3. CHAPTER II

Regulation of cxcl13 and cxcr5 gene transcription during breast cancer
3.1. Introduction

Gene expression is regulated by transcription factors (TFs). TFs mainly bind to the promoter regions and influence the transcription of respective genes either positively or negatively. By binding to promoter DNA sequence, TFs regulate RNA polymerase recruitment [Latchman DS, 1997; Karin M, 1990]. Promoters are regulatory regions of DNA, located upstream (towards the 5′ region) of gene coding sequences, providing a control point for regulated gene transcription.

Besides, regulation by binding of TFs, promoter activity sometimes is regulated by epigenetic DNA methylation modifications. Gene promoters must be easily reachable to TFs and/or other regulatory DNA regions such as enhancers [Watt F and Molloy PL, 1998]. DNA methylation can directly prevent transcription factor binding and lead to changes in chromatin structure that restrict access of transcription factors to the gene promoter [Moore LD et al, 2013; Handy DE et al, 2011]

DNA methylation is the process of covalent methyl group (−CH₃) addition to the base cytosine (C) in the dinucleotide 5′-CpG-3′ [Razin A and Kantor B, 2005; Deaton AM and Bird A, 2011]. Occasionally, methylated cytosines are considered as the fifth nucleotide. The term CpG defines that a cytosine (C) is linked to the base guanine (G) through a phosphate bond in the DNA nucleotide sequence [Razin A and Kantor B, 2005]. The majority of human genomic CpG dinucleotides are methylated. Unmethylated CpGs are usually grouped together in the promoter region of many genes known as ‘CpG islands’ [Razin A and Kantor B, 2005; Bird AP, 1986; Ehrlich M et al, 1982]. CpG islands in the promoters are differently methylated in cancer cells. Methylation of promoters of tumor suppressors often leads to tumor formation [Esteller M, 2002; Lopez-Serra L et al, 2008; Irimia M et al, 2004; Lujambio A et al, 2010]. Conversely, withdrawal of some promoter-CpG methylation favors expression of tumor promoting genes [Esteller M, 2002; Lopez-Serra L et al, 2008; Irimia M et al, 2004; Lujambio A et al, 2010]. In general, but not always, DNA methylation is associated with loss of gene expression.

Initially, we have reported that co-expression of CXCL13 and CXCR5 is associated with poor prognosis [Biswas S et al, 2014]. It is important to understand the gene regulation of both cxcl13 and cxcr5. Based on bioinformatics studies, we were keen to understand the potential of RelA and Nrf2 to regulate cxcl13 transcription.

Fig. 3.1. Nucleotide sequences of cxcl13 promoter and its position in the genome.
Members of the NFκB family can also act as oncogenes and are very often constantly activated in tumor cells, contributing to malignant phenotype [Hoesel B and Schmid JA, 2013]. Direct inhibition of the NFκB-Rel activity in breast cancer cells induces apoptosis [Hoesel B and Schmid JA, 2013; Sovak MA et al, 1997], whereas, NFκB family protein RelA (p65) acts as a transcription factor and regulates expression of many genes during breast cancer progression. Involvement of RelA in breast cancer progression is well documented [Sovak MA et al, 1997; Helbig G et al, 2003]. Recent evidences suggest that
RelA regulates expression of different chemokine ligands and receptors. For example, NFκB decreases transcription of CXCL1 and CXCL2 transcription [Bachmeier BE et al, 2008]. On the other hand, NFκB regulates the motility of breast cancer cells by directly up-regulating the expression of CXCR4 [Helbig G et al, 2003; Luker KE and Luker GD, 2005]. Overexpression of the inhibitor of IκB in breast cancer cells with constitutive NFκB activity downregulates CXCR4 expression and a resultant loss of CXCL12-mediated migration in vitro [Helbig G et al, 2003]. Introduction of CXCR4 cDNA into IκB-expressing cells restored the CXCL12-mediated migration [Helbig G et al, 2003]. Electrophoretic mobility shift assays and transient transfection assays revealed that the NFκB subunits, p65 and p50 bind directly to sequences within the -66 to +7 region of the CXCR4 promoter and activate transcription [Helbig G et al, 2003].

