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Up-regulation of miR-124 inhibits invasion and proliferation of prostate cancer cells through mediating JAK-STAT3 signaling pathway

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Abstract. – OBJECTIVE: Signal transducer and activator of transcription 3 (STAT3) is an important protein in Janus kinase (JAK)-STAT signaling pathway, and can facilitate expression of Bcl-2 and Cyclin D1 gene, thus playing a role in tumor pathogenesis. Bioinformatics analysis revealed targeted binding sites between mircroRNA-124 (miR-124) and 3'-UTR of STAT3 mRNA. This study aims to investigate the role of miR-124 in regulating STAT3 expression and proliferation, cycle, apoptosis and invasion of prostate cancer cells.

MATERIALS AND METHODS: Dual lucif porter gene assay demonstrated targeted la- tion between miR-124 and STAT3. Expressi f miR-124, STAT3, p-STAT3, Bcl-2 and Cyclin D1 compared between normal human prostate epith lial cell RWPE-1 and prostate ca DU145. *In vitro* cultured DU145 cells we ith miR-124 mimic and/or si-STAT3, t pare ression of STAT3, phosphorylated 3 (p-ST B-cell lymphoma-2 (Bcl-2) and D1. try detected cell apopt is llowed by clonal formation and swell o test malignant proliferation a ell invasion

RESULTS: Ta regulation d between miR-12 TAT3. Comp ing to RWPE-1, DU ells ver miR-124 expression, G0/G hase ratio, apoptosis, plus higher ex sion of STAT3, AT3, Bcl-2 and Cyclin D atio of S or G2/M ph e. Transfection of mi 4 mim nd/or si-STAT3 remarkably dec ed ge expression, weakened clonal f on invas ratio of S and G2/M phase, ptosis ncreased G0/G1 ratio.

CONCL S: M 24 up-regulation signifi- supp AT3, pSTAT3 and downstre Bcl-2 a lin D1 expression, weakens cell sion or malignant proliferation potency, ind hase arrest, and facilitates cell a osis.

Words:
24, STAT3, Cell apoptosis, Cell cycle, Invasion, Pro on, Prostate cancer.

Introduction

state cancer (PCa) is one common malint tumor in m urinary-reproductive system, is the sixth pular cancer in males[1]. PCa uently occu aged males, as more than 9 tients w between 60 and 80 years old. Geo bution of PCa patients showed specific patterns, as Western countries had significantly higher incidence than China[2]. Janus AK)-signal transducer and activator of ans tion (STAT) signal transduction pathway is widely involved in regulating cell proliferation, apoptosis, migration and invasion, thus is closely correlated with tumor occurrence[3]. STAT3 is the most important member of STAT protein family. As one transcription factor, STAT3 can facilitate expression of genes involving in cell proliferation, cell cycle, apoptosis, invasion and degradation of extracellular matrix (ECM), and is thus closely correlated with occurrence, progression and distal metastasis of multiple tumors including pancreatic carcinoma[4], colon cancer[5] and breast cancer[6]. A previous study showed that anti-apoptotic factor B-cell lymphoma-2 (Bcl-2)[7,8] and Cyclin D1[9,10] were all targeted genes of STAT3 transcriptional factor. By enhancing gene transcription and expression, STAT3 participates in facilitating cell proliferation and cycle progression, and also in antagonizing cell apoptosis; thus, it is one STAT protein with most close correlation with human tumor pathogenesis. Recent studies[11,12] showed the important role of enhanced STAT3 expression or functional activity in facilitating PCa pathogenesis. MicroRNA (miR) is one endogenous small RNA molecule in eukaryotic cells, and can regulate target gene expression via complementary binding on 3'-untranslated region (3'-UTR)

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of target gene mRNA to degrade mRNA or inhibit mRNA translation, modulating cell proliferation, differentiation and migration. The role of abnormal expression of function of mRNA in tumor onset has drawn increasing research focus[13]. Studies showed significantly decreased miR-124 expression in PCa tissues/cells[14,15], suggesting its potential role as tumor suppressor gene in PCa occurrence. Bioinformatics analysis showed the existence of complementary binding sites between miR-124 and 3'-UTR of STAT3. This study investigated the role of miR-124 in regulating STAT3 and downstream target genes Bcl-2 and Cyclin D, and in affecting proliferation, cycle, apoptosis and invasion of PCa cells.

