Molecular Human Reproduction, Vol.15, No.10 pp. 587–607, 2009
Advanced Access publication on August 3, 2009 doi:10.1093/molehr/gap064

MHR Basic science of reproductive medicine

NEW RESEARCH HORIZON ***Review***

Epigenetics of endometriosis

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ABSTRACT: Endometriosis is a common gynecologic disorder with an enigmatic etiopathogenesis. Although it has been proposed that endometriosis is a hormonal disease, an autoimmune disease, a genetic disease, and a disease caused by exposure to environmental toxins, our understanding of its etiopathogenesis is still inadequate, as reflected by recent apparent setbacks in clinical trials on endometriosis. In the last 5 years, evidence has emerged that endometriosis may be an epigenetic disease. In this article, the evidence in support of this hypothesis is reviewed, and its diagnostic, therapeutic and prognostic implications discussed. Publications, up to the end of June 2009, pertaining to epigenetic aberration in endometriosis were identified through PubMed. In addition, publications on related studies were also retrieved and reviewed. Epigenetics appears to be a common denominator for hormonal and immunological aberrations in endometriosis. Epigenetics also appears to have a better explanatory power than genetics. There is accumulating evidence that various epigenetic aberrations exist in endometriosis. *In vitro* studies show that histone deacetylase inhibitors may be promising therapeutics for treating endometriosis. In conclusion, several lines of evidence suggest that epigenetics plays a definite role in the pathogenesis and pathophysiology of endometriosis. As such, endometriosis is possibly treatable by rectifying epigenetic aberrations through pharmacological means. DNA methylation markers may also be useful for diagnostic and prognostic purposes. It is also possible that the delineation of the epigenetic changes accompanied by the genesis and progression of endometriosis could lead to interventions that reduce the risk of developing endometriosis.

Key words: endometriosis / epigenetics / methylation / micro RNA / therapeutics

Introduction

Endometriosis, characterized by the presence and growth of functional endometrial-like tissues outside the uterine cavity, is a common and benign gynecological disorder with a poorly understood and somewhat enigmatic etiopathogenesis and pathophysiology (Giudice and Kao, 2004). It is a leading cause of disability in women of reproductive age, responsible for dysmenorrhea, pelvic pain and subfertility (Farquhar, 2000).

In treating women with endometriosis, the efficacy has been measured by means of assessment of pain and infertility (Olive and Pritts, 2001). The current treatment modalities include medical, surgical or a combination of both, with surgery being the treatment of choice. However, the recurrence risk after surgery is high: 7–30% of patients reported recurrences 3 years after laparoscopic surgery (Weedon *et al*., 2008). The risk increases to 40–50% 5 years after surgery (Wheeler and Malinak, 1983; Guo, 2009a). Since repeated surgeries are positively associated with increased morbidity and health care costs and, in endometriosis, with damage to ovarian reserve (Hachisuga and Kawarabayashi, 2002; Somigliana *et al*., 2003; Candiani *et al*., 2005; Ragni *et al*., 2005; Somigliana *et al*., 2006), the risk for reoperation poses a serious challenge to the effective management of endometriosis. Therefore, non-surgical medical therapy is needed.

Non-surgical medical therapy is also used as a first-line therapy for treating endometriosis, and can be used in conjunction with those patients who undergo surgical therapy for pain. The current medical treatment for endometriosis has so far focused on the hormonal alteration of the menstrual cycle to produce a pseudo-pregnancy, pseudo-menopause or chronic anovulation, creating an acyclic, hypoestrogenic environment (Olive and Pritts, 2001). This is achieved either by blocking ovarian estrogen secretion [GnRH agonists (GnRH-a)], by inducing pseudo-pregnancy (progestins), or by locally inhibiting estrogenic stimulation of the ectopic endometrium (progestins, androgenic progestins) (Lessey, 2000; Olive and Pritts, 2001; Valle and Sciarra, 2003). Although all hormonal treatments are more or less equally effective in relieving pains (Kennedy *et al*., 2005), the relief, however, appears to be relatively short-term (Waller and Shaw, 1993). Given the lack of long-term efficacious medical therapy for endometriosis-associated pelvic pain and for minimizing recurrence risk, as well as for endometriosis-associated subfertility, there is a clear need for novel medical therapies with more tolerable side-effects and cost profiles (Guo, 2008).

In response to this need, numerous encouraging preclinical studies of potential therapeutics for endometriosis have been reported in the last decade. A handful of these have undergone phase II/III clinical trials. Unfortunately, most of the finished trials remain unpublished (Guo *et al*., 2009). For those trials that have been published, the

efficacy turns out to be far less impressive than that found in preclinical studies (Guo, 2008).

These setbacks could well be temporary, but may also signal our current inadequate understanding of the molecular mechanisms underlying endometriosis. In this article, our current knowledge of the epigenetic changes in endometriosis is reviewed, and their implications for delineating the molecular mechanisms discussed, together with clinical diagnosis, therapeutics and intervention aimed at reducing recurrence risks of endometriosis.

Methods

A systematic and comprehensive search of PUBMED was performed for all studies published up to 30 June 2009, using the following search terms: 'endometriosis', 'epigenetics', 'methylation', 'histone acetylation', 'histone phosphorylation', 'histone ubiquitylation', 'histone sumoylation', 'micro RNA', 'post-translational modifications (PTMs)' or a combination of them. The studies had to report epigenetic aberrations in endometriosis. The search was limited to publications written in English. Evidence for endometriosis epigenetics was included, and its therapeutical, diagnostic and prognostic implications were discussed.

Why epigenetics?

