Effect of the knockdown of death-associated protein 1 expression on cell adhesion, growth and migration in breast cancer cells

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Abstract. Death-associated protein 1 (DAP1) is a highly conserved phosphoprotein involved in the regulation of autophagy. A previous clinical study by our group suggested an association between low DAP1 expression and clinicopathological parameters of human breast cancer. In the present study, we aimed to determine the role of DAP1 in cancer cell behaviour in the context of human breast cancer. We developed knockdown sublines of MCF7 and MDA-MB-231, and performed growth, adhesion and invasion assays and electric cell-substrate impedance sensing (ECIS) studies of the post-wound migration of cells. In addition, we studied the mRNA expression of caspase 8 and 9, DELE, IPS1, cyclin D1 and p21 in the control and knockdown sublines. Knockdown was associated with increased adhesion and migration, significantly so in the MDA-MB-231^{DAP1kd} cell subline (p=0.029 and p=0.001, respectively). Growth in MCF7 cells showed a significant suppression on day 3 (p=0.029), followed by an increase in growth matching the controls on day 5. While no change in the apoptotic response to serum starvation could be attributed to DAP1 knockdown, the expression of known components of the apoptosis pathway (caspase 8) and cell cycle (p21) was significantly reduced in the MCF7^{DAP1kd} cell subline ($p \leq 0.05$), while in MDA-MB-231^{DAP1kd} the expression of a pro-apoptotic molecule, IPS1, was suppressed ($p \leq 0.05$). DAP1 may have an important role in cell adhesion, migration and growth in the context of breast cancer and has significant associations with the apoptosis pathway. Furthermore, we believe that delayed increase in growth observed in the MCF7^{DAP1kd} cell subline may indicate activation of a strongly pro-oncogenic pathway downstream of DAP1.

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Introduction

Death-associated protein 1 (DAP1) is characterised as a ubiquitous, highly conserved protein weighing 15 kDa, which exists as a phosphoprotein *in vivo*. It is transcribed from the *DAP1* gene located in chromosome 5 band p15.2 (1).

DAP1 was initially identified along with other molecules with the same designation in HeLa cells. The HeLa cells were subjected to a continuous apoptotic stimulus (specifically, exposure to interferon γ). DAP1 was identified along with DAP kinase, DAP3 and DAP5. However, due to a lack of any identifiable functional motifs, no function was initially ascribed to DAP1 (2).

Recently, further studies have suggested a role for DAP1 in the regulation of autophagy. In times of starvation, the UNC51-like kinase 1 (ULK1) complex is freed from the inhibitory effect of the mammalian target of rapamycin complex 1 (mTORC1). This triggers the autophagy pathway. It has recently been determined that DAP1 is similarly co-stimulated with ULK1. However, DAP1 has an inhibitory effect on autophagy (2).

The mechanisms controlling autophagy are still subject to a significant degree of speculation. However, while the downstream effectors of DAP1 are as yet unknown, the pathways affected by DAP1 can be extrapolated from the known downstream effectors of ULK1. The pathways known to be impacted by the focal adhesion kinase family interacting protein of 200 kDa (FIP200), the active component of the ULK1 complex, include the cell cycle, embryogenesis, autophagosome formation, cell migration, the mTOR pathway and apoptosis. It could be speculated that DAP1 would have an effect on the above enumerated pathways in breast cancer cells (3).

Therefore, we hypothesised that DAP1 would serve as an anti-oncogenic molecule, and would most likely exert its protective effect as a pro-apoptotic protein. Previously, we published our findings regarding the mRNA expression of *DAP1* in a breast cancer cohort of 127 patients. The present

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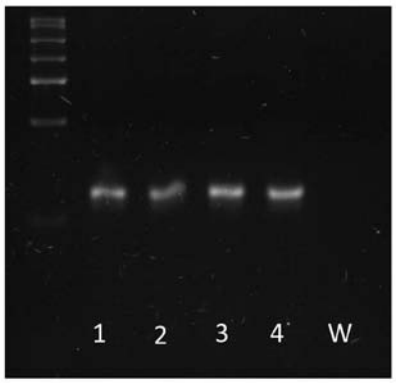

Legend	Sub-line	Area	Mean	Min	Max
1	MCF7pEF6	0.026	630.699	300	1033
2	MCF7DAP1kd	0.026	565.680	300	896
3	MDA-MB-231pEF6	0.026	707.085	310	1605
4	MDA-MB-231DAP1kd	0.026	665.405	319	1111
W	Water				

