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Research Article

Olfactomedin III expression contributes to anoikis-resistance in clonal variants of a human lung squamous carcinoma cell line

Joanne Keenan[a,*], *Helena Joyce*[a,c], *Sinead Aherne*[a,c], *Shirley O'Dea*[b], *Padraig Doolan*[a], *Vincent Lynch*[a], *Martin Clynes*[a]

[a]*National Institute for Cellular Biotechnology (NICB), Dublin City University, Glasnevin, Dublin 9, Ireland*
[b]*Institute of Immunology, Biology Department, National University of Ireland, Maynooth, Co. Kildare, Ireland*
[c]*Molecular Therapeutics for Cancer Ireland, c/o NICB, Ireland*

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ABSTRACT

Three clonal subpopulations of DLKP, a poorly differentiated squamous lung carcinoma cell line, display striking differences in ability to survive in suspension (anoikis resistance). DLKP-SQ is anoikis resistant (7.5% anoikis at 24 h). In contrast, DLKP-M and DLKP-I are sensitive to anoikis (49.2% and 42.6% respectively). DLKP-I shows increased apoptosis consistently over all time points tested while DLKP-M appear to slow down metabolically and perhaps delays onset of anoikis by undergoing autophagy. Expression microarray analysis identified pronounced differential expression of Olfactomedin 3 (OLFM3) between the clones. High expression of OLFM3 was confirmed at the RNA level by qRT-PCR in DLKP-SQ and at the protein level by Western blotting (within the cell and secreted). Little or no OLFM3 was detected in the other two clones (DLKP-M and DLKP-I). Following siRNA knockdown of OLFM3 in DLKP-SQ, anoikis was increased 2.8-fold to 21% which was intermediate between the anoikis levels in DLKP-SQ and DLKP-M or DLKP-I. This knockdown correlated with increased apoptosis in suspension but not in attached culture conditions. Addition of recombinant OLFM3 reduced anoikis in DLKP-I. This is the first instance of OLFM3 being linked with anoikis resistance in a human cancer cell line.

Introduction

Cellular heterogeneity within tumours could provide protection against a changing environment, such as exposure to hypoxia or chemotherapeutic drugs or the opportunity for invasion to a more suitable site. Subpopulations can arise by mutation or by alterations in the differentiation state, such as epithelial to mesenchymal and mesenchymal to epithelial transitions (EMT–MET) or by differentiating cancer stem cells [1]. Lung cancer, although the most preventable cancer, kills more people than the next top three cancers (breast, prostate and colon) [2]. Lung cancer is heterogeneous, consisting of two main groups: small cell lung cancer and non-small cell lung cancer (adenocarcinoma, large cell lung cancer and squamous cell carcinoma) and even within these groups there is morphological, functional, genetic as well as proteomic heterogeneity [3–5]. Subpopulations have been found to have differences in their drug resistance profiles and /or in their invasive potential [6,7] and this can impact critically on

* *Corresponding author at:* National Institute for Cellular Biotechnology, Dublin City University, Dublin, Ireland. Fax: +353 1 7005484.
E-mail address: Joanne.keenan@dcu.ie (J. Keenan).

Abbreviations: OLFM, Olfactomedin; 2D, two dimensional; siRNA, small interfering RNA; poly-Hema, poly-2-hydroxyethyl methacrylate; CM, conditioned Media.

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treatment [8]. Understanding the biology of these subpopulations and how they interact may provide opportunities to improve treatment or produce new diagnostic markers.

In previous work in our laboratory, three subpopulations (DLKP-SQ, DLKP-M and DLKP-I) of a poorly differentiated squamous lung carcinoma line, DLKP, were isolated and characterised with respect to epithelial, mesenchymal and other lung differentiation markers [4]. In this paper, we explore differences in anoikis-resistance between these clonal subpopulations, and investigate the role of Olfactomedin-3 in regulation of anoikis-resistance.

Materials and methods

Chemicals

All chemicals (unless otherwise stated), FBS, glutamine and cell culture media were obtained from Sigma (Poole, UK). Recombinant OLFM3 (cat. H00118427-P01) was obtained from Abnova (Taiwan).

Cell lines

DLKP is a poorly differentiated human squamous carcinoma cell line established in this laboratory [9] from which the three subclones DLKP-SQ, DLKP-M and DLKP-I were isolated [4]. DLKP-SQ is squamous-like, DLKP-M is mesenchymal cell-like and DLKP-I exhibits an intermediate morphology. DLKP variants were maintained in DMEM/Hams F12 (1:1) supplemented with 5% FBS and 1% L-glutamine. MCF-7, SKBR3 and RPMI-2650 were obtained from the ATCC. RPMI-2650tx was previously obtained by continuous exposure to taxol [10]. MCF-7 and SKBR3 were maintained in RPMI-1640 supplemented with 10% FBS and 1% L-glutamine. RPMI-2650 and RPMI-2650txl were maintained in MEM supplemented with 5% FBS and 1% L-glutamine, 1% sodium pyruvate and 1% NEAA. All cell lines were mycoplasma negative. A frozen cell pellet of 2×10^6 cells of DLKP and variants was submitted to LGC Limited (Middlesex, UK) for DNA fingerprinting. A database search of over 3000 cell lines showed no match with DLKP indicating that it was unique and the sub-clones were confirmed to be derived from DLKP.

qRT-PCR for OLFM3

Total RNA extracted from cell lysates (MirVana miRNA Isolation Kit, cat AM1560, Applied Biosystems) were quantified using the Nanodrop (ND-1000 spectrophotometer), reverse-transcribed using (High capacity RNA-to-cDNA kit, cat 4387406, Ambion) and requantified using the Nanodrop. TaqMan gene expression experiments were performed in 20 µl reactions containing 10 µl of TaqMan® Fast Universal Master Mix (2X), No AmpErase® UNG, 9 µl of cDNA template (80 ng) and 1 µl of TaqMan gene expression assay (20x). The following thermal cycling specifications were performed on the ABI 7500 Fast Real-Time PCR system (Applied Biosystems) 20 s at 95 °C and 40 cycles each for 3 s at 95 °C and 30 s at 60 °C. Expression values were calculated using the comparative threshold cycle (C_t) method. Briefly, this technique uses the formula $2^{-\text{delta deltaC}}t$ to calculate the expression of target genes normalised to a calibrator sample [11]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was selected as the endogenous control.

