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Functional interrelationship between the WASF3 and KISS1 metastasis associated genes in breast cancer cells

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Abstract

Loss of *WASF3* function in breast cancer cells results in loss of invasion phenotypes and reduced metastatic potential. Using oligonucleotide arrays we now demonstrate that knockdown of *WASF3* leads to the upregulation of the *KISS1* metastasis suppressor gene with concomitant reduced invasion and loss of MMP9 activity. Using a luciferase reporter, *KISS1* transcription is significantly increased in the absence of *WASF3*. Knockdown of *KISS1* in *WASF3* silenced cells resulted in the recovery of the invasion phenotype. *WASF3* knockdown also resulted in elevated I κ B α levels in the cytoplasm and reduced levels of NF κ B p65/50 subunits in the nucleus. TNF α has been associated with cell invasion through induction of MMP9 production via *KISS1* regulation of the NF κ B pathway. When *WASF3* knockdown cells are treated with TNF α , no effect is seen on invasion or nuclear translocation of NF κ B. Thus, coordinated expression patterns of the *WASF3* metastasis promoter gene and the *KISS1* metastasis suppressor gene appear to exert their influence through inhibition of NF κ B signaling which in turn regulates MMP9 production facilitating invasion.

Keywords

Metastasis; *WASF3*; *KISS1*; Matrix metalloproteinase; NF κ B

Introduction

The Wiscott-Aldrich syndrome protein (WASP) was the founding member of a family of proteins that now includes N-WASP and three members of the *WASF/WAVE* family (1). This family of proteins is defined by a highly conserved group of motifs at their C-terminal ends; a WASP-homology-2 domain (WHD/V), a cofilin-homology domain (C) and an acidic domain (A). This VCA domain facilitates actin polymerization through binding monomeric actin and the ARP2/3 complex. We recently showed that inactivation of the *WASF3/WAVE3* gene in breast cancer cells results in loss of cell motility and invasion in vitro (2) and metastasis in vivo (3). The loss of cell motility is accompanied by a reduction in lamellipodia formation, even in the presence of normal expression levels of the *WAVE1/2* proteins. Thus, the metastasis-promoting function of the *WASF3* gene plays a critical and distinct role in coordinating actin dynamics related to cell motility, invasion and metastasis independently of the other *WASF* family members (2-3). The *WASF* proteins are normally maintained in an inactivated state in resting cells through the binding of a protein complex

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including PIR121, HSPC300, Abi1, and Nap125 to the WHD (1). Upon stimulation with growth factors such as PDGF, the WASF complex breaks down allowing binding and activation of the Arp2/3 complex of proteins, resulting in actin polymerization (4-6).

Consistent with its role in promoting metastasis, WASF3 levels are elevated in advanced stage cancers compared to lower grade tumors and normal tissue, and knockdown of *WASF3* in breast (7) and prostate cancer (8) cells results in decreased expression and activity of matrix metalloproteinases (MMP). These observations suggest a direct relationship between MMP activity and the metastasis promoting function of WASF3 (7), since in some tumors MMPs have been shown to be related to ECM degradation and cellular invasion (9-10). In particular, MMP9, a type IV collagenase, is essential for destruction of the basement membrane and is associated with tumor progression (11). Although MMP9 has been implicated in the invasion/metastatic phenotype of many cancers, and is regarded as a prognostic indicator in breast cancer patients, the regulation of its expression is still not fully understood (12) and neither is the role of WASF3 in the regulation of its activity.

To obtain a better understanding of molecular mechanisms of action of WASF3, we have undertaken a global survey of gene expression changes following *WASF3* knockdown in MDA-MB-231 cells. These studies have shown altered expression levels in a wide range of genes known to be implicated in cell invasion and metastasis and, in particular, revealed an up regulation of *KISS1* expression levels with a concomitant decrease in MMP9 levels. *KISS1* is a member of the metastasis suppressor family of genes (13-17). Functional analysis now indicates that one of the molecular mechanisms driving WASF3-mediated breast cancer cell migration and invasion is through the suppression of KISS1-mediated, NF κ B-dependent MMP9 activation.

MATERIALS AND METHODS

Cell culture and plasmids

MDA-MB-231, SkBr3, BT474, MCF7, MDA-MB-157 and PC3 cell lines were purchased from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). MDA-MB-231 cells were subjected to aCGH analysis (06/2009) and revealed copy number changes characteristic of this cell line. The full-length open reading frame of the human *WASF3* gene was amplified and inserted into the pcDNA3.1 vector to generate the WASF3 overexpression vector. The 4kb KISS1 promoter was cloned from BAC clone RP11-203F10 (accession AL592114) and inserted upstream of a luciferase gene in pGL3 (Promega, Madison, WI) using Kpn I/Xho I sites. The primers used to construct the plasmid of pGL-KISS1₄₀₀₀ are as follows: forward- 5' GGGGTACCAGTTCGAGAACAGACTAGCC 3', reverse- 5' CCGCTCGAGGTTCTCCCCAGCTCCCT 3'. The pNF κ B-TA-Luc vector (Clontech, NC) acts as a signal transduction reporter vector that monitors activation of NF κ B signal transduction. Lipofectamine (Invitrogen, Carlsbad, CA) was used to deliver the vectors into cells.

Gene silencing by RNA interference

Cell lines were transiently transfected with small interfering RNA (siRNA) using DharmaFECT (Dharmacon RNA Technologies, Lafayette, CO). 21 bp siRNA duplexes targeting WASF1, WASF2, WASF3 (siWASF1, siWASF2 and siWASF3) and a standard control (Dharmacon siCONTROL non-targeting siRNA) were synthesized by Dharmacon. Stealth KISS1 siRNA (siKISS1, HSS142730) and the stealth RNAi negative control duplexes were purchased from Invitrogen (Carlsbad, CA). To generate *WASF3* stable knockdown MDA-MB-231 cells, the pSM2 retroviral vectors containing a short hairpin

RNA against *WASF3* (shRNA1 - V2LHS 261688; shRNA2-V2LHS90825; Open Biosystems, Huntsville, AL) were used for infection. As controls the empty pSM2 vector and an shRNA targeting GFP, were used. Virally infected cells were selected in a medium containing 1.0 µg/ml puromycin (Sigma, NY) for 2 weeks and individual drug-resistant clones were collected and expanded.

