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Original article

Role of KDM2B epigenetic factor in regulating calcium signaling in prostate cancer cells

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ABSTRACT

KDM2B, a histone lysine demethylase, is expressed in a plethora of cancers. Earlier studies from our group, have showcased that overexpression of KDM2B in the human prostate cancer cell line DU-145 is associated with cell adhesion, actin reorganization, and improved cancer cell migration. In addition, we have previously examined changes of cytosolic Ca^{2+}, regulated by the pore-forming proteins ORAI and the Ca^{2+} sensing stromal interaction molecules (STIM), via store-operated Ca^{2+} entry (SOCE) in wild-type DU-145. This study sought to evaluate the impact of KDM2B overexpression on the expression of key molecules (*SGK1*, *Nhe1*, *Orai1, Stim1*) and SOCE. Furthermore, this is the first study to evaluate KDM2B expression in circulating tumor cells (CTCs) from patients with prostate cancer. mRNA levels for *SGK1*, *Nhe1*, *Orai1, and Stim1* were quantified by RT-PCR. Calcium signals were measured in KDM2B-overexpressing DU-145 cells, loaded with Fura-2. Blood samples from 22 prostate cancer cases were scrutinized for KDM2B expression using immunofluorescence staining and the VyCAP system. KDM2B overexpression in DU-145 cells increased *Orai1*, *Stim1,* and *Nhe1* mRNA levels and significantly decreased Ca^{2+} release. KDM2B expression was examined in 22 prostate cancer patients. CTCs were identified in 45 % of these patients. 80 % of the cytokeratin (CK)-positive patients and 63 % of the total examined CTCs exhibited the (CK + KDM2B + CD45−) phenotype. To conclude, this study is the first to report increased expression of KDM2B in CTCs from patients with prostate cancer, bridging *in vitro* and preclinical assessments on the potentially crucial role of KDM2B on migration, invasiveness, and ultimately metastasis in prostate cancer.

1. Introduction

Ca^{2+} is crucial for numerous cell functions including cellular proliferation and migration. Free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in resting cells is approximately 100 nM and can increase around 10-fold following stimulation. However, a sustained rise in free Ca^{2+} in cytosol can cause cell death and it therefore needs appropriate regulation (Kappel et al., 2017, Bootman and Bultynck, 2020). Regulation of intracellular Ca^{2+} involves regulation of entry of Ca^{2+} across the cell membrane, Ca^{2+} release from intracellular stores, and of Ca^{2+} removal from the cytosol.

Store-operated Ca^{2+} entry (SOCE), the process of refilling Ca^{2+} in intracellular stores, maintains Ca^{2+} homeostasis and regulates various cellular functions. SOCE is mediated by two players: the Ca^{2+} sensing stromal interaction molecules (STIM), which sense store depletion in the endoplasmic reticulum (ER), and the pore-forming proteins ORAI, which comprise channels in the cell membrane. STIM family consists of two isoforms, STIM1-2, with modest structural yet important functional differences such as a weaker ability of STIM2 to activate SOCE (Emrich et al., 2021, Tiffner and Derler, 2021, Collins et al., 2022, Johnson et al.,

Abbreviations: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CTCs, circulating tumor cells; DAPI, 4′,6-diamidino-2-phenylindole; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum; FBS, fetal bovine serum; NSCLC, non-small cell lung cancer; *Orai*/ORAI, calcium release-activated calcium channel protein; PBMCs, peripheral blood mononuclear cells; SERCA, sarco/endoplasmatic reticulum Ca^{2+}-ATPase; SGK1, serum and glucocorticoid inducible kinase-1; SOCE, store-operated Ca^{2+} entry; *Stim*/STIM, stromal-interacting molecule.

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2022). The ORAI family has three isoforms, namely ORAI1-3, which form heterohexamers (Emrich et al., 2021, Tiffner and Derler, 2021, Collins et al., 2022). The segments in their sequences that are not conserved play a crucial role in causing isoform-specific differences in structural and functional properties that ORAI channels exert, including the decreased SOCE associated with the expression of ORAI2 and/or ORAI3 (Tiffner and Derler, 2021, Johnson et al., 2022). STIM1 and ORAI1 have been considered the primary mediators of SOCE, since their identification. The current notion suggests a role for all five evolutionarily highly conserved proteins which can function together to regulate Ca^{2+} signaling in reaction to both physiological and pathological stimuli (Emrich et al., 2021, Collins et al., 2022).

We have previously shown that the placental growth factor enhances the expression of both ORAI1 and STIM1 proteins, along with the increase in SOCE in ovary carcinoma cells (Abdelazeem et al., 2019). ORAI1, STIM1 expression and functional SOCE, have been extensively investigated in cell lines of prostate cancer (Xu et al., 2015, Kappel et al., 2017, Stagno et al., 2017, Zhou et al., 2017). Our group has previously studied SOCE in the presence and absence of istaroxamine (a Na^+/K^+ ATPase inhibitor) in wild-type DU-145 prostate cancer cells (Stagno et al., 2017). Istaroxamine was shown to decrease both mRNA levels of *Orai1* and *Stim1*, along with ORAI1 protein expression. Istaroxamine was also reported to decrease SOCE. Blockage of Orai1 by 2-aminoethoxydiphenyl borate significantly decreased migration and enhanced istaroxime-induced blocking of cell migration (Stagno et al., 2017).

