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Effect of Silencing Neutrophil Gelatinase-Associated Lipocalin in Ovarian Cancer Cells on Epithelio-Mesenchymal Transition

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Abstract

Objectives: Previously, we reported that neutrophil gelatinase-associated lipocalin (NGAL) is present in epithelial ovarian cancer cells, but not mesenchymal cancer cells. Furthermore, we reported that epithelial growth factor (EGF) induced epithelio-mesenchymal transition (EMT) in epithelial ovarian cancer cells and concomitantly suppressed NGAL expression. We hypothesised that inhibition of NGAL expression induces EMT in these cells.

Methods: NGAL knockdown was achieved using siRNA in ovarian cancer cell line OVCA 429. NGAL siRNA-treated cells [n=3] were assayed for EMT markers such as migration and invasion assays, and E-cadherin, N-cadherin, Snail, β-catenin and vimentin expression by Western blot.

Results: There were no significant differences between NGAL siRNA-treated cells and controls for the protein markers. There was an increase in cell migration for NGAL siRNA-treated cells compared to control (96 h post transfection), however there was no change in cell invasiveness between treatments.

Conclusions: We conclude that direct NGAL inhibition by siRNA does not induce a mesenchymal transition of ovarian cancer cells.

Keywords: Ovarian cancer; Epithelio-mesenchymal transition; Neutrophil gelatinase-associated lipocalin

Introduction

Epithelial ovarian cancer is the most lethal gynaecological cancer and the fifth most common cause of cancer death in women in the Western world [1]. It has an overall five-year survival of 42%, due to most cases being diagnosed at an advanced stage of disease [2]. There is a paucity of information concerning the pathophysiology of ovarian cancer. It is by understanding the pathways that are intrinsic to disease progression and metastasis that new insights are realised and improvements in patient management and treatment affected.

Epithelial-mesenchymal transition (EMT) has recently been recognised as a mechanism for cancer progression and malignant transformation. EMT by cancer cells does not follow an orderly program and is different from physiological and developmental EMT [3]. EMT induced by growth factors and cytokines requires reprogramming of epithelial cells in order to reshape for locomotion and degradation of the extracellular matrix (ECM). Epithelial growth factor (EGF) has been described as a powerful mitogen for human ovarian surface epithelium (OSE) [4], where 90% of all ovarian carcinomas originate. The over-expression of EGF receptors (EGFRs) is common in human ovarian carcinoma-derived cell lines and tumours, thereby suggesting that the growth factor family plays a critical role in ovarian tumour aetiology and progression [5]. EGF has been shown to induce EMT in epithelial cells [6] and epithelial breast cancer cells [7].

Loss of the epithelial phenotype is characteristic of EMT; the shift from E-cadherin to N-cadherin is a central event mediating EMT [8]. Crucial to E-cadherin activity is its interaction with β-catenin, which links E-cadherin to the actin cytoskeleton. Two members of the Snail family of transcription factors, Snail and Slug, have been shown to down-regulate E-cadherin by binding the E-boxes present in its promoter.

During EMT, Snail up-regulates mesenchymal molecules such as fibronectin and vimentin. The mesenchymal N-cadherin interacts with members of the fibroblast growth factor receptor (FGFR) family, thereby inducing pro-migratory and invasive signalling cascades [9]. This promotion of cell motility and migration is the opposite effect of E-cadherin. Thus, hallmarks of EMT include the loss of E-cadherin and β-catenin, and the gain of N-cadherin, Snail and vimentin.

Previously, we have reported that normal ovaries lack neutrophil gelatinase-associated lipocalin (NGAL) expression and that NGAL expression occurs in benign tumours and increases in early grade ovarian tumours [10]. Furthermore, it was also demonstrated that compared to normal healthy women and women with advanced-stage disease, NGAL concentration is higher in the serum of patients with benign and grade 1 tumours. The mechanisms and biology of NGAL in ovarian cancer, however, are not known. Our previous study established that NGAL is only expressed in epithelial ovarian cancer cells and the expression of NGAL is suppressed in ovarian cancer cells induced with EGF to undergo EMT [10]. The aims of this current study were to assess the effect of NGAL knockdown by siRNA transfection

on the expression of EMT markers in ovarian cancer cell line OVCA 429. As EGF is an inducer of EMT in ovarian cancer cells and NGAL expression was decreased in EGF-treated cells, NGAL may play a role in the EMT of ovarian cancer cells. Thus, the hypothesis to be tested in this study was that inhibition of NGAL expression in OVCA 429 cells induces EMT.

