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Down-Regulation of Nfatc1 Suppresses Proliferation, Migration, Invasion, and Warburg Effect in Prostate Cancer Cells

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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Background: Prostate cancer (PCa), accounting for 28% of all male cancer cases, is the second leading cause of cancer-related death among men. NFATc1, belonging to the NFAT family, is overexpressed in PCa and is correlated with the risk of recurrence after radical prostatectomy.

Material/Methods: In the present study, the expression of NFATc, c-myc, and PKM2 in PCa cells was regulated by lentiviruses and then detected by real-time PCR and Western blot analysis. Further, proliferation, invasion, and migration assays were performed. The glucose consumption and lactate production were assessed by biochemical detection.

Results: We found that NFATc1 down-regulation significantly suppressed the proliferation and Warburg effect of PCa cells, concurrent with a decrease of c-myc and PKM2 expression. Likewise, the abilities of migration and invasion were also inhibited in NFATc1-silenced PCa cells. In addition, NFATc1 down-regulation-induced inhibition of cell proliferation, migration, invasion, and Warburg effect were counteracted by up-regulation of c-myc or PKM2. The expression of PKM2 was positively regulated by NFATc1 and c-myc expression.

Conclusions: These results indicate that NFATc1 down-regulation can suppress the proliferation, Warburg effect, and migration and invasion abilities of PCa cells, probably by regulating c-myc and PKM2 expression. NFATc1 may be a potential therapeutic target for PCa and could be used as a diagnosis or prognosis indicator of PCa.

MeSH Keywords: Cell Migration Inhibition • Cell Proliferation • Neoplasm Invasiveness • NFATC Transcription Factors • Prostatic Neoplasms

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Background

Prostate cancer (PCa), the second leading cause of cancer-related death among men, has become the most important form of genitourinary cancer in men, accounting for 28% of all male cancer cases [1]. Most PCa patients die from tumor metastases rather than tumor growth at the primary site. A study has shown that bone metastasis is closely related to morbidity and mortality of PCa. Bone is the preferred site of PCa metastasis, and almost all patients who die of PCa had extensive bone metastases [2]. PCa metastasis is a complex and multi-step process involving various independent and continuous cell behaviors. Although the mechanism of this process has not yet been elucidated, it is clear that cell migration and invasion are the most important components of the tumor metastasis [3].

Nuclear factor of activated T-cells (NFAT), a transcription factor in immune response, was originally identified as an activator of T lymphocytes [4,5]. Previous studies have related NFAT expression to cell growth, survival, angiogenesis, and tumorigenesis [6,7]. NFATc1 (NFAT2) is a distinct member of the NFAT family, and acquisition of NFATc1 expression down-regulates E-cadherin and contributes to the tumor progression [8]. Studies revealed that NFATc1 promotes the migration and invasion of multiple cancer cells, such as breast, colon, and ovarian cancer [9,10]. NFATc1 down-regulation inhibits the growth of bladder cancer [11]. In addition, high expression of NFATc1 is observed in specimens of pancreatic cancer and pancreatic cancer cell lines. NFATc1 cooperation with STAT3 promotes *KrasG12D*-driven carcinogenesis, giving rise to highly aggressive pancreatic cancer [12]. Highly expressed NFAT enhances malignancy of pancreatic cancer primarily by increasing the transcriptional activity of the c-myc oncogene, and this potentiation can be inhibited by blocking the Ca2+/calcineurin/NFAT signaling pathway or disrupting NFAT expression [13]. Further, NFATc1 promotes the growth and tumorigenesis of ovarian cancer cells by up-regulating c-myc through ERK1/2/p38/MAPK activation [14].

Warburg effect (also known as aerobic glycolysis), exhibiting elevated glucose consumption and increased lactate production, is common in cancer cells [15,16]. The Warburg effect meets the demands for cell proliferation during cancer development, such as macromolecular synthesis and energy production [17,18]. Pyruvate kinase M2 isoform (PKM2) is an important isoenzyme in Warburg effect in cancer cells. High expression of PKM2 was observed in serum of patients with urological tumors [19], cervical cancer [20], and colon cancer [21], and nuclear translocation of PKM2 is reported to impair oxidative phosphorylation and PCa metastasis [22]. However, the effect and molecular mechanism of NFATc1, in cooperation with c-myc and PKM2, of PCa proliferation and metastasis remains unclear.

Table 1. NFATc1 interference target design results.

Name	Sequences
NFATc1 target site 1 (833)	GCCTGTACCACAACAATAA
NFATc1 target site 2 (1556)	CCGAAGACTACTCCTCTTT
NFATc1 target site 1 (2093)	GGAAAGGAGAGACGGACAT
Negative control	CCTAAGGTTAAGTCGCCCTCG

In this study, down-regulation of NFATc1 inhibited the proliferation, migration, and invasion of DU145 and PC-3 cells, concurrent with a decrease of c-myc and PKM2 protein levels, accompanied with decreased glucose consumption and lactate production. Furthermore, the effects of NFATc1 down-regulation on PCa cells were significantly counteracted by c-myc and PKM2 up-regulation, and the expression of PKM2 in PCa cells was positively regulated by c-myc and NFATc1 expression. These findings suggest that NFATc1 down-regulation suppresses PCa cell proliferation and metastasis, probably by regulating c-myc and PKM2 expression.

Material and Methods

Cell culture

Two human PCa cell lines – DU145 and PC-3 – were purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). DU145 and PC-3 cells were cultured with RPMI-1640 medium (HyClone, SH30809.01B, USA) supplemented with 10% fetal bovine serum (FBS, GIBCO, 16000-044, USA) and 1% double antibiotics (penicillin and streptomycin, Solarbio, P1400-100, China), incubated in a 5% CO_2 incubator at 37°C. According to cell demands, the medium was refreshed every day or every other day.

