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RelA driven co-expression of CXCL13 and CXCR5 is governed by a multifaceted transcriptional program regulating breast cancer progression

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ABSTRACT

Lethal metastasis of primary breast tumors to lymph nodes has been found to be associated with the co-expression of chemokine CXCL13 and its receptor CXCR5. To date, however, the precise molecular events regulating the co-expression of CXCL13 and CXCR5 in the context of breast cancer progression have not been identified. Therefore, to extend our understanding of the drivers of breast cancer metastasis, we undertook a line of investigation in this study in which we demonstrate that the transcriptional regulation of CXCL13 is mediated by the reciprocal activity of RelA and Nrf2, while CXCR5 is transcriptionally silenced by CpG island methylation within its promoter. Critically, we show that intra-tumoral CXCL13 and CXCR5 mRNA expression is positively correlated with intra-tumoral RelA expression within the primary tumor of breast cancer (BCa) patients (n = 98). We demonstrate a role for Nrf2 in the negative transcriptional regulation of cxcl13. Furthermore, using a luciferase assay and deletion analysis of the cxcl13 gene promoter, we demonstrate that RelA and Nrf2 directly act upon the cxcl13 promoter to regulate transcription. Chromatin immunoprecipitation PCR, supported by *in silico* docking analyses, confirmed that RelA and Nrf2 both occupy multiple positions within the cxcl13 promoter. Collectively, in RelA high conditions, low Nrf2 and lack of cxcr5 promoter DNA-methylation govern CXCL13-CXCR5 co-expression within breast tumors, and thus drive disease progression and metastasis.

1. Introduction

The high mortality rate of breast cancer (BCa) is mainly due to tumor cell dissemination from the primary lesion, leading to metastatic disease [1]. Metastasis is largely driven by the activity of chemokines, which are chemo-attracting small cytokine molecules (8–10 kDa) [2,3]. CXCL13 is a widely-studied chemokine that functions through its receptor CXCR5 [4,5], and is known to promote downstream function through the activation of PI3K, Akt, and Src [6,7]. Crucially, CXCL13 participates in the processes of invasion and migration in various cancers [4,6,8]. Several cell types have been implicated as potential sources of CXCL13, namely follicular dendritic cells (fDCs) [9], macrophages [10], germinal center-T cells [11] as well as T follicular helper cells (TFH) [12]. Interestingly, it has been reported that primary breast tumor cells also secrete CXCL13 [13].

When CXCL13 and CXCR5 are co-expressed within breast tumors, this ligand-receptor pair forms an autocrine loop that favors epithelial-to-mesenchymal transition (EMT), elevated expression and activation of matrix metalloproteinase 9 (MMP9), which drives increased cellular migration and lymph node metastasis (LNM) [7]. Therefore, it is of extreme importance to unveil the molecular mechanisms that govern CXCL13-CXCR5 co-expression to broaden our understanding of the process of BCa metastasis.

CXCR5 expression is positively regulated by p65/RelA through its physical interaction with specific binding-sequences in the cxcr5 promoter [14]. Involvement of RelA subunit of NFκB in BCa progression has been well documented [15–18]. Direct inhibition of the NFκB-RelA activity in BCa cells induces apoptosis [17,19]. A recent report has shown that transcriptional repression of RelA by reducing nuclear phosphorylation of p65 and inhibition of NFκB signaling impedes BCa progression [20]. Moreover, RelA regulates transcription of different EMT-related genes during BCa progression [21], however the molecular events driving co-expression of cxcr5 and cxcl13 remain unknown. On the other hand, Nrf2 induces cellular stress signals and also regulates

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transcriptional activities of many genes by binding on to the anti-oxidant response element (ARE) within their promoters [22,23]. It has sufficiently been addressed in literature that Nrf2 is involved in the regulation of cellular growth, apoptosis and tumorigenesis [24,25]. Interestingly, whether Nrf2 could be considered as a tumor suppressor or an oncogene is debated [23], suggesting both pro-oncogenic and anti-oncogenic activities of Nrf2 in BCa.

In this work, we have identified a large CpG island within the cxcr5 promoter, which we show, upon methylation, negatively regulates transcription of this gene. Furthermore, through deletion analysis of the cxcl13 promoter, we identified binding sites of RelA and Nrf2 within the cxcl13 promoter, this occupancy was confirmed through ChIP-PCR analysis. Therefore, we have delineated the molecular events that regulate CXCL13-CXCR5 co-expression in BCa. Precisely, we revealed that RelA positively regulates gene-transcription of both cxcl13 and cxcr5, while transcription of each gene is negatively regulated independently; cxcl13 by Nrf2 and cxcr5 by promoter-DNA-methylation.

2. Materials and methods

2.1. Bioinformatics analyses

Promoter sequences for cxcl13 (GRCh37.p13:78432107-78433207) and cxcr5 (GRCh38.p7:118882966-118884066) were obtained from the official website of National Center for Biotechnology Information. Probable transcription factors (TFs) and their putative binding sites within the promoter regions were predicted using the online tool Mapper2.0.

2.2. Clinical samples

Primary tumor samples and associated healthy tissues were collected from 98 patients diagnosed with infiltrating duct carcinoma (IDC) of breast. Parts of freshly operated tissues were immediately processed for DNA, RNA and protein extraction, and rest of tissues was used for flow-cytometry. Clinicopathological data were collected from the data inventory of Saroj Gupta Cancer Centre & Research Institute (SGCC&RI). Approval was obtained from IEC of SGCC&RI [ECR/250/Inst/WB/2013] and informed consents were taken from patients.

2.3. Cell lines, culture and treatment

Human BCa cell lines, MDA-MB-231 and T47D were procured from National Centre for Cell Science (NCCS), India. Cells were cultured in DMEM (Invitrogen:#12800017), supplemented with 10% FBS (Gibco:#26140087), 100 U/mL penicillin and streptomycin (Gibco:#15140122), and maintained in a cell culture incubator at 37 °C with 5% CO_2. Both cell lines were authenticated by NCCS in 2017 using short-tandem repeat polymorphisms analysis. Cells were transfected using lipofectamine-3000™ (Invitrogen:#L3000015), according to manufacturer's recommendations. For indirect co-culture experiments, 24 well cell culture inserts (Falcon:#353095) of 0.4 μ pore size were used. According to combinations, T47D or MDA-MB-231 cells (5×10^4) were seeded in either lower or upper chamber. Proteins from cells of the lower chamber were extracted after 48 h. Conditioned medium were collected for performing Enzyme-linked immunosorbent assay (ELISA) of CXCL13. Experiments were performed in duplicate.

2.4. Molecular cloning and plasmid DNA preparation

Coding sequences of RelA, Nrf2 and CXCR5 were PCR-amplified from MDA-MB-231 cDNA and cloned in mammalian expression vector pcDNA3.1(+). Promoter region of cxcl13 along with nine deletion variants were PCR-amplified from genomic DNA and cloned in pGL3 basic reporter vector. Q5-high fidelity DNA polymerase [New England Biolabs (NEB):#M0491S] and specific primer pairs were used. Each cloned plasmid was confirmed by restriction digestion and PCR amplification. Cloned ORF of CXCL13 (Origene:#SC321077) was purchased and used for CXCL13-overexpression.