Materials and Methods

Major Reagent and Materials

Human prostate cancer cell line DU145 and normal prostate epithelial cell line RWPE-1 were purchased from Shengbo Biomed (Zhanjiang, China). Dulbecco's Modified Eagle's medium (DMEM), Keratinocyte-serum-free media (SFM) [illegible] re medium, fetal bovine serum (FBS) ar[illegible] tomycin-penicillin were purchased from [illegible]co (Rockville, MD, USA). Trizol and Lipofecta[illegible] 2000 were purchased from Invitrogen/Life Te[illegible] nologies (Carlsbad, CA, USA). QuantiTect SYB[illegible] Green RT-PCR Kit was purch[illegible] Qiagen (Hilden, Germany). miR-124[illegible]mic a[illegible]miR-NC nucleotide fragments were[illegible]signed [illegible]synthesized by Ruibo Biotech[illegible] Co[illegible] gzhou, China). siRN[illegible] seq[illegible]d negative control sequence we[illegible]nthesiz[illegible]GE Pharmakon (Milpitas, CA[illegible]A). Mouse a[illegible]AT3 and p-STAT3 was p[illegible]from Abcam ([illegible]mbridge, MA, USA). R[illegible]t an[illegible]3 and Cyclin D1 were purchased f[illegible]om GeneTex[illegible]rvine, CA, USA). Transwell[illegible]mber was purc[illegible] from Greiner Bio-On[illegible]ickenhausen, Germany). Matrigel was purch[illegible]a from[illegible]-Rad Laboratories (Hercules, CA[illegible]A). D[illegible]ucifera[illegible]gene reporter plasmid pLUC[illegible]e vecto[illegible]s purchased from Am-bi[illegible]n (Ca[illegible]CA[illegible]A). Dual-Luciferase Re-Assay[illegible]as purchased from Promega (Ma[illegible]n, WI, [illegible]. PI dye, cell apoptosis assay reag[illegible]nd RIPA lysis buffer were purchased from B[illegible]g, China).

[illegible] Culture

[illegible]45 cells were cultured in Dulbecco's Modifie[illegible]agle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and were kept in a humidifi[illegible] with 5% CO_2 at 37°C. RWPE-1 cell[illegible]e kep[illegible] Keratinocyte-SFM medium cont[illegible]g 5 ng/mL EGF, 0.05 mg/mL bovine pituit[illegible]xtract (BPE) in a humidified chamber with 5[illegible]at 37°C. Cells at log-growth phase [illegible]satisfa[illegible]tatus were used for further ex[illegible]ents.

Luciferase Report[illegible]Gene [illegible]struct

Full length or m[illegible]frag[illegible]t of 3'[illegible]R of STAT3 gene wa[illegible]b-c[illegible]nto pL[illegible]Lucifera-se vector, w[illegible]was na[illegible]pL[illegible]-STAT3-wt. Lucifera[illegible]porter vecto[illegible]ng mutant form of 3[illegible]STAT3 gene[illegible]as also constructed[illegible]pLU[illegible]AT3-mut. Lipofectamine 2000 was used to tra[illegible]pLUC-STAT3-wt (or pLU[illegible]T3-mut) and [illegible]24 mimic (or miR-1[illegible]nhibitor, or miR-NC) into HEK293T cells. [illegible]er 48 h, dual l[illegible]iferase activity assay kit was [illegible]d to test dua[illegible]ciferase activity. Nucleotide [illegible]ences were[illegible]R-NC, 5'-ACUAC UGAGU C[illegible]G UAGA[illegible] miR-124 mimic, 5'-GGCAU UCA[illegible] CCUUA-3'; miR-124 inhibitor, 5'-UAAGG CACGC GGUGA AUGCC-3'.

[illegible]sfection and Grouping

In vitro cultured DU145 cells were divided into five groups: miR-NC transfection group, miR-124 mimic transfection group, si-NC transfection group, si-STAT3 group, and miR-124 mimic + si-STAT3 transfection group. 72 h after transfection, cells were collected for gene and protein expression assay. Nucleotide sequences for transfection were: si-STAT3 sense, 5'-CAUCU GCCUA GAUCG GCUA-3'; si-STAT3 anti-sense: 5'-UAGCC GAUCU AGGCA GAUG-3'; si-NC sense: 5'-UUCUC CGAAC GUGUC ACGU-3'; si-NC anti-sense, 5'-ACGUG ACACG UUCGG AGAA-3'.

