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The Association Between WAVE1 and -3 and the ARP2/3 Complex in PC 3 Cells

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Abstract. *Background: Actin polymerisation is stimulated by the actin-related protein (ARP) 2/3 complex and drives cell migration. This complex is activated by Wiskott-Aldrich syndrome protein family (WASP) verprolin homologous protein (WAVE) proteins. WAVE1 and -3 have been implicated in the aggressiveness of metastatic prostate cancer cells. Materials and Methods: Cell growth, motility and invasion were analyzed in WAVE1- and WAVE3-knockdown PC-3 cells along with the ARP2/3 inhibitor, CK-0944636. Confocal microscopy was adopted to examine protein co-localisation. Immunoprecipitation approaches were used to determine protein tyrosine phosphorylation. Results: Cell growth suppression was observed with WAVE3 knockdown and ARP2/3 inhibition. Reduced cell invasion effects observed with WAVE1 knockdown appeared to be rescued by ARP2/3 inhibition. WAVE1 and WAVE3 and ARP2 co-localisation was lost in PC-3 WAVE-knockdown cells, while increased ARP2 tyrosine phosphorylation was observed with WAVE3 knockdown. Conclusion: These results implicate a contributory role of WAVE1 and -3 to the metastatic phenotype of PC-3 cells through their interaction with the ARP2/3 complex.*

Actin polymerisation is the mechanism that underlies multiple cellular processes that rely on the dynamic restructuring of the cytoskeleton. This process is dependent on the Actin-related protein (ARP) 2/3 complex that is unable to elicit this function without stimulation by its nucleation-promoting factors (NPF). The spontaneous assembly of pure actin monomers into filaments is kinetically unfavourable due to the relative instability of actin dimers (1). However, NPFs such as members of the Wiskott-Aldrich syndrome protein family (WASP) verprolin-homologous protein (WAVE) family can induce a conformational change that brings the ARP2 and ARP3 subunits into proximity to form an actin pseudo-dimer, thus forming an important preliminary 'seed' in actin polymerisation (2). This process promotes rapid actin polymerisation at the cell leading edge and is a primary principle underpinning cell migration. Although this order of events and its associated cell function are well defined, it is still not clear whether auxiliary proteins are involved in this complex mechanism and ultimately which are these collaborating proteins.

Increasing evidence points towards an association between WAVE and several human cancer types. In particular, carcinomas that progressed to an advanced stage were demonstrated to exhibit aberrant WAVE expression compared to normal tissues or benign cancer (3-6). Metastatic prostate cancer has been linked to a general trend of increase in WAVE1 and -3 levels (7, 8). These studies highlight the clinical importance of WAVE and potentially their role as a therapeutic target.

The metastatic potential of cancer cells is not solely limited to their motile ability but also to their capacity to proliferate and establish secondary tumours. Cancer metastasis also depends on the ability of tumour cells to resist apoptosis and induce angiogenesis. It is apparent that cancer metastasis is a multistep process that involves a complex network of protein interactions. Whilst the contribution of WAVE proteins to cell motility is well documented (9), exploring the role of WAVE could provide insight into the contribution of these proteins to other cellular functions in the context of a metastatic prostate cancer cell line.

Furthermore, as humans express three WAVE isoforms, it would be interesting to investigate whether any functional redundancy exists between these protein members and whether these different WAVE isoforms interact in a similar manner with the ARP2/3 complex. It is unclear whether actin polymerisation occurs in response to the interaction solely of

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Table I. *Primers used for polymerase chain reaction to analyze mRNA expression.*

Gene	Primer	Primer sequence 5'-3'
WAVE1	Forward	CCTCCTCCACCACCTCTTC
	Reverse	GCACACTCCTGGCATCAC
WAVE3	Forward	TACTCTTGCCGCTATCATACG
	Reverse	TGCCATCATATTCCACTCCTG
ARP2	Forward	ATTGAGCAAGAGCAGAAACT
	Reverse	ACTGAACCTGACCGTACATTCTGGTGCTTCAAATCTCT
ARP3	Forward	AGAAGTAGGAATCCCTCCCTCCAG
	Reverse	ACTGAACCTGACCGTACATTAATCCATTTTGACCCATC
GAPDH	Forward	GGCTGCTTTTAACTCTGGTA
	Reverse	GACTGTGGTCATGAGTCCTT

ARP2/3 or whether additional proteins are required. With these questions in mind, this study aimed to determine the effect on ARP2 and -3 expression of knockdown of *WAVE1*, and *WAVE3* expression in the metastatic prostate cancer cell line, PC-3. Following this, cell function assays were utilised to investigate the effect of ARP2/3 complex inhibitor.

