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Annexin-A1 controls an ERK-RhoA–NFκB activation loop in breast cancer cells

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ABSTRACT

Wound healing is critical for normal development and pathological processes including cancer cell metastasis. MAPK, Rho-GTPases and NFκB are important regulators of wound healing, but mechanisms for their integration are incompletely understood. Annexin-A1 (ANXA1) is upregulated in invasive breast cancer cells resulting in constitutive activation of NFκB. We show here that silencing ANXA1 increases the formation of stress fibers and focal adhesions, which may inhibit wound healing. ANXA1 regulated wound healing is dependent on the activation of ERK1/2. ANXA1 increases the activation of RhoA, which is dependent on ERK activation. Furthermore, active RhoA is important in NF-κB activation, where constitutively active RhoA potentiates NFκB activation, while dominant negative RhoA inhibits NFκB activation in response to CXCL12 stimulation and active MEKK plasmids. These findings establish a central role for ANXA1 in the cell migration through the activation of NFκB, ERK1/2 and RhoA.

1. Introduction

The high mortality and morbidity rates of breast cancer is predominantly contributed by the development of distant metastases in secondary sites [1]. An increase in motility or migratory capacity of tumor cells serves as a tangible signal for metastatic progression. The microenvironment of secondary metastases is often characterized by enriched localized secretion of chemokines [2]. Therefore, chemokines can play a crucial role in tumor progression by mediating tumor metastasis and growth [3]. Specifically, the stromal cell-derived factor-1α (SDF1α, or CXCL12) modulates site specific metastasis of breast, liver and lung cancer [4]. Its receptor CXCR4 is highly expressed in metastatic breast cancer cells, while CXCL12 is expressed in organs representing the main sites of breast cancer metastasis, such as bone and lung [5].

The aberrant activation of NF-κB has been observed in various human cancers including breast cancer [6,7]. The activation of NF-κB is associated with an increased expression of chemokine receptors including CXCR4 in metastatic tumors [6]. The Rho family of GTPases, which include Rac, Cdc42, and Rho has been shown to regulate cell cycle progression, apoptosis/survival, morphology, cell polarity, cell adhesion, and membrane trafficking [8]. Overexpression of either RhoA itself or its upstream or downstream signaling elements have been detected in several human tumors, including breast cancer, where levels of RhoA correlate with level of aggressiveness [9]. RhoA can also affect gene expression through activation of transcription factors such as NF-κB [10]. The integration of Rho family GTPase and extracellular signal-regulated kinase (ERK) signaling is important for cell motility. ERK can promote Rho-dependent focal adhesion formation [11], and both pathways have cross talks in cell migration [12] while Rho kinase inhibitors can target both Rho-ROCK and ERK signaling pathways [13]. In addition, ERK signaling has been implicated in the disassembly of focal adhesions [14]. Aberrant expression of Annexin-1 (ANXA1) has been reported in multiple cancer types [15]. Levels of ANXA1 are highly upregulated in basal cell or triple negative carcinomas of the breast [16]. We have recently shown that ANXA1 regulates NF-κB activation and enhanced metastatic capability in metastatic breast cancer cells [17]. ANXA1 has been implicated in a variety of physiological

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events including apoptosis, cell proliferation, cell transformation and membrane trafficking [18–20], and regulation of the actin cytoskeleton, which is central for cell migration [21–23]. Furthermore, ANXA1 is a modulator of the MAP kinase pathway, and has been shown to modulate ERK [24,25] and p38 and JNK [26] activation. However, whether and how ANXA1 is involved in Rho-regulated cell migration is unknown.

An enhanced understanding of the signal transduction pathways underlying cancer migration is an important step towards identifying novel targets for therapy and prevention of tumor metastasis. In the present study, we tested the hypothesis that the highly invasive phenotype of breast cancer cells was mediated, at least in part, through a highly active RhoA-ERK–NF–κB pathway, through the induction of ANXA1.

2. Material and methods

2.1. Cell lines and culture

Experimental cultures of the MCF-7 breast cancer cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD) and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (GIBCO Laboratories) and 1% Penicillin-streptomycin (GIBCO Laboratories) at 37 °C in a 5% CO_2. Cells were split one day prior to any treatment to ensure log phase growth.

