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Down-regulation of vimentin expression inhibits carcinoma cell migration and adhesion

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Abstract

Vimentin is a type III Intermediate filament protein that is expressed frequently in epithelial carcinomas correlating with invasiveness and poor prognosis. We have analysed migration and adhesion to collagenous matrix of a panel of carcinoma cell lines. In vitro invasiveness was highest in vimentin-positive SW480 colon cancer and MDA-MB-231 breast cancer cells and the role of vimentin in these cell lines was investigated by RNA interference. Down-regulation of vimentin expression resulted in impaired migration in both scratch-wound experiments and in invasion assays through cell culture inserts coated with collagen gel. Compromised migration was observed in both cell lines, whereas cell attachment assays revealed impaired adhesion to fibrillar collagen in MDA-MB-231 cells while the adhesion of vimentin-ablated SW480 cells, that express both vimentin and keratin intermediate filaments was not affected. In conclusion, ablation of vimentin expression inhibits migration and invasion of colon and breast cancer cell lines.

Keywords: Intermediate filaments; Vimentin; RNA interference; Invasion; Cell migration; Cell adhesion

Vimentin is a predominant intermediate filament (IF) protein in mesenchymal cells [1]. A classical view of IF cytoskeleton, named after their 10–12 nm diameter, describes the role of these filaments as structural proteins that maintain cytoarchitecture and tissue integrity, but a growing body of evidence implicates a role for IFs in regulation of various cellular functions [2,3]. Especially interesting is the role of vimentin in cell migration and epithelial–mesenchymal transition of epithelial carcinomas. Early studies investigating vimentin during embryogenesis noted an association of vimentin expression and developmental cell movements [1]. Subsequent generation of vimentin null mice revealed a phenotype where migration and contractability of fibroblasts is compromised, which is reflected by impaired healing of full-thickness skin wounds [4,5]. Recently, in vivo migration and diapedesis across endothelium of peripheral blood mononuclear cells has been shown to be reduced in vimentin null mice [6].

Studies of human epithelial carcinoma cell lines have demonstrated that vimentin expression is induced in invasive cell lines [7–9]. In breast cancers vimentin expression is, furthermore, correlated with poor prognosis [10]. Likewise, tissue microarray analysis has established that occurrence of metastasis in hepatocellular carcinoma is significantly associated with over-expression of vimentin [11]. Intriguingly, the expression of vimentin in breast cancer cells has been shown to be downstream of several signalling proteins implicated in carcinogenesis. Smad-interacting protein1, SIP1, a transcription factor implicated in epithelial–mesenchymal transition, up-regulates vimentin expression [12]. Likewise, β-Catenin/TCF complex can directly transactivate vimentin promoter [13]. Finally, over-expression of Tiam-1, a guanine nucleotide exchange factor (GEF), results in a increased invasiveness and vimentin expression in colon carcinoma cells [14]. However, none of the above studies has conclusively established, whether vimentin expression is only a marker of epithelial–mesenchymal transition (EMT)-like changes or if vimentin expression is required for the invasive behaviour of the cells.

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Ongoing elucidation of molecular interactions of vimentin give clues of the potential functions of vimentin in migration and invasion. Unlike keratin intermediate filaments that are connected to cellular junctions promoting adherence and tissue integrity, hemidesmosomes and desmosomes, vimentin can be localised with transient, actin-rich adhesion sites that play a role in cell migration. For example, in macrophages vimentin co-localises with fimbrin (T-plastin) in podosomes [15], whereas in endothelial cells, vimentin filaments associate with to αvβ3-positive focal contacts. Finally, vimentin is phosphorylated by PKCε on vesicles that recycle β1 integrins in mouse embryonic fibroblasts and expression of mutant vimentin harbouring PKC targets that are replaced with phosphomimetic amino acid residues results in a rescue of cell migration in PKCε null cells [16].

In this study, we have investigated the effect of vimentin ablation in invasive colon and breast carcinoma cell lines. We show that siRNA mediated knock-down of vimentin results in impaired migration and adhesion. These results support the hypothesis that vimentin expression is not only a marker associated with the invasive phenotype but required for carcinoma cell motility. Moreover, it can be concluded that methods based on RNA interference targeting of vimentin are potentially effective in modulating cell migration and carcinoma cell invasion.