Endometriosis has been regarded as an ultimate hormonal disease, owing much to its estrogen-dependency and aberrations in estrogen production and metabolism (Bulun *et al*., 2002; Kitawaki *et al*., 2002; Gurates and Bulun, 2003). It also has been viewed as an immunological disease due to a myriad immunological aberration in endometriosis (Paul Dmowski and Braun, 2004; Ulukus and Arici, 2005). In addition, it has been thought of a disease caused by exposure to environmental pollution and toxins (Rier *et al*., 1993; Rier, 2002) although so far there are no solid human data (Guo, 2004). Finally, it has been regarded as a genetic disease (Simpson *et al*., 2003; Barlow and Kennedy, 2005), due, apparently, to its reported familial aggregation. Yet even the reported familial aggregation, when examined closely, may be debatable (Di and Guo, 2007), and there has been little progress regarding the identification of genetic variants that predispose women to endometriosis (Di and Guo, 2007; Falconer *et al*., 2007; Guo, 2009b).

Endometriosis is undoubtedly a hormonal disease and certainly entails an array of immunological aberrations. Although so far there is no solid evidence linking dioxin exposure to endometriosis, it may still be plausible that dioxin exposure, at right time and dosage, might precipitate the initiation or progression of endometriosis through interaction with estrogen receptors (ERs) (Ohtake *et al*., 2003) or suppressing expression of progesterone receptors (PRs) (Igarashi *et al*., 2005). So what is the common denominator for a disease that is hormonal, immunological and possibly environmental and genetic?

In the last decade, numerous large-scale gene expression profiling studies have demonstrated, unequivocally, that many genes are deregulated in endometriosis (Carson *et al*., 2002; Arimoto *et al*., 2003; Kao *et al*., 2003; Matsuzaki *et al*., 2005; Wu *et al*., 2006a; Burney *et al*., 2007; Chand *et al*., 2007; Eyster *et al*., 2007; Flores *et al*., 2007; Hever *et al*., 2007; Konno *et al*., 2007; Mettler *et al*., 2007; Van Langendonckt *et al*., 2007; Hull *et al*., 2008; Sherwin *et al*., 2008; Zafrakas *et al*., 2008; Pelch *et al*., 2009; Umezawa *et al*., 2009). It also has been shown that a single focus of endometriotic lesion originates from a single progenitor cell (Wu *et al*., 2003), forming a cellular lineage. During their development from single progenitor cells to endometriotic lesions leading to various symptoms, endometriotic cells presumably need to make a series of sequential, perhaps dichotomous and irrevocable cell fate choices. These choices are likely to be made without any change in DNA sequences. This cellular lineage, or identity, inevitably requires that cells transcribe, or enable transcription of, specific sets of genes whereas at the same time repress others. To maintain cellular identity, the gene expression program must be iterated through cell divisions in a heritable fashion by epigenetic processes.

Indeed, transcription is regulated, in part, by the assembly of a plethora of complexes of transcription factors on regulatory regions of genes, and can be regulated at various levels: DNA modifications (both chemical and structural), post-transcriptional modifications and PTMs. These involve chemical modification of DNA (methylation), histone modification and various machineries, such as specific factors, repressors, activators, general transcriptional factors, enhancers, micro RNAs (miRNAs) (Ambros, 2004; Bartel, 2004) and recently discovered, double-stranded, non-coding RNAs (ncRNAs) (Kurokawa *et al*., 2009). These levels are either part of the epigenetic regulation (DNA methylation, histone modifications, miRNA) or closely related. After the DNA is transcribed and mRNA formed, there are extra levels of regulation on how much the mRNA is translated into proteins. PTMs of protein products, localization and higher order interactions with other transcription factors, coactivators or corepressors are one set of mechanisms through which transcription can be controlled at another level.

In light of these, epigenetics is likely to be involved in maintaining cellular identity in ectopic endometrial cells. This is the view that was first expressed in Wu *et al*. (Wu *et al*., 2005) that 'endometriosis, like neoplasia, may also be an epigenetic disease', after realizing that epigenetic aberration, as a more general biological phenomenon and possibly one major mechanism for gene deregulation, should not be exclusively confined only in cancers or developmental diseases.

What is epigenetics?

Epigenetics refers to the stable inheritance of phenotypes of cells and organisms without changes in DNA sequence or DNA content, which involves nuclear processes such as DNA methylation, histone modifications and transcription factor network (Holliday, 1994). An expanded definition of epigenetics may also include regulatory circuits at the cellular or even tissue level—any aspects other than DNA sequences, in keeping with the initial notion of C.H. Waddington who coined the word 'epigenetics' (epi-, in Greek, means 'over' or 'above', hence epigenetics means 'on top of' or 'in addition to' genetics). Epigenetic processes are known to be involved in development, health, disease and aging, and responsible for phenomena such as X-chromosome inactivation and genomic imprinting (Robertson and Wolffe, 2000; Rodenhiser and Mann, 2006).

The human genome consists of just over 3 billion DNA base pairs. It contains about 24 000 protein-coding genes (Stein, 2004), not many more than the roundworm *Caenorhabditis elegans*, which has about 20 000 genes even though its genome size is about 1/30 of that in

humans. Recent analyses of the human and animal genomes reveal that the majority of RNA transcripts in humans are not protein coding RNAs (i.e. mRNAs), but ncRNAs (Szymanski *et al*., 2003; Mattick and Makunin, 2006). In humans, about 50% of genomic DNA is transcribed into RNA transcripts, of which merely 2% is translated into proteins—the remaining 98% is ncRNAs (Erdmann *et al*., 2001; Storz, 2002; Szymanski *et al*., 2003; Mattick and Makunin, 2005, 2006).

One important way that genes are regulated is through the remodeling of chromatin. The nucleosome, a basic unit of chromatin, consists of an octamer formed of two copies of each of the four core histones (H2A, H2B, H3 and H4) around which 147 bp DNA is wrapped in 1.65 left-handed superhelical turns (Luger *et al*., 1997). The neighboring nucleosomes are connected by a stretch of free DNA called 'linker DNA'. The nucleosomes and histones are organized into chromatin. Dynamic changes in chromatin structure influence gene expression and are affected by chemical modifications of histone proteins such as methylation (DNA and histone) and acetylation (histone), and by non-histone, DNA-binding proteins (Li, 2002). Enzymes involved in this process include DNA methyltransferases (DNMTs), histone deacetylases (HDACs), histone acetylases (HATs), histone methyltransferases (HMTs) and the methyl-CpG-binding protein MeCP2.