The protein domains of WAVE are integral to their ability to interact with ARP2/3 and their association with the other four subunits that comprise the WAVE regulatory complex. While the domains of WAVE proteins do not appear to confer the ability of the protein to phosphorylate downstream targets, the effects of WAVE knockdown on the phosphorylation state of ARP2 and 3 were investigated. It is hoped that this will provide insight into the mode of action utilised by WAVE in the context of prostate cancer metastasis.

Materials and Methods

Materials. The PC-3 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). All cells were maintained in Dulbecco's modified Eagle's medium (DMEM)-F12 medium supplemented with 10% foetal bovine serum and antibiotics. Antibodies against WAVE1, ARP2, ARP3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and WAVE3 antibody was obtained from R&D Systems (Abingdon, UK). Other kits and reagents were purchased from Sigma-Aldrich. For ARP2/3 inhibitor treatment groups, the small-molecular inhibitor, CK-0944636 was used at a concentration of 200 nM (Sigma-Aldrich, Poole, Dorset, UK) based on preliminary cytotoxic assays (data not shown).

Production of ribozyme transgenes. Ribozyme transgenes were designed to specifically target either *WAVE1* or *WAVE3* messenger RNA transcripts to down-regulate their expression. These ribozyme constructs were prepared as previously described (7, 8). In brief, the ribozyme transgenes were designed based on the secondary structure of *WAVE1* and *WAVE3* transcripts as predicted with Zuker's RNA mFold software (10). The ribozymes were subsequently synthesised and cloned into the pEF6/V5-His TOPO TA Expression Kit (Invitrogen, Paisley, UK) following the manufacturer's protocol. PC-3 cells were transfected with these ribozyme transgenes or empty plasmids to generate *WAVE* knockdown and control cell lines, respectively. The transfected cells were subjected to blasticidin selection for 5 days and transfectants verified for *WAVE1* and *WAVE3* knockdown were selected for the study.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR). RNA was obtained using total RNA isolation reagent. Reverse transcriptase-polymerase chain reaction was carried out according to manufacturer's protocol (Applied Biosystems Life Technologies Ltd, Paisley, UK) and was followed by PCR with GoTaq Green master mix (Promega, Southhampton, UK). The primers used are listed in Table I. The PCR parameters were 94˚C for 5 min, followed by 34 cycles of 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 40 sec, and a final extension at 72˚C for 10 min. PCR products were visualised on a 0.8% agarose gel stained with SYBR® SAFE (Invitrogen).

In vitro cell-growth assay. Three thousand cells were seeded into each well of a 96-plate before incubation for 24- and 120-h periods. After this time, cells were formalin fixed and stained with crystal violet. The addition of 10% acetic acid was followed by spectrophotometric absorbance readings. The percentage change in absorbance compared to PC-3 ^{pEF6} control cells was calculated for *WAVE1* and *WAVE3* knockdown cell lines.

In vitro cell-motility assay. Cell motility was assessed by incubating 10^6 cells with Cytodex-2 beads overnight. After two washes to remove non-adherent or dead cells, the beads were resuspended and transferred into 96-well plates and incubated for an additional 4 hours. Any cells that had migrated from the beads to the bottom of the well were fixed with formalin and stained with crystal violet. The stain was extracted with the addition of 10% acetic acid for spectrophotometric absorbance readings.

In vitro cell-invasion assay. Twenty thousand cells were seeded in transwell inserts with 8 μm pores pre-coated with 50 μg of Matrigel (BD Biosciences, Oxford, UK). After an incubation period of 72 h, cells that had invaded through the Matrigel layer and migrated

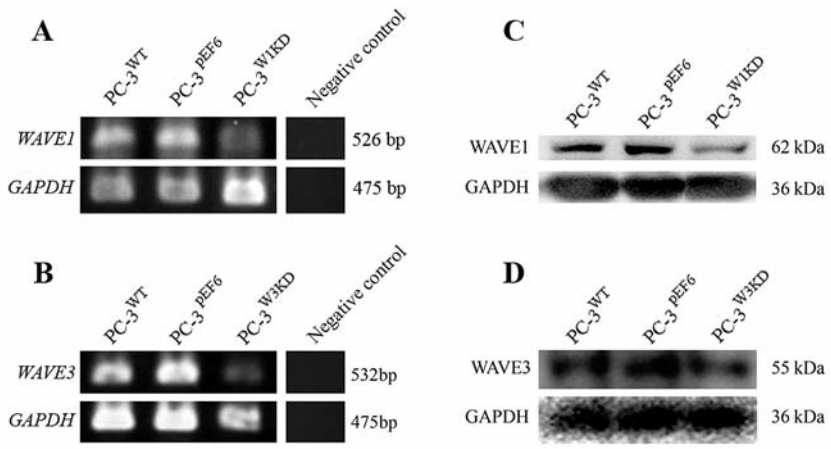

Figure 1. *(A) WAVE1 knockdown (W1KD) following targeted transfection of PC-3 wild type cells at the mRNA level. (B) WAVE3 knockdown (W3KD) at the mRNA level. (C) WAVE1 knockdown at the protein level. (D) WAVE3 knockdown at the protein level.*

through the 8 μm pores were fixed with formalin and stained with crystal violet for visualisation and cell counting under a microscope at ×200 magnification.