2.5. Genomic DNA isolation, RNA isolation, reverse-transcription and PCR

Genomic DNA was isolated using genomic DNA purification kit (Invitrogen:#K180001). RNA was extracted using TRIzol reagent (Invitrogen:#15596018) applying laboratory optimized protocol [7]. Total RNA was reverse-transcribed into cDNA using MMLV reverse transcriptase (Invitrogen:#28025013). PCR were performed to analyze mRNA expression and target amplification for cloning. End-point and qPCR were performed in triplicate using Taq-master mix (Xcelris:#XG334A) and SYBR-Green master mix (ThermoFisher:#4367659), respectively. 18S rRNA was used as internal control. Primer sequences are listed in Supplementary Table I. Fold changes in qPCR were calculated as relative values normalized to control and quantified in terms of $2_T^{-\Delta\Delta C}$.

2.6. Western blotting

Western blots were performed using denatured total proteins with standardized protocol [26]. Briefly, proteins were resolved by SDS-PAGE gels and immunoblotted in PVDF membranes (Millipore:#IPVH00010; PALL Corporation:#PVM020C-099) overnight at 4 °C with primary antibodies: anti-RelA (Cell Signaling Technology [CST]:#8242S), anti-Nrf2 (CST:#12721S), anti-CXCL13 (Abcam:#ab112521), anti-CXCR5 (Epitomics:#6558-1), anti-Src (CST:#2109S), anti-phospho-Src (CST:#6943S), and anti-β-actin (Abcam:#ab8227) followed by 2 h incubation with anti-rabbit HRP-conjugated secondary antibodies (CST:#7074S) at room temperature (RT). Bands were developed using luminol substrate.

2.7. Immunohistochemistry (IHC)

Immunohistochemistry for RelA were performed on formalin-fixed, paraffin-embedded tissue sections of 3–5 μM. Slides were de-paraffinized, rehydrated, blocked and incubated overnight with anti-RelA primary antibody (CST:#8242S) at 4 °C. HRP-conjugated secondary antibody (CST:#7074S) was added at 1:250 dilutions. Slides were developed using DAB chromogen (ThermoFisher:#34001), counterstained with hematoxylin (Merck), dehydrated and mounted.

2.8. Flow cytometry

Cultured MDA-MB-231 and T47D cells were harvested through detachment using trypsin-EDTA (Gibco:#25300054). Single cell suspensions (1×10^6) were incubated with PE-conjugated anti-CXCR5 (BioLegend:#356904) and APC-conjugated anti-CXCL13 (ThermoFisher:#MA5–23629) antibodies. Fc-blocker (BioLegend:#422301) was used to minimize non-specific antibody binding. Experiments were performed with appropriate controls. Data were acquired in BD-AccuriC6 and data were analyzed using FlowJo_v10 software.

2.9. ELISA

Sandwich ELISA (Ray Biotech:#ELH-BLC-1) was performed in triplicate for quantitative determination of CXCL13 protein with standardized protocol [26]. Briefly, a standard curve was prepared using serial dilution of human recombinant CXCL13 protein. Concentrations of CXCL13 in BCa cell line-lysates and culture-conditioned mediums were estimated using the standard curve. A biotinylated-detection antibody against CXCL13 was added to the wells. After further washings, HRP-conjugated streptavidin was added, followed by tetramethylbenzidine (TMB) substrate reagent. Lastly, stop solution was

added to the wells and absorbance of the colored solution was measured using an ELISA reader (ThermoFisher, MultiskanGo) at 450 nm. Sensitivity threshold of the test was 1.5 pg/mL.

2.10. Promoter DNA-methylation assay

Promoter region of cxcr5 (GRCh38.p7:118882966-118884066) encompasses a 305 nucleotide long CpG island, identified by Methyl Primer Express™ software. Methylation specific primers (MSP) were designed using the same software. End point PCR was performed using tumor-extracted genomic DNA templates. Bands were visualized by agarose gel electrophoresis and analyzed for cxcr5 promoter methylation.

2.11. Luciferase assay

MDA-MB-231 and T47D cells were transfected with different promoter variants of cxcl13 in different treatment combinations. Luciferase mRNA fold changes were quantified by qPCR in triplicate considering as the indicator of relative promoter activity. Transfection with empty pGL3 vector was considered as control.

2.12. Chromatin immunoprecipitation (ChIP)

Conventional ChIP was performed as per laboratory optimized protocol [26] and recommendation of manufacturer (Millipore:#17-295). Briefly, sonicated cross-linked protein-DNA complexes were incubated with ChIP-grade antibodies: anti-RelA (CST:#8242S), anti-Nrf2 (CST:#12721S), and precipitated with ProteinA-Agarose/Salmon sperm DNA. After eluting protein-DNA complexes and DNA purification, target DNA was quantified by qPCR. Input C_T was adjusted to 100% and specific binding was calculated using the following formula Percentage of input = 100 × 2^[Adjusted input - Ct (IP)].

2.13. Molecular docking

In silico prediction for interaction of cxcl13 promoter-DNA-region (GRCh37.p13:78432107-78433207) with RelA (NP_068810.3) and Nrf2 (NP_006155.2) and construction of PDB file was done by 'tfmodeller'. Output data were analyzed and the 3D models were prepared by pyMOL Quad 3D.

2.14. Statistical analyses

Distribution of variables was tested by Kolmogorov-Smirnov test. Association between gene expressions was analyzed by Pearson's correlation. One-way analysis of variance (ANOVA) (Bonferroni correction) was performed to assess the level of significance among paired data sets. Statistical analyses were performed using GraphPad Prism7.0 and OriginPro8. All data are presented as mean ± SEM and p value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Intra-tumoral RelA expression is positively correlated with intratumoral CXCL13 and CXCR5 expression

Reportedly, co-expression of CXCL13 and CXCR5 positively correlates with LNM during BCa progression [7], and RelA/p65 positively regulates cxcr5-transcription through direct interaction with the cxcr5 promoter [14]. Furthermore, through investigation of the cxcl13 promoter, we identified putative RelA binding sites (Supplementary Fig. S1). Therefore, to understand the relevance of RelA expression with CXCL13-CXCR5 co-expression, we first correlated intra-tumoral RelA expression with that of CXCL13 and CXCR5 ($n = 98$) (Table 1 and Table 2; Fig. 1A-D). Pearson's correlation analysis showed that RelA mRNA expression has a strong positive correlation with both CXCL13 ($r = +0.7403$) (Fig. 1C) and CXCR5 ($r = +0.6982$) (Fig. 1D), suggesting that RelA may function as a common positive regulator of both. Notably, in spite of high RelA expression, mRNA level of CXCL13 and CXCR5 is low in ~25% and ~38% of samples, respectively, suggesting negative regulation beyond RelA-mediated positive regulation (Fig. 1C and D). Correlation analysis comparing CXCL13 and CXCR5 in "RelA-high" ($2^{-\Delta\Delta C}_T > 10$) samples ($r = +0.0276$, $p = 0.837$, $n = 58$) further suggested that CXCL13 and CXCR5 have dissimilar negative regulation mechanisms (Fig. 1E). In addition, highly correlated ($r = +0.8527$, $p < 0.0001$, $n = 40$) but low expression of both molecules in "RelA-low" ($2^{-\Delta\Delta C}_T < 10$) samples confirmed that RelA is essential for both CXCL13 and CXCR5 expression (Fig. 1F).