Serum and glucocorticoid inducible kinase-1 (SGK1) has been reported to modulate cell survival and inhibit cell death partly by upregulating a vast number of ion channels, including ORAI1 and its stimulator STIM1 and transporters, such as the Na^+/H^+ exchanger Nhe1 (Lang et al., 2018a, Lang et al., 2018b, Hosseinzadeh et al., 2020). For ORAI1/STIM1 this is partly achieved by NFκB, which is also upregulated by SGK1 (Lang et al., 2018a). SGK1 is expressed in many tumors like colorectal (Liang et al., 2017, Chen et al., 2020), non-small cell lung cancer (NSCLC) (Guerriero et al., 2020, Kale et al., 2023), breast (Godbole et al., 2018, Al-Alem et al., 2023, Zhang et al., 2023) and prostate (Liu et al., 2017, Liu et al., 2018).

KDM2B, the epigenetic factor, is a lysine-specific histone demethylase which demethylates H3K4me3 and H3K36me2 inducing transcription regulation. Whilst suppression of transcription is more common by demethylases and KDM2B, there are also increasing examples of upregulated factors (Yan et al., 2018, De Nicola et al., 2020), which is what will be presented in the results herein. We (Zacharopoulou et al., 2018a, Zacharopoulou et al., 2018b) and others (Kuang et al., 2017, Zhang et al., 2022) have previously shown that KDM2B overexpression increased Ezh2 expression, the histone methyltransferase, in colon, prostate, ovarian, and NSCLC cells. KDM2B has been reported to be expressed in many tumors (Yan et al., 2018). Regulation of KDM2B expression in various tumors by the basic fibroblast growth factor has been demonstrated to promote cell proliferation, migration, and invasiveness. More specifically, KDM2B has been reported to affect cervical (Peta et al., 2018), ovarian (Kuang et al., 2017), and prostate (Zacharopoulou et al., 2018a, Zacharopoulou et al., 2020) cancer cell migration.

Expression of biomarkers and identification of epigenetic changes are now easily trackable with the advent and utilization of liquid biopsy and evaluation of circulating tumor cells (CTCs), accountable for metastatic spread, in the blood of cancer patients (Pantel and Alix-Panabieres, 2022, Koinis et al., 2023, Strati et al., 2023).

The current study aimed to explore the impact of KDM2B overexpression on the molecules related to the Calcium signal transduction pathway such as *Orai1* and *Stim1*, along with its effect on the level of intracellular Ca^{2+}. Furthermore considering the role of KDM2B in cancer cell migration, we conducted the first-ever examination of the expression of this epigenetic factor in prostate cancer patients' CTCs.

2. Material and methods

2.1. Cell culture

DU-145 cells, obtained by ATCC (Manassas, VA, USA), underwent cultivation in RPMI 1640 medium (Invitrogen/Life Technologies, Carlsbad, CA, USA). The culture medium was enriched with heat-inactivated fetal bovine serum (10 % FBS; Bioline Reagents Limited, London, UK) and penicillin/streptomycin (1 %; Invitrogen/Life Technologies, Carlsbad, CA, USA). The cells were maintained under humidification with 5 % CO_2 incubated at 37 °C (Zacharopoulou et al., 2018a, Zacharopoulou et al., 2018b, Zacharopoulou et al., 2020). Cells were infected with either the pBABEpuro KDM2B (for overexpression of KDM2B) or pBABEpuro (control vector, empty) retroviral vectors, as demonstrated formerly (Zacharopoulou et al., 2018a, Zacharopoulou et al., 2018b). Verification of the successful overexpression of KDM2B, following retroviral infection, has been presented in previous work at the mRNA (approx. 3-fold higher than control) and protein (>25-fold higher compared to control) levels (Zacharopoulou et al., 2018a).

2.2. Isolation of RNA, synthesis of cDNA, and quantitative real-time PCR

Total cell RNA was isolated using Trizol (Invitrogen/Life Technologies, Carlsbad, CA, USA) and oligo-dT priming and the SuperScript II Reverse Transcriptase kit was used to reverse transcribe 1 μg of total RNA (Invitrogen/Life Technologies, Carlsbad, CA, USA), both processes were done following the manufacturer's instructions (Zacharopoulou et al., 2018a). KAPA SYBR Green/ROX qPCR Master Mix was used to perform real-time PCR (Fermentas, Maryland, USA) and amplifications were performed on a 7500 Real-Time System (Applied Biosystems) (Zacharopoulou et al., 2018a). Relative gene expression quantification was obtained through Δct method. Normalization was conducted using β-actin. The primers are listed in Table 1.