Materials and methods

Cell culture and siRNA transfections. Colon carcinoma HT-29. SW948 and SW480 and breast carcinoma MCF-7 and MDA-MB-231 cell lines were obtained from the European Collection of Cell Cultures ECACC, Porton Down UK. Cells were cultured in DMEM/NUT.MixF-12 (Gibco, UK), supplemented with 10% FCS (Sigma–Aldrich, UK), 50 U/ml penicillin and 50 μg/ml streptomycin. Double-stranded siRNA oligonucleotides for vimentin and a negative control siRNA were purchased from Ambion and transfected (60 pmol siRNA per 24-well) using Oligofectamine reagent as recommended by the manufacturer (Invitrogen). The sequence for vimentin siRNA oligonucleotide (sense strand) was GCAAGUAUCCAACCAACUUtt. Cells were processed for immunofluorescence or protein extraction 72 h after transfection.

Cell migration and adhesion assays. Scratch-wound assays were performed as described previously for MCF-7 cells [17]. For the invasion assays through collagen gel, cells were serum starved for 24 h and seeded (1×10^5 cells per well) on 24-well cell culture inserts with 8 μm pore size, (Thincerts Greiner). Collagen gels were prepared by adding 50 μl of fibrillar collagen (2.1 mg/ml, First Link UK) diluted in PBS on the insert and allowing to gel 4 h at 37 °C. Lower chambers contained 10% fetal calf serum as a chemoattractant. Invasion was stopped at indicated time points by removing non-invaded cells in the upper chamber using swabs and washing with PBS. Invaded cells were fixed with ice cold methanol and stained with Dapi (Molecular Probes). It was observed that invaded cells always attached to the bottom of the membrane and did not float to the bottom of the plate. Membranes were removed from the cell culture inserts with a scalpel and mounted onto slides where three fields of triplicate membranes were counted.

For the cell attachment assays 500,000 cells were seeded on collagen-coated 12-well plates (Greiner) 72 h after siRNA transfection. Non-adherent cells were removed by three times gentle washing with PBS. CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega) was modified to assess the number of attached cells. Three 100 μl aliquots from each well was read at 490 nm with a spectrophotometric plate reader (Anthoslucy1).

Indirect immunofluorescence and immunoblotting. Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 10 min, permeabilised in 0.02% Triton X-100 (Sigma) and blocked for 40 min with 0.2% (w/v) fish skin gelatin (Sigma). The primary antibodies used were Cy3 conjugated mouse monoclonal antibody (clone V9, 1:1000 dilution) against vimentin (Sigma) and goat anti-plectin polyclonal antibody (C20, 1:200 dilution, Santa Cruz). Confocal images were obtained with Zeiss LSM 510