Microarray hybridization and data analysis

RNAs were extracted using Trizol reagent (Invitrogen) and the Qiagen RNeasy kit (Qiagen, CA) according to manufacturer's instructions and then evaluated using a Bioanalyzer to assess the quality of the sample. All samples passing this quality control were hybridized to a GeneChip® Human Genome U133A 2.0 Array (Affymetrix, CA) probing 18,400 transcripts and variants. The probe was generated using the Affymetrix 3' IVT Express protocol. Gene Expression values were created by normalizing the array data using a Quantile Normalization in Partek Genomics Suite and statistical analysis was performed using NIA Array Analysis (<http://lgusun.grc.nia.nih.gov/ANOVA/>). The log₂ ratios were converted to fold-change values and considered significant if the change from control cells was >2 or < -2 and statistical significance showed a p-value <0.05.

RT-PCR and quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript II RT-PCR kit (Invitrogen) with 3 µg of RNA and PCR was performed using gene specific primers (Supplemental Table S1). β-actin was used as a loading control. Quantitative real-time RT-PCR was performed using a BioRad iCycler (Bio-Rad, CA) using BioRad iQ SYBR Green Supermix (Bio-Rad). Gene expression levels were normalized against β-actin. In this assay, primers for human *WASF3* were obtained from Sabiosciences (Frederick, MD).

Luciferase reporter gene analysis

Cells were co-transfected with pRL-TK and pGL-KISS1₄₀₀₀ or p-NF-κB-TA-Luc. After 24 or 48 hours, the expression of luciferase gene was determined and normalized using Dual-Luciferase reporter assays (Promega) according to the manufacturer's protocol. Luminescence was measured using a Synergy2 plate reader luminescence module (BioTek).

Matrigel invasion and chemoinvasion assay

Matrigel invasion assays were performed as described previously (18) using transwells (BD biosciences, CA) with 8-µm pore size filters. For the chemoinvasion assay, 5×10^4 cells were placed in the upper chamber which had been precoated with gelatin (0.1%, w/v) and RPMI medium containing 5% FBS with or without TNFα (50 ng/ml, ProSec-TechnoGene Ltd.) was added to the lower chamber. After 20 hrs incubation, the cells were fixed, stained and counted. Each experiment was performed in triplicate.

Nuclear and cytoplasmic protein fractionation and immunoblotting

For nuclear and cytoplasmic extracts, cells were lysed in NE-PER extraction reagent (Pierce, IL) according to the manufacturer's protocol. Protein concentration was determined using the BCA assay. For immunoblotting, 50 µg of cell lysates were resolved in 8-12 % SDS-PAGE gels, and transferred to PVDF membranes (BioRad, CA). Membranes were incubated with the individual primary antibodies at 4°C overnight. Anti-WASF3, anti-MMP9 and anti-IκB were purchased from Cell Signaling Inc., anti-PCNA and anti-β-actin were purchased from Sigma. Anti-NF-κB p50 and anti-NF-κB p65 and anti-KISS1 were purchased from Santa Cruz Biotechnology. The KISS1 antibody recognizes the full length 145 amino acid protein giving a size of ~15 kD on Western blots. Equal protein loading was confirmed by

probing for β -actin or PCNA. To remove bound antibody, membranes were incubated in Restore Western blot stripping buffer (Pierce) according to the manufacturer's protocol.

Immunofluorescence microscopy

Immunofluorescence staining was used to determine the translocation of p65 in MDA-MB-231 cells with or without WASF3 shRNA expression after 1 h post TNF α treatment. Briefly, cells were fixed with 4 % paraformaldehyde and permeabilized using 0.1% Triton X-100 in PBS. After washing and blocking, the cells were incubated with a primary antibody against p65 overnight at 4°C. After three washes with PBS, the cells were incubated with an Alexa-conjugated secondary antibody in the dark for 1 h. Slides were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing the DAPI nuclear stain before examination under a fluorescence microscope.

Pro and active matrix metalloproteinase 9 (MMP9) detection

MDA-MB-231 cells were seeded in 12-well plates and grown to 70-80% confluence in 1 ml of DMEM with 2% FBS. After 24 hrs, the media were collected in tubes and centrifuged for 10 min at 10,000g. The pro and active forms of MMP-9 levels released into the media were measured using a Fluorokine MAP human MMP9 kit (R&D Systems, MN) according to the manufacturer's instructions. The fluorescent signal in the sample was determined using a BioRad analyzer at excitation/emission wavelengths of 340 nm/465 nm.

Results

Loss of WASF3 leads to a change in expression of genes associated with cell motility and metastasis

In a preliminary screen, we used two different WASF3 shRNA targeting constructs (see Materials and Methods). Both showed 60-70% knockdown of the WASF3 mRNA (Figure 1) compared with cells expressing the empty vector or an shRNA targeting GFP. Stable clones expressing either shRNA1 or shRNA2 all showed WASF3 knockdown (Figure 1 and Supplemental figures S1 and S2). Boyden chamber invasion assays for individual clones from each shRNA series showed a proportional loss of invasion in the WASF3 knockdown cells (Supplemental Figures S1 and S2). These data demonstrate the specificity of the targeting shRNA constructs (which produce the same phenotype) making off-target effects unlikely. The knockdown of WASF3 protein was more pronounced in the clones from the shRNA1 series and so these were analyzed more extensively in subsequent experiments. In these clones, knockdown of WASF3 also showed reduced cell motility in the scratch wound assay and reduced anchorage independent growth ability compared with control clones (Supplemental Figure S1). Three shRNA1 clones (#3, #6, and #8) which did not show knockdown and three clones (#5, #12, and #18) which showed high level knockdown (Figure 1) were then selected for gene expression analyses and subsequent studies.