Confocal microscopy. Protein co-localisation and cell morphology were investigated using confocal microscopy. Cells were seeded into each chamber of a Millicell® EZ Slide (Merck Millipore, Darmstadt, Germany) before incubation overnight. Once cells were fixed with formalin, primary antibody for each protein being invested for co-localisation was added followed by the appropriate secondary antibodies. A cover slide was mounted using FluorSave Reagent (Merck Millipore) for cell visualisation using an Olympus Fluoview FV10i confocal laser-scanning microscope under ×60 magnification and analyzed with the accompanying manufacturer's software (Olympus, Southend-on-Sea, UK).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting. Proteins were separated with SDS-PAGE and were subsequently blotted onto nitrocellulose membrane. Proteins were probed with the WAVE1, WAVE3, ARP2, ARP3 or GAPDH primary and peroxidase conjugated secondary antibodies using the SNAP id.® Protein Detection System (Merck Millipore). Protein band visualisation was carried out using the Luminata Forte Western HRP Substrate (Merck Millipore) and UVITec imager (UVItec Cambridge, Ltd, UK). To study protein tyrosine phosphorylation, proteins were first immunoprecipiated with a phosphotyrosine antibody before being pulled out with AG agarose beads following manufacturer's instructions (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical analysis. Statistical analysis was performed using SigmaPlot 11.0 statistical software (Systat Software Inc, London, UK). Data were analysed using a two-sample, two-tailed *t*-test. Normality of data to perform these parametric tests was assessed by Sigmaplot software (Systat softare Inc., London, UK) and if deemed non-parametric, Mann–Whitney test was performed. Each assay was performed at least three times. *p*-Value of less than 0.05 were considered statistically significant.

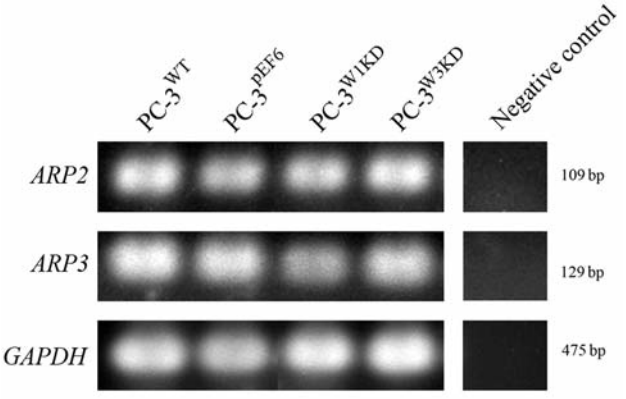

Figure 2. *Actin related protein 2 (ARP2) and ARP3 mRNA expression analysis following knockdown of either Wiskott-Aldrich syndrome protein family (WASP) verprolin homologous protein 1 (WAVE1) (PC-3^{W1KD}) or WAVE3 (PC-3^{W3KD}) in prostate cancer cells revealed no significant effect on levels of ARP2 or ARP3 expressed in PC-3 cells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression remained unaffected regardless of WAVE knockdown, whilst the negative control revealed no signs of contamination.*

Results

Manipulation of WAVE1 and WAVE3 expression by ribozyme transgenes. Previously published work identified high levels of WAVE1 and WAVE3 in prostate cancer cell lines compared to prostate epithelial cell lines. Here, these *WAVE* isoforms were independently targeted in the PC-3 cell line with ribozyme transgenes. Transfection of PC-3 cells with *WAVE1* ribozyme transgenes were seen to reduce *WAVE1* mRNA expression (PC-3^{W1KD}) compared to PC-3 wild-type

