Chapter 01

Review of literature
1.1. INTRODUCTION

Endometriosis is an estrogen dependent gynecological disorder that affects ~10% of reproductive women worldwide. Endometriosis develops in the peritoneum mainly at the sites of ovary, fallopian tube, outer surface of uterus, cul-de-sac, bladder, and rectal walls (Bulun 2009), where endometrium-like glands and stroma appear. Similar to normal endometrium, endometriosis undergoes hormone-dependent periodic menstrual cycle, which causes hemorrhage, blood and iron deposition at the ectopic sites leading to inflammatory conditions and oxidative stress responses (Burney and Giudice 2012). This inflammatory disease results in infertility (Wheeler 1989) and severe pain responses, like dysmenorrhea, dyspareunia, dysuria and chronic pelvic pain, which are the typical symptoms for the disease diagnosis (Berkley et al. 2005). Unfortunately, these pain responses are not completely exclusive for endometriosis (Howard 2003), thus the only conclusive evidence comes from laparoscopic surgery and histology of the specimen (Vercellini et al. 2014). The etiology for endometriosis remains unknown, however, according to Sampson’s hypothesis, endometriosis develops from efflux of endometrial debris through ‘retrograde menstruation’ (Sampson 1927). Being an estrogen dependent disease, the proper therapeutics for the disease remains incomplete. Clinicians widely use GnRH (gonadotropin-releasing hormone)-based hormonal therapy for endometriosis management, which obviously has severe side effects on normal physiology (Vercellini et al. 2014). Additionally, COX-2 inhibitors are used for associated pain management (Hayes and Rock 2002; Olive and Pritts 2001). The viable options for endometriosis management is surgical removal of the ectopic tissues, however, the incident of recurring is quite common.

The ectopic growth and periodical remodeling of endometriosis requires special group of peptidases, known as matrix metalloproteinase (MMP). MMP are zinc dependent endopeptidases that are principally involved in extracellular matrix (ECM) degradation. MMP was first discovered in the tail of amphibian’s tadpole (Brinckerhoff and Matrisian 2002) and since then MMPs have been reported in numerous physiological and pathological events. In pathological conditions e.g. endometriosis, the major role of MMPs are ECM degradation. Apart from that, MMPs are involved in different cellular responses, which include cellular proliferation, apoptosis, invasion, differentiation etc (Page-McCaw et al. 2007). Moreover, MMPs are reported to be involved in metastatic responses in cancer progression (Egeblad and Werb 2002). Majority of the MMPs are
secreted as latent pro-form which gets activated by proteolytic removal of the pro-domain. MMPs are mainly regulated by its endogenous inhibitors, known as tissue inhibitors of metalloproteinases (TIMPs). TIMPs are structurally conserved family of small proteins and only four TIMPs (1-4) are reported till date (Brew and Nagase 2010).

Disease like endometriosis exclusively occurs in human and few primates, thus to develop a rodent model for endometriosis is quite inappropriate (Slayden 2013). However, studies have managed to develop ‘endometriosis-like’ disease model in rodents which are a necessity for therapeutic studies (Grümmer 2006). In this regard, the role of curcumin, a natural spice with multiple attributes worth investigating. Curcumin has anti-oxidant and anti-inflammatory activities (Hatcher et al. 2008; Maheshwari et al. 2006)and its role as a preventive factor for endometriosis remains unknown.

1.2. ENDOMETRIOSIS

The word ‘endometriosis’ defines ‘a disease related to inside womb’. However, endometriosis actually develops outside uterus, at the peritoneum where it contains the structure of endometrium. Endometriosis most frequently develops at the site of ovary, and relatively less frequently at other peritoneal part including cul-de-sac, uteroscaral ligaments, fallopian tube, bladder, intestinal wall, etc and very rarely at the site of lung, or other parts of the body (Cohen et al. 1990). Histological study of endometriosis shows development of dysregulated but functional endometrial glands and stroma. Similar to the normal endometrium, endometriotic lesion undergoes proliferative, secretary and menstrual phase in a monthly periodic manner; however unlike uterus, no exit pathway exists for the removal of blood or endometriotic debris for the ectopic implants. Thus, ectopic implants develop into endometriotic chocolate cysts, spots or patches, which may grow and reseed as the menstrual cycle continues, leading to increased inflammatory millue and local iron-mediated oxidative stress responses (Burney and Giudice 2012). Hence, endometriosis is a progressive disorder of reproductive women, it finally affects fertility (Wheeler 1989). Although, infertility and endometriosis is well associated, the exact reason for infertility remains uncertain. One direct link that correlates endometriosis with infertility is the site of disease development. Indeed, ovarian endometriosis can directly affect normal ovulation cycle; however extra-ovarian endometriosis also affects fertility (Ozkan et al. 2008). Reports suggest that infertility in endometriosis resulted from not due to reduced uterine receptivity potential (Sakhel et al.
2008), but because of the decreased number and quality of oocytes (Gauché-Cazalis et al. 2012). However, presence of endometriosis does effect uterine functions, specially in relation to progesterone responses (Brosens et al. 2012). Endometriosis has a prevalence of 0.5-5% in fertile and 25-40% in infertile women (Ozkan et al. 2008), and in 25-70% of women with chronic pelvic pain and dysmenorrhea (Osteen et al. 2005). Epidemiological data also revealed that several conditions are associated with increased risk for endometriosis, including early menarche, heavy menstrual bleeding, lower body weight and alcohol use (Barbieri 1990; Hediger et al. 2005; Vitonis et al. 2010).

### 1.2.1. Symptoms and diagnosis

The symptoms for endometriosis mainly consist of pain responses in women. Chronic pelvic pain is the most common phenotype of endometriosis (Berkley et al. 2005). Specific pain responses associated with endometriosis are (i) dysmenorrhea–pain and disabling cramps during menstruation; (ii) dyspareunia– pain during sex and (iii) dysuria– pain during urination etc (Vercellini et al. 2007).

No confirmatory non-invasive markers for diagnosis of endometriosis are still available (Hsu et al. 2010). Specific pelvic pain responses are considered as the most predictive factor for endometriosis, even though the disease severity and pain responses can vary depending upon individual patients (Stratton and Berkley 2011). Pelvic examination and ultra-sonographical identification of endometriosis cysts is the second step. The gold standard for endometriosis detection is laparoscopic surgery, which allows the surgeon to examine the exact condition and location of endometriosis on surface of the uterus, fallopian tubes, ovaries, and other pelvic organs. The severity of endometriosis is also evaluated during laparoscopy (Brosens et al. 2004).