Materials and Methods

Cell culture experiments

Cell lines: The ovarian cancer cell line OVCA 429 has been described previously [11]. Cell culture media consisted of a 1:1 ratio of medium 199 and MCDB 105 (Sigma Aldrich, MO, USA), with 10% FBS and supplemented with 2.4 mM L-glutamine and 60 U/ml penicillin/streptomycin solution. All cells were maintained at 37°C in the presence of 5% CO_2. Viability of the cells was checked routinely by the trypan blue exclusion method using a haemocytometer.

siRNA Transfection of ovarian cancer cells

Gene knockdown of NGAL in OVCA 429 ovarian cancer cells (n=3) was achieved by using siRNA transfections. siRNA against NGAL was used, along with a Negative control siRNA and β-actin siRNA (Qiagen, MD, USA). Mock transfected cells consisted of cells treated with Lipofectamine 2000 (Invitrogen, CA, USA) but not siRNA. Ovarian cancer cells were grown in media free of antibiotics in 6 well plates until 30% confluent. For a total transfection complex of 200 μl per well, 2 μl Lipofectamine 2000 was added dropwise to pre-warmed OPTI-MEM I medium, vortexed and incubated at room temperature for 10 min. siRNA was added to diluted Lipofectamine 2000 transfection reagent for a final concentration of 150 nM and incubated at room temperature for 15 min. The volume of normal growth medium in each well was adjusted to 800 μl and the siRNA complex was then added. Cells were incubated for 24 h then washed in sterile PBS. Fresh complete medium was added and cells were incubated for up to 72 h. Cells were collected and lysates prepared and frozen until analysis by Western blot or subjected to wounding or invasion assays, whereby data was collected 72 or 96 h post transfection.

Wounding assay

siRNA transfected cells were serum-starved for 24 h before a wound was created with the tip of a sterile 1000 μl pipette. The wound was marked and measurements were taken of distances between wound edges using an ocular micrometer. Six representative fields were marked and measured. Wounds were measured after 24 and 48 h (72 and 96 h post transfection). Three independent experiments were performed.

Invasion assay

The invasive potential of siRNA treated OVCA 429 cells was determined by the ability of the cells to invade a synthetic basement membrane [12]. Briefly, polycarbonate filters (8 μm pore size) pre-coated with Matrigel were placed in a modified Boyden chamber. OVCA 429 cells transfected with siRNA for 48 h were trypsinized and resuspended in SFM. Culture medium containing 5% FBS as a chemoattractant was added to each well of a 24 well plate. Additional wells contained SFM as a negative control. Inserts were placed in the wells and resuspended cells were added. After 24 h, non-invading cells were removed from each membrane by running a sterile cotton tip over the membrane on the inside of the chamber; invading cells appeared on the outer side of the membrane and were fixed in 100% methanol, stained with Diff-Quik and mounted on slides. Total numbers of invading cells were counted using a light microscope. Each experiment was performed in triplicate.

Western blotting

Cell lysates were prepared by adding 150 μl RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Igepal, 0.1% SDS, 0.25% Na deoxycholate, 1 mM EDTA, pH 7.4, 1mM PMSF and 10 μl/ml aprotinin) to cell pellets, vortexed, incubated at 4°C for 1 h, then centrifuged at 10,000 X g for 20 min. Supernatants were collected as cell lysates. Equal amounts of protein were electrophoresed on 7.5% and 12% SDS-PAGE gels. Western blotting was performed as described previously [10]. The following antibodies and concentrations were used: NGAL (Clone 211-1 [13], at 0.2 μg/ml), E-cadherin (BD Biosciences, at 50 ng/ml), Snail (Abcam, at 0.5 μg/ml), β-catenin (BD Biosciences, at 50 ng/ml), vimentin (Dako, at 2 μg/ml) and β-actin (Sigma Aldrich, at 0.1 μg/ml). Western blots were the product of at least two independent experiments.

