidos, bem como as respectivas metodologias e discussão, estão apresentados em formato de artigo no subtópico “Manuscrito” a seguir:

4.1. Manuscrito:

MELK silencing alters molecular pathways that impact survival and tumorigenic capacity of prostate cancer cells *in vitro*

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ABSTRACT

Prostate cancer (PCa) is the second most common type of cancer and the fifth leading cause of cancer deaths in men worldwide. Even with advances in therapeutic strategies used in the treatment of PCa, it is necessary to search for new molecular targets that can offer a more personalized and effective treatment, especially in advanced and metastatic cases. Among the several proteins that have been highlighted as a possible target in tumors, MELK (Maternal Embryonic Leucine Zipper Kinase) is overexpressed in several types of cancers, including PCa, and is related to proliferative, metastatic and resistance to conventional treatments. In this sense, the aim of this work is to evaluate the effect of *MELK* silencing in radiotherapy and chemotherapy with Docetaxel, as well as to investigate new cell signaling pathways in which MELK participates and whose silencing affects. For this, *MELK* gene knockdown was performed in DU-145 PCa cells, followed by functional assays of cell viability and clonogenic capacity in vitro, with exposure to radiotherapy or chemotherapy with Docetaxel. Molecular changes caused by *MELK* silencing in PCa DU-145 and LNCaP cell lines were also evaluated through proteomic analysis, followed by enrichment of possible signaling pathways. It was observed that the inhibition of *MELK* expression, alone or associated with radiotherapy and chemotherapy with Docetaxel in DU-145 cells, significantly decreases colony formation and cell viability. Proteomic analysis of LNCaP and DU-145 cells with *MELK* knockdown enriched important pathways related to cell cycle control, tumor progression and recurrence, and metastasis, such as AKT-mTOR, MYC, AR, AURKA/AURKB and SYK. Until then, the understanding of the role of MELK in cell physiology is mainly linked to the FOXM1 pathway in neural tumors, making the molecular results obtained in the present study bring greater clarity to the role of MELK in PCa. In this way, MELK presents itself as a potential therapeutic target for PCa in the context of targeted therapies.

INTRODUCTION

Prostate cancer (PCa) is the second type that most affects the male population worldwide[1] and the fifth type that most kills men when the cause is cancer[2]. In 2020, approximately 1,4 million new cases and 375.000 deaths were reported[2]. Circulating androgens are essential in the normal development of the prostate, as well as in the initial development of prostate cancer through the interactions with cytoplasmic androgen receptor (AR)[3,4]. Currently, prostatectomy and radiotherapy are the standard treatments in the early stages of PC, in addition to androgen deprivation therapy from the intermediate stages (grade III)[5]. However, androgen deprivation is associated with castration resistance and prostate cancer recurrence, due to AR genomic mutations[5], including activation of non-canonical androgen signaling pathways that result in increased proliferation and cell survival, migration and inhibition of apoptosis[4,5].

In the course of the disease the presence of cells with a neuroendocrine phenotype in the tumor tissue is associated with cancer recurrence after prostatectomy and antiandrogen therapy in 30% of the cases[6–8]. The cells with neuroendocrine phenotype are the most prevalent histological variant in castration-resistant prostate cancer (CRPC) and metastatic prostate tumors[9]. One of the most important alterations is the frequent loss of AR expression, which impairs the therapeutic strategy imposed by conventional treatments[9]. Thus, the identification of new markers and targets related to the progression of prostate cancer is essential to the development of new treatment strategies.

Maternal Embryonic Leucine Zipper Kinase (MELK) is a protein that belongs to the snf1/AMPK family, which, under normal tissue conditions, acts on cell cycle regulation, apoptosis and stem cell proliferation[10–12]. In cancer, MELK has been related to the initiation and progression of several tumors, and its high expression is related to poor prognosis in patients diagnosed with breast, colon, pancreas, ovarian, brain, prostate and other cancers[12–18]. In addition, increased MELK expression is also involved in malignant transformation[11], cell invasion and metastatic capacity of stem cells[19]. In the context of PCa, MELK has relevance as a potential therapeutic target for more advanced stages of the disease, including CRPC[20].

Due to these characteristics, MELK has become an interesting target of study, both for the understanding of its participation in tumorigenic processes and for the development of new antitumor drugs capable of inhibiting its activity[19]. MELK acts on the activation of FOXM1 (Forkhead Box M1), a fundamental transcription factor in tumor maintenance and evolution, a pathway that is much more elucidated in cancers of neural origin[10,21–23] than in prostatic tumors.

Therefore, the aim of the present study was to evaluate the effects of MELK inhibition on the effectiveness of conventional radio- and chemotherapy used in PCa, as well as to characterize signaling pathways with MELK participation and its relationship with factors involved in tumor development.

MATERIAL AND METHODS

1. Cell Culture

LNCaP and DU-145 cells were obtained from American Type Cell Culture. LNCaP was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 50 µg/ml penicillin, 50 µg/ml streptomycin and 0.5 µg/ml amphotericin B (pH 7.2-7.4). DU-145 was cultured in DMEN medium supplemented with 10% fetal bovine serum, 50 µg/ml penicillin, 50 µg/ml streptomycin and 0.5 µg/ml amphotericin B (pH 7.2-7.4). All cells were kept in an 5% CO2 incubator with a humid atmosphere at 37ºC.

2. Silencing of MELK gene expression by siRNA

LNCaP and DU145 cells were plated in 6-well plates using RPMI and DMEM medium, respectively, supplemented with fetal bovine serum. The following steps were carried out with corresponding medium from each cell line supplemented with 0.5% fetal bovine serum. The cell transfection protocol was performed using the Lipofectamine® RNAiMAX Transfection Reagent and Silencer® Select Pre-designed siRNA kit against MELK, according to the manufacturer's instructions. Negative controls were performed using Silencer® Select Negative control from Invitrogen® (siRNA scramble) and lipofectamine alone. For silencing, 30 nmol of siRNA-MELK was standardized. Cells were incubated for 48 hours for transfection and after this period they were used for different experiments.

3. mRNA and RT-PCR

Total RNAs from cultured cells were extracted using the RNeasy mini kit extraction kit (QIAGEN, Crawley, UK) according to the manufacturer's protocol. Quantification of extracted total RNA was performed in Nanovue (GE, USA) and based on optical density (OD) with absorbance readings at 260nm and spectrophotometric ratios at 260 and 280 nm. cDNA was synthesized using the High Capacity® RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). RT-PCR reactions were performed in triplicate for the MELK gene in QuantStudio 12K flex (Applied Biosystems). The relative quantification of each gene was performed using the $2^{-\Delta\Delta CT}$ method[24]. The values obtained for all samples were normalized by the ratio obtained between the informative gene and the reference gene of the beta-actin protein.

4. Drug and treatments

4.1. Docetaxel

Docetaxel was purchased from TargetMol, Boston, USA, dissolved at 5 mM in DMSO and stored at -20°C until use.

4.2. Radiotherapy

Treatments with ionizing radiation were carried out in the facilities of the Radiotherapy sector of the HCFMB/UNESP-Botucatu, in a CGR/MEV telecobalt therapy unit - model Alcyon-II. The 60Co beam energy of the device is 1.25 MeV. The dose applied to each sample was calculated from the irradiation times and the dose rate with a value of 0.98 cGy/s. The field size adopted was 30 cm² and the dose-isocenter distance was 80 cm. To prepare the calculations and correction factors for the radiometric parameters, the IAEA Dosimetry Protocol (TRS-398) [25] was used. Also, to confirm the calculation and dosimetric verification, absorbed radiation measurements were performed using an ionization chamber model PTW – TN 30013 series 006901 and a Sun Nuclear electrometer, model PC Electrometer, provided by the company Nucleata Radiometria from Araçatuba, SP, Brazil.