2.3. Calcium measurements

Calcium measurements were conducted following an established protocol (Stagno et al., 2017, Sukkar et al., 2018, Abdelazeem et al., 2019). Fura-2 indicator (2 μM Fura-2, AM; Invitrogen, Goettingen, Germany) was used to load DU-145 cells (wild-type or overexpressing KDM2B) at 37 °C for about 30 min. SOCE was determined following store depletion by inhibiting the sarco/endoplasmatic reticulum Ca^{2+}-ATPase (SERCA) with thapsigargin (1 μM, Invitrogen) under conditions lacking extracellular Ca^{2+} and successive Ca^{2+} re-introduction with thapsigargin also present. Assays were carried out in Ringer solution [125 mM NaCl, 1.2 mM $MgSO_4$, 5 mM KCl, 2 mM $CaCl_2$, 2 mM Na_2HPO_4, 32 mM HEPES, 5 mM glucose (pH 7.4)]. To establish Ca^{2+} free conditions, assays were conducted by utilizing Ringer solution that was Ca^{2+}-free and contained all the above, excluding $CaCl_2$, but including EGTA 0.5. Fluorescence (detected at 505 nm with alternating excitation at 340/380 nm) was recorded using an inverted phase contrast microscope (Axiovert 100, Zeiss, Oberkochen, Germany) via an objective (Fluor 40 × /1.30 oil). Data were obtained with the Metafluor software (Universal Imaging, Downingtown, USA). Cytosolic Ca^{2+} signals were measured

Table 1
List of primer used.

Primers	Sequence (5′-3′)
β-Actin, forward:	CGGCATCGTCACCAACTG
β-Actin, reverse:	GGCACACGCAGCTCATTG
Nhe 1, forward:	ACCTGGTTCATCAACAAGTTCCG
Nhe 1, reverse:	TTCACAGCCAACAGGTCTACCA
Orai1, forward:	TGATGAGCCTCAACGAGCACTCCATG
Orai1, reverse:	TGCTGATCATGAGCGCAAACAGGTG
Stim1, forward:	CCTGTGGAAGGCATGGAAGT
Stim1, reverse:	CTGAGGCAGCTCCACATATGT

from 340/380 nm fluorescence ratios. To quantify the entry of Ca^{2+}, we computed the slope (delta ratio/s) and peak (delta ratio) after the reintroduction of Ca^{2+}.

2.4. Patients and preparation of cytospins

Twenty-two patients with prostate cancer participated in the study. Samples were collected at the Metropolitan General Hospital and the University General Hospital of Larissa between February 2021 and June 2023. All patients' samples were analyzed at the University of Patras.

Clinical data were available for 22 patients, and their clinical characteristics are presented in Table 2. Blood samples were collected before the commencement of the initial line of therapy. Exclusion criteria were: a. Age < 18 (years), b. Primary prostate cancer cases without histological and cytological evidence, c. Having previously received a minimum of one round of treatment, and d. Failure to provide a signed informed consent. The inclusion criteria were rigorously adhered to, with all participants providing written informed consent. The Declaration of Helsinki was followed and Ethical Committees of all participating institutes approved: Metropolitan General Hospital, 15,562 Athens, Greece (35/00-03/16) and University General Hospital of Larissa, 41,334 Larissa, Greece (32710/3-8-20).

Ten ml of venous blood were collected in tubes with EDTA, after removing the first 5 ml to prevent adulteration from epithelial cells of skin while collecting sample. Ficoll-Hypaque density centrifugation at 1800 rpm was utilized to isolate peripheral blood mononuclear cells (PBMC) for 30 min at RT. Washing of samples with PBS twice and centrifugation at 1500 rpm for 10 min was performed. Furthermore, centrifugation of aliquots with 500,000 cells was carried out for 2 min at 2000 rpm on glass slides. The resulting cytospins were air-dried and kept at −80 °C. Two slides from each patient were defrosted at Room Temperature before the staining process for about 20 min. Afterward, the slides were rehydrated with PBS 1X for ten minutes and proceeded to the following staining procedure.

2.5. Triple immunofluorescence staining

Experiments with triple immunofluorescence staining for cytokeratin (CK)/KDM2B/CD45 were performed in patients' samples. 1000 H1299 (lung non-small cell carcinoma) cells were spiked in 100,000 normal donors' PBMCs and were utilized as controls to mimic patients' samples (Roumeliotou et al., 2022). Negative controls were free from primary antibodies, whereas IgG secondary antibodies were included. It is standard procedure to perform staining tests in spiked samples to investigate the biomarker expression of interest before assessing it in the "precious" patients' samples. Similar conditions of exposure time have been implemented, to allow the respective figures to be comparable. Cytomorphological criteria like high nuclear to cytoplasmic ratio and bigger cell size than leukocytes were utilized to detect CTCs, CK-positive cells (Meng et al., 2004).

Patients' samples and control cytospins underwent fixation with a cold mixture of methanol and acetone (1:9 ratio), and thereafter 10 % FBS for overnight blocking. Detection of KDM2B expression was done with a rabbit anti-KDM2B antibody (1:150; EMD Millipore) and an Alexa Fluor 488 secondary anti-rabbit antibody (1:600; Life Technologies, Carlsbad, CA, USA). CD45 antibody coupled with Alexa Fluor 647 (1:100; Novus Biologicals, USA) was employed to detect potential ectopic expression of CK in hematopoietic cells. CKs, serving as markers for cancer cells i.e. CTCs in the blood, were identified using the primary antibody A45-B/B3 (1:100; Amgen, City, Thousand Oaks, CA, USA) and a secondary antibody anti-mouse with Alexa Fluor 555 (1:500; Life Technologies, Carlsbad, CA, USA). The A45-B/B3 antibody is a pan-CK antibody that detects CK8, CK18, and CK19. Finally, cells were mounted on an anti-fade reagent containing 4′,6-diamidino-2-phenylindole (DAPI). Subsequently, samples were examined using VyCAP (VyCAP B. V., Enschede, the Netherlands) (Roumeliotou et al., 2022, Vardas et al., 2023).