2.2. Wound healing assay

Cells were seeded in 24 well plates and allowed to adhere overnight. A scratch was performed using a white tip and was viewed under a microscope (Olympus IX81, 10× magnification) at specific time points after the scratch. The extent of wound closure was measured using Image J software at three different points along the chosen field of view. Percentage gap closure per field of view was calculated using the average gap distance per field of view at the respective time intervals divided by the average gap distance of that field of view at 0 h.

2.3. Western blot

Equal amount of samples were subjected to 15% SDS-PAGE electrophoresis and then electrophoretically transferred to nitrocellulose membranes (Bio-Rad Laboratories) using a wet transfer apparatus (Bio-Rad Laboratories). The membranes were exposed to appropriate primary antibodies overnight at 4 °C, washed and incubated with the appropriate horseradish peroxidase-labeled secondary antibodies for 1 h at room temperature. The antibody-reactive bands were revealed by chemiluminescence based detection using West Pico Substrate (Pierce Biotechnology). Protein expression was normalized to actin or tubulin levels. Antibodies against phospho-p38, p38, phospho-MEK, MEK, phospho-ERK1/2, ERK1/2, ROCK were purchased from Cell Signaling (Danvers, MA). Antibodies against phospho-AKT, AKT, FAK, RhoA, paxillin and actin, as well as Bay 11-7082 (NF-κB inhibitor) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.4. Luciferase assays

Cells were transfected with 100 ng of NF-kB luciferase reporter plasmid (Stratagene, La Jolla, CA, USA), and 10 pg of Renilla plasmid in serum-free DMEM medium. Luciferase activity was measured using dual luciferase reporter kit from Promega (Madison, WI, USA). The results are expressed as relative NF-kB activity compared to controls after normalizing for Renilla activity.

2.5. Rho activity assay

Cells were harvested and lysed in RBD lysis buffer. The lysates were clarified by centrifuging at 13,000 g, 4 °C for 10 min. Fusion proteins were added to the samples and tumbled at 4 °C for 45 min before bound fusion proteins were collected. Pull-down RhoA proteins were eluted and heated at 100 °C for 5 min. RBD samples were probed with primary anti-RhoA antibody to detect pulldown level of active RhoA, while the input lysate proteins were probed for total RhoA protein levels and actin levels.

2.6. Immunofluoresence microscopy

Cells were seeded on coverslips in a six-well plate and stained for immunofluorescence using confocal fluorescence microscopy. Images were collected using a Leica microscope equipped with a 63× lens. Focal adhesions and Stress fiber density were analyzed using NIH Image J 1.42q by first converting the image to 8-bit grayscale and subtracting the background. Focal adhesions per cell were obtained by creating a region of interest around the cell and analyzing and counting particles over a certain size (2 μm) Stress fibers were quantified by drawing a line perpendicular to the stress fibers and a plot profile was generated. The number of peaks was counted and plotted against μm measured. Stress fiber density was normalized to number of stress fibers/μm.

2.7. Statistical analysis

Data are represented as mean ± SEM of independent experiments. Statistical analysis was performed using ANOVA (single factor) and student's T-test (two-tailed). P values below 0.05 were considered significant.

3. Results

3.1. ANXA1 is required for stress fiber and focal adhesion formation

We first investigated if ANXA1 was involved in focal adhesion formation, which is important for directed cell migration. Murine mammary cells obtained from ANXA1 deficient (−/−) or ANXA1 heterozygous (+/−) mice, previously described in Ref. [27] were stained with vinculin and f-actin (Fig. 1A,B). We show that a two-fold higher number of focal adhesions are formed in ANXA1 −/− vs ANXA1 +/− cells (Fig. 1C). In addition, ANXA1 −/− cells exhibit significantly higher stress fiber formation in untreated conditions (Fig. 1D). Stress fiber density was quantified per cell and ANXA1 −/− macrophages appear to have more stress fibres per cell than ANXA1 +/− cells (Fig. 1E,F). To confirm this in human breast cancer cells, MCF-7 cells were either transfected with a scrambled or ANXA1 siRNA and stained with paxillin, another focal adhesion protein (Fig. 2A,B). Once again, MCF-7 cells silenced for ANXA1 exhibited more focal adhesions when compared to scrambled siRNA transfected MCF7 cells. This data indicate that ANXA1 may regulate stress fiber and focal adhesion formation, and a high expression of ANXA1 results in lower focal adhesions.