Construction of the lentivirus

Three shRNA targeting NFATc1 (NM_172390.2, Table 1) and a negative control shRNA were designed to construct NFATc1 interference lentivirus, and the shRNA construct was inserted into AgeI/EcoI restriction sites of a pLKO.1-Puro vector. The coding DNA sequence (CDS) region of c-myc (NM_002467.5) or PKM2 (NM_002654.5), synthesized by Genewiz Company (Shanghai, China), was inserted into EcoR I/BamH I restriction sites of a pLVX-Puro vector. The synthesized core plasmid was confirmed by DNA sequencing (Majorbio, Shanghai). Subsequently, pLKO.1-Puro-shNFATc1, pLVX-Puro-c-myc, and pLVX-Puro-PKM2 were co-transfected with psPAX2 and pMD2.G (Addgen, USA) viral packaging plasmids into 293 cells by Lipofectamine 2000 (Invitrogen). After 48 h of infection, the lentivirus particles in the supernatant were collected.

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Experimental grouping

To down-regulate NFATc1 expression by lentivirus infection, DU145 or PC-3 cells were divided to infect with NFATc1 interference lentivirus (shNFATc1-1, shNFATc1-2 and shNFATc1-3)/negative control (shNC), while the cells treated with medium were used as control. After 48 h of infection, NFATc1 expression was quantified by real-time PCR and Western blot. Later, assays of cell proliferation, migration, and invasion, biochemical detections of glucose consumption and lactate production, as well as Western blot for associated-gene expression, were performed to explore the effect of NFATc1 on PCa cells.

To further study the effect of c-myc and PKM2 in PCa cells, PC-3 cells were divided to infect with lentivirus of c-myc overexpression (oe-c-myc)/Vector and PKM2 overexpression (oe-PKM2)/Vector, while the cells treated with medium served as control. Real-time PCR and Western blot were used to quantify NFATc1 expression 48 h later. Subsequently, PC-3 cells were divided to infect with shNC, Vector, shNFATc1, shNC + c-myc, shNFATc1 + c-myc, shNC + PKM2, and shNFATc1 + PKM2. The cell proliferation, glucose consumption, lactate production, and PKM2 protein level were detected.

Cell proliferation assay

The Cell Counting Kit-8 (CCK-8, SAB, CP002) was applied to assess cell proliferation rate. NFATc1-silenced DU145 or PC-3 cells were digested with 0.25% trypsin (Solarbio, T1300-100). Cell suspension was seeded at a density of 3×10^3 cells/well in 96-well culture plates with 3 identical wells as duplicate wells, followed by incubated in a 5% CO_2 humidified incubator at 37°C overnight. The next day, 100 µl of CCK-8 solution (CCK-8: serum-free medium=1: 10) was added and incubated for 1 h. The optical density (OD) of the absorbance at 450 nm was measured by a microplate reader (Perlong, Beijing). NFATc1-silenced PC-3 cells infected with oe-c-myc or oePKM2 lentivirus were seeded in 96-well plates at equal numbers, and cell proliferation was measure at 0, 24, 48, and 72 h.

Real-time polymerase chain reaction (RT-PCR)

Total RNA from lentivirus-infected DU145 or PC-3 cells was extracted by Trizol reagent (Invitrogen, 1596-026) and quantified, and the integrity of RNA was confirmed by 1% electrophoresis. Portions of the RNA were reversed into cDNA using a reverse transcriptase kit (Fermentas, #K1622). Later, RT-PCR reactions with cDNA as template were conducted on a real-time detector (ABI, ABI-7300, USA) using a SYBR Green PCR kit (Thermo, #K0223). Finally, using the method of $2^{-\Delta\Delta CT}$, the mRNA levels of NFATc1, c-myc and PKM2 normalized to GAPDH in DU145 or PC-3 cells were analyzed. The primers were as follows: NFATc1, 5' CACCGCATCACAGGGAAGAC 3' and 5' GCACAGTCAATGACGGCTC 3'; c-myc, 5' GGCTCCTGGCAAAAGGTCA 3' and 5' CTGCGTAGTTGTGCTGATGT 3'; PKM2, 5' ATGTCGAAG CCCCATAGTGAA 3' and 5' TGGGTGGTGAATCAATGTCCA 3'; GAPDH, 5' CACCCACTCCTCCACCTTTG 3' and 5' CCACCACCCTGTTGCTGTAG 3'. In addition, RT-PCR procedure as follows: 95°C, 10 min (95°C, 15 s; 60°C, 45 s)×40; 95°C, 15 s; 60°C, 1 min; 95°C, 15 s; and 60°C, 15 s [23].

Western blot analysis

After 48 h of infection, DU145 or PC-3 cells were harvested to lyse in RIPA buffer (Solarbio, R0010, Beijing, China) complemented with protease and phosphatase inhibitors, and incubated on ice for 30 min to fully homogenize. After centrifuging at 12 000 g at 4°C for 10 min, the protein in the supernatant was collected and quantified by a BCA protein kit (Thermo Fisher Scientific, PICPI23223). Portions of protein were separated by 10% SDS-PAGE electrophoresis, and then transferred semi-dry to polyvinylidene fluoride (PVDF) microporous membrane (Millipore, HATF00010, Bradford, MA, USA). The blots were blocked in 5% skim milk (BD Biosciences, BYL40422, USA), followed by incubation with primary antibodies against NFATc1 (1: 1000, Cell Signaling Technology [CST], #8032, Danvers, MA, USA), c-myc (1: 1000, Abcam, Ab39688, Cambridge, MA, USA), PKM2 (1: 1000, Abcam, Ab38237), GAPDH (1: 2000, CST, #5174) at 4°C with gentle shaking overnight. The next day, after washing 5–6 times, the blots were incubated with corresponding secondary antibodies (1: 1000, Beyotime, Shanghai, China) at room temperature for 1 h. After washing again and incubation with chemiluminescent reagent (Millipore, WBKLS0100) for 5 min, the blots were visualized by an ECL imaging system (Tanon, Tanon-5200) and the protein levels were calculated by Image J software (version 1.47v, Bethesda, MD, USA).