The VyCAP system is an imaging system based on a Nikon Ti-2 inverted fluorescence microscope. It enables the automatic scanning of 8 patient slides simultaneously. Particularly cytospins slides are scanned automatically, at 20× or 40× magnification in four channels, and the corresponding frames are analyzed based on different cytomorphological parameters, depending on our research needs. Three points define the area for the automatic scanning on each slide and the focus is adjusted. The system is calibrated using the spiked controls and an expression threshold is defined. Frames for each channel are captured to allow for the enumeration and phenotypic characterization of CTCs. A relocation function allows for real-time observation and confirmation by the user.

2.6. Statistical analysis

GraphPad Prism 4 (GraphPad Software, La Jolla, CA, USA) was used for data analysis. All values and data for three independent experiments are expressed as means ± SD or SEM. For comparison, the Unpaired Student's *t*-test was used. A normality test (Kolmogorov–Smirnov test) was also performed for patients' samples.

Table 2
Clinical characteristics of 22 prostate cancer patients.

Characteristics	*n* (%)
Age (years) for 14 patients with known age	
Median	70
Range	42–80
Histological type	
Adenocarcinoma	11 (50)
Unknown	11 (50)
Best response	
Stable disease	4 (18)
Unknown	18 (82)
Distant metastasis (some patients have more than one sites of metastasis)	
Bones	7 (32)
Lymph nodes	6 (27)
Lungs	2 (9)
Marrow	1 (5)
Unknown	10 (45)
Smoking	
Yes	5 (23)
No	13 (59)
Unknown	4 (18)
Family history of cancer	
Yes	3 (14)
No	5 (23)
Unknown	14 (64)

3. Results

3.1. KDM2B controls the expression of Ca^{2+} signaling players

The present study initially explored whether KDM2B overexpression in the prostate cancer cell line DU-145 affected *SGK1* mRNA expression. A slight decrease was observed, but it was not statistically significant (Fig. 1A). We then investigated the consequence of KDM2B overexpression on the mRNA levels of key players of ion transport which have been reported to be associated with SGK1 pathway, such as *Orai1*, *Stim1* and *Nhe1*. Interestingly, as illustrated in Fig. 1B-D, all genes were significantly increased in DU-145 cells overexpressing KDM2B ($p < 0.01$). Since *SGK1* expression was unaltered, effects can be attributed to a distinct pathway regulated by the epigenetic factor KDM2B.

3.2. Effect of KDM2B overexpression on intracellular Ca^{2+} release and store-operated Ca^{2+} entry

To examine whether increased *Orai1* and *Stim1* mRNA levels were translated into alterations in $[Ca^{2+}]_i$, cell were loaded with Fura-2. Cells

