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miRNA-21 inhibition enhances RANTES and IP-10 release in MCF-7 via PIAS3 and STAT3 signalling and causes increased lymphocyte migration

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ABSTRACT

MicroRNAs (miRNAs) are a class of small endogenous gene regulators that have been implicated in various developmental and pathological processes. However, the precise identities and functions of miRNAs involved in antitumor immunity are not yet well understood. miRNA-21 is an oncogenic miRNA that can be detected in various tumours. In this study, we report that a miRNA-21 inhibitor enhances the release of chemoattractants RANTES and IP-10 in the MCF-7 breast cancer cell line and results in increased lymphocyte migration. Thus, miRNA-21 is a potential therapeutic target for cancer immunotherapy. We further demonstrated that PIAS3, a protein inhibitor of activated STAT3, is a target of miRNA-21 in MCF-7. Thus, miRNA-21 is a novel miRNA regulating immune cell recruitment, which acts at least in part via its inhibition of PIAS3 expression and oncogenic STAT3 signalling in tumour cells.

1. Introduction

Cancer immunotherapy has developed into a first-line therapeutic regimen, particularly in cancers refractory to chemotherapy. However, immune-mediated cancer regression preferentially occurs in the minority of patients who have a T cell-inflamed tumour environment [1], suggesting that infiltration of immune effectors into tumour locations is required for tumour eradication. Thus, a pre-existing T cell inflamed tumour microenvironment may be predictive of clinical outcome to immunotherapy [2,3]. Previous studies have demonstrated that infiltration of CD8+ effector T cells in inflamed tissues depend on adhesion molecules and specific chemokines such as RANTES and IP-10 [4,5]. Several intracellular signalling pathways, including signal transducer and activator of transcription 3 (STAT3), Notch, β-catenin and PI3K, regulate the expression of inflammatory cytokines and chemokines [6–9]. Correlations amongst these signalling pathways, together with various inflammatory mediators of adhesion molecules, cytokines and chemokines, form a complex immune regulatory network. The absence of chemokines may present a major barrier to cancer immunotherapy in a subset of patients. Further investigation of the molecular mechanisms underlying immune cell infiltration and recruitment to tumours is urgently required to design new therapeutic strategies and improve clinical outcomes for cancer immunotherapy.

MicroRNAs (miRNAs) are small, endogenous, noncoding RNAs that target the 3′-untranslated region (3′-UTR) of specific mRNAs and result in degradation or repression of translation [10]. miRNAs regulate important cellular processes such as cell differentiation, proliferation, apoptosis and tumourigenesis. miRNAs are also involved in cancer-related inflammatory responses, and certain miRNAs display important modulation of the migration and immunogenicity of immune cells. Mark et al. have shown that induced miRNA-146 expression negatively regulates IL-8 and RANTES release in human alveolar epithelial cells [11]. They further demonstrated that divergent intracellular signalling pathways are involved in the regulation of chemokine production by miRNA-146 [12]. miRNA-125b has been reported to regulate the expression of cytokines and chemokines by targeting tumour necrosis factor α (TNF-α) transcription in mouse Raw 264.7 macrophages [13].

miRNA-21 is an oncogenic miRNA that has been reported to regulate tumour cell proliferation, tumourigenicity, migration and invasion, apoptosis and chemoresistance by repressing expression of various target molecules [14–18]. STAT3 has recently been predicted via bio-informatics as another target of miRNA-21 [19]. Xiong et al. [20] demonstrated that PIAS3, a protein inhibitor of activated STAT3, is a direct target of miRNA-21 and contributes to the oncogenic function of miRNA-21 in myeloma cells. STAT3 is constitutively activated in many types of human malignancies and is capable of inducing a large number of genes that are crucial

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for cancer inflammation [21,22]. Blocking STAT3 signalling in tumour cells leads to the production of multiple chemoattractants that induce migration of various immune cells [23]. Based on these findings, we tested whether miRNA-21 regulates release of chemokines and affects lymphocyte migration. We present here the first evidence that downregulation of miRNA-21 increases chemokine release in MCF-7 human breast cancer cells in vitro and results in increased lymphocyte migration, partially due to targeting PIAS3, which regulates the STAT3 signalling pathway. These results represent a novel mechanism in immunoresistance.