To identify altered gene expression patterns in the knockdown cells, we compared gene expression levels between three different knockdown and three different control clones. 246 genes were identified that showed consistent $\pm$ 2 fold expression level changes, of which, ~35 were associated with cell movement and migration (Figure 1B and Supplementary Table S2). For example, the three members of the trefoil factor family (*TFF1*, *TFF2* and *TFF3*) were downregulated as well as *MUC1*. Downregulation of these genes was confirmed using semi-quantitative RT-PCR (Figure 1C) and QRT-PCR (Figure 1D, lower panel). Two other genes important in cell movement, *IL8* and *PDGFA*, were also analyzed using RT-PCR (Figure 1C), again confirming their decreased expression in the *WASF3* knockdown cells. An especially intriguing observation was the 4-fold up regulation of the *KISS1* gene (Figure 1D, upper panel), which has been defined as a metastasis suppressor gene because

its overexpression reduces tumor metastasis in breast, ovarian and melanoma cancer cells (13-15).

KISS1 is down regulated in WASF3 knockdown breast and prostate cancer cell lines

The inverse relationship between expression of a metastasis suppressor gene, *KISS1*, and a metastasis promoter gene, *WASF3*, suggested a common regulatory interaction between these two genes. To determine whether the same inverse relationship in expression levels was more widespread, we compared endogenous *WASF3* and *KISS1* protein levels in four breast cancer cell lines (MDA-MB231, SkBr3, MDA-MB-157, and MCF-7). Both MDA-MB-231 and SkBr3 breast cancer cell lines expressed robust *WASF3* protein levels compared to MDA-MB-157 and MCF-7 cells. MDA-MB-231 and SkBr3 cells showed relatively low *KISS1* expression levels, whereas the MDA-MB-157 and MCF-7 cell lines expressed relatively stronger *KISS1* protein levels (Figure 2A). To investigate this possibility further, we overexpressed *WASF3* in MDA-MB-231 cells. As anticipated, increased *WASF3* protein levels were accompanied by a corresponding reduction of *KISS1* protein levels (Figure 2A). In the MCF7 breast cancer cell line, which expresses higher endogenous *KISS1* levels (Figure 1A), overexpressing *WASF3* leads to an even more pronounced downregulation (Figure 1A), demonstrating that this effect is not limited to the MDA-MB-231 cells. Knockdown of *WASF3* in SkBR3 and BT474 cell lines also led to increased expression of *KISS1* (Figure 2B). These observations confirmed the inverse relationship between *WASF3* and *KISS1* expression levels in a wider variety of breast cancer cell lines. We recently also showed that down regulation of *WASF3* in prostate cancer cells leads to a loss of invasion and metastasis (8). Although the focus of the current study was breast cancer cells, we also examined this *WASF3/KISS1* relationship in the PC3 prostate cancer cell line where, following *WASF3* knockdown, *KISS1* expression was even more upregulated (Figure 2B). To determine whether the reverse was true, we knocked down *KISS1* in breast and prostate cancer cells but this did not result in any expression level changes of *WASF3* in these cells (Supplemental Figure S3). Thus, while *WASF* can influence *KISS1* expression, the reverse does not appear to be the case, implying that *KISS1* lies downstream of the effects of *WASF3*.

Using RT-PCR we also demonstrated that cell lines used in this study all express the GPR54 *KISS1* receptor gene (Supplemental Figure S3), and so are competent to respond to the *KISS1* signal.

Upregulation of KISS1 transcription in WASF3 knockdown cells

To determine the mechanism of *KISS1* upregulation in *WASF3* knockdown cells, we introduced a luciferase reporter construct for the *KISS1* promoter into MDA-MB-231 cells which showed stable knockdown of *WASF3* and compared promoter activity to the parental cells and control clone #8. As shown in Figure 2C, *KISS1* promoter activity in the absence of *WASF3* is significantly increased compared with the parental cells and control clone #8. These experiments clearly demonstrate that the elevated *KISS1* expression levels seen in this system are due to enhanced transcription, which is somehow influenced by *WASF3*.

KISS1 upregulation is specifically associated with knockdown of the WASF3 family member

The experiments described above demonstrate that knockdown of *WASF3* leads to increased *KISS1* expression. To determine whether this was specific to the *WASF3* family member, we also knocked down the *WASF1* and *WASF2* genes in MDA-MB-231 cells using siRNA and measured *KISS1* expression levels (Figure 2D). In these experiments it was clear that a significant change in *KISS1* expression was only observed when *WASF3* was down regulated, demonstrating the specificity of the relationship to this particular family member.

Knockdown of *WASF3* is associated with reduced MMP9 expression

Our previous studies (2), demonstrated that knockdown of *WASF3* was associated with loss of MMP9 activity. This relationship was confirmed in the present series of clones using RT-PCR analysis of MDA-MB-231 cells after stable shRNA knockdown of *WASF3* (Figure 3A). Western blot analysis of the *WASF3* knockdown clones (#12 and #18) also demonstrated a corresponding increase of KISS1 and decrease of MMP9 proteins (Figure 3B). To explore the potential role of MMP9 on the invasion phenotype of MDA-MB-231 cells, we determined the pro- and active levels of MMP9 secretion in cell supernatants from actively growing parental, control and knockdown cells. MMP9 enzyme concentration was reduced in supernatants from the *WASF3* knockdown cells after 24 hours, indicating that knockdown of *WASF3* expression is also associated with reduced MMP9 release from these cells (Figure 3C).

Knockdown of *WASF3* is associated with higher cytoplasmic I κ B α levels and reduced nuclear localization of the p65/p50 subunits of NF κ B

KISS1 inhibits MMP9 activity by decreasing nuclear translocation of both the p50 and p65 subunits of NF κ B, which bind to the *MMP9* promoter (19). Our data suggests that *WASF3* promotes MMP9 expression as a result of decreased KISS1 expression. We, therefore, examined how KISS1 expression is affected by NF κ B in the stable *WASF3* knockdown cells. I κ B α (inhibitor of NF κ B) is one of the NF κ B inhibitory proteins, which functions by masking the nuclear localization signals of NF κ B transcription factors and so blocking the ability to bind their target DNA (20-21). Elevated levels of I κ B α lead to a reduction of p65/p50 in the nuclear fraction in KISS1 transfectants (20). In *WASF3* knockdown cells, Western blot analysis revealed higher levels of cytoplasmic I κ B α (Figure 3D). Based on this relationship, we next determined NF κ B subunit protein levels in both the cytoplasmic and nuclear compartments in control and *WASF3* knockdown cells. Little change was noted in either the p65 or p50 subunit levels in the cytoplasmic fraction in the control cells (Figure 3D), whereas, the levels of both the p65 and p50 subunits were greatly reduced in the nuclear compartment in *WASF3* knockdown cells (Figure 3D).