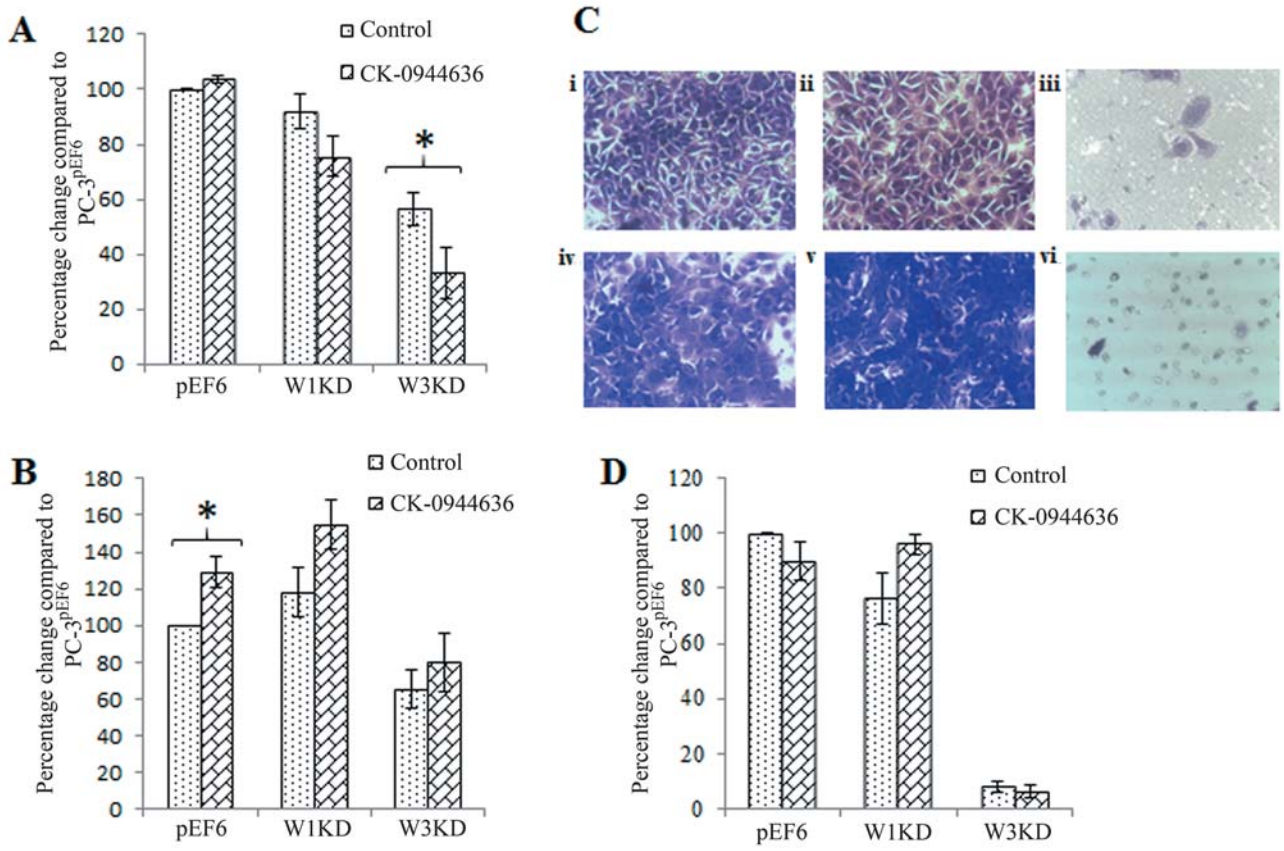

Figure 3. *Investigating the effect of actin related protein 2/3 (ARP2/3) inhibition in Wiskott-Aldrich syndrome protein family (WASP) verprolin homologous protein 1 (WAVE1) (W1KD) and WAVE3 (W3KD) knockdown PC-3 cells. A: ARP2/3 inhibition had little effect on cell growth in pEF6-transfected cells; however, a moderate decrease was observed with WAVE1 knockdown and a significant decrease with WAVE3 knockdown. B: ARP2/3 inhibition increased cell motility in pEF6-transfected cells and WAVE1 and WAVE3 knockdown PC-3 cells. C: Images acquired from cell invasion assay (i) PC-3 ^{pEF6}/control (ii) WAVE1 knockdown/control (iii) WAVE3 knockdown/control (iv) pEF6/ARP inhibition (v) WAVE1 knockdown/ARP inhibition (vi) WAVE3 knockdown/ARP inhibition. (D) Cell invasion was moderately reduced in pEF6-transfected cells with ARP2/3 inhibition whilst an increase was observed in WAVE1-knockdown cells. WAVE3 knockdown was not found to affect cell invasion with ARP2/3 inhibition. Images acquired at 200X magnification. Mean data from a minimum of three independent repeats and values represent the percentage change to pEF6 cells without treatment (control). Error bars represent SEM. *$p<0.05$.*

cells (PC-3^{WT}) or empty-plasmid control cells (PC-3^{pEF6}). Similarly, knockdown of *WAVE3* mRNA levels was observed with ribozyme transgenes that targeted *WAVE3* in PC-3 cells (PC-3^{W3KD}) (Figure 1). Expression analysis with western blotting also showed a decrease of WAVE1 and WAVE3 protein expression corresponding with targeted ribozyme transgenes in PC-3 cells. Furthermore, mRNA and protein expression levels in PC-3^{WT} and PC-3^{pEF6} cells were observed to be similar (Figure 1).