3.2. Nrf2 negatively regulates RelA-induced CXCL13 expression, but not CXCR5 expression

Mapper2.0-based analyses have indicated that besides three putative RelA-binding sites, cxcl13 promoter contains two putative binding sites for Nrf2 (Supplementary Fig. S1), a known transcriptional regulator [22,23]. Thus, in order to dissect the mechanism of CXCL13 and CXCR5 transcriptional regulation, we cloned the coding sequences of RelA, Nrf2 and CXCR5 (Supplementary Fig. S2) and ectopically expressed them in two BCa cell lines, MDA-MB-231 and T47D. RelA-overexpression significantly ($p < 0.05$) induced mRNA expressions of CXCL13 ($p = 0.000027$ and 0.0000059 in MDA-MB-231 and T47D cells, respectively) and CXCR5 ($p = 0.0000253$ and 0.000000014 in MDA-MB-231 and T47D cells, respectively) in both cell lines (Fig. 2A). Importantly, we observed significantly decreased CXCL13-mRNA expression in BCa cells ($p = 0.00604$ and $p = 0.00177$ in MDA-MB-231 and T47D cells, respectively) induced with simultaneous RelA and Nrf2-overexpression, compared to only RelA-overexpressed cells (Fig. 2A). Moreover, in MDA-MB-231 cells, a significant drop ($p = 0.00516$ and $p = 0.082$ in MDA-MB-231 and T47D cells, respectively) of CXCL13 mRNA expression in Nrf2-overexpressed cells compared to untreated control cells was detected (Figure 2Ai). No significant change in CXCR5 mRNA expression was observed when compared between dual-overexpressed and only RelA-overexpressed cells ($p = 0.81012$ and 0.64394 in MDA-MB-231 and T47D cells, respectively) or between only Nrf2-overexpressed and control cells ($p = 0.57592$ and 0.3739 in MDA-MB-231 and T47D cells, respectively) (Fig. 2A). Immunoblots and densitometry analyses confirm that Nrf2-mediates significant repression ($p < 0.05$) of RelA-induced CXCL13 protein expression (Fig. 2B and C). Notably, no significant ($p > 0.05$) impact of Nrf2 in RelA-induced CXCR5 protein expression was detected.

Further, significantly increased ($p < 0.05$) surface-expressions of CXCR5 were observed in RelA-overexpressed BCa cell lines, irrespective of co-induction with Nrf2-overexpression (Fig. 2D and E). Conversely, we observed significant elevation in intracellular CXCL13 ($p < 0.05$) upon RelA-overexpression, which is reversed by Nrf2 co-overexpression (Fig. 2D–F). However, direct CXCR5-overexpression did not alter CXCL13 expression, which confirms that RelA-mediated elevation of CXCL13 and CXCR5 expression are independent events (Fig. 2D and E). Basal concentration of intracellular CXCL13 was found to decrease significantly ($p < 0.05$) in Nrf2-overexpressed MDA-MB-231 cells. Collectively, Nrf2 has an overall negative influence in cxcl13 transcription *in vitro,* and no significant effect in cxcr5 transcription.

3.3. Nrf2 has negative correlation with CXCL13 in "RelA-high" primary tumors

To validate the *in vitro* negative regulatory function of Nrf2 in cxcl13 transcription, intra-tumoral Nrf2 mRNA expression was correlated with that of CXCL13 and CXCR5 (Table 2) in "RelA-high" primary tumor samples ($2^{-\Delta\Delta C}_T > 10$, $n = 58$). Correspondingly, we observed a negative correlation of Nrf2 with CXCL13 ($r = -0.9141$), while,

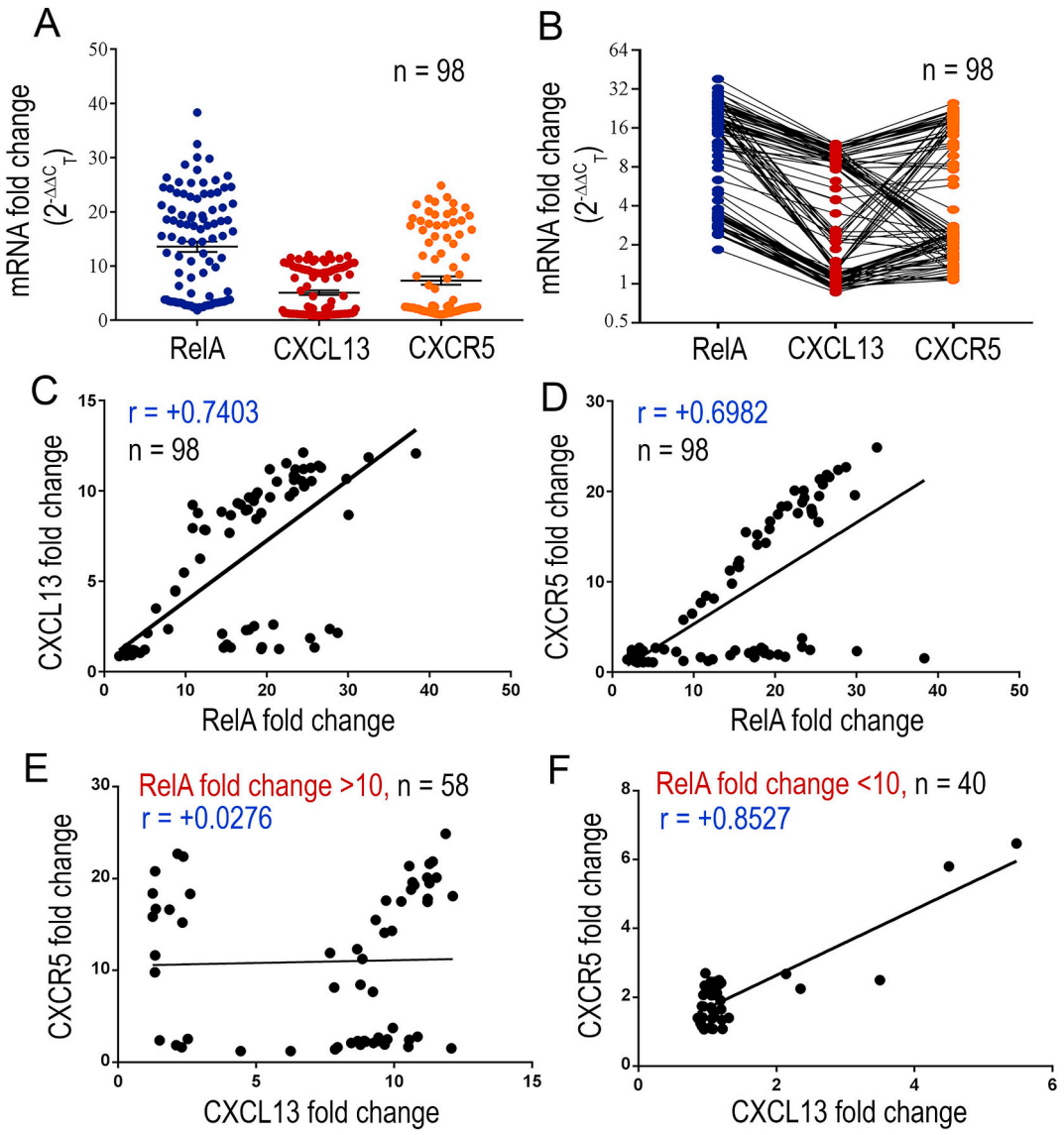

Fig. 1. Intra-tumoral expression analyses of RelA, CXCL13, CXCR5 mRNAs and correlation. (A) Scatter plot of RelA, CXCL13 and CXCR5 mRNA fold changes in primary tumor tissues (n = 98) compared to healthy breast tissues. (B) Pairwise comparison graph showing mRNA fold changes of RelA, CXCL13 and CXCR5 in individual samples (n = 98). (C) Graph showing Pearson's correlation between RelA and CXCL13 mRNA fold changes in primary tumors (n = 98). (D) Graph showing Pearson's correlation between RelA and CXCR5 mRNA fold changes in primary tumors (n = 98). (E) Graph showing Pearson's correlation between CXCL13 and CXCR5 in primary tumors with RelA mRNA fold changes > 10 (n = 58) (F) Graph showing Pearson's correlation between CXCL13 and CXCR5 in primary tumors with RelA mRNA fold changes < 10 (n = 40).