SiRNA transfection

Transfection assays were carried out with NeoFX transfection reagent (siPORT NeoFX, AM4511, Ambion Inc) and olfactomedin 3 validated siRNAs S26707, Ambion Inc (OLFM3(1)) and S26708, Ambion Inc (OLFM3(2)) with scrambled control 2 (AM4613, Applied Biosystems) in OptiMEM medium (Gibco™) [12]. Cells in log phase of growth were fed prior to setting up transfection at the following conditions: 2×10^5 cells per well (in 1 ml for transfection) and 2 ul NeoFX. Cells were fed after overnight exposure to transfection with 2 ml fresh medium. The cells were allowed to grow for three days at 37 °C and 5%CO_2, over which time, microscopic observations were used to look for changes in morphology and to assess the efficiency of transfection (by looking at the growth in kinesin control (KIF-II, Ambion, Inc). At 72 h the cells were trypsinized, counted and anoikis assays were set up. A duplicate plate was washed in PBS A and lysed with 2D lysis buffer for protein analysis.

Western blotting

Western blotting was performed on cell lysates. Samples with 20–30 µg of protein per well, were separated on a 12% SDS gel [13]. After Western blotting [14], blots with primary antibodies (OLFM3 (Santa Cruz, Germany), Caspase-3 and FAK (BD, Europe, cat 610322 and 610087), phospho-FAK and phospho-PAX (Cell Signalling Cat 8556, 2541) or alpha-tubulin, LC3 (Sigma, cat T6199) were incubated overnight at 4 °C. Secondary antibodies conjugated to horse-radish peroxidase (Sigma, Poole, UK) were detected by enhanced chemiluminesence (Luminol, Santa Cruz, CA, USA).

Anoikis assays

The base of wells in a 24-well tissue culture plate (Costar, Cat 3524) were coated with 200 µl of poly-2-hydroxyethyl methacrylate (poly-HEMA, 12 mg/ml dissolved in 95% ethanol, Sigma P3932) and allowed to dry overnight. Coating was repeated. Immediately before use, the coated wells were washed twice with sterile PBS A. A 1 ml volume of cell suspension at 1x10E5 cells/ml was added to control (no poly-HEMA) and coated wells (3 per assay). The cells were incubated for specified times at 37 °C and 5% CO_2. Three hour prior to the end of the assay, 100 µl Almar Blue (Serotec BUF012B), an indicator dye was added. Metabolically active cells convert Almar Blue (resazurin) to a fluorescent and colormetric indicator (resorufin). Colour development was measured on a Bio-Tek plate reader at 570 nm with reference wavelength of 600 nm. Alternatively at the end of the incubation, cell viability was assessed by trypan blue exclusion dye of cells growing in poly-HEMA coated plates. Apoptosis (Guava Nexin cat 4500–0450) and Cell cycle analysis (Guava Cell cycle reagent 4500-0220) were assessed on a bench top flow cytometer (Guava EasyCyte Instrument).

Collection of conditioned media

Cells were grown in 175 cm2 flasks until about 40–50% confluent. Following extensive washing with fresh medium (no serum), cells were grown for 72 h to generate conditioned medium (CM). The CM was removed and centrifuged at 1000 rpm for 5 min, filter-

sterilised through a 0.22 μm low-protein binding filter and concentrated (to 10x of the starting volume of the CM) in a Vivaspin concentrator (Vivaspin-20, cat Vs2012) with a 5 kDa cut-off and the retentate was filter-sterilised.

Statistics

Statistics were carried out using the Student *t*-test assuming a two-tailed distribution and two samples of unequal variance. Values of $p < 0.05$ were considered as statistically significant.

Results

Anoikis resistance in DLKP clones

The presence of 'floaters' (viable cells that are suspended in the medium without the need for attachment to a surface) in sub-confluent flasks of DLKP-SQ, suggest that either DLKP-SQ or a sub-population of the clone could be resistant to anoikis. These floating cells if placed in a fresh flask attach and grow in the same fashion as DLKP-SQ. In contrast, DLKP-M and DLKP-I have very few floaters, and most of the cells floating were dead. As a result of these observations, the three clones were tested individually for anoikis resistance (Fig. 1). Microscopically, DLKP-SQ in suspension consists mostly of single cells, not forming clumps. DLKP-M and DLKP-I had very few loose single cells and formed aggregates which became more tightly clumped with time (Fig. 1F). When cells were grown for prolonged periods in polyhema-coated flasks (up to 10 passages), DLKP-SQ continued to grow with no lag-phase, the percentage viability remained high and when seeded in normal flasks, the cells attached and grew with the same morphology as the original DLKP-SQ. DLKP-M and DLKP-I, on the other hand, struggled and after three days showed only 42% and 56% viability respectively (data not shown).

Anoikis was measured by monitoring cell counts of attached and suspended cells, cell growth and viability in suspension and the Almar Blue assay (a measure of the metabolic activity of cells). After 24 h in suspension, DLKP-SQ undergo only 7.5% cell death as measured by viable cell counts and Almar Blue measurements (Figs. 1A and B) and with no significant changes in cell viability (Fig. 1E). At 48 h, although there is a slight reduction in growth for suspension cells compared to attached cells and Almar Blue measurement, there is no change in viability. While Annexin V staining suggest some apoptosis is occurring (Fig. 2A), DLKP-SQ is the only clone that continues to grow in suspension (Fig. 1D). These results suggest that no significant adaptation is required to survive anoikis conditions and that the bulk DLKP-SQ population rather than a sub-population is anoikis resistant.