3.2. ANXA1 enhances wound healing and inhibits proliferation in breast cancer cells

As lower focal adhesions may lead to faster migration ability, we confirmed that overexpression of ANXA1 would be necessary for the wound healing of MCF7 cells to CXCL12. In accordance to our

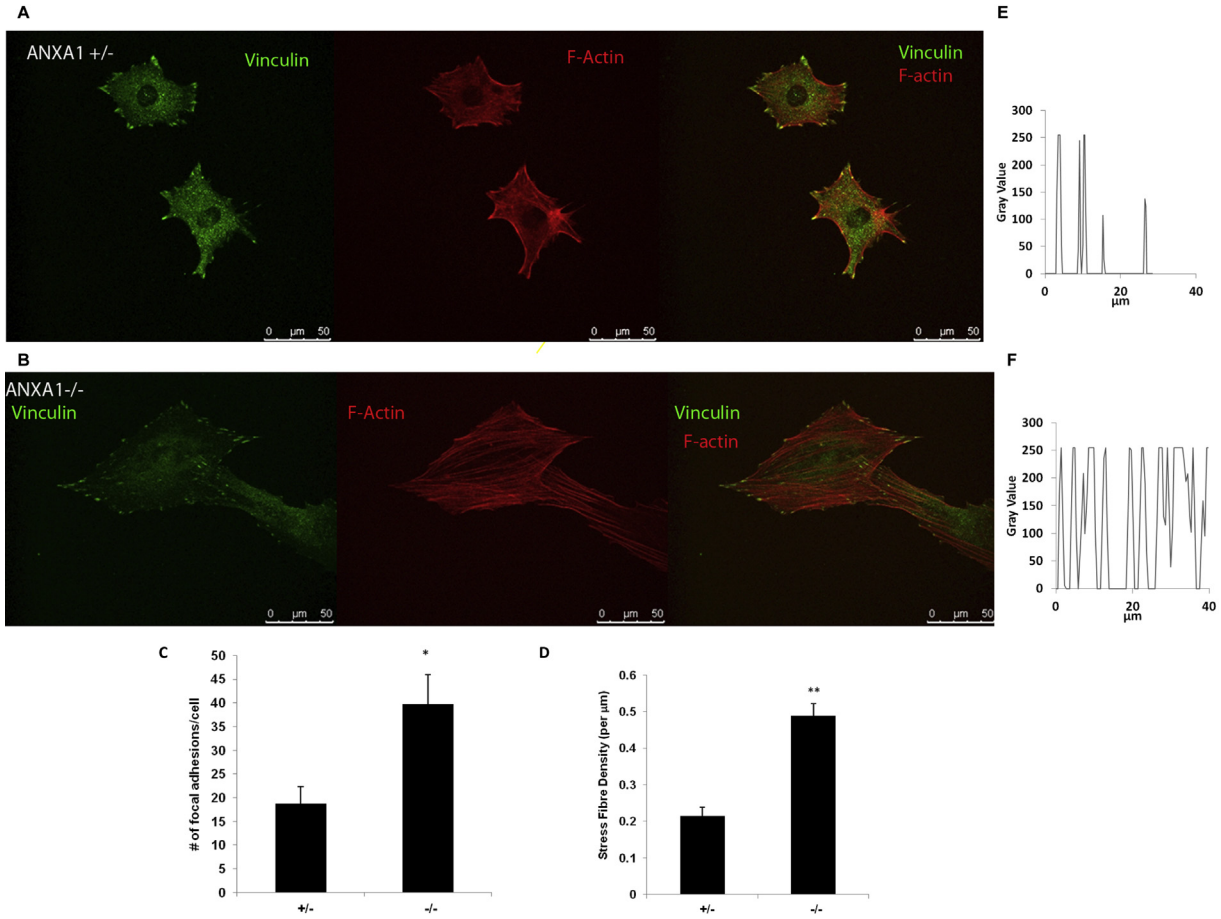

Fig. 1. ANXA1 deficient murine mammary cells exhibit higher focal adhesions and stress fiber formation. ANXA1 heterozygous (+/−) or deficient (−/−) mouse mammary gland cells were fixed and stained with the indicated antibodies (vinculin, f-actin). 2 separate fields of view are shown for (A) ANXA1 +/− and (B) −/− cells. Magnification 630×, Scale bar 50 μm. All image exposures were enhanced 1.25×. C. Number of focal adhesions was measured per cell (average of 3–5 cells). D. Stress fiber density was measured as described in the materials and methods with examples for (E) ANXA1 +/− and (F) −/− represented. *p < 0.05 vs +/− cells.

published results [17], control MCF-7 cells did not respond well to CXCL12 induction (Fig. 3A). However, MCF-7 cells stably overexpressing ANXA1 (pcDNA3.1-A1) displayed a significantly higher degree of wound closure (53 ± 4.5%, Fig. 3A). In contrast, MDA-MB231 cells exhibited high wound healing capacity in response to CXCL12 and siRNA silencing of ANXA1, which we have previously shown to be highly expressed in MDA-MB231 cells, resulted in an inhibition of CXCL12-induced wound healing (Fig. 3B). These results confirm our previous data and shows that ANXA1 can promote migration and/or wound healing in breast cancer cells.

To assess if ERK could be involved in CXCL12-induced migration, pcDNA3.1 control and pcDNA3.1-A1 MCF7 cells were pretreated with U0126, a selective inhibitor of MEK1/2. Treatment with U0126 resulted in a significant inhibition of wound closure, particular in pcDNA3.1-A1 cells, indicating that enhancement of migration by ANXA1 is ERK1/2 dependent (Fig. 3C). Similarly, treatment with U0126 in control ANXA1-siRNA silenced MDA-MB231 cells resulted in a complete inhibition of wound closure. This data demonstrates that ANXA1 enhances cell migration capacity and this is regulated by ERK1/2 activation.