Statistical analyses

Statistical analysis of the data was performed using commercially available statistical software (Statgraphic Plus version 3.1, statistical graphics corporation, Rockville, Maryland, USA). The data are presented as the mean ± standard error of the mean (SEM). Statistical significance was assigned to $P<0.05$, unless otherwise stated. The data were initially analysed for homogeneity of variance using Bartlett's test and when significant ($P<0.05$) the data were logarithmically transformed and homogeneity of variance confirmed before further analysis. Where logarithmic transformation of the data was necessary, the mean values before transformation have been presented. In cases where homogeneity was established, two-sample comparisons were performed using Student's t-tests, and three or more sample analysis was performed using one-way analysis of variance (ANOVA).

Results

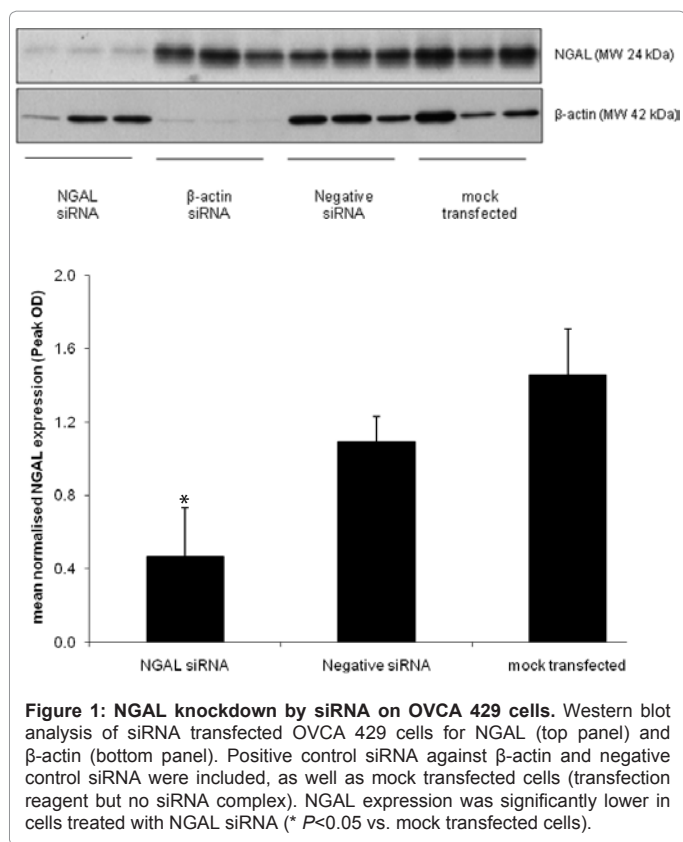### Effect of NGAL knockdown by siRNA on EMT markers

siRNA Transfection of OVCA 429 cells: OVCA 429 cells were transfected with NGAL **siRNA,** Negative control siRNA or no siRNA (mock cells). Western blot showed that NGAL protein expression was unaffected by β-actin and negative control siRNA-treated cells (Figure 1). NGAL siRNA successfully decreased NGAL protein expression in OVCA 429 cells compared to mock transfected cells by ~70% ($P<0.05$). Likewise, a decrease in β-actin protein expression was found in cells treated with β-actin siRNA; however, there was no effect on β-actin protein expression on the NGAL siRNA-treated cells.

Protein expression of EMT markers

The effect of NGAL knockdown in OVCA 429 cells on markers of EMT was assessed by Western blot. The EMT markers investigated were E-cadherin, β-catenin and Snail (Figure 2). Blots were also probed for β-actin. A431 lysate and HEY day 4 spheroid cell lysates were included as positive controls [14]. Human recombinant Snail protein provided a positive control for the Snail antibody. The NGAL siRNA-treated cells were compared to negative and mock siRNA treated cells and there was no effect for E-cadherin, β-catenin or Snail proteins. There was also no change for N-cadherin and vimentin proteins [data not shown].

Cell motility

The invasive potential of siRNA-treated OVCA 429 cells was explored using a wounding assay (Figure 3). The average percentage

Figure 1: NGAL knockdown by siRNA on OVCA 429 cells. Western blot analysis of siRNA transfected OVCA 429 cells for NGAL (top panel) and β-actin (bottom panel). Positive control siRNA against β-actin and negative control siRNA were included, as well as mock transfected cells (transfection reagent but no siRNA complex). NGAL expression was significantly lower in cells treated with NGAL siRNA (* $P<0.05$ vs. mock transfected cells).

wound closure after 72 h [24 h post wound)]for NGAL siRNA-treated cells was 26 ± 6; after 96 h (48 h post wound) it was 55 ± 3. For mock transfected cells, the wound closure after 72 and 96 h was 21 ± 1 and 42 ± 3 respectively. There was a statistical significance between 72 and 96 h for each treatment ($P<0.05$), but there was no difference between treatments at 72 h. However, at 96 h, NGAL siRNA-treated cells had a greater percentage wound closure compared to mock transfected cells ($P<0.05$).