Knockdown of KISS1 increases MMP9 expression and secretion while reversing the migration and invasive phenotype in *WASF3* knockdown cells

We have clearly demonstrated that *WASF3*-depleted cells lose their ability to migrate and invade (Supplementary Figures S1 and S2), and it is known that *KISS1* is an important tumor metastasis suppressor gene in a variety of cancer cells (13-15). Additionally, we have evidence that KISS1 blocks MMP9 activity by up-regulating I κ B, which subsequently inhibits NF κ B activation and nuclear translocation. Thus, it is possible that *WASF3* and KISS1 may physically and/or functionally interact to regulate the invasion phenotype. To explore this potential functional relationship between *WASF3* and KISS1 further, *KISS1* expression was transiently knocked down in two of the stable *WASF3*-silenced clones (#12 and #18) using siRNA. As shown in Figure 4A, KISS1 expression and protein levels were significantly reduced in both of these clones following exposure to the KISS1 siRNA. Knockdown of *KISS1* expression did not alter cell proliferation rate as determined by the MTT assay (data not shown). Using the wound closure assay, inhibiting *KISS1* using siRNA had little effect on cell movement in control cells with normal *WASF3* expression, although in *WASF3* knockdown cells, loss of *KISS1* expression significantly promoted cell motility (Figure 4B). This observation further suggests that *WASF3* can suppress KISS1 function. The Matrigel Clustering Assay measures the ability of cells to form a homotypic cluster when embedded in Matrigel as a measure of invasion potential. Invasive cells will break down the cluster and cells will migrate from the edges. Cells lacking *WASF3* could not break the cluster, unlike control cells. Knockdown of *KISS1* in *WASF3* knockdown cells also resulted in breakdown of the homotypic cluster and scattering of the cells beyond the colony

(Supplementary Figure S3). Using the transwell invasion assay, loss of *KISS1* in WASF3-silenced cells is accompanied by increased cell invasion (Figure 4B). Collectively, these results provide convincing evidence that loss of *KISS1* enhances cell migration and invasive potential in WASF3-depleted cells, which is consistent with its role as a metastasis suppressor.

The observations described above raised the question whether the increased invasiveness associated with loss of *KISS1* was related to MMP9 activity. We demonstrated earlier that *WASF3* knockdown led to reduced *MMP9* expression. To determine whether knockdown of *KISS1* alone also affected *MMP9* transcriptional levels, as well as the concentration of released MMP9 (activity) in the absence of WASF3, we used siRNA to inhibit *KISS1* expression in *WASF3* knockdown clones #12 and #18. In both clones, *MMP9* expression (Figure 4C) as well as its concentration in the supernatant (Figure 4C) was increased following knockdown of *KISS1*. These observations further support the suggestion that *KISS1* inhibits both *MMP9* expression and its enzymatic activity in WASF3-silenced cells.

Next we used the pNFkB-TA-Luc signal transduction reporter vector to investigate the role of *KISS1* in controlling NFkB signal transduction in cells with and without WASF3 knockdown. Knockdown of *KISS1* in cells expressing WASF3 show no effect on the activation levels of NFkB. In contrast, in cells where WASF3 is knocked down, reduction in *KISS1* levels result in a significant increase in NFkB activation (Figure 4D) which demonstrates the ability of *KISS1* to control this signaling pathway.

WASF3 knockdown attenuates the migration and invasion response to TNF α

Treatment of various tumor cell lines with TNF α typically results in elevated levels of MMP9 (22). In the WASF3 expressing cell clone #8, TNF α -induced *MMP9* transcription in a dose-dependent manner but, in WASF3-silenced cells (#12), TNF α treatment only raised MMP9 levels to minimally detectable levels (Figure 5A, upper panel). To determine whether TNF α treatment can induce cell invasion in WASF3-silenced cells, we used the chemo-invasion transwell assay (23) where cells are challenged to migrate through a gelatin matrix, an important substrate of MMP9. In WASF3 expressing cells (#8), exposure to TNF α significantly increased cell invasion (Figure 5A). In contrast, invasion of the WASF3 knockdown cells was not significant whether or not TNF α was added (Figure 5A), suggesting that, even though TNF α can still induce minimal *MMP9* transcription levels after WASF3 knockdown, other mechanisms must be involved in mediating this inhibition of TNF α -induced cell migration. TNF α also induces NFkB activation in breast cancer cells (23). Since *KISS1* inhibits this pathway and suppresses cell migration by inhibiting TNF α -induced NFkB nuclear translocation (20,23,24), it is possible that the relationship between WASF3 and *KISS1* expression, and subsequently modulation of *MMP9* transcription, is regulated through NFkB binding to the MMP9 promoter. This action is mediated through I κ B α binding to NFkB. Western blot analysis of I κ B α levels in the parental cells and control clone #8 following TNF α treatment demonstrates reduced protein levels (Figure 5B). In contrast, WASF3 knockdown cells (#12 and #18) show no change in I κ B α levels following TNF α stimulation suggesting a reduced activity of NFkB in these cells and hence reduced MMP9 production. Using the p-NF-kB-TA-Luc reporter construct, cells expressing WASF3 show increased NFkB activity, whereas cells that do not express WASF3 show no significant change in NFkB activity. These data support the role of I κ B α in the TNF α response only in WASF3 competent cells (Figure 5B).

KISS1 blocks TNF α -induced NFkB nuclear translocation in MDA-MB-231 cells (23). Because *KISS1* was up-regulated in the absence of WASF3, it was possible that *KISS1* may regulate MMP9 through its action on NFkB. Immunofluorescence staining revealed a distinct reduction of p65 nuclear translocation in WASF3 knockdown clones (#12 and #18)

compared to that seen in the parental cells and control clone #8 (Supplemental figure S4). If nuclear translocation of these subunits is reduced, then their activity on the *MMP9* promoter could be compromised, possibly suggesting why up-regulation of *KISS1* expression leads to loss of *MMP9* expression following *WASF3* knockdown.