As both migration and proliferation contributes to wound healing, we next examined if ANXA1 could regulate the proliferation of MCF-7 cells. Stable transfection of MCF-7 cells with an ANXA1 expression plasmid resulted in a corresponding reduction in proliferation, suggesting that ANXA1 plays an anti-proliferative role in MCF7 cells (Fig. 3E). Conversely, silencing ANXA1 using siRNA in MCF-7 cells (which already express low levels of ANXA1) resulted in a slight but significant increase in proliferation (Fig. 3F). These results demonstrate that although ANXA1 has pro-migratory properties, it is also anti-proliferative, suggesting that the observed effect of ANXA1 in enhancing wound healing is due to a higher migratory capacity.

3.3. ANXA1 regulates CXCL12-induced ERK and Rho activation

We next assessed if ERK1/2 phosphorylation induced by CXCL12 could be regulated by ANXA1. After MCF7 cells were stimulated with CXCL12, a biphasic trend of ERK1/2 phosphorylation was observed (Fig. 4A). However, in MCF7 cells overexpressing ANXA1, a sustained activation of ERK1/2 was observed after CXCL12 stimulation. This suggests that high levels of ANXA1 may induce a sustained ERK activation in response to CXCL12. This observation is specific to ERK1/2 as no change in p38 activation was observed in MCF7 or MCF7-A1 cells after CXCL12 treatment.

We next explored the role of ANXA1 in the regulation of Rho/Rac, which are important signaling molecules in cell migration and focal adhesion formation. The Rho GTPase GST-RBD assay was performed, to which only the active GTP bound forms of Rho and Rac/Cdc42 specifically bind. Basal and CXCL12-stimulated RhoA activation was substantially higher in cells stably overexpressing ANXA1 when compared to MCF7 cells (Fig. 4B). We investigated if this increase in RhoA activation by ANXA1 was mediated by ERK.