The clones, DLKP-I and DLKP-M, showed 42–50% anoikis by Almar Blue measurement and show similar morphology under anoikis conditions but variations in viable cell counts suggest different mechanisms.

DLKP-I maintained in suspension show a continuous loss of viable cell numbers and viability (Figs. 1A, D–E) over time which is reflected in the metabolic state of the cells (Figs. 1B–C) and correlates with increased apoptosis. Annexin V staining revealed DLKP-I cells to be more susceptible to apoptosis under normal attached conditions (at 13%, higher than DLKP-SQ or DLKP-M at 6% and 8% respectively). In suspension, there was a significant (2.8-fold) increase in apoptosis in DLKP-I, with 33% of the cells being apoptotic at 24 and 48 h (Fig. 2B). Procaspase-3 levels were reduced in DLKP-I in suspension due to cleavage, indicating apoptosis (Fig. 2C). In addition there is a loss of phosphorylated FAK and phosphorylated Paxillin when DLKP-I are maintained in suspension (Fig. 2C).

For DLKP-M, there was a lag phase between the loss of metabolic activity and the reduction in viable cell number (Figs. 1A–C). While there was a slight increase in annexin V staining (Fig. 2B) this was not significant.

We analysed cell cycle progression to see if this could explain the lag phase. For the clones, cell cycle analysis revealed some changes between the clones under attached conditions, notably for DLKP-I, there was a higher proportion of cells in the G2/M phase than the other clones. However at 24 h, comparison of suspension cells to attached cells for each of the clones showed no significant differences, suggesting for this time point, cell cycle analysis was not a determining factor in the lag phase for DLKP-M (Fig. 2A). At 48 h, there was a more distinct increase in the proportion of DLKP-M cells in G0/G1 arrest (Fig. 2A).

At 24 h, an increase in the ratio the LC3-II to LC3-I peptides of the autophagy marker LC3 was observed (Fig. 2C). This in conjunction with the Almar Blue measurements and viable cell counts suggests that DLKP-M may be undergoing autophagy under anoikis conditions during the first 24 h.

Olfactomedin 3 in DLKP clones

Microarray analysis of the three clones of DLKP (Helena Joyce and Padraig Doolan, unpublished results) shows that one of the most highly differentially expressed mRNA among the clones was olfactomedin 3. The levels of OLFM3 in DLKP-I were 6% of those in DLKP-SQ (p-value <0.002) and in DLKP-M were less than 1% of those in DLKP-SQ (p-value <0.003)(Fig. 3A). Other members of the olfactomedin family (OLFM1, 2 and 4) were not differentially or highly expressed in the three clones at the mRNA level from microarray studies. Quantitative RT-PCR was carried out on the clones (Fig. 3B) using GAPDH as the internal control (shown not to be differentially expressed on the mRNA level in microarray results). OLFM3 was shown to be highly expressed in the DLKP-SQ compared to the two clones DLKP-M and DLKP-I. The levels of OLFM3 in DLKP-I were 3.6% of those in DLKP-SQ (p-value 2x10E-13) and in DLKP-M, were less than 1% of those in DLKP-SQ (p-value $<$ 2x10E-12).

Western blotting demonstrated high levels of OLFM3 protein in the DLKP-SQ cells, with little or no expression in the other two clones (Fig. 3C). Densitometry of Western blots for OLFM3 using alpha-tubulin as the loading control (shown not to be differentially regulated at least at transcript level in the microarray data) revealed DLKP-M and DLKP-I to express 1.3% and 1.2% of the OLFM3 expressed in DLKP-SQ (p-values <0.005) (Fig. 3D). OLFM3 is a secreted protein and may be present in the conditioned media (CM). Western blotting showed expression of OLFM3 in DLKP-SQ CM and none in DLKP-M or DLKP-I (Fig. 3E). There was little or no alpha- tubulin in the CM, indicating a lack of intracellular contaminants.

RNAi downregulation of Olfactomedin 3 in DLKP-SQ

Two validated siRNAs targeting OLFM3 were selected (OLFM3(1) and OLFM3(2)) and DLKP-SQ in log phase of growth were