correlation with CXCR5 and Nrf2 (r = +0.1788) (Fig. 3A and B). Together, these observations led us to consider Nrf2 as a negative regulator of CXCL13 expression, in spite of high RelA expression. Additionally, this also confirmed that CXCR5 expression is not sensitive to Nrf2 and indicated that CXCR5 expression to be negatively regulated by independent mechanisms during "RelA-high" conditions. Notably, our observation supports tumor suppressor function of Nrf2 that could potentially uncouple CXCL13-CXCR5 co-expression.

3.4. DNA methylation of cxcr5 promoter abolishes RelA-induced transcriptional up-regulation of CXCR5

Reportedly, p53 is a negative regulator of cxcr5 transcription, which is indirectly driven by p53-mediated downregulation of RelA [14]. In order to identify the mechanism that governs CXCR5 transcription, we identified a 305 nucleotide long CpG-island that encompasses the transcription start site (TSS) (Supplementary Fig. S3). Hence, we hypothesized that methylation of the cxcr5 promoter within this CpG-island result in silencing of cxcr5 transcription and therefore, could possibly be a reason for low CXCR5 in some "RelA-high" tumors. A nested region in the methylation island was PCR-amplified using methylation-specific primer pairs (MSP) and genomic DNA templates extracted from "RelA-high" samples and analyzed. Positive PCR bands indicating cxcr5-promoter-DNA-methylation were correlated with CXCR5 mRNA fold changes in RelA-high tumors. High negative correlation (r = −0.7555) between CXCR5 expression and presence of cxcr5 promoter methylation were observed (Fig. 3 Ci). We further validated our observation by examining CXCR5 protein expression in representative RelA-high samples and comparing MSP-PCR gel bands (Figure 3Cii and 3Ciii). Conversely, cxcl13 promoter does not contain any potential CpG island (Supplementary Fig. S4). Together, these results signify that CXCR5 and CXCL13 have different mechanisms of negative regulation though commonly regulated positively by RelA.