Cell invasiveness

In addition to the wounding assay to measure the invasive potential of siRNA-treated OVCA 429 cells, an invasion chamber assay was performed. This assay measures the number of cells migrating through pores of a polycarbonate filter, simulating a basement membrane. After 24 h (72 h post transfection), the number of cells on the underside of each membrane was counted and the mean calculated. As a negative control, no chemoattractant was added to wells of mock transfected cells; the average number of invading cells ($\pm$ SEM) was 2 ± 1 (data not shown). With the chemoattractant, the mean number of mock cells that invaded the membrane was 21 ± 7 (Figure 4). The mean number of NGAL siRNA-treated cells that migrated through the membrane was 13 ± 2. No significant difference was found between NGAL siRNA-treated cells and mock cells. NGAL knockdown of these cells were confirmed by Western blot [data not shown].
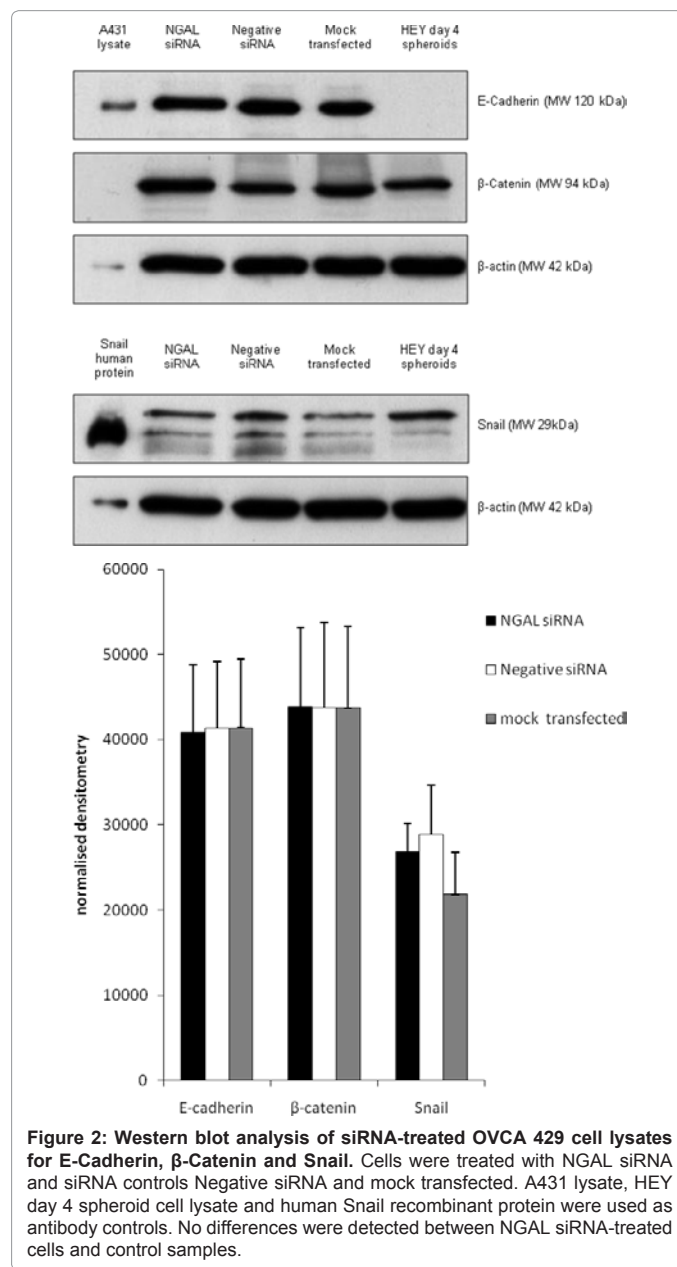
Discussion

The aim of this study was to test the hypothesis that inhibition of NGAL expression in OVCA 429 cells induces EMT. NGAL expression was effectively suppressed by 70% using siRNA knockdown. Known phenotypic markers of EMT, including E-cadherin, β-catenin,

Snail, vimentin, N-cadherin and cell migration metrics were used as experimental endpoints. The data obtained establish that inhibition of NGAL expression in OVCA 429 cells is not obligate for EMT.