qRT-PCR for Gene Expression

Trizol reagent kit was used to extract RNA following manual instruction. QuantiTest SYBR Green RT-PCR Kit was used to test gene expression by one-step qRT-PCR. In a 20 μL qRT-PCR system, there were 10 μL 2×QuantiTect SYBR Green RT-PCR Master Mix, 1.0 μL of forward and reverse primer (0.5 μm/L), 2 μg template RNA, 0.5 μL QuantiTect RT Mix, and ddH_2O. Primer sequences used were: miR-124P_F: 5'-CGGTA AGGCA CGCGG TGA-3'; miR-124P_R: 5'-AGTGC GAACT GTGGC GAT-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC

GCTTC ACGAA TTTG-3'; STAT3P$_F$: 5'-ATCAC GCCTT CTACA GACTG C-3'; STAT3P$_R$: 5'-CA-TCC TGGAG ATTCT CTACC ACT-3'; Bcl-2P$_F$: 5'-GGTGG GGTCA TGTGT GTGG-3'; Bcl-2P$_R$: 5'-CGGTT CAGGT ACTCA GTCAT CC-3'; CyclinD1P$_F$: 5'-CAATG ACCCC GCACG ATT-TC-3'; CyclinD1P$_R$: 5'-CATGG AGGGC GGATT GGAA-3'; β-actinP$_F$: 5'-GAACC CTAAG GC-CAA C-3'; β-actinP$_R$: 5'-TGTCA CGCAC GATTT CC-3'. PCR conditions were: 95°C pre-denature for 15 min, followed by 40 cycles each containing 94°C denature for 15 s, 60°C annealing for 30 s, and 72°C elongation for 30 s. Gene expression was examined on ABI ViiA™ 7 fluorescent quantitative polymerase chain reaction (PCR) cycler.

Western Blot

Radioimmunoprecipitation assay (RIPA) lysis buffer was used to extract protein. A total of 40 μg samples was separated in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and was then transferred to polyvinylidene fluoride (PVDF) membrane, which was blocked in 5% defatted milk powder at room temperature incubation. Primary antibody (STAT3 at 1:300, p-S T3 at 1:100, Bcl-2 at 1:200, Cyclin D1 at 1:200 at 1:800) was added for 4°C overnight incu n. After phosphate buffered saline tween-20 (P) rinsing, horse radish peroxidase (HRP) conjuga secondary antibody (1:5000 dilution) was add for 60 min of incubation. The was rin-sed in phosphate buffered sa weer (PBST) and incubated using Enha Chemil nescence (ECL, Amersham Bio es, L UK) method. After dar xpo velopment, the film was scanned analyz

Clonal Forma ay for Mal ant Growth Po y

Cells fro an transfec oups were inoculated into m diameter cu ish at 100 cells dens ells were incubated or 14-21 weeks. After t, cells e fixed in paraformaldehyde for ng w iemsa e. Clones were counted u agnifi n microscope. Clonal f mation = (number/inoculate cell r) ×10

Tra ell Assay for Cell Invasion Potency

y was employed to test cell invasion potency. In brief, 1×10^5 cells were inoculated the upper chamber containing Matrigel and s free Dulbecco's Modified Eagle Medium (DM). DMEM containing 10% fetal bovine serum (FBS) was added to the bottom chamber. After 48 h, un-penetrated cells wer Chambers were then fixed in meth and s ned with 0.1% crystal violet. C umber was counted under five randomly d high-magnification fields.

Cell Apoptosis Assay

Cells were collected digest d with try and were re-suspen m bin buffer. 5 μL Annexin V-fluoresc thi ate (F) and 5 μL propidium ide ining h r were sequentially a . Flow c try used to test cell apo

PI Stai g for Cycle

Cells were digeste trypsin and rinsed in pho buffered salin S). After 70% etha-n ation overnight and phosphate buffered saline (PBS) washi PI was added for staining in dark at 37°C 30 min. Flow cytometry was to detect ce cle.

Sta ysis

SPSS18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement presented as mean ± standard deviation D). dent *t*-test was used to compare measurement data between groups. Statistical significance was defined when $p<0.05$.