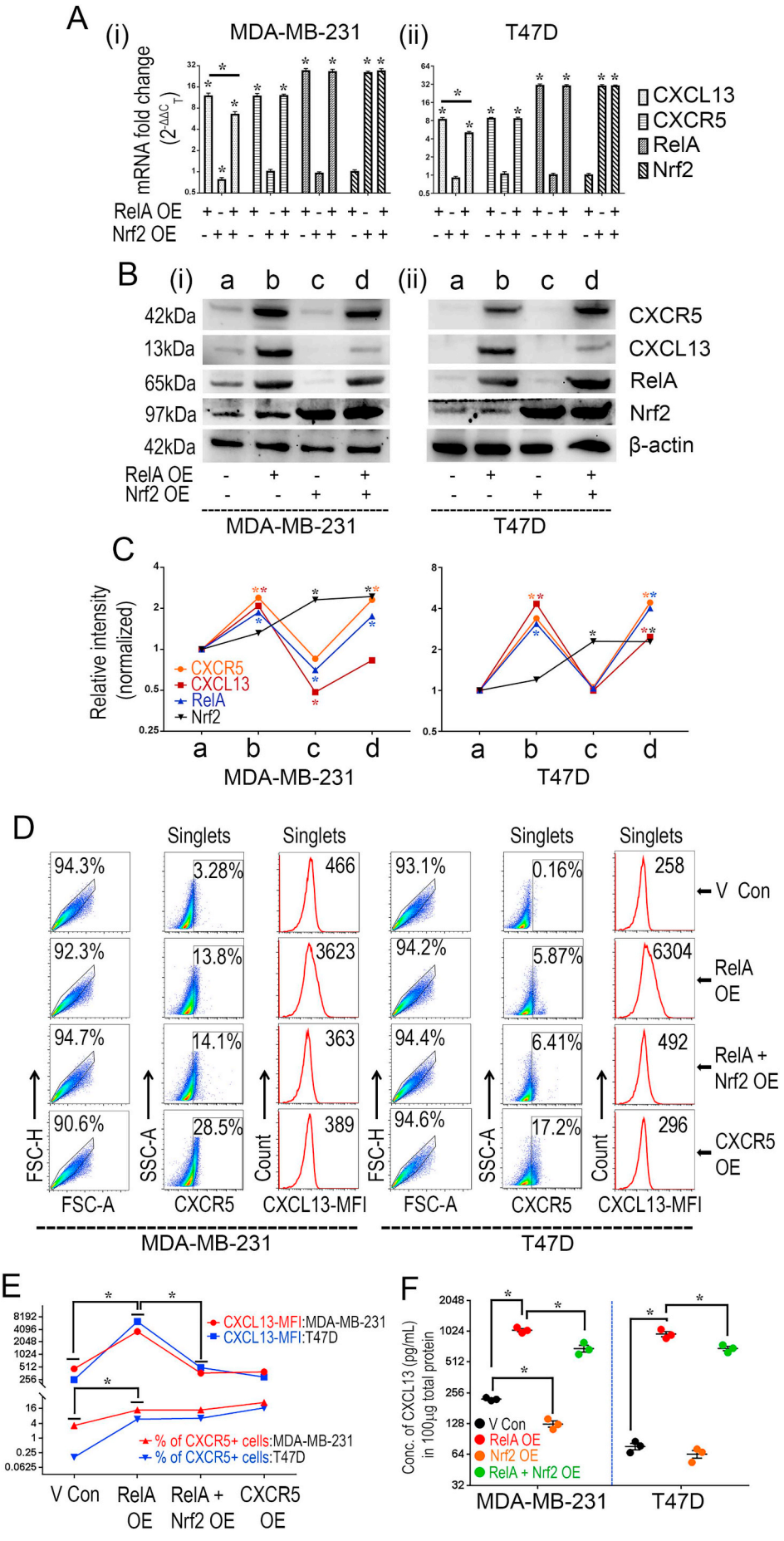

Fig. 2. Effects of RelA- and Nrf2-transductions in BCa cell lines for CXCL13 and CXCR5 expression. (A) Quantitative real-time PCR for CXCL13, CXCR5, RelA and Nrf2. Fold changes are represented as relative values normalized to the 18S rRNA control. (B) Western blot of CXCL13, CXCR5, RelA and Nrf2. β-actin used as loading control. (C) Densitometry analyses of the WB bands. Relative intensities, normalized to the β-actin loading control, calculated using ImageJ software and are shown in line graphs. (D) Flow cytometry analyses of CXCR5 and CXCL13 in transduced MDA-MB-231 and T47D cell lines, with PE-conjugated anti-CXCR5 and APC- conjugated anti-CXCL13 antibodies. Singlet population was gated from total cells using FSC-A *vs* FSC-H dot plots. Percentages of CXCR5-positive cells and mean fluorescence intensity (MFI) values of intracellular CXCL13 expressions in total singlet populations are indicated. (E) Line graphs showing percentages of CXCR5-positive cells and MFI of CXCL13 in singlet populations of MDA-MB-231 and T47D cells. (F) Quantitative intracellular CXCL13 protein ELISA in 100 µg of total lysate was measured by represented as grouped-scattered graphs. 'RelA OE', 'Nrf2 OE', 'V con', 'CXCR5 OE' represents cells transfected with RelA-overexpression plasmid, Nrf2-overexpression plasmid, empty pcDNA3.1 vector, and CXCR5-overexpression plasmid, respectively. 'RelA + Nrf2 OE' represents cells co-transfected with RelA-overexpression plasmid and Nrf2-overexpression simultaneously. Results are representative of two independent experiments and are represented as mean ± SEM. One-way ANOVA with Bonferroni correction was performed to determine statistical significance, where $^*p \leq 0.05$.

Table 1
Clinicopathological characteristics of patients with expressions of RelA, CXCL13, CXCR5 and Nrf2.

Characteristics	Number of patients per group
Total	98
Age (median and range) in years	47 (24–75)
pT status	
pT_1	18
pT_2	46
pT_3	26
pT_4	8
pN status	
pN_0	42
pN_1	15
pN_2	24
pN_3	17
M status	
M_0	83
M_1	8
M_X	7
Stage	
I	18
II	39
III	33
IV	8
Tumor differentiation grade	
Well (I)	14
Moderate (II)	45
Poor (III)	39
RelA fold change ($2_T^{-\Delta\Delta C}$)	
< 4	33
≥ 4 to < 10	7
≥ 10 to < 20	30
≥ 20	28
Nrf2 fold change ($2_T^{-\Delta\Delta C}$)	
< 4	12
≥ 4 to < 10	45
≥ 10 to < 20	14
≥ 20	27
CXCL13 fold change ($2_T^{-\Delta\Delta C}$)	
< 2	43
≥ 2 to < 4	10
≥ 4 to < 10	27
≥ 10	18
CXCR5 fold change ($2_T^{-\Delta\Delta C}$)	
< 2	31
≥ 2 to < 4	30
≥ 4 to < 10	6
≥ 10	31

Table 2
Expressions of CXCL13 and CXCR5 mRNA in breast tumors grouped according to intra-tumoral RelA mRNA expressions (upper); and in "RelA–high" tumors according to intra-tumoral Nrf2 mRNA expressions (lower).

Characteristics			
CXCL13 mRNA fold change ($2_T^{-\Delta\Delta C}$)	CXCR5 mRNA fold change ($2^{-\Delta\Delta C}{}_T$)	RelA mRNA fold change ($2_T^{-\Delta\Delta C}$) ≥ 10	RelA mRNA fold change ($2_T^{-\Delta\Delta C}$) < 10
< 4	< 4	4	38
≥ 4	≥ 4	24	2
< 4	≥ 4	11	0
≥ 4	< 4	19	0
CXCL13 mRNA fold change ($2_T^{-\Delta\Delta C}$)	CXCR5 mRNA fold change ($2^{-\Delta\Delta C}{}_T$)	Nrf2 mRNA fold change ($2_T^{-\Delta\Delta C}$) ≥ 8	Nrf2 mRNA fold change ($2_T^{-\Delta\Delta C}$) < 8
< 4	< 4	4	0
≥ 4	≥ 4	1	23
< 4	≥ 4	11	0
≥ 4	< 4	0	19

3.5. Functionally active CXCL13 is secreted by RelA-induced BCa cells

Like other chemokines, CXCL13 functions once it is secreted extracellular and interacts with CXCR5 on plasma membrane of recipient cells [6,7]. Active CXCL13 signals through intracellular Src-phosphorylation [6,7]. We have performed co-culture experiments to verify the functionality of CXCL13, secreted from RelA-induced BCa cells (Fig. 4A). Target cells were made CXCR5 positive ectopically. Immunoblot analyses showed a significant increase in Src-phosphorylation in CXCR5-positive target cells (Fig. 4B and C). Moreover, results suggested that CXCL13 expressed by RelA-induced MDA-MB-231 cells can activate Src in T47D cells and *vice versa*. Ectopic overexpression of CXCL13 in donor cells was considered as positive controls (Figure 4Ae and 4 Ah). Notably, we observed a significant increase in secreted CXCL13, when donor cells were induced for RelA-overexpression ($p = 0.00344$ and 0.00188 when donor cells were MDA-MB-231 and T47D, respectively) (Fig. 4D). Cumulatively, these results demonstrate that RelA-induced BCa cells secrete CXCL13 in a functionally active form.

3.6. The promoter region of cxcl13 has RelA and Nrf2 responsive regions

To understand the mechanism of RelA- and Nrf2-mediated cxcl13 transcription, we have sub-cloned the genomic fragment of the cxcl13 promoter ($-800/+301$) in pGL3 luciferase vector (Fig. 4E, Supplementary Fig. S5). In RelA-overexpressing BCa cells, we observed a significant ($p < 0.05$) increase in luciferase mRNA fold changes (20.87 and 18.77 fold in MDA-MB-231 and T47D cells, respectively) (Fig. 4F). Further, when cells were co-transduced with Nrf2, we observed a significant ($p < 0.05$) drop in luciferase mRNA fold changes (6.45 and 9.31 fold in MDA-MB-231 and T47D cells, respectively) (Fig. 4F). In order to identify the location RelA and Nrf2 responsive regions of the cxcl13 promoter, we strategically designed nine deletion variants (Del1 –800/–688; Del2 –800/–564; Del3 –800/–462; Del4 –800/–319; Del5 –800/–218; Del6 –800/–109; Del7 –800/–15; Del8 –800/+148; Del9 –800/+217) (Fig. 4E, Supplementary Fig. S5). The first, second and third RelA binding sites are positioned in the deleted regions of Del2, Del4 and Del9, respectively; whereas, the first and second Nrf2 binding sites are positioned in the deleted region of Del2 and Del8, respectively. Analyses from different deletion variants and in differently induced BCa cell lines have indicated that Del2 has no significant effect in luciferase activities when cells were transduced only with RelA, but significantly increased in cells co-transduced with RelA and Nrf2 or induced only with Nrf2 (Fig. 4F). The deleted region between Del1 and Del2 encompasses first putative binding sites of each of RelA and Nrf2 (Fig. 4E), which suggests that Nrf2 has, but not RelA, significant influence in cxcl13 transcription through this site. Del4, which covers second putative binding site of RelA (Fig. 4E), showed a significant and drastic fall of luciferase activities in all combinations (Fig. 4F), implying this to be a potent RelA binding site. Deleted regions of Del8 and Del9 include third putative binding sits of Nrf2 and RelA, respectively (Fig. 4E). Though we observed a drastic and maximum fall through Del8 and Del9 in all treatment combinations, we cannot infer anything significant because these sites are positioned post-TSS. Together, the luciferase assay results indicated that cxcl13 promoter DNA contains RelA and Nrf2 responsive regions, sensitive to intracellular levels of RelA and Nrf2, respectively.