Previously, we reported that transformation to a more invasive mesenchymal phenotype was associated with a decrease in NGAL expression in epithelial ovarian cancer cell lines [10]. In this study, however, the effects of independent suppression of NGAL did not induce EMT.

NGAL activity has been studied in colon carcinoma cells [15]. Colon carcinoma cell lines were stably transfected with NGAL (for over-expression) or antisense NGAL (under-expression). Western blot analysis of cell lysates showed no changes in E-cadherin and β-catenin expression, mirroring the results of this study. A change in both proteins, however, was found by immunofluorescence staining; localisation of E-cadherin was found to be critical for maintaining
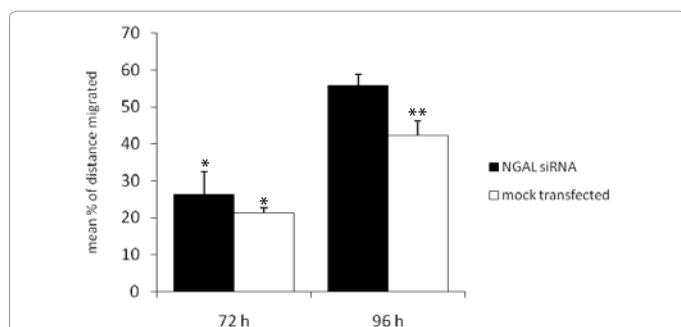

Figure 2: Western blot analysis of siRNA-treated OVCA 429 cell lysates for E-Cadherin, β-Catenin and Snail. Cells were treated with NGAL siRNA and siRNA controls Negative siRNA and mock transfected. A431 lysate, HEY day 4 spheroid cell lysate and human Snail recombinant protein were used as antibody controls. No differences were detected between NGAL siRNA-treated cells and control samples.

Figure 3: Wounding Assay of siRNA-transfected OVCA 429 cells. NGAL siRNA-treated cells and mock transfected cells were grown in a 6-well plate and wounded, as described in Materials and Methods. Measurements were taken between wound edges at time of wound (0 h) and 24 h and 48 h after wound (72 and 96 h post transfection). The percentage wound closure was calculated for both time points compared to 0 h. For both NGAL siRNA and mock transfected cells, there was a significant increase in wound closure between 72 and 96 h (*$P<0.05$ vs. 96 h mock transfected). At 96 h there was a significant increase in wound closure for NGAL siRNA-treated cells (**$P<0.05$ vs. 96 h NGAL siRNA).
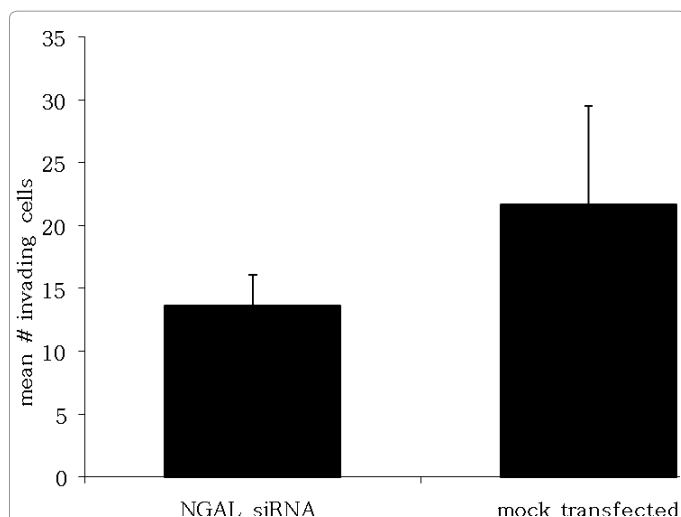

Figure 4: Invasion chamber assay for siRNA-transfected OVCA 429 cells. NGAL siRNA-treated cells and mock transfected cells were put into modified Boyden chambers to determine the ability of treated cells to invade a synthetic membrane. The total number of invading cells were counted after 24 h (72 h post transfection) and the mean calculated. No significant difference was detected between NGAL siRNA-treated cells and mock transfected cells.

normal epithelial structure. This study explored the effects of NGAL on small Rho GTPases, such as Rac1, as they are implicated in the establishment and maintenance of E-cadherin-mediated cell-cell adhesion as well as in the migration and invasion of epithelial tumour cells [16]. NGAL over-expression in the colon cancer cell line decreased E- cadherin-mediated cell-cell adhesion and increased cell motility and invasion through the alteration of the subcellular localisation of Rac1 [15]. NGAL suppression also decreased colon cancer cell invasion, independent of MMP-9. It was suggested NGAL contributed to colon cancer pathophysiology through the signalling of intracellular small GTPases in cancer migration and invasion. Applying these finding to our own results, the lack of change between NGAL siRNA and control cells for EMT markers may be due to using the total cell lysate and not looking at localisation of proteins. Only further studies will clarify if NGAL has a role in ovarian cancer progression that is different to colon cancer.

NGAL expression has also been identified in breast cancer cells, with a recent study demonstrating that NGAL promotes breast cancer progression by inducing EMT [17]. NGAL over-expressed in human breast cancer cells up-regulated mesenchymal markers vimentin and fibronectin, down-regulated epithelial marker E-cadherin and increased cell motility and invasiveness. NGAL silencing in aggressive breast cancer cells inhibited cell migration and the mesenchymal phenotype. It has been noted that NGAL expression is highest in advanced breast cancer stages [18], contrary to our findings which showed that NGAL expression was highest in well-differentiated ovarian cancers and decreased with tumour grade [10]. Similarly, NGAL has also been characterised in pancreatic cancer (PaCa) cells [19], being highly expressed in early dysplastic lesions in the pancreas. Stable NGAL over-expression in PaCa cells significantly blocked cancer cell adhesion and invasion, with the opposite effects occurring in the NGAL under-expressing PaCa cells. The differences in the results observed in ovarian cancer cells compared to the findings on breast and colon cancer cells may suggest different mechanistic pathways relevant to the physiology of different types of carcinomas.

Although these results are not conclusive in determining the role of NGAL in the metastasis of epithelial ovarian cancer cells, studies in the literature indicate that NGAL does play some part in disease progression. To further our study, it would be beneficial to explore the effect of NGAL inhibition in other ovarian cancer cell lines, and to determine the effects of NGAL over-expression. We demonstrated that EGF was able to induce EMT in ovarian cancer cell lines, and that NGAL expression also decreased in these treated cells, leading to our hypothesis that NGAL was able to induce EMT; however our results suggest that NGAL expression decreased as a result of EMT, i.e. in EGF-treated cells. Our results may best be explained by the recent study by Tong et al. [20], which demonstrated that EGF blocked NGAL expression in pancreatic ductal adenocarcinoma (PDAC). They showed that EGF exposure decreased NGAL expression, an effect that was abolished when cells were further treated with EGF-inhibitor AG1478, the same result as seen in our ovarian cancer cells [10]. E-cadherin expression was also decreased in EGF-treated cells, again in accordance to our previous study [10]. Tong concluded that EGF down-regulated E-cadherin and NGAL by activating EGFR and the mitogen-activated protein kinase (MAPK)-ERK signalling pathway in PDAC cells. Our studies have shown that NGAL is present in epithelial ovarian cancer cell lines, rather than the more aggressive mesenchymal cell lines, and that EMT induced by EGF decreases NGAL expression. While our hypothesis was drawn from these results, that NGAL inhibition is akin to the transition of the mesenchymal phenotype, we found that there were no differences in NGAL siRNA treated cells for typical EMT markers such as vimentin, E-Cadherin, N-cadherin, Snail and β-catenin. There was an increase in cell migration for NGAL siRNA-treated cells however there was no change in the invasion chamber assay to indicate a change in cell invasiveness, which is contrary to our hypothesis. Studies have shown that NGAL knockdown decreased cell invasion in colon carcinoma cells [15] and breast cancer models [21]. While NGAL has been demonstrated to be involved in numerous human tumour types, conflicting results have contributed to the debate of what role NGAL plays in cancer pathophysiology, as reviewed by Bolignano et al. [22]. NGAL can have a pro-tumour effect, as seen in breast, esophageal and gastric cancer, but it can also have an anti-tumour effect, as seen in our studies of ovarian cancer, pancreatic and colon carcinomas. It appears that the effects of NGAL are neoplasia-specific and influenced by multiple mechanisms. The exact role of NGAL during ovarian carcinogenesis is still unclear, however our results suggests that NGAL does not regulate EMT in ovarian cancer cells.

Acknowledgements

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