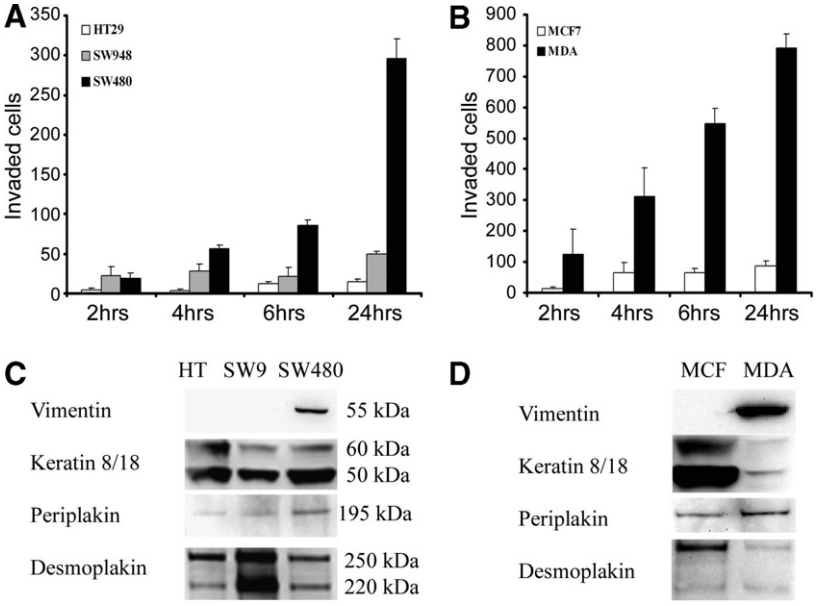

Fig. 1. Invasiveness and expression of cytoskeletal proteins in a panel of colon and breast carcinoma cells. (A) Invasion assay of colon carcinoma cells through collagen-coated cell culture inserts. Mean invaded cells/microscopic field from three independent experiments are shown. Error bars denote s.d. (B) Migration assay of breast carcinoma cells (C) Immunoblotting analysis of expression of intermediate filament proteins keratin 8/18 and vimentin, and cytoskeletal linker proteins desmoplakin I/II, and periplakin in colon carcinoma cell lines. HT, HT-29 cells; SW9, SW948 cells. (D) Expression of keratin 8/18, vimentin, desmoplakin I/II and periplakin in breast carcinoma cells. MCF, MCF-7 cells; MDA, MDA-MB-231 cells.

Confocal microscope using 40× N/A1.3 oil immersion objective. Composite panels were generated using Adobe Photoshop 7.0 (Adobe Systems) and LSM510 image browser software (Carl Zeiss). Immunoblotting of cytoskeletal proteins was carried out otherwise as described [17] apart from that rabbit polyclonal antibody 3052 (gift from Professor Roy Quinlan, University of Durham)) against vimentin was used for vimentin immunoblots.

Results and discussion

Vimentin expression is associated with increased migration in a panel of colon carcinoma cells

In order to investigate the role of intermediate filaments and associated cytolinker proteins in carcinoma cell migration, we first established, how invasive cells in a panel of colon carcinoma (Fig. 1A) and breast carcinoma (Fig. 1B) cell lines are in a migration assay through collagen-coated cell culture inserts towards 10% fetal calf serum as a chemoattractant. Only a low number of HT29 (derived from Duke's grade I/II colon cancer) and SW948 (derived from a grade III tumour) migrated through the collagen-coated membrane over a 24 h period, whereas SW480 cells (derived from grade III/IV colon cancer) were the most motile of the three cell lines with approximately six times more migrating cells in a 24 h period than SW948 cells (Fig. 1A). A similar difference was observed in the experiments using two different breast carcinoma cell lines. MCF-7 breast adenocarcinoma cells that migrate as a collective epithelial sheet [17] were on average eight times less motile than MDA-MB-231 cells in the collagen migration assay (Fig. 1B).

In the case of both colon and breast carcinoma cell lines, the most invasive cell lines, SW480 and MDA-MB-231, were vimentin-positive (Fig. 1C and D), whereas no vimentin expression was detected in the other studied cell lines. SW480 cells (Fig. 1C), unlike MDA-MB-231 cells (Fig. 1D) had also retained relatively high expression of keratin 8/18 intermediate filaments and desmoplakin, the major cytolinker protein connecting keratin intermediate filaments to desmosomes [18]. Thus, we were able to evaluate in subsequent experiments the effect of vimentin ablation in cells with or without intact keratin cytoskeleton. Co-expression of vimentin and keratin intermediate filaments in the same cells is intriguing, as previous experiments have shown increased motility in vimentin-positive melanoma cells that were transfected with keratin 8 and 18 constructs [19]. Expression of vimentin in MDA-MB-231 cells has been reported previously [20] but this is the first study to compare the vimentin and keratin expression in the studied panel of colon carcinoma cells.

Another plakin cytolinker protein, periplakin, that plays a role in organising keratin filament during the epithelial migration of MCF-7 sheets [17] was slightly up-regulated in the most motile carcinoma cells. Periplakin can interact with both keratins and vimentin [21,22] and unlike desmoplakin it has wider plasma membrane distribution than desmosomal junctions only [17,23]. Thus, it is possible that periplakin participates in the organisation of vimentin cytoskeleton in SW480 and MDA-MB-231 cells.