### 1.2.2. Staging of endometriosis

Endometriosis is staged (from I–IV) depending on the severity as per the revised classification of the American Society of Reproductive Medicine (rASRM) on endometriosis guidelines (Canis et al. 1997). The process is a complex point system that assesses lesions and adhesions in the pelvic organs (Fig. 1); however it assesses physical disease only, not the level of pain or infertility. A patient with stage I endometriosis may have little disease and severe pain, while a patient with stage IV endometriosis may have severe disease and no pain or vice versa (dell'Endometriosi 2001).
Stage I (Minimal): Findings restricted to only superficial lesions and possibly a few filmy adhesions. Stage II (Mild): In addition to the above, some deep lesions are present in the cul-de-sac. Stage III (Moderate): In addition to the above, presence of endometriomas on the ovary and more adhesions. Stage IV (Severe): In addition to symptoms for moderate endometriosis, presence of large endometriomas, extensive adhesions. The rASRM staging system describes the extent of endometriosis, adhesions, and a score of 1-15 indicates minimal or mild endometriosis and a score of 16 or higher indicates moderate to severe disease (Canis et al. 1997).

Fig. 1: Staging of endometriosis as per revised American Society for Reproductive Medicine (rASRM). Endometriosis is classified and scored into four stages (I-minimal, II-mild, III-moderate, and IV-severe) depending on location, extent, and depth of endometriosis implants; presence and severity of adhesions; and presence and size of ovarian endometriomas (Canis et al. 1997).
1.2.3. Theories for the origin of endometriosis

The origin of endometriosis is still not well understood. Following theories are proposed for genesis of endometriosis (Vinatier et al. 2001).

**Retrograde menstruation**: The most widely accepted hypothesis for origin of endometriosis was proposed by John A. Sampson in 1926. According to the hypothesis, endometriosis develops from the remnants of endometrial debris through retrograde menstruation. The reflux of uterus helps the menstrual debris to reach the peritoneum through the fallopian tube pathway (Sampson 1927). The hypothesis is well accepted for certain reasons. Firstly, highest incidences of endometriosis occur at the ovary (Jenkins et al. 1986), the site where the endometrial debris first gets exposed within peritoneum. Secondly, because the cells are originally from endometrium, it gets influenced by peritoneal hormonal milieu and responds to the menstrual cycle upon transplantation. Moreover, endometriosis is reported to occur only during reproductive ages and regresses after menopause (Barbieri 1990). Thirdly, higher prevalence of fragments of shed basalis in menstrual blood of women with endometriosis than in healthy controls (Leyendecker et al. 2002). However, the hypothesis is unable to explain how only 10-15% of the women are suffering from endometriosis, while almost 90% of the menstrual women is affected by retrograde menstruation (Halme et al. 1984).

**Müllerianosis**: Mullarian theory for origin proposes that endometriosis develops from specific cells (now considered as pluripotent stem cells) that are laid down in tracts during embryonic development and organogenesis (Batt et al. 2007). These tracts follow the female reproductive (Mullerian) tract as it migrates caudally during 8–10 weeks of embryonic life. These cells differentiate into endometrial cells at the peritoneum developing the disease in the later stage of life. This theory is supported by foetal autopsy (Signorile et al. 2009).

**Coelomic metaplasia**: According to this theory, coelomic epithelium is the common ancestor of endometrial and peritoneal cells and hypothesizes that later metaplasia (transformation) from one type of cell to the other develops the disease (Suginami 1991; Vinatier et al. 2001). The germinal epithelium of the ovary and mesothelial layer of the peritoneum are considered to transform into endometrial cells (Matsuura et al. 1999).
Several other hypotheses like genetic predisposition, dysfunctional immune responses and environmental contaminants also exist. It is also believed that not one, but more than one factors or theories are involved in pathogenesis of endometriosis (Nap 2012).

1.2.4. Management

The current approved treatments for endometriosis relies on pain management and hormone-base therapies that obviously have side effects (Olive and Pritts 2001). Apart from the therapeutic approaches, surgical removal is the best course of action till date (Hsu et al. 2010).

**Surgical recession of endometriosis:** According to rASRM guidelines for management of endometriosis, removal of lesion through laparoscopic surgery is required after a certain amount of growth (more than 2cm in ovarian lesions) (Canis et al. 1997); however, this procedure is far from ideal due to implied risks associated with all surgical interventions. Furthermore, it was demonstrated that 20% of patients who underwent laparoscopic surgery did not report an improvement of pain (Abbott et al. 2004).

**Medical treatments:** Depending upon the condition and requirement of the patients, priority is given on fertility or pain management in endometriosis. Generally, medical treatments aim to relieve pain in the first line with improvement of fertility as a secondary objective. The medical treatment focuses on suppression of the disease rather than removal of lesions.

**Progesterone:** Progesterone counteracts estrogen and inhibits the growth of the endometrium. Thus, progesterone therapy can reduce or eliminate menstruation in a controlled and reversible fashion. Chemical variants of natural progesterone, like progestins are used for endometriosis treatment and proved to be effective in pain management (Gezer and Oral 2015). Long term usage of hybrid progestin like dienogest is effective in endometriosis management, however reported to have side effects like irregular menstrual problems (Schindler 2011).

**Hormone contraception therapy:** Oral contraceptives are used as a long term approach for endometriosis; it reduces the menstrual pain associated with endometriosis (Harada et al. 2008). It may function by reducing or eliminating menstrual flow and providing estrogen support. Continuous hormonal contraception consists of the use of combined oral contraceptive pills are reported to be helpful for disease management in patients who have already completed family planning (Harada et al. 2008).
Danazol (Danocrine) is a suppressive steroid with some androgenic activity that inhibits enzymes in the steroidogenic pathway and increases free testosterone concentrations by displacement of testosterone from sex hormone-binding globulin (Olive and Pritts 2001). Treatment results in anovulation with hypoestrogenism and hyperandrogenism, thus relieving pain symptoms. Although danazol is reported to inhibit the growth of endometriosis successfully, it has major side effects like hirsutism and voice changes (Farquhar et al. 2007).

Gonadotropin Releasing Hormone (GnRH) agonist: GnRH agonists are majorly used for endometriosis treatment because it can repress the hypothalamic-pituitary-ovary axis. The GnRH agonists bind to GnRH receptors and result in stimulation of gonadotropin release. However, because of the remarkably longer half life of GnRH agonists (than natural GnRHs), the pituitary is exposed to continuous GnRH stimulation resulting negative feedback loop and downregulation of LH and FSH secretion (Olive and Pritts 2001). Consequently, secretion of ovarian steroids like estradiol (E2) is blocked; resulting in postmenopausal E2 levels and pain relief is achieved in most cases (Batzer 2006). However, side effects include unpleasant menopausal symptoms, and might lead to osteoporosis. To counteract the side effects some estrogen are given through add-back therapy (Küpker et al. 2002).

Non-steroidal anti-inflammatory drugs (NSAIDs): NSAIDs are common therapeutics for endometriosis treatment, however, generally used in combination with other therapies. Cyclooxygenase-2 (COX-2) is an important enzyme for prostaglandin pathway and reported to get influenced through estrogen production in endometriotic stromal cells (Noble et al. 1997). COX-2 inhibition is utilized for suppression of endometriosis growth and pain management. Different in vivo and in vitro models showed significant affectivity of NSAIDs for endometriosis regression (Banu et al. 2008; Dogan et al. 2004). Long term usage of celecoxib, rofecoxib are reported to attenuate dysmenorrheal pain responses in endometriosis (Hayes and Rock 2002); however, is associated with gastric and cardiovascular side effects (Green 2001; Hochman and Shah 2006).

Anti-angiogenesis drugs: Because ectopic development of endometriosis relies on angiogenesis, inhibition of angiogenesis is a strategic approach against the disease (Rocha et al. 2013). Different approaches were used for inhibition of angiogenesis in endometriosis animal models through endogenous angiostatin, endostatin or vascular endothelial growth factor (VEGF) antagonist, VEGF receptor antagonists, small molecular inhibitors etc in variant in vivo models (Djokovic and Calhaz-Jorge 2014).
Bevacizumab significantly decreased VEGF in surgically induced endometriotic model for BALB/c mice (Ricci et al. 2011). Combined inhibition of VEGF-A, basic fibroblast growth factor (bFGF) and platelet-derived growth factor B (PDGF-B) receptors much effectively regressed angiogenesis in animal model of endometriosis, in comparison to only VEGF-A inhibition (Laschke et al. 2006). Similarly, sorafenib, a multi-kinase inhibitor targeting VEGF-R2, VEGF-R3, B-Raf and other tyrosine kinase receptors, was highly efficient against murine model of induced endometriosis(Ozer et al. 2013). Although anti-angiogenic drugs are promising in non-human endometriosis models, further validations are necessary before clinical trials(Becker and D’Amato 2007).

**Others:** Aromatase inhibitors, peroxisome proliferator-activated receptor (PPAR) agonists and immunomodulatory drugs are gaining importance for treatment of endometriosis(Vercellini et al. 2014). Apart from the conventional medicine, studies have reported other molecules are effective in regressing endometriosis in animal models. One such small molecule, melatonin showed significant promise in regression of endometriosis in mice model (Paul et al. 2010; Paul et al. 2008). Moreover, clinical phase II trial for melatonin showed significant promise on pain management in endometriosis patients(Schwertner et al. 2013).

### 1.3. MATRIX METALLOPROTEINASES

**1.3.1. Structure of MMPs**

MMP was first discovered in the tail of a tadpole undergoing metamorphosis(Grinckerhoff and Matrisian 2002). To date, 26 different vertebrate MMPs have been identified, of which 24 are found in humans (including one gene duplication, these genes encode 23 unique MMP proteins)(Khokha et al. 2013; Puente et al. 2003). MMPs are a group of zinc containing calcium dependent endopeptidases that commonly consist of a prodomain, a catalytic domain, a hinge region, and a hemopexin domain (Fig. 2). The pro-peptide domain is ~80 amino acids long and contains a cysteine-switch motif. The catalytic domain is ~170 amino acids long and contains two zinc ions (one catalytic and one structural). The catalytic zinc is bound by three histidine residues found in the conserved zinc-binding motif $\text{HE}xx\text{HxxGxxH}$. The catalytic domain also includes a ‘Met-turn’ structure eight residues past the catalytic zinc ion, which stabilizes it. The cysteine residue in the pro-peptide domain interacts with the catalytic zinc ion, which prevents its association with a water molecule and, thus, remain
inactivated until the pro-peptide domain is removed. The catalytic and haemopexin domains are attached by a linker region of variable length. The C-terminal haemopexin domain is ~200 amino acids long and forms a propeller blade structure. The haemopexin domain confers substrate specificity to the MMP (Khokha et al. 2013). Some members of the MMP family have slight differences in their subunit organization; MMP-2 and MMP-9 both contain three repeats of a fibronectin-like motif that bind gelatin (Nagase et al. 2006). MMP-23 has a unique cysteine-rich region and an immunoglobulin-like domain in place of the haemopexin domain. Membrane-type MMPs (MT-MMPs) contain a transmembrane domain or a glycosylphosphatidylinositol anchor (Visse and Nagase 2003).

![Fig. 2: The structure of MMPs](image)

The activity minimal-required domains are N-terminal signal sequence (S), a propeptide (Pro) and a catalytic domain with a zinc-binding site (Zn). Other MMPs contain a linker hinge region and a propeller blade structured hemopexin-like domain. Additionally, gelatinases contain fibronectin type-II repeats at the catalytic domain. The Membrane-type MMPs (MT-MMPs) contain a transmembrane domain or a glycosylphosphatidylinositol anchor. MMP-23 has a unique cysteine-rich region and an immunoglobulin-like domain in place of the hemopexin domain. The proprotein convertase (furin) recognition sequence containing MMPs are activated intracellularly. Other MMPs are secreted as pro-form and activated through proteolytic removal of the prodomain (adapted from Khokha et al. 2013).
1.3.2. Classification of MMPs

MMPs are grouped into six classes depending upon substrate specificity: gelatinases, collagenases, strepomlysin, matrilysin, membrane bound MMPs and others (Nagase et al. 2006).

**Gelatinases:** Gelatinase A (MMP-2) and gelatinase B (MMP-9) can readily digest the denatured collagens, gelatins. The gelatin-specificity of these enzymes comes from three repeats of a type II fibronectin domain inserted in the catalytic domain. MMP-2, but not MMP-9, digests type I, II, and III collagens (Aimes and Quigley 1995; Patterson et al. 2001). Mutations in human MMP-2 results in multicentric osteolysis, a rare autosomal recessive disorder, that causes destruction and resorption of the affected bones (Martignetti et al. 2001).

**Stromelysins:** MMP-3, MMP-10 and MMP-11 belong to this group. While MMP-3 and MMP-10 have similar structure and substrate specificity, MMP-11 is distantly related in terms of sequence (Nagase et al. 2006). MMP-11 contains a furin recognition motif RX[R/K]R at the C-terminal end of the propeptide and the substrate specificity also diverge from the other two (Visse and Nagase 2003).

**Collagenases:** MMP-1, MMP-8, MMP-13, and MMP-18 (Xenopus) are in this group. These MMPs mainly cleave interstitial collagens I, II, and III at a specific site three-fourths from the N-terminus. Collagenases can also digest a number of other ECM and non-ECM molecules (Visse and Nagase 2003).

**Matrilysins:** The matrilysins (MMP-7, MMP-26) are characterized by the lack of a hemopexin domain. Alongwith ECM components, MMP-7 can process a number of different cell surface molecules, including pro-α-defensin, Fas-ligand, pro–tumor necrosis factor (TNF)-α, and E-cadherin etc (Page-McCaw et al. 2007).

**Membrane-Type MMPs:** Membrane-type MMPs (MT-MMPs) containing six MMPs, among them four are type I transmembrane proteins (MMP-14, MMP-15, MMP-16, and MMP-24), and two are glycosylphosphatidylinositol (GPI) anchored proteins (MMP-17 and MMP-25). They all have a furin recognition sequence RX[R/K]R at the C-terminus of the propeptide and activate intracellularly (Visse and Nagase 2003). All MT-MMPs, except MT4-MMP (MMP-17) can activate proMMP-2. MT1-MMP play important role in angiogenesis and MT1MMP null mice exhibit skeletal abnormalities during postnatal development (Holmbeck et al. 1999). MT5-MMP is brain specific and is mainly expressed in the cerebellum (Sekine-Aizawa et al. 2001). MT6-MMP (MMP-25) is
expressed almost exclusively in peripheral blood leukocytes (Pei 1999) and in anaplastic astrocytomas and glioblastomas (Velasco et al. 2000).

**Others:** Seven MMPs are not classified in the above categories. MMP-12, MMP-20 and MMP-27 have similar structures and chromosome location as stromelysins. Metalloelastase (MMP-12) is mainly expressed in macrophages (Shapiro et al. 1993). MMP-19 was identified as a T-cell derived auto-antigen from patients with rheumatoid arthritis (Kolb et al. 1997b). Mutations at MMP-20 cleavage sites results in Amelogenin imperfecta, a genetic disorder caused by defective enamel formation (Li et al. 2001). The function of MMP-22 is unknown. Instead of the haemopexin domain, MMP-23 contains a cysteine-rich domain followed by an immunoglobulin-like domain and a furin recognition motif in the propeptide domain. MMP-28 (epilysin) mainly expressed in keratinocytes might function in tissue homeostasis and wound repair (Visse and Nagase 2003).

### 1.3.3. Regulation of MMPs

MMPs regulate cellular homeostasis by maintaining ECM degradation, thus require tight regulation over its activity. MMPs are mainly regulated at the steps of transcription, pro-zymogen activation and inhibition (Page-McCaw et al. 2007). In addition, regulations are implied on the steps of protein synthesis, and at levels of secretion, intracellular trafficking, subcellular or extracellular localization etc (Fig. 3). These regulations act coordinately maintaining the required levels of MMP activity at proper sites (Overall and Lopez-Otin 2002).

**Transcriptional regulation of MMPs:** The MMP promoter harbors a large number of cis-acting elements (Fig. 4) that regulate MMP genes by a diverse set of trans-activators including AP-1 (activator protein 1), TCF4 (transcription factor 4), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), SP-1 (specificity protein 1) and PEA3 etc (Yan and Boyd 2007). AP-1 proteins include the JUN, FOS, ATF (activating transcription factor) and MAF (musculoaponeurotic fibrosarcoma) protein families, which can form homodimers and heterodimers through their leucine-zipper domains. The different dimer combinations recognize different sequence elements in the promoters and enhancers of target genes (Eferl and Wagner 2003). Majority of MMPs contain TATA box (-30bp) and AP-1 site at approximately -70 bp upstream of the transcriptional start site plays a dominant role in the transcriptional activation of the MMP promoters (Fig. 4). AP-1 is reported to regulate different MMPs, including MMP-1, MMP-3, and MMP-9 by...
several external stimuli including growth factors, cytokines, and cellular stress etc (Yan and Boyd 2007). Other cis acting elements like PAE3 acts cooperatively on AP-1 for transcriptional regulation of MMP (Benbow and Brinckerhoff 1997). In some MMPs (MMP-8,-11,-21) the TATA box is present, however proximal AP-1 site (-70bp) is absent, distal AP-1 is present. Other MMPs that completely lack AP-1 site at their promoter region (MMP-2,-14,-28) is mainly regulated by ubiquitous family of SP-1 transcription factors which bind to the proximal GC box. These MMPs are regulation is mainly constitutive, although sometimes moderately inducible by different growth factors (Chakraborti et al. 2003). AP-2 is an important transcription regulator for MMP-2 (Qin et al. 1999). In addition to AP-1 and PEA3, TCF-4 is an important regulator for MMP-7,-14,-12,-26 (Yan and Boyd 2007). TCF is mainly reported to regulate MMP through Wnt-β-catenin mediated signaling pathway (Ilyas 2005), whereas NFκB mediated signaling pathway contribute to MMP-9, -3 and -11 expressions (Overall and Lopez-Otin 2002).

Fig. 3: Regulation of MMPs. Being an important regulator for ECM homeostasis, MMP activities are tightly regulated at several steps. The extents of MMPs are being regulated at the steps of (1) RNA transcription and (2) protein synthesis. In addition, MMP functions can be regulated at the levels of (3) secretion, intracellular trafficking, (4) subcellular or extracellular localization, (5) activation of the zymogen form, (6) expression of their endogenous protein inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) and α2-macroglobulin, and (7) protease degradation (adapted from Page-McCaw et al 2007).
**Fig. 4:** Regulatory elements in the promoter regions of different MMPs. Bent arrow indicates transcription start site and different transcription-factor-binding sites placed within boxes. Based on the composition of cis-elements, MMP promoters can be roughly grouped into three categories. (A) The first group, includes majority of the MMP, contains a TATA box at approximately -30 bp and an AP-1-binding site at approximately -70 bp. (B) The MMP promoters in the second group (MMP-8, -11, and -21) also contain a TATA box, but lack a proximal AP-1 site. (C) The last group of promoters (MMP-2, -14, and -28) does not harbor a TATA box, and expectedly, transcription from these promoters start at multiple sites (Yan and Boyd, 2007).

**Activation of MMPs:** MMPs are synthesized as pre-pro-form; signal peptide domain is subsequently removed during translation generating the proMMP form. Thirteen MMPs are secreted as inactive pro-form, which was further activated by the proteolytic removal of the pro-domain (Nagase et al. 2006). The pro-domain contains a conserved ‘cysteine switch’ sequence motif whose free cysteine residue interacts with the catalytic zinc ion to maintain enzyme latency (Fig. 5A). The activation of the MMP occurs when a conformational change in the pro-domain pulls out the cysteine residue and enable water to interact with the zinc ion in the active site (Visse and Nagase 2003). The activation can result from (i) removal of the pro-domain by direct cleavage of another endopeptidases; (ii) allosteric reconformation of the pro-domain; and (iii) chemical modifications of the free cysteine by reactive oxygen species or treatment with mercurial compounds, SH reagents and chaotropic agents etc (Löffek et al. 2011). Eleven MMPs contain furin-like pro-protein convertase recognition sequence RX[K/R]R at the end of the propeptide and they are likely to be activated intracellularly and active enzymes are delivered as secreted...
or cell surface-bound forms (Fig. 2). These MMPs can immediately start their catalytic action when appearing on the cell surface or being secreted in the pericellular environment (Vercellini et al. 2014). MMPs can also be activated by other proteases including, MMPs and serine proteases, like chymase, plasmin etc (Fig. 5B). For example, plasmin has been reported to activate several MMPs (Lijnen 2001); MMP-14 is involved in activation of pro-MMP-2 through cooperative action with TIMP-2 (Bernardo and Fridman 2003).

**Endogenous inhibitors of MMPs:** The activities of MMPs are regulated *in vivo* by two major inhibitors (i) α2-macroglobulin and (ii) TIMP (tissue inhibitor of metalloproteinases-1-4). The α2-macroglobulin is a broad spectrum proteinase inhibitor.
of tissue fluids and blood. This homo-tetrameric macromolecule of 725 kDa inhibits almost all classes of endopeptidases by entrapping the whole enzyme, rapidly clearing through LDL receptor related protein-1 mediated endocytosis (Baker et al. 2002).

TIMPs, one other hand, are natural inhibitors for MMPs that bind MMPs in a 1:1 stoichiometry. TIMPs are 184-194 amino acids long and consist of two distinct domains: a larger N-terminal and a smaller C-terminal domain, each one is stabilized by three conserved disulfide bonds. TIMP molecule has a ‘wedge-like’ structure and the N-terminal domain alone can fold independently. N-terminal four residues Cys1-Thr-Cys-Val4 are linked by a disulfide bond from a contiguous ridge that slots into the active site of the MMPs. The catalytic zinc atom is bidentately chelated by the N-terminal amino group and the carbonyl group of Cys1, which expels the water molecule bound to the zinc atom. The function of the C-terminal domain is not fully understood, but it has been shown that it can bind tightly to the haemopexin domain of latent MMPs (Brew and Nagase 2010). The four human TIMPs are broad-spectrum inhibitors for all 24 human MMPs, although there are differences in specificity and affinity. TIMP-1 is more restricted in its inhibitory range than the other three TIMPs, having a relatively low affinity for MMP-14, MMP-16, MMP-24 and MMP-19 (Murphy 2011). TIMP-2 and -3 are weaker inhibitors than TIMP-1 for MMP-3 and MMP-7, in comparison to their affinities for other MMPs (Hamze et al. 2007). Unlike other TIMPs, TIMP-3 has ECM-binding property (Yu et al. 2000) and can inhibit a broader array of metalloproteinases including several members of the ADAM and ADAMTS families (Brew and Nagase 2010). TIMP-4 was also reported to inhibit ADAM28 (Mochizuki et al. 2004). Knockout of TIMP-1 or TIMP-2 in mice show limited abnormalities (listed in Table 1), however, loss of TIMP-3 in mice is associated with enhanced apoptosis in mammary gland duct epithelial cells (Fata et al. 2001) and pulmonary alveolar enlargement (Gill et al. 2003).

Few other small proteins are also reported to inhibit MMP. Tissue factor pathway inhibitor-2 is a serine protease inhibitor that inhibits MMPs (Herman et al. 2001). A C-terminal fragment of the procollagen, C-terminal proteinase enhancer protein has been shown to inhibit MMP-2 (Mott et al. 2000). Membrane bound β-amyloid precursor protein (secreted form) has also been reported to inhibit MMP-2 activity (Higashi and Miyazaki 2003).
1.3.4. Functions of MMPs

MMPs are involved in degradation of ECM proteins, including collagen, gelatin, fibronectin, laminin, elastin, fibrin, aggrecan etc. Apart from the degradation of ECM components, MMPs are involved in different signaling pathways and cellular events (Fig. 6) by means of proteolytic cleavage of receptors, ligands and growth factors etc (Egeblad and Werb 2002).

Apoptosis: MMPs have both apoptotic and anti-apoptotic actions by cleaving adhesion molecules (Herren et al. 1998; ILAN et al. 2001; Steinhusen et al. 2001). Degradation of laminin by MMP-3 enhances apoptosis in mammary epithelial cells (Sympson et al. 1994). MMP-7 releases the Fas ligand from the membrane which then induces apoptosis of neighboring cells, or decreases cancer-cell apoptosis (Powell et al. 1999b). MMPs might also negatively regulate cancer-cell growth, by means of activation of TGF-β or generation of pro-apoptotic molecules such as Fas ligand or TNF-α. Moreover, MMP-11 inhibits cancer cell apoptosis in tumor xenografts (Wu et al. 2001). In contrast, MMP11-null mice show a higher rate of apoptosis compared to wild-type when challenged with cancer cells (Baserga 2000). Although MMP-9 and -11 decrease cancer cell apoptosis, they increase apoptosis during development (Bergers et al. 2000a; Swarnakar et al. 2011; Wu et al. 2001).

Angiogenesis: Angiogenesis, the formation of new capillaries from pre-existing vessels, is associated with several physiological processes as well as pathological conditions. The activation of angiogenesis is governed by VEGF along with activated MMPs which degrade collagen and ECM proteins aiding in the migration of endothelial cells. Cleavage of collagen type I allows endothelial cells to invade the tumor stroma during vessel formation (Seandel et al. 2001). Among all MMPs, MMP-2, -9 and -14 are directly involved in angiogenesis (Kolb et al. 1997a). Tumor angiogenesis is significantly inhibited in mice deficient in MMP-2 in comparison with wild type mice (Itoh et al. 1998). Cleavage of collagen type IV by MMP-2 exposes a cryptic, αvβ3 integrin binding site within collagen which promotes migration of endothelial cells both in in vitro and in vivo models (Xu et al. 2001). Both MMP-14 and MMP-9 null mice have impaired angiogenesis during development (Vu et al. 1998; Zhou et al. 2000a). MMPs are involved in generation of endostatin, which inhibits endothelial-cell invasion by acting as an inhibitor of MMP-14 and -2 (Kim et al. 2000). MMP-14 promotes cell endothelial cell invasion and angiogenesis directly and by means of activation of MMP-
2. In contrast, MMP-12 inhibits tumor angiogenesis by inhibiting endothelial cell invasion via a different pathway that mediated by urokinase-type plasminogen activator receptor.

**Cellular proliferation:** MMPs play important roles in cellular proliferation by acting on growth factors. For example, membrane-bound precursors of some growth factors, e.g. TGF-β, are released by MMPs or ADAMs (Yu and Stamenkovic 2000). Degradation of growth factors by MMPs makes them available in pericellular space, e.g., MMPs can cleave IGF-BP to release IGF (Mañes et al. 1997). By producing heparin-binding epidermal growth factor (HBEGF) from the latent form, i.e. pro-HBEGF, MMP-7 promotes cell survival. Cell proliferation by growth factors also occurs through integrin signaling (Agrez et al. 1994). Cleavage of plasminogen by MMP-2, -3, -7, -9 and -12 generates angiostatin (Cornelius et al. 1998), and MMP-3, -9, -12, -13 and -20 are involved in the generation of endostatin, a C-terminal fragment of the basement membrane collagen type XVIII (Dong et al. 1997; Gorrin-Rivas et al. 2000). Both angiostatin and endostatin inhibit endothelial cell proliferation (O'Reilly et al. 1994).

**Invasion and metastasis:** MMPs act as a pathclearing enzyme for invasive cells by degradation of ECM components and the cellular invasionness is regulated by localized MMP activities. The role of MMPs in metastasis was evidenced by *in vitro* invasion assays and *in vivo* xenograft metastasis models. Reduced metastatic responses were observed in the MMP-2 and MMP-9 null mice. Cleavage of cell-adhesion molecules like E-cadherin by MMP-3 or -7 is associated with increased cellular invasiveness (Noe et al. 2001). Moreover, the released fragment of E-cadherin promotes tumor-cell invasion in a paracrine manner *in vitro* (Löchter et al. 1997; Sternlicht et al. 1999). MMP-3 is involved in cellular transition processes, like epithelial to mesenchymal transition (Radisky et al. 2005). Moreover, MMP-2 is recruited to invadopodia by either binding to α5β3 integrin (Brooks et al. 1996) or by binding to MMP-14. MMP-14 is recruited to invadopodia by means of its transmembrane and cytoplasmic domains (Nakahara et al. 1997). Overexpression of MMP-2 or MMP-14 increases cellular invasiveness of cancer cells in metastasis assay (Deryugina et al. 1997; Tsunezuka et al. 1996). Furthermore, docking of metastatic cells at the secondary sites also involves MMP activity.

**Immune responses:** MMPs mediate cleavage of various components, which are involved in modulation of immune responses (Coussens and Werb 2001). While
immune system is capable of recognizing and attacking cancer cells. MMPs are involved in the escape mechanisms for cancer cells. MMP-9 can cleave interleukin-2 receptor (IL-2R)-α and thereby suppress the proliferation of the T lymphocytes (Sheu et al. 2001). MMP-2 cleaves the monocyte chemoattractant protein-3, and the cleaved fragment acts as an antagonist to the receptors (McQuibban et al. 2002). Furthermore, CXCL12 (also known as stromal-cell-derived factor 1) is cleaved and inactivated by MMP-1, -3, -9, -13 and -14 (McQuibban et al. 2000). CXCL12 is a ligand for the CXC chemokine receptor 4 (CXCR4) on leukocytes. MMP-11 acts on α1-proteinase-inhibitor and the cleaved product altered sensitivity of tumor cells towards natural killer cells (Kataoka et al. 1999). Moreover, few MMPs also activate TGF-β an important inhibitor of the T-lymphocyte response against tumors (Yu and Stamenkovic 2000).

**Fig. 6: Different functions of MMPs.**
Apart from ECM degradation for cellular motility (A), MMPs are involved in different functions, including cellular invasion through cleavage of junctional proteins (B) and basement membrane (C), proteolytic activation of other members of MMPs (D), cleavage of different receptors, ligands that mediate different signaling pathways for cellular proliferation, apoptosis, differentiation etc (E,F) (adapted from Page-McCaw 2007).
### Table 1 | Selected MMP and TIMP null mutant phenotypes (adapted from Page-McCaw 2007).

<table>
<thead>
<tr>
<th>MMP gene</th>
<th>Null mutant phenotype in mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td>Reduced neovascularization; decreased primary ductal invasion in the mammary gland; reduced lung saccular development</td>
</tr>
<tr>
<td>MMP-3</td>
<td>Altered structure of neuromuscular junctions; reduced purse stringing during wound healing; altered secondary branching morphogenesis in the mammary gland</td>
</tr>
<tr>
<td>MMP-7</td>
<td>Innate immunity defects; decreased re-epithelialization after lung injury</td>
</tr>
<tr>
<td>MMP-8</td>
<td>Increased skin tumours; resistance to tumour necrosis factor (TNF)-induced lethal hepatitis</td>
</tr>
<tr>
<td>MMP-9</td>
<td>Bone-development defects; defective neuronal remyelination after nerve injury; delayed healing of bone fractures; impaired vascular remodelling; impaired angiogenesis</td>
</tr>
<tr>
<td>MMP-10</td>
<td>Increased inflammation and increased mortality in response to infection or wounding</td>
</tr>
<tr>
<td>MMP-11</td>
<td>Delayed mammary tumorigenesis</td>
</tr>
<tr>
<td>MMP-12</td>
<td>Diminished recovery from spinal cord crush; increased angiogenesis due to decreased angiostatin</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Bone remodelling defects; reduced hepatic fibrosis; increased collagen accumulation in atherosclerotic plaques</td>
</tr>
<tr>
<td>MMP-14</td>
<td>Skeletal remodelling defects; angiogenesis defects; inhibition of tooth eruption and root elongation; defects in lung and submandibular gland</td>
</tr>
<tr>
<td>MMP-19</td>
<td>Obesity</td>
</tr>
<tr>
<td>MMP-20</td>
<td>Defects in tooth enamel</td>
</tr>
<tr>
<td>MMP-24</td>
<td>Abnormal response to sciatic nerve injury</td>
</tr>
<tr>
<td>MMP-28</td>
<td>Increased inflammatory response</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>Accelerated endometrial gland formation; impaired learning and memory; accelerated hepatocyte cell-cycle progression</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>Motor defects</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>Accelerated apoptosis in mammary glands; impaired bronchiole branching; enhanced metastatic dissemination</td>
</tr>
</tbody>
</table>
1.4. PATHOPHYSIOLOGY OF ENDOMETRIOSIS

Endometriosis is an estrogen dependent, inflammatory proliferative disease, associated with increased cellular proliferation, decreased apoptosis, increased inflammatory milieu, oxidative stress, altered hormonal responses, angiogenesis and increased cellular invasiveness (Fig. 7).

1.4.1. Inflammation: Endometriosis is an inflammatory disease and several inflammatory cytokines and interleukins elevate in endometriosis patients (Burney and Giudice 2012; Harada et al. 2001). TNF-α is reported to be elevated in peritoneal fluids and serum of endometriosis patients (Ilie and Ilie 2013), and involved in activation of other interleukins like IL-8 (Iwabe et al. 2000). TNF-α-mediated signaling also involves in activation of transcription factor NFκB (Wieser et al. 2005), which regulates expressions of many inflammatory genes, including cytokines and MMP etc (Grund et al. 2008). IL-8 produced by human endometrium and endometriotic tissues are involved in stromal cell proliferation (Hirata et al. 2008). Recent study with molecular network analysis of endometriosis patients revealed essential role for c-Jun-regulated macrophage activation for inflammatory network (Beste et al. 2014a).

1.4.2. Apoptosis: Patients with endometriosis show reduced apoptotic responses (Harada et al. 2004); studies reported that endometriosis patients have lower numbers of apoptotic cells in sloughed endometrium (Gebel et al. 1998), as well as glandular epithelium (Dmowski et al. 2001). Dmowski et al also found higher stages of endometriosis is associated with reduced numbers of apoptotic cells. Reduced apoptosis in endometrial slough retain higher chances of implantation, whereas decreased apoptosis in endometriotic implants facilitate the ectopic growth. Moreover, Bcl-2 expression elevate in endometriotic lesions, especially in the stromal cells (Jones et al. 1998) and in the eutopic endometrium of women with endometriosis (Meresman et al. 2000). FasL expressions increased in serum and peritoneal fluids of endometriosis patients; higher level of soluble FasL may contribute increased cellular death to Fas bearing immune cells in the peritoneum (Garcia-Velasco et al. 2002). Study with knock out mouse proved TNF-α-mediated MMP-9 activity contribute to formation of the 70-kDa SRC-1 C-terminal isoform that is involved with apoptotic resistance and increased cellular invasion in mouse model of endometriosis (Han et al. 2012). Selective induction of apoptosis in endometriotic tissues is a strategic intervention and studies have already
reported using small pro-apoptotic peptides in Baboon model of endometriosis (Sugihara et al. 2014).

**1.4.3. Cellular proliferation:** Endometriosis is a progressive disease and majorly relies on the estrogenic milieu of the peritonium (Bulun 2009). Endometriosis lesions prevalently express estrogen receptor(ER)-β that plays unique role with apoptotic machinery to prevent TNF-α-induced apoptotic responses and enhance proliferative signals in endometriosis (Han et al. 2015). Moreover, aromatase, which is usually absent in eutopic endometrium, present in elevated levels in endometriosis and constitutively catalyzes the production of estrone and estradiol (Munksgaard and Blaakaer 2012). Endometriotic tissues contain 17β-HSD (hydroxysteroid dehydrogenase) type-1, instead of the 17β-HSD type-2, which is more potent in converting estrone to estradiol, and thus locally supports the proliferative microenvironment of endometriotic cells (Zeitoun et al. 1998). Self-sufficient estrogenic milieu of endometriosis promotes cellular growth and invasion. Progesterone receptor (PR) isoforms are believed play the critical role for insensitivity to anti-proliferative signal. Endometriosis prevalently expresses the PR-A isoform, instead of the stimulatory PR-B isoform, which might result for insensitivity to anti-proliferative signal (Attia et al. 2000). Moreover, depending upon lesion nature endometriosis showed differential expression for p27Kip1, which is positive for red lesions and negative in black lesions of peritoneal endometriosis along with the expression of Ki67 (Matsuzaki et al. 2001).