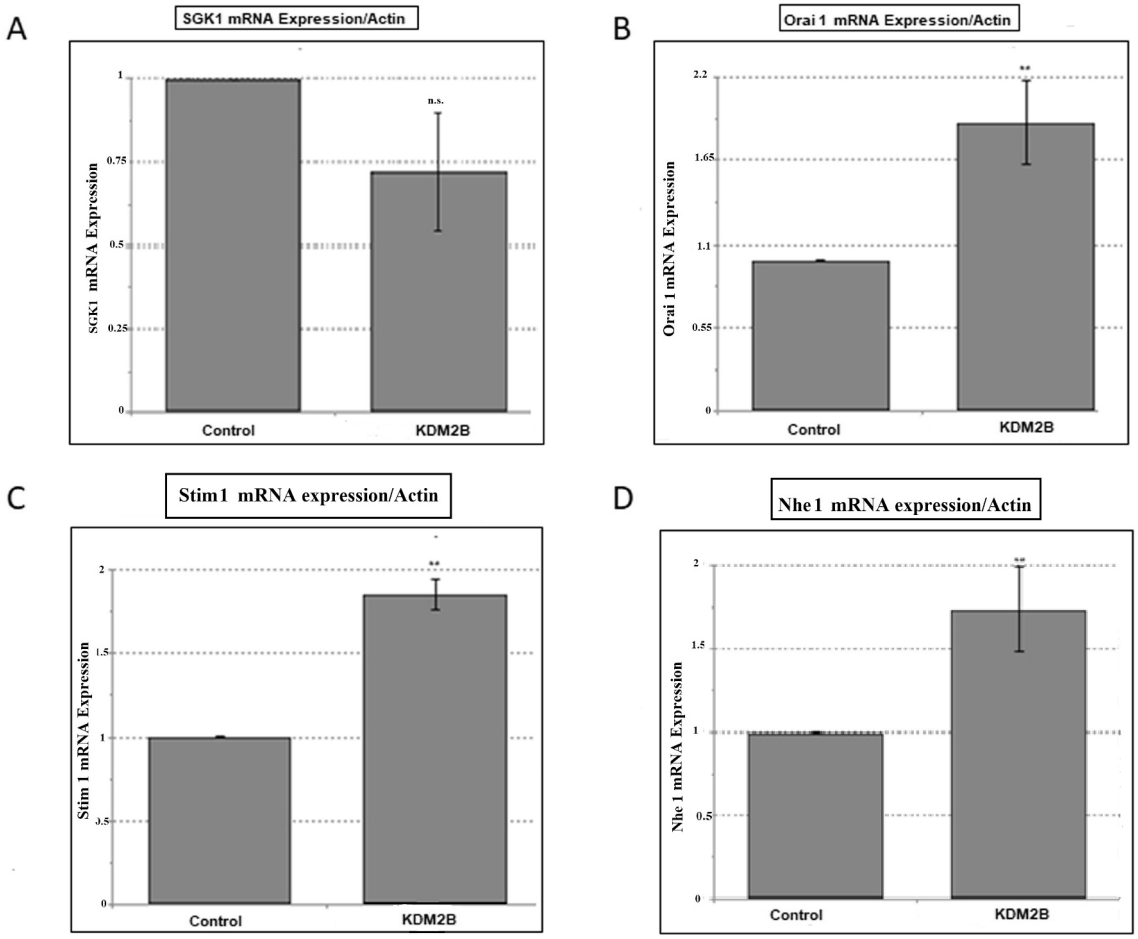

Fig. 1. Effect of KDM2B overexpression on *SGK1*, *Orai1*, *Stim1*, and *Nhe1* transcription in the prostate cancer cell line DU-145. mRNA expression of *SGK1* (A), *Orai1* (B), *Stim1* (C), and *Nhe1* (D) was measured by RT-PCR in DU-145 cells overexpressing KDM2B compared to wild-type cells. Data are presented as ratios of the respective genes with β-actin, with control cells arbitrarily set to 1. Data are shown as mean ± SD and n = 3 represents the independent experiments. **p < 0.01 signifies a difference that is statistically significant (unpaired Student *t*-test). n.s.: non-statistically significant.

were exposed to Ca^{2+}-free solution in the presence of thapsigargin, a SERCA inhibitor. This resulted in intracellular Ca^{2+} store depletion. The reintroduction of extracellular Ca^{2+} while thapsigargin was still present led to a swift elevation in fluorescence, indicative of SOCE (Fig. 2A). While overexpression of KDM2B caused a substantial drop in the magnitude of intracellular Ca^{2+} release ($p < 0.01$, Fig. 2B-C), it did not affect either peak or slope of Ca^{2+} entry (Fig. 2D-E). This suggests that overexpression of the epigenetic factor KDM2B could potentially be affecting the store Ca^{2+} content.

3.3. KDM2B expression in CTCs derived from prostate cancer patients

Twenty-two prostate cancer patients were enrolled in this study. Ten out of 22 total prostate cancer patients (45 %) were positive for CTCs. The phenotype (CK+/KDM2B+/CD45−) (Fig. 3A) was the most common with a frequency of 80 % in the patients' cohort (8 out of 10 of the CK-positive patients). The (CK+/KDM2B−/CD45−) phenotype was less frequent (50 %, 5 out of 10 CK-positive patients). It is also interesting that 5 out of 10 patients with upregulated expression of KDM2B exclusively harbored these cancer cells in their blood. Table 3 shows the absolute frequency of CTCs/patient and phenotype.

Furthermore, analysis of the average proportion of detected CTCs/patient showed that most of the isolated CTCs belonged to the (CK+/KDM2B+/CD45−) phenotype (63 %), while the (CK+/KDM2B−/CD45−) phenotype was less frequent (37 %; Fig. 3B). Representative photos of a CTC from a prostate cancer patient stained for CK, KDM2B and CD45 are shown in Fig. 3C, while control experiments are shown in Supplementary data 1.

4. Discussion

We have previously shown KDM2B expression in the prostate cancer cell line DU-145. In fact, KDM2B basal gene expression was higher in DU-145 cells compared to MDA-MB-231 breast and HCT116 colon cancer cell line (Zacharopoulou et al., 2018a). KDM2B was also higher in terms of protein levels in DU-145 compared to HCT116 cells (Zacharopoulou et al., 2018b). Furthermore, overexpression of KDM2B, evident as both up-regulation of gene and protein levels, has been previously verified in DU-145 cells (Zacharopoulou et al., 2018a). While KDM2B overexpression did not affect viability (Zacharopoulou et al., 2020), it led to the increased migration of DU-145 prostate cancer and HCT166 colon cancer cells in contrast to wild-type cells (Zacharopoulou et al., 2018a, Zacharopoulou et al., 2018b, Zacharopoulou et al., 2020).

Overexpression of KDM2B has been observed to participates in the activation of signaling cascades of pFAK and p85 subunit of PI3K in prostate cancer cells. Furthermore, this pathway has been evidenced to be related to high cancer cell migration and invasion (Zacharopoulou et al., 2018a, Zacharopoulou et al., 2020). The prominence of KDM2B in cancer cell migration directed the current study. We have extensively studied the activation of FAK and PI3K in CTCs from patients affected with breast cancer (Kallergi et al., 2007, Kallergi et al., 2008), however, KDM2B has not been studied so far in any neoplasm.