2. Materials and methods

2.1. Cell culture

The MCF-7 breast cancer cell line was obtained from the Shanghai Institute of Cell Biology, China Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) with high glucose (Hyclone, Beijing, China) supplemented with 10% foetal calf serum and 1% penicillin/streptomycin at 37 °C, 5% CO_2.

2.2. RNA extraction and qRT-PCR

Total RNA was extracted from MCF-7 cells using Trizol Reagent (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcription of microRNA was performed using the SYBR PrimeScript miRNA RT-PCR Kit (TaKaRa, Dalian, China). Reverse transcription of mRNA was performed according to the protocol of the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). qPCR was performed as described in the SYBR premix Ex Taq instructions (TaKaRa, Dalian, China) with a LightCycler 480 (Roche, USA). U6 snRNA or GAPDH mRNA levels were used for normalisation.

The primer sets for PCR were designed as follows [20]: 5′-GCCGCTAGCTTATCAGACTGATGT-3′ for miRNA-21 forward primer, 5′-GCGCGTCGTGAAGCGTTC-3′ for U6 snRNA forward primer. PIAS3-F: 5′-GCCGACATGGACGTGTCCTGTG-3′, PIAS3-R: 5′-TTCC CTCCTGGACTGCGCTGTAC-3′, GAPDH-F: 5′-AGAAGGCTGGGGCT CATTTG-3′, GAPDH-R: 5′-AGGGGCCATCCACAGTCTTC-3′. PCR product quality was assessed by performing a melting curve analysis. The relative expression levels of miRNA-21 and PIAS3 mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method [24]. PCR was performed in triplicate.

2.3. Oligonucleotide transfection

The miRNA-21 inhibitor (miR-21-In: 5′-UCAACAUCAGUCUGA UAAGCUA-3′), a negative control miRNA (In-NC: 5′-CAG-UACUUUUGUGUAGUACAA-3′), PIAS3 siRNA (sense: 5′-GG AGC-CAAAUGUGAUUAUAUU-3′, antisense: 5′-UAUAAUCACAUU UGGCUCCUU-3′) and a negative control siRNA (sense: 5′-UUCUCC GAACGUGUCACGUTT-3′, antisense: 5′-ACGUGACACGUUCGGAGA-ATT-3′) [20] were chemically synthesised by Shanghai GenePharma Company (Shanghai, China). MCF-7 breast cancer cells were plated in 6-well plates (5×10^5 cells/well) and transfected with 100 nM of the miRNA-21 inhibitor, PIAS3 siRNA or negative controls by Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. To confirm the efficiency of transfection, the same amount of negative controls labelled with FAM was also transfected. Cells were harvested for RNA (48 h) or protein (72 h) extraction post-transfection for qRT-PCR and western blot analysis.

2.4. Western blot analysis

Protein extracts (30 μg) prepared with RIPA lysis buffer were resolved on a 10% SDS–PAGE gel and then transferred onto an Immobilon-P PVDF membrane. After blocking with 5% non-fat milk, membranes were probed with mouse anti-PIAS3 monoclonal antibody, mouse anti-STAT3-pY705 monoclonal antibody, and rabbit anti-STAT3 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were then incubated with peroxidase-conjugated anti-mouse or anti-rabbit IgG (Beyotime, China) for 1 h at room temperature at a 1:1000 dilution. Band detection via enzyme-linked chemiluminescence was performed according to the manufacturer's protocol (Millipore, USA).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The levels of RANTES (CCL5) and IP-10 (CXCL-10) in the supernatants of MCF-7 cells transfected with miRNA-21 inhibitor and/or si-PIAS3 or the negative controls were measured using ELISA kits purchased from Bostar Ltd. (Wuhan, China).