![Fig. 3.3. Analyses of putative binding sites for RelA and Nrf2 within the cxcl13 promoter.](image-url)
Nrf2 is a TF that put together cellular stress signals. Nrf2 is able to respond through directing various transcriptional programs. Keap1 is the known negative regulator of Nrf2 [Gañán-Gómez I et al, 2013]. There is a controversy that whether NRF2 is a tumor suppressor or an oncogene [Kensler TW and Wakabayashi N, 2010]. During cellular homeostasis, Nrf2 is generally sequestered by its inhibitor, Keap1 in the cytoplasm, by interacting with Nrf2 N-terminal domain Neh2 [Itoh K et al, 1999; Katoh Y et al, 2005]. Subsequently, Nrf2 is degraded by 26S proteasome [Nguyen T et al, 2003; Kobayashi A et al, 2004]. However, during oxidative or electrophilic stress conditions, Keap1 releases Nrf2 [Kobayashi A et al, 2006; Rachakonda G et al, 2008]. Nrf2 then translocates into the nucleus and function as TF [Itoh K et al, 2003; McMahon M et al, 2003]. It has been well documented that Nrf2 takes part in the regulation of cell growth, apoptosis and tumorigenesis [Gañán-Gómez I et al, 2013; Jaramillo MC and Zhang DD, 2013].

![Fig. 3.4. Confirmation of RelA and Nrf2 expression clones: digestion of the cloned plasmids with BamH1 and Xba1. Double digestion of the cloned plasmids resulted in the release of linear vector of 5.4 kb and inserts of either RelA of ~1.6 kb or Nrf2 of ~1.8 kb size.](image)

In a collaborative study with our collaborator, Prof. Dmitry V. Kuprash and his group, we have demonstrated that how RelA regulates basic promoter activity of cxcr5 in MCF7 breast cancer cell line and identified specific RelA binding sites within the cxcr5 promoter [Mitkin NA et al, 2015]. Our approach in this study was to examine whether RelA is a common transcription factor of cxcl13 and cxcr5 and if so, then what are the other conditions that influence RelA-mediated transcription regulation. Sequential deletion of promoter region and luciferase assay has indicated the possible RelA-inducible sites. Moreover, we also aimed to find out mechanism of negative regulation of both cxcl13 and cxcr5 transcription. We have
found cxcr5 promoter is methylated in many patients associated with very low CXCR5 level and Nrf2 plays the role of negative TF for cxcl13 transcription.

![Nucleotide sequence of cxcl13 promoter](image)

**Fig. 3.5.** Nucleotide sequence of cxcl13 promoter, showing putative binding sites of RelA and Nrf2, and complementary binding sequences of primers designed for cloning of cxcl13 promoter in pGL3 basic reporter vector including various deletions. Red and blue colored sequences are putative binding sites for RelA and Nrf2, respectively. Highlighted sequences are primer binding sites. From top to bottom chronologically the sequences are for forward primer of “Full” promoter, forward primer of Del1 promoter, forward primer of Del2 promoter, forward primer of Del3 promoter, forward primer of Del4 promoter, forward primer of Del5 promoter, forward primer of Del6 promoter, forward primer of Del7 promoter, forward primer of Del8 promoter, forward primer of Del 9 promoter and the last one is the binding sequence for common reverse primer.

### 3.2. Materials and Methods

#### 3.2.1. Cell lines and culture

Human breast cancer cell lines MDA-MB-231 and T47D were procured from National Centre for Cell Science, India. Cells were cultured in DMEM (Gibco, Invitrogen, CA, US), supplemented with 10% FBS (Gibco, Invitrogen, CA, US), 100 U ml⁻¹ penicillin and streptomycin (Gibco, Invitrogen, CA, US) and maintained in a cell culture incubator at 37°C.
with 5% CO₂. Cells were transfected with lipofectamine 3000™ (Invitrogen, CA, US), according to manufacturer’s recommendations.

![Fig. 3.6. Confirmation of promoter deletion constructs in pGL3 basic luciferase reporter vector. (a) Double digestion of cloned plasmids with NheI and KpnI resulted in the release of linear pGL3 vector of 4.8 kb and inserts. (b) PCR amplification taking cloned plasmids as templates and using cloning primers of respective inserts. Gel bands at respective positions confirmed successful cloning of all the constructs.](image)

### 3.2.2. Clinical Samples

Post-operative primary tumor samples and associated healthy tissues were collected from 98 patients, diagnosed with IDC of breast. Permission was obtained from IEC of SGCC&RI [ECR/250/Inst/WB/2013] and informed consents were taken from patients. Tumors were graded by RB–protocol [Bloom HJ and Richardson WW, 1957], and staged by UICC-TNM classification [Brierley JD et al, 2016] (Table 2.1 and Table 2.2). Parts of freshly operated
tissues were immediately processed for RNA and protein extraction, and rest of tissues was used for flow-cytometry.