The most studied and the best understood epigenetic modification is DNA methylation, which involves the addition of a methyl group to specific dinucleotide sites along the genome, i.e. cytosines 5′ of guanines, or at CpG sites (Laird and Jaenisch, 1996). In general, promoter hypo- and hyper-methylation is associated with gene expression and silencing, respectively. The patterns of DNA methylation are initiated and maintained by DNMTs.

Besides DNA methylation, covalent histone modifications such as acetylation, methylation, ubiquitinization and sumoylation can also orchestrate DNA organization and gene expression (Peterson and Laniel, 2004). These modifications result in subsequent changes in chromatin structure, making it either accessible or inaccessible for transcription (Fig. 1). DNA methylation typically works in concert with histone modifications, dynamically controlling the chromatin structure and gene expression (Vaissiere *et al*., 2008).

The dynamic change of epigenetic modifications

One notable feature in epigenetics is that all epigenetic modifications are reversible and dynamic. The epigenome, the collection of DNA methylation states and histone modifications, can be influenced and modified by environmental and lifestyle factors throughout the entire lifespan (Jaenisch and Bird, 2003). Aging, for example, is associated with changes in DNA methylation patterns (Issa, 2003). In addition, it is associated with a decline in global CpG methylation (Fraga and Esteller, 2007).

Diet also can impact on gene expression through epigenetic modifications. Folate deficiency is linked with open neural tube defects (Tamura and Picciano, 2006), likely due to the essential role that folate plays in converting methionine to S-adenosylmethionine, the main methyl group donor in DNA methylation reactions. Folate therapy restores the normal state of DNA methylation, normalizing gene expression (Ingrosso *et al*., 2003).

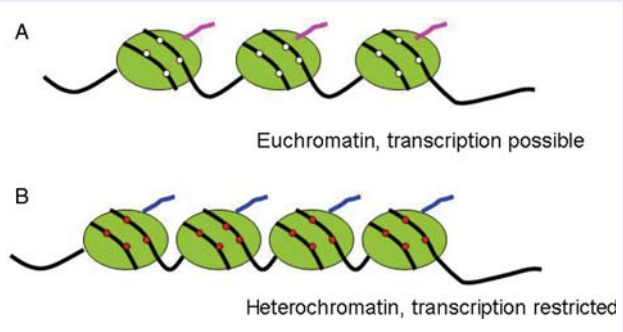

Figure 1 Schematic illustration of close interactions between DNA methylation and histone modifications.

Strands of DNA (black) are wrapped around histone octamers (olive green), collectively constituting nucleosomes. The neighboring nucleosomes are connected by linker DNAs (black). The nucleosomes, along with linker DNAs, are organized into chromatin, the building blocks of chromosomes. (**a**) Euchromatin, characterized by unmethylated CpG sites (white dots) in the promoter and characteristic chemical modifications of the histone tails (pink), is extended chromatin accessible for transcription. The gene is 'switched on'—expressed. (**b**) Heterochromatin, characterized by methylated CpG sites (red dots) in the promoter and characteristic chemical modifications of the histone tails (blue), is condensed chromatin restricted or inaccessible for transcription. The gene is 'switched off'—unexpressed.

Steroid nuclear receptors, coregulators and epigenetic regulation

Endometriosis is an estrogen-dependent disease. One class of medication in treating endometriosis is GnRH agonists which suppress luteinizing hormone and follicle-stimulating hormone, which, in turn, suppresses ovarian production of estrogens, resulting in an acyclic, hypoestrogenic environment (Olive and Pritts, 2001) and thus striping away the fuel for proliferation and possibly pain. Two other classes involve progesterone (progestins) and androgen (Danazol). Since the action of steroids such as estrogen, progesterone and androgen are mediated through their respective receptors—ERs, PRs and androgen receptor (AR)—these receptors must be and have shown to be intimately involved in the pathogenesis of endometriosis. ERs, PRs and AR, along with glucocorticoid receptor and mineralocorticoid receptor, form the steroid receptors (SRs) family, which is one of three members of the nuclear receptor (NR) superfamily of transcription factors (Bain *et al*., 2007). Besides the SR family, other members of the NR superfamily, such as vitamin D receptor (Agic *et al*., 2007), retinoic acid receptor (Sawatsri *et al*., 2000), and peroxisome proliferator-activated receptor (Lebovic *et al*., 2004) may also be involved in endometriosis.

SRs are ligand (hormone)-dependent transcription factors. Upon activation with the specific hormone they can interact with hormone response elements (HREs) in the promoter of target genes. They can also activate genes lacking specific HREs by interacting with other sequence-specific transcription factors bound to their target sequences (Wolf *et al*., 1992). Besides these 'genomic effects', hormones can also produce non-genomic effects, characterized by their insensitivity to inhibitors of transcription and protein synthesis and,

most notably, by their rapid onset of action very similar to those elicited by growth factors (Falkenstein *et al*., 2000).

SRs, like the rest of the members of the NR superfamily, have a modular structure consisting of four main conserved domains: a highly conserved and centrally located DNA-binding domain (DBD), a moderately conserved C-terminal ligand-binding domain (LBD), a weakly conserved N-terminal activation function (AF) domain, and the hinge that links LBD and DBD (Bain *et al*., 2007). The DBD is the site of many protein–protein and hormone interactions, whereas the LBD binds hormones. SRs, and NRs are part of multi-protein complexes including transcription factors and chromatin-modifying coactivator proteins which include HMTs, kinases, ubiquitinases, deacetylases, HMTs and demethylases (Taylor *et al*., 1984). ERα, AR and PRs can be directly modified by kinases, HATs and small ubiquitin-like modifiers and these modifications modify SRs' DNA binding, transcriptional activity and hormone sensitivity (Fu *et al*., 2000; Lange *et al*., 2000; Sato *et al*., 2000; Du *et al*., 2001; Wang *et al*., 2001; Chauchereau *et al*., 2003; Kim *et al*., 2006).