Detection of glucose consumption and lactate production

DU145 or PC-3 cells were infected with lentivirus for 48 h. Glucose consumption was evaluated by a glucose test kit using glucose oxidase-peroxidase method, while lactate production was evaluated by a lactic acid kit. Following the instructions, the samples were prepared and the OD of absorbance at 505 nm (glucose) or 530 nm (lactate) was measured by a spectrophotometer.

Cell migration and invasion assays

Transwell assays using a modified Boyden chamber (Transwell, Costar, 3422, Cambridge, MA) were used to measure migrating or invasive cells as previous described [24]. Lentivirus-infected cells were cultured with serum-free medium for 24 h. Before inoculation, Transwell chambers and 24-well plates were soaked with 1×PBS for 5 min (invasion experiments required 1 more step: 80 µl of matrigel was placed in small chambers and clotted in a 37°C incubator for 30 min). The cells were digested to

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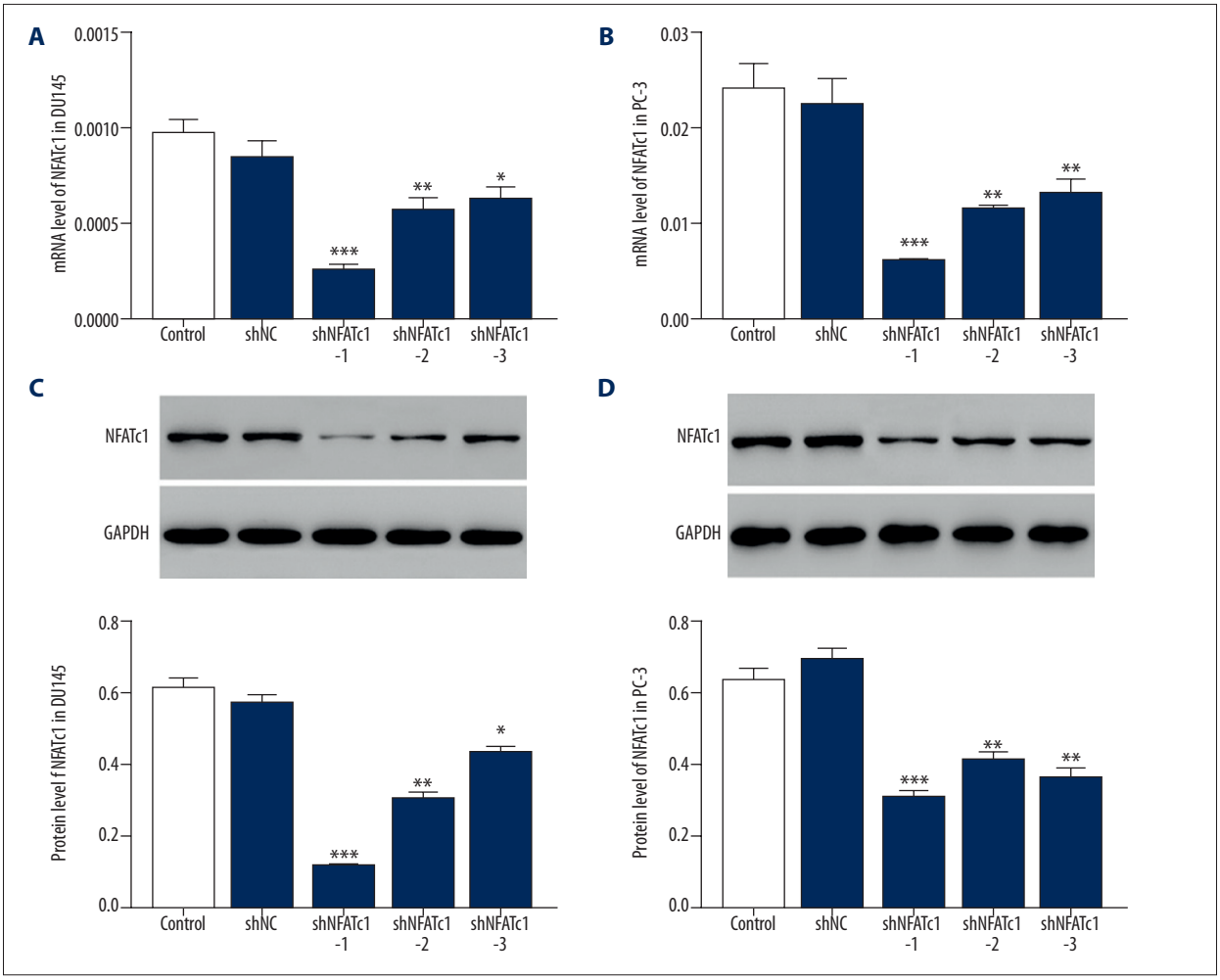

Figure 1. Down-regulation of NFATc1 in PCa cells by lentivirus infection. PCa cells were infected with lentivirus of shNC, shNFATc1-1, shNFATc1-2 and shNFATc1-3, while cells treated with medium were as Control. After 48 h, (**A, C**) the mRNA and protein levels of NFATc1 in DU145 cells were respectively quantified by RT-PCR and western blot analysis. (**B, D**) NFATc1 expression in PC-3 cells was detected. Data were shown as mean ±SD, * P<0.05, ** P<0.01, *** P<0.001 compared to shNC.

inoculate into Transwell chambers and 24-well plates (soaked with 1×PBS for 5 min), and to the lower surface of the 24-well plate we added 0.7 ml of RPMI-1640 medium containing 10% FBS as a chemoattractant with 3 repeated wells to incubate in a 37°C incubator for 48 h. Cells were fixed with 1 ml/well of 4% formaldehyde for 10 min, and then we removed the adhering cells, followed by washing once with 1×PBS. Subsequently, the cells were stained with 1 ml/well 0.5% crystal violet for 30 min, washed 3 times with 1×PBS, and dried. The migrating or invading cell numbers were counted by a 200×microscope.