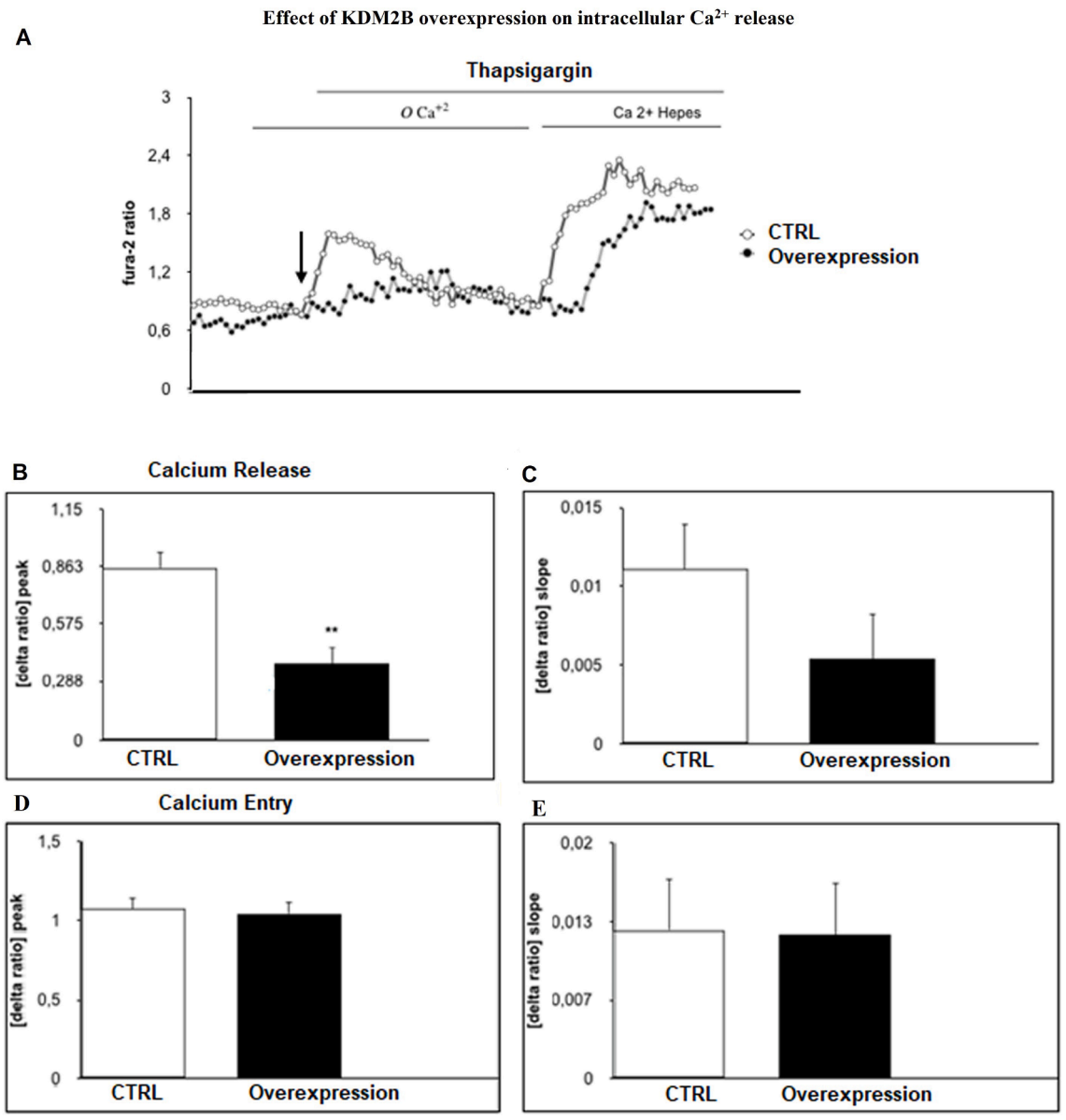

Fig. 2. KDM2B overexpression's effect on Ca^{2+} release and SOCE in the prostate cancer cell line DU-145. A) Representative trace of Fura-2 fluorescence ratio following extracellular Ca^{2+} removal, add-on of thapsigargin (line and arrow above trace), and re-introduction of extracellular Ca^{2+} in DU-145 overexpressing KDM2B (black circles) compared to wild-type cells (white circles). Arithmetic means (±SEM, n = 3) of peak (B) and slope (C) elevation in Ca^{2+} from intracellular stores after addition of thapsigargin (1 μM) and of peak (D) and slope (E) increase of SOCE after re-addition of extracellular $Ca2^{+}$ in DU-145 cells overexpressing KDM2B (black bars) compared to wild-type cells (white bars). **$p < 0.01$ signifies a difference that is statistically significant.

SOCE, the major Ca^{2+} influx mechanism for cells, is synchronized by a store-operated Ca^{2+} channel consisting of ORAI and STIM. ORAI/STIM have gained attention owing to their oncogenicity, as inhibiting either of these proteins affected proliferation, migration, and invasion of cancer cells both *in vitro* and *in vivo*. This study capitalized on our previous work on KDM2B and SOCE (Stagno et al., 2017, Zacharopoulou et al., 2018a, Zacharopoulou et al., 2018b, Zacharopoulou et al., 2020) and aimed at initially reporting on the expression of SOCE players upon overexpression of KDM2B. Interestingly, overexpression of KDM2B significantly upregulated transcription of *Orai1* and *Stim1*. It also upregulated mRNA expression of *Nhe1*, the Na^{+}/H^{+} exchanger, but the role of Nhe1 was not further studied. A common denominator of *Orai1*, *Stim1,* and *Nhe1* is that they have been reported to be upregulated by SGK1. We have previously reported the significance of SGK1 in upregulating SOCE and in migration and cell survival (Lang et al., 2010, Lang and Stournaras, 2013, Lang et al., 2018a). As SGK1 transcript levels were not affected following KDM2B overexpression, it could be suggested that the effects seen on *Orai1*, *Stim1,* and *Nhe1* expression can be attributed to KDM2B overexpression using an alternative pathway. The involvement of SGK1 cannot be completely ruled out but needs further examination. Indeed, SGK1 has been suggested to up-regulate NFκB activity (Lang et al., 2010, Lang and Stournaras, 2013, Lang et al., 2018a). We have previously reported that lithium can significantly enhance transcription and protein levels of ORAI1 and STIM1, as well as increase SOCE. These effects were reversed following treatment with wogonin, an NFκB inhibitor (Sukkar et al., 2018), hence suggesting that this pathway could be of interest in a future undertaking.