Transfection with siRNA oligonucleotides results in effective down-regulation of vimentin expression

To investigate the role of vimentin in the most invasive cell lines, SW480 and MDA-MB-231, we downregulated vimentin expression by transient siRNA transfections using as a control 'scrambled' siRNA with no human target mRNAs. Forty-eight hours after transfection, siRNA

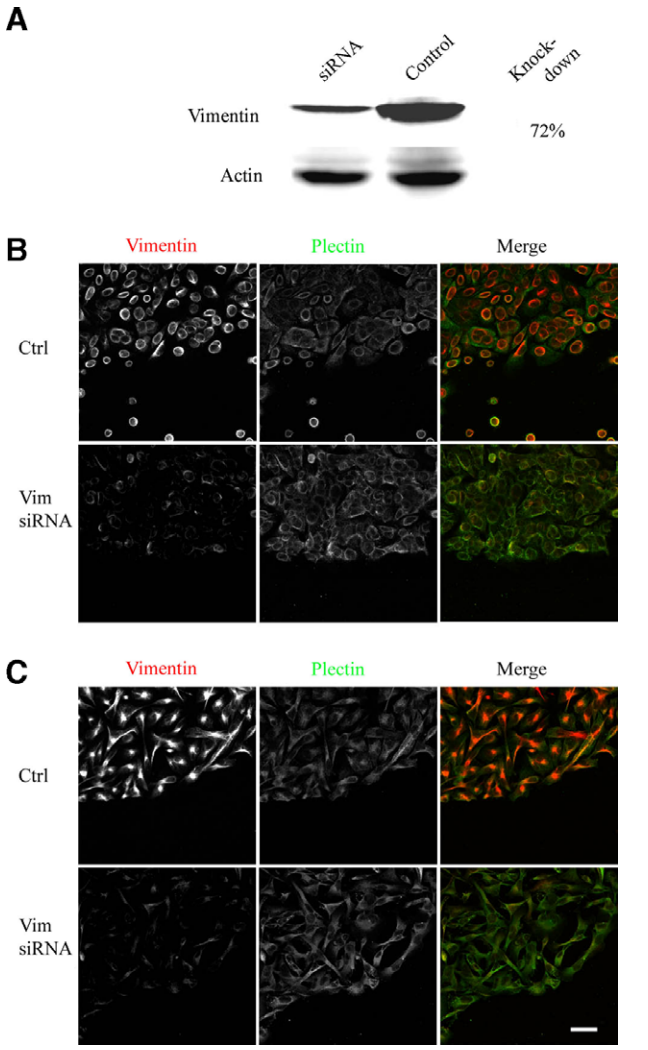

Fig. 2. Knock-down of vimentin expression by siRNA transfections. (A) Immunoblotting of vimentin expression in SW480 cells transfected with either vimentin siRNA oligonucleotides or control oligonucleotides. Knock-down efficiency was calculated from densitometric analysis of the protein bands. Actin expression was used as a loading control. (B) Vimentin (red channel) and plectin (green channel, merged image on the right) immunofluorescence staining of vimentin siRNA or control siRNA transfected SW480 monolayers that were scratch-wounded 48 h after transfection. (C) Vimentin and plectin immunostainings of siRNA transfected and scratch-wounded MDA-MB-231 cell monolayers. Note in both (B) and (C) that vimentin siRNA transfection leads to uniform down-regulation of vimentin expression. Scalebar corresponds to 20 μm for all panels.

transfections had reduced vimentin expression levels by over 70% compared to control siRNA transfections (Fig. 2A). This extend of down-regulation is similar to that seen in experiments with another intermediate filament, keratin 8 [17]. Indirect immunofluorescence and confocal microscopy confirmed that vimentin expression was uniformly downregulated in scratch-wound migration experiments of both SW480 (Fig. 2B) and MDA-MB-231 cells (Fig. 2C). Thus, siRNA transfections were efficient in causing a consistent and reproducible down-regulation of vimentin expression. Immunostaining with antibodies against plectin, a member of plakin family cytolinkers proteins that interacts with vimentin, demonstrated that vimentin ablation did not cause major changes in cell morphology (Fig. 2B and C). However, we noticed that in scratch-wound experiments, there was markedly less SW480 cells that quickly escaped wound edge and started to fill in the wound space (Fig. 2B). This observation was a further indication that cell migration could be affected by absence of vimentin and prompted us to study in detail migration and adhesion of vimentin-ablated cells.