SRs function as transcription factors that govern transcription of their target genes. It is now known that the regulation of gene transcription by these SRs requires the recruitment of proteins characterized as coregulators, which bind to modify target gene expression through protein complex formation. These interactions facilitate either coactivation or corepression of gene expression. Coactivators act either through recruiting protein complexes to function as a bridge linking SRs and the transcriptional machinery or through their histone modifying capabilities (Taylor *et al*., 1984; Rosenfeld and Glass, 2001; Smith and O'Malley, 2004). SR corepressors, on the other hand, typically interact with unliganded SR and recruit other histone modifying proteins such as HDACs to suppress target gene expression (Taylor *et al*., 1984; Smith and O'Malley, 2004).

Coactivators can be roughly grouped into two categories (with some overlaps): those that are components of the basal transcriptional apparatus, or in contact with the apparatus, and those that remodel or modify the structure of chromatin (Featherstone, 2002). The latter includes ATP-dependent chromatin-remodeling complexes such as SWI/SNF and complexes with HAT activity such as CBP/p300.

Depending on the type and level of co-regulators recruited, the SR may display different transactivation behaviors, even though the same ligand is used (Kale *et al*., 1971; Smith and O'Malley, 2004). PR is a member of the SR subfamily and is expressed as two different sized proteins from a single gene; PR-A and PR-B. The two PR isoforms are identical in their DBD and C-terminal LBD, differing only in the N-terminal domain that is truncated in PR-A. PR also contains two autonomous transcription activation domains, ligand-dependent AF-2 in the C-terminus and constitutive AF-1 in the N-terminus. The opposing transcriptional activity of the two isoforms of PR has been shown to be due to differential co-regulator binding (Giangrande *et al*., 2000). One recent study found that one PR co-activator, Hic-5, is expressed in endometrium but its expression is significantly lower in endometrium from women with endometriosis, suggesting that progesterone resistance in endometrium from women with endometriosis may derive, in part, from impaired expression of the PR co-activator, Hic-5 (Aghajanova *et al*., 2009).

Coregulators participate in gene transcription by acting as ATP-dependent enzymes that remodel the chromatin landscape of particular gene sections, even though they themselves are unable to bind DNA (Clapier and Cairns, 2009). Different classes of chromatin-modifying coregulators include proteins that covalently modify histones by acetylation, methylation, phosphorylation, ubiquitylation or sumoylation (Couture and Trievel, 2006). The post-translationally modified histones regulate gene transcription by either facilitating or hindering the binding of other transcription factors, structural proteins or the basic transcriptional machinery (Jenuwein and Allis, 2001).

SR coactivators such as SRC-1 and p300/CBP are reportedly expressed in endometrium (Gregory *et al*., 2002; Shiozawa *et al*., 2003; Kershah *et al*., 2004). Corepressors such as NcoR and SMRT are found to be expressed in a cycle-dependent fashion in endometrium (Wieser *et al*., 2002; Shiozawa *et al*., 2003; Vienonen *et al*., 2004). The expression patterns in SR coregulators appear to be different between normal and pathological endometrium (Gregory *et al*., 2002; Uchikawa *et al*., 2003; Kershah *et al*., 2004; Quezada *et al*., 2006). One promising drug candidate for treating endometriosis, asoprisnil (a selective PR modulator), apparently recruits coregulators differently from antiprogestins (Madauss *et al*., 2007).

One piece of evidence that co-activators may be involved in endometriosis in the context of steroid production is the report that treatment of endometriotic stromal cells with PGE_2 resulted in increased H3 acetylation bound to the promoter of steroidogenic acute regulatory (StAR) protein, with concomitant induction of CREB-recruited CBP/p300 (Sun *et al*., 2003). StAR regulates the first committed step of 17β-estradiol biosynthesis by promoting the translocation of cholesterol from the cytosol to the inner mitochondrial membrane via a yet undetermined mechanism (Arakane *et al*., 1998; Bose *et al*., 2002). It is possible that PGE_2 stimulation results in increased recruitment of CBP/p300, which, as a HAT, acetylates StAR-bound H3.

Taken together, these discussions suggest that epigenetic regulation plays a very important role in regulating the activity of SRs, and that it is biologically plausible that some coregulators must be involved in endometriosis.

Epigenetics and immunology

There is ample evidence indicating that alterations in both cell-mediated and humoral immunity contribute to the pathogenesis of endometriosis. Increased number and activation of peritoneal macrophages, decreased T cell and natural killer (NK) cell cytotoxicities are the alterations in cellular immunity, yielding diminished removal of ectopic endometrial cells from the peritoneal cavity (Ulukus and Arici, 2005). In addition, increased levels of several proinflammatory cytokines and growth factors produced by immune and endometrial cells (Wu and Ho, 2003; Umezawa *et al*., 2008) are likely to be involved in facilitating implantation and growth of ectopic endometrial cells by promoting proliferation, inflammation and angiogenesis. Besides the impaired capacity of the immune cells to mediate endometrial cell removal, endometriotic cells may have inherent resistance against immune cells, or evade detection and thus contribute additional factors in the pathogenesis of endometriosis.

Epigenetic mechanisms have shown to be involved in regulating normal immune response (Sawalha, 2008). Evidence is accumulating that epigenetic regulation is involved in several key immune processes including antigen presentation, T-cell differentiation, cytokine expression, effector function and memory (Fitzpatrick and Wilson,

2003). Various epigenetic mechanisms may contribute to the establishment of transcriptional thresholds that vary between genes and T cell types. For example, a small region in IL-2 promoter in T cells is demethylated soon after activation, resulting in IL-2 production (Bruniquel and Schwartz, 2003). In T cells also, IL-4 gene regulation involves not only certain transcription factors but also DNMTs (Makar *et al*., 2003).