**Fig. 3.7.** (a) Nucleotide sequence of cxcr5 promoter and its position in the genome. (b) Analysis report of CpG island within cxcr5 promoter studied by Methyl Primer Express™ software. Promoter of cxcr5 encompasses a 305 nucleotide long CpG island starting from 796th nucleotide to 1100th nucleotide of the sequence.
3.2.3. Bioinformatics analyses

The promoter sequence of cxcl13 was acquired from official website of NCBI. The genomic position of cxcl13 promoter was found to be from Gch37: 78432107 to 78433207. This covers -800 and +300, upstream and downstream of transcription start site (TSS), respectively (Fig. 3.1). Therefore, the size of cxcl13 promoter that we have considered for our experiments was 1101 nucleotide long.

Probable TFs for cxcl13 was identified using the online tool “Genomatix MatInspector” (Fig. 3.2). Prediction of putative RelA (Fig. 3.3a) and Nrf2 binding sites (Fig. 3.3b) within the cxcl13 promoter region was done by Mapper2.0.

3.2.4. Molecular cloning

RelA and Nrf2 coding sequences (CDS) were cloned in mammalian expression vector pcDNA 3.1 (+) vector (Invitrogen, CA, US) (Fig. 3.4). First, the CDS of RelA and Nfr2 were amplified by PCR from c-DNA templates using Q5-high fidelity DNA polymerase (New England Biolabs). Amplified products and vector were double digested using BamHI (New England Biolabs) and XbaI (New England Biolabs) restriction enzymes. Before overnight ligation with T4 DNA ligase (New England Biolabs), alkaline phosphatase (Thermo Scientific) treatment was carried out to minimize vector re-circularization and negative colony formation. For overexpression of CXCL13, cloned ORF of CXCL13 was used (OriGene Technologies, Inc., US). Region of cxcl13 promoter (Gch37: 78432107-78433207) was cloned into pGL3 basic luciferase reporter vector (Promega). Moreover, cxcl13 promoter variants containing sequential deletions were generated using PCR amplifications from genomic DNA using specific primers (Fig. 3.5). All variants of cxcl13 promoter and pGL3 vector were digested using NheI and KpnI before ligation with T4-DNA ligase. Escherichia coli DH5-α bacterial strain was used as host for all the cloning carried out. Full and all the 9 deletion variants of cxcl13 promoter were confirmed by restriction digestion and PCR amplification (Fig. 3.6).

3.2.5. Reverse transcription and PCR

Total RNA (5 µg) was reverse transcribed into c-DNA, using MMLV-RT (Invitrogen, CA, US), random hexamer (Thermo Scientific) and dNTPs (Promega). End point PCR and quantitative real-time PCR were performed to analyze mRNA expressions of CXCL13,
CXCR5, RelA, Nrf2, Snail, Slug, Vimentin, N-cadherin and MMP9. 18s rRNA was used as internal control. Luciferase mRNA expression was also quantified by real time PCR. Fold changes in qPCR are represented as relative values normalized to control and quantified in the terms of 2^ΔΔC_T. Experiments were performed thrice in triplicate.

**Fig. 3.8.** Analyses of CXCL13 and CXCR5 expression in T47D and MDA-MB-231 cell lines induced with or without RelA and Nrf2 overexpression. CXCR5 and CXCL13 mRNA levels were assessed by end point PCR, followed by agarose gel electrophoresis. 18s r-RNA was used as internal control. Gel bands show increased CXCL13 and CXCR5 expression in RelA-overexpressed cells. Decreased CXCL13 expression in RelA-Nrf2 cotransfected cells as compared to cells induced only with RelA overexpression.

### 3.2.6. Western Blotting

WB analyses were performed using 60 µg of denatured protein samples. Membranes were immunoblotted with primary antibodies: anti-p65 (Cell Signaling Technology), anti-Nrf2 (Cell Signaling Technology), anti-CXCL13, (Abcam), anti-CXCR5 (Epitomics), anti-β-actin (Santa Cruz Biotechnology), overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) at room temperature for 2 hr. Bands were developed using substrate luminol (Sigma Aldrich), developer solution (Kodak) and fixer solution (Kodak).