2.6. In vitro migration assay

Lymphocyte migration assays were performed using 24-well Transwell™ supports (Corning, Amsterdam, Netherlands) with 3 μm pore membranes. Spleens were harvested from 6-week-old BALB/c mice and splenocytes were prepared in a single cell suspension. The splenocytes were carefully layered on the surface of lymphocyte separation medium (Tianjin haematology Institute, Chinese Academy of Medical Sciences, Tianjin, China). After centrifugation at 1200 g for 20 min, the white cloudy band of splenocytes was collected. RBC lysis buffer (BioLegend, USA) was used to remove RBCs. Lymphocytes were then seeded onto the upper chamber of the Transwell™. Conditioned medium from MCF-7 cells transfected with miRNA-21 inhibitor or the negative control was added into the lower chamber. After an incubation of 12 h, the number of lymphocytes in the lower chamber was measured by a WST-8 Cell Counting Kit-8 (Beyotime, China) as described previously [25]. Absorbance was measured at 450 nm using a microplate reader (Bio Tek, USA).

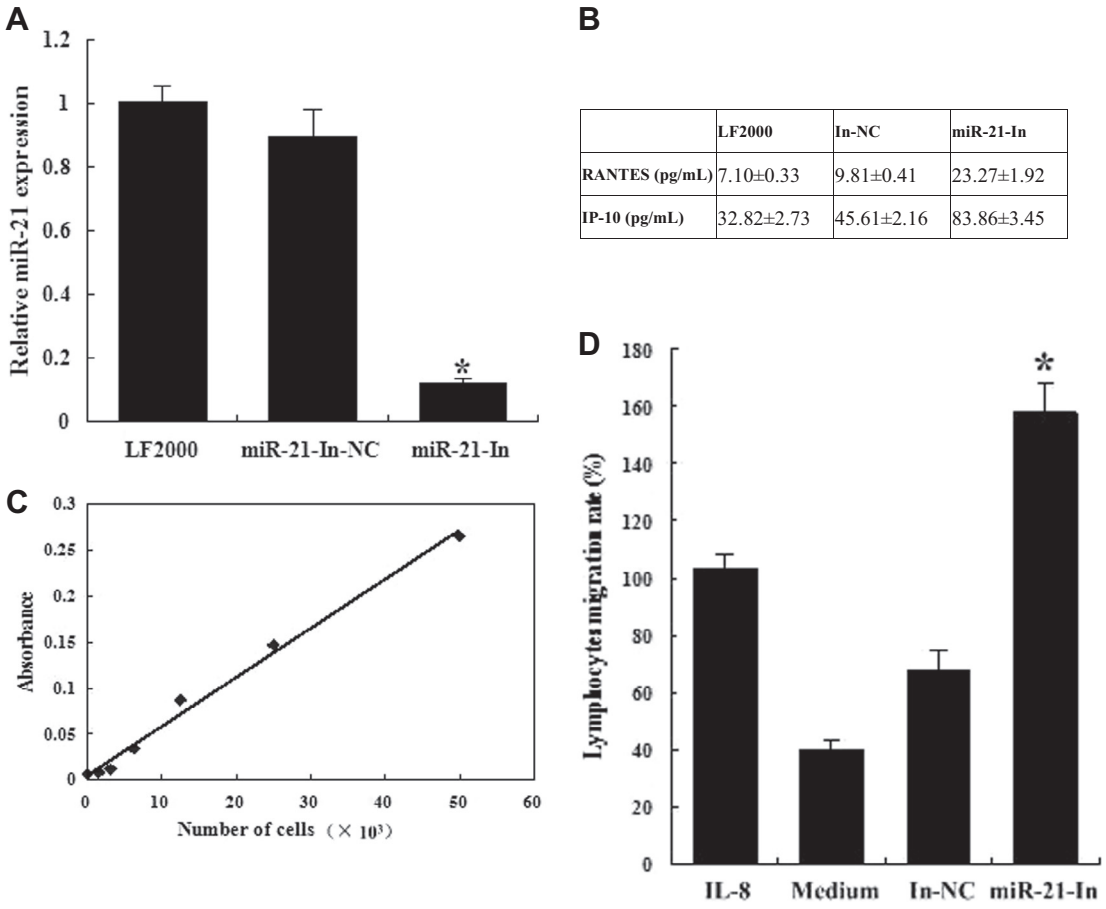
2.7. Statistical analysis

All values are expressed as the mean ± SEM. A two-tailed student's *t*-test was used to evaluate the significance of differences between two groups. The statistical significance level was set to $p < 0.05$.

3. Results and discussion

3.1. miRNA-21 inhibition enhances RANTES and IP-10 release and lymphocyte migration

miRNA-21 is overexpressed in various tumours and has been reported to be associated with multiple cancer-related processes including proliferation, apoptosis, invasion and metastasis, and chemoresistance [14–18]. In a recent study, miRNA-21 was found to suppress T cell priming and impair responses triggered by BCG vaccination by targeting IL-12 and Bcl-2 [26]. To further address the possible biological function of miRNA-21 in regulating chemokine expression and immune cell migration, we used a synthesised miRNA-21 inhibitor to downregulate miRNA-21 in the human breast cancer cell line MCF-7. As shown in Fig. 1A, transfection of the miRNA-21 inhibitor reduced miRNA-21 levels more than 80% compared with untreated MCF-7 cells or cells transfected with a negative control. miRNA-21 inhibition significantly increased secreted protein levels of chemokines RANTES and IP-10 (Fig. 1B), which are best known as T cell chemoattractants capable of stimulating the migration of multiple immune cell types [27,28].



	LF2000	In-NC	miR-21-In
RANTES (pg/mL)	7.10±0.33	9.81±0.41	23.27±1.92
IP-10 (pg/mL)	32.82±2.73	45.61±2.16	83.86±3.45

Fig. 1. Effect of miR-21 inhibition on MCF-7 cells. (A) Real time RT-PCR analysis of miR-21 expression levels in MCF-7 cells transfected with miR-21 inhibitor or negative control. U6 snRNA was used as an internal standard. Data were shown as a ratio of miR-21 inhibitor transfected cells to control transfected cells. (B) The concentration of secreted RANTES and IP-10 was measured by ELISA of miR-21-inhibitor transfected MCF-7 cell supernatant. RANTES and IP-10 concentrations are shown in pg/ml CM from 5×10^5 MCF-7 cells. (C)(D) Lymphocyte migration induced by CM from miR-21 inhibitor transfected MCF-7 cells as determined by CCK-8. (C) The established standard curve for absorbance and 2-fold dilutions of lymphocytes. (D) Quantification of lymphocyte migration induced by CM from MCF-7 cells transfected with miR-21-inhibitor. Bars represent the mean ± SD of three independent experiments; $^*p < 0.05$.