It is plausible that epigenetics may also play a role in the immunology of endometriosis. At the very least, one transcription factor, NF-κB, which is known to play a critical role in induction of various genes in immunity and inflammation (Kumar *et al*., 2004) and has been shown to be involved in endometriosis also (Gonzalez-Ramos *et al*., 2007), is intimately involved with ubiquitylation, a form of epigenetic control (see below). As of now, little, if any, work has been published regarding the epigenetics of immunology in endometriosis.

Evidence in support that endometriosis is an epigenetic disease

The first piece of evidence came from the study showing that the putative promoter of HOXA10 in endometrium from women with endometriosis is hypermethylated as compared with that from women without endometriosis (Wu *et al*., 2005). HOXA10 is a member of a family of homeobox genes that serve as transcription factors during development and has been shown to be important for uterine function. It is expressed in human endometrium, and its expression is dramatically increased during the midsecretory phase of the menstrual cycle, corresponding to the time of implantation and increased circulating progesterone (Troiano and Taylor, 1998). This suggests that HOXA10 may have an important function in regulating endometrial development during the menstrual cycle and in establishing conditions necessary for implantation (Taylor *et al*., 1998).

In endometrium of women with endometriosis, however, HOXA10 gene expression is significantly reduced, indicating some defects in uterine receptivity (Gui *et al*., 1999; Taylor *et al*., 1999), which may be responsible for reduced fertility in women with endometriosis. As promoter hypermethylation is generally associated with gene silencing, the observed HOXA10 promoter hypermethylation provides a plausible explanation as why HOXA10 gene expression is reduced in endometrium of women with endometriosis (Wu *et al*., 2005). The HOXA10 promoter hypermethylation coinciding with reduced HOXA10 expression has been demonstrated also in induced endometriosis in baboons (Kim *et al*., 2007), and in mouse (Lee *et al*., 2009). Hoxa10 hypermethylation, accompanied by overexpression of Dnmt1 and Dnmt3b, has been reported recently in mice prenatally exposed to diethylstilbestrol (DES) (Bromer *et al*., 2009). This aberrant methylation seems to be a novel mechanism of altered developmental programming induced by *in utero* DES exposure.

The second piece of evidence came from the study demonstrating that the promoter of PR-B is hypermethylated in endometriosis (Wu *et al*., 2006b). In addition, the PR-B promoter hypermethylation is concomitant with reduced PR-B expression, providing support for the role of epigenetic aberration in PR-B down-regulation. It is well-known that there is a general tendency of progesterone resistance in endometriosis (Giudice and Kao, 2004). It is also known that PR-B is down-regulated in endometriosis (Attia *et al*., 2000) and may be responsible for, at least in part, progesterone resistance since progesterone is mediated through its receptors, including PR-B. PR-B promoter hypermethylation thus provides a plausible explanation as why PR-B is persistently down-regulated in endometriosis.

Perhaps the most important piece of evidence showing that endometriosis is an epigenetic disease comes from a study demonstrating that DNMT1, DNMT3A and DNMT3B, the three genes coding for DNMTs that are involved in genomic DNA methylation, are all overexpressed in endometriosis (Wu *et al*., 2007b). Since these genes are involved in *de novo* as well as maintenance methylation, their aberrant expression suggests that aberrant methylation may be wide-spread in endometriosis. As methylation is closely linked with chromatin remodeling, the aberrant expression of these genes may also signal that there are aberrant epigenetic changes, other than methylation, in endometriosis.

Consistent with this view, several very recent studies provide further evidence for epigenetic changes in endometriosis. Steroidogenic factor-1 (SF-1), a transcriptional factor essential for activation of multiple steroidogenic genes for estrogen biosynthesis, is usually undetectable in normal endometrial stromal cells but is aberrantly expressed in endometriotic stromal cells. Xue *et al*. (2007b) showed that SF-1 promoter has increased methylation in endometrial cells yet in endometriotic cells it is hypomethylated. They also found that ERß promoter is hypomethylated in endometriotic cells, which accounts for its overexpression (Xue *et al*., 2007a). Izawa *et al*. (2008) also showed that the treatment of endometrial stromal cells, which normally do not express aromatase, with a demethylation agent (DMA), 5-aza-deoxycytidine, dramatically increased the aromatase mRNA expression. Since endometriotic cells use the same aromatase promoters, promoter II, 1.3 and 1.6, as in endometrial cells, their finding appears to suggest an epigenetic regulatory mechanism in deregulating aromatase expression in endometriosis. One lingering puzzle, however, is whether aromatase is hypomethylated in ectopic endometrium, and if so, whether treatment with either histone deacetylase inhibitors (HDACIs) or DMAs can inhibit aromatase gene expression.

Endometriotic cells are found to lack the intercellular adhesion protein E-cadherin, a known metastasis-suppressor protein in epithelial tumor cells whose deregulation also seems to be associated with invasiveness of endometriotic cells (Starzinski-Powitz *et al*., 1998, 2001). In two immortalized endometriotic cell lines, E-cadherin was found to be hypermethylated, and the treatment with the HDACI, trichostatin A (TSA) resulted in its reactivated expression with concomitant attenuated invasion. (Wu *et al*., 2007a) This seems to suggest that, at least in endometriotic cell lines, E-cadherin silenced by methylation is associated with invasiveness.

Our *in silico* study based on large-scale gene expression profiling of paired ectopic and eutopic endometrium also suggests a theme of PTM and histone deacetylation (Wren *et al*., 2007), again supporting the role of epigenetics in endometriosis. Table I provides a complete list of genes with various epigenetic aberrations identified so far.