### 3.2.7. Immunohistochemistry

Immunohistochemistry of RelA and CXCR5 were performed on formalin-fixed, paraffin-embedded tissue sections of 3-5 µM. The sections were de-paraffinized, rehydrated, blocked and incubated overnight with primary antibodies at 4°C. HRP-conjugated secondary
antibodies were added at 1:250 dilutions. The slides were developed using DAB chromogen (Millipore) and counterstained with hematoxylin (Merck).

### 3.2.8. Flow cytometry

Cultured MDA-MB-231 and T47D cells were harvested through detachment using trypsin-EDTA. After washing the cell pellets, single cell suspensions (1 × 10^6) were incubated with anti-CXCR5 (Biolegend) antibody, conjugated with FITC. Flow cytometry was performed in BD-AccuriC6. Fc blocker (Biolegend) was used to minimize non-specific antibody binding. Experiments were performed with taking appropriate isotype control. Analyses were done using BD-AccuriC6 software.

![Fig. 3.9](image.png)

**Fig. 3.9.** Quantitative analyses of CXCL13 and CXCR5 mRNA expression by real time PCR in T47D and MDA-MB-231 cell lines transfected with or without RelA and Nrf2. Fold changes are represented as relative values normalized with 18s r-RNA and quantified. CXCL13 direct overexpression is considered as positive control for CXCL13 expression. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. One-way ANOVA (Bonferroni correction) was performed, where significance level stands for *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

### 3.2.9. Enzyme-linked immunosorbent assay

Quantitative determination of CXCL13 was performed using a commercially available kit (Ray Biotech) for sandwich Enzyme-linked immunosorbent assay (ELISA) that makes use of a capture antibody against human CXCL13 precoated onto a microplate. Human recombinant CXCL13 protein at serial concentrations was used to prepare a standard curve. Level of CXCL13 in lysates of breast cancer cell lines was estimated using the standard curve. A detection antibody conjugated with biotin specifically directed against CXCL13 protein was added to the wells. After further washings, HRP-conjugated streptavidin was added followed...
by addition of tetramethylbenzidine (TMB) substrate reagent. Finally stop solution was added to the wells and absorbance of the colored produced was measured using an ELISA reader (Multiskan™ GO, Thermo Scientific) at 450 nm. The sensitivity threshold of the test was 1.5 pg/mL.

**Fig. 3.10.** CXCL13 and CXCR5 protein expression analyzed by ELISA and flow cytometry, respectively. (a) Bar graphs showing intracellular CXCL13 concentration in pg/ml per 100 µg of total protein. It shows significantly increased CXCL13 protein levels in both cell lines with either transfection combination of RelA and Nrf2. CXCL13 direct overexpression is considered as positive control. (b) Histograms of fluorescence intensities of FL1 (FITC) showing changes in quantitative surface expression of CXCR5 in control and transfected T47D and MDA-MB-231 cell lines. Black colored histograms represent fluorescence intensity of isotype control sets. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. One-way ANOVA (Bonferroni correction) was performed, where significance level stands for *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
3.2.10. Luciferase assay

The mRNA expression of firefly luciferase was quantitatively measured by real-time PCR using specific primer. MDA-MB-231 and T47D cells were transfected with cloned plasmids of different deletion variants of cxcl13 promoter. Cells were co-transfected with or without overexpression clone of RelA. Relative luciferase mRNA expression was considered as indicator of relative CXCL13 promoter activity. Transfection with empty pGL3 vector was considered as negative control.

3.2.11. Methylation-Bi-sulfite conversion assay

Promoter region of cxcr5 (GRCh38.p7: 118882966 to 118884066) (Fig. 3.7a) encompasses a 305 nucleotide long CpG island (Fig. 3.7b), identified by Methyl Primer Express™ software (Applied Biosystems). Methylation specific primer (MSP) and bi-sulfite specific primer (BSP) were designed using the same software. Genomic DNA (500ng) extracted from primary breast tumors were bi-sulfite converted using Methyl Edge® Bisulfite Conversion System (Promega). End point PCR was performed with unconverted and bi-sulfite converted genomic DNA templates and agarose gel bands were analyzed for CpG-methylation.

3.2.12. Statistical analyses

Association between gene expression and clinicopathological characteristics was analyzed by Fisher’s exact test. 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 SPSS Statistics 17.0 and OriginPro8. All data are presented as mean ± SD and p-value of ≤ 0.05 was considered statistically significant.