Finally, we used the chemoinvasion assay to determine whether knockdown of both *WASF3* and *KISS1* affected TNF α -induced migration. As expected, in response to TNF α stimulation, cell invasion was increased after simultaneously downregulating both *WASF3* and *KISS1* (Figure 5C). Taken together, these data support the concept that up-regulation of *KISS1* expression in *WASF3* knockdown cells contributes to the inhibition of cell metastases and invasion in breast cancer cells, as an important example of the synchronization of interactions between metastasis suppressors and metastasis promoters, where an imbalance of either leads to abnormal cell behavior.

Discussion

The metastatic phenotype is clearly influenced by expression levels of individual genes (26). Since the demonstration that introduction of specific whole chromosomes from normal cells into cancer cells could suppress the invasion phenotype (26-27), an increasing number of genes have now been described that achieve the same outcome (28). These genes are described as metastasis suppressor genes (29). Other genes, such as *WASF3*, promote the invasion phenotype independently of the malignant phenotype (2) and their downregulation leads directly to a loss of the ability to metastasize (3). In many cases the action of these genes on metastasis has been studied in isolation in individual cell systems but, as we have shown here, and as previously described for the *NM23* metastasis suppressor gene (30), it is likely that their influence on metastasis is exerted through the intersection of common pathways. Here we provide evidence supporting this idea, which links the different functions of the metastasis promoting *WASF3* gene and the *KISS1* metastasis suppressor gene to regulate *MMP9* production through dysregulation of the NF κ B pathway. This model provides at least a partial explanation for the observed effect that *WASF3* has on *MMP9* expression. Down regulation of *WASF3* leads to up regulation of *KISS1*, which suppresses the nuclear translocation of the NF κ B subunits (p65/p50), through upregulation of I κ B α , leading to reduced activation of NF κ B responsive genes such as the *MMP9*. This effect has been shown to result from the reduced interaction of NF κ B with the *MMP9* promoter (20). Molecular and cellular analysis shows reduced nuclear trafficking of NF κ B subunits in *WASF3* knockdown cells. In HT-1080 cells, *KISS1* overexpression also led to a decrease in nuclear translocation of NF κ B leading to a specific down regulation of *MMP9*. This effect appears to be somewhat specific for *MMP9*, since in HT1080 cells (20) and MDA-MB-231 cells (7), *MMP2* expression levels were unaffected by dysregulation of either *KISS1* or *WASF3* respectively.

KISS1 was initially discovered as a tumor metastasis suppressor gene (13-15,30-31) and overexpression of *KISS1* reduces invasion and migration in MDA-MB-435 cells (13). Loss of *KISS1* expression has been noted in metastatic breast, pancreatic, bladder, and esophageal cancers (30-32). In transient transfection assays using luciferase reporter constructs, *KISS1* expression is driven through the binding of the AP-2 α and SP1 transcription factors to its promoter (34). In our studies, using a promoter reporter construct, upregulation of *KISS1* in *WASF3* depleted cells resulted from increased transcription.

The primary *KISS1* transcript produces a 145 amino acid protein known as Kisspeptin. Following proteolytic processing smaller peptides (Kp54, Kp14, and Kp13) are generated which are defined by their amino acid length and constitute the family of kisspeptins (32,35). A synthetic peptide, Kp-10, which retains receptor binding activity (35) appears to

inhibit tumor angiogenesis by blocking Sp1-mediated *VEGF* expression (36). The correlation between downregulation of *WASF3* and upregulation of *KISS1* suggests one mechanism for the control of *VEGF*. Knockdown of *WASF3* was shown to result in reduced *VEGF* expression in vivo, where the number and size of microvessels in the tumors that formed from these cells were significantly reduced (3). This observation also provides an explanation for the reduced tumorigenicity in *WASF3* depleted cells. Our gene expression analysis identified >35 dysregulated genes involved in tumor invasion and metastasis as a result of knockdown of *WASF3*. Some of these events are probably consequences of the disruption of NFκB signaling. For example, *IL8* is transcriptionally responsive to NFκB, and it is frequently up regulated in metastatic cells. Overexpression of exogenous *IL8* promotes the metastatic phenotype in breast cancer cells (37). *IL8* has other effects on tumor progression and has been shown to promote neovascularization in tumors, which may contribute to the hypovascularization in tumors from the *WASF3* knock down cells. Loss of *WASF3* showed significant changes in many other genes, some of which, such as *ICAM1*, *IL8*, *IL1B*, and *TGM2*, have been shown to be direct targets of NFκB (<http://bioinfo.liffr.fr/NF-KB/>) and these genes are also down regulated in the knockdown cells. Interestingly, *IL11* is also a target of NFκB but shows up regulation, suggesting an independent control mechanism in this system. Other genes down regulated in response to *WASF3* knockdown include the TFF family which have been shown to promote metastasis (38), in this case apparently independently of NFκB, and their down regulation may contribute to loss of invasion seen in *WASF3* depleted MDA-MB-231 cells. The analysis of the TFF genes was beyond the scope of the present report.

TNFα is a proinflammatory cytokine, whose chronic elevated expression has been directly linked to the promotion and progression of human cancer (25). There is strong evidence implicating the chronic production of TNFα with invasion and motility of cancer cells through induction of *MMP9* expression activated through the NFκB pathway (20,25). TNFα is also highly expressed in the tumor microenvironment and modulates NFκB, transcriptional activation of apoptosis, angiogenesis, invasion, and metastasis (20). NFκB is found in a complex with IκB in the cytoplasm of unstimulated cells. Following stimulation with phorbol esters, or cytokines such as TNFα and interleukin 1, NFκB dissociates from IκB and can then migrate into the nucleus, where it regulates transcription of its target genes. Consistent with this observation is the upregulation of *KISS1* in *WASF3* knockdown cells, which in turn interferes with TNFα-induced NFκB nuclear translocation, resulting in a decrease in *MMP9* promoter activation, which could account for the negligible change seen in cell invasion following TNFα stimulation in these cells. Interestingly, the KISS1 protein forms a complex with pro-MMP and, following activation cleaves the KISS1 protein (39) suggesting a feedback loop exists between these two factors.