Epigenetics and MicroRNA

MicroRNAs (miRNAs) are a large class of endogenous, single-stranded, short, ncRNA of approximately 22-nucleotides in length,

Table I Genes identified so far to have epigenetic aberrations in endometriosis

Year of the first report	Gene name	Major finding	Reference
2005	HOXA10	Hypermethylated in eutopic endometrium	Wu *et al.* (2005), Kim *et al.* (2007), Lee *et al.* (2009)
2006	PR-B	Hypermethylated in ectopic endometrium	Wu *et al.* (2006b)
2007	Aromatase	Endometriotic cells secreted more aromatase than endometrial cells with added testosterone, yet when treated with a DMA, endometrial cells increased the secretion	Izawa *et al.* (2008)
2007	ERβ	Hypomethylated in ectopic endometrium	Xue *et al.* (2007a)
2007	SF-1	Hypomethylated in ectopic endometrium	Xue *et al.* (2007b)
2007	E-cadherin	Methylated and inactivated in an endometriotic epithelial-like cell line, and can be demethylated and reactivated by the treatment with the HDACI, TSA	Wu *et al.* (2007a)

that play a key role in regulating gene expression through interaction with mRNA of protein-coding genes. Evolutionarily highly conserved, miRNAs account for 2–3% of the human genome and collectively regulate about 30% of the human genes (Lewis *et al.*, 2003). MiRNAs may be located within introns of coding genes or in intergenic regions, but also can be found, albeit more rarely, in coding exons (Lai *et al.*, 2003; Lim *et al.*, 2003; Rodriguez *et al.*, 2004). As with DNA methylation or histone modifications, they also regulate gene expression without any change in DNA sequences, which is achieved through either inhibiting mRNA translation or, less frequently, inducing mRNA degradation (Fig. 2). Because of this, miRNAs are components of epigenetics regulation.

Discovered at the turn of the century (Reinhart *et al.*, 2000; Lau *et al.*, 2001; Lee and Ambros, 2001), miRNA's essential role in development and its significance in health and disease soon became evident and is now an active research field (He and Hannon, 2004). Growing evidence shows that miRNA expression is deregulated in cancer, and that aberrant miRNA expression correlates with aberrant expression of tumor suppressor genes and oncogenes, and thus can be of diagnostic and prognostic values (Calin *et al.*, 2005; Croce and Calin, 2005; Lu *et al.*, 2005; Lujambio *et al.*, 2008). More recent research indicates that miRNA is not merely a suppressor of gene expression; it also plays a more diverse role in gene regulation (Takamizawa *et al.*, 2004). Ørom *et al.* (2008) report that one specific miRNA, miR-10a, can interact with the 5′ untranslated region of mRNAs encoding ribosomal proteins and *enhance* their translation.

At the time of writing, there are only three published miRNA studies of endometriosis. One miRNA expression profiling study identified 48 out of 287 miRNAs that are differentially expressed with progressive decline in expression level in endometrium from women without endometriosis (EN), paired eutopic and ectopic endometrium (EU and EC), and ectopic endometrium from women with endometriosis (EE) (Pan *et al.*, 2007). The target genes of these identified miRNAs include many genes known to be involved in endometriosis, such as ERα, ERβ, PR and TGF-β, suggesting that miRNA deregulation may be involved in endometriosis.

A follow-up study conducted by the same group further evaluated the expression of four miRNAs, identified previously to be differentially expressed in ectopic and eutopic endometrium, and their predicted target genes, StAR protein, aromatase and COX-2, and further assessed the influence of ovarian steroids on their expression in endometrial cells (Toloubeydokhti *et al.*, 2008). An inverse correlation between miRNA and their putative gene expression levels was found, and the expression of these miRNAs and genes were differentially regulated by 17β-estradiol, medroxyprogesterone acetate, ER antagonist ICI-182780 and RU486 (Toloubeydokhti *et al.*, 2008). Thus, by showing that the altered expression of specific miRNAs affects the stability of their target genes known to be involved in endometriosis, the role of these miRNAs in pathogenesis of endometriosis is demonstrated.

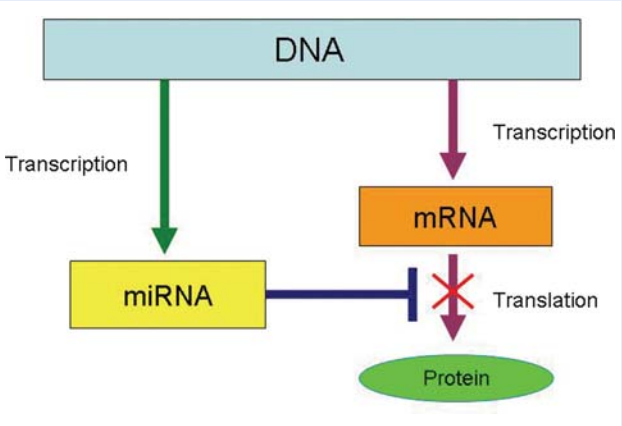

Figure 2 Schematic illustration of the canonical mechanism of protein suppression by miRNA.

MiRNAs are transcribed mainly through RNA pol II, occasionally through RNA pol III, from DNA, processed in the nucleus by Drosha to give rise to pre-miRNA, and then exported to the cytoplasm, where pre-miRNA matures into miRNA:miRNA duplex. The duplex is then recognized and cleavaged by Dicer and the resultant miRNA strand is integrated in a complex called miRISC, which by binding to target mRNA molecules inhibits translation or induces mRNA degradation. See (Bartel, 2004).