3.3. Results

3.3.1. RelA induces cxcl13 transcription in T47D and MDA-MB-231 cells

Bioinformatics analyses of cxcl13 promoter region (Gch37: 78432107- 78433207) (Fig. 3.1) indicated that RelA could be a potential TF for cxcl13 transcription (Fig. 3.2). From analyses in Mapper 2.0 database, we have identified three putative binding sites of RelA within the cxcl13 promoter (Fig. 3.3). To examine the potential of RelA to regulate cxcl13 transcription, T47D and MDA-MB-231 cells were transfected with expression clone of RelA. We used direct overexpression of CXCL13 as positive control. End-point PCR gel bands have
indicated higher CXCL13 mRNA expression in RelA-overexpressed cells of both cell types (Fig. 3.8). Further, we have performed quantitative real time PCR and observed significantly ($p < 0.05$) elevated mRNA fold change in RelA-induced cells. Increase of CXCL13 mRNA expression in RelA-induced T47D and MDA-MB-231 cells were 8.4 and 12.6 fold, respectively (Fig. 3.9). Correspondingly, mRNA fold change in positive control sets was even significantly higher than that of RelA-induced cells. Results signify that RelA positively regulates cxcl13 transcription.

To confirm the previous report that RelA is a positive regulator of cxcr5 transcription, we also verified CXCR5 mRNA expression in RelA-induced cells. As expected, significantly raised CXCR5 mRNA expression was observed in gel bands as well as in qPCR. CXCR5 mRNA fold changes were 9.2 and 11.3 fold in T47D and MDA-MB231 cells, respectively. However, direct induced overexpression of CXCL13 did not show any significant effect in cxcr5 transcription.

3.3.2. Nrf2 is a negative regulator of RelA-induced cxcl13 transcription

Sufficient RelA translocation does not necessarily mean that it is associated with significant expression of both CXCL13 and CXCR5 within primary tumor tissues, though our results suggest that RelA is a positive regulator of CXCL13 expression and previously we have reported that RelA positively regulates CXCR5 expression. Therefore, there should be some other means of regulation present inside the cell which can affect cxcl13 and cxcr5 gene transcription. Genomatix matinspector analyses have indicated that Nrf2 (alternate name NFE2L2) could also be a TF of cxcl13 (Fig. 3.2) and we learned from Mapper 2.0 database that two putative binding sites of Nrf2 are present within the cxcl13 promoter (Fig. 3.3). Therefore, we have investigated that whether Nrf2 can modulate RelA-induced cxcl13-transcription in vitro.

T47D and MDA-MB-231 cells were co-induced with Nrf2 and RelA-overexpression. Interestingly, we have found a significant reduction ($p < 0.05$) in cxcl13 transcription, when cells were co-induced with RelA and Nrf2 rather than sole induction with RelA (Fig. 3.8 and 3.9). However, CXCL13 mRNA expression was still significantly higher compared to control cells (Fig. 3.9). Fold changes of CXCL13 mRNA in co-transfected cells were 5.2 and 7.8 in T47D and MDA-MB-231 cells, respectively (Fig. 3.9). No significant change in cxcr5 transcription was detected in either breast cancer cell types.
Fig. 3.11. Relative expression of luciferase mRNA in T47D and MDA-MB-231 cells transfected with cxcl13 promoter constructs cloned into pGL3 basic luciferase reporter vector. “Fu” denotes pGL3 construct with full cxcl13 promoter, “D1-D9” denotes deletion constructs from 1 to 9. Bar graphs are showing relative luciferase mRNA expression. Amplification plots and Melt curves of the real time PCR are included. Amplification plots clearly indicating the difference in luciferase mRNA level in empty vector-transfected, full cxcl13 promoter-cloned-transfected and del4-cloned-transfected cells. Results are representative of three independent experiments performed in triplicate and are represented as mean ± SD. One-way ANOVA (Bonferroni correction) was performed, where significance level stands for *p ≤ 0.05.