Interestingly, KDM2B overexpression did not significantly alter the peak of SOCE elicited by Ca^{2+} stores exhaustion with the SERCA inhibitor, thapsigargin. The absence of effect on SOCE halted further evaluation of ORAI1 and STIM1 at the protein level. Peak surge of fura-2-fluorescence ratio, from intracellular stores, was significantly

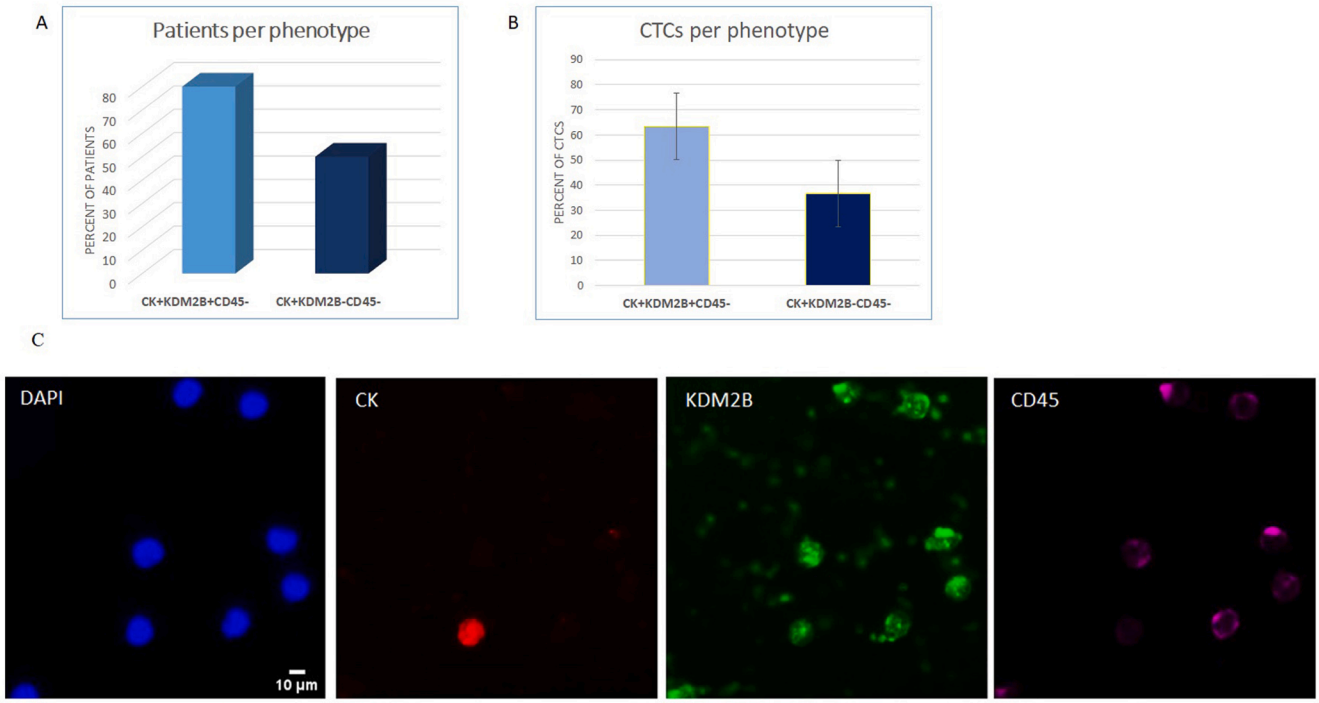

Fig. 3. Expression of KDM2B in prostate cancer patients' blood samples. A) Proportion of cytokeratin CTCs-positive patients with (CK+/KDM2B+/CD45−) and (CK+/KDM2B−/CD45−) phenotypes. B) Mean proportions of overall CTCs exhibiting the phenotypes (CK+/KDM2B+/CD45−) and (CK+/KDM2B−/CD45−). C) Representative photos of patient's CTCs expressing CK (red), an epithelial CTC marker, KDM2B (green), the biomarker of *interest,* and CD45 (purple), a leukocyte marker, in samples isolated from prostate cancer patients. DAPI was used for staining the nuclei (blue). Magnification 20×. Scale bar = 10 μm.

Table 3
Frequency of CTCs per phenotype in all prostate cancer patients.