In another study, several miRNAs also have recently been identified as playing a role in epigenetics of endometriosis (Ohlsson Teague *et al*., 2009). By functional analysis of predicted target genes based on the identified miRNAs, DNMT3A and HADC4 were found to be among the target genes of miR-29c and miR-1, respectively (Ohlsson Teague *et al*., 2009). One unsolved puzzle, however, is that, since miR-29c overexpression has been shown to be correlated with higher expression of DNMT3A in lung cancer (Fabbri *et al*., 2007), the overexpression of miR-29c in ectopic endometrium as reported would imply the down-regulation of DNMT3A. But this would be at odds with previously reported DNMT3A up-regulation in endometriosis (Wu *et al*., 2007b). Whether this apparent discrepancy is due to the difference in phenotype (lung cancer versus endometriosis) or in methods [laser capture microdissected endometriotic epithelial cells in Wu *et al*. (2007b) versus just endometrial cells as in Ohlsson Teague *et al*. (2009)], or simply due to the many-to-one relationship in miRNA-mediated gene regulation (i.e. one gene can be regulated by several miRNAs) is unclear. Further research should clarify this discrepancy.

The apparent miRNA deregulation in endometriosis as recently reported begs an obvious question as how they are deregulated. It turns out that one mechanism is identical to that in gene regulation, i.e. through miRNA methylation. It has been shown that treatment of breast cancer cell line with a HDACI rapidly induced significant changes in the miRNA expression profile (Scott *et al*., 2006). Treatment with HDACIs and DMAs has been shown to restore miR-127 expression (Saito *et al*., 2006), and genetic disruption by homologous recombination of DNMTs is reported to restore epigenetically silenced miR-124 (Lujambio *et al*., 2007).

So far no report on methylation and miRNA in endometriosis has been published. It is interesting to note that miR-34c has been shown to be under-expressed in endometriosis (Ohlsson Teague *et al*., 2009), yet epigenetic silencing of miR-34c has also been shown to be associated with CpG island methylation which can be restored by treatment with a DMA in colorectal cancer (Toyota *et al*., 2008). Thus, an obvious question is whether miR-34c expression also could be restored by treatment with either HDACIs, DMAs or both.

MiRNAs have been shown to play important roles in modulating innate and adaptive immune responses, T-cell differentiation and activation, and B cell differentiation (Baltimore *et al*., 2008; Bi *et al*., 2009). MiRNAs also respond to steroid hormones and interact with SRs (Vreugdenhil *et al*., 2009; Wickramasinghe *et al*., 2009). Hence they may also be involved in SR regulation and in immunological aspects of endometriosis, neither of which has been characterized so far.

It should be noted that miRNAs are not merely unidirectional negative regulators of gene expression. Vasudevan *et al*. (2007) demonstrate that miRNA can also increase translation. Recent research also demonstrates that many miRNAs interact closely with transcription factors, often forming a network for gene regulation. Human granulocytic differentiation, for example, is shown to be controlled by a regulatory circuitry involving miR-223 and two transcriptional factors, NFI-A and C/EBPα (Fazi *et al*., 2005). The two factors compete for binding to the miR-223 promoter: NFI-A maintains miR-223 at low levels, whereas its replacement by C/EBPα, following retinoic acid-induced differentiation, up-regulates miR-223 expression. The competition by C/EBPα and the granulocytic differentiation are favored by a negative-feedback loop in which miR-223 represses NFI-A translation. The interactions between miRNA and transcription factors can yield either a negative-feedback loop (Burk *et al*., 2008) or a feedforward loop (Sylvestre *et al*., 2007). This adds another layer of complexity in gene regulation.

As a new class of post-transcriptional regulators of gene expression, the importance of miRNAs in the pathogenesis of endometriosis is only beginning to be unveiled. Existing evidence, mostly from cancer research, has shown that miRNAs are involved in numerous cellular processes, including differentiation, cell cycle progression, apoptosis, embryogenesis, angiogenesis, oncogenesis and immune responses (Cobb *et al*., 2006; Song and Tuan, 2006; Rodriguez *et al*., 2007; Dykxhoorn *et al*., 2008; Kuehbacher *et al*., 2008). MiRNAs also have been found to be both regulators and targets of methylation and acetylation processes. It is thus reasonable to expect that miRNAs may similarly wear both hats of regulators and targets in endometriosis as well and are involved in endometriosis pathogenesis. Thus, in light of their potentially important roles in endometriosis, miRNAs may well become promising therapeutic targets as their roles and mode of actions are unraveled in future studies. This enthusiasm is buoyed, perhaps in no small part, by the recognition that there are far less miRNAs (predicted to be around 1000) than mRNAs (~24 000 genes, plus numerous splicing variants) and also by the recent reports of successful and well-tolerated use of anti-miRs in animal studies (Esau *et al*., 2006; Elmen *et al*., 2008a, b; Stenvang *et al*., 2008), as well as the news of a stage I human trial on the use of anti-miR-122 in treating hepatitis C infection (www.fiercebiotech.com/story/first-mirna-drug-enters-human-studies/2008-05-28, accessed on 4 July 2009).

However, this enthusiasm is not unguarded and certainly not unbounded. Aside from various technical hurdles inevitably espoused with new technology, the seemingly vast gulf between the exciting preclinical findings and somewhat disappointing clinical trials as seen recently in endometriosis serves as a sobering and even humbling reminder as just how challenging it is for translational research (Guo and Olive, 2007; Guo *et al*., 2009). Indeed, anti-miRNAs may have global, non-specific effects, causing collateral damage and untoward side effects. It has been reported that cardiac-specific knockout of Dicer, a gene encoding a RNase III endonuclease essential for miRNA processing, leads to post-natal lethality (Chen *et al*., 2008). Therefore, more research will be needed.

Post-translational modifications

As proteins, histones can, as alluded to above, undergo various kinds of PTMs which alter their interaction with DNA and nuclear proteins. The H3 and H4 histones, in particular, have long tails protruding from the nucleosome and can be covalently modified post-translationally at various residuals and in various ways. Histone modifications, primarily on the N-terminal tail, include acetylation, methylation, phosphorylation, ubiquitination, sumoylation, citrullination and ADP-ribosylation. The core of the histones H2A and H3 can also be modified. Thus, histone PTM can take place in different histones (e.g. H3 or H4), histone variants (e.g. H3.3) and histone residuals (e.g. argine, lysine and serine). The modification can involve different chemical groups (e.g. acetyl, methyl and phosphate), and, for methylation, there can be different degrees (e.g. mono-, di- and tri-methylation). These

combinations of histone PTMs influence the interaction of histones with DNA and nuclear proteins, and act in gene regulation. Thus, the combinations of modifications are proposed to constitute a code for gene expression, the so-called 'histone code' (Strahl and Allis, 2000; Jenuwein and Allis, 2001).