Results

miR-124 Targeted and Inhibited STAT3 Expression

Bioinformatics analysis showed the existence of complementary binding sites between miR-124 and 3'-UTR of STAT3 mRNA (Figure 1A). Dual luciferase gene reporter assay showed that transfection of miR-124 mimic and miR-124 inhibitor remarkably decreased or increased relative luciferase activity in HEK293T cells, respectively (Figure 1B), suggesting that miR-124 could target 3'-UTR of STAT3 mRNA and inhibited its expression. qRT-PCR results showed that transfection of miR-124 mimic and miR-124 inhibitor remarkably decreased and potentiated STAT3 mRNA expression in DU145 cells, respectively (Figure 1C).

MiR-124 Down-Regulation and STAT3 up-Regulation in DU145 Cells

qRT-PCR results showed that, compared to RWPE-1 cells, DU145 cells had significant-

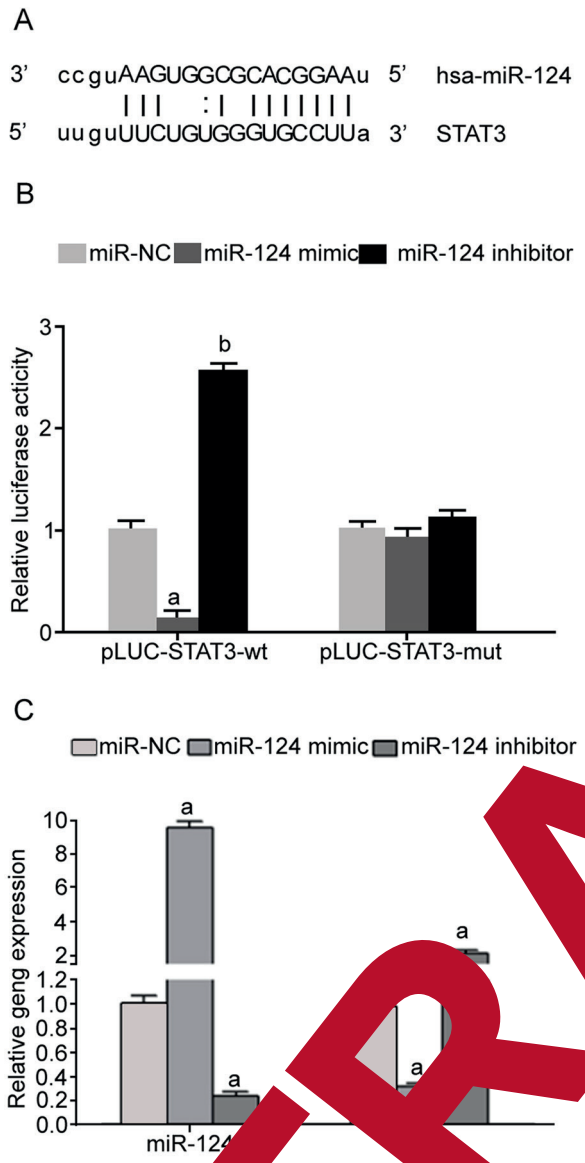

Figure 1. miR-12 and inhibited S expression. ***(A)*** Functi te miR-124 and 3'-UTR of STAT3 mRNA; (Dual luci ene reporter assay; ***(C)*** qRT-PCR fo 145 cell gene e a, $p<0.05$ comparing betwe R-124 mimic and i ; b, $p<0.05$ comparing b n miR-124 inhibitor and miR-NC.

ly de iR-12 pression, plus lower expressio STAT Bcl-2, Cyclin D1 mRNA e 2A). olot results showed significa higher tein expression of STAT3, p-S 3, Bcl-2 and Cyclin D1 in DU145 cells RWPE-1 cells (Figure 2B). Cell e test showed significantly higher S phase and G2/M phase ratio in DU145 cells than cells, whilst G0/G1 phase ratio was lowe DU145 cells (Figure 2C). Flow cytometry results showed significantly lower basal apoptotic rate of DU145 cells c RWPE-1 cells (Figure 2D).

MiR-124 up-Regulation In d STAT3 Expression, Cell invasion or eration, Induced cell Apoptosis d Cy est

Transfection of miR mimic a STAT3 significantly eased xpressio STAT3, p-STAT3, B and C D1 in DU145 cells (Figure 3A), w d tional f ation ability (Figure 3 dec ell inva potency (Figure 3C) potenti ll tosis (Figure 3D) or 1 phase arre e 3E).