3.7. RelA and Nrf2 interacts with specific binding sites within CXCL13 promoter

To verify *in vivo* binding of RelA and Nrf2 to cxcl13 promoter, we performed a ChIP-PCR assay. Primers for different 6 amplicons were designed (Supplementary Table I, Fig. 5A). Among RelA-specific amplicons, amplicon3 and amplicon6 produced significantly ($p < 0.05$) stronger signals than amplicon1 for all treatment conditions in both cell

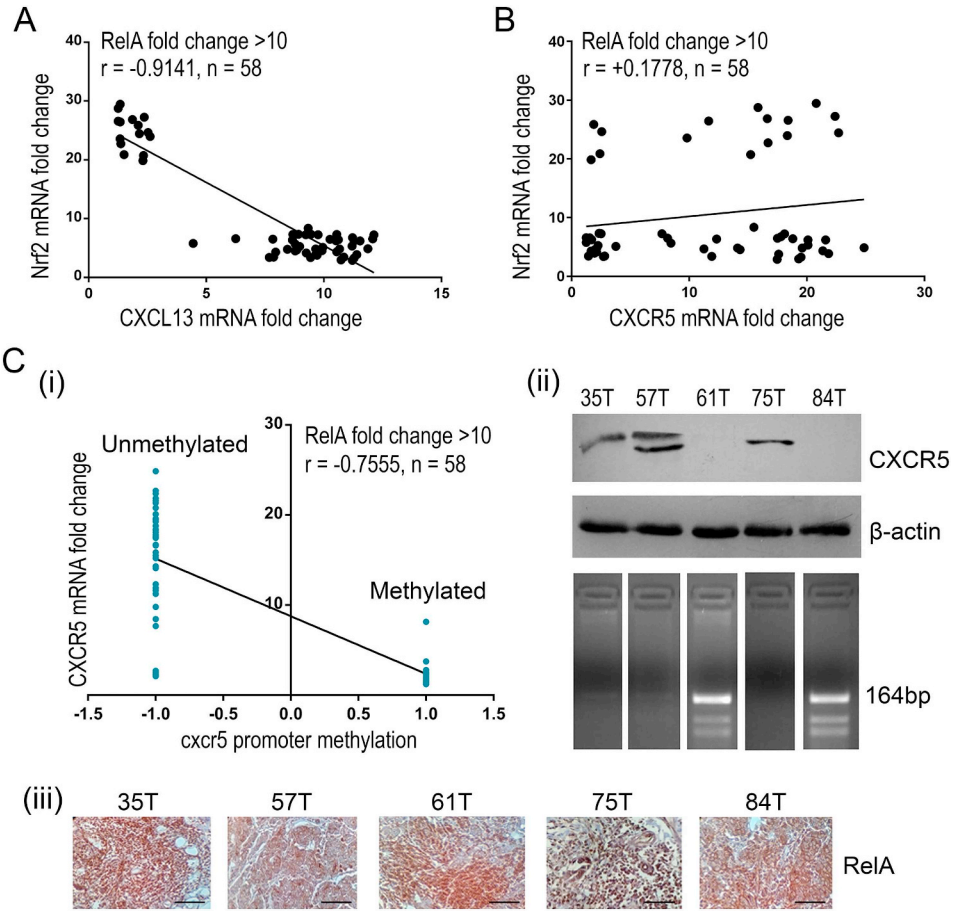

Fig. 3. Correlation studies between Nrf2 and CXCL13, CXCR5 expressions; and Cxcr5 promoter-methylation analyses of in "RelA-high" tumors. (A) Pearson's correlation between Nrf2 and CXCL13 in primary tumor samples where RelA mRNA fold change is > 10 (n = 58). (B) Pearson's correlation between Nrf2 and CXCR5 in primary tumor samples where RelA mRNA fold change is > 10 (n = 58). (C) (i) Pearson's correlation between mRNA fold changes of CXCR5 and methylation status of cxcr5 promoter in primary tumor samples where RelA mRNA fold change is > 10 (n = 58). Positive MSP-PCR gel band was given a + 1 score whereas negative PCR was given a − 1. (C) (ii) Immunoblots showing CXCR5 protein expressions in representative "RelA-high" samples (up) and Agarose gel bands showing PCR amplification using MSP-primers representing methylation status of the cxcr5 promoter in the samples (bottom). β-actin used as loading control for immunoblot analyses. (iii) Representative IHC staining of RelA in samples with RelA mRNA fold changes > 10.

lines when genomic DNA was immunoprecipitated with anti-RelA antibody (Fig. 5B). This observation supports our observations from the luciferase assay, and also suggests that RelA has strongest affinity for its third binding site within the cxcl13 promoter. On the other hand, both Nrf2 specific amplicons showed significant signals in all treatment conditions when genomic DNA was immunoprecipitated (IP) with anti-Nrf2 antibody. Comparatively, amplicon2 has shown stronger signals than amplicon5 (Fig. 5B). Amplicon4 produced no considerable signal in any treatment conditions and with any IPs (Fig. 5B). Together, our ChIP-PCR results confirm our hypothesis that RelA and Nrf2 maintain occupancy of the cxcl13 promoter and mediate transcription regulation.

3.8. RelA and Nrf2 dock on cxcl13 promoter in silico

To complement our hypothesis, we have performed molecular docking of RelA and Nrf2 on the cxcl13 promoter regions under study. *In silico* Docked DNA-protein conformations were analyzed and evaluated. Models show possible molecular interactions between cxcl13 promoter and RelA (Fig. 5C), or cxcl13 promoter and Nrf2 (Fig. 5D). Analyses revealed intimate interactions of RelA and Nrf2 with cxcl13 promoter within < 4A° and undoubtedly suggest possible hydrogen bond formation and hydrophobic interactions. Collectively, these models support our ChIP data and overall findings.

4. Discussion

CXCL13 and its associated signaling have gained immense importance in connection to BCa progression [5,7,12]. Besides fDCs, macrophages and TFH cells [9,10,12], researchers have also shown that primary breast tumor epithelial cells secrete CXCL13 [13]. CXCL13 has been found to drive a poorer prognosis during BCa progression when it is co-expressed with its receptor CXCR5 within primary tumors [7]. Co-expression of this chemokine ligand-receptor pair forms possible autocrine loop which favors EMT, migration and metastasis of BCa cells [7]. Therefore, it is utmost important to understand the molecular mechanisms that drive co-expression of CXCL13 and CXCR5. Previously, it has been demonstrated in MCF7 BCa cells that RelA facilitates cxcr5 transcription by binding to specific sequences; this in turn is negatively regulated by p53 [14]. RelA, being a multifunctional TF, regulates transcription of many genes during BCa progression [17,18,21]. In this study, we have demonstrated RelA binding to cxcl13 promoter drives its transcription. We sought to verify whether elevated RelA within primary tumor is significantly associated with CXCL13-CXCR5 co-expression. Analyses of tumor samples indicated that RelA overexpression is obligatory, though it does not exclusively correlate with CXCL13-CXCR5 co-expression (Fig. 1). This observation had raised the possibilities for negative regulation of cxcl13 and cxcr5 transcription beyond RelA-mediated positive induction. Careful analyses of "RelA overexpressed CXCL13-CXCR5 non-co-expressed" samples indicated differential negative regulation for cxcl13 and cxcr5.