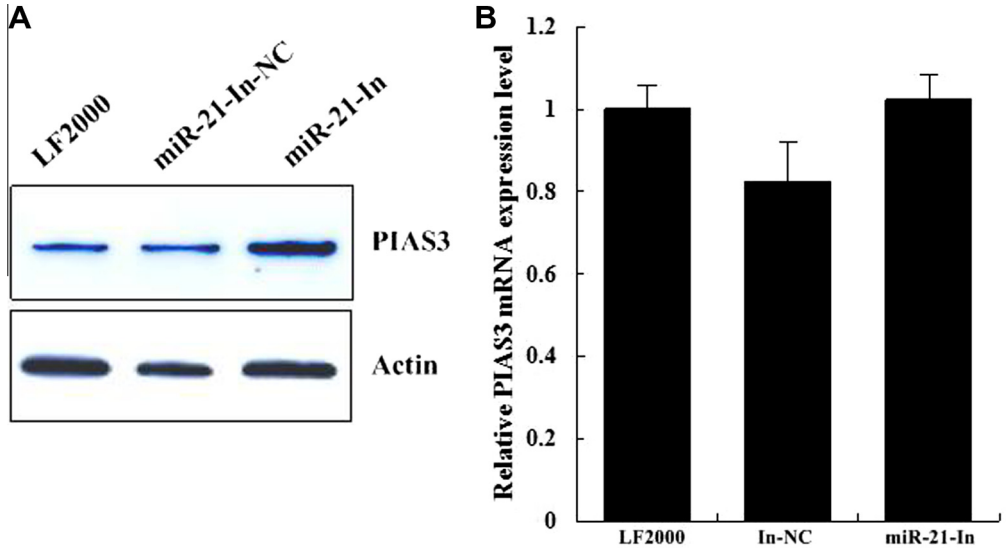
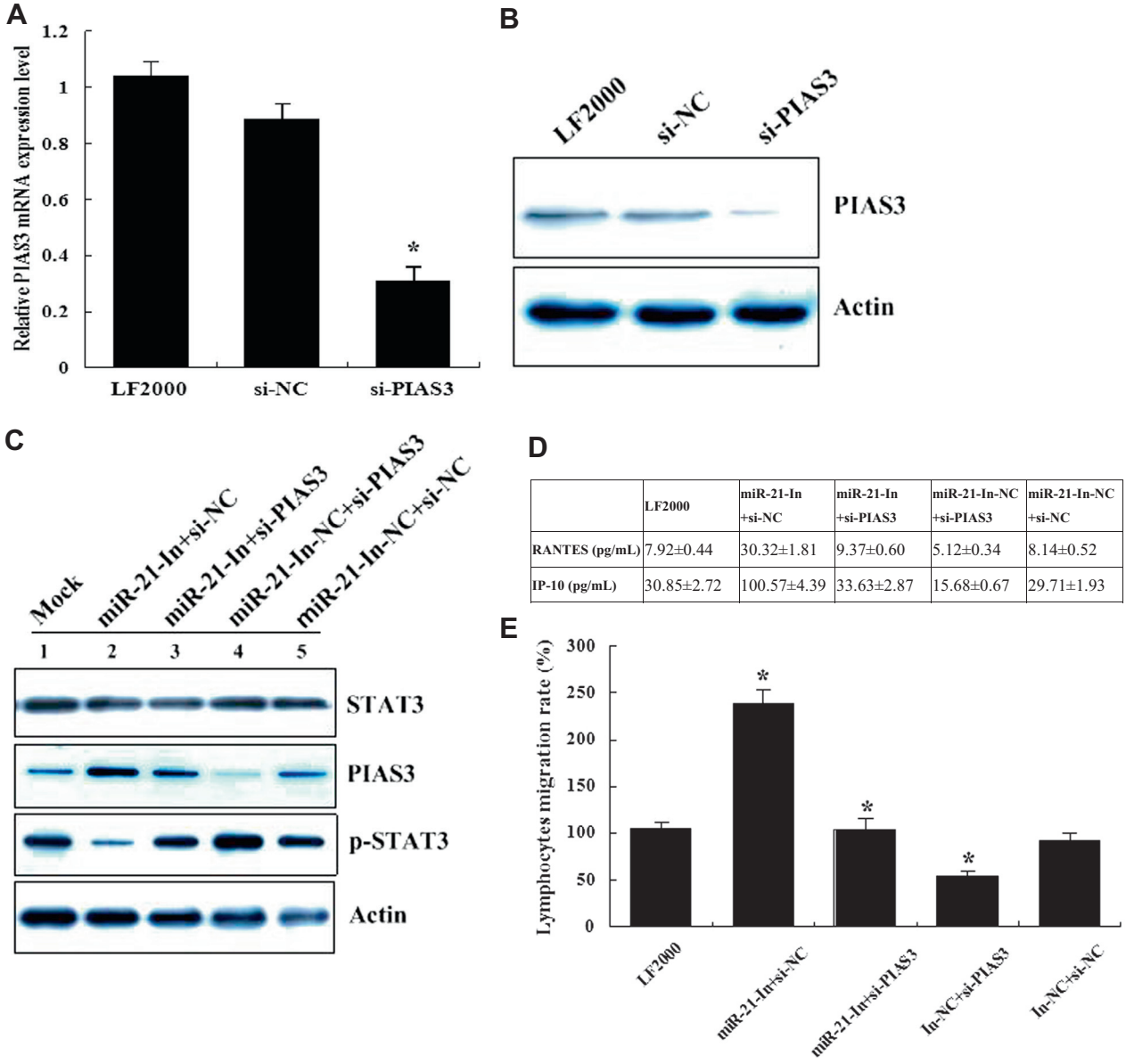


Fig. 2. miR-21 negatively regulates PIAS3 protein expression in MCF-7 cells. (A) MCF-7 cell lysates were prepared and western blot of PIAS3 was performed 72 h post transfection. miR-21 inhibition increased the expression of PIAS3 protein. (B) Real time RT-PCR of PIAS3 mRNA in MCF-7 cells transfected with miR-21 inhibitor. Data were normalised to *GAPDH* expression.