Histone acetylation, methylation and phosphorylation are the most investigated histone PTMs. In general, histone acetylation is associated with transcriptional activation whereas deacetylation, transcriptional repression (Jenuwein and Allis, 2001; Bernstein *et al*., 2007). In contrast, the effect of histone methylation depends on the histone residuals, their positions and degrees (Jenuwein and Allis, 2001; Bernstein *et al*., 2007).

Work on histone PTMs and their roles in endometriosis has been scanty. Animal studies seem to suggest that HDAC2 is aberrantly expressed in ectopic endometrium (Liu *et al*., unpublished data). The only published work related with histone PTM is the one by Sun *et al*. (2003) who reported that the treatment of endometriotic stromal cells with PGE_2 resulted in increased H3 acetylation bound to the StAR promoter, with concomitant induction of CREB-recruited CBP/p300. Taken together, histone PTM is likely to be involved in the pathogenesis of endometriosis.

As with histone PTMs, the research on possible involvement of protein PTMs, other than phosphorylation, in endometriosis also has been scanty, even though they are almost surely involved. For example, ubiquitylation is proposed to interact with acetylation and methylation (Osley *et al*., 2006). Similarly, sumoylation, a process known to be involved in various cellular processes such as nuclear-cytosolic transport and protein stability (Hay, 2005), is thought to be involved in DNMT3a regulation (Ling *et al*., 2004) and HDAC-mediated transcriptional repression (Yang and Sharrocks, 2004). Sumoylation also is reported to be involved in the repression of SF-1 (Lee *et al*., 2005; Campbell *et al*., 2008), a gene recently reported to be hypomethylated in endometriosis (Xue *et al*., 2007b).

As an important transcription factor involved in inflammation, apoptosis and other cellular processes, NF-κB was once suspected to play a pivotal role in the pathogenesis of endometriosis (Guo, 2006b) and soon its involvement in endometriosis was demonstrated (Gonzalez-Ramos *et al*., 2007; Lousse *et al*., 2008). Yet the NF-κB pathway is intimately involved with ubiquitylation, i.e. the degradation of IκB proteins, processing of NF-κB precursors and the activation of IKK (Chen, 2005). In addition, although quite a number of genes contain NF-κB-responsive an element in their regulatory regions, their expression pattern varies significantly, due possibly to environmental and differentiative cues. Recent research indicates that chromatin structure and epigenetic settings appear to be the ultimate integration sites of both environmental and differentiative inputs, determining proper expression of each NF-κB-dependent gene (Vanden Berghe *et al*., 2006). It is unclear as whether or not such integration also occurs in the development of endometriosis.

One tantalizing observation suggesting the involvement of PTM in endometriosis is the report of increased soluble IL-1 receptor type II (sIL-1RII) proteolysis in the endometrium of women with endometriosis (Bellehumeur *et al*., 2005). The released sIL-1RII binds with high affinity to proinflammatory cytokine IL-1, especially IL-1β which is known to be involved in endometriosis, and neutralizes its biological effects (Laurincova, 2000). It is reported that marked degradation of sIL-1RII in the culture medium of endometriosis women-derived endometrial tissue correlates significantly with the concentration of MMP-9, a protease with limited proteolytic activity (Laurincova, 2000). On the other hand, TIMP-1, an indigenous specific inhibitor of several MMP including MMP-9, partially inhibited sIL-1RII degradation. The observation appears to suggest a PTM mechanism, possibly ubiquitylation, by which proteases may contribute to the decreased IL-1RII stability and its decreased protein levels in the endometrial tissue of endometriosis women.

Cause or consequence?

Given the reported epigenetic aberrations in endometriosis, one question is whether these aberrations are the cause or merely the consequence of endometriosis. Since most, if not all, human studies reporting epigenetic aberrations in endometriosis are carried out cross-sectionally, the reported aberration may be a cause for, but also could be a consequence of, endometriosis. In a linearly causal relationship, the cause and consequence can be clearly defined, with temporal sequences, and necessary and sufficient cause distinguished. In a complex system, such as endometriosis which appears to be a system-wide disease (Leyendecker, 2000; Vinatier *et al*., 2000), in which there are usually many interconnected parts, a linearly causal relationship may be rare. In many ways, a complex transcription network often has a highly optimized tolerance featuring high efficiency, performance and robustness to designed-for-uncertainties yet hypersensitive to design flaws and unanticipated perturbations (Carlson and Doyle, 2000). In such a system, the demarcation of cause and consequence could be difficult since the removal of one part may affect other parts of the system, especially when the system is redundant. Such complex systems often display emergent properties. Therefore, it may be difficult to prove that in endometriosis aberrant methylation is a cause rather than a consequence.

Despite this challenge, it is known that methylation can be induced by various factors. Aging (Wilson and Jones, 1983; Toyota and Issa, 1999; Richardson, 2002), diet (Jacob *et al*., 1998), chronic inflammation (Hsieh *et al*., 1998; Issa *et al*., 2001), prolonged transcriptional suppression (Song *et al*., 2002; Stirzaker *et al*., 2004) and even maternal care (Champagne *et al*., 2006). In endometriosis, it has been shown that prolonged stimulation of an endometriotic epithelial-like cell line by tumor necrosing factor (TNFα), which has been shown to have increased production in endometriosis, resulted in at least partial methylation in the PR-B promoter (Wu *et al*., 2008b). This provides evidence that certain phenotypic changes in endometriosis, such as increased production of proinflammatