Regulation of MMP-1 and TIMP-3 in the development of ovarian endometriosis
3.1. INTRODUCTION

Inflammation is intriguingly associated with endometriosis and different inflammatory cytokines are elevated in endometriosis patients (Harada et al. 2001). The proinflammatory cytokines TNF-α and IL1β play essential roles in initiating the cascade of cytokine production in the inflammatory response (Ilie and Ilie 2013). TNF-α and IL1β levels are significantly increased in the peritoneal fluid of women with endometriosis and are also positively correlated with menstrual pain (Scholl et al. 2009). TNF-α mediated pathways are reported to promote NFκB-dependent activation (Wieser et al. 2005), positively modulating the proinflammatory cytokines like macrophage migration inhibitory factor (MIF), IL-8, IL-6, TNF-alpha, intercellular adhesion molecule (ICAM)-1, granulocyte macrophage colony-stimulating factor etc (Gonzalez-Ramos et al. 2010).

MMP-1 is a member of collagenase family, is reported to cleave mainly cleave interstitial collagens I, II and III into characteristic 3/4 and 1/4 fragments but they can also digest other ECM molecules and soluble proteins (Aimes and Quigley 1995; Page-McCaw et al. 2007). MMP-1 is involved in many diseases including fibrosis as well as cancer (Duarte et al. 2015; Liu et al. 2012). Studies documented involvement of MMP-1 in endometriosis patients (Gottschal et al. 2002). In vitro study found TNF-α mediated upregulation of MMP-1 responses in endometriotic stromal cells (Pino et al. 2009). Studies suggested that MMP-1 is not exclusively regulated by inflammatory factors; but non-inflammatory factors like prostaglandin E2 can also upregulate MMP-1 in condrocytes (Guan et al. 2015). In prostaglandin E synthase-1 null mouse, reduced MMP responses was reported upon IL1β mediated inflammation (Gosset et al. 2010).

Transcriptional regulation of MMP-1 is mediated via AP-1 mediated signaling responses, which are activated through several external stimuli including growth factors, cytokines and cellular stress signals (Yan and Boyd 2007). Moreover, other cis-acting elements like PAE3 acts cooperatively on AP-1 for transcriptional regulation of MMP-1 (Benbow and Brinckerhoff 1997). MMP-1 contains a promoter polymorphism at position -1607 resulting 2G allele together with adjacent nucleotide, which creates a binding site for ETS-1 and reported to have increased transcriptional activity for 2G allele (Rutter et al. 1998). The NFκB is also reported to contribute to the regulation of MMP-1 expression, even though no functional NFκB-binding site is present within the proximal promoter region of MMP-1 gene (Vincenti and Brinckerhoff 2002). Endometriosis is reported to
have decreased expressions of TIMP-3 with disease progression (Paul et al. 2010). Unlike other TIMPs, TIMP-3 is reported to have anti-inflammatory responses by blocking the release of soluble TNF-α (Black 2004). Moreover, evidences indicate the important role of TIMP-3 in promoting apoptotic responses (Ahonen et al. 2003). TIMP-3 is the only MMP reported to have ECM binding properties (Yu et al. 2000) and reported to inhibit MMP and ADAM family members of proteins (Brew and Nagase 2010). This present chapter highlights the role and regulation of MMP-1 in endometriosis both in clinical samples and in vitro study. The results of the current study provide a mechanistic basis for MMP-1 upregulation in endometriosis via different factors associated with the disease. Involvement of TIMP-3 in regulation of MMP-1 is also elucidated in the present study.

3.2. OBJECTIVES

The objective of the present study is to elucidate the involvement of MMP-1 and TIMP-3 in pathogenesis of ovarian endometriosis. The other objective is to understand the regulation of MMP-1 expression by interleukin1β and protaglandinE2 mediated responses in cultured endometriotic cells.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals: Gelatin, Triton X-100, chemical inhibitors, prostaglandin E2, protease inhibitors mixture, Masson’s Trichrome staining kit, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, 3,3’,5,5’-Tetramethylbenzidine and chemical inhibitors were obtained from Sigma Aldrich Inc, St. Louis, MO, USA. Recombinant IL1β was purchased from Invitrogen, USA. ELSA kit for human TNF-α (DY210), CXCL/IL-8 (DY208), MMP-1 (DY901) was purchased from R&D system, USA. Antibodies, siRNA for control (SC-37007), MMP-1(SC-41552), TIMP-3(SC-37022), cJUN (SC-29223) and transfection medium and reagents were obtained from Santa Cruz Biotechnology Inc, California, USA. The Trizol reagent, molecular biology products (RNase H, Taq polymerase, dNTPs) were purchased from Invitrogen, USA. DMEM, FBS and antibiotics are purchased from Gibco, Thermo Fisher Scientific, USA. All other chemicals were purchased from Sisco Research Laboratories, Mumbai, India.