B

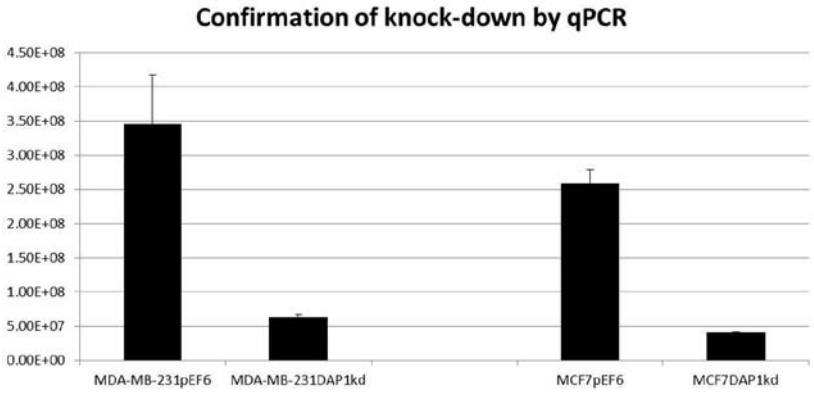

Figure 1. Demonstration of the knockdown of DAP1 mRNA expression in MCF7 and MDA-MB-231 sublines using conventional and quantitative PCR. (A) PCR gel with densitometry by ImageJ; (B) knockdown demonstrated by quantitative real-time PCR. DAP1, death-associated protein 1.

study suggested that *DAP1* was associated with favourable prognosis in the context of human breast cancer. Specifically, low expression levels of *DAP1* were found to be significantly associated with adverse clinical outcomes, advanced clinical stage, and higher scores according to the Nottingham Prognostic Index (NPI). However, a significant association was not observed between *DAP1* expression and tumour grade (4).

The results from the breast cancer cohort support the hypothesis that DAP1 is a favourable marker of prognosis in human breast cancer. Therefore, it could be hypothesised that DAP1 would have inhibitory effects on cell migration and apoptosis.

We aimed to study these effects on breast cancer tumour cells after knocking down DAP1 expression in the cells by transfecting them with a plasmid bearing a ribozyme transgene designed for this purpose. Furthermore, we studied the effects of DAP1 knockdown on the mRNA expression levels of a panel of molecules involved in apoptosis and the cell cycle. The molecules studied as surrogates for apoptosis were death ligand signal enhancer (DELE), IFN-β promoter stimulator-1 (IPS1), caspase 8 and 9. Cyclin D1 and p21 were studied as surrogates for the cell cycle (5,6).

Materials and methods

Cells and media. The human breast cancer cell lines, MCF7 and MDA-MB-231, were purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA) and routinely maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal calf serum (FCS) and antibiotics. The cells were incubated at 37˚C in 5% CO_2 at 95% humidity.

Generation of MCF-7 and MDA-MB-231 cells displaying knockdown of DAP1. DAP1 expression was suppressed in the MCF7 and MDA-MB-231 cells through transfection of a pEF6/V5-His-TOPO plasmid (Invitrogen, Paisley, UK) containing ribozyme transgenes which specifically recognise and cleave DAP1 mRNA transcript. These techniques have been widely implemented by our laboratories and are outlined by Jiang *et al* (7). The purified plasmids were sequenced in order to verify the presence and sequence of the construct (MWG, Milton Keynes, UK). MCF7 and MDA-MB-231 cells were transfected with plasmids containing the ribozyme transgenes or an empty pEF6 control plasmid. Cells were then subjected to selection with blasticidin. Cells containing the control pEF6 plasmid were designated as $MCF7^{pEF6}$ and $MDA\text{-}MB\text{-}231^{pEF6}$, respectively, and those containing the ribozyme transgenes were labelled $MCF7^{DAP1kd}$ and $MDA\text{-}MB\text{-}231^{DAP1kd}$, respectively. Knockdown was demonstrated using quantitative and conventional polymerase chain reaction assays (Fig. 1).

Conventional PCR was performed using primers as detailed in Table I, and subjected to the following conditions: 95˚C for 5 min, then 30 cycles consisting of 94˚C for 40 sec, 55˚C for 40 sec and 72˚C for 1 min, followed by 72˚C for 10 min. PCR by-products were separated on a 2% agarose gel, visualised under blue light, photographed using a Unisave camera (Wolf

Table I. Primers used in the polymerase chain reaction.

Gene	Sequence (5'-3')
PDPL F8	GAATCATCGTTGTGGTTATG
PDPL ZR1	ACTGAACCTGACCGTACACTTTCATTTGCCTATCACAT
DAP1 F1	ATGGACAAGCATCCTTCC
DAP1 ZR1	ACTGAACCTGACCGTACACTCTGTCAGGGAAATACCAA
Caspase 8 F1	AGAAAGGAGGAGATGGAAAG
Caspase 8 ZR1	ACTGAACCTGACCGTACAGACCTCAATTCTGATCTGCT
Caspase 9 F1	AAGCCCAAGCTCTTTTTC
Caspase 9 ZR1	ACTGAACCTGACCGTACAGTTACTGCCAGGGGACTC
Caspase 9 F2	ATATGATCGAGGACATCCAG
Caspase 9 ZR2	ACTGAACCTGACCGTACACAGGAGATGAACAAAGGAAG
DELE F1	GTCATGAGCATGGCAGAG
DELE ZR1	ACTGAACCTGACCGTACAACCTGGCATAGCGCTACT
IPS1 F1	AGAGAAGGAGCCAAGTTACC
IPS1 ZR1	ACTGAACCTGACCGTACAATTCCTTGGGATGGCTCT
P21 F1	CGGGATGAGTTGGGAGGAG
P21 ZR1	ACTGAACCTGACCGTACAACAGGTCCACATGGTCTTCC
Cyclin D1 F1	CGGTGTCCTSCTTCAAATGT
Cyclin D1 ZR1	ACTGAACCTGACCGTACAAAGCGGTCCAGGTAGTTC
GAPDH F1	AAGGTCATCCATGACAACTT
GAPDH ZR1	ACTGAACCTGACCGTACAGCCATCCACAGTCTTCTG
GAPDH F2	CTGAGTACGTCGTGGAGTC
GAPDH ZR2	ACTGAACCTGACCGTACACAGAGATGATGACCCTTTTG

PDPL, podoplanin; DAP1, death-associated protein 1; DELE, death ligand signal enhancer; IPS1, IFN-β promoter stimulator-1; p21, protein 21; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Laboratories), and analysed by ImageJ (National Institutes of Health, Bethesda, MD, USA).

The plasmids were previously produced and provided by Dr Y. Jia and colleagues, who also had successfully demonstrated knockdown of DAP1 in transfected mammalian cells using qPCR and western blotting (8).

Quantitative analysis of the transcripts in the cells. Total RNA was extracted from cell pellets using TRI reagent (Sigma-Aldrich). Total RNA was extracted and purified from the cells using a modified phenol-chloroform phase separation technique as per the manufacturer's instructions. Equal amounts of RNA were reverse transcribed into complementary DNA (cDNA) using a first-strand reverse transcription (RT) kit from AbGene (Surrey, UK). For quantitative analysis of the DAP1 transcripts, we employed a real-time quantitative PCR assay, as previously reported (9). Briefly, primers (Table I) were designed using the Beacon Designer software. The Amplifluor Uniprimer system was used as the probe system.

cDNA from cells and tissues, along with a set of standards were amplified simultaneously, on an ICycler iQ5 system (Bio-Rad). The concentration of the respective transcript was calculated from the standard curve, which was simultaneously generated. The levels of the transcripts are shown here as the respective transcript/glyceraldehyde 3-phosphate dehydrogenase (GAPDH) ratio.

Growth assay for the breast cancer cell lines. The growth of the breast cancer cell lines was assessed using a colorimetric-based method (10). On day one (D1), three (D3) and five (D5), plates were fixed and stained with crystal violet. Cells (3,000/well) were added. Cell density and growth were measured on a BioTek ELx800 multiplate reader (BioTek Instruments, Inc., Winooski, VT, USA).

Adhesion assay for the breast cancer cell lines. The adhesion assay was based on a previously published protocol (9). In brief, Matrigel basement membrane matrix (BD Biosciences, UK) was used as an adhesion sheet at 5 μg/well. Cells (45,000/well) were added. After a 45-min incubation, the wells were vigorously washed to remove unbound cells. Adherent cells were then fixed and stained with crystal violet. Stained cells were later counted in a number of random fields under a x40 objective.

Invasion assay for the breast cancer cell lines. Invasion assays were undertaken using inserts with 8-μm pores (BD Biosciences, Oxford, UK) in 24-well tissue culture plates. Each insert was first coated with Matrigel. After rehydration, 15,000 cells were seeded into each insert. After three days of incubation, the inserts were fixed with formalin and stained in crystal violet. Cells stained with crystal violet were counted and observed under the microscope for possible invasion as described by Jiang *et al* (11).