Nrf2 expression has been known to be associated with better outcome for BCa [27], and it regulates transcription of multiple genes when it gets translocated into the nucleus after its release from inhibitory Keap1 [28,29]. Promoter of cxcl13, but not cxcr5, possesses putative Nrf2 binding sites. We have demonstrated that Nrf2 significantly abrogates the positive inducing effect of RelA for cxcl13 transcription *in vitro* (Fig. 2). However, we did not witness any significant effect of Nrf2 for cxcr5 transcription (Fig. 2). In "RelA-high" samples, CXCL13 mRNA expression is negatively correlated with Nrf2 expression (Fig. 3A). This is important because high Nrf2 could uncouple co-expression of CXCL13 with CXCR5 in "RelA-high" conditions. Literature suggests that estrogen signaling negatively regulates Nrf2

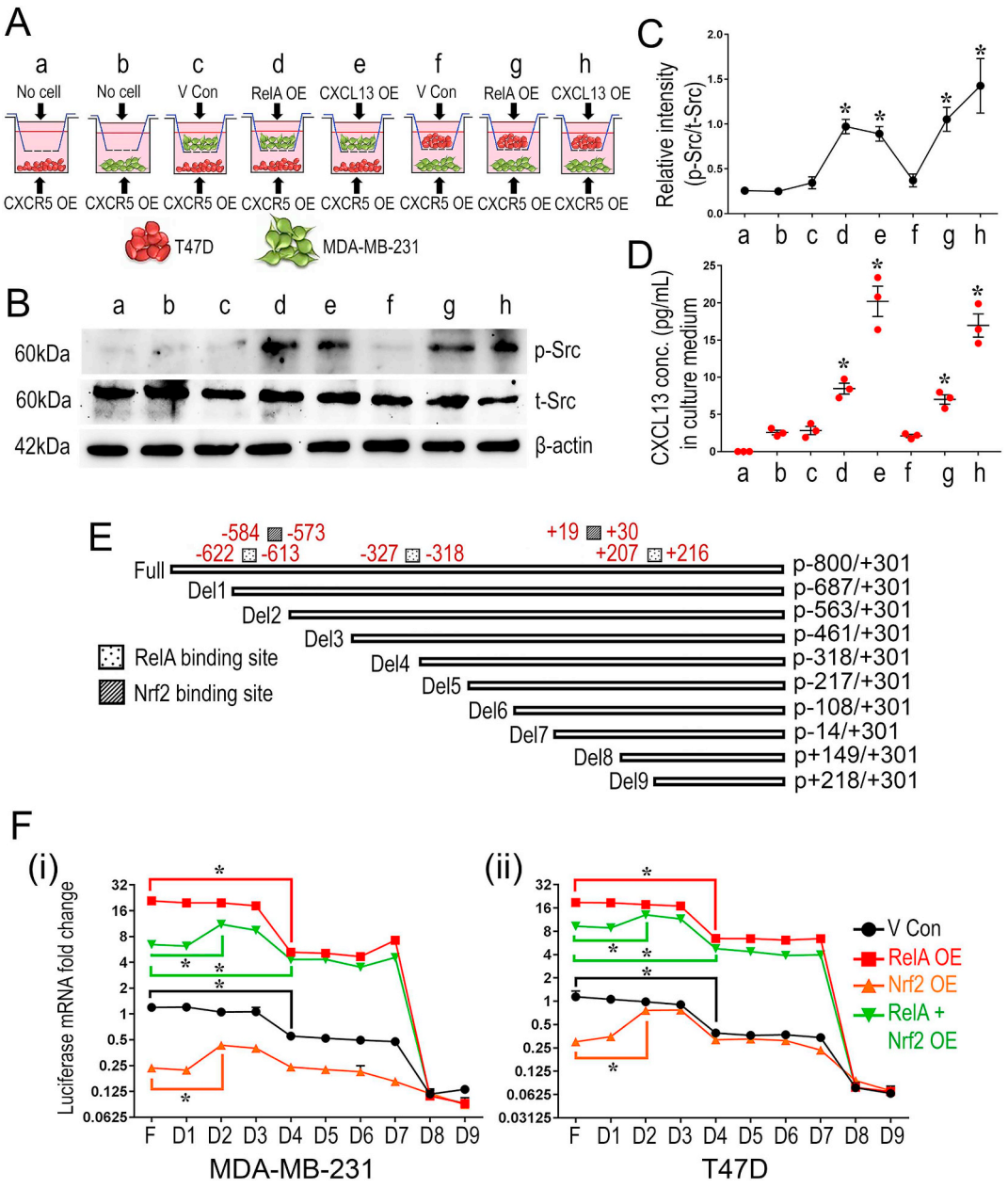

Fig. 4. Functionality assay for CXCL13, and luciferase assay. (A) Strategy used for in-direct co-culture assay between differently transduced MDA-MB-231 and T47D cells. Donor cells were placed in upper 0.4 μ transwell inserts and recipients in bottom wells of 24 well tissue culture plates. In combinations a, c, d and e, T47D cells were used as recipient and in combinations 'b', 'f', 'g', 'h', MDA-MB-231 cells were used as recipient. All recipient cells were transfected for CXCR5 overexpression. Combination 'a' and 'b' has no donor cells. In combinations 'c', 'd' and 'e' donor MDA-MB-231 cells were transfected with empty vector, RelA overexpression vector and CXCL13 overexpression vector, respectively. In combinations 'f', 'g' and 'h' donor T47D cells were transfected with empty vector, RelA overexpression vector and CXCL13 overexpression vector, respectively. (B) Immunoblot analyses for total-Src and phosphorylated-Src using lysates from recipient cells. β-actin was used as loading control. (C) Densitometry analyses of the WB bands. p-Src/t-Src intensities, relative to the β-actin loading control, were calculated using ImageJ software. (D) CXCL13 protein concentrations in the co-culture medium, measured by ELISA, and are represented as grouped-scattered graphs. (E) Positions of putative binding sites of RelA (white) and Nrf2 (black) are indicated as boxes, and locations of all nine deletion variant on the cxcl13 promoter. (F) Functional analysis of cxcl13 promoter by deletion scanning. Luciferase mRNA fold-changes in MDA-MB-231 (i) and T47D (ii) cells are represented in bar graphs. 'V con', 'RelA OE', 'Nrf2 OE' represents cells transfected with empty pcDNA3.1 vector, RelA-overexpression plasmid, and Nrf2-overexpression plasmid, respectively. 'RelA +Nrf2 OE' represents cells co-transfected with RelA-overexpression plasmid and Nrf2-overexpression simultaneously. Results are representative of two independent experiments and are represented as mean ± SEM. One-way ANOVA with Bonferroni correction was performed to determine statistical significance, where $^{*}p \leq 0.05$.

expression and a significant proportion of breast tumors are ER positive [30]. In our study population, we observed higher Nrf2 expression in ER negative samples (Supplementary Fig. S6). Correspondingly, ER positive samples with lower Nrf2 are more prone to co-express CXCL13 and CXCR5.