5. Cell viability assay by Prestoblue©

5.1. Silencing of MELK and treatment with Docetaxel

$4x10^5$ DU-145 cells were plated per well in 6-well plates. After 48 hours, the wells were divided into three treatment groups: one group of cells treated with siRNA-MELK, another group was exposed only to lipofectamine and the third group received only culture medium, and the treatment followed the silencing protocol described in item 2 of Material and Methods. After 48 hours, the cells were trypsinized, re-plated in a 96-well plate at a density of $8 x10^3$ cells/well and kept in culture for 30 hours to ensure total cell adhesion. At this point, the culture medium was changed and the cells treated with Docetaxel (4, 8 and 16 nM). After 40 hours, the medium was removed, and 200 µl of Prestoblue diluted in medium was added to each well, as recommended by the manufacturer. After 2h of incubation at 37ºC, the microplate was read with fluorescence excitation/emission in 560-590 nm to determine the cell viability indices. The experiment was carried out in triplicate.

6. Clonogenic Survival

The clonogenic assay is based on the ability of a cell to form colonies, defining these as groups of more than 50 cells. This test serves to assess the reproductive capacity of cells after treatments[26]. For the clonogenic assay, $4x10^5$ DU-145 cells were plated per well in 6-well plates. After 48 hours, the wells were divided into three treatment groups: one group of cells treated with siRNA-MELK, another group only with lipofectamine and the third group only with culture medium, and the treatment followed the silencing protocol described in item 2 of the methodology. After 48 hours, cells were trypsinized and re-plated, in duplicate, at a confluence of 2000 cells/well in a 6-well plate. After 30 hours, the cells were subjected to doses of ionizing radiation or Docetaxel and kept incubated at 37°C for 10 days. After this period, the colonies formed were fixed with methanol and stained with a 3% Giemsa solution. Colonies were counted with the aid of a magnifying glass, only those with at least 50 cells being considered. From the colony count, the Placing Efficiency and the Survival Fraction (SF) were calculated, expressed as a percentage, following the protocol defined by Franken and collaborators[26].

6.1. Radiotherapy Doses in the Clonogenic Assay

In the clonogenic assay, exposures with ionizing radiation were carried out at 4 different doses (0 Gy, 2 Gy, 4 Gy and 6Gy) in the same apparatus, as described in item 4.2. of Materials and Methods.

6.2. Docetaxel Doses in the Clonogenic Assay

Treatment with Docetaxel in the clonogenic survival assay was carried out with doses of 0.25 and 0.5 nM of Docetaxel, as well as controls with only the DMSO vehicle and culture medium.

7. Statistical Analysis of Functional Assay

The results obtained in the functional tests are presented as mean and standard deviation. Significant difference analyzes were performed using one-way analysis of variance (ANOVA), followed by Bonferroni's post-test, and Student's t test. All analyzes were analyzed using GraphPad 6.0 software (San Diego, CA) with a significance level of $p<0.05$.

8. Proteomic analysis by ShotGun after MELK silencing

8.1. MELK silencing and protein extraction

For Proteomic Analysis, $4x10^5$ DU-145 and LNCaP cells were plated in 6-well plates. After 48 hours, the wells were divided into three treatment groups: one group of cells treated with siRNA-MELK, another group was exposed only to lipofectamine and the third group received only culture medium, and the treatment followed the silencing protocol described in item 2 of Material and Methods. After silencing, cells were collected and protein extraction was performed using 200µL RIPA® lysis buffer (Sigma Aldrich Co., Saint Louis, MO, USA) with protease and phosphatase inhibitors according to manufacturer's guidance. Protein concentration was determined by Bradford's method[27].

8.2. Enzymatic digestion of proteins.

Protein digestion was performed according to Dias and collaborators[28]. Fifty micrograms of total proteins were diluted in 50 mM ammonium bicarbonate and RapiGest SF surfactant (code 186001861, Waters) according to manufactur's protocol. Then, the samples were submitted to reduction and alkylation steps, using 10 mM DTT and 45 mM iodoacetamide, respectively. Enzymatic digestion in solution was performed with trypsin 1:50 solubilized in an ammonium bicarbonate buffer, followed by hydrolysis for 18h at 37ºC. After this period, the samples were centrifuged and the tryptic digests contained in the samples were submitted to Peptide Cleanup C18 Spin (Agilent Technologies) desalination columns, according to the manufacturer's recommendations.

8.3. Mass spectrometry analysis

Mass spectrometry analysis was performed using the Ultimate 3000 LC liquid nanochromatography equipment (Dionex, Germering, Germany) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) according to Cavalcante and collaborators[29]. The mobile phases used were formic acid in LCMS water and formic acid in acetonitrile. The peptides were loaded onto a C18 pre-column, 30µm x 5mm (ThermoFisher Scientific) and desalted in an isocratic gradient. Then, the peptides were fractionated by Reprosil-Pur C18-AQ (PICOCHIP) analytical column using linear gradient. Ionization was obtained using Nanospray ion source (PICOCHIP). The operating mode was positive ionization using the Data Dependent Acquisition (DDA) method. MS spectra were acquired from m/z 200 to m/z 2000, resolution of 70,000 and 100 ms injection time. The fragmentation chamber was conditioned with collision energy between 29 to 35% with a resolution of 17,500, 50 ms injection time, 4.0 m/z isolation window and dynamic exclusion of 10 s.

8.4. Data analysis

Raw MS files were obtained by Thermo Xcalibur software (version 4.0.27.19, ThermoFisher Scientific) and submitted to PatternLab for Proteomics v4 for protein identification and quantification[30]. Spectrum counts were analyzed in pairs and the fold change values (Log2FC) were calculated using T-test using MetaboAnalyst[31]. Common proteins to all groups were analyzed by discriminant analysis by partial least

squares analysis (PLS-DA) and Variable Importance Projection (VIP) Score using MetaboAnalyst software to identify the proteins responsible for the difference among the samples[31].

RESULTS

RNAi-MELK promotes MELK gene silencing in PCa cell lines.

In order to analyze the relationship of MELK in molecular pathways and in tumor development processes, mRNA silencing was performed using specific siRNA against MELK (si-MELK) in PCa cell lines. Gene expression results indicated significant (*p<0.05) MELK silencing in cells exposed to si-MELK when compared to control-lipo after 48 hours of treatment initiation. Furthermore, the maintenance of silencing was observed 30 hours after the end of the si-MELK treatment.

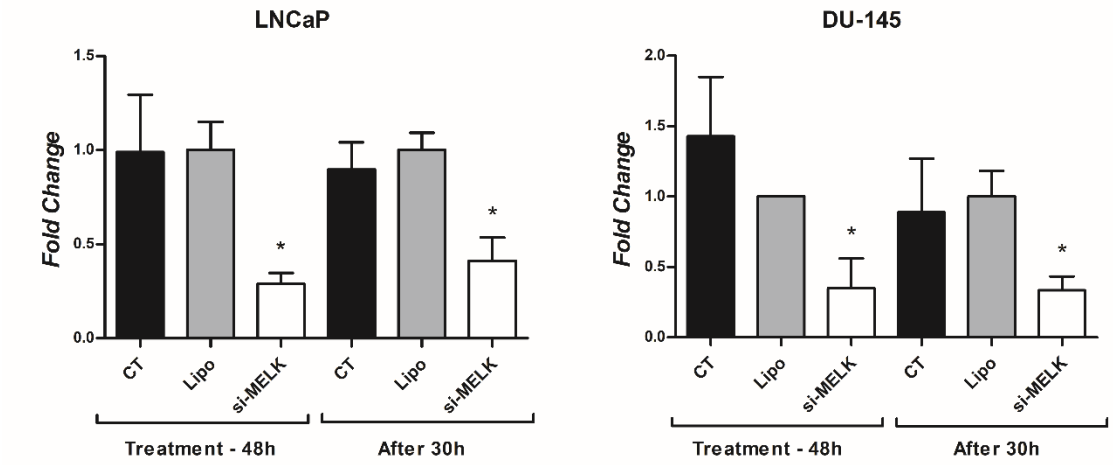

Figure 1: MELK gene expression after siRNA-MELK treatment in CaP cells. MELK expression levels were quantified, by qRT-PCR, after 48h treatment with siRNA-MELK (48h treatment) and 30h after the end of the treatment (After 30h treatment), in LNCaP and DU-145 tumor lines. CT: experimental control cells; Lipo: treatment control cells; siMELK: treated cells with siRNA against MELK. Expression values are given in fold change decimal scale. *p<0.05, in relation to lipofectamine control (Lipo).

MELK silencing reduces clonogenicity but does not favor the chemotherapeutic effect in PCa cell lines.

From the clonogenic assay, it was possible to evaluate the tumorigenic capacity of DU-145 cells with silenced MELK after exposure to chemotherapy with Docetaxel. It was observed the effect of decreasing MELK levels in combination with chemotherapy on cell colony formation, as shown in Figure 2A. The number of colonies formed was used to calculate the specific SF for each treatment, expressed in the graph of Figure 2B. MELK silencing, alone or combined with Docetaxel, significantly reduced cell clonogenicity when compared to the control group ($p < 0.05$). In the DMSO group, si-MELK cells showed 35% reduction compared to MELK positive (Lipo) cells. In the 0.5 nM Docetaxel group, MELK positive cells showed a 36% reduction in colonies when compared to the Lipo+DMSO control cell group (MELK positive and without chemotherapy). Meanwhile, si-MELK cells exposed to 0.5 nM Docetaxel had 57% fewer colonies formed, showing a sum effect of MELK silencing and chemotherapy on PCa cells.

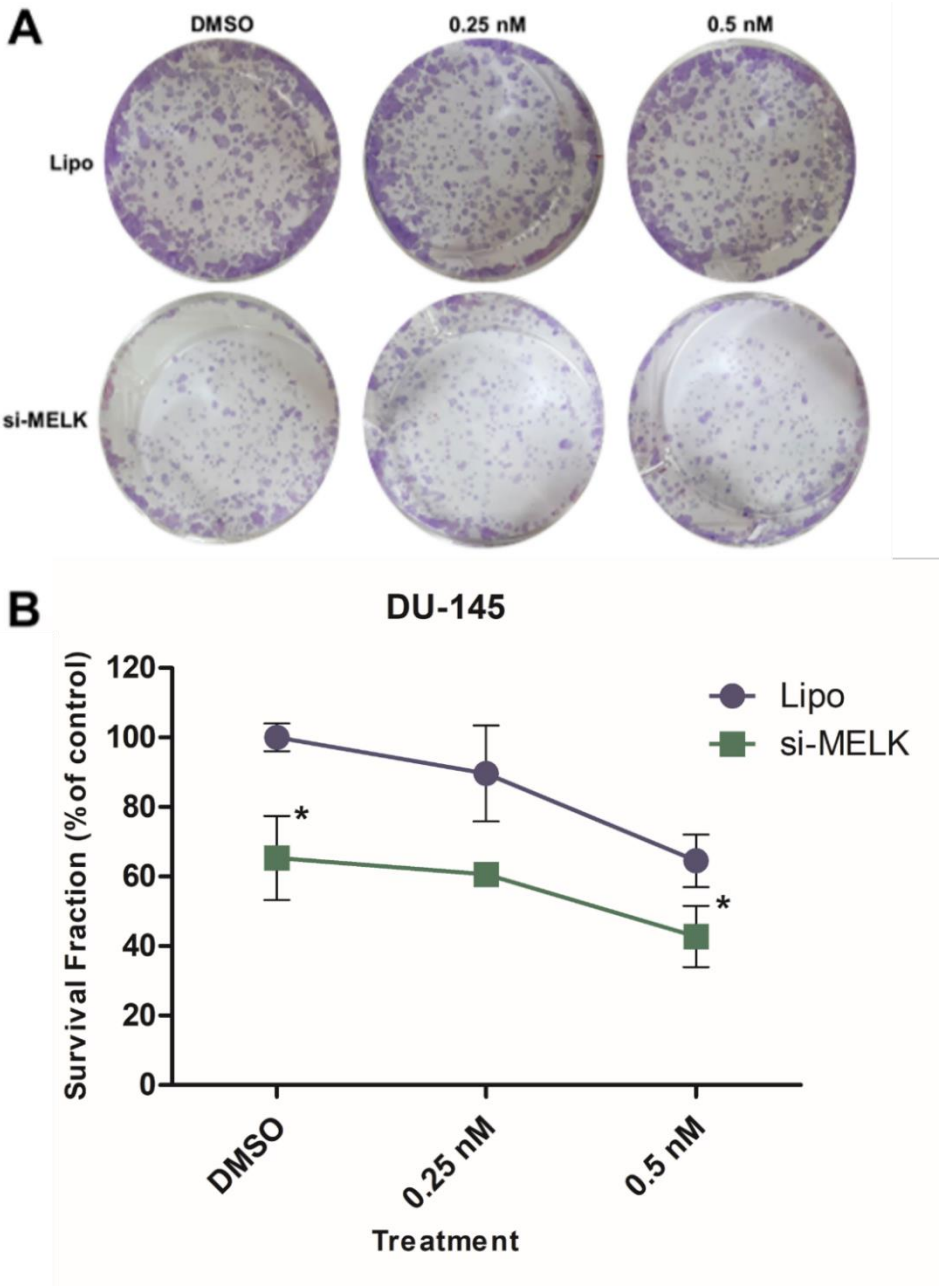

Figure 2. MELK silencing reduces clonogenicity but does not favor the chemotherapeutic effect in PCa cell lines. DU-145 cells were incubated with si-MELK for 48h previously and treated with Docetaxel during 40h. After the treatment, the cells were kept incubated for 10 days to colony formation. Cells with lipofectamine (Lipo) alone are controls for MELK silencing, while the group treated only with DMSO serves as control for treatment with Docetaxel. The colonies formed were normalized to the plating efficiency (FP) and the survival fraction obtained was compared to the control DMSO + Lipo. Comparison between Lipo and si-MELK within the Docetaxel dose subgroups was also performed. *$p<0.05$ indicates a significant difference between Lipo and si-MELK cells within each Docetaxel subgroup alone.

MELK silencing reduces PCa cell viability but does not favor the effect of chemotherapy drug Docetaxel in vitro.

To evaluate the effect of MELK silencing in PCa DU-145 cells, as well as the response of these cells to the chemotherapy drug Docetaxel in a condition without MELK, cell viability assay was performed. The results indicate that MELK silencing per se reduces cell viability between 28 and 32% ($p<0.05$), approximately, compared to MELK positive cells. Furthermore, when combining MELK silencing with Docetaxel chemotherapy treatment, it is possible to observe an increase in the effect of the chemotherapy in reducing the viability of DU-145 cells, but without statistical significance ($p<0.05$) when compared to the DU-145 MELK group positive (figure 3).

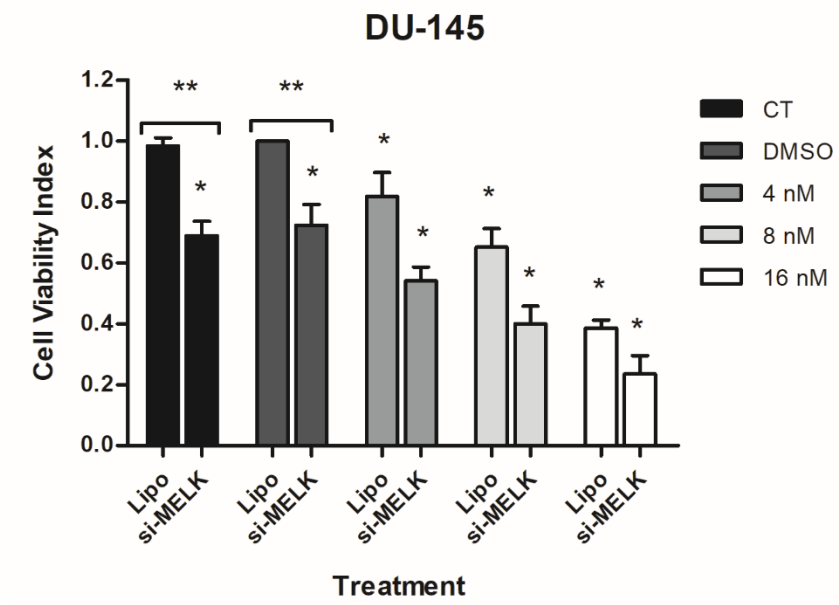

Figure 3. MELK silencing reduces viability of DU-145 PCa cells. Analysis of the cell viability index after treatment of DU-145 cell line with increasing concentrations of Docetaxel (0-16nM), for 40h, with (Lipo) or without (si) MELK expression. Proliferation was quantified by the Prestoblue© method. The plot shows the mean ± standard deviation of three independent experiments. * $p<0.05$ indicates a significant difference between the treated cells and the DMSO+Lipo control. **$p<0.05$ indicates a significant difference between Lipo cells and si-MELK cells within each Docetaxel subgroup alone.