Statistical analysis

All statistical analyses were performed using GraphPad prism 7.0 software (GraphPad Software, USA). Quantitative data are shown as mean ±SD with 3 independent experiments. We used the *t* test to conduct the comparisons between 2 groups. Comparisons among multiple groups were conducted by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison. P value less than 0.05 in all data were considered significant.

Results

Down-regulation of NFATc1 in PCa cells by lentivirus infection

Previous study of immunohistochemistry has revealed that NFATc1 expression is markedly increased in PCa [25,26]. Here, *in vitro*, the expression of NFATc1 in DU145 and PC-3 cells was down-regulated by lentivirus infection. The results Figure 1 show the mRNA (Figure 1A) and protein (Figure 1C) levels of NFATc1 were significantly down-regulated by NFATc1 lentivirus infection in DU145, and the effect of shNFATc1-1 was much better than the other 2.

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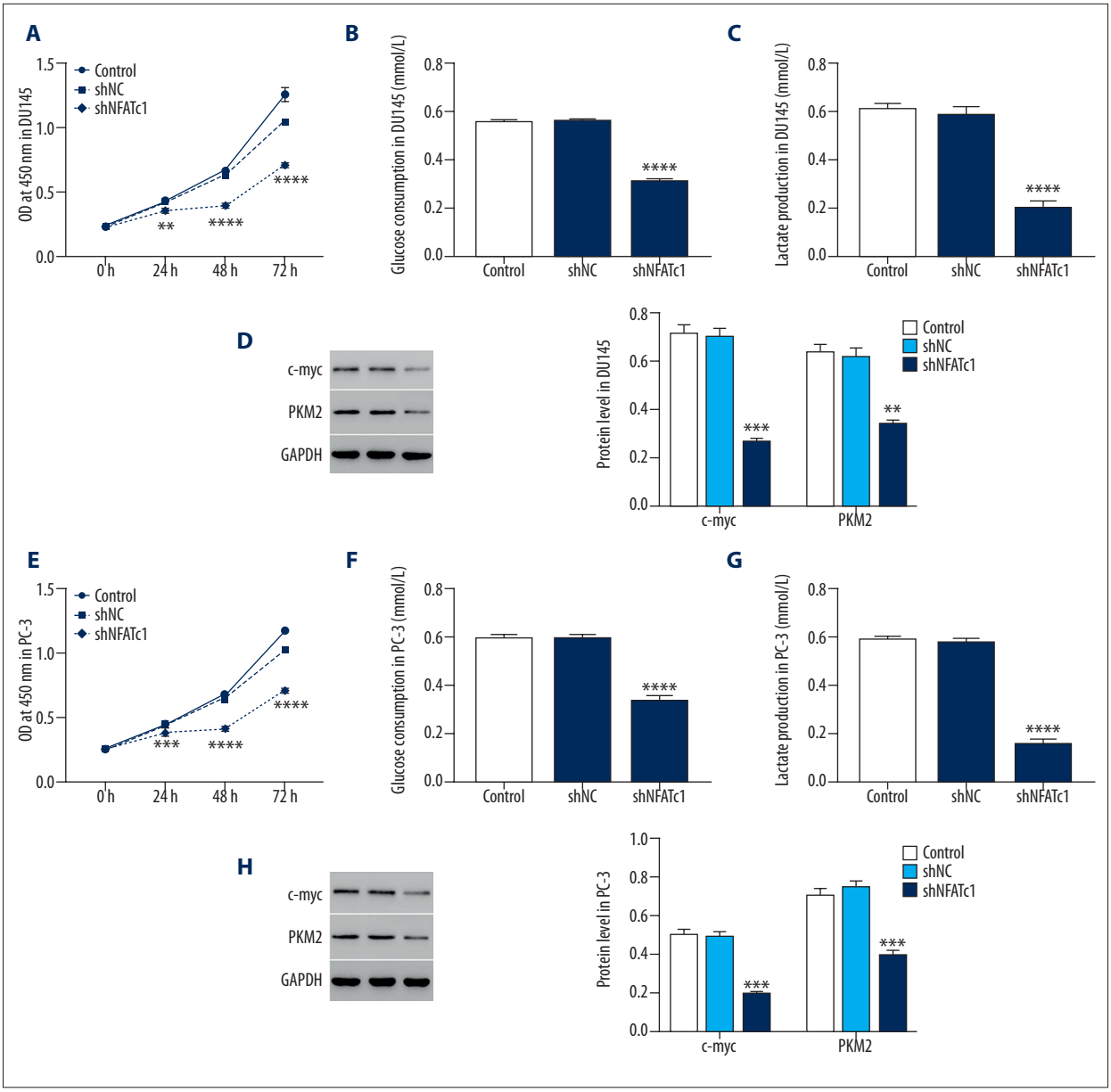

Figure 2. Down-regulation of NFATc1 inhibited proliferation and Warburg effect in PCa cells. PCa cells were divided to respectively infect with lentiviruses of shNC and shNFATc1. (**A, E**) the CCK-8 assays were performed at 0, 24, 48 and 72 h to evaluated the cell proliferation of DU145 or PC-3 cells. (**B, F**) The glucose consumption in DU145 or PC-3 cells was measured by biochemical detections. (**C ,G**) The lactate production in DU145 or PC-3 was also measured. (**D, H**) c-myc and PKM2 protein levels were detected by western blot analysis. All results were expressed as mean ±SD, ** P<0.01, *** P<0.001, **** P<0.0001 compared to shNC.

Likewise, the NFATc1 expression in PC-3 was also markedly down-regulated (Figure 1B, 1D). Therefore, lentivirus of shNFATc1-1 was selected for subsequent experiments due to it effective action.