Vimentin-ablated cells show impaired adhesion and migration

The effect of vimentin knock-down on carcinoma cell migration was investigated by collagen gel invasion assays and scratch-wound migration assays (Fig. 3). In the invasion assay, cells migrated from cell culture inserts through collagen gel-coated filters to 24-well plate wells with 10% FCS as chemoattractant. Migrated cells were visualised by Dapi staining and counted. A representative experiment (Fig. 3A) demonstrates a marked reduction in the migration of both MDA-MB-231 and SW480 cells after vimentin siRNA transfection compared with control siRNA transfection. The results were quantified from three independent experiments confirming significant decrease in the number of vimentin-ablated MDA-MB-231 (Fig. 3B) and SW480 (Fig. 3C) migrating through collagen.

Scratch-wound assays on confluent monolayers were utilised as an additional way of investigating cell migration. Control transfected MDA-MB-231 cells migrated particularly rapidly in this assay filling the empty wound space in about 6 h whereas vimentin knock-down wounds remained largely open at that time point (Fig. 3D). SW480 cells migrated more slowly than MDA-MB-231 cells but the difference between control transfected and vimentin siRNA transfected cells was evident at the 6 h time point (Fig. 3D) and confirmed by measurements of the relative course of the wound in three independent transfected wounds (Fig. 3E). MDA-MB-231 wound closure was quantified at 2, 4 and 6 h time points demonstrating significantly slower closure at all studied time points. Notably, two out of three control wounds were completely closed after 6 h, whereas all vimentin siRNA transfected wounds remained almost 80% open (Fig. 3F).

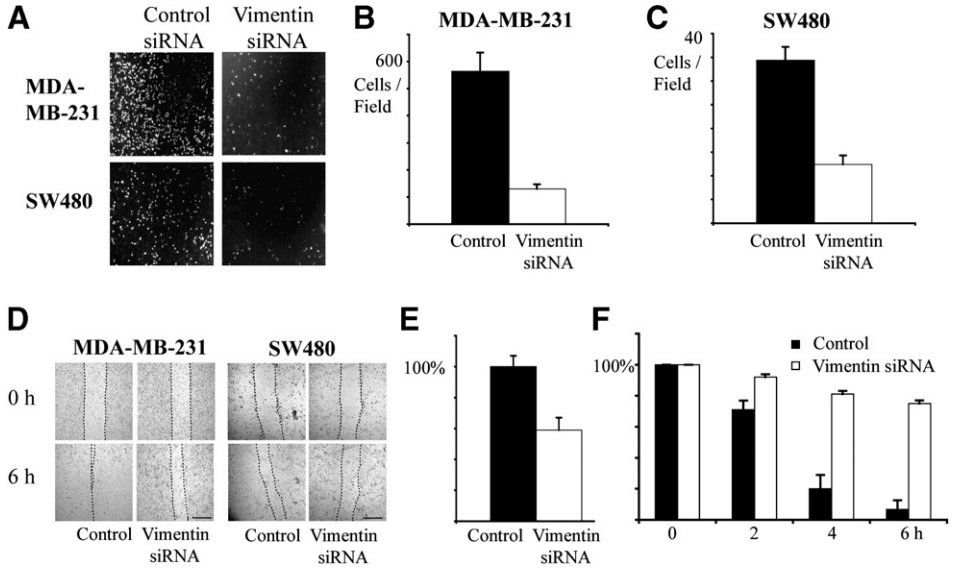

Fig. 3. Impaired migration of vimentin siRNA transfected cells. (A) Dapi stainings to visualise cell migrated through collagen-coated filters towards 10% FCS in a collagen invasion assay. Cells were transfected with control scrambled siRNA or vimentin siRNA 48 h prior to the assay. MDA-MB-231 images show migrated cells after 6 h and SW480 images represent a 12 h migration experiment. (B) Quantification of MDA-MB-231 migration. Dapi stained nuclei were counted from three independent migration experiments at 6 h time point. (C) Quantification of SW480 migration at 6 h time point. In (B) and (C) the graphs show average number of cells per microscopic field and standard error. (D) Representative phase contrast micrographs of closure of scratch-wounded confluent cultures of control siRNA or vimentin siRNA transfected MDA-MB-231 and SW480 cells at time point immediately after wounding and 6 h post wounding. Scratch-wound edges are marked by dotted lines. Scale bar represents 500 μm. (E) Relative closure of SW480 wounds. The average distance migrated by the control transfected wound edge cells (in pixels) was designated as 100% and the average (and standard error) migration of vimentin siRNA transfected cells in three independent wounds is shown relative to control. (F) Open wound distance of MDA-MB-231 wounds. Three locations of three independent control and vimentin siRNA transfected wounds were photographed at 0, 2 4 and 6 h. Open wound distance at the start of the experiment designated as 100% and closure of the wounds is shown relation to that. Error bars denote standard error.