Interestingly, among "RelA-high Nrf2-low" samples, we also identified tumors where high CXCL13 does not accompanied by high CXCR5, suggesting a possible negative regulation of cxcr5 transcription. Cxcr5 promoter has a CpG-island and we observed lack of CXCR5 expression in "RelA-high" tumors where cxcr5 promoter is methylated, showing a high negative correlation (Fig. 3C). Notably, RelA-induced BCa cells not only produce significantly increased CXCL13, but also secrete it in functionally active form (Fig. 4A–D). Moreover, RelA-induced BCa cells express CXCR5 on the cell surface (Fig. 2D and E). We

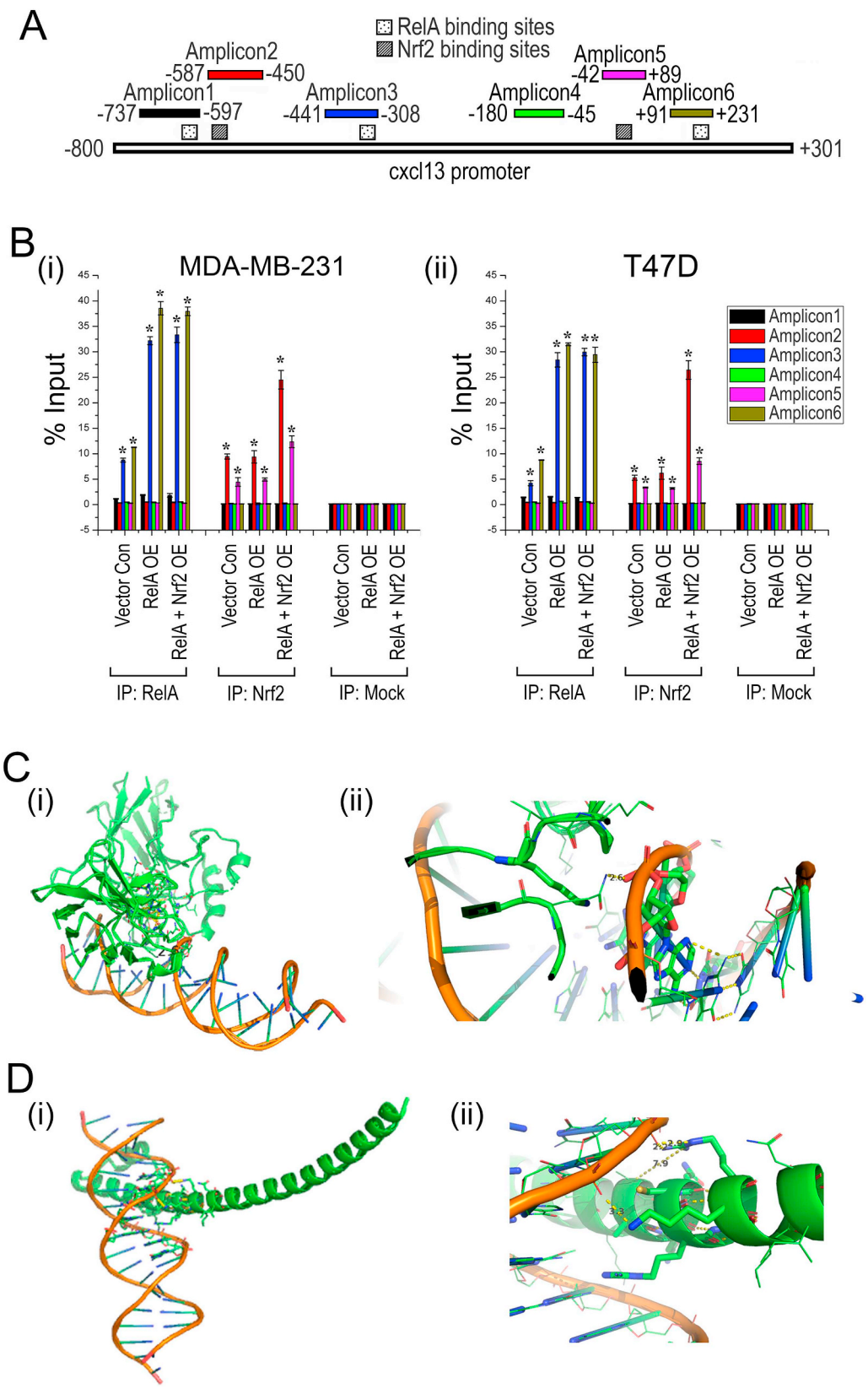

Fig. 5. ChIP assay and DNA-protein docking models showing DNA-protein interactions between cxcl13-promoter, RelA and Nrf2. (A) Map of the cxcl13 promoter showing predicted RelA and Nrf2 binding sites and PCR products to be amplified in ChIP assay (Amplicons 1 to 6). Amplicon1, amplicon3 and amplicon6 comprise the first, second and third putative binding sites of RelA respectively. Amplicon2 and amplicon5 comprise first and second putative binding sites of Nrf2 respectively. Amplicon4 does not contain any binding site of either of the molecules (B) IP-cross-linked DNA fragments of cxcl13 promoter from anti-RelA, anti-Nrf2, isotype-IgG and input samples were amplified by qPCR and percentage input were calculated from C_T values, represented as bar graphs. 'V con', 'RelA OE', 'Nrf2 OE' represents cells transfected with empty pcDNA3.1 vector, RelA-overexpression plasmid, and Nrf2-overexpression plasmid, respectively. 'RelA + Nrf2 OE' represents cells co-transfected with RelA-overexpression plasmid and Nrf2-overexpression simultaneously. Results are representative of two independent experiments and are represented as mean ± SEM. One-way ANOVA with Bonferroni correction was performed to determine statistical significance, where $^*p \leq 0.05$. (C) Models depicting interaction of p65/RelA (NP_068810.3) with cxcl13 promoter (−800/+301). (D) Models depicting interaction of Nrf2 (NP_006155.2) with cxcl13 promoter (−800/+301). (C)(i) and (D)(i) are presenting overall complexes whereas, (C)(ii) and (D)(ii) are displaying magnified interaction from one angle at 8A° sphere.

have also identified regions in cxcl13 promoter for RelA and Nrf2 binding (Fig. 4E and F), and confirmed that both RelA and Nrf2 bind to cxcl13 promoter in the chromosome (Fig. 5A and B). Additionally, occupancy of Nrf2 on cxcl13 promoter remained unaltered upon RelA overexpression as compared to the Vector control, which is evident from the unaltered signals for Amplicon 2 and Amplicon 5 in the Nrf2-immunoprecipitates. Similarly, simultaneous overexpression of Nrf2 with RelA did not alter occupancy of RelA on cxcl13 promoter as compared to the RelA-overexpressed cells, reflected by the unaltered Amplicon 1, Amplicon 3 and Amplicon 6 signals in the RelA-

immunoprecipitates (Fig. 5B). These observations suggested that binding of RelA and Nrf2 within the cxcl13 promoter is independent of each other.

In summary, our findings corroborate that RelA positively regulates both cxcl13 and cxcr5 transcription. Individual negative regulation of cxcl13 and cxcr5 transcription, mediated by Nrf2 and promoter-DNA-methylation, respectively, determines their co-expression in conditions where RelA is highly expressed. Tumors with high RelA, low Nrf2 and un-methylated cxcr5 promoter have the strongest possibility of CXCL13-CXCR5 co-expression.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.12.002.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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Author contributions

Study designed by SB, SRC and AB. Major experiments were performed by SB and GM. SP and GM performed the immunohistochemistry. SB, SRC, GM and AB analyzed and interpreted results. AG provided tumor samples. SB, SRC, GM and AB drafted manuscript. All authors revised the manuscript and approved.

Conflict of interest statement

Authors declare no potential conflicts of interest.

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