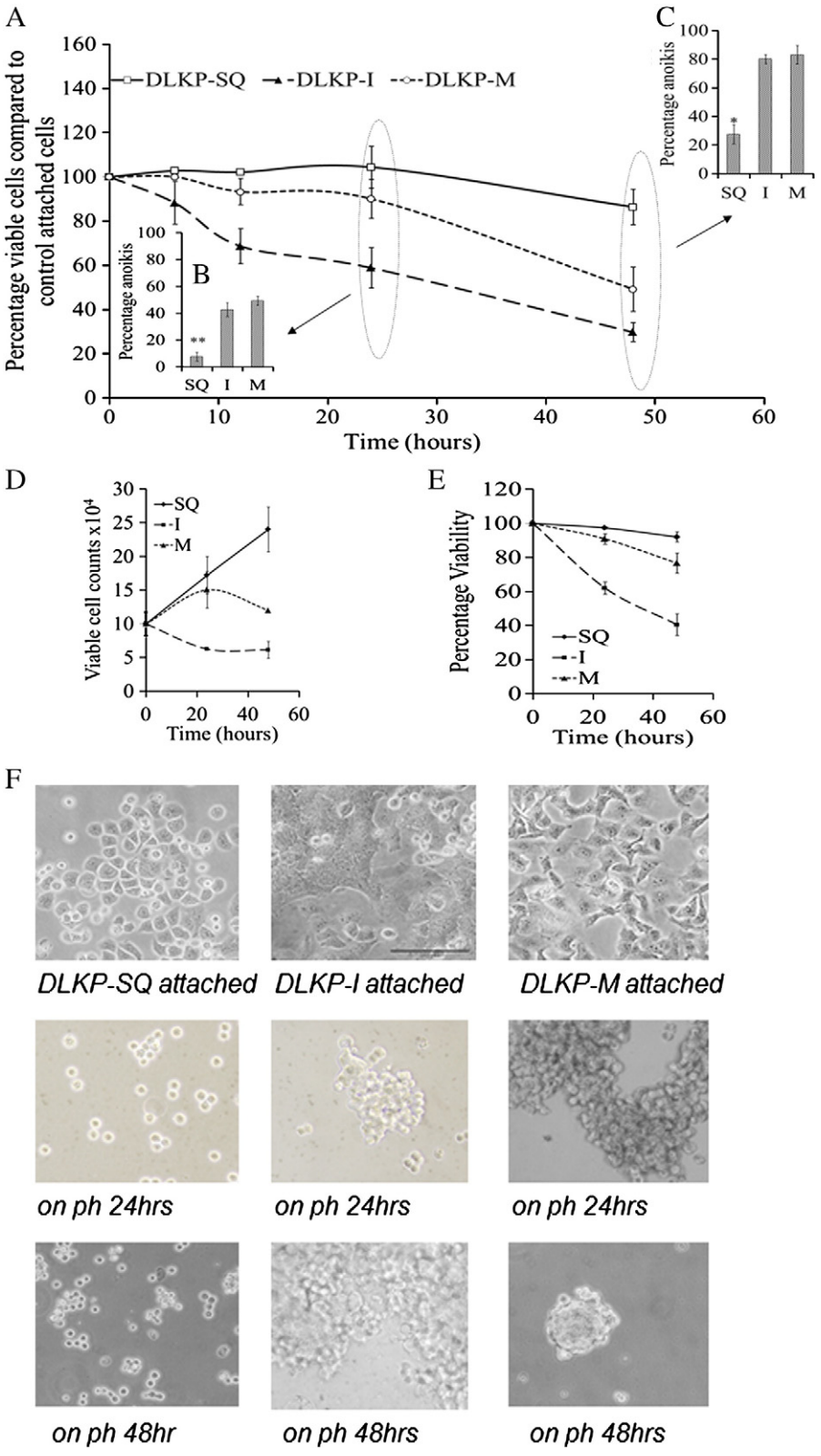

Fig. 1 – Effect of anoikis conditions on cell growth, Almar blue measurements, viability and morphological differences in DLKP clones (DLKP-SQ, DLKP-I and DLKP-M). (A) Anoikis measured as the percentage of cell death when grown in polyhema-coated wells over a 24 hour period (n = 4) compared to control cells in untreated wells. B and C show the results of Almar Blue measurements at 24-hour and 48-hour in polyhema-coated versus control plates respectively. * or ** indicates a p-value of <0.05 or <0.01 comparing DLKP-SQ to either of the other two clones; ph indicates in polyhema-coated wells. D shows the Viable cell number of the cells in the polyhema-coated plates while E shows the viability of those cells. (F) Morphological differences between DLKP clones viewed under 200× magnification at 24 h and 48 h.

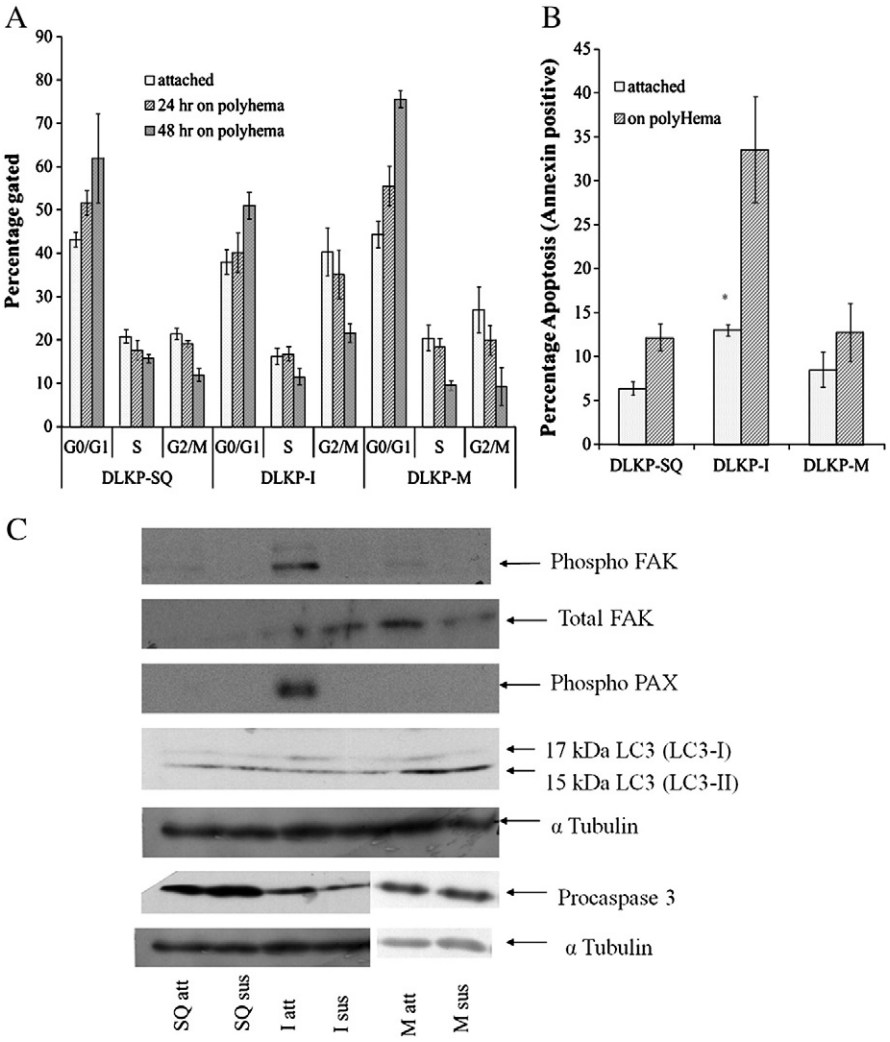

Fig. 2 – Effect of growth under anoikis conditions on cell cycle and apoptosis. (A) Cell cycle analysis on DLKP clones comparing attached versus suspension cells at 24 h and 48 h. (B) Apoptosis in DLKP clones as measured by Annexin-V staining in attached versus suspension cells at 24 h. * indicates a p-value of <0.05 DLKP-I in suspension to DLKP-I attached. For all experiments, results are the average of at least 3 separate repeats. (C) Western blotting of selected proteins in DLKP clones either attached (att) or in suspension (sus).

transfected with OLFM3 siRNA (kinesin siRNA (positive) or scrambled siRNA (negative) were used as controls). Microscopically, transfection of kinesin siRNA was seen to reduce growth as would be expected (by 90%, data not shown) indicating good siRNA transfection conditions. There was no morphological change in the DLKP-SQ as visualised microscopically following OLFM3 siRNA transfection, suggesting that OLFM3 was not a decisive factor in determining the morphological differences between DLKP-SQ and the other clones. At 72 h, cells counts (using trypan blue dye exclusion to look at viable cell numbers) showed no significant change in the total viable cell counts of siRNA-treated DLKP-SQ cells.

Knockdown of OLFM3 in DLKP-SQ had a significant impact on anoikis resistance (Fig. 4A), resulting in a 2.8-fold increase in anoikis compared to the scrambled control which was significant for both siRNAs ($p<0.01$ and $p<0.05$ for OLFM3(1) and OLFM3(2) respectively). Analysis of the protein levels of OLFM3 lysates in transfected samples showed that levels of OLFM3 were reduced by about 80% in the siRNA transfected cells (Figs. 4B and D). The level of anoikis in DLKP-SQ transfected cells was not as high as that seen with DLKP-M or DLKP-I suggesting that other factors may be having an impact on anoikis resistance, or that the remaining levels of OLFM3 are sufficient to maintain some level of anoikis resistance.

Loss of OLFM3 correlates with increased annexin V staining (Fig. 4D) suggesting anoikis may be occurring by apoptosis. There was no significant change in cell cycle profiles on loss of OLFM3 following siRNA transfection (data not shown).

Effect of recombinant OLFM3 on Anoikis sensitive clones

As OLFM3 appears to have a protective role against anoikis in DLKP-SQ, recombinant OLFM3 was tested on DLKP-I and DLKP-M anoikis sensitive cell lines. Recombinant OLFM3 at 1 ng/ml reduced anoikis in DLKP-I significantly ($p<0.001$) by 1.9-fold but not in DLKP-M, supporting a protective role for OLFM3 in apoptosis (Fig. 5). It is interesting to note that DLKP-I is the intermediate clonal type in the interconversion of the DLKP clones. It appears to

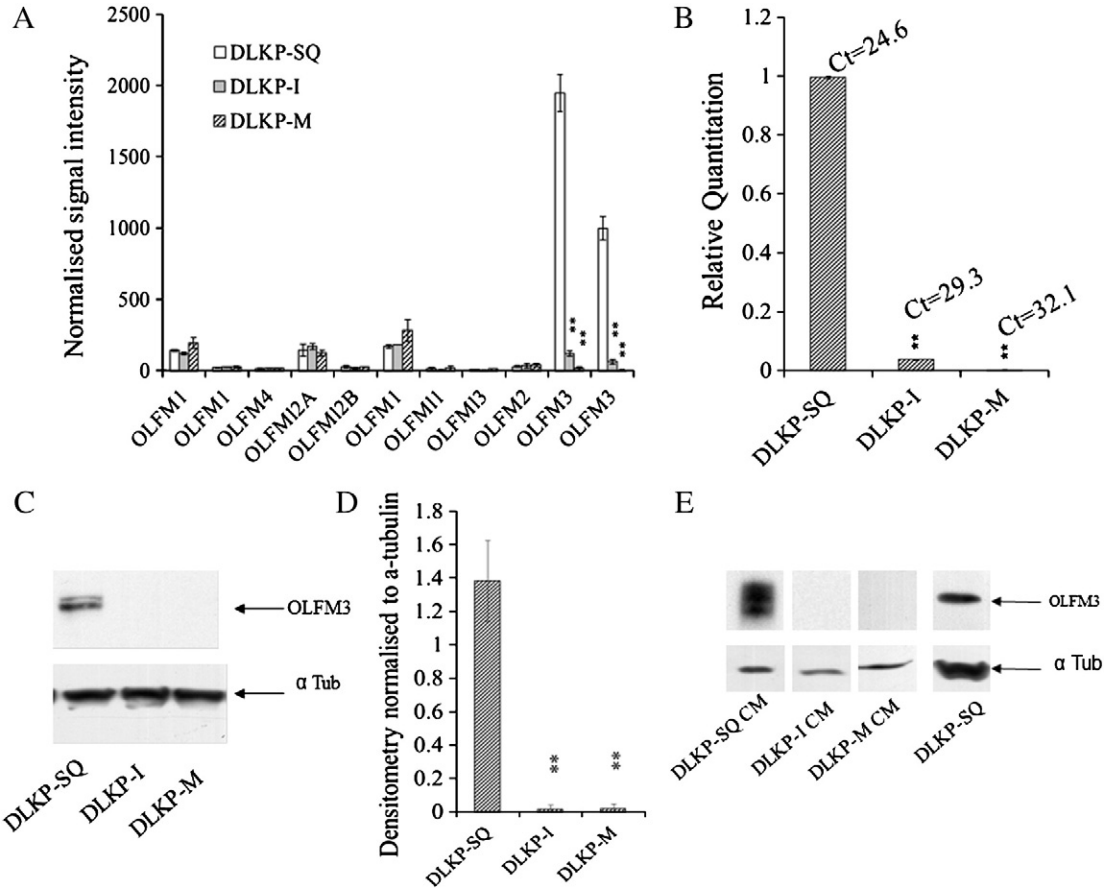

Fig. 3 – Expression of OLFM3. (A) Normalised signal intensity for OLFM species from microarrays. Standard *t*-test with p-value <0.003 compared to DLKP-SQ for DLKP-I or DLKP-M. OLFMl1 refers to OLFM-like 1. (B) Relative quantitation of OLFM3 compared to DLKP-SQ and normalised to GAPDH by qRT-PCR. Results are the average and standard deviation for three biological repeats. Standard *t*-test with p-value <2x10E-12 compared to DLKP-SQ for DLKP-I or DLKP-M. (C) Protein expression of OLFM3 and alpha tubulin as loading control in DLKP clones with (D) densitometry normalised to alpha tubulin. Results are the average and standard deviation for four biological repeats. ** represents Student *t*-test with p-value <0.002 for DLKP-SQ compared to DLKP-M and DLKP-I. (E) Western blot of OLFM3 in conditioned medium from DLKP clones. Image shows average blots from three biological repeats. A typical Western blot for OLFM3 in DLKP-SQ (labelled DLKP-SQ con) and control alpha tubulin is shown.

retain OLFM3 sensitivity without producing it, while DLKP-M not only does not produce OLFM3 but has lost sensitivity to it.

Expression of OLFM3 in other anoikis resistant cell lines

We looked at expression of OLFM3 in number of cancer cell lines with different anoikis resistance levels (Fig. 6). In the nasal carcinoma, the anoikis-sensitive parent RPMI-2650 expressed little or no OLFM3 but the taxol-resistant variant which was anoikis-resistant expressed OLFM3. In breast cell lines, anoikis-resistant SKBR-3 express OLFM3 while anoikis-sensitive MCF-7 does not. These results together with the results for SQ-CM and recombinant OLFM3 suggest that OLFM3 expression may have a role in anoikis resistance.

Discussion

In this work, the presence and possible role in anoikis of Olfactomedin 3 was investigated in clones of a poorly differentiated squamous lung carcinoma cell line. Affymetrix expression microarray studies in our laboratory showing OLFM3 to be one of the most highly differentially expressed mRNAs between the DLKP clones DLKP-SQ (high) and DLKP-M and DLKP-I (both low) were confirmed by qRT-PCR. Western blotting confirmed the presence of OLFM3 at the protein level in DLKP-SQ while the two other clones had little or no expression of OLFM3.

SiRNA knockdown of OLFM3 showed no effect on total viable cell growth or in morphology in the siRNA-transfected DLKP-SQ compared to the parental DLKP-SQ. Knockdown of OLFM3 resulted in increased anoikis (which is a form of cell death resulting from a disruption in the ECM signals). Knockdown of OLFM3 did not alter cell cycle but showed increased apoptosis as measured by annexin V expression (Fig. 4D). While the knockdown of OLFM3 was significant as detected by Western blotting (Fig. 4), the level of anoikis in the siRNA-transfected cells was still less than that observed in the DLKP-M or DLKP-I cells, suggesting that reduction in OLFM3 levels was not sufficiently complete for the time period or that other factors are involved in anoikis resistance in DLKP-SQ compared to DLKP-M and DLKP-I.