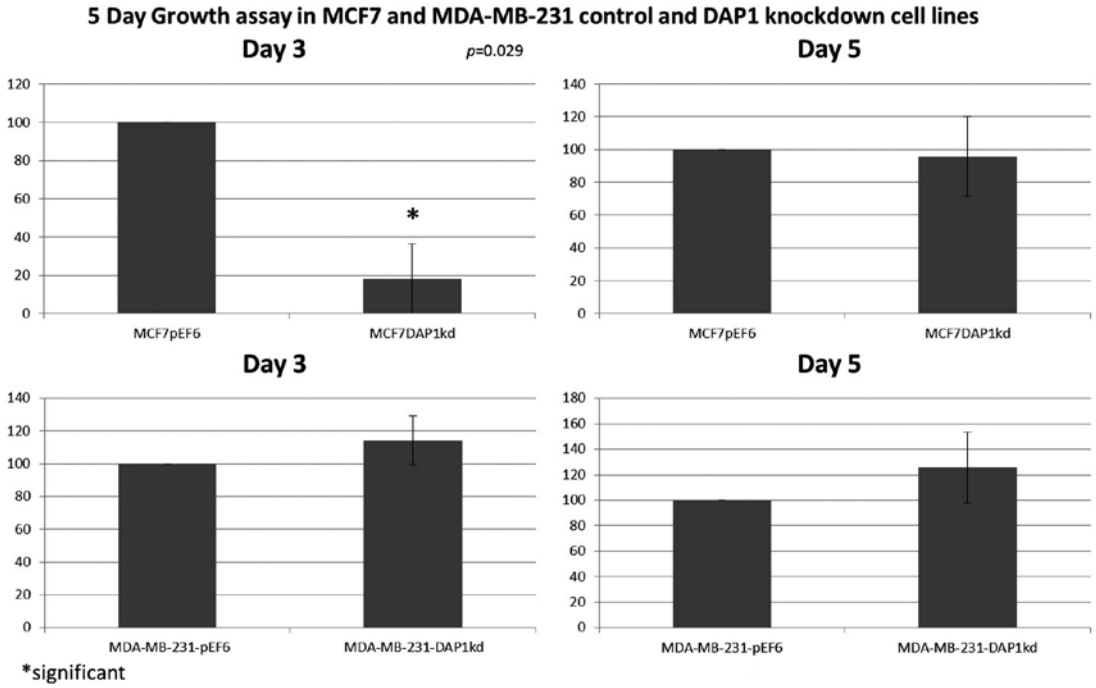

Figure 2. Growth assay comparing controls (pEF6) and DAP1 knockdown sublines of MCF7 and MDA-MB-231. The results of a 5-day growth assay, with plates fixed on day 1 (D1; reference), day 3 (D3) and day 5 (D5). Each cell line had 5 repeats (n=4) (*significant associations, Mann-Whitney U test). DAP1, death-associated protein 1.

Electric cell-substrate impedance sensing (ECIS)-based cell adhesion and cell migration assays. An ECIS Zθ device and 96W1E arrays were used (Applied BioPhysics, Inc., Troy, NY, USA) (12). Prior to seeding, the wells were instilled with serum-free medium, and the array was subjected to the 'stabilisation' function of the ECIS Zθ machine. A plate plan was made after thus identifying non-functional wells.

An identical number of MCF7 or MDA-MB-231 cells of the control or knockdown sublines was added into each well. Cell adhesion was recorded immediately following addition of the cells, at multiple frequencies, for up to 3 h. For the cell migration assay, confluent cells were wounded after at least 10 h of growth. Wounding conditions were optimised for each cell line (for MDA-MB-231, 20 sec, 3,000 μA, 60,000 Hz; for MCF7, 20 sec, 1,400 μA, 60,000 Hz). The migration of the cells was immediately traced following wounding for up to 4 h, again with multiple frequencies. Knockdown sublines were compared to the controls. Cell migration is shown here as the change in resistance.

Analysis of apoptosis using flow cytometry. Cells ($2x10^5$) were seeded into 25 cm^2 flasks and underwent different treatments. For each subline, one flask was maintained in normal nutrient medium as a control and another in serum-free medium for 3-4 days (depending on the cell line used). Both the adherent cells and floating cells were collected. Following cell collection, experiments were carried out using the Annexin V kit (Santa Cruz Biotechnology) and analysed using Partec CyFlow® SL flow cytometry and the accompanying FloMax software package (Partec GmbH, Münster, Germany).

Statistical analysis. Analysis of the data was performed using SigmaPlot 11 (Systat Software, San Jose, CA, USA). The software automatically determines the underlying distribution and advised accordingly. Medians were compared using the Mann-Whitney U test, while means were compared using the two-sample t-test. Percentage control was employed to compensate for variations between repeats of assays likely due to passage, variations in the quality of material and performance of the technique.

After consultation with the university statistician, it was found to be appropriate to apply random effects modelling to the results of a representative instance of the ECIS-based migration assay in order to ascertain the significance of the results.

Results

Increased growth is observed in the MCF7^{DAP1kd} cell subline compared to controls after an initial inhibition. The experiment was repeated successfully four times (n=4). Triplicate plates were made for D1 (reference plate), D3 and D5. During each run, each cell line was tested in eight repeats, of whom an average was taken. The percentage increase over the readings taken on D1 was calculated for D3 and D5.

The results of each run of the experiment were subjected to percentage control, with the control normalised to 100. The underlying distribution of the results was determined. Depending on the distribution, either the Mann-Whitney U test or the independent t-test was employed.

Compared to the controls (MCF7^{pEF6}), the DAP1 knockdown strain (MCF7^{DAP1kd}) was found to show less growth on D3 (100 vs. 18.2; p=0.029). However, the difference in the percentage of growth between cell lines was indistinguishable on D5. No statistically significant difference was observed in the MDA-MB-231 sublines (Fig. 2).