Di ion

-STAT signal transduction pathway can re- nd to multiple tracellular growth factors and ogen stimuli der the existence of activator AK-STAT si pathway, member receptor n dergo erization, which can further phos d activate JAK kinase to phosphorylate eceptor tyrosine, facilitating the re- uitment of STAT onto tyrosine phosphorylation eptor complex via SH2 domain. Under s sc ario, JAK kinase can phosphorylate and activate STAT protein with spatial proximity, separating it from receptor complex to form dimer, which is transported from cytoplasm to nucleus, where it can facilitate transcription and expression of genes related to cell proliferation, cycler, and apoptotic regulation[16]. STAT3 is the most important member of STAT protein family (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6). Previous studies attributed anti-apoptotic factor B-cell lymphoma-2 (Bcl-2)[7,8] and Cyclin D1[9,10] as important target genes under regulation by STAT3 transcriptional factor. By enhancing gene transcription and expression, STAT3 participates in facilitating cell proliferation and cycle progression, and modulating/antagonizing cell apoptosis, makes it one STAT protein with closest correlation with human tumor pathogenesis. A previous study[17] showed elevated STAT3 expression in PCa patient tumor tissues, indicating its tumor-facilitating role in PCa. Reports[14,15] also indicated significantly lower miR-124 expression in PCa tumor tissues/cells, indicating its possible role as tumor suppressor gene in PCa pathogenesis. Bioinformatics analysis showed complementary binding sites between miR-124 and 3'-UTR of STAT3. This work thus investigated if miR-124

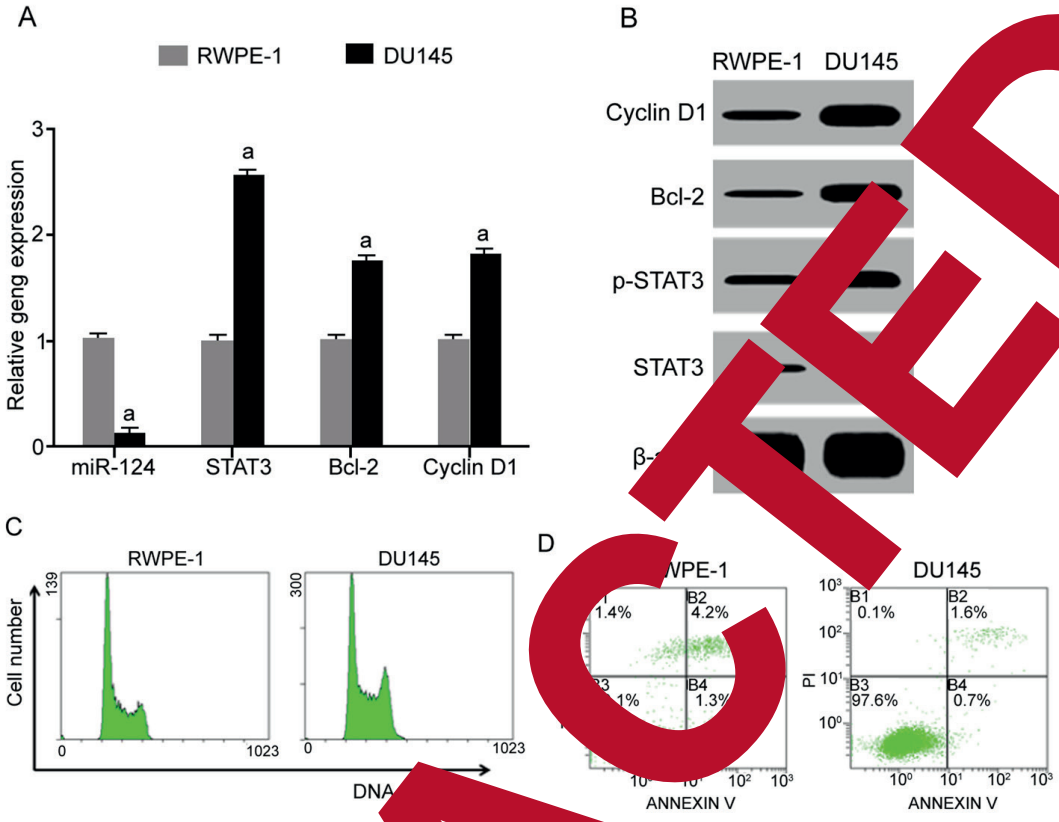

Figure 2. Higher miR-124 and lower STAT3 expressi... DU1... ...qRT-PCR for gene expression; ***(B)*** Western blot for protein expression; ***(C)*** Flow cytometry for cell cycle; ***(D)*** ...etry for cell apoptosis. a, $p<0.05$ comparing to RWPE-1 cells.