Expression of ARP2 and ARP3 in WAVE1 and WAVE3 knockdown PC-3 cells. PC-3^{W1KD} and PC-3^{W3KD} cells displayed no significant change in *ARP2* or *ARP3* mRNA expression (Figure 2).

Coupling WAVE3 knockdown with ARP2/3 inhibition synergistically affects cell growth. In the current study, knockdown of *WAVE1* or *WAVE3* was shown to suppress cell growth in PC-3 cells compared to empty vector PC-3 control cells after a 120-hour period (Figure 3A). To investigate the relationship between the WAVE proteins and the ARP2/3 complex, cell growth in PC-3 cells was compared with and without the small-molecule ARP2/3 inhibitor, CK-0944636. While ARP2/3 inhibition in PC-3^{pEF6} cells appeared to have no effect on cell growth (p=0.109), CK-0944363 treatment was shown to suppress cell proliferation in PC-3^{W3KD} cells (p=0.045). Although an overall trend of reduced cell proliferation was observed in PC-3^{W1KD} cells, it was not significant (p=0.182). ARP2/3 inhibition coupled with

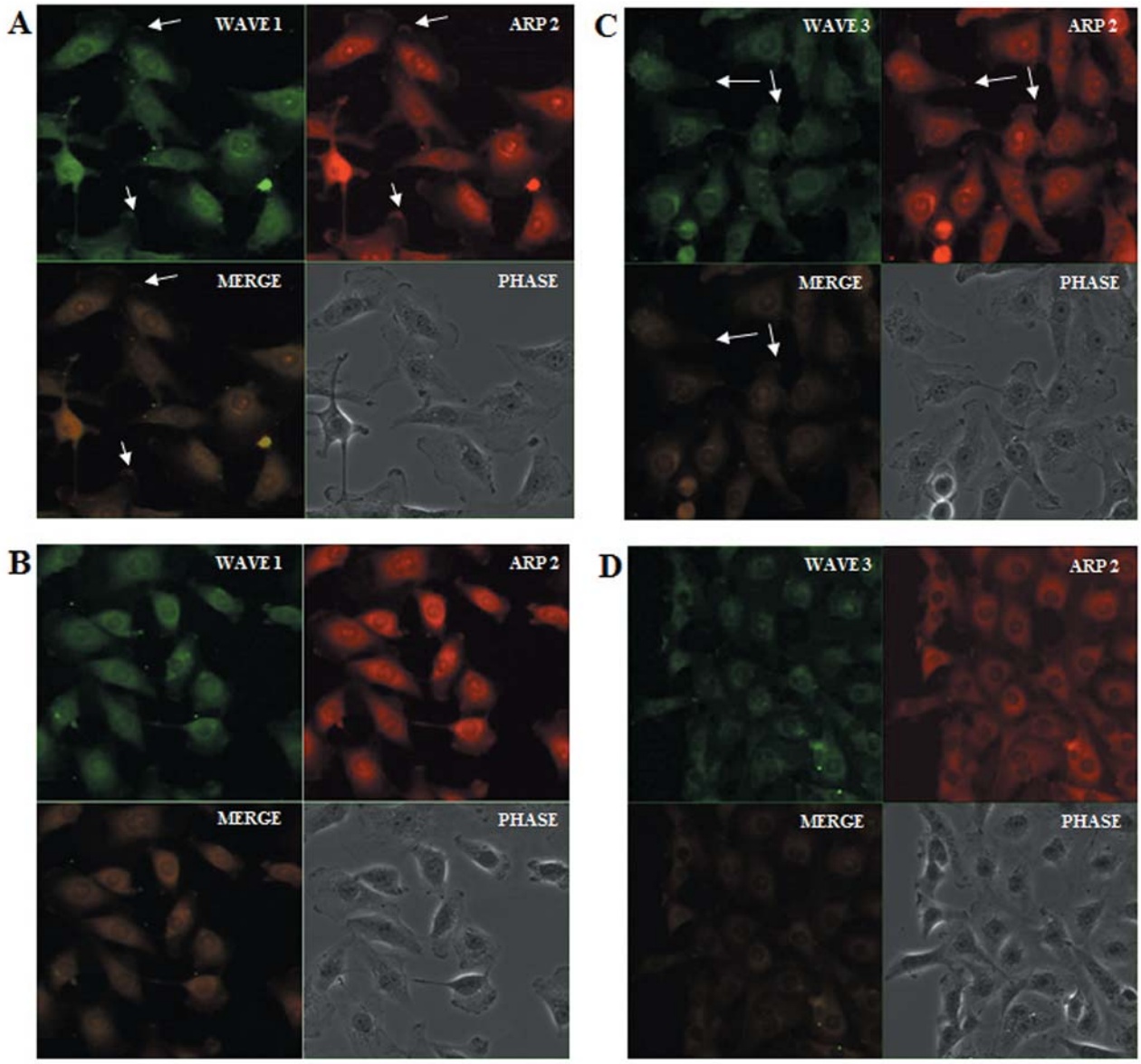

Figure 4. *Confocal microscopy images of PC-3 cells. A: pEF6-transfected cells stained for Wiskott-Aldrich syndrome protein family (WASP) verprolin homologous protein1 (WAVE1) (fluorescein isothiocyanate; FITC) and actin related protein 2 (ARP2) tetramethyl rhodamine isothiocyanate; TRITC) reveal co-localisation of these proteins (arrows). B: WAVE1 knockdown cells stained for WAVE1 (FITC) and ARP2 (TRITC). C: pEF6-transfected cells stained for WAVE3 (FITC) and ARP2 (TRITC) reveal co-localisation of these proteins (arrows). D: WAVE3 knockdown cells stained for WAVE3 (FITC) and ARP2 (TRITC). Representative images at 60x magnification are shown.*

WAVE3 knockdown led to the greatest suppression of cell proliferation compared to *WAVE1* or *WAVE3* knockdown alone in PC-3 cells.

ARP2/3 inhibitor treatment increases motility of PC-3 WAVE1-knockdown cells. An overall trend of increased cell motility was observed following a 4-h treatment with the ARP2/3 inhibitor, CK-0944636 with PC-3^{W1KD} cells (p=0.078). This trend was also observed in PC-3^{pEF6} cells (p=<0.001) (Figure 3B). No significant change was observed with PC-3^{W3KD} cells (p=0.421).

ARP2/3 inhibitor treatment affects cell invasion in PC-3 cells with WAVE1 knockdown but not with WAVE3 knockdown. A reduction in cell invasion was observed in PC-3 cells exhibiting either *WAVE1* or *WAVE3* knockdown compared to wild-type or

PC-3^{pEF6} cells (Figure 3C and D). CK-0944636 treatment of PC-3^{pEF6} cells was shown to reduce the number of invading cells (p=0.214). No significant change in cell invasion was observed following ARP2/3 inhibition of PC-3^{W3KD} cells (p=0.652). Interestingly, ARP2/3 inhibition was shown to rescue the cell invasion-suppressive effect seen with *WAVE1* knockdown in PC-3^{W1KD} cells to levels similar to that of PC-3^{pEF6} cells without ARP2/3 inhibition (p=0.216).

ARP2 co-localises with WAVE1 and WAVE3 in PC-3 cells. Confocal microscopy approaches were employed to investigate the intracellular relationship of WAVE and ARP proteins in PC-3 cells. The acquired images revealed co-localisation of WAVE1 and ARP2 to the lamellipodia perimeter of PC-3^{pEF6} cells (Figure 4A). Similarly, this co-localisation pattern was also evident for WAVE3 and ARP2 (Figure 4C).

In contrast, analysis of PC-3^{W1KD} cells revealed a weaker co-localisation of WAVE1 and ARP2, while an even less prominent relationship was observed between ARP2 and WAVE3 in PC-3^{W3KD} cells (Figure 4B and D, respectively). Moreover, the area and boundary encompassed by the lamellipodia of PC-3^{pEF6} cells was larger, as well as more pronounced, than that seen for both PC-3^{W1KD} and PC-3^{W3KD} cells.

WAVE3 knockdown increases ARP2 tyrosine phosphorylation in PC-3 cells. A higher level of phosphorylated tyrosine of ARP2 was observed in PC-3^{W3KD} cells relative to both PC-3^{WT} and PC-3^{pEF6} cells. Levels of tyrosine phosphorylation of ARP2 in WAVE3 knockdown cells were comparable to the positive control (Figure 5A). In contrast, PC-3 cells expressing lower levels of WAVE1 were found to have similar levels of tyrosine phosphorylation of ARP2 as the PC-3^{WT} and PC-3^{pEF6} cells. Similar levels of ARP2 were observed in the raw lysate for all PC-3 protein samples.

The same techniques revealed no changes in tyrosine phosphorylation of ARP3 levels following *WAVE1* or *WAVE3* knockdown in PC-3 cells. Moreover, the levels of tyrosine phosphorylation of ARP3 in PC-3^{WT,} PC-3^{pEF6}, PC-3^{W1KD} and PC-3^{W3KD} cells were seen to be lower than those of the positive control (Figure 5B). Overall, ARP3 levels were demonstrated to be the same in the cell lysates examined.

Discussion

Uncontrolled cell motility is a factor contributory to the spread of aggressive cancer to both local and distant sites. By further studying the possible mechanism underlying the relationship between WAVE and ARP2/3 complex, it was hoped that this would enable a better understanding of how their association contributes to metastatic prostate cancer through use of the PC-3 cell line.

WAVE1 and *WAVE3* knockdown had no significant effect on ARP2 or ARP3 expression. This implies that the regulation of WAVE and ARP expression are independent of each other. Therefore, the role of WAVE1 and WAVE3 in this actin polymerisation pathway is to regulate activation of ARP2/3 and not the expression of these subunits.

An investigation into the effects of *WAVE1* and *WAVE3* knockdown was found to have implications for the proliferative and invasive ability of PC-3 cells. Treatment of PC-3^{pEF6} cells with the ARP2/3 inhibitor CK-0944636 was found to have little effect on cell growth; this is in contrast to the findings of suppressed cell proliferation in PC-3 cells with either *WAVE1* or *WAVE3* knockdown. It is worth noting that these antiproliferative effects were most dramatic when coupling PC-3^{W3KD} cells with ARP2/3 inhibition and were not significant in PC-3^{W1KD} cells. These observations suggest that both WAVE1 and WAVE3 along with ARP2/3 play a role in cell growth, although the influence of *WAVE* on proliferation is greater than that of the ARP2/3 complex. These findings highlight a complexity of the cell proliferation pathway that extends beyond both WAVE and ARP2/3, involving auxiliary proteins. As the treatment of the pEF6 cells with the ARP2/3 inhibitor showed little change in cell growth, this implies that WAVE may target downstream proteins in a pathway independent of ARP2/3 to regulate proliferation of PC-3 cells. Furthermore, it is likely that the ARP2/3 complex is influenced by an upstream regulator separately from WAVE, as inhibition of ARP2/3 activity reduced cell proliferation further when coupled to *WAVE* knockdown. This upstream protein could be WASP or N-WASP as they are both known activators of the ARP2/3 complex (11).

PC-3 cells exhibited increased motility with ARP2/3 inhibitor treatment, regardless of whether *WAVE* was knocked-down or not and regardless of which *WAVE* was targeted. However, this increase was only significant for PC-3^{pEF6} and PC-3^{W1KD} cells, not for PC-3^{W3KD}. These findings could be explained by the interplay of alternative cell motility signaling pathways that are independent of WAVE as the trend for increased motility was observed in both control and *WAVE* knockdown cells. It could be postulated that the role of ARP2/3 is to drive cell motility *via* actin polymerisation while regulating it to an appropriate level by suppressing these alternative cell motility pathways.

A moderate decrease in cell invasion was demonstrated in the pEF6 cells treated with the ARP2/3 inhibitor and very little change in PC-3^{W3KD} cells. On the contrary, the suppression of cell invasion observed in response to *WAVE1* knockdown was seen to be rescued by ARP2/3 inhibition to levels comparable with that of untreated pEF6 cells. This highlights a distinction between WAVE1 and -3 functions. It would not be unreasonable to interpret these contrasting effects of ARP2/3 inhibitor treatment, when comparing *WAVE1*- and *WAVE3*-knockdown cells, as a consequence of distinct and different roles served by these proteins in the cell.

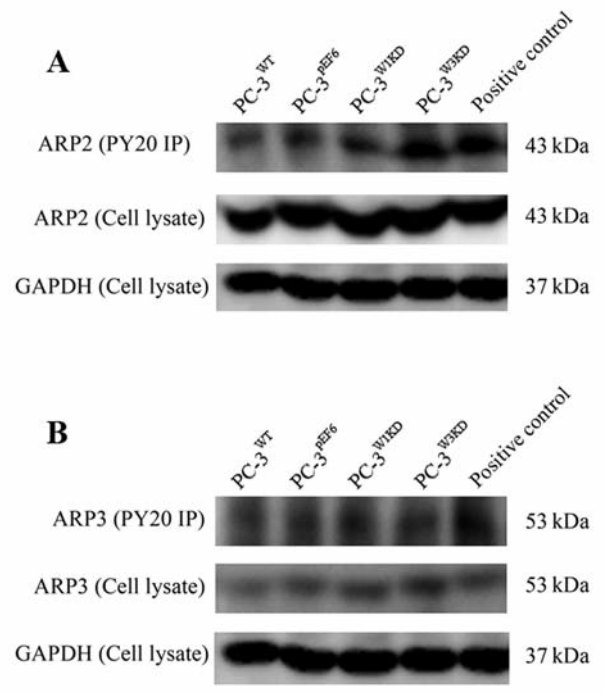

Figure 5. *Proteins immunoprecipitated with the anti-phosphotyrosine antibody, PY20, revealed increased tyrosine phosphorylation of actin related protein (ARP2) in the Wiskott-Aldrich syndrome protein family (WASP) verprolin homologous protein3 (WAVE3) knockdown cells (PC-3^{W3KD}). B: No obvious changes in tyrosine phosphorylation of ARP3 was observed with WAVE1 or WAVE3 knockdown in PC-3 cells.*