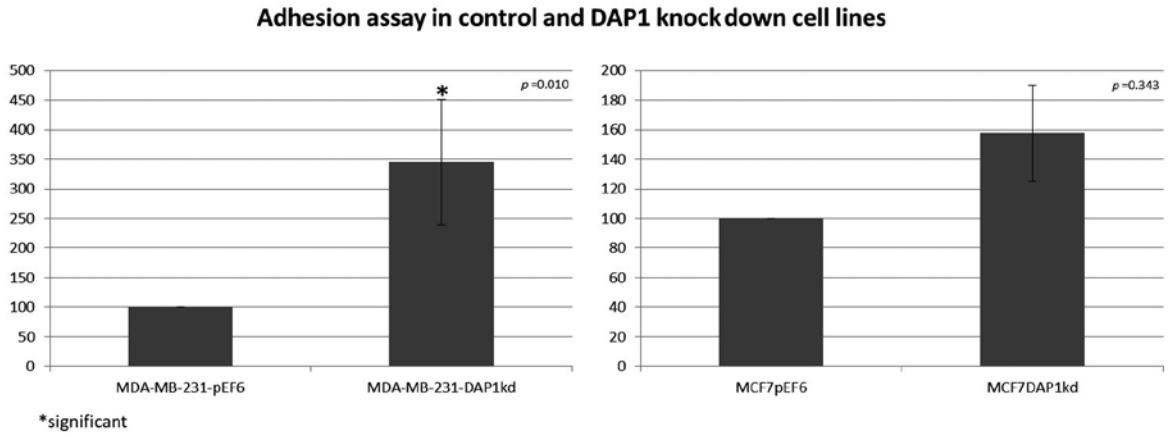

Figure 3. Adhesion assay comparing controls (pEF6) and DAP1 knockdown sublines of MCF7 and MDA-MB-231. The assay was performed on Matrigel-coated wells, with 6 repeats/cell line. The plate was incubated for 45 min before fixation in 4% formalin (n=4) (*significant associations, Mann-Whitney U test). DAP1, death-associated protein 1.

Increased adhesion is observed in the MDA-MB-231^{DAP1kd} cell subline compared to MDA-MB-231^{pEF6}. The adhesion assay was completed four times (n=4), with eight repeats for each subline studied. As detailed in the Materials and methods section, the cells were allowed to adhere for 45 min within the incubator, after which the non-adherent cells were discarded and washed away. The cells were fixed and stained, in order to be counted to assess adhesion.

The results of each run of the experiment were subjected to percentage control, with the control normalised to 100. The underlying distribution of the results was determined. Depending on the distribution, either the Mann-Whitney U test or the independent t-test was employed.

In the MCF7 cell lines, DAP1 knockdown resulted in a consistently higher ability to adhere as compared to the controls. However, this trend did not achieve statistical significance (p=0.34). As was observed in the MCF7 sublines, the MDA-MB-231^{DAP1kd} subline showed a consistent trend promoting adhesion when compared to MDA-MB-231^{pEF6}. This trend was much more pronounced (350% vs. controls), and was found to be statistically significant (p=0.01) (Fig. 3).

The Matrigel-based invasion was performed repeatedly in both cell lines (n=3 for MCF7 and n=4 for MDA-MB-231). However, no clear change in invasive ability was observed in the knockdown strains when compared to the controls in either cell line (data not shown).

No observable change is observed in response to serum starvation. The effects of serum starvation on the controls and knockdown sublines were studied using Annexin V and propidium iodine dyes to differentially mark healthy, early apoptotic and late apoptotic cells. However, no discernible difference was observed between the control and knockdown sublines (data not shown).

Reduction in expression of pro-apoptotic molecules is observed in the MCF7^{DAP1kd} and MDA-MB-231^{DAP1kd} sublines. After knockdown was confirmed, the mRNA expression levels of the following molecules were studied in the cDNA libraries derived from the transfected MCF7 and MDA-MB-231 sublines: caspase 8, caspase 9, IPS1, DELE, p21 and cyclin D1.

MCF7^{DAP1kd} demonstrated a statistically significant decrease in the mRNA expression of caspase 8 (p=0.004), which is an apical caspase associated with the external apoptosis pathway. Furthermore, the expression of IPS1 was also suppressed in the MDA-MB-231^{DAP1kd} subline vs. the controls (p=0.004). In addition, the expression of p21 was depressed in the MCF7^{DAP1kd} (p=0.029) and MDA-MB-231^{DAP1kd} cell sublines (p=0.051) when compared to the controls (Fig. 4). Finally, no statistically significant difference was noted in the expression of DELE, caspase 9 or cyclin D1 (data not shown).

In vitro migration assay using ECIS. Changes in the propensity for migration after wounding in the knockdown sublines vs. the controls were studied using ECIS, in which the changes in the impedance measured in the seeded wells were measured after the cell culture was subjected to wounding by electroporation.

When compared to the controls (MCF7^{pEF6}), the DAP1 knockdown subline demonstrated consistently greater attachment and migration across at least three repeats.

Presented here are the results of a representative instance of the ECIS assay. The migration over 4-h post-wounding in the MCF7^{pEF6} and MCF7^{DAP1kd} cell lines was compared using a random effects model, in which an average curve was calculated, and then were compared for significant differences.

The curve for MCF7^{pEF6} was calculated as follows:

$$\text{Reading} = -42.04 + 20.65 \times \text{time} + 18.52 \times \text{time}^2$$

In the case of MCF7^{DAP1kd}, the equation was calculated as follows:

$$\text{Readings} = -42.04 + 82.40 + 20.65 \times \text{time} + 18.52 \times \text{time}^2$$

The difference in the readings in the two groups failed to achieve statistical significance (p=0.081).