played a role in mediating S... 3 exp... ion and affecting proliferation, cyc... apoptos... d inva-sion of PCa cells. Dual... rase assay showed that tra... fecti... -124 mimic significantly depres... elative ... se activity, whilst miR-124 i... or elevated ... e lucife-rase activity, in... rgeted regula... y corre-lation betwee... R-12... STAT3. Transfection of miR-124 mimic and i... 4 inhibitor remar-kably in... ed and decrea... TAT3 mRNA expres... in DU145 cells, respectively, further dem... ating t... ted regulation between miR-12... STAT... 0/G1 p... e ratio of DU145 cells was s... y lowe... n that of RWPE-1 cel-l... whilst ... of S... se and G2/M phase were indi... d cell cycle progression. Flo... tometry ... ults showed that comparing to R... E-1 cells, DU145 cells had significantly lo... c rate. Further assay found decre-... miR-124 expression in DU145 cells, whilst ...ession of STAT3, p-STAT3, Bcl-2 and Cyclin D... elevated. These findings showed possible role ... miR-124 down-regulation up-regulating STAT3 and p-STAT3, facilitating downstream gene Bcl-2 and Cyclin D1 expression, and facilitating proliferation of PCa cells for suppressing their apoptosis. Chu et al[18] showed hyper-methylation of miR-124 gene promoter region in PCa cells, leading to suppression of miR-124 expression. Shi et al[15] found that comparing to prostate epithelium RWPE-1, PCa cell lines including 22Rv1, LNCaP, LAPC-4, cds2 and C4-2B, all had decreased miR-124 expression. Moreover, miR-124 expression level in PCa tissues was also lower than benign prostate tissue hyperplasia[15]. Falzarano et al[19] observed significantly elevated miR-124 expression in PCa patients after treatment, indicating that miR-124 expression was one important mechanism governing PCa pathogenesis. In this study, miR-124 was down-regulated in prostate cells, indicating its role in PCa pathogenesis, as consistent with Chu et al[18], Shi et al[15], and Falzarano et al[19]. Abdulghani et al[17] showed significantly elevated STAT3 expression in PCa tumor tissues, with higher expression level in those with bone or lung metastasis, indicating the correlation betwe-

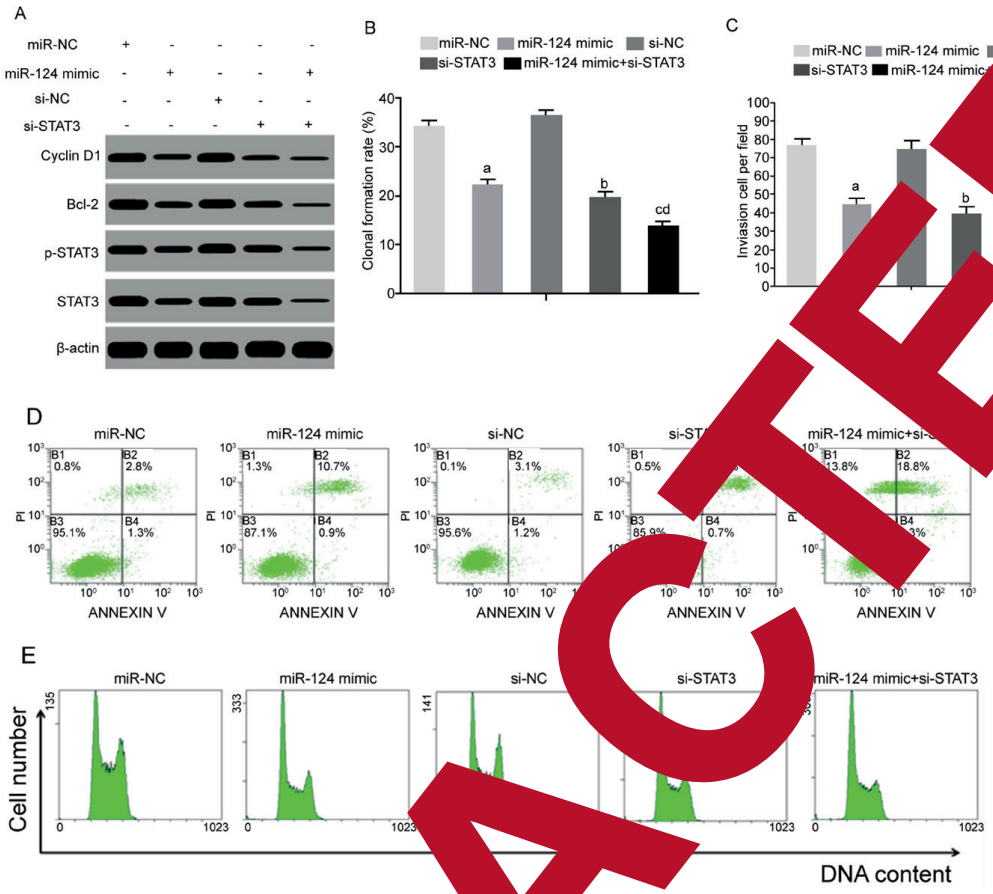

Figure 3. Elevated miR-124 expression inhibited STAT3 expression, cell invasion or proliferation, and induced cell apoptosis or cycle arrest. ***(A)*** Western blot for protein expression; ***(B)*** Clonal formation assay; ***(C)*** Transwell assay for cell invasion potency; ***(D)*** Flow cytometry for cell apoptosis; ***(E)*** Flow cytometry for cell cycle. a, $p<0.05$ comparing between miR-124 and miR-NC group; b, $p<0.05$ comparing between si-STAT3 and si-NC group; c, $p<0.05$ comparing between miR-124 mimic + si-STAT3 and miR-NC group; d, $p<0.05$ comparing between miR-124 mimic + si-STT3 and si-NC group.

en STAT3 expres and both o d distal metastasis pote Ca. In this st STAT3 expression w gnifi higher than normal human pros te epitheliu icating the role of STAT3 u gulation in PCa genesis, as similar w Abdulghani et al[17]. F ther researches foun t trans on of miR-124 mimic and/or si-S 3 sig antly wn-regulated STAT3, p-STA ownstr Bcl-2 and Cyclin D1 expressio U145 ls, thus weakening cell n an nt proliferation potency, indu cell c arrest at G0/G1 phase, and faci ng cell apoptosis. Shi et al[15] found that ov of miR-124 remarkably weakened proliferation potency of PCa cells. Chu et al showed that miR-124 up-regulation may suppress *vitro* proliferation and invasion of PCa cells targeted inhibition on androgen receptor expression, and weakening the tumor formation potency in recipient animals. Kang et al[20] revealed that miR-124 could target and inhibit PACE4 expression to exert anti-cancer effects for antagonizing PCa cell proliferation. Qin et al[21] showed that miR-124 up-regulation can inhibit PCa cell motility, migration and invasion via targeted inhibition on Slug gene expression to weaken the epithelial-mesenchymal transition (EMT) process. Shi et al[22] found that over-expression of miR-124 may suppress PCa cell proliferation, and potentiate their drug sensitivity towards enzalutamide induction. Moreover, intravenous injection of miR-124 significantly inhibited *in vivo* growth of PCa tissues and facilitated tumor cell apoptosis[23]. Abdulghami et al[17] demostated that over-expression of STAT3 significantly enhanced *in vitro* DU145 cell motility and distal metastasis *in vivo*. After

activation of STAT3 by JAK kinase inhibitor, PCa cell had significantly weakened cell motility and migration potency. All these studies revealed the role of miR-124 up-regulation in weakening malignant biological features of PCa cells, as supported by our results. In this study, STAT3 down-regulation weakened proliferation or invasion of PCa cells, as consistent with Abdulghani et al[17]. This work revealed the role of miR-124 down-regulation in inducing STAT3 up-expression and in facilitating PCa occurrence, whilst miR-124 up-regulation could weaken PCa proliferation, invasion or apoptosis resistance via targeted inhibition of STAT3 expression. All these results have not been reported before.

Conclusions

MiR-124 up-regulation significantly decreases STAT3, p-STAT3 and downstream Bcl-2 or Cyclin D1 expression in DU145 cells, whose invasion and malignant proliferation potency are weakened, along with induction of cell cycle arrest at G0/G1 phase to facilitate cell apoptosis.

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Conflict of interest

The authors declare no con of inter

R ences

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