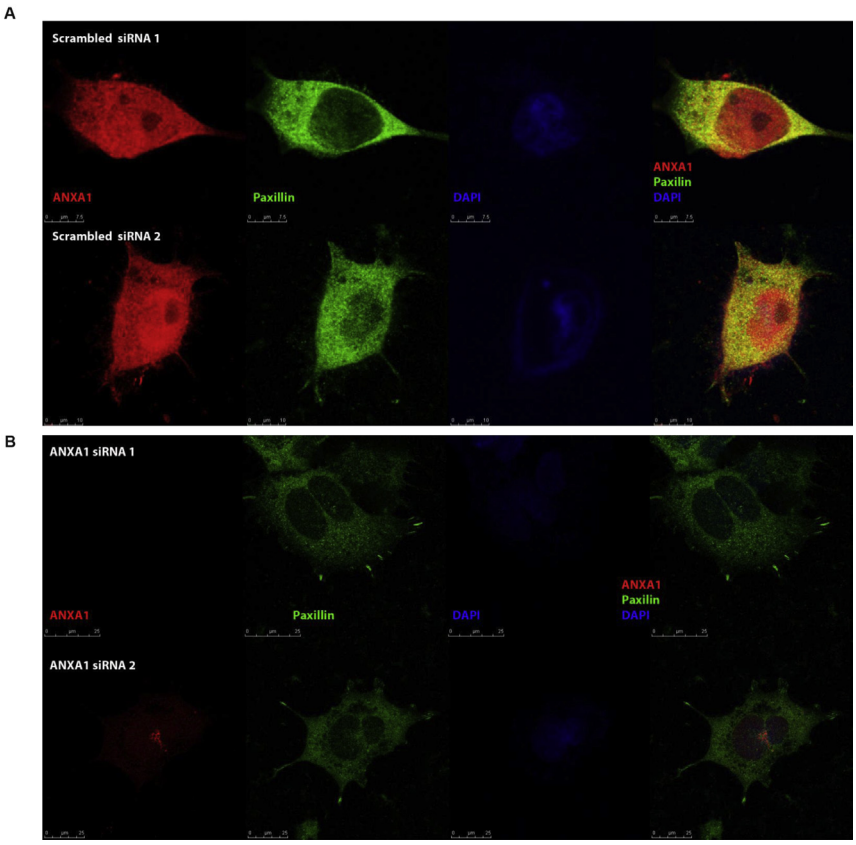

Fig. 2. Silencing ANXA1 enhances focal adhesion formation. (A) MCF7 cells transfected with scramble siRNA or (B) ANXA1 siRNA and stained with antibodies against the indicated antibodies (ANXA1, Paxillin, Dapi). Magnification 630×, Scale bar 7.5 μm.

Treatment of CXCL12-activated MCF7 cells with U0126 resulted in lower levels of active RhoA (Fig. 4B). However, MCF7-A1 cells were not responsive to UO126 treatment. This suggests that the active RhoA induced by ANXA1 may not be dependent on ERK.

3.4. ERK regulates ANXA1-dependent NF-κB activation

We previously demonstrated that ANXA1 promotes NF-κB activation in breast cancer cells [17]. We next investigated if ERK could regulate ANXA1-dependent NF-κB activation induced by CXCL12. MCF-7 cells stimulated with CXCL12 displayed minimal NF-κB activation, while MCF7-A1 cells, which we have previously shown to have higher levels of CXCR4, exhibited higher levels of NF-κB activation after CXCL12 stimulation (Fig. 4C). In addition, stimulation of MDA-MB231 cells with CXCL12 induced a significant enhancement in NF-κB activation (Fig. 4C). Treatment of cells with UO126 completely inhibited NF-κB activation induced by CXCL12 stimulation in MCF7-A1 and MDA-MB231 cells, indicating that CXCL12-induced NF-κB activation is dependent on ERK activation, and that NF-κB activation which is enhanced by ANXA1 is also dependent on the activation of ERK.