MDA-MB-231 sublines were similarly studied. When compared to the controls (MDA-MB-231^{pEF6}), the

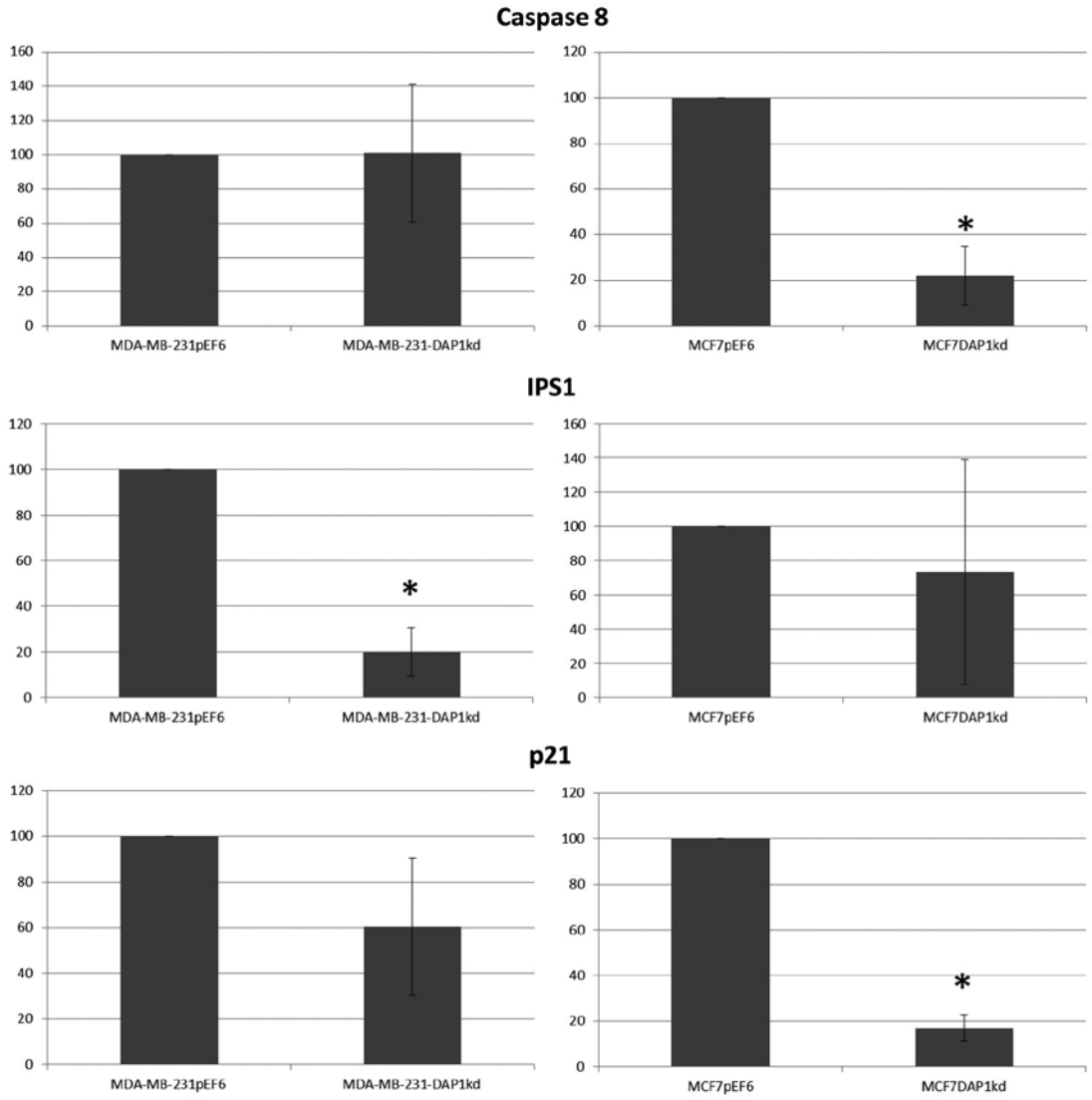

Figure 4. mRNA expression of IFN-β promoter stimulator-1 (IPS1), caspase 8 and p21 in the control and DAP1 knockdown sublines (*significant associations, Mann-Whitney U test). DAP1, death-associated protein 1.

MDA-MB-231^{pEF6} knockdown subline demonstrated consistently greater attachment and migration across at least three repeats. These effects were much more pronounced in comparison to those observed in the MCF7 sublines.

The curve for MDA-MB-231^{pEF6} was calculated as follows:

$$\text{Readings} = 1.43 + 26.55 \times \text{time} - 4.48 \times \text{time}^2$$

The curve for MDA-MB-231^{DAP1kd} was calculated as follows:

$$\text{Readings} = 1.43 - 0.83 + (26.55 + 381.4087) \times \text{time} + (-4.48 - 55.78125) \times \text{time}^2$$

There is strong evidence that the readings' growth in the two groups differed substantially ($p<0.001$) (Fig. 5).