MELK silencing reduces colony formation but does not favor the effect of radiotherapy on PCa cells in vitro.

Through clonogenic assay, it was observed the effect of decreasing MELK levels in combination with radiotherapy on the colony formation, as shown in Figure 4A. SF calculated from the number of colonies are shown in the graph in Figure 4B. When comparing the silenced cells (siMELK) with control cells (Lipo), within each radiotherapy treatment group, it was observed, at 2 and 4 Gy doses ($p < 0.05$), a smaller number of colonies formed in DU-145 cells with silenced MELK. Still, MELK silencing did not potentiate radiotherapy, however, a sum effect of si-MELK and therapy on colony formation was observed.

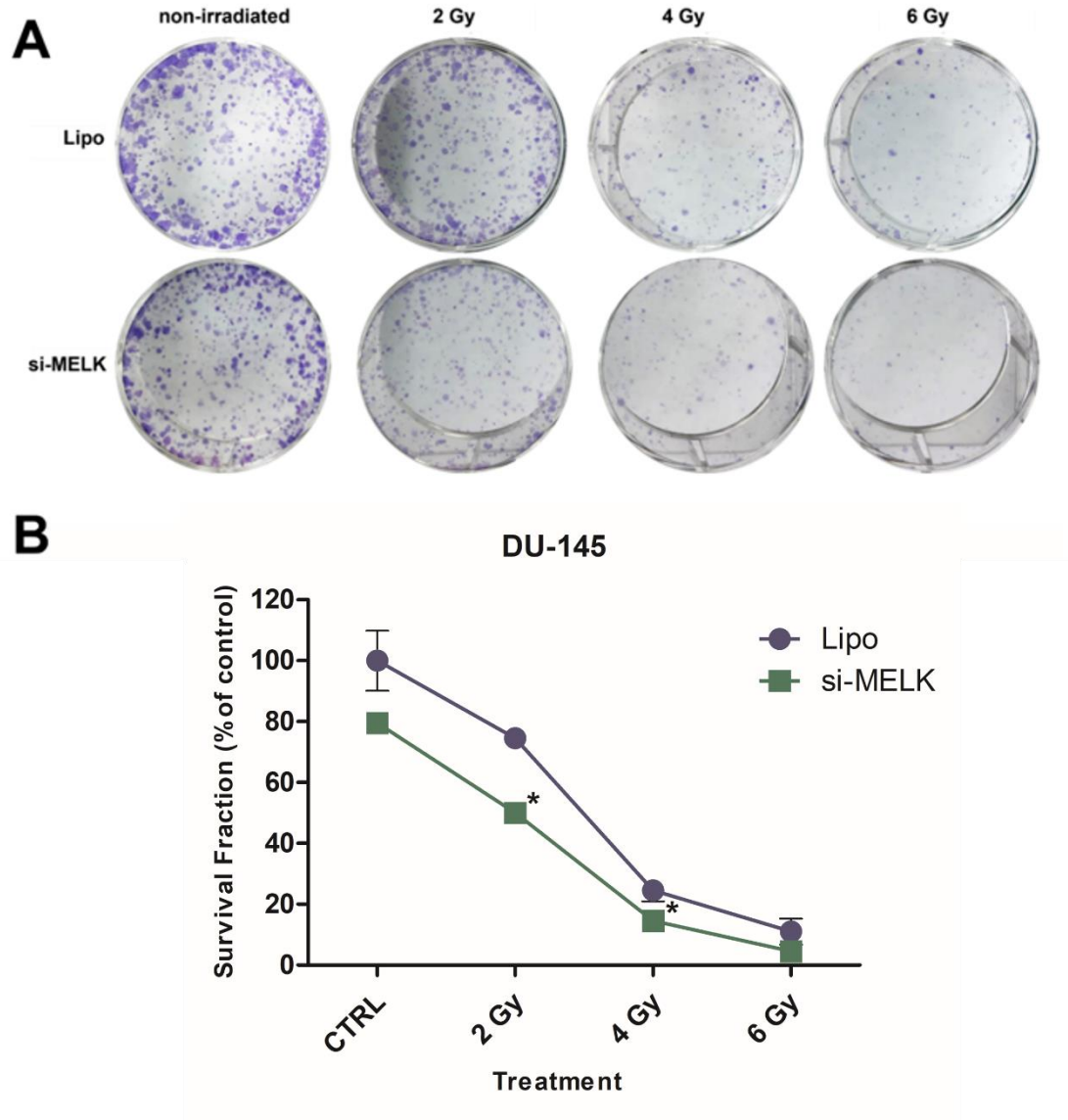

Figure 4. MELK silencing reduces colony formation but does not favor the effect of radiotherapy on PCa cells in vitro. (A) DU-145 cells were incubated with si-MELK

for 48h previously and treated with ionizing radiation. After the treatment, the cells were kept incubated for 10 days to colony formation. Cells with lipofectamine (Lipo) alone are controls for MELK silencing, while the non-irradiated group serves as control for conditions without ionizing radiation. (B) The colonies formed were normalized to the plating efficiency (FP) and the survival fraction obtained was compared to the control non-irradiated + Lipo. A comparison between Lipo and si-MELK within the ionizing radiation dose subgroups was also performed. *$p<0.05$ indicates a significant difference between Lipo cells and si-MELK cells within each radiotherapy subgroup alone.

Altered molecular pathways in MELK silenced prostate tumor cells.

Proteomic analysis identified 72 proteins downregulated (Table S1 and S2) and increased 29 proteins upregulated in DU-145-MELK silenced cells (Table S3 and S4). In the LNCaP cell line, 75 proteins were downregulated (Table S5 and S6) and 146 upregulated (Table S7 and S8) after a knockdown. From these results, functional enrichment was performed, represented in figure 5-6. The blue and red bars show the enriched terms for the downregulated and upregulated proteins, respectively. In the DU-145 cell line, downregulated proteins enriched terms related to targets of MYC, mTORC1, AKT1, MET, SYK, FGFR3, DIRK3, among others, with special emphasis on the chemical inhibition term of MELK; in turn, the upregulated proteins enriched the EGFR and MAPK14 terms (figure 5).