As with its other family members, *WASF3* has been implicated in actin cytoskeleton dynamics through exposure of the binding sites for the Arp2/3 complex following external stimulation by growth factors such as PDGF. As a result, monomeric actin is recruited, leading to actin polymerization. The WASP family of proteins are activated through the Rho/cdc42 intermediates. Activation of the WASF proteins is thought to be through activation of Rac (40), which for *WASF3* results in increased lamellipodia formation facilitating cell movement or initiation of metastasis (2). It was recently shown that KISS1 inhibits TNFα-induced breast cancer cell invasion by suppressing RhoA-mediated NFκB activation (23), suggesting that *WASF3* and KISS1 function may be mediated through activation of different signaling intermediates, although activation of the NFκB pathway is a common outcome.

WASF3 was shown to interact with the p85 component of PKC and evidence was presented to suggest downstream activation of the p38 pathway (2). Our current data show that

knockdown of *WASF3* inhibits *JNK* activation but, consistent with previous reports, had no effect on ERK (unpublished data). *MMP9* has been shown to be regulated partly by both *ERK* and *JNK*-dependent signaling pathways (20,41). We found that transcription of *MMP9* is attenuated, although still responsive to, *TNFα* stimulation in *WASF3*-silenced cells, consistent with the existence of a redundant pathway controlling *MMP9* expression, in addition to its regulation by *KISS1*. This observation raises the possibility that *MMP9* expression can be promoted through *TNFα*-induced ERK activation when *WASF3* levels are repressed. The high expression of *IL-8* in invasive cancer cells requires a complex cooperation between NFκB and other transcription factors (37). The corresponding elevation of *KISS1* expression seen in *WASF3* stable knockdown MDA-MB-231 cells, supports the concept of a direct interplay between *WASF3* and the regulation of *MMP9* and other metastasis related genes through its effects on *KISS1*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Takenawa T, Suetsugu S. The WASP-WAVE protein network: Connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol.* 2007; 8:37–48. [PubMed: 17183359]
2. Sossey-Alaoui K, Li X, Ranalli TA, Cowell JK. WAVE3-mediated cell migration and lamellipodia formation are regulated downstream of phosphatidylinositol 3-Kinase. *J Biol Chem.* 2005; 280:21748–55. [PubMed: 15826941]
3. Sossey-Alaoui K, Safina A, Li X, Vaughan MM, Hicks DG, Bakin AV, Cowell JK. Down-regulation of WAVE3, a metastasis promoter gene, inhibits invasion and metastasis of breast cancer cells. *Am J Pathol.* 2007; 170:2112–21. [PubMed: 17525277]
4. Miki H, Suetsugu S, Takenawa T. WAVE, a novel WASP-family protein involved in actin reorganization induced by Rac. *EMBO J.* 1998; 17:6932–41. [PubMed: 9843499]
5. Gautreau A, Ho HY, Li J, Steen H, Gygi SP, Kirschner MW. Purification and architecture of the ubiquitous Wave complex. *Proc Natl Acad Sci.* 2004; 101:4379–83. [PubMed: 15070726]
6. Derivery E, Lombard B, Loew D, Gautreau A. The Wave complex is intrinsically inactive. *Cell Motil Cytoskel.* 2009; 66:777–90.
7. Sossey-Alaoui K, Ranalli TA, Li X, Bakin AV, Cowell JK. WAVE3 promotes cell motility and invasion through the regulation of MMP-1, MMP-3, and MMP-9 expression. *Exp Cell Res.* 2005; 308:135–45. [PubMed: 15907837]
8. Teng Y, Ren M, Cheney R, Sharma S, Cowell JK. Inactivation of the *WASF3* gene in prostate cancer cells leads to suppression of tumorigenicity and metastases. *Br J Cancer.* 2010; 103:1066–75. [PubMed: 20717117]
9. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell.* 2010; 141:52–67. [PubMed: 20371345]
10. Schnaeker EM, Ossig R, Ludwig T, Dreier R, Oberleithner H, Wilhelmi M, Schneider SW. Microtubule-dependent matrix metalloproteinase-2/matrix metalloproteinase-9 exocytosis: prerequisite in human melanoma cell invasion. *Cancer Res.* 2004; 64:8924–31. [PubMed: 15604254]