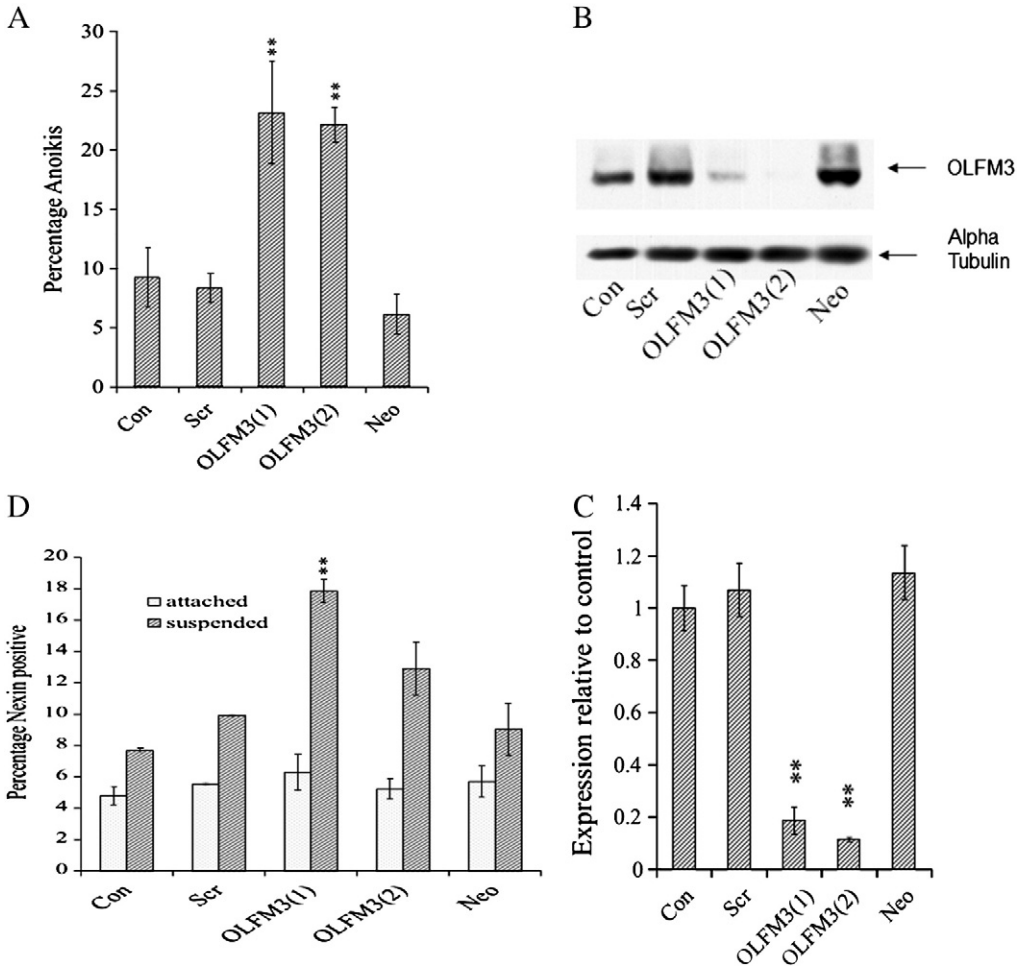

Fig. 4 – Effect of siRNA knockdown on anoikis in DLKP-SQ cells. After siRNA transfection and growth for 72 h, cells were trypsinised and set up under anoikis conditions, allowed to grow for a further 24 h and then counted. Samples were taken for Western blotting at 72 h. (A) Effect of siRNA knock-down on anoikis in DLKP-SQ as measured using Almar blue assay. (B) Western blotting for OLFM3 expression of siRNA-transfected DLKP-SQ and (C) Densitometry of Western blot. (D) Apoptosis assay as measured by Annexin V staining. Results are the average of at least 3 separate experiments. ** denotes Student *t*-test with a p-value of <0.005 compared to the scrambled control.

When recombinant OLFM3 was added to DLKP-I cells in suspension, anoikis was significantly reduced (Fig. 5). This is the first time, to our knowledge, that OLFM3 has been linked with anoikis resistance.

Olfactomedin 3 belongs to a family of glycoproteins containing a conserved C terminal olfactomedin domain, with four main groups OLFM1 to OLFM4 and at least 13 isoforms in mammals [15,16]. Identified first in the olfactory neuroepithelium of the bull frog in the 1990s [17], olfactomedins have been found in diverse species ranging from *C. elegans* to humans. The fact that the olfactomedins have been identified only in multicellular organisms suggests that they play a role in cell–cell interaction and signalling. Olfactomedins are involved in normal development and are associated with several diseases including open-angle glaucoma and cancer [18–22]. OLFM1 and 3 are expressed mainly in the brain, OLFM2 in the pancreas and prostate, and OLFM4 in the bone marrow, small intestine, colon and prostate [15]. Although originally identified in neural tissue, OLFM3 expression has been reported in the lung and spinal cord [23–25] and commercially available OLFM3 antibodies have been shown to stain lung cancer tissue. Lung cancers have also been observed to express OLFM1 and OLFM4 [19,22]. In this study, we also observed OLFM3 expression in lung as well as breast and nasal cancer cell lines that were anoikis resistant. None of the other olfactomedin proteins were differentially regulated at the mRNA level and subsequently were not investigated.

OLFM3, first identified in rat eye tissue and named optimedin [24] is a very conserved protein. OLFM3 forms homodimers (N-terminal important) and heterodimers (C-terminal important) with other olfactomedin domain containing proteins. OLFM3 is often localised to the Golgi and is a secreted protein. In mouse lens, the Pax6 transcription factor directly targets OLFM3 expression [25]. However, while Pax6 was detected in our microarray studies, the mRNA levels were slightly lower in DLKP-SQ than in DLKP-I or DLKP-M.

Olfactomedin 3 is known to interact with myocilin, a protein with cytoskeletal function and is associated with hereditary juvenile-onset open-angle glaucoma [26]. Microarray analysis showed very low levels of myocilin mRNA to be expressed in the clones and no difference was seen in the expression levels.

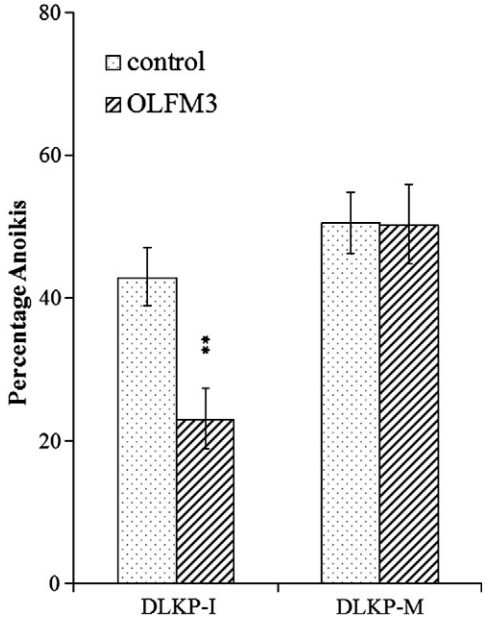

Fig. 5 – Effect of recombinant OLFM3 on DLKP-I and DLKP-M. Results are the average of at least 3 separate experiments. ** denotes Student *t*-test with a p-value of <0.001 compared to DLKP-I.

From the literature, it has been previously found that OLFM3 increased growth rate [27], and modulated cytoskeletal organisation, cell adhesion and migration [15]. Expression of OLFM3 in PC cells increased expression of N-cadherin, alpha-catenin and beta-catenin [27]. When Lee and Tomerav [27] knocked down expression of N-cadherin in OptH cells, the decreased levels of N-cadherin led to beta-catenin destabilisation, reduced formation of aggregates and increased the number of single cells in culture.

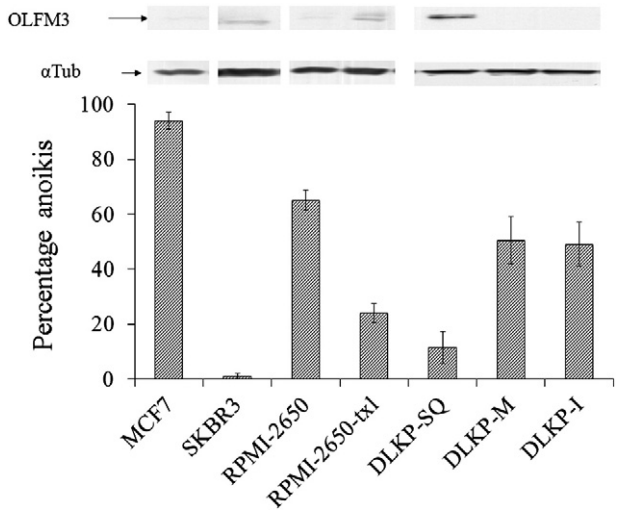

Fig. 6 – Correlation of expression of OLFM3 in anoikis resistant and anoikis sensitive pairs. Results for anoikis are the average of at least 3 separate experiments. Western blots shown are representative of three separate repeats with alpha tubulin being used as the loading control.