Discussion

Clinical evidence regarding the role of DAP1 in human oncogenesis is limited. As per Koren *et al* (13), DAP1 was found to be a substrate of mTORC1, inactive in a phosphorylated state along with the ULK1 complex. In the absence of the effect of mTORC1, both of these molecules are stimulated. While ULK1 initiates autophagy and autophagosome formation, DAP1 was determined to be an inhibitor of autophagy. As apparent from the discussion by Gan and Guan regarding FIP200, such a role would entail control and coordination of several critical cellular mechanisms in addition to the direct inhibition of autophagy (3).

Dysregulation of autophagy has long been known to play a role in carcinogenesis. We hypothesised that DAP1 silencing or knockdown would increase the survival of cancer cells by aiding their survival. Consequently, we surmised that DAP1 would have a prognostically favourable role in human breast cancer (14). This was borne out by the results of our previous study of mRNA expression in a breast cancer cohort, in which a significant inverse relationship was noted between *DAP1* mRNA expression and the Nottingham Prognostic Index (NPI), TNM stage (TNM1 vs. TNM4, $p=0.0039$) and clinical outcome (15).

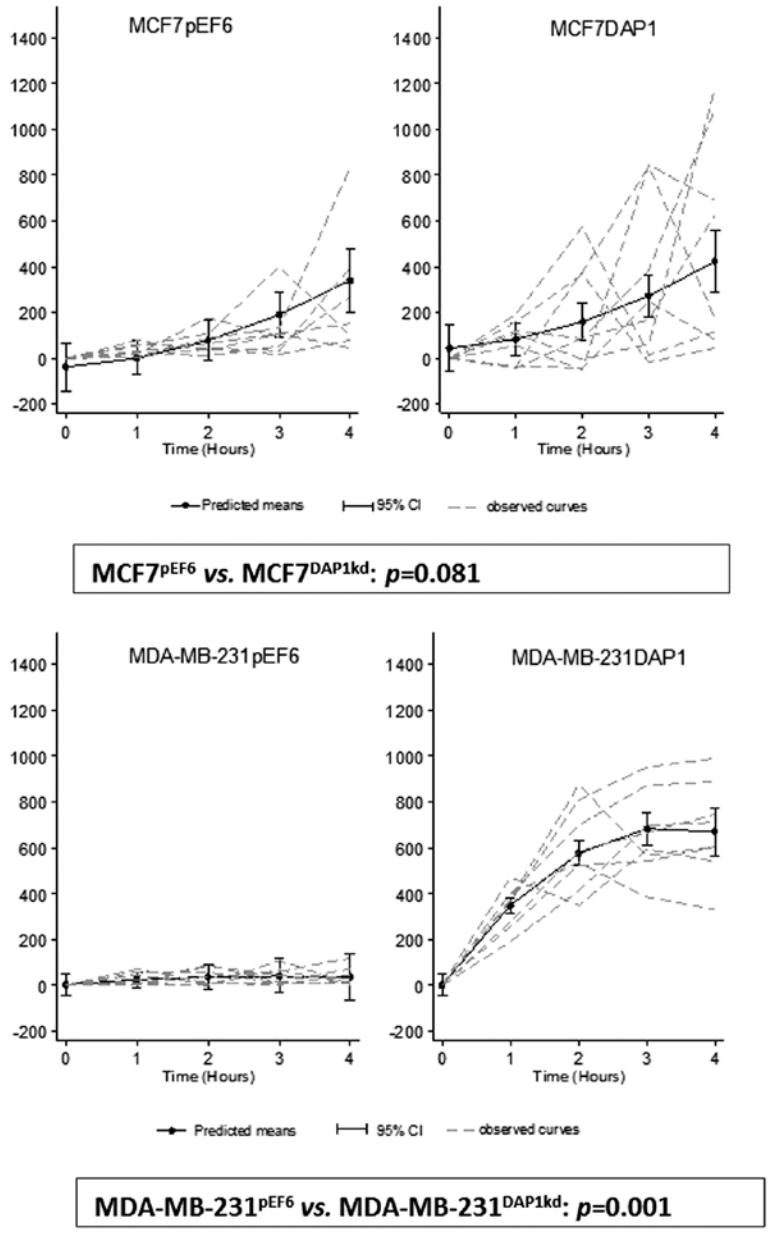

Figure 5. ECIS-based post-wounding migration assay of the DAP1 knockdown and control sublines. The x-axis represents change in impedance after successful wounding of the cell monolayer (*significant associations). DAP1, death-associated protein 1.

On the basis of the clinical data and the above cited literature, we hypothesised that knockdown or silencing of DAP1 would increase the aggressiveness of cancer cells by increasing the ability of the cells to grow, adhere, invade and migrate. We also hypothesised that part of the role of DAP1 may be due to a pro-apoptotic function, which may be direct or via interaction with the canonical apoptosis pathway (16-18).