3.5. Rho enhances NF-κB activation in response to CXCL12

We next investigated if RhoGTPases were involved in CXCL12 induced NF-κB activation. In untreated MDA-MB231 cells, overexpression of RhoA and Rac increased NF-κB activation by 3-fold, while Cdc42 induced NF-κB activation by over 20-fold (Fig. 4D), indicating that CDC42 may be upstream of NF-κB. Stimulation of MCF7 cells with CXCL12 increased NF-κB activation, and overexpression of Rho and CDC42 in these cells resulted in a high level of NF-κB activation, but not Rac. This indicated to us that CXCL12-induced NF-κB activation is regulated by RhoA. To further confirm our findings whether CXCL12-induced RhoA was necessary for the increased NF-κB activity, MDA-231 were transiently transfected with a dominant-negative form of RhoA (RhoT17N). No significant change in NF-κB reporter activity was observed after transfection with the RhoT17N in control cells (Fig. 4E). In addition, cells transfected with RhoT17N and stimulated with CXCL12 did not exhibit significant NF-κB activation, indicating that CXCL12-induced NF-κB activation is mediated via RhoA.

Next, to determine if there is an interplay between RhoA and MEKK in NF-κB activation, we transfected MCF7 cells with MEKK WT, constitutively active MEKK R4F and dominant negative mutant MEKK 8E. MEKK WT and R4F constructs induced NF-κB activation, which was not observed with MEKK 8E mutant (Fig. 4F). Co-transfection of dominant negative RhoT17N construct inhibited MEKK WT induced NF-κB activation, indicating that Rho is upstream of MEKK and capable of regulating NF-κB activation induced by WT MEKK, but not its mutants which are already active or inhibited.

4. Discussion

Basal type mammary carcinomas such as MDA-MB231, which express high levels of constitutively active NF-κB, are highly invasive and usually ER-negative [28]. These cancer types have been shown to be express high levels of ANXA1, in this study and in previous studies [16,17], and we show here that RhoA and ERK activity also correlates with high levels of ANXA1. The upregulation of RhoA expression has been described in breast cancer, and is