To assess whether secreted RANTES and IP-10 following miRNA-21 inhibition could attract lymphocytes, in vitro migration assays were performed. A cell chemotaxis assay demonstrated enhanced lymphocyte migration towards conditioned medium from MCF-7



	LF2000	miR-21-In +si-NC	miR-21-In +si-PIAS3	miR-21-In-NC +si-PIAS3	miR-21-In-NC +si-NC
RANTES (pg/mL)	7.92±0.44	30.32±1.81	9.37±0.60	5.12±0.34	8.14±0.52
IP-10 (pg/mL)	30.85±2.72	100.57±4.39	33.63±2.87	15.68±0.67	29.71±1.93

Fig. 3. PIAS3 siRNA partially reverses the effects of miR-21 inhibition on MCF-7 cells. (A) Real time RT-PCR of PIAS3 mRNA expression 48 h after transfection of MCF-7 cells with PIAS3 siRNA or si-NC. (B) Western blot of PIAS3 expression 72 h after transfection of MCF-7 cells with PIAS3 siRNA. (C) miR-21 inhibition decreased phosphorylated STAT3 in MCF-7 cells and PIAS3 siRNA reversed the effect of miR-21 inhibition on STAT3 phosphorylation. Cell lysates were analysed for PIAS3 and total and phosphorylated STAT3. (D)(E) Silencing PIAS3 partially abrogated miRNA-21 inhibitor induced (D) soluble chemoattractant expression and (E) lymphocyte migration. Bars represent the mean ± SD of three independent experiments; $^{*}p < 0.05$.

cells transfected with the miRNA-21 inhibitor compared with cells transfected with the negative control or untreated cells. Quantitative analysis indicated that the total number of lymphocytes in the conditioned medium from MCF-7 cells transfected with the miRNA-21 inhibitor was significantly higher (p <0.05; Fig. 1C and D) than in the negative control transfected conditioned medium. These data demonstrate for the first time the properties of miRNA-21 in regulating expression of tumour cell chemoattractants and induced migration of immune cells.

3.2. PIAS3 is a target of miRNA-21 in MCF-7 breast cancer cells

Next, we investigated the molecular mechanism by which miRNA-21 may regulate chemokine expression. miRNA-21 is an oncogenic miRNA that can be detected in various tumours. Previous studies have demonstrated that the target genes of miRNA-21 include programmed cell death 4 (PDCD4) [14], tissue inhibitor of metalloproteinase 3 (TIMP3) [15], reversion-inducing cysteine-rich protein with kazal motifs (RECK) [15], myristoylated alanine-rich c-kinase substrate (MARCKS) [17], tensin homolog deleted on chromosome 10 (PTEN) [18], ras homolog family member B (RhoB) [29], SWI/SNF related, matrix associated, actin dependent regulator of chromatin subfamily a member 4 (SMARCA4) and acidic (leucine-rich) nuclear phosphoprotein 32 family member A (ANP32A) [30]. We hypothesise that miRNA-21 could exert its activity by targeting PIAS3, a negative regulator of STAT3 activation, a process that is involved in immune cell recruitment. PIAS3 has been shown to be targeted by miRNA-21 in myeloma cells to regulate cell growth, cell cycle and apoptosis [20]. Whether regulation of PIAS3 induced by miRNA-21 is involved in chemoattractant expression and immune cell migration remains unclear. To determine whether miRNA-21 can regulate PIAS3 expression in human

breast cancer cells, we transfected the miRNA-21 inhibitor into MCF-7 cells and assessed PIAS3 protein and mRNA expression by immunoblotting and qRT-PCR, respectively. Compared with control MCF-7 cells, the level of PIAS3 protein expression in MCF-7 cells transfected with miRNA-21 inhibitor significantly increased (Fig. 2A), although no significant change was observed in PIAS3 mRNA expression levels (Fig. 2B). These results suggest that PIAS3 was negatively regulated by miRNA-21 in MCF-7 cells mainly through translational inhibition rather than mRNA degradation. Our data provide further evidence that PIAS3 is a target of miRNA-21 and support a recent report that shows miRNA-21 enhances myeloma cell growth and apoptosis by directly regulating PIAS3 [20].