Although cell invasion was significantly suppressed by *WAVE1* knockdown, when ARP2/3 complex activity was blocked with ARP2/3 inhibitor, the repression was lifted, restoring cell invasion to a level similar to that observed for pEF6 control cells without ARP inhibitor treatment. While *WAVE1* or *WAVE3* knockdown was shown to suppress the ability of PC-3 cells to invade the Matrigel layer in the *in vitro* invasion assay, it is apparent that an independent mechanism is utilised by WAVE1 during cell invasion from that used by WAVE3.

Previous studies have highlighted the clinical importance of WAVE and ARP proteins in human cancer. Immunofluorescence analysis of PC-3^{pEF6} cells in this study demonstrated the co-localisation of ARP2 with both WAVE1 and -3 to the outer boundaries of the cell lamellipodia. Proteins responsible for actin polymerisation are commonly recruited to these cell protrusions to cope with the dynamic nature of cytoskeleton remodelling in order to facilitate cell migration (12). The co-localisation of ARP2 with both WAVE1 and -3 at the PC-3^{pEF6} cell edge in this study corresponds with previous findings. The PC-3 cell line was derived from a patient presenting aggressive metastatic prostate cancer and the finding that these proteins, known for their role in cell migration by co-localising at the cell edge, is unsurprising. Furthermore, the observation of a reduced area encompassed by the cell in *WAVE1*- and *WAVE3*-knockdown cells implies a suppressed ability of cells to generate cell protrusions. Coupling this observation with reduced ARP2/WAVE co-localisation implies that by knocking-down *WAVE1* or *WAVE3* expression, these proteins are unable to activate the ARP2/3 complex to stimulate actin polymerisation.

Protein tyrosine kinases are integral to regulating intracellular signaling transduction pathways. Their activity influences several cellular properties, including proliferation and survival. With such an essential role in the cell, it is unsurprising to find deregulated cell traits such as uncontrolled growth attributed to aberrant tyrosine kinase activity in human cancer (13). A knockdown in *WAVE3* expression was shown to increase the levels of tyrosine phosphorylation of ARP2 in PC-3 cells, whereas *WAVE1* knockdown led to no change. These observations suggest WAVE3 functions upstream of a protein regulator of a tyrosine kinase that can target ARP2 of the ARP2/3 complex. At present, six sites of tyrosine phosphorylation have been identified in ARP2: Y22, Y72, Y91, Y202, Y225 and Y378. The majority of these modifications are associated with different manifestations of leukaemia and lymphoma (14). Recently, a study demonstrated the importance of phosphorylating Y202 of the ARP2 subunit for ARP2/3 activity (15). Even though it is uncertain in this study which tyrosine residues were phosphorylated, it is evident that *WAVE3* knockdown has an influence on the phosphorylation of tyrosine residues in ARP2, which may affect its activity.

The effects of *WAVE1* and *WAVE3* knockdown coupled with ARP2/3 inhibitor treatment has revealed their influence on several cell traits. Whilst some of the trends observed were similar for both isoforms, the intriguing finding that ARP2/3 inhibitor treatment was able to restore cell-invasive properties in response to *WAVE1* knockdown was not found to be the same with *WAVE3* knockdown. Although it was not explored here, the alternative method of using siRNA to target ARP2/3 could be utilised to gain a clearer and more complete picture of the relationship between WAVE and ARP2/3.

Additionally, *WAVE3* but not *WAVE1* knockdown was shown to affect ARP2 tyrosine phosphorylation. Overall, the *in vitro* cell experiments described, along with phospho-immunoprecipitation approaches, both emphasise shared and distinct roles for WAVE1 and -3 in PC-3 cells. More importantly, the use of a small-protein inhibitor targeting the ARP2/3 complex highlights the relationship of WAVE1 and -3 with ARP2/3, involving a multiplex and elaborate network of proteins. Ideally, future work will aim to identify these collaborating proteins. Although the current study examined the co-localisation of WAVE1 and WAVE3 with ARP2,

future work could aim to explore their relationship with ARP3 in PC-3 cells. It is hypothesised that a similar pattern would be observed for ARP3 to that for ARP2 due to the close association of these subunits in the ARP2/3 complex. The work presented here revealed additional paths showing that WAVE1 and -3 are able to regulate ARP2/3 and have consequential effects on cell properties.

Conflicts of Interest

The Authors declare no conflicts of interest.

Acknowledgements

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