The recently recognised role for vimentin in the recycling of integrin heterodimers [16] and reports demonstrating requirement of vimentin for focal adhesion complex stability in endothelial cells [24] prompted us to investigate the consequences of vimentin ablation on cell-matrix adhesion. We used fibrillar collagen as substrate and measured attached cells after 5, 10, 20 and 40 min of incubation. In MDA-MB-231 breast cancer cells, siRNA ablation of vimentin resulted in a significant decrease in adhesion to collagen (Fig. 4A). Impaired adhesion was evident already in the 10 min time point and the difference in the adherent cells was even more pronounced in the later time points (Fig. 4A). On the contrary, vimentin ablation had only a modest effect on cell adhesion in SW480 cells with no clear trend observed in comparison with control siRNA transfected cells (Fig. 4B). Thus, the effect of vimentin on cell adhesion is cell type specific. It is possible that carcinoma cells that, like SW480 cells, have retained expression of keratin intermediate filaments are able to circumvent the requirement of vimentin for integrin targeting.

To summarise, our results indicate that siRNA mediated down-regulation of vimentin can inhibit migration of epithelial carcinoma cells that have acquired vimentin expression as a part of epithelial–mesenchymal transition. This inhibition can be seen both in a cell line that continues to express keratin intermediate filament and in a cell line (MDA-MB-231) that has lost virtually all keratins. Our results support the other recent functional studies on vimentin. Reduced migration and invasion is consistent with a role for vimentin in controlling recycling of β1 integrins [16] and reduced size of focal contacts in vimentin-ablated endothelial cells [24]. In future studies it would be of interest to elucidate molecular interactions of vimentin that are required for invasion and migration. A putative candidate for mediating the effects of vimentin is plectin, a versatile cytoskeletal linker protein, that can connect vimentin to other cytoskeletal networks and, via certain plectin N-terminal splice variants, to focal adhesion sites [25]. Notably, a 70% inhibition of vimentin expression is sufficient to impair migration and invasion, which suggests that it could be plausible to investigate strategies to reduce vimentin expression in vivo.

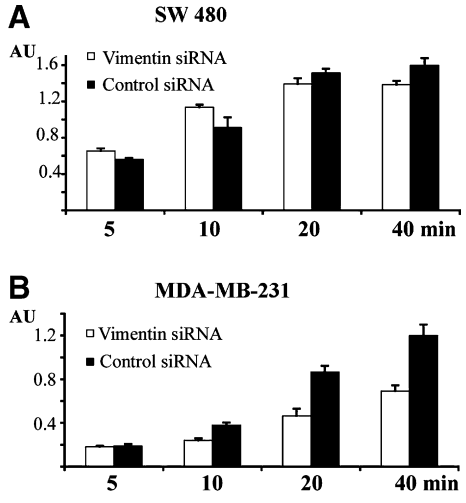

Fig. 4. Vimentin ablation impairs MDA-MB-231 cell adhesion. Cell attachment assay of (A) SW 480 cells and (B) MDA-MB-231 cells. Vimentin siRNA (white bars) or control siRNA (black bars) cells were suspended 48 h after transfection and let to attach to collagen-coated tissue-culture wells. Non-adherent cells were washed away at indicated time points and the adherent cells were quantified by colorimetric cell viability kit (measured by absorbance units at 490 nm). Mean and standard error of mean of three independent experiments (Three repeats each) are shown. The difference between control and vimentin siRNA transfected cells was statistically significant (t-test, $p < 0.05$) at 10, 20 and 40 min time points.

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