Our observation clearly indicates a negative regulatory effect of Nrf2 in RelA-induced cxcl13 transcription. This finding is very important as the role of Nrf2 in cancer is still a matter of debate [Kensler TW and Wakabayashi N, 2010]. However, it has been unanimously documented that expression of CXCL13 is associated with cancer promotion. Therefore, in our case, Nrf2 may be playing the role of a tumor suppressor.
3.3.3. **RelA overexpression significantly increases intracellular CXCL13 protein level, sensitive to Nrf2 overexpression**

Additionally, we have performed intracellular CXCL13 protein estimation by sandwich ELISA to validate our findings in mRNA level. Correspondingly, we have found significantly ($p < 0.001$) elevated CXCL13 protein level in RelA-induced cell lines. Moreover, similar to mRNA expression, Nrf2 overexpression also significantly ($p < 0.05$) decreases RelA-induced CXCL13 protein expression. The intracellular concentration of CXCL13 protein in control, RelA overexpressed, RelA-Nrf2 co-overexpressed and CXCL13 overexpressed cells were 221.5 pg/ml, 1116.2 pg/ml, 792.6 pg/ml and 2865.2 pg/ml, respectively, in MDA-MB-231 cells, and 85.8 pg/ml, 1065.6 pg/ml, 752.4 pg/ml and 2932.5 pg/ml, respectively in T47D cells, estimated in 100 ug of total protein (Fig. 3.10a).

3.3.4. **Increased surface expression of CXCR5 in RelA-induced breast cancer cells**

We further looked at the surface expression of CXCR5 upon induction with RelA and Nrf2-overexpression because functionality of CXCR5 depends on its’ expression on cell surface. Surface expression CXCR5 in control and induced breast cancer cell lines were analyzed by performing flow cytometry using FITC-conjugated anti-CXCR5 antibody without making the cells permeable. We have observed increased surface expression of CXCR5 upon RelA overexpression. No significant effect of Nrf2 was observed for surface expression ($p > 0.05$) of CXCR5 in RelA-induced cells. Mean fluorescence intensities (MFI) values of control, RelA overexpressed, RelA-Nrf2 co-overexpressed and CXCR5 overexpressed (positive control) cells were 1706.79, 7953.36 (4.66 fold), 8081.02 (4.73 fold) and 21184.27 (12.41 fold), respectively, in T47D cells, and 2234.27, 5676.01 (2.54 fold), 5787.13 (2.59 fold), 30298.28 (13.56 fold), respectively, in MDA-MB-231 cells (Fig. 3.10b).

3.3.5. **RelA increases activity of cxcl13 promoter**

To ascertain about the direct influence of RelA in cxcl13 transcription, we have cloned cxcl13 promoter region in pGL3 basic luciferase reporter vector (Fig. 3.5 and 3.6) and co-transfected with or without RelA in breast cancer cell lines. Empty pGL3 vector was transfected as negative control. Promoter activity of cxcl13 was measured by quantifying luciferase mRNA by qPCR. Results clearly suggested for a significantly ($p < 0.05$) increased
cxcl13 promoter activity in RelA overexpressed cells (4.78 and 4.68 fold in T47D and MDA-MB-231 cells, respectively). Further, we aimed to verify whether RelA actually accommodates itself in any of the three predicted putative binding sites within the cxcl13 promoter, and for that, we have performed nine sequential deletion of the selected promoter region of cxcl13 (Fig. 3.6).

Fig. 3.12. CpG methylation analyses of cxcr5 promoter in genomic DNA samples from tumor tissues. (a) IHC sections showing high RelA expression in primary tumor tissues from patients bearing serial numbers 35, 57, 61, 75, 84. 'T' denotes tumor. (b) WB analyses of CXCR5 in same set of tumor lysates. It shows positive CXCR5 expression in 35T, 57T, 75T and no CXCR5 expression in 61T and 84T. (c) PCR from genomic DNA samples from these patients using MSP, specific for CpG-methylation of cxcr5 promoter. Positive bands in 61T and 84T indicate CpG-methylation of cxcr5 promoter in these tumors.