11. Kupferman ME, Fini ME, Muller WJ, Weber R, Cheng Y, Muschel RJ. Matrix Metalloproteinase 9 Promoter Activity Is Induced Coincident with Invasion during Tumor Progression. *Am J Pathol.* 2000; 157:1777–83. [PubMed: 11106549]
12. Mylona E, Nomikos A, Magkou C, Kamberou M, Papassideri I, Keramopoulos A, Nakopoulou L. The clinicopathological and prognostic significance of membrane type 1 matrix metalloproteinase (MT1-MMP) and MMP-9 according to their localization in invasive breast carcinoma. *Histopathology.* 2007; 50:338–47. [PubMed: 17257129]
13. Lee JH, Welch DR. Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. *Cancer Res.* 1997; 57:2384–87. [PubMed: 9192814]
14. Jiang Y, Berk M, Singh LS, Tan H, Yin L, Powell CT, Xu Y. KiSS1 suppresses metastasis in human ovarian cancer via inhibition of protein kinase C alpha. *Clin Exp Metastasis.* 2005; 22:369–76. [PubMed: 16283480]
15. Goldberg SF, Miele ME, Hatta N, Takata M, Paquette-Straub C, Freedman LP, Welch DR. Melanoma metastasis suppression by chromosome 6: evidence for a pathway regulated by CRSP3 and TXNIP. *Cancer Res.* 2003; 63:432–40. [PubMed: 12543799]
16. Shirasaki F, Takata M, Hatta N, Takehara K. Loss of expression of the metastasis suppressor gene KISS1 during melanoma progression and its association with LOH of chromosome 6q16.3-q23. *Cancer Res.* 2001; 61:7422–25. [PubMed: 11606374]
17. Sanchez-Carbayo M, Capodiceci P, Cordon-Cardo C. Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. *Am J Pathol.* 2003; 162:609–17. [PubMed: 12547718]
18. Lo KC, Rossi MR, LaDuca J, Hicks DG, Turpaz Y, Hawthorn L, Cowell JK. Candidate glioblastoma development gene identification using concordance between copy number abnormalities and gene expression level changes. *Genes Chromosomes Cancer.* 2007; 46:875–94. [PubMed: 17620294]
19. Kunapuli P, Chitta KS, Cowell JK. Suppression of the cell proliferation and invasion phenotypes in glioma cells by the LGI1 gene. *Oncogene.* 2003; 22:3985–91. [PubMed: 12821932]
20. Yan C, Wang H, Boyd DD. KiSS-1 represses 92-kDa Type IV collagenase expression by down-regulating NF- κ B binding to the promoter as a consequence of I κ B α -induced block of p65/p50 nuclear translocation. *J Biol Chem.* 2001; 276:1164–72. [PubMed: 11060311]
21. Karin M. Nuclear factor- κ B in cancer development and progression. *Nature.* 2006; 441:431–36. [PubMed: 16724054]
22. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit Rev Biochem Mol Biol.* 2002; 37:375–536. [PubMed: 12540195]
23. Cho SG, Li D, Stafford LJ, Luo J, Rodriguez-Villanueva M, Wang Y, Liu M. KISS1 suppresses TNF α -induced breast cancer cell invasion via an inhibition of RhoA-mediated NF κ B activation. *J Cell Biochem.* 2009; 107:1139–49. [PubMed: 19533666]
24. Adli M, Merkhofer E, Cogswell P, Baldwin AS. IKK α and IKK β each function to regulate NF- κ B activation in the TNF-Induced/Canonical pathway. *PLoS ONE.* 2010; 5:e9428. [PubMed: 20195534]
25. Balkwill F. TNF- α in promotion and progression of cancer. *Cancer Met Rev.* 2006; 25:409–16.
26. Mareel M, Oliveira MJ, Madani I. Cancer invasion and metastasis: interacting ecosystems. *Virchows Arch.* 2009; 454:599–622. [PubMed: 19471961]
27. Ichikawa T, Ichikawa Y, Dong J, Hawkins AL, Griffin CA, Isaacs WB, Oshimura M, Barrett JC, Isaacs JT. Localization of metastasis suppressor gene(s) for prostatic cancer to the short arm of human chromosome 11. *Cancer Res.* 1992; 52:3486–90. [PubMed: 1596907]
28. Welch DR, Chen P, Miele ME, McGary CT, Bower JM, Stanbridge EJ, Weissman BE. Microcell-mediated transfer of chromosome 6 into metastatic human C8161 melanoma cells suppresses metastasis but does not inhibit tumorigenicity. *Oncogene.* 1994; 9:255–62. [PubMed: 8302587]
29. Horak CE, Lee JH, Marshall JC, Shreeve SM, Steeg PS. The role of metastasis suppressor genes in metastatic dormancy. *APMIS.* 2008; 116:586–601. [PubMed: 18834404]

30. Steeg PS. Metastasis suppressors alter the signal transduction of cancer cells. *Nat Rev Cancer*. 2003; 3:55–63. [PubMed: 12509767]
31. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, Welch DR. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst*. 1996; 88:1731–37. [PubMed: 8944003]
32. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, Terao Y, Kumano S, Takatsu Y, Masuda Y, Ishibashi Y, Watanabe T, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature*. 2001; 411:613–17. [PubMed: 11385580]
33. Makri A, Pissimissis N, Lembessis P, Polychronakos C, Koutsilieris M. The kisspeptin (KiSS-1)/GPR54 system in cancer biology. *Cancer Treat Rev*. 2008; 34:682–92. [PubMed: 18583061]
34. Mitchell DC, Abdelrahim M, Weng J, Stafford LJ, Safe S, Bar-Eli M, Liu M. Regulation of KiSS-1 metastasis suppressor gene expression in breast cancer cells by direct interaction of transcription factors activator protein-2a and specificity protein-1. *J Biol Chem*. 2006; 281:51–8. [PubMed: 16260418]
35. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, Brézillon S, Tyldesley R, Suarez-Huerta N, Vandeput F, Blanpain C, Schiffmann SN, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem*. 2001; 276:34631–6. [PubMed: 11457843]
36. Cho SG, Yi Z, Pang X, Yi T, Wang Y, Luo J, Wu Z, Li D, Liu M. Kisspeptin-10, a KiSS-derived decapeptide, inhibits tumor angiogenesis by suppressing Sp1-mediated VEGF expression and FAK/Rho GTPase Activation. *Cancer Res*. 2009; 69:7062–70. [PubMed: 19671799]
37. Freund A, Jolivel V, Durand S, Kersual N, Chalbos D, Chavey C, Vignon F, Lazennec G. Mechanisms underlying differential expression of interleukin-8 in breast cancer cells. *Oncogene*. 2004; 23:6105–14. [PubMed: 15208657]
38. Oertel M, Graness A, Thim L, Bühlung F, Kalbacher H, Hoffmann W. Trefoil factor family-peptides promote migration of human bronchial epithelial cells: synergistic effect with epidermal growth factor. *Am J Respir Cell Mol Biol*. 2001; 25:418–24. [PubMed: 11694446]
39. Takino T, Koshikawa N, Miyamori H, Tanaka M, Sasaki T, Okada Y, Seiki M, Sato H. Cleavage of metastasis suppressor gene product KiSS-1 protein/metastin by matrix metalloproteinases. *Oncogene*. 2003; 22:4617–26. [PubMed: 12879005]
40. Shakir MA, Jiang K, Struckhoff EC, Demarco RS, Patel FB, Soto MC, Lundquist EA. The Arp2/3 activators WAVE and WASP have distinct genetic interactions with Rac GTPases in *Caenorhabditis elegans* axon guidance. *Genetics*. 2008; 179:1957–71. [PubMed: 18689885]
41. McCawley LJ, Li S, Wattenberg EV, Hudson LG. Sustained Activation of the Mitogen-activated Protein Kinase Pathway: A mechanism underlying receptor tyrosine kinase specificity for matrix metalloproteinase-9 induction and cell migration. *J Biol Chem*. 1999; 274:4347–53. [PubMed: 9933637]