Interestingly, for DLKP-SQ which express OLFM3 but not N-cadherin (unpublished), the cells readily grow as single cells in suspension.

While the functions of OLFM3 are mostly unknown, several of the other olfactomedin proteins show similarities to the effects seen with DLKP-SQ. Both OLFM1 and OLFM4 have been implicated in apoptosis. Mutations in OLFM1 [28] and silencing of OLFM1 resulted in increased apoptosis [29]. OLFM4 may have differing roles depending on the tissue, being antiapoptotic in gastric cancers [30] and growth promoting in pancreas and lung cancer cells [31,21]. Recently, OLFM4 in prostate cancer, suppressed growth and metastasis and exerted a negative impact on autophagy through interactions with Cathepsin D and stromal-derived factor 1 [31].

The mechanism by which OLFM3 may regulate anoikis in DLKP-SQ is currently being investigated. Anoikis, meaning 'homelessness' in Greek, describes the induction of programmed cell death due to inappropriate expression of or loss of cell surface attachment to the extracellular matrix, and as such, provides a mechanism for preventing dissemination of cancer cells. There are two major cell death pathways involved in anoikis, apoptosis and autophagy [32]. Apoptosis from the literature appears as the main mechanism of anoikis with autophagy becoming important where apoptosis is blocked as a means of cell death. In autophagy, macromolecules and organelles especially mitochondria are targeted for degradation by lysozymal proteins to recycle nutrients within the cell. As such, autophagy may be a self-conservation response in times of stress. If the stress is excessive, autophagy can lead to cell death and there is extensive cross-talk between the two processes [32].

Mechanisms of anoikis resistance include augmentation of anti-apoptotic signals (such as Bcl-2 or Bcl-Xl [33]) or dampening of extracellular signals. Reducing extracellular signals occur at several levels, through cell surface receptors (such as integrins, cadherins and IGF-IR [34–37]), through the signalling cascades emanating from those receptors (such as the PI3K/Akt and ERK signalling pathways) and through the signalling molecules connecting receptors to pathways (such as Src family kinases and FAK [38]). EMT has been linked to suppression of anoikis via depletion of E-cadherin and expression N-cadherin to protect cells against anoikis [39,40].

It is possible that the activity of OLFM3 in reducing anoikis in DLKP-SQ is related to intracellular interaction with apoptotic machinery within the cell. For example, OLFM4 was found to interact with and inhibit the apoptosis promoting factor GRIM-19 [41]. Alternatively, secreted OLFM3 may interact with cell surface receptors or secreted proteins to prevent anoikis signalling. Secreted OLFM1 interacts with the Wnt signalling system through WIF-1 to regulate apoptosis through a variety of mechanisms [42].

In the clones, phosphorylated FAK is low in DLKP-SQ and DLKP-M suggesting reduced sensitivity to extracellular anoikis signals (Fig. 2C). In DLKP-I, the high levels of phosphorylated FAK in attached cells, are lost when the cells are maintained in suspension together with a loss of phosphorylated Paxillin (Fig. 2C), suggesting that integrin signalling through FAK is active and a source of anoikis signalling in DLKP-I.

There are several interacting proteins that have been previously identified to bind to OLFM3 including N-cadherin, Pax and Myoc [27,23,24]. Interestingly, N-cadherin is the only one of the above connectors that is significantly differentially regulated at the

mRNA level in the DLKP-clones, being up in DLKP-I and low or absent in DLKP-M and DLKP-SQ respectively (also in protein levels as determined by Western blotting (data not shown)).

Given that recombinant OLFM3 was able to reduce anoikis in DLKP-I but not back to the levels of DLKP-SQ and that loss of OLFM3 did not increase anoikis in DLKP-SQ to levels seen in the anoikis sensitive clones, suggests that other factors are involved in anoikis resistance in addition to OLFM3. Further, that FAK is not significantly affected by DLKP-SQ between attached and suspended cells supports more than one mechanism of anoikis resistance. This is not surprising as changes in apoptosis and/or autophagy may result in anoikis resistance. For apoptosis, many sets of molecules interact at the cell surface, inducing different downstream signalling cascades that lead to intracellular machinery controlling apoptosis [43]. In addition, the signalling molecules connecting the cytoskeleton and cytoplasm to the extracellular matrix can exhibit extensive cross-talk between the different signalling pathways [44].

Whether the expression of OLFM3 is a common mechanism is uncertain. Limited analysis has shown some correlation in breast cell lines with expression detected in the anoikis resistant SKBR3 but not in the anoikis sensitive MCF-7. Similarly OLFM3 was shown to be expressed in the anoikis resistant nasal cell line RPMI-2650-Txl variant while not in the anoikis sensitive parent, RPMI-2650.

Recently, both OLFM1 and OLFM4 expression have been linked to early stage lung cancer, while OLFM4 expression has also been implicated in early stages of gastric, colon and breast cancer [19–21]. It may be that expression of OLFM3 in DLKP-SQ reflects an early stage cancer.

In conclusion, this paper shows that expression of OLFM3 contributes to the increased anoikis resistance of DLKP-SQ and reduces anoikis in DLKP-I by reducing apoptosis. These results suggest that OLFM3 expression may have a role in anoikis resistance.

Conflict of interest

The authors declare no conflict of interest.

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