Patient	CK + KDM2B + CD45–	CK + KDM2B–CD45–
1	0	0
2	1	1
3	1	0
4	1	0
5	0	2
6	4	6
7	0	0
8	0	1
9	0	0
10	0	0
11	4	5
12	1	0
13	0	0
14	0	0
15	0	0
16	0	0
17	1	0
18	0	0
19	0	0
20	0	0
21	0	0
22	1	0

decreased in KDM2B-overexpressing DU-145 cells, suggestive of an effect on the size of releasable Ca^{2+} in the store as shown in Fig. 2A. Given the lack of significant changes in SOCE, which is the major replenishing pathway for ER Ca^{2+} store, the more likely reasons underlying the reduced store Ca^{2+} content could be due to potentially increased levels of Sarcoendoplasmic Reticulum Calcium ATPase (SERCA). The SERCA plays a pivotal role in cellular calcium homeostasis by transporting calcium ions from the cytosol back into the sarcoplasmic reticulum (SR). In line with this assumption, the expression of SERCA could be further examined in the future. However, the current study was not further concentrated on the evaluation of the dynamics of Ca^{2+} signaling, instead, it was directed towards addressing the significance of KDM2B in real clinical samples and mainly in cells participating in migration and invasion of tumors in the bloodstream.

In this novel study, we are the first who appraised the overexpression of KDM2B in prostate cancer patients' CTCs. CTCs are the main cause of cancer metastasis. They also hold prognostic value for almost every type of cancer, including prostate. CTCs' enumeration, phenotypic characterization, and expression of prostate-derived transcripts in CTCs have been suggested to facilitate prognosis (Giunta et al., 2021, Yang et al., 2021, Crocetto et al., 2022). Increased CTCs counts have been correlated with worse overall survival and progression-free survival in prostate cancer patients (Giunta et al., 2021). Similar attempts to correlate CTCs' phenotype heterogeneity with clinical outcome have also been reported (Mizuno and Beltran, 2022). Our results revealed that 45 % of the total patients possessed CTCs which is in line with other studies (Klusa et al., 2023). KDM2B was highly expressed in prostate cancer patients; 80 % of CK-positive patients and 63 % of the total examined CTCs had the (CK+/KDM2B+/CD45−) phenotype, representing the highest percentage of the total examined CTCs. Whereas, only 37 % of the isolated tumor cells belonged to the (CK+/KDM2B−/CD45−) phenotype. Therefore, increased expression of KDM2B in patients' CTCs confirmed our previous *in vitro* results regarding the implication of KDM2B in cancer cell migration. This increased expression of KDM2B in CTCs could also have a major clinical impact since a potential drug inhibition of this histone lysine demethylase could serve as an interesting therapeutic target for preventing metastatic dissemination of tumor cells. It is widely accepted that most of the drugs focus on primary tumors and they ignore metastatic mechanisms. Therefore, new regiments targeting metastatic cells could open a different window to cancer therapeutic approaches.

Owing to the pilot nature of the study, the sample was rather small, and hence correlation with clinical outcome has not been attempted. Considering the increasing importance of KDM2B and its association with many important signaling molecules, including Ca2+, expanding this research to include a larger group of patients and relevant proteins

such as ORI1 and STIM1 in CTCs would be crucial for further evaluating their clinical significance. In a recent investigation, the analysis of circulating tumor DNA in individuals facing relapsed or refractory classical Hodgkin lymphoma uncovered KDM2B mutations in 17 % of cases demonstrating acquired resistance to anti-PD-1 therapy (Shi et al., 2020).

Epithelial-to-mesenchymal transition (EMT), a process in which cells undergo a transformation, leading to the loss of their epithelial characteristics and the acquisition of more mesenchymal traits, is responsible for the invasive properties that cells acquire and it requires reorganization of the cytoskeleton (Zacharopoulou et al., 2018a). The importance of KDM2B in cell-to-cell adhesion has been previously demonstrated by evaluating E-Cadherin and ZO-1 expression, the epithelial biomarkers. More specifically, gene and protein expression of both markers was markedly reduced in DU-145 with KDM2B overexpression (Zacharopoulou et al., 2018a). We have also shown that EMT phenotypes are upregulated in CTCs, promoting their migration into the bloodstream (Kallergi et al., 2011, Pantazaka et al., 2021, Vardas et al., 2022). KDM2B is involved in various signaling pathways crucial in tumorigenesis (Yan et al., 2018). Elucidation of the pathways through which KDM2B acts could provide valuable insight regarding the progression of prostate cancer.

5. Conclusions

To conclude, KDM2B overexpression in the DU-145 prostate cancer cell line *in vitro* seems to affect *Orai1/Stim1* expression and the store Ca^{2+} content. In addition, KDM2B is expressed in the majority of prostate patients' CTCs, suggestive of the important role of this epigenetic factor in the metastatic process. As epigenetic proteins can potentially be detected in a non-invasive manner using liquid biopsy, this would improve prostate cancer screening, early detection, and prognosis. More studies could further highlight the role of KDM2B as a useful biomarker for potential targeted therapies.

CRediT authorship contribution statement

Evangelia Pantazaka: Data curation, Writing – original draft, Writing – review & editing. **Saad Alkahtani:** Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing. **Saud Alarifi:** Resources, Writing – original draft, Writing – review & editing. **Abdullah A. Alkahtane:** Resources, Writing – original draft, Writing – review & editing. **Christos Stournaras:** Conceptualization, Resources, Supervision, Writing – original draft, Writing – review & editing. **Galatea Kallergi:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsps.2024.102109.

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