Figure 1. Knockdown of WASF3 affects expression levels of genes associated with cell motility and metastasis

(A) qRT-PCR analysis (left) of MDA-MB-231 cells transfected with two different shRNAs (shRNA1 and 2) show ~70% downregulation of WASF3 mRNA levels compared with cells transfected with the empty vector or an shRNA targeting GFP, which show the same expression levels at the parental (WT) cells. Both RT-PCR and Western blot analysis (right) confirmed the loss of

WASF3 RNA (above) and protein (below) in selected MDA-MB-231 stable knockdown cell clones (#5, #12, and #18) compared to WASF3-expressing control clones (#3, #6, and #8), and the parental cell lines (WT). β -actin was used as a loading control for both assays. (B) Summary of expression level changes for selected genes associated with cell movement in WASF3 knockdown cells. (C) RTPCR analysis of PDGFA, TFF1, TFF2, and IL8 confirms transcriptional down regulation in WASF3 knockdown clones compared with clones showing no knockdown (#3, #6, #8). (D) qRT-PCR analysis of KISS1 and TFF3 gene expression further demonstrates their altered expression levels in WASF3 knockdown clones which were identical to those seen in the microarray experiment. Relative expression levels (mean $\pm$ S.E. from three clones, three repeats for each clone) are presented as a percentage of control expression levels (** $p < 0.01$).

Figure 2. KISS1 expression is inversely correlated with WASF3 expression in breast and prostate cancer cell lines

(A) Western blot analyses show an inverse relationship between endogenous KISS1 and WASF3 protein levels in four breast cancer cell lines (left). Overexpression of WASF3 is coincident with down-regulation of KISS1 in both MCF7 and MDA-MB-231 cells (right). (B) Both qRT-PCR (left) and Western blot analysis (right) shows that siRNA silencing of WASF3 results in up-regulation of KISS1 expression in SkBr3 and BT474 breast cancer cells as well as PC3 prostate cancer cells. (C) When a KISS1 promoter reporter construct was introduced in cells expressing WASF3 (parental cells (WT) and a control (#8) clone), luciferase activity was low. In contrast, in cells (#12 and #18) where WASF3 was significantly downregulated, KISS1 promoter activity increased. Data presented are the mean $\pm$ S.E. of triplicate experiments (* $p < 0.05$ and ** $p < 0.01$ by Student's t -test). (D) To determine the consequences to KISS1 expression following WASF1 and WASF2 knockdown was assessed. In these experiments, in contrast to knockdown of WASF3 where KISS1 expression increases, there is no change in KISS1 expression following knockdown of WASF1 or WASF2.

Figure 3. Effect of WASF3 on MMP9 and NFκB activity

(A) RT-PCR analysis shows that knockdown of WASF3 results in reduced MMP9 expression levels. (B) Western blot analysis of representative WASF3-silenced clones (#12 and #18) shows reduced MMP9 protein levels with concomitant increases in KISS1 protein levels. β-actin was used as the loading control in this assay. (C) Quantification of the pro- and active forms of MMP9 in the supernatants of actively growing cells was determined using the Fluorokine MAP assay procedure. Cells showing knockdown of WASF3 expression (#12 and #18) show reduced MMP9 release from MDA-MB-231 cells. (D) Western blot analysis demonstrates increased IκBα levels in the cytoplasm of WASF3-knockdown cell clones (#12 and #18) compared to WASF3-expressing cells (#8). Western blot analysis of nuclear fractions from the same clones and parental cells (WT) demonstrates

reduced nuclear levels of NF κ B proteins (p50 and p65) in cells without WASF3 expression (below).

Figure 4. Knockdown of KISS1 in WASF3 depleted cells increases invasion and MMP activity

(A) RT-PCR analysis of knockdown of KISS1 (left) demonstrates reduction to below detectable levels after 72 hours in parental cells. Under these conditions, western blot analysis of WASF3 knockdown cells shows significant loss of KISS1 protein (right). (B) Wound healing assays show increased mobility of WASF3 knockdown clones #12 and #18 after down regulation of KISS1 expression (left), while no significant change was found in control clone #8, with or without silencing KISS1 expression (* $p < 0.05$). Transwell invasion assays show increased invasion after double knockdown of the KISS1 and WASF3 genes (right). (C) Loss of KISS1 in WASF3-depleted cells leads to increased transcription of MMP9 and increased activity of MMP9 in the supernatant. RT-PCR analysis shows that knockdown of KISS1 is associated with up-regulation of MMP9 expression levels in the WASF3-depleted cells (upper panel). Measurement of MMP9 concentration indicates increased pro- and active forms of MMP9 are secreted into the supernatant in WASF3 and KISS1 double knockdown cells compared with the single WASF3 knockdown cells (lower panel). (D) Using a luciferase reporter assay, knockdown of KISS1 in WASF3 depleted cells (#12 and #18) results in increased NF κ B activity compared with WASF3-expressing clones (#8).

Figure 5. Knockdown of WASF3 is associated with down regulation of MMP9 expression, as well as cell migration and invasion response to TNFα

(A) RT-PCR analysis shows that MMP9 expression levels are up-regulated in a dose-dependent manner following TNFα stimulation in control clone #8, compared with WASF3 knockdown clone #12 (above). Using the chemoinvasion assay (below), TNFα-stimulated cell invasiveness was suppressed in cells showing knockdown of WASF3, whereas the TNFα stimulated control clone (#8) significantly promotes cell invasion. Data (mean ± S.E.) are expressed as the percentage of invasion rate of TNFα-untreated cells (**p<0.01). (B) Western blot analysis (upper panel) demonstrates a TNFα-induced reduction in IκBα levels in parental (WT) and control cells (#8), whereas no significant decrease in IκBα protein

levels was seen in WASF3 knockdown cells (#12 and #18). Using a luciferase reporter for NF κ B activity (lower panel), WASF3 expressing cells showing reduced I κ B α show a concomitant increase in NF κ B activity whereas WASF3 depleted cells do not. (C) The chemoinvasion assay indicates that double knockdown cells increase their invasion potential in response to TNF α stimulation. Results (mean $\pm$ S.E.) are expressed as the percentage of cell invasion rate of TNF α -untreated cells.