3.3.2. Human Study: As described in chapter 2.
3.3.3. **In vitro study:** Endometriotic cell Hs832 was purchased from ATCC, USA. MDAMB-231 breast cancer cells were procured from NCCS, Pune, India. Hs-832, and MDAMB-231 cells were grown in plastic cell culture dishes in 95% air/5% CO₂ in DMEM supplied with 20 mM HEPES, 10% heat-inactivated FBS, 2 mM-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

HS832 cells were treated with recombinant IL1β (0.1-10 ng/ml), prostaglandin E2 (0.1-10 μM/L) or both for 24 hr or 2 hr. Inhibitor for MEK (PD184352), ERK (PD98059), JNK (SB39063), P38 kinase (SP600125) were treated along with IL1β (1 ng/ml) or PGE2 (1 μM/L) or both treatments for 24 hr in experimental medium.

SiRNA experiment was performed in MDAMB-231 cells using 6 well plates with 60% confluent cells grown under antibiotic-free condition. SiRNA from Santa Cruz Biotechnology Inc for MMP-1, TIMP-3, cJUN and control were treated as per the manufacturers’ protocol. Treatment with IL1β (1 ng/ml) + PGE2 (1 μM) and invasion and migration assay was performed after 48 hr of siRNA transfection with conformation of silencing. Cell supernatant was used for ELISA, gelatin zymography and cell extract was used for immunoblotting.

3.3.4. **Cell and tissue extraction and protein estimation:** As described in chapter 2.

For preparation of nuclear extract, cells or tissues were minced in ice cold PBS and centrifuged at 1000g for 5 min. Pellets were resuspended in low salt buffer (10 mM HEPES pH 7.9, 1.5 mM MgCl₂ and 10 mM KCl) and vigorously mixed after addition of 20 ml of 10% NP-40. Nuclei were collected followed by centrifugation at 12,500g and resuspending in 50 ml of high salt buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂ and 0.2 mM EDTA and 25% glycerol). Proteins were estimated either by Lowry method or Bradford assay.

3.3.5. **Enzyme-Linked Immunosorbent Assay (ELISA):** ELISA was performed using R&D system ELISA kit for cell supernatant, human serum and ectopic tissue extracts as per the user manual instructions. In brief, 96-well microplate was coated with prescribed dilution of the capture antibody for overnight in room temperature. Each well was aspirated with Wash Buffer for a total of three times and blocked with plates by adding 300 µl reagent diluent for 1 hr followed by 3 times washing. Then samples (serum/cell sup 100 µl, tissue 300 µg/per well) or standards in reagent diluents, or an appropriate
diluents, was added and incubated for 2 hr followed by 3 times washing. 100 μl of the detection antibody, diluted in reagent diluents was added to each well and incubated for 2 hours at RT followed by 3 times washing. Next 100 μl of the working dilution of streptavidin-HRP was added to each well for 20min in dark followed by washing. 100 μl of TMB (3,3′,5,5′-Tetramethylbenzidine) substrate Solution was added for colour development followed by addition of 50 μl of stop solution (2N sulfuric acid) to each well. OD was measured at 450 nm using a ELISA reader with reference corrections of 570 nm.

3.3.6. Viability assay: 10^4 cells were seeded into each well of a 96 well plate after containing fresh media and then the plate was incubated at 37°C in presence of 5% CO₂ overnight to reach confluency. Cells were treated with PGE2 (0.1-10μmol/l), IL1β (0.1-10ng/ml), or both in experimental media. After 24hr, the media was discarded and working MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution was then added to each well and incubated for 3 hr. MTT solution was then discarded and dimethyl sulphoxide (DMSO) was added. The absorbance was measured at 570 nm with reference wavelength of 660 nm in a microtiter plate reader. Experiments were performed in triplicates.

3.3.7. Migration assay: Scratch/wound healing assays were performed using endometriotic cell Hs832, MDAMB231 cells. Wounds with a constant diameter were made. Cells were treated with PGE2 (0.1-10μmol/l), IL1β(0.1-10ng/ml), both and/or treated with inhibitors for, MEK, ERK, JNK, p38 kinase, or/and pretreated with SiRNAs for control, MMP-1, TIMP-3, and cJUN. After 24 h, images were captured in Olympus microscope using Camedia software (Chicago, MI, USA) (E-20P 5.0 megapixel) and processed using Adobe Photoshop version 7.0.

3.3.8. Invasion assay: As described in chapter 2. The invasion assays were performed with transwell Boyden chamber assay kit (BD Biosciences, MA. The invasion assays were performed with transwell invasion assay kit (Corning, NY,USA), using endometriotic cell Hs832, MDAMB231 cells. 25000 cells were added to upper chamber of the well with or without inhibitor or stimulators in a serum free condition. The lower chamber contained complete media (with 10% FBS). For siRNA treatments, SiRNA pretreated MDAMB-231 cells were used with IL1β+PGE2 treatments. After 24 hr, cells
from the upper chamber were removed and lower chamber cells were fixed and stained with 1% crystal violate. Images were captured in Olympus microscope using Camedia software (Chicago, MI, USA) (E-20P 5.0 megapixel) and processed using Adobe Photoshop version 7.0. Data are represented as the average of four counts ± SE.

3.3.9. Gelatin Zymography: As described in chapter 2.

3.3.10. Immunofluorescence: As described in chapter 2. Antibodies used for the present chapters are MMP-1(SC-12348), TIMP-3 (SC-6836), IL-1β (SC-7884), pJNK (SC-12882), cJUN (Sc-1694), ETS-1(SC-22802), Rabbit anti-goat IgG conjugated with FITC (SC-2777) or TR (SC-3919), goat anti-rabbit IgG conjugated with FITC (SC-2012) or TR (SC-2780).

3.3.11. Immunohistochemistry: As described in chapter 2. Antibodies used for the present chapters are MMP-1(SC-12348), rabbit anti-goat IgG HRP (SC-2768).

3.3.12. Collagen staining: Masson’s Trchorme staing kit (HT-15) was obtained from Sigma, USA. After deparaffinization, human tissue slides were heated at 70°C for 10 min in picric acid solution and washed in tap water for 5 minutes. Then Weigert's hematoxylin was added for 10 minutes and washed in running tap water for 5 minutes followed by addition of Biebrich scarlet solution for 5 minutes.After wasing in tap water for 5min, Phosphotungstic/phosphomolybdic acid (1:1 ratio) was added for 10 minutes. Then the slides were transferred directly into aniline blue solution for 5 minutes followed by washing in 1% acetic acid for 1 minute. Slides were dehydrated and fixed in DPX mounting medium. Images were captured at 10x and 40x magnifications in Olympus microscope using Camedia software (Chicago, MI, USA) (E-20P 5.0 megapixel) and processed using Adobe Photoshop version 7.0.

3.3.13. Western blotting: As described in chapter 2. Antibodies used for the present chapters were MMP-1(SC-12348), TIMP-3 (SC-6836), IL-1β (SC-7884), pJNK (SC-6254), JNK (SC-1648), pERK (SC-7676), ERK (SC-292838), pP38(SC-7975R), P38(SC-33688), pcJUN (SC-7981), cFOS (SC-7202), cJUN (SC-1694), ETS-1(SC-22802), GAPDH (SC-48167), H3 histone(SC-10809), β-actin (SC-69879), β-tubulin(SC-9935). Secondary antibodies used are ALP conjugated anti-goat(SC-105218), anti-mouse (SC-105207) and anti-rabbit IgG(SC-105206).

3.3.14. RNA isolation: For isolation of total cellular RNA, cell/ tissues were kept in 500 μl of Trizol reagent in sterilized centrifuge tubes and stored at -70°C. During RNA
extraction the samples were homogenized properly at 4°C with electrical homogenizer. 100 μl of chloroform (Trizol: Chloroform = 5:1) was next added and shaken vigorously by hand for 30 sec and incubated at room temperature for 5 min. The samples were then centrifuged at 10,000x g for 15 min at 4°C. This separates the mixture into a lower red phenol-chloroform phase containing protein, an white interphase containing DNA and a colorless upper aqueous phase containing RNA. The upper aqueous phase was then transferred to a sterile centrifuge tube and equal volume of isopropyl alcohol (1:1) was added followed by proper mixing followed by centrifugation at 10,000x g for 15 min at 4°C. The precipitated RNA was washed properly with 500 μl of ice cold 75% ethanol and was centrifuged at 10,000x g for 5 min at 4°C. The pellet was air-dried partially for 15 min at room temperature and carefully dissolved in RNase free sterile 0.1% DEPC treated water by incubating for 10 min at 55°C. The purity of extracted RNA was judged by 260/280 ratio and was quantified by measuring the absorbance at 260 nm through Nanodrop 200c (Thermo Scientific, USA). The dissolved RNA was then stored at -80°C.

3.3.15. Reverse transcriptase-PCR: Complementary (c)DNA (1μl) was prepared using iscript cDNA synthesis kit (Bio-Rad, USA) and amplified in 20 μl of total reaction volumes using 10 pmoles of each primer (primers were designed with Primer 3 software), 0.2 mM of each dNTPs, 1.5 mM of MgCl2, 1U of Taq polymerase in a standard PCR buffer (Invitrogen, USA). PCR was carried out for 35 cycles of: (i) denaturation for 1 min at 94°C, (ii) primer-template DNA annealing for 1 min at 55-59°C, and (iii) DNA synthesis at 72°C for a time based on expected fragment size (1 min/kb) using the following primers (F,R): MMP-1(190bp), 5’CTACCCGGAAGTTGAGCTC3’, 5’CTAGGGAAGCCAAAGGAGCT3’; TIMP-3 (240bp), 5’AGACACTACCCTCCATGCC-3’, 5’CTTCCCTCTCCGCTCC AAAGA3’; cFOS(180bp), 5’TGTCTGTGGCTTCCCTTGAT3’, 5’ATCAAGGGGC TCGGTCTTCA3’; cJUN(180bp), 5’CGGAGAGGAAGCGCATGA3’, 5’CCTGTT CCCTGAGCATGTGTTG3’; GAPDH(230bp), 5’AGGTGGGTCAACGGATTT3’, 5’ATCTCGGCTCTGGAAAGGATG3’. The PCR products were analyzed by electrophoresis in 2% agarose gels and visualized by ethidium bromide staining. PCR product sizes were estimated by 100bp marker (Invitrogen) in each case.

3.3.16. Statistical analysis: Experiments were repeated for at least three times independently. Protein band intensities were quantified by densitometric analysis using
Lab image software. The statistical analysis of the data was done using GraphPad Instat-3 software. Comparison between groups was done using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test. Data were fitted using Sigma plot represented as means ± SEM. p < 0.05 was accepted as level of significance; *** highly significant p < 0.001; ** significant p < 0.01; * less significant p < 0.05; NS not significant for p > 0.05.

3.4. RESULTS

3.4.1. Involvement of inflammatory markers in endometriosis: To understand how inflammatory cytokines are involved in pathogenesis of endometriosis, we looked into TNF-α and IL-8 levels in serum of endometriosis patients of different stages (Fig. 1). A total of 88 patients and 15 control samples were used for the study and we found women with endometriosis show significantly elevated serum TNF-α level than control women (without endometriosis). Moreover, ELISA data revealed gradual upregulation of TNF-α levels in the endometriosis population with increased disease severity (Fig. 1A). Similar to TNF-α status, serum IL-8 level showed significantly increased expression for endometriosis patients in comparison to serum of control women (Fig. 1B). Furthermore, levels of IL-8 upregulated with the advancement of endometriosis. Endometriosis patients showed ~4-6 fold increased level of TNF-α in serum, while ~6-8 fold elevated level of IL-8 in comparison to serum of control women (Fig. 1A, B).

![Fig. 1: Involvement of inflammatory cytokines with the progression of ovarian endometriosis.](image)

(A) TNF-α and (B) IL-8 expressions were quantified using ELISA for serum samples of endometriosis and control samples. Number of patients stage1 (n=19), stage2 (n=14), stage3 (n=24), stage4 (n=31). HC represents healthy control (n=15).
3.4.2. Involvement of collagenase-1 (MMP-1) in endometriosis: Although MMP-1 was reported to be present in endometriosis (Gottschal et al. 2002), its involvement with severity of the disease is not well investigated. We looked into MMP-1 expression in the serum and ectopic tissues of endometriosis patients as well as control women through ELISA (Fig. 2). Our study found gradual increase in MMP-1 expression in serum samples of endometriosis patients with disease severity. In comparison to control women, the early stages of endometriosis patients showed no significant upregulation of MMP-1 levels in serum. However, in late stages of the disease serum MMP-1 level was significantly elevated compared to the control samples (Fig. 2A). To understand tissue specific expression, MMP-1 expression was quantified in ectopic ovarian endometriosis extract. In comparison to the minimal endometriosis, the ectopic expression of MMP-1 increased with advancement of the disease; and upregulated significantly at advanced stages (III-IV) of the disease (Fig. 2B). We confirmed our study with immunohistochemistry of MMP-1 at ectopic endometriosis. As depicted in Fig 2C, the MMP-1 expression elevated with increased severity of the disease and localization of MMP-1 is mainly restricted to the stromal regions, however at higher stages both stromal and glandular regions are MMP-1 positive (Fig. 2C).

Fig. 2: Expression of MMP-1 with the progression of ovarian endometriosis. MMP-1 expression was quantified using ELISA for serum (A) and ectopic tissue samples (B). Immunohistochemistry for MMP-1 in different stages of ovarian endometriosis (n=3). Number of patients stage I (n=19), stage II (n=14), stage III (n=24), stage IV (n=31). HC represents healthy control (n=15).
3.4.3. Status of TIMP-3 in ovarian endometriosis: To understand the status of endogenous inhibitors of MMP-1, we looked into TIMP-3 expressions. Unlike other TIMPs, TIMP-3 has matrix binding properties, which makes the molecule localized for its responses (Yu et al. 2000). Furthermore, TIMP-3 is involved in modulation of apoptotic responses, TIMP-3 deficient mice develops severe apoptotic responses (Fata et al. 2001). The expression profiles for TIMP-3 downregulated with advancement of disease; in comparison to the minimal endometriosis group (stage I), the expression significantly reduced with moderate and severe stages of endometriosis (stage III and IV) (Fig. 3A,B). Immunofluorescence study of TIMP-3 showed localization of TIMP-3 is mainly present at the glandular regions of endometriosis, which are minimally present in late stages of endometriosis. Figure C shows the representative blots for MMP-1 and TIMP-3. Interestingly, when MMP-1/TIMP-3 ratio was quantified with the mean value of each group (Fig. 3D), we found elevated values of the ratio, which suggested that reduced expression of TIMP-3 allow increased activity MMP-1 during progression of the disease.

Fig. 3: Expression of TIMP-3 with the progression of ovarian endometriosis. (A) TIMP-3 expression was quantified using western blotting for ectopic tissue samples stage I (n=19), stage II (n=14), stage III (n=24), stage IV (n=31). (B) Immunofluorescence study for TIMP-3 in early and late stage of endometriosis (n=3). (C) Representative western blot for MMP-1, TIMP-3 and (D) ratio of cumulative mean values for MMP-1 and TIMP-3 expressions in different groups.
3.4.4. **Status of collagen in ectopic ovarian endometriosis:** The major component of extracellular matrix, collagen is mainly produced by fibroblasts. Dysregulated collagen responses are associated with many inflammatory diseases, including fibrosis, cancer (Bateman et al. 2009; Nelson and Bissell 2006). Since, the quantity and quality of collagen can be grieving factor for inflammatory disease like endometriosis, we looked into collagen status in ovarian endometriosis in a stage dependent manner through Masson’s trichome staining (Fig. 4). We report increased collagen (in blue stain) in the ectopic sites of endometriosis. In early stages of endometriosis (with minimal and mild progression of the disease) collagen is minimally present. However, the extent of the collagen deposition gradually increased with advancement of disease. The increased levels of collagen in higher stages of endometriosis might result from upregulated production of collagen, and/or dysregulated collagenase activities. Interestingly, we found the nature of collagen also changed with disease severity. A closer look into the collagen (lower panel) will show increased deposition of febrile collagen with fragmented and irregular depositions (Fig. 4). This is liable for the increased stiffness of the tissues, which can be an important factor for elevated cellular invasiveness of endometriotic cells.

**Fig. 4: Evaluation of collagen levels in ovarian endometriosis.** Messon’s trichrome staining was performed for collagen staining in a stage dependent manner (n=3) in formalin-fixed ectopic endometriosis tissues. Red stain represents the muscle cells, whereas blue stains indicate the collagen. The lower panel magnifies the boxed area of the upper panel.
3.4.5. Effect of interleukin1β and prostaglandin E2 on gelatinases in endometriotic cell Hs832. The purpose of the present study was to understand how inflammation influences MMPs in disease specific cell line Hs832. We were also curious about PGE2, because of its role for upregulation of MMP-2 in human endothelial cells. To understand the roles of PGE2 and IL1β, we treated endometriotic cell Hs832 with PGE2 and recombinant IL-1β in a dose dependent manner for 24 hr. Gelatin zymography was performed to assess the MMP-2 and MMP-9 activity from the cell supernatant(Fig. 5). Interestingly, we found elevated active MMP-2 activities, but no active MMP-9 upregulation upon PGE2 treatment. The effect was similar to HUVEC cells, indicating the role of PGE2 over MMP-2 is not exclusive for endothelial cells. Moreover, IL1β treatment showed increased active MMP-9 responses, but no significant changes were observed for active MMP-2 activities, which indicate that inflammation principally influences MMP-9(Fig. 5). The upregulation for MMP-9 and MMP-2 by IL1β and PGE2 respectively also suggest that these factors regulate the two gelatinases in a differential manner. To our surprise, when we treated Hs832 with IL1β and PGE2 together, both active MMP-9 and -2 activities elevated. Similar upregulation of MMP-9 and -2 was observed in ectopic tissues of endometriosis; suggesting that in pathogenesis of endometriosis inflammatory and angiogenic factor influence together.

Fig. 5: Effects of PGE2 and IL1β on gelatinases for Hs832. Endometriotic cell Hs832 were treated with prostaglandin E2 (0.1-1µM) or interleukin 1β (0.1-1ng/ml) or both in a dose dependent manner for 24hr and gelatine zymography was performed using 10µl of cell supernatant (A). Quantification of zymogram for MMP-9 (B) and MMP-2 (C) activities.
3.4.6. Effect of interleukin 1β and prostaglandin E2 on MMP-1 in endometriotic cells. To understand the roles of PGE2 and IL1β on MMP-1 expression, we treated endometriotic cell Hs832 with PGE2 and recombinant IL1β in a dose dependent manner (0.1-10µM/L or ng/ml) for 24 hr. MMP-1 expression was quantified with ELISA from the cell supernatant. To our surprise, we found both IL1β and PGE2 was able to upregulate MMP-1 level in a dose dependent manner. More interestingly, treatment with both IL1β and PGE2 synergistically effected MMP-1, upregulating the expressions(Fig. 6A).

**Fig. 6: Effects of prostaglandin E2 and interleukin 1β on MMP-1 and endometriotic cell behavior.** Hs832 were treated with prostaglandin E2 (0.1-10µM) or interleukin 1β(0.1-10 ng/ml) or both for 24hr and MMP-1 expression was quantified using cell supernatant/ELISA (A). MTT assay was performed for cellular viability for all treatments (B). Effect of prostaglandin E2 or interleukin 1β or both treatments on cellular migration (C) and invasion (D).

Furthermore, cellular viability was significantly reduced with higher doses of IL1β (10ng/ml) treatments, while no significant changes in viability were observed upon PGE2 treatments. Interestingly, PGE2 alongwith higher doses of IL1β rescued reduced viability (Fig. 6B), suggesting that the presence of an angiogenic factor with
inflammation influences cellular viability and might promote apoptotic resistance in endometriosis. Cellular migration assay was performed for 24 hr with IL1β and PGE2 treatments and found to be increased percent of migrated cells for lower doses (0.1-1ng/ml) of IL1β treatments. Higher doses(10ng/ml) of IL1β significantly inhibited cellular migration in Hs832, whereas PGE2 dose dependently elevated cellular migration in endometriotic cells(Fig. 6C). Moreover, transwell invasion assay with selective dose showed elevated cellular invasiveness for IL1β and PGE2 treatments (Fig. 6D). Treatment with both IL1β and PGE2 has cumulative responses over cellular migration and invasion. PGE2 significantly elevated percentage of cellular migration even in higher dose of IL1β treatment, which suggested that presence of increased PGE2 in inflammatory diseases like endometriosis aggrave disease progression by promoting cellular invasion and viability.

3.4.7. MAPK signaling pathway is involved with MMP-1 upregulation. Total cell extract were immunoblotted for recombinant IL1β (1ng/ml) and PGE2 (1µM/L)treatment to look into MMP-1 expressions. Similar to ELISA, we found increased MMP-1 expressions upon both treatments, moreover, together the MMP-1 expression upregulated more (Fig.7A,B). We looked into TIMP-3 expression in cell extract, and found that TIMP-3 expression significantly downregulated upon PGE2 treatments with or without IL1β in Hs832 cells(Fig.7A,B). IL1β only treatment also downregulated TIMP-3 expressions, however the reduction was relatively less significant, which might result due to anti-inflammatory responses of TIMP-3.
To understand the cellular signaling pathways that were involved with MMP-1, we looked into MAPK (mitogen-activated protein kinases) pathways. HS832 endometriotic cells were treated with IL1β, PGE2 or both in a time dependent manner for up to 2hr and whole cell extract were subjected for Western blotting (Fig. 7C). Time dependent response for pERK (phosphorylated extracellular signal-regulated kinases) showed increased expressions for all treatments. For IL1β and IL1β+PGE2 treatments, the pJNK (phosphorylated cJUN N-terminal kinase) expression upregulated significantly. We also found increased phosphorylated P38 expressions for IL1β and IL1β+PGE2 treatments(Fig. 7C). The present result indicated involvement of pP38 and pJNK responses involved with the IL1β+PGE2 treatments and further modulated the downstream transcription factor that regulates MMP-1 expressions. As the MAPK signaling pathway is reported to directly affect cFOS and cJUN mediated AP-1 transcription factor for MMP-1 upregulation(Nelson et al. 2006), phosphorylated cJUN and cFOS expressions were evaluated from nuclear extract. The pcJUN and cFOS expressions significantly upregulated in IL1β and IL1β+PGE2 treatments (Fig.7D). Interestingly, IL1β+PGE2 treatment also significantly upregulated ETS-1 expressions in nuclear extract (Fig.7D). PEA3, which is a subfamily of ETS transcription factors, present and acts as a co-activator of AP-1 on MMP-1 promoter and co-ordinately regulate MMP-1 expressions(Benbow and Brinckerhoff 1997).

3.4.8. Inhibition of JNK attenuated MMP-1 expression and cellular invasion in endometriotic cell. To explore further, the HS832 cells were treated with chemical inhibitors for MEK, ERK, JNK, and P38 upon IL1β+PGE2 treatment(Fig.8). Among specific inhibitors for MAPK pathways, inhibition for JNK most significantly attenuated MMP-1 upregulation. The inhibition of P38 also reduced MMP-1 expressions significantly. The expressions for TIMP-3 showed negative correlation with MMP-1(Fig.8A). Interestingly, inhibition of P38 kinase was found to significantly downregulate ETS-1 expression (Fig.8C). Similar to MMP-1 results, inhibition for JNK showed significantly reduced cellular invasion of endometriotic cells(Fig.8D). To further understand the contribution of PGE2 and IL1β in regulation of MAPK pathway, we
treated cells with MAPK inhibitors with only IL1β or PGE2 treatments. We found PGE2 mediated MMP-1 was regulated through ERK and JNK pathways, whereas IL1β mediated MMP-1 expressions were regulated mainly through JNK-mediated pathway (Fig. 8E).

**Fig. 8: Involvement of MAPK kinase pathways for MMP-1 in endometriotic cells.** Inhibitors for MAP kinase pathways were treated along with IL1β(1ng/ml)+PGE2(1µM/L) treatments for 24 hr. Cell lysate were used for quantification of MMP-1, TIMP-3 by Western blotting (A,B). Nuclear extract were used for assessing transcription factor expressions(C). Invasion assay was performed for 24hr upon inhibitor treatments(D). Inhibitors for MAP Kinase pathways were treated along with either IL1β(1ng/ml) or PGE2(1µM/L) treatments for 24 hr(E).

### 3.4.9. cJUN involved in JNK-mediated MMP-1 upregulation and cellular invasiveness.

From the previous study, we identified involvement of JNK and pcJUN mediated responses for MMP-1. However, to confirm further knock down of MMP-1 was performed through specific siRNAs transfection in MDA MB-231 cell line. After conformation of inhibition of MMP-1, cells were treated with IL1β (1ng/ml)+PGE2(1µmol/L) for 24 hr and status of MMP-1 was assessed through immunoblotting. Silencing of MMP-1 significantly reduced cellular invasion (Fig.9).

Moreover, silencing of TIMP-3 by siRNA showed significant upregulation for MMP-1 expression, although, failed to reach significant level for cellular invasion in comparison to control siRNA treatment (Fig.9A,C-E). The involvement of cJUN on MMP-1 expression was confirmed through siRNA-mediated silencing of cJUN. Knock down of
cJUN significantly inhibited MMP-1 expression (Fig. 9A, B), confirming its involvement on MMP-1 transcription. Knock down of cJUN also significantly inhibited cellular invasion, through downregulated MMP-1 expression (Fig. 9D-G).

Fig. 9: Involvement of cJUN pathways for MMP-1 expressions. Cells were transfected with specific siRNAs for MMP-1, TIMP-3 and cJUN. After inhibition was confirmed, cells were treated with IL1β (1ng/ml)+PGE2 (1µM/L) for 24 hr. Whole cell lysate were used for Western blotting (A-C). Transwell assays were performed for 24hr to assess cellular invasiveness upon SiRNA treatments (D,E).

3.4.10. Involvement of JNK-cJUN pathway in human ovarian endometriosis. The present study elucidates the important roles of AP-1 for MMP-1 expression through JNK-mediated phosphorylation of cJUN in endometriotic cell Hs832. To confirm the involvement for these molecules in endometriosis samples, immunofluorescence was performed (Fig. 10A). Elevated IL1β expressions were observed in late stages of endometriosis, localized at the stromal regions. Increased expressions for phosphorylated JNK and phosphorylated cJUN were observed with advancement of the disease. In addition, pcJUN was mainly localized at the glandular regions, while pJNK was positive for both stromal and glandular regions. ETS-1 expression was found to be elevated in late stages of endometriosis, and localized specially at the glandular regions. We further confirm the elevated responses of these genes through RTPCR with disease dependent ectopic ovarian endometriosis, where mRNA levels for MMP-1, cJUN and cFOS elevated with disease progression, while TIMP-3 expression decreased with disease severity (Fig. 10B).
3.5. DISCUSSION

Endometriosis is associated with increased levels of pro-inflammatory cytokines. The present study reassured the fact that increased levels of TNF-α and IL-8 are involved in the pathogenesis of endometriosis in a severity dependent manner. It is not well established whether MMP-1 can be marked as a pro-inflammatory marker and till now, limited studies have reported the presence of MMP-1 in endometriosis. Moreover, the roles and regulation of MMP-1 in the disease progression remain completely unexplored. The present study found MMP-1 expression significantly upregulated both in serum and ectopic tissues of ovarian endometriosis patients with the advancement of the disease. Furthermore, one of the endogenous inhibitors of MMPs, TIMP-3 expression downregulated with increased severity of the disease, suggesting reduced inhibition over MMP activities during progression of endometriosis. Because, MMP-1 is a member of collagenase family, and principally degrades collagen I, II, III and we looked into collagen status in endometriosis. We report increased collagen deposition with endometriosis progression, which might result from increased collagen production or less removal of collagens by means of dysregulated proteolytic responses. Abnormal collagen responses are associated with pathogenesis of different diseases (Bateman et al. 2009) and we found the deposited collagen in higher stages of endometriosis are more like...
fibrile collagen in nature. Disease like fibrosis are often associated with fibrile collagens, resulting in increase of tissue stiffness (Carver and Goldsmith 2013). Tissue stiffness is an important regulator for increased cellular invasiveness and often associated to cellular transition, like EMT (Kim et al. 2006). Recent studies reported increased cellular invasiveness (Guan et al. 2016) and EMT-like transition are associated with endometriosis (Matsuzaki and Darcha 2012) which may result from increased tissue stiffness due to dysregulated collagen deposition.

Inflammation mediated upregulation of MMP-1 was reported in endometrial-derived primary cells from endometriosis patients (Pino et al. 2009). To understand how MMP-1 is regulated in endometriosis, disease specific endometriotic cell (Hs832) was treated with recombinant IL1β and PGE2. The result show upregulated MMP-1 expressions for each treatment and proved that MMP-1 is not exclusively regulated by inflammation, but also by pro-angiogenic factor like PGE2. In support to our study, other diseases also showed elevated MMP-1 responses upon PGE2 and IL1β treatments (Guan et al. 2015). Interestingly, PGE2 and IL1β also upregulated MMP-2 and MMP-9 activity respectively, indicating gelatinases are differentially regulated by PGE2 and IL1β treatments. Because endometriosis is both progressive and inflammatory disease, when endometriotic cells were treated with IL1β and PGE2 together, MMP-1 expression not only increased, but also significantly enhanced cellular migration and invasion. In support of our result, Gosset et al found reduced IL1β-induced MMP responses in prostaglandin E synthease-1 null mouse(Gosset et al. 2010). Interestingly, Guan et al suggested IL1β induced elevation of MMP-1 mediate through PGE2 production in condrocytes (Guan et al. 2015), which can be regarded as one of the rationale for the augmentation of MMP-1 expressions in the present study. However, our study also found increase in both MMP-2 and -9 activities upon IL1β+PGE2 treatments, which should also attribute to increased cellular invasiveness. Upon increased doses of IL1β (10ng/ml), we found inhibited cellular migration alongwith decreased cellular viability. Interestingly, the inhibitory effects of higher doses of IL1β over cellular migration and viability were rescued with PGE2 treatments, indicating that presence of angiogenic factor with inflammatory responses aggravate the disease progression by elevating cellular viability and invasiveness. TIMP-3 expressions did not completely suppress by only IL1β treatment, however PGE2 treatments significantly downregulated the expressions. Unlike other TIMPs, TIMP-3 has localized responses due to ECM binding properties (Yu et al.
2000) and also confer anti-inflammatory responses, which might be the reason for elevated levels of TIMP-3 for only IL1β-treatment. TIMP-3 also acts as a regulator of inflammation by blocking the release of TNF-α (Black 2004). Moreover, evidences indicate the important role of TIMP-3 in promoting cell death by apoptosis (Ahonen et al. 2003). Attenuated TIMP-3 expressions augment MMP-1 responses for cellular invasiveness and disease progression.

Because, MMP-1 is well reported to be regulated via AP-1 transcription factor (Benbow and Brinckerhoff 1997), we looked into MAP kinase pathway and AP-1 components cFOS and cJUN. Herein, IL1β plus PGE2 treatments significantly elevated pcJUN, cFOS expressions and subsequently upregulated MMP-1 expressions. By blocking specific MAPK pathways, we confirmed that MMP-1 responses in endometriosis govern through JNK-pcJUN pathway. Similar to our findings, Nelson et al claimed that under redox dependent condition, MMP-1 is reported to be regulated via JNK through ETS and AP-1 promoter motif (Nelson et al. 2006). We found, ETS-1 expression upregulated significantly in vitro and clinically, suggesting its possible role in endometriosis. Studies indentified PEA3, which is a subfamily of ETS transcription factors, present and act as a co-activator of AP-1 on MMP-1 promoter and coordinately regulated MMP-1 expression (Rutter et al. 1998; Yan and Boyd 2007). Interestingly, inhibition of P38 pathway reduced ETS expression as well as MMP-1, suggesting involvement of P38 and ETS in MMP-1 expression in endometriotic cells. Knock down of cJUN further confirmed the JNK-cJUN mediated transcriptional regulation over MMP-1 and subsequent cellular invasiveness. Furthermore, recent molecular network analysis study revealed that inflammation in endometriosis mediates through cJUN–regulated macrophage activation (Beste et al. 2014b).

In summary, the present chapter explored that elevated MMP-1 and reduced TIMP-3 expressions are involved with ovarian endometriosis progression. In vitro experiments with Hs832 cells showed that IL1β plus PGE2 treatments upregulated MMP-1 expression along with increased cellular migration and invasion, while TIMP-3 expression decreased. The upregulation of MMP-1 resulted from JNK-pcJUN mediated AP-1 responses. Knock down through siRNA reconfirmed our findings for cJUN mediated MMP-1 expression and TIMP-3 mediated regulation of MMP-1.