**1.4.4. Angiogenesis:** Endometriosis is an angiogenesis-dependent disease. Endometriosis patients show elevated levels of VEGF in systemic fluid and endometriosis lesions (Donnez et al. 1998). Elevated VEGF receptor (VEGFR-2) expressions were reported with endometriosis progression (Rocha et al. 2013). The pathological angiogenesis in endometriosis occurs through inflammatory molecules (Lin et al. 2006) and local growth factors millue guides angiogenic tip cells to invade in to the implant (Folkman 2007). Apart from VEGF, there are plenty of other pro-angiogenic factors which were reported elevated in endometriosis patients, including hepatocyte growth factor, IL-8, IL-15, macrophage migration inhibitory factor, neutrophil-activating factor, TNF-α, erythropoietin and angiogenin etc (Artini et al. 2012; Rocha et al. 2013).

**1.4.5. Tissue invasion:** Ectopic development of endometriosis relies on invasion through peritoneal basement membrane and reported to spread from one location to another within peritoneum during the growth of transplants (Burney and Giudice 2012).
Endometriotic niche contains of several proteases, including MMPs and ADAMs, which cleave different ECM components (Paul et al. 2010). Moreover, MMPs can cleave different cellular adherent molecules, including E-cadherin, which promote cellular invasiveness by releasing cells from its basement membranes (Page-McCaw et al. 2007).

Fig. 7: Common pathological features associated with endometriosis. Endometriosis, believed to develop from retrograde menstruation, affects almost 10% of reproductive women worldwide. Endometriosis is estrogen dependent, inflammatory proliferative disease, associated with increased cellular proliferation, decreased apoptosis, increased inflammatory milieu and oxidative stress, altered hormonal responses, angiogenesis and increased cellular invasiveness. Endometriosis is also correlated with early menarche, short menstrual cycle, family history and exposure to environmental toxicants etc. Inset shows schematic depiction of ovarian endometrioma (adapted from Vercellini et al, 2014).

1.5. ROLE OF MMPs IN ENDOMETRIOSIS

The roles of MMPs in different cellular events, like invasion, angiogenesis, proliferation, apoptotic responses are well reported in different diseases. However, the involvement of specific MMPs in pathogenesis of endometriosis is still not well investigated. Reports have identified several MMPs in animal models of endometriosis as well as in clinical studies. Till now, MMP-1, -3, -9, -2, -7, -14 etc are the most reported MMPs, associated with endometriosis (Osteen et al. 2003; Pitsos and Kanakas 2009). MMP-1 and MMP-9 were reported to be expressed from endometrial stromal cells under the influence inflammatory molecules (Pino et al. 2009). The increased levels of MMP-9 and MMP-3...
in endometriosis were also confirmed by previous works from our laboratory, where the MMP activities increased with disease progression (Paul et al. 2010; Paul et al. 2008). The study also found increased levels of MMP-9 responses in eutopic endometrium of the endometriosis patients, compared to control endometrium (Paul et al. 2008). In mouse model of endometriosis, AP-1 mediated signaling responses were reported to be upregulated during early phases of endometriosis progression (Paul et al. 2010). MMP-2 and -9 are reported to increase cellular invasiveness in endometrial stromal cells (Wang et al. 2010). Moreover, MMP-9 activity conferred generation of ~70kDa SRC fragment that inhibit apoptotic responses in endometriosis alongwith increased cellular invasiveness and EMT phenomenon (Han et al. 2012). The presence of MMP-3 and -7 were reported in nude mice model of induced endometriosis (Bruner-Tran et al. 2002). The presence of MMP-2 and MMP-14 were also identified in endometriosis (Aresu et al. 2012). The levels of MMP-2 were positively correlated with 17β-estrogen levels in human serum and peritoneum fluids (Huang et al. 2004). Inhibition of total MMPs regressed endometriosis in mouse model (Bruner et al. 1997) as well as in chick chorio-allantoic membrane model (Nap et al. 2004).

1.6. ALTERNATIVE THERAPY FOR ENDOMETRIOSIS—CURCUMIN

Because of the absence of any specific therapeutics for endometriosis treatment, the development of an alternative therapy is a necessity. Treatments of compounds with therapeutic values which are still not considered as conventional drugs are considered here as alternative therapy. In the recent past, our laboratory had looked into one such molecule, melatonin, which was significantly able to prevent endometriosis in murine model through modulation of MMP-9/TIMP-1 expressions (Paul et al. 2008). This endogenous molecule showed anti-oxidant, anti-inflammatory responses against endometriosis and was successful to regress endometriosis through induction of apoptotic responses in mouse model of endometriosis (Paul et al. 2010). Later, melatonin was used as a phase II, randomized, double-blind, placebo-controlled trial, where melatonin successfully proved its role in endometriosis management through improved sleep quality, reduced pain responses and brain-derived neurotrophic factor levels in endometriosis patients than the placebo group (Schwertner et al. 2013).

Curcumin (diferuloylmethane) the major constituent of turmeric, is widely used as spice in Indian continent. Reports suggest it contains anti-oxidant, anti-inflammatory, anti-viral, anti-bacterial, antifungal activities (He et al. 2015), for which it has been used for
different diseases including, skin diseases, pulmonary, and gastrointestinal disease, wounds, cardiovascular disease, cancer and liver disorders etc (Hatcher et al. 2008). These effects are mediated by regulation of different growth factors, transcription factors, cytokines, interleukins and inflammatory molecules etc (Hatcher et al. 2008). Curcumin inhibits TNF-α mediated NFκB activation and subsequent inflammatory genes (Singh and Aggarwal 1995). It is reported as a potent antioxidant and modulator of different anti-oxidant enzymes (Menon and Sudheer 2007). Curcumin is reported to rescue NSAID-induced gastric ulcer in mice model through MMP-9 mediated pathway (Swarnakar et al. 2005). Moreover, it inhibits angiogenesis and COX-2 activity in different inflammatory disease models (Menon and Sudheer 2007). Curcumin also induced cell cycle arrest, apoptotic responses in cancer cells as well as in vivo cancer models (Anand et al. 2008; Sa and Das 2008). Previous studies on curcumin make it an ideal candidate for therapeutic use against endometriosis in mouse model. Moreover, being used as an ingredient for daily food gives the compound an advantage for becoming a preventive measure against the disease.

**Fig. 8: Multiple signaling pathways modulated by curcumin:** Curcumin modulates several cellular events including migration, apoptosis, proliferation via regulation of different signaling pathways. Blunt-head lines (orange) indicate that these molecules can be down-regulated by curcumin, where as arrow-head lines indicate molecules up-regulated by curcumin (adapted from Sa et al, 2008).