Down-regulation of NFATc1 inhibited proliferation and Warburg effect in PCa cells

After NFATc1 down-regulation, we investigated the effect of NFATc1 on proliferation and Warburg effect of PCa cells. As shown in Figure 2, down-regulation of NFATc1 significantly inhibited the proliferation of DU145 (Figure 2A) and PC-3 (Figure 2E) cells. Studies have revealed that glucose consumption and lactate production are remarkably elevated in cancer cells [15,27], consistent with our observation that there was a significant decrease of glucose consumption (Figure 2B, 2F) and lactate production (Figure 2C, 2G) in NFATc1-silenced PCa cells, concurrent with obviously reduced c-myc and PKM2 protein levels (Figure 2D, 2H). These results indicate that NFATc1

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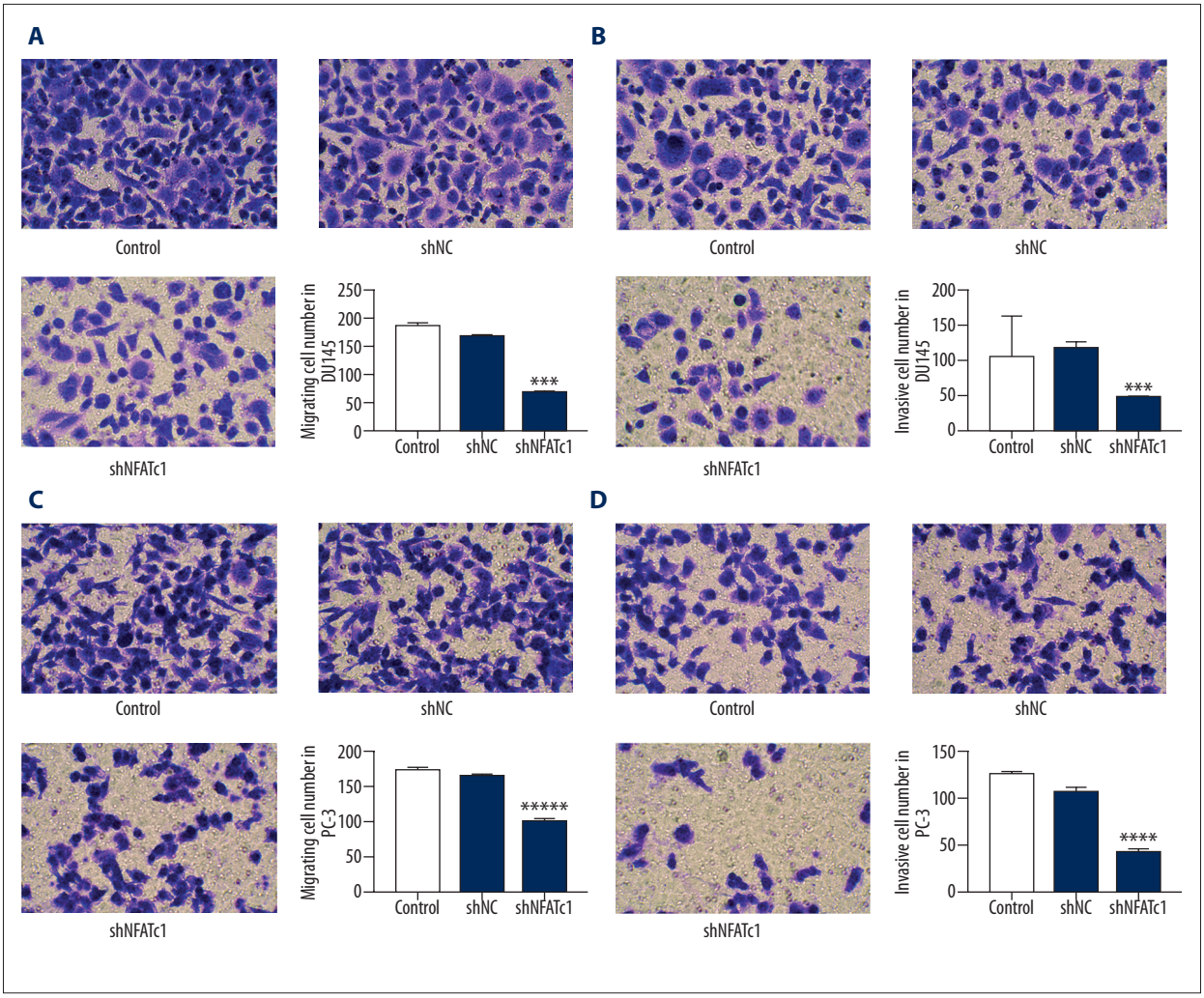

Figure 3. Down-regulation of NFATc1 suppressed migration and invasion of PCa cells. After infected with shNFATc1/shNC, the migration and invasion abilities of DU145 and PC-3 cells were assessed by Transwell assays. (**A, B**) The migrated or invaded cell numbers in NFATc1-silenced DU145 cells. (**C, D**) The migrated or invaded cell numbers NFATc1-silenced PC-3 cells. The data were presented as mean ±SD, *** P<0.001, **** P<0.0001 compared to shNC.

down-regulation potently suppressed proliferation and Warburg effect in PCa cells, probably by regulating c-myc and PKM2 expression.

Down-regulation of NFATc1 suppressed migration and invasion of PCa cells

In addition to its role in regulating cell proliferation and the Warburg effect, studies in recent years have also reported that NFAT plays a key role in tumor metastasis, such as in ovarian cancer [10]. Here, the abilities of migration and invasion in NFATc1-silenced DU145 and PC-3 cells were evaluated. The results presented in Figure 3 show that NFATc1 down-regulation potently suppressed the cell migration activities in both DU145 (Figure 3A) and PC-3 (Figure 3C) cells, and NFATc1-silenced DU145 (Figure 3B) and PC-3 (Figure 3D) cells were also less invasion. These results demonstrated the inhibitory effect of NFATc1 down-regulation on migration and invasion abilities of PCa cells.