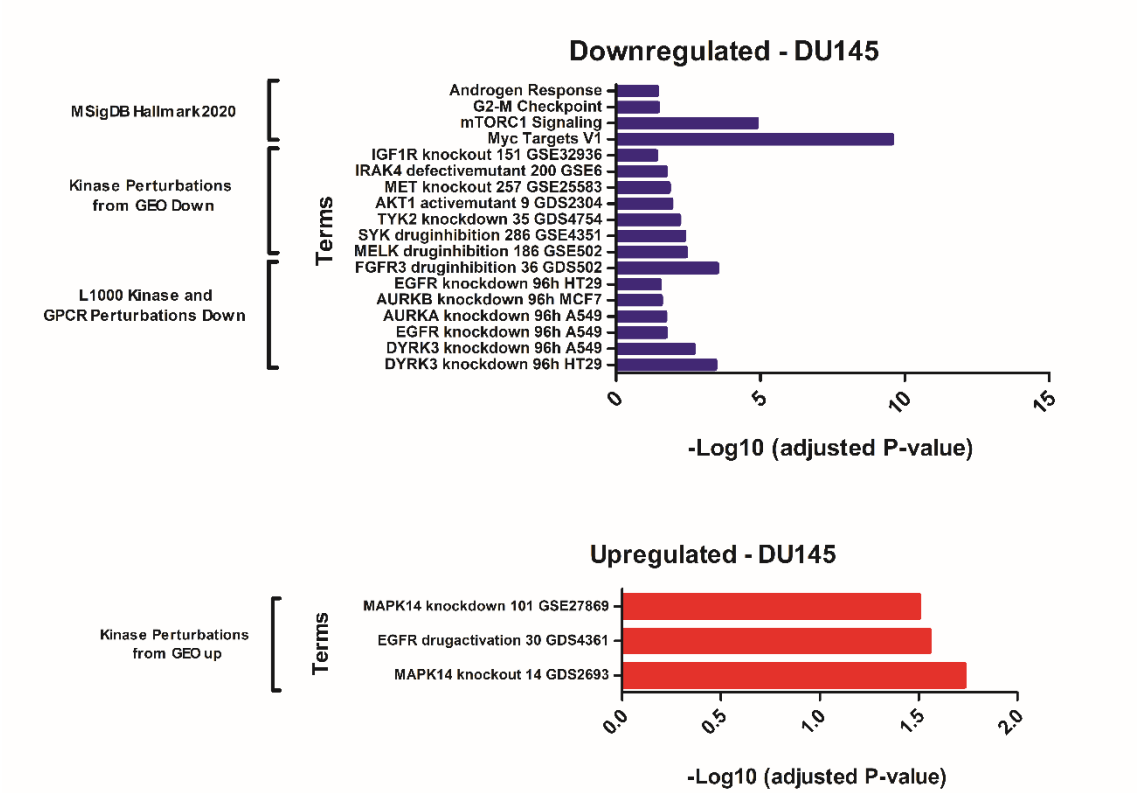

Figure 5. Enriched terms after MELK silencing in DU-145 PCa cell line. Ontological enrichment of downregulated (blue) and upregulated (red) proteins on DU-145 by Enrichr. All data were expressed as -Log10 (adjusted P-value).

In the LNCaP cell line, the downregulated proteins enriched terms linked to the mTOR and mTORC1 signaling pathway, metabolic reprogramming in cancer and to targets of MYC, mTORC1, SYK, MET, FGFR3, AKT1, GSK3A, SRC, among others, as a term of chemical inhibition of MELK, like that observed in the DU-145 cell line. The upregulated proteins showed a similar enrichment pattern, such as the mTOR, AKT1, SYK and MET signaling pathways; further, enrichments of EGFR, IRAK4 and ILK signaling pathways were observed (figure 6).

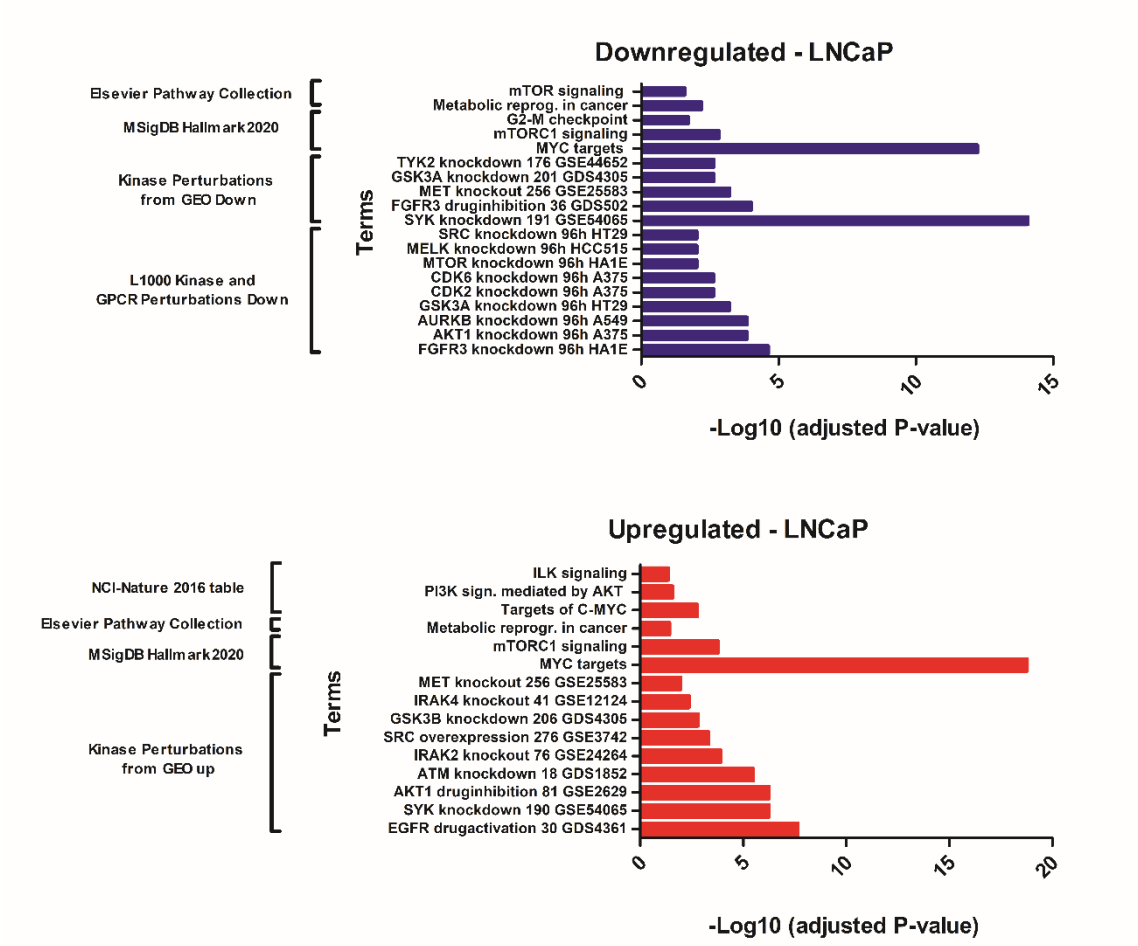

Figure 6. Enriched terms after MELK silencing in LNCaP PCa cell line. Ontological enrichment of downregulated (blue) and upregulated (red) proteins on DU-145 by Enrichr. All data were expressed as -Log10 (adjusted P-value).

DISCUSSION

The elevated expression of MELK is marked in several tumors, including relapsed and metastatic cases. In addition, MELK is also a biomarker of poor prognosis, being related to cell proliferation, inhibition of apoptotic mechanisms and tumor progression[32]. Thus, MELK inhibitors capable of blocking kinase activity and reducing cell proliferation *in vitro*, *in vivo*, preclinical and clinical trials are being developed and tested[19,33–36], such as the OTS167 inhibitor, which has shown promising results[32,37]. However, in a recent study, OTS167 showed an antitumor effect in MELK-deleted cells, suggesting an off-target action against other important kinases in the tumor growth pathway[38].

On the other hand, MELK gene silencing causes cell cycle arrest by inducing p21 independent of p53, through the stabilization of FoxO family proteins (*Forkhead box O*)[39]. Another study with *MELK* silencing indicated, *a priori*, inhibition of the proliferation of tumor cells that presented increased MELK, which was corrected by the same group, when stating that MELK correlates with the mitotic activity of any cell, without being necessary for tumoral growth[40]. In turn, Wang et al. highlight the existence of a conditional dependence by MELK on cell proliferation, being more evident in conditions of low cell density *in vitro*[41].

To elucidate the mechanisms of action and signaling of MELK in prostate tumor cells, the experiments carried out in this study were modeled on two cell lines, LNCaP and DU-145, which have distinct molecular profiles and are widely used in cell culture research. LNCaP cells were isolated from a lymph node metastatic prostate adenocarcinoma, are androgen responsive due to mutated AR expression, conferring promiscuous steroid binding, and display unmutated p53. DU-145 was isolated from a prostate tumor with brain metastasis, does not have AR expression and activity, does not respond to hormonal stimulation, and has mutated p53 and PTEN[42,43]. In addition, DU-145 expresses neuroendocrine differentiation markers, such as Chromogranin A, similar to neuroendocrine prostatic tumors, unlike LNCaP, which maintains a profile closer to that of adenocarcinoma[43,44]. In this way, LNCaP resembles non-metastatic CSPC, while DU-145 has characteristics closer to metastatic CRPC, with some neuroendocrine features.