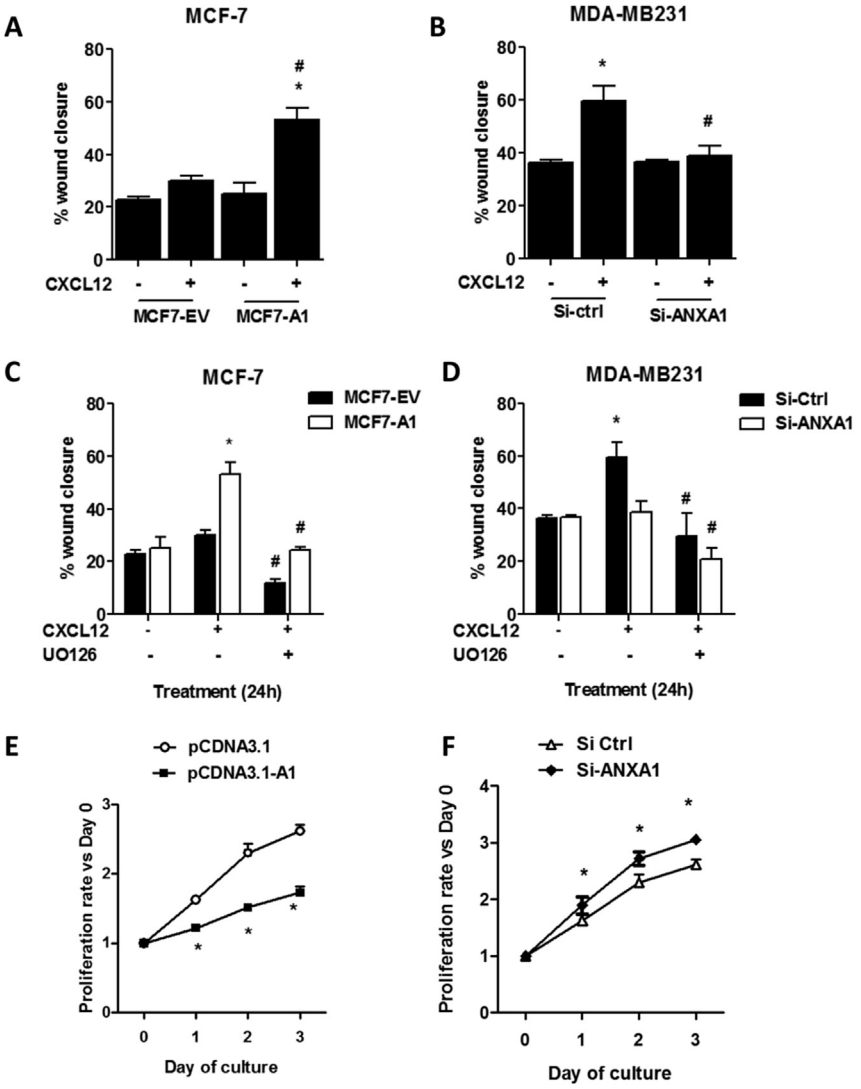

Fig. 3. CXCL12-induced wound healing is enhanced by ANXA1 and requires active ERK. (A) MCF7 and MCF7-ANXA1 stable overexpressing cells (A1) or (B) control −siRNA or ANXA1-siRNA transfected MDA-MB231 cells were co-treated with or without CXCL12 (100 ng/ml), and wound healing assay was performed. Percentage of wound closure was monitored at 24 h. (C) MCF7 and MCF7-ANXA1 stable overexpressing cells (A1) or (D) control −siRNA or ANXA1-siRNA transfected MDA-MB231 cells were co-treated with CXCL12 with or without U0126 (10 μM), and wound healing assay was performed as above. (E) MCF7 and MCF7-ANXA1 stable overexpressing cells (A1) or (F) control −siRNA or ANXA1-siRNA transfected MCF7 cells were allowed to grow for up to 3 days and proliferation was measured using Cell Titer Reagent and normalized to Day 0.

correlated with malignancy [29], while high levels of ERK expression and activation has been described in a number of cancers [30,31]. We have recently shown that ANXA1 can regulate NF-κB activity in breast cancer cells, which results in the modulation of cancer cell migration and metastasis [17]. Here we have evaluated this regulation further, where ERK signaling synergizes with RhoA and NF-κB activation.

The CXCL12/CXCR4 axis is important in site-specific metastasis in breast cancer [4], where CXCR4 is highly expressed on metastatic cells, and its ligand, CXCL12 is secreted in secondary sites of cancer metastasis [5]. We have previously shown that CXCL12 increases MCF-7 wound healing ability in MCF-7 cells, and even more so in MCF-7-A1 cells overexpressing ANXA1 [25]. CXCL12 stimulation resulted in a biphasic activation of ERK in MCF-7, while in MCF7-A1 cells, a sustained activation of ERK was observed, suggesting that ANXA1 can regulate CXCL12 induced ERK activation. CXCL12 activation has previously been shown to involve ERK signaling [32,33]. The sustained activation of ERK seen in MCF7-A1 and MDA-MB231 cells may be attributed to ZAP70, which is associated with enhanced ability to respond to migratory signals [34] [35] or SLP-76 scaffold protein [35]. It may be possible that ANXA1 may regulate these regulators to induce sustained activation of ERK.

Crosstalk of these pathways may suggest that the ERK/RhoA pathway may be a key modulator of NFKB-driven tumor metastasis. Although our finding that RhoA activation is dependent on Erk is novel, several studies have suggested that the two pathways can cross-talk and/or cooperate. Stimulation of cell migration induced by active MEK1 was totally blocked by either a dominant-negative RhoA or the ROCK inhibitor, Y-27632 [12]. However, inhibition of ERK activation did not affect the migration of cells induced by active RhoA. These results suggest that ERK is upstream of RhoA in inducing cell migration and are in agreement with our findings, yet we also show that dominant negative mutant of Rho can inhibit ERK-induced activation of NFKB, indicating that Rho can also act upstream of ERK.