Up-regulation of c-myc and PKM2 in PC-3 cells by lentivirus infection

To further explore the effects of c-myc and PKM2 *in vitro*, lentiviruses of oe-c-myc and oe-PKM2 were used to up-regulate the expression of c-myc and PKM2 in PC-3 cells. Data shown in Figure 4 suggest that c-myc mRNA (Figure 4A) and protein (Figure 4C) expression was significantly up-regulated in PC-3 cells by oe-c-myc lentivirus infection. Similarly, PKM2 expression in PC-3 cells was also up-regulated by oe-PKM2 (Figure 4B, 4D). Thus, the lentiviruses of c-myc and PKM2 were utilized for further studies.

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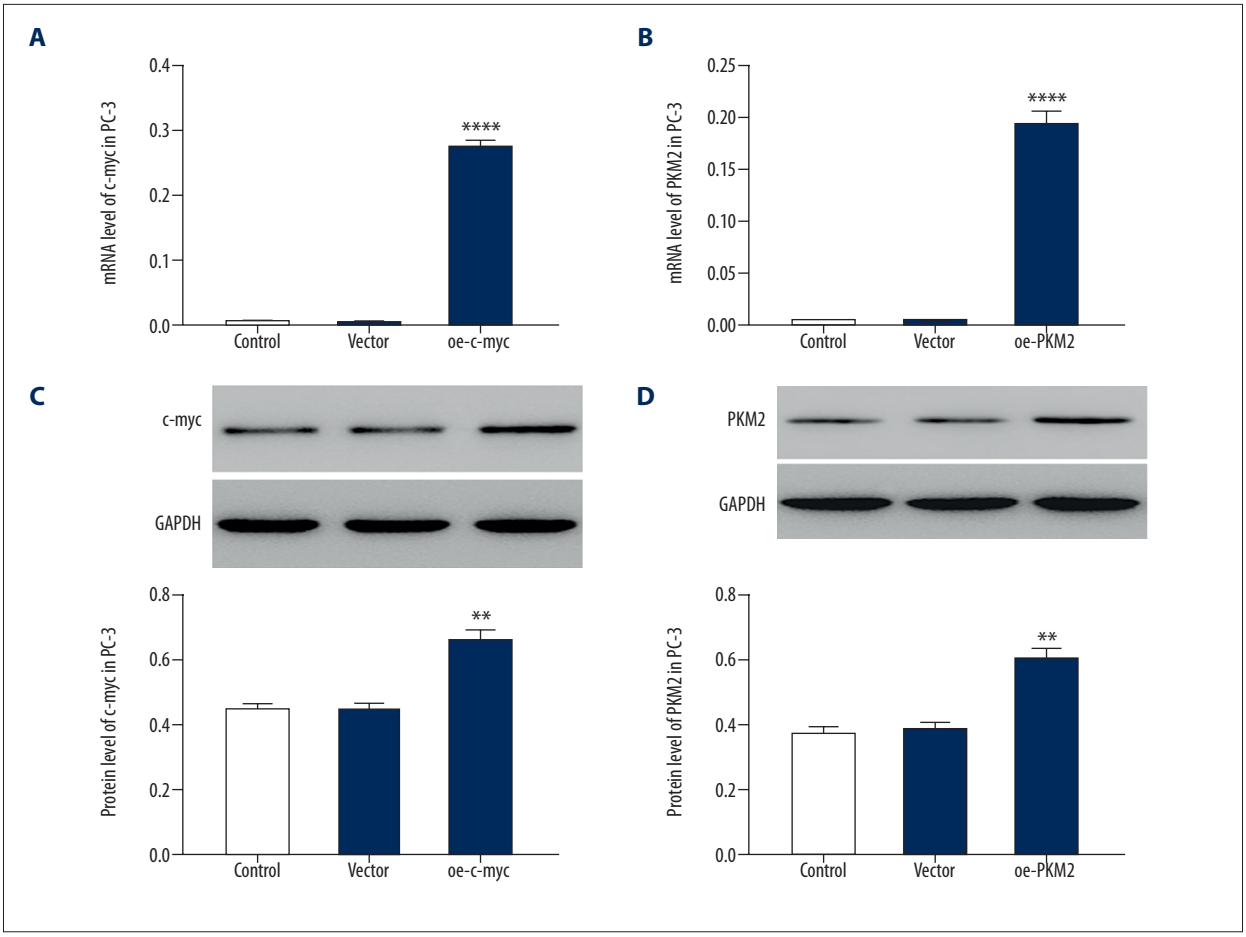

Figure 4. Up-regulation of c-myc and PKM2 in PC-3 cells by lentivirus infection. PC-3 cells were infected with lentivirus of Vector/oe-c-myc and Vector/oe-PKM2, while cells treated with medium were as Control. (**A, C**) The mRNA and protein levels of c-myc were respectively quantified by RT-PCR and western blot analysis. (**B, D**) The PKM2 mRNA and protein expression was detected. Data were shown as mean ± SD, * P<0.05, ** P<0.01, *** P<0.001 compared to Vector.

NFATc1 suppressed the cell proliferation and Warburg effect via regulating PKM2 and c-myc

Further studies on the mechanism of NFATc1 in PCa found that NFATc1 down-regulation-induced inhibition of Warburg effect (Figure 5A, 5B) and cell proliferation (Figure 5C), migration (Figure 5E), and invasion (Figure 5F) were significantly counteracted by c-myc and PKM2 up-regulation. In addition, PKM2 expression was positively regulated by the expression of NFATc1 (Figure 5D). It agreement with previous studies showing that constitutively low PKM2 activity is closely related to Warburg effect [28,29], and c-myc located downstream of NFAT) is a master regulator of cellular proliferation and metabolism [30]. These confirmed that NFATc1 regulated the cell proliferation and Warburg effect of PCa possible through the regulation of c-myc and PKM2 expression.