3.3. PIAS3 siRNA reverses the effects of miRNA-21 inhibitor on STAT3 phosphorylation, chemokine expression and lymphocyte migration

Downregulation of miRNA-21 expression in MCF-7 cells could facilitate upregulation of PIAS3 and other potential phenotype-related miRNA-21 target molecules, thus promoting MCF-7 chemokine release. To investigate whether PIAS3 knockdown could reverse the effect of miRNA-21 inhibitor on STAT3 activation, chemokine expression and lymphocyte migration, MCF-7 cells were cotransfected with the miRNA-21 inhibitor and PIAS3 siRNA to inhibit miRNA-21 and PIAS3 expression. Transfection with siRNA against PIAS3 resulted in a significant reduction in both PIAS3 mRNA and protein expression (Fig. 3A and B). As shown in Fig. 3C, a significant decrease in STAT3-pTyr705 was observed in miRNA-21 inhibitor transfected cells (lane 2) relative to the negative control, while an increase in STAT3-pTyr705 was seen in MCF-7 cells cotransfected with PIAS3 siRNA and miRNA-21 inhibitor when compared to cells transfected with only the miRNA-21 inhibitor (lane 3). Furthermore, cotransfection of MCF-7 cells with PIAS3 siRNA and miRNA-21 inhibitor partially abrogated miRNA-21 inhibitor induced soluble RANTES and IP-10 expression and lymphocyte migration (Fig. 3D and E). We further investigated the role of STAT3 in chemokine expression and lymphocyte migration in MCF-7 by RNAi. Western blotting confirmed a reduction in the expression levels of STAT3 and phospho-STAT3 (pY705) proteins in STAT3 siRNA transfected MCF-7 cells (Fig. S1A, Supporting Information). As shown in Figure S1B, suppression of STAT3 led to an elevated expression of chemoattractants RANTES and IP-10 in MCF-7 and an increased migration of lymphocytes (Fig. S1C), consistent with previous studies. Taken together, these data demonstrate for the first time that miRNA-21 inhibits RANTES and IP-10 release in MCF-7 at least in part by directly regulating the expression of PIAS3. The role of miRNA-21 in regulating immune cell infiltration of tumours in vivo is the subject of further investigation.

In the present study, we found that downregulation of miRNA-21 enhances chemokine release in MCF-7 human breast cancer cells and leads to increased lymphocyte migration. A previous report identified PIAS3, a protein inhibitor of activated STAT3, as a direct target of miRNA-21, and a positive reciprocal regulation of miRNA-21 and STAT3 has been suggested in U266 myeloma cells [20]. Here, we confirmed that PIAS3 is a target of miRNA-21 in MCF-7 cells. This PIAS3 targeting at least partially explains the function of miRNA-21 in regulating chemokine expression and immune cell migration.

Because STAT3 has been predicted to be a target of miRNA-21 [19], we further tested whether miRNA-21 directly modulates STAT3 protein expression. We found no significant change of mRNA expression or total STAT3 protein expression in MCF-7 cells transfected with either miRNA-21 mimics or inhibitor compared to control transfected cells (data not shown). In contrast, miRNA-21 inhibition significantly decreased phosphorylated STAT3 protein levels. Taken together, these findings indicate that miRNA-21 targets PIAS3 and STAT3 phosphorylation but does not directly regulate STAT3 protein expression in MCF-7 cells. The increased RANTES and IP-10 expression levels and lymphocyte migration observed in MCF-7 cells cotransfected with miRNA-21 inhibitor and STAT3 siRNA compared to cells transfected with only a miRNA-21 inhibitor or STAT3 siRNA were of particular interest and suggested a positive reciprocal relationship between STAT3 and miRNA-21 for regulation of chemokine expression. This relationship is consistent with results from previous studies [20,31].

The STAT3 signalling pathway is known to affect tumour infiltration by immune effectors, promote expression of many immunosuppressive factors, and exert immune suppressive effects. miRNA-21 is an oncogenic miRNA that can be detected in various tumours and has emerged as a key regulator of oncogenic processes as well as drug resistance. The positive reciprocal relationship between STAT3 and miRNA-21 could aggravate immune suppression in the tumour microenvironment, thus leading to immunoresistance to cancer immunotherapy [32]. Recent advances in miRNA delivery and targeting suggest that miRNA replacement therapy with miRNA-21 may be a feasible supplementary approach to traditional cancer immunotherapy [33].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.072.

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