In the present study, it was observed that the inhibition of MELK gene expression associated with radiotherapy had a combined effect when compared to radiotherapy alone, reducing DU-145 cells' growth. Similar results were found in glioma stem cells[21], in triple negative breast cancer cells in vitro and in vivo[45] and in glioblastoma[46]. MELK favors radioresistance in glioblastoma through the MELK/FOXM1/EZH2 pathway, with EZH2 being a fundamental factor in the protection of tumor cells against radiotherapy, which was evidenced by the reduction of EZH2 expression and tumor radiosensitization after MELK inhibition[21]. Recently, MELK was identified as a regulator of EZH2 levels by a FOXM1-independent mechanism. This process takes place through the control of EZH2 ubiquitination and turnover, where MELK acts by phosphorylating EZH2, thus blocking its marking for degradation[47]. EZH2 participates in initiation, proliferation, invasion, tumor metastasis, in addition to

drug resistance, and EZH2 inhibition combined with radiotherapy has shown promising results in clinical trials with several types of cancers[48], giving even more relevance to the MELK/FOXM1/EZH2 in tumor biology and the development of new therapies.

In the same sense, a greater antitumorigenic effect was observed when Docetaxel was combined with MELK silencing, compared to Docetaxel alone in DU-145 cells. In a study with Docetaxel-resistant triple-negative breast cancer cells, it was observed that the use of Maslinic Acid affects the MELK-FOXM1 interaction, and thus reduces the expression of ABCB1 (*ATP Binding Cassette Subfamily B Member 1*), a protein related to multidrug resistance, thus recovering sensitivity to Docetaxel[49]. In uterine leiomyosarcoma cells, MELK was related to chemoresistance to doxorubicin, through the activation of anti-apoptotic machinery via the JAK2/STAT3 pathway[50]. MELK silencing also had a significant effect ($p<0.05$) on the viability of DU-145 cells, even when cultured at high cell density, a condition in which MELK inhibition does not appear to impact cell proliferation in other cell lines, as mentioned previously.[41]. Thus, the clonogenic and viability results presented here also indicate a relationship between MELK and tumorigenicity in DU-145 cells, as observed in hepatocarcinoma[51] and neuroblastoma[12], evidencing its relevance as a potential therapeutic target in advanced PCa.

The understanding of cell signaling pathways related and affected by MELK is fundamental for the advancement of this kinase as a therapeutic target. From the proteomic analysis, MELK silencing in LNCaP and DU-145 cells enriched for the term “mTOR downregulated” and “mTORC1 signaling”. In a study with endometrial carcinoma cells, MELK is shown to regulate the mTOR pathway, through interaction with the MSLT8 subunit of the mTORC1 and mTORC2 complex, thus promoting its activation[18]. mTOR has a broad relationship with tumor biology, mainly through the PI3K-AKT-mTOR pathway and activation of tumor progression mechanisms, such as cell proliferation, cell survival and metabolic reprogramming[52]. The databases enriched in the present study also bring “AKT activemutant” and “AKT knockdown”, in addition to “Metabolic reprogramming” in LNCaP, which is further evidence of alteration of this axis. In this context, PI3K/AKT/mTOR is altered in 100% of metastatic PCa[53], which shows its relevance in the evolution and maintenance of CRCP. *In silico* data also point to the role of the PI3K/AKT pathway in the resistance of PCa to Docetaxel[54], which is reversed when PI3K/AKT inhibitors are combined with Docetaxel in CRCP[55], which

may favor tumor regression[56]. At this point, MELK is relevant by participating in the orchestration of this axis, as well as in the progression of PCa and its response to conventional chemotherapy.

Another enriched term was “MYC targets”, indicating alteration of this pathway in LNCaP and DU-145 cells with silenced *MELK*. MYC is increased in several types of cancers and its activation is described as contributing to several processes of tumor development, such as proliferation, cell survival, metabolic reprogramming, invasiveness and escape from defense mechanisms[57]. In PCa, MYC is shown to be involved in tumor initiation and, later, in disease recurrence, through repression of AR transcription, favoring resistance to antiandrogen therapies and progression to CRPC[58]. The alteration in AR pathway after MELK knockdown reinforces this MYC-AR relationship in this cancer type. Another interesting association between MYC and MELK, it is described in neuroblastoma, in which MYCN, a member of the MYC family protein, regulates MELK transcription[12,59]. MYCN acts on the transcription of PLK1, which in turn upregulates the MYC pathway[60]. Interestingly, MELK is described to regulate PLK1 in lung carcinoma[61], which may explain the alteration of the MYC pathway after *MELK* knockdown in PCa cells and possibly indicates the existence of a self-regulating MYC/MELK/PLK1 pathway. This relationship is further strengthened by the fact that PLK1 silencing potentiates the effect of the MELK inhibitor OTSSP167, in addition to showing that MELK participates in the G2/M transition of the cell cycle, regulating the PLK1/CDC25C/CDK1 pathway[61].

In the context of the cell cycle, MELK silencing enriched the terms “AURKB knockdown” in DU-145 and LNCaP lines and “AURKA knockdown” in LNCaP cells. AURKA and AURKB are two proteins of the Aurora Kinase family, the first being related to the G2/M transition by activating CDK1-Cyclin B, while the second has a fundamental role in stabilizing the kinetochore-microtubule binding and positioning of the chromosomes during metaphase[62]. In this way, the participation of AURKA and AURKB in the cell cycle contributes to the correct chromosomal segregation, avoiding aneuploidies and genomic instabilities that can lead to tumorigenic behavior[62]. In cancer, AURKA and AURKB are identified as oncogenes and are overexpressed in several types of tumors, including PCa[62,63]. The tumor role of AURKA is better understood than AURKB, performing the modulation of mitotic regulators, tumor suppressors, and other oncogenes, which favors cell proliferation, malignant

transformation and resistance to radio and chemotherapeutics[64]. In turn, the overexpression of AURKB is described as favoring oncogenicity, the epithelial-mesenchymal transition (EMT) and cell survival, the latter by phosphorylation and activation of MYC[62]. By relating AURKA, AURKB, and MELK, a recent study observed that MELK silencing delays the activation of AURKs as well as CDK1, decelerating G2/M cell cycle transition[65]. A possible explanation for MELK silencing affects G2/M progression is through FOXM1, a transcription factor activated by MELK[66,67], which acts on the expression of *AURKA*[64] and *AURKB*[68]. In addition, FOXM1 regulates the expression of *PLK1*[68], which, as discussed earlier, acts on MYC activation[60]. Thus, FOXM1 and AURKA/B may be involved in the MYC/MELK/PLK1 axis already described, forming a possible regulatory network, as hypothesized in Figure 7, evidencing the relevance of MELK in tumor formation and progression to the metastatic stage.

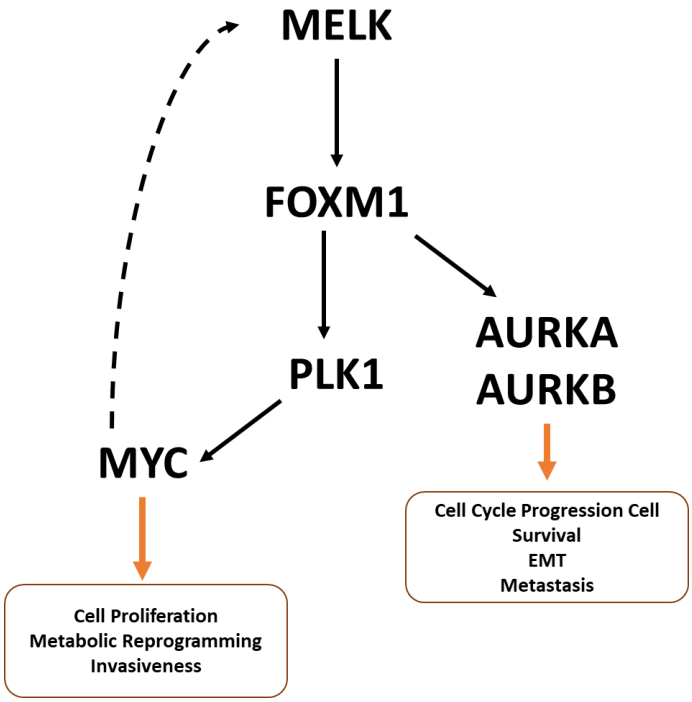

Figure 7. Hypothetical MELK signaling pathway in MYC, AURKA and AURKB activation. From the results of proteomic enrichment and the analysis of the literature, it is possible to observe a possible regulatory network where MELK acts in the activation of FOXM1, which in turn controls the gene expression of PLK1, AURKA and AURKB. PLK1 also contributes to the enhancement of FOXM1 activity. In addition, PLK1 acts on the activation of the MYC pathway. MYC in turn regulates MELK and PLK1, in a positive loop signaling mechanism.

The terms “SYK knockdown” and “SYK druginhibition” were also observed in the enriched proteomic of both cell lines, DU-145-siMELK and LNCaP-siMELK, but with greater prominence in the latter. In the cancer scenario, SYK has a dual function, mainly in tumors of hematopoietic and epithelial origin, acting as a tumor suppressor in some and an oncogenic promoter in others[69,70]. In PCa, SYK is described as a promoter, acting in the maintenance of proliferation and migration of tumor cells, with greater relevance in advanced and metastatic tumors[71], and may even be related to resistance to Docetaxel, as observed in a previous study[54]. In addition, SYK activity is fundamental in colony formation, while its inhibition reduces clonogenic growth[71], a behavior that may explain the relationship between MELK silencing affecting SYK and the decrease in colonies in the present work. The participation of SYK in tumorigenesis occurs through the PI3K/AKT pathway, with consequent positive regulation of anti-apoptotic factors and negative regulation of pro-apoptotic factors, in addition to the activation of MYC, mTOR[69] and NF-κB[70]. There is little knowledge about the possible interaction between MELK and SYK, which guarantees the need for future studies, and highlights the relevance of MELK as a potential therapeutic target in prostate tumors, especially in CRPC.

MELK has been widely studied since 2005, mainly with the aim of elucidating its role and influence in different types of cancers[32]. Although many results show the importance of MELK, placing it as a new therapeutic target, recent works point to a scenario where cancer cells do not depend on MELK in terms of tumor development and progression[72]. Even with controversial functions so far, the search for understanding the mechanisms of action and regulation of MELK in cancer biology continues to be explored. The results obtained and discussed in the present work provide evidence that, in the context of PCa, MELK has an expressive role in terms of tumor cell growth and survival, evidence that supports the search for understanding its molecular and tumorigenic action, as well as its potential as a therapeutic target.

CONCLUSION

MELK silencing, alone or combined with chemo and radiotherapy, had an antitumor effect on advanced PCa cells in vitro, by reducing the viability and the number of colonies formed. MELK inhibition mainly affected molecular pathways related to AKT-mTOR, MYC, AR, AURKA/AURKB and, SYK, and these alterations

may explain the results observed in the in vitro assays. Further studies are needed in order to elucidate the mechanisms of MELK regulation in PCa, as well as to confirm the pathways hypothesized in the present work. Understanding such aspects can contribute to the development of new treatments, as well as the promotion of more effective and targeted therapies.

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SUPLEMENTARY RESULTS

Table S1

Downregulated – DU-145-siMELK sample

Entry	Protein Name	p value	Log2 (Fold Change)
A0A0B4J2C3	TPT1	0,0013963	-1,4467
P17096	HMGA1	0,000038654	-1,2326
P62820	RAB1A	0,002228	-1,0024
E9PB61	ALYREF	0,002228	-1,0024
E7EMB3	CALM2	0,00099258	-0,93598
Q5TEC6	H3-2	0,000043615	-0,89985
P06748	NPM1	0,0004779	-0,70713
P84243	H3-3A	0,001592	-0,68709
P12277	CKB	0,0004779	-0,51751
P62805	H4C1	0,001592	-0,51578
P62701	RPS4X	0,00084861	-0,50458
P63104	YWHAZ	0,00013834	-0,44031
P45880	VDAC2	0,000063027	-0,32332

Table S2

Proteins detected exclusively in DU-145 control sample

Entry	Protein Name
P60903	S100A10
P62906	RPL10A
Q9P0S9	TMEM14C
P16401	HIST1H1B
F8VVM2	SLC25A3
Q15843	NEDD8
J3QRS3	MYL12A
P62280	RPS11
Q02543	RPL18A
Q00325	SLC25A3
P11413	G6PD
P16949	STMN1
P26373	RPL13
P62316	SNRPD2
Q99584	S100A13
P43487	RANBP1
P12429	ANXA3
P84103	SRSF3
P62241	RPS8
A0A0A6YYL6	RPL17-C18orf32
M0QYS1	RPL13A
A8MU27	SUMO3
Q01130	SRSF2
Q14240	EIF4A2
F5GXR3	PTMS
Q96AG4	LRRC59
Q16629	SRSF7
P68871	HBB
Q9UQ80	PA2G4
P62753	RPS6
P52597	HNRNPF
O43175	PHGDH
P30153	PPP2R1A
Q92688	ANP32B
P61026	RAB10
P02786	TFRC
P62917	RPL8
P52272	HNRNPM
P55209	NAP1L1
Q9BWD1	ACAT2
P31948	STIP1
P52209	PGD
P11586	MTHFD1
P04843	RPN1
O75390	CS
P49720	PSMB3
O75874	IDH1
P13010	XRCC5
P00491	PNP

Q16881	TXNRD1
P31040	SDHA
Q15008	PSMD6
P06737	PYGL
Q04637	EIF4G1
Q12906	ILF3
B5ME19	EIF3CL
E9PAV3	NACA
A0A0D9SGF6	SPTAN1
Q09161	NCBP1

Table S3

Upregulated – DU-145-siMELK sample

Entry	Protein Name	p value	Log2 (Fold Change)
P15121	AKR1B1	0,0035003	0,17632
Q562R1	ACTBL2	0,0033165	0,18415
C9JIZ6	PSAP	0,00030878	0,3854
A0A5K1VW95	MDH1	0,0000052942	0,40486
P17844	DDX5	0,00398	0,58254
P50914	RPL14	0,0010624	0,73454
P53396	ACLY	0,0004891	0,94732
P62269	RPS18	0,0029632	0,96282
Q99497	PARK7	0,0018428	1,1238
P35232	PHB1	0,0048754	1,5958

Table S4

Proteins detected exclusively in DU-145-siMELK sample

Entry	Protein Name
Q04695	S100A10
P22090	RPS4Y1
A0A0C4DG17	RPSA
P27695	APEX1
H0YKD8	RPL28
P27816	MAP4
H0Y449	YBX1
J3KPF3	SLC3A2
Q01844	EWSR1
G5E9Q6	PFN2
Q9Y678	COPG1
Q04941	PLP2
P42704	LRPPRC
P05455	SSB
O75533	SF3B1
P22102	GART
P54727	RAD23B
P53999	SUB1
P07741	APRT

Table S5

Downregulated – LNCaP-siMELK sample

Entry	Protein Name	p value	Log2 (Fold Change)
P30153	PPP2R1A	0,0013963	-1,4825
Q3ZCM7	TUBB8	0,0036888	-1,3392
P05455	SSB	0,0000018053	-1,2486
P12956	XRCC6	0,0000000000029162	-1,1782
P62701	RPS4X	0,00094704	-1,1031
Q00796	SORD	0,0088675	-0,97222
Q13509	TUBB3	0,00026087	-0,96918
P61313	RPL1	0,0036348	-0,96538
P78371	CCT2	0,000073341	-0,94979
P49006	MARCKSL1	0,000010375	-0,92671
H0YJ66	DHRS7	0,000010375	-0,92671
P49368	CCT3	0,000010375	-0,92671
P68431	HIST1H3A	0,019655	-0,89436
Q8TC12	RDH11	0,029765	-0,88904
A0A5F9ZHL1	ACAT1	0,0015961	-0,76297
J3QQ67	RPL18	0,000070774	-0,66367
Q71DI3	HIST2H3C	0,0096893	-0,63621
P51148	RAB5C	0,00070331	-0,62786
P04406	GAPDH	0,0000027975	-0,62299
Q5TEC6	HIST2H3PS2	0,036761	-0,58206
Q9NR30	DDX21	0,036761	-0,58206
Q04917	YWHAH	0,0080133	-0,54058
A0A2R8Y5Y7	RPL9	0,00030161	-0,51167
P62424	RPL7A	0,00030161	-0,51167
A0A2U3TZU2	GPI	0,00000074699	-0,50652
P13804	ETFA	0,043257	-0,49271
P07437	TUBB	0,0065989	-0,46332
P68371	TUBB4B	0,014179	-0,46066
P84243	H3F3A	0,027619	-0,44455
P49327	FASN	0,001797	-0,44244
Q06830	PRDX1	0,000047311	-0,41815
P0CG39	POTEJ	0,00076165	-0,39741
P25705	ATP5A1	0,034887	-0,37065
Q12905	ILF2	0,0024899	-0,34174
P30084	ECHS1	0,0024899	-0,34174
P13639	EEF2	0,00025364	-0,33551
P60174	TPI1	0,031776	-0,33413
P14618	PKM	0,025594	-0,32358
P23528	CFL1	0,0043059	-0,30522
P06748	NPM1	0,024163	-0,29082
P63261	ACTG1	0,000042371	-0,27988
P55072	VCP	0,0088893	-0,26784
P11142	HSP70	0,0045713	-0,22326
P62805	HIST1H4I	0,00000000000023816	0,1590
A0A0C4DG17	RPSA	0,036576	-0,1358
P62269	RPS18	0,036576	-0,1358
P07237	P4HB	0,036576	-0,1358
P62263	RPS14	0,036576	-0,1358

P35232	PHB	0,00001093	-0,12755
Q32Q12	NME1-NME2	0,0096085	-0,091486
P68032	ACTC1	0,00075468	-0,086361
P61978	HNRNPK	0,046964	-0,069945

Table S6

Proteins detected exclusively in LNCaP-control sample

Entry	Protein Name
Q71UI9	H2AZ2
P25787	PSMA2
A0A087WZM5	FKBP1A
P20700	LMNB1
E7EVA0	MAP4
F8W0W8	PPP1CC
J3KRY1	ARHGDIA
Q9H9B4	SFXN1
Q7Z4W1	DCXR
G3V5Z7	PSMA6
A8MXP9	MATR3
P00505	GOT2
Q04637	EIF4G1
A8MU27	SUMO3
B5ME19	EIF3CL
P37802	TAGLN2
P14174	MIF
P62314	SNRPD1
Q15388	TOMM20
Q13200	PSMD2
P62753	RPS6
P23246	SFPQ
P38606	ATP6V1A

Table S7

Upregulated – LNCaP-siMELK sample

Entry	Protein Name	p value	Log2 (Fold Change)
P68363	TUBA1B	0,043606	0,078855
P68366	TUBA4A	0,017233	0,17969
P23284	PPIB	0,000069312	0,23044
Q32P51	HNRNPA1L2	0,000069312	0,23044
Q07021	C1QBP	0,000069312	0,23044
M0R0R2	RPS5	0,000069312	0,23044
P16402	HIST1H1D	0,00016605	0,26994
P50991	CCT4	0,001539	0,27924
Q96KP4	CNDP2	0,001539	0,27924
P05387	RPLP2	0,001539	0,27924
P08238	HSP90AB1	0,00000000000068544	0,30936
P07900	HSP90AA1	0,0000058972	0,31612
P05141	SLC25A5	0,0000022167	0,34665

P12236	SLC25A6	0,0000022167	0,34665
P39687	ANP32A	0,01082	0,37214
O43390	PHGDH	0,01082	0,37214
P27348	YWHAQ	0,00090095	0,42122
P63244	RACK1	0,017767	0,42514
O75367	H2AFY	0,022268	0,45942
P61604	HSPE1	0,00030662	0,45976
P07954	FH	0,000407	0,48833
Q562R1	ACTBL2	0,0060919	0,52837
O75874	IDH1	0,00000017042	0,64548
P62244	RPS15A	0,000076855	0,65826
P30101	PDIA3	0,000036029	0,75137
P55060	CSE1L	0,0053801	0,76561
P0CG48	UBC	0,0035142	0,84756
Q6FI13	HIST2H2AA3	0,00075963	0,85313
P16104	H2AFX	0,0030058	0,86155
P08758	ANXA5	0,000014359	0,88065
Q14974	KPNB1	0,000000000011213	0,93732
E9PF18	HADH	0,0000045088	1,0733
A6NHT5	HMX3	0,0000045088	1,0733
P35579	MYH9	0,0000045088	1,0733
P05783	KRT18	0,000000001754	1,154
H0Y449	YBX1	0,017855	1,2809
Q15366	PCBP2	0,00010622	1,5012
P20962	PTMS	0,00083956	1,729
P04844	RPN2	0,0085271	1,9289

Table S8

Proteins detected exclusively in LNCaP-siMELK sample

Entry	Protein Name
A0A0A6YYJ8	LUC7L2
A0A0B4J2C3	TPT1
A0A0D9SF53	DDX3X
A0A2R8Y5A3	CTNNB1
A0A3B3IUB5	HM13
A0A5H1ZRQ2	DDX17
A0A669KBE1	CAPN3
B3KS98	EIF3H
C9J8T6	COX17
E5RHG6	TBCA
E9PRY8	EEF1D
F8WAR4	CHCHD3
G8JLB6	HNRNPH1
H7C2N1	PTMA
K7ELC2	RPS15
K7ELC7	RPL27
O00231	PSMD11
O75915	ARL6IP5
O75964	ATP5L
O76003	GLRX3
O95831	AIFM1

P00403	MT-CO2
P04040	CAT
P05023	ATP1A1
P05198	EIF2S1
P06732	CKM
P07814	EPRS1
P07910	HNRNPC
P08133	ANXA6
P0C7P4	UQCRFS1P1
P12081	HARS1
P12268	IMPDH2
P12532	CKMT1A
P13010	XRCC5
P13073	COX4I1
P18077	RPL35A
P18206	VCL
P19367	HK1
P22307	SCP2
P24534	EEF1B2
P26373	RPL13
P26440	IVD
P26640	VARS1
P29966	MARCKS
P30048	PRDX3
P31930	UQCRC1
P31939	ATIC
P31949	S100A11
P35030	PRSS3
P35221	CTNNA1
P36542	ATP5F1C
P37108	SRP14
P39023	RPL3
P41091	EIF2S3
P42704	LRPPRC
P43490	NAMPT
P43686	PSMC4
P49189	ALDH9A1
P49588	AARS1
P49755	TMED10
P50395	GDI2
P51858	HDGF
P51991	HNRNPA3
P52907	CAPZA1
P53004	BLVRA
P53396	ACLY
P54577	YARS1
P54819	AK2
P62241	RPS8
P62820	RAB1A
P62829	RPL23

P62906	RPL10A
P62913	RPL11
P63220	RPS21
P68036	UBE2L3
P68871	HBB
P69905	HBA1
P78527	PRKDC
P84103	SRSF3
Q01518	CAP1
Q01844	EWSR1
Q02790	FKBP4
Q12792	TWF1
Q13011	ECH1
Q13185	CBX3
Q13242	SRSF9
Q13838	DDX39B
Q14103	HNRNPD
Q14444	CAPRIN1
Q14697	GANAB
Q15819	UBE2V2
Q5JXB2	UBE2NL
Q6IPX4	RPS16
Q7KZF4	SND1
Q7Z4V5	HDGFL2
Q7Z5H4	VN1R5
Q8NC51	SERBP1
Q92804	TAF15
Q92945	KHSRP
Q99988	GDF15
Q9H2U2	PPA2
Q9H444	CHMP4B
Q9P2E9	RRBP1
Q9UJZ1	STOML2
Q9UNX3	RPL26L1
Q9Y230	RUVBL2

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7. ANEXOS

7.1. Anexo I

Declaração de que o trabalho não versou sobre pesquisa envolvendo seres humanos, animais, patrimônio genético ou temas afetos a biossegurança.

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DECLARAÇÃO

Em observância ao **§5º do Artigo 1º da Informação CCPG-UNICAMP/001/15**, referente a Bioética e Biossegurança, declaro que o conteúdo de minha Dissertação de Mestrado, intitulada "***EFEITOS MOLECULARES E FUNCIONAIS DA INIBIÇÃO DE MELK EM LINHAGENS DE CÂNCER DE PRÓSTATA: ESTUDO IN VITRO***", desenvolvida no Programa de Pós-Graduação em Biologia Celular e Estrutural do Instituto de Biologia da Unicamp, não versa sobre pesquisa envolvendo seres humanos, animais ou temas afetos a Biossegurança.

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Data: 16/12/2022

7.2. Anexo II

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