Discussion

NFATc1, as an independent prognosticator of positivity in carcinomas, is related to malignancy of several cancers. For example, it is reported that NFATc1 overexpression is a predictor of poor prognosis of patients with upper urinary tract urothelial carcinoma [31], and overexpression of NFATc1 in ovarian cancer is correlated with poor prognosis at the clinical tissue level [32]. NFATc1 silencing reduced proliferation, migration, and invasion of ovarian cancer cells and decreased the tumorigenesis of ovarian cancer in nude mice [10]. In addition, high expression of NFATc1 is observed in PCa and there is a correlation between NFATc1 and the risk of recurrence after radical prostatectomy, suggesting the critical important of NFATc1 in PCa outgrowth [25]. In the present study, we found that NFATc1 regulated cell proliferation, migration, invasion, and Warburg effect in PCa, probably through modulating c-myc and PKM2 expression.

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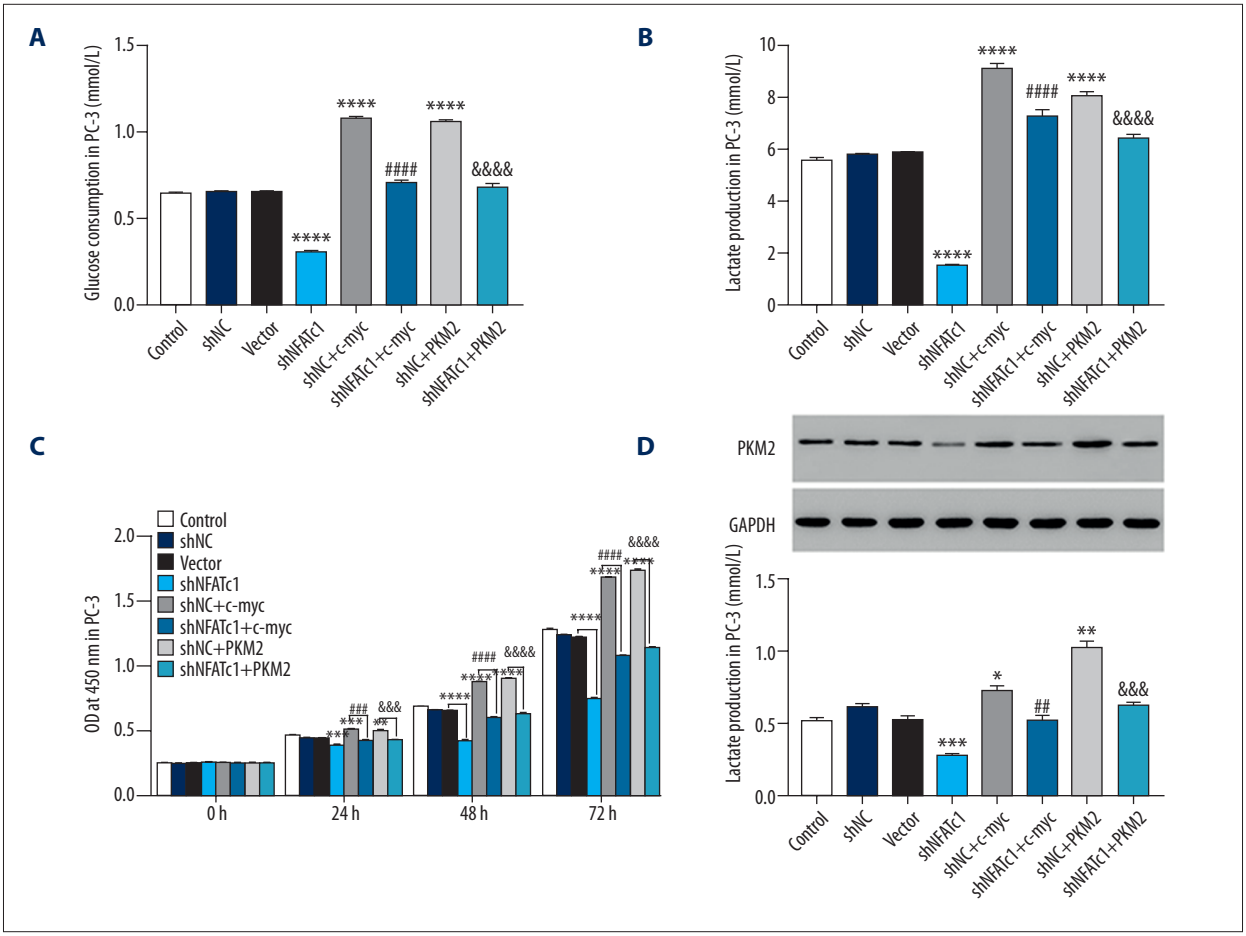
A previous study has reported that the cellular proliferation regulation process is closely related to the cellular metabolism process [30]. The proto-oncogene c-myc, located downstream of NFAT, plays a primary role in these processes in tumors [25,30]. Recent studies have revealed that the glucose metabolism in tumors is potently influenced by c-myc expression [33]. PKM2 is a special glycolytic enzyme that regulates the final rate-limiting step of glycolysis, contributing to the growth, survival, and metabolism of cancer cells [34–37], in agreement with our *in vitro* finding that down-regulation of NFATc1 significantly suppressed the proliferation and Warburg effect of PCa cells, concurrent with a decrease of c-myc and PKM2 expression. PKM2 is reported to promote tumor angiogenesis and the expression of PKM2 coincides with cancer cell glycolysis addiction and is associated with chromosome segregation and mitosis in tumor cells, and decreasing PKM2 can partly inhibit the growth of liver cancer cells [18,38]. Besides proliferation and Warburg effect, we found that the ability of migration and invasion in NFATc1-silenced PCa cells were also markedly inhibited, which was consistent with previous observations that cyclosporine A and tacrolimus suppress proliferation, migration, and invasion of PCa cells through inhibiting NFATc1 expression [34]. Further, we found that NFATc1 down-regulation-induced inhibition of Warburg effect and cell proliferation, migration, and invasion were significantly counteracted by c-myc and PKM2 up-regulation, and the expression of PKM2 was positively regulated by NFATc1 expression. In pancreatic cancer cells, NFATc1, as a transcription factor, is suggested to be an upstream regulator of c-myc [13]. Therefore, we inferred that down-regulation of NAFTc1 inhibited the transcription of c-myc and PKM2 in PCa. However, further studies are needed to explore the detailed mechanisms. These results indicated that down-regulation of NFATc1 suppresses the cell proliferation, Warburg effect, migration, and invasion abilities of PCa cells, possible by down-regulating c-myc and PKM2 expression.

Conclusions

In summary, we demonstrated the inhibitory effect of NFATc1 down-regulation on the cell proliferation and Warburg effect, as well as migration and invasion abilities, of PCa cells, possibly by down-regulating the expression of c-myc and PKM2. Thus, NFATc1 may be a therapeutic target for PCa and down-regulating NFATc1 probably contributes to PCa treatment.



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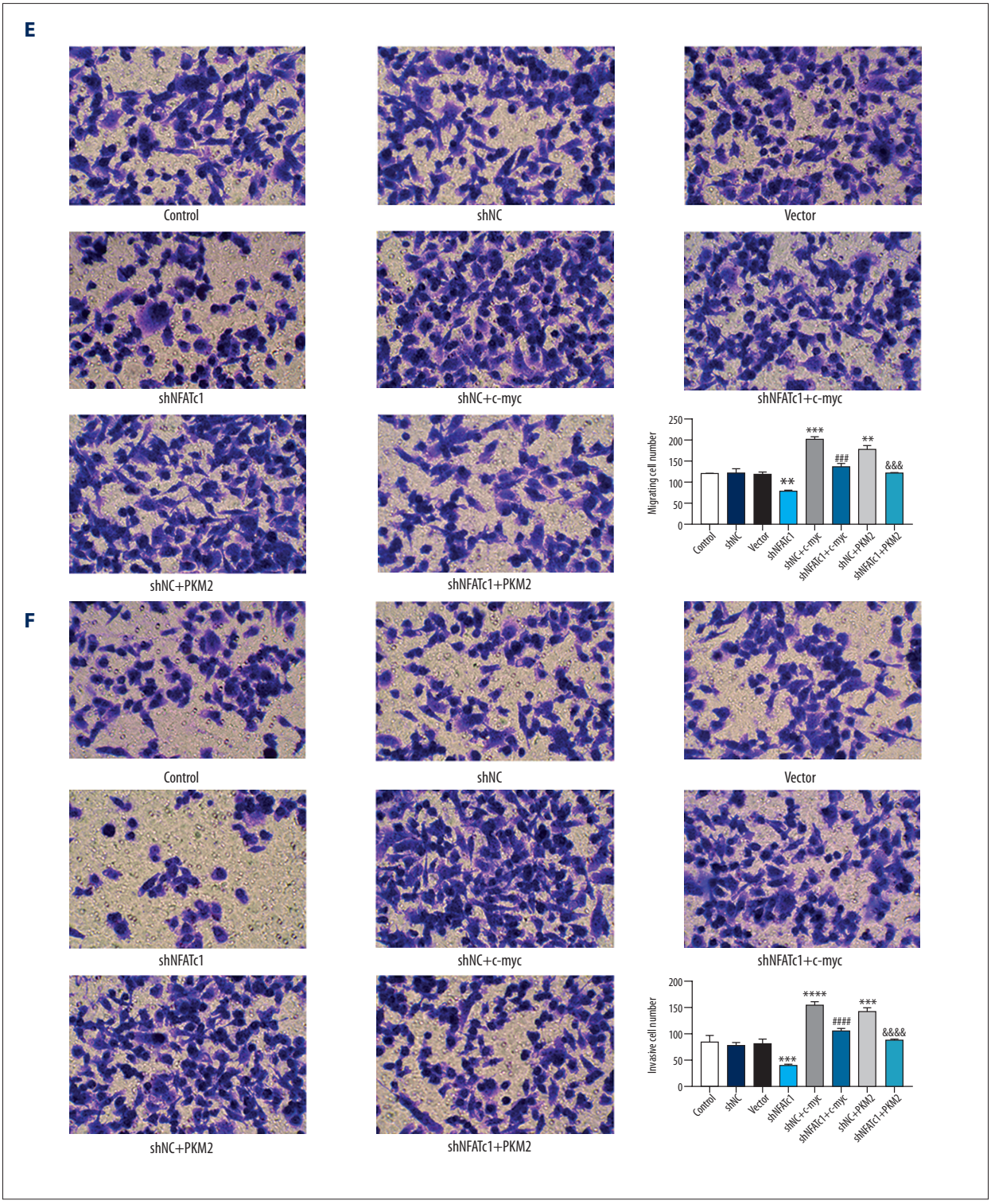

Figure 5. (**A–F**) FNFATc1 suppressed the cell proliferation and Warburg effect via regulating PKM2 and c-myc. PC-3 cells were divided to respectively infect with lentivirus of shNC, Vector, shNFATc1, shNC + c-myc, shNFATc1 + c-myc, shNC + PKM2, shNFATc1 + PKM2, while the cells treated with medium were as Control. (**A**) The glucose consumption in infected PC-3 cells was assessed. (**B**) The lactate production was detected. (**C**) The cell proliferation was evaluated at 0, 24, 48, and 72 h. (**D**) PKM2 protein level was detected. The data were presented as mean ±SD, * P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001 compared to shNC, ## P<0.01, ### P<0.001, #### P<0.0001 compared to shNFATc1, &&& P<0.001, &&&& P<0.0001 compared to shNFATc1.

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