Analyses of luciferase mRNA level indicated that deletion of the first RelA binding site (CDS position -94735 to -94726) which is positioned between del1 and del2, has no significant impact on RelA-mediated cxcl13 promoter activity (Fig. 3.5 and 3.11). However, a significantly decreased ($p < 0.05$) promoter activity was observed when the region encompassing the second binding site (CDS position -93907 to -93898) is deleted (transfection with del4, fold change 2.45 fold and 2.89 fold in T47D and MDA-MB-231 cells, respectively). Notably, a further fall in luciferase mRNA expression ($p < 0.05$) was found in del9-transfected cells and the third RelA binding site (CDS position -94440 to -94431) is positioned between del8 and del9 (Fig. 3.5 and 3.11). Fold changes in luciferase mRNA expression in del9 transfected cells were 1.13 and 0.96 fold, respectively.
3.3.6. Promoter of cxcr5 is CpG-methylated in patients with high RelA and low CXCR5 expression within the primary tumor

We have examined the intra-tumoral RelA expression by IHC and WB in representative patient samples and analyzed RelA expression with CXCL13 and CXCR5 expression scores. Interestingly, we have found that in many of the primary tumors where significant intra-tumoral RelA expression is present, lack a considerable CXCR5 expression (Fig. 3.12). Through examining the promoter region of cxcr5 gene (Fig. 3.7a), we have identified a 305 nucleotide long CpG island (Fig. 3.7b).

It is well known that methylation in promoter-CpG island is associated with decreased gene transcription. Therefore, we have analyzed patients’ genomic DNA for CpG methylation in the promoter of cxcr5. Significantly, we observed an inverse association between CpG-methylation and CXCR5 expression within the primary breast tumor. For instances, high RelA expression is found in primary tumor of patients numbers 35, 57, 61, 75 and 84. Among these, positive CXCR5 expression is present in tumors 35, 57, 75. No CXCR5 expression is found in tumors 61 and 84 in WB. Correspondingly, highly positive band (164bp) with MSP is detected for samples 61 and 84 (Fig. 3.12). This clearly indicates that promoter CpG-methylation within cxcr5 promoter can negatively regulate intra-tumoral cxcr5 transcription, at least true for patients of Indian origin.

3.4. Discussion

CXCL13 is overexpressed in the primary tumor tissues of breast cancer. Earlier, it has been shown that CXCL13-CXCR5 ligand-receptor pair is important because it is associated with cancer progression and metastasis [Panse J et al, 2008; El Haibi CP et al, 2010]. We have reported that CXCL13 induces EMT of breast cancer cells [Biswas S et al, 2014]. Therefore, comprehensive understanding of the molecular mechanism of both cxcl13 and cxcr5 transcription regulation is utmost important. In a joint effort, we have already shown that how p53 and RelA regulates cxcr5 gene transcription [Mitkin NA et al, 2015]. However, transcription regulation of cxcl13 is not well documented. As cxcl13 promoter region also encompasses putative RelA binding sites (Fig. 3.3), we aimed to investigate whether RelA is also a TF of cxcl13. Our data suggests that RelA positively regulates cxcl13 transcription. Moreover, we have identified that out of the three putative binding sites, two are important for RelA-induced cxcl13 transcription.
Fig. 3.13. Schematic representations of cxcl13 and cxcr5 transcription regulation. (a) It represents how positive regulation of cxcl13 transcription is mediated by RelA and how Nrf2 performs as a negative regulator of cxcl13 transcription. (b) It represents how RelA-mediated positive regulation of cxcr5 transcription is inhibited by methylation at promoter CpG island.

Additionally, we have explored the suppressive function of Nrf2 in cxcl13 transcription. Nrf2 is a positive regulator of cytoprotective genes which are involved in the cellular defense mechanisms against oxidative stress [Ma Q, 2013]. Although, recent studies have demonstrated that high levels of Nrf2 protein in several human malignancies might be advantageous for cell growth and chemical resistance, and is finally associated with either good or worse prognosis in these patients, but still, the pathological and clinical significance of Nrf2 has remained largely unknown in breast cancer [Kensler TW and Wakabayashi N, 2010]. However, our results suggest that Nrf2 is playing tumor suppressive function by downregulating cxcl13 transcription.

In addition to that, we have also pointed a probable inhibitory mechanism of cxcr5 transcription regulation. Our discovery that promoter of cxcr5 is CpG-methylated and is inversely associated with CXCR5 level is novel. Uniqueness of the study lies where it
proposes RelA as a common TF of both cxcl13 and cxcr5. Additionally, it is the first report where we have examined the role of Nrf2 in cxcl13 transcription.

In summary, RelA positively regulates transcription of both cxcl13-cxcr5 ligand receptor pair (Fig. 3.13). However, negative regulation mechanisms are different for cxcr5 and cxcl13. Nrf2 plays the role of inhibitory TF for cxcl13 transcription (Fig. 3.13a). Conversely, cxcr5 transcription is inhibited by methylation of the promoter-CpG island (Fig. 3.13b).