A significant finding in the cell assays was the increase in adhesion. This trend was statistically significant in the case of the MDA-MB-231^{DAP1kd} subline, while a similar trend noted in the MCF7^{DAP1kd} subline vs. the controls did not attain statistical significance.

Furthermore, the ECIS-based migration assay demonstrated a consistent increased migratory tendency in the knockdown sublines, which was highly significant in the MDA-MB-231 cells and approached significance in the MCF7 cells. In comparison to the conventional assay, the cell monolayer was monitored more extensively, with continuous non-invasive impedance sensing rather than arbitrarily spaced interval photography.

Increased adhesion and migration noted in the cells with silenced DAP1 expression is suggestive of an impact on the expression of cell membrane proteins involved in cell-cell and cell-matrix interactions. The most likely effector may be focal adhesion kinase (FAK). Such changes would be necessary to enable metastatic cells to seed locations distant from the primary site, and would be a prerequisite for more aggressive disease (17).

However, the Matrigel-based invasion assay failed to show a significant difference in the invasive potential in the knockdown strains when compared to the controls.

In the present study, we were unable to demonstrate a direct pro-apoptotic effect in response to serum-starvation in the knockdown sublines when compared to the controls.

However, the effects of DAP1 knockdown on the expression of key components of the apoptosis pathway are suggestive of significant interactions between DAP1 and the said pathway. Suppression of DAP1 was associated with a decrease in caspase 8 expression, which is suggestive of an association with the external apoptosis pathway (19). A similar relationship was noted between DAP1 and IPS1. IPS1 is part of the external apoptosis pathway, and is particularly related to TRAIL-related apoptosis via DAP3 (20). IPS1 is also involved in virus-related innate immunity, anoikis and inflammation (21).

Finally, the suppression of p21 expression in the MCF7^{DAP1kd} subline is suggestive of a direct relationship with the cell cycle. p21 plays a significant role in cell cycle arrest and cell senescence (22).

The results of the growth assays in the MCF7 cells can be said to be very compelling from the point of view of discerning DAP1's place in cellular pathways. A statistically significant suppression of growth was noted on D3. However, by D5, the growth in the knockdown strains caught up to the controls, thus erasing the earlier short-fall in growth. However, as the assay was limited to 5 days, we were unable to determine the continued trajectory of the growth in the MCF7^{DAP1kd} subline vs. the controls. With caveats, it would be reasonable to extrapolate that the spurt of growth noted between D3 and D5 could have caused MCF7^{DAP1kd} to overtake the controls if further measurements were technically feasible. It can be speculated that this may be due to deactivation of an initial pathway which would mediate growth in the presence of DAP1, which is then over-compensated for by a late acting positive feedback loop.

As the substrates of DAP1 are as yet unknown, any conjecture with regards to the specific pathways which would account for these results would be speculative at this point in time. The literature regarding ULK1 does not suggest a substrate which may account for the short term slowing of growth. However, the mTORC1 pathway via tuberous sclerosis complex 1 (TSC1) does fit the requisite description of the late acting positive feedback. If DAP1 is taken to act as a functional agonist of FIP200 in every instance, then the presence of DAP1 would result in inhibition of mTORC1 via activation of TSC1. It is possible that the antagonistic inputs of FIP200 and DAP1 via TSC1 would form part of a dynamic equilibrium controlling the strong proliferative effects of mTORC1, which would be disrupted by knockdown of DAP1, leaving only the mTORC1-activating effect of the ULK1 complex (23,24).

In conclusion and for future directions. We believe that our results are highly suggestive of an important role for DAP1 silencing in the increase in cell adhesion and migration in human breast cancer cells, which would contribute to the aggressiveness of the disease.

Furthermore, while a direct role in inducing apoptosis was not demonstrated in this set of experiments, qPCR revealed significant associations with the external apoptosis pathway. This and the results pertaining to p21 would hopefully suggest the future direction for investigations regarding the downstream effectors of DAP1. Furthermore, one may make the observation that the extrapolation of the functions of DAP1 from those of FIP200 has been by and large borne out by the results presented in this manuscript (2,3).

The hypothetical model proposed above regarding the increased late growth in the MCF7^{DAP1kd} subline would require further testing with mechanistic studies involving the development of cell lines with knockdown expression for components of the mTOR pathway in addition to DAP1, or incubation of the present sublines in appropriate mTOR inhibitors. In view of current efforts toward the therapeutic targeting of the mTOR pathway in human breast cancer, this proposed model warrants further investigation (25).

The literature and our results are suggestive of a locus for DAP1 at the juncture of several critical cellular pathways. Such a locus can be characterised as more typical of a significant regulatory molecule rather than that of a principal effector. Therefore, its knockdown would result in several disruptions of moderate magnitude rather than a limited number of large effects. Discernment of the downstream substrates of DAP1 would be necessary for a more nuanced understanding of the mechanism involved in coordinating these critical cellular pathways.

Acknowledgements

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