Our recent studies using quantitative proteomics to study the role of ANXA1 in murine mammary gland cells demonstrated that ANXA1 −/− cells were more adhesive and less migratory when

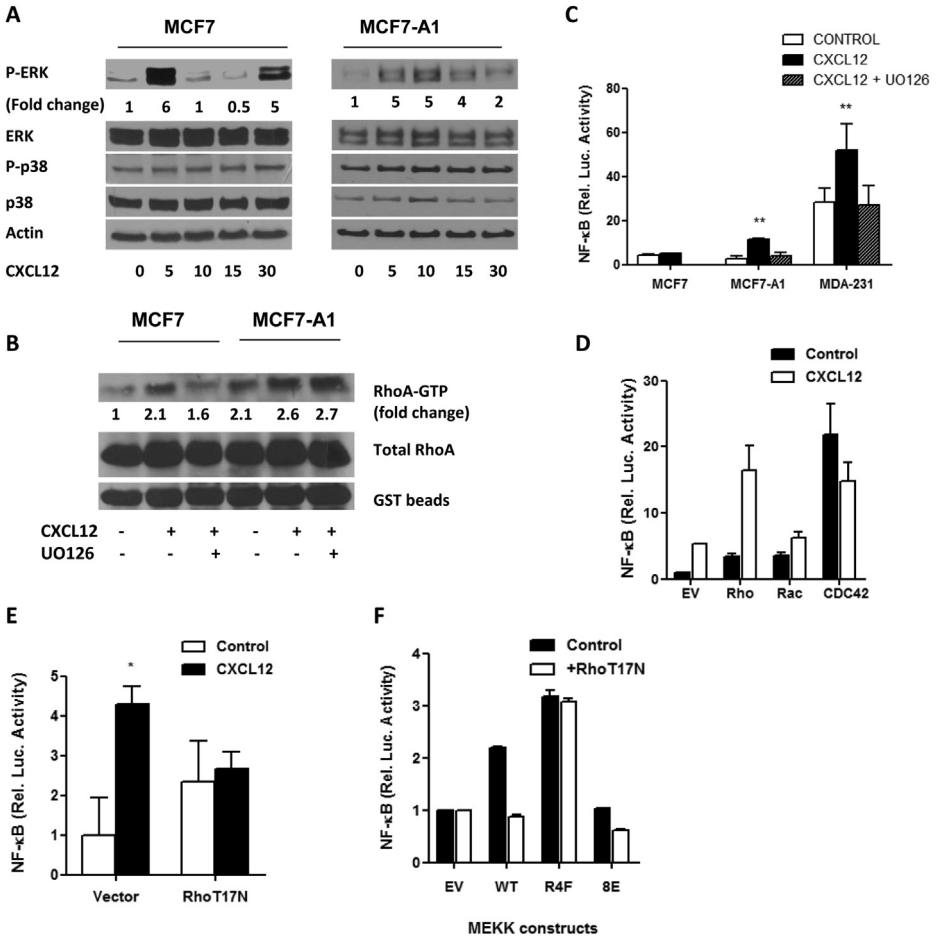

Fig. 4. ANXA1/ERK/RhoA regulate NF-κB activity in breast cancer cells. (A) Cells were incubated with CXCL12 (100 ng/ml) for indicated time intervals, and protein levels were determined by SDS-page immunoblotting. (B) Cells were incubated with CXCL12 for 24 h with or without UO126 inhibitor pretreatment and RhoA-GTP pull-down assay was performed (C). Cells were transfected with NF-κB-luciferase, renilla prior to treatment with CXCL12 and/or UO126 and NF-κB promoter activity was determined. $^{**}p < 0.01$ vs control. (D–F). Cells were co-transfected with NF-κB-luciferase, renilla and the indicated expression plasmids and NF-κB promoter activity was determined. Cells were treated with CXCL12 (100 ng/ml) was used for 6 h. $^{*}p < 0.05$ vs vector control.

compared to ANXA1 +/− cells [27]. This is in line with this present study, where ANXA1 −/− cells were shown to have more focal adhesions. We have confirmed this with MCF7 cells, where silencing ANXA1 also resulted in higher focal adhesions. ANXA1 negatively regulated adhesive proteins and focal adhesion proteins such as parvin and integrin α11 (*ITGN11*), as well as collagens (*Col1a1*, *Col5a2*) [27]. This could explain why cells lacking ANXA1 are less migratory and possess less wound healing abilities due to their high adhesive characteristics.

In summary, we show that ANXA1 inhibits focal adhesion formation. We also show that ANXA1 enhances wound healing in response to CXCL12. This is a result of enhanced ERK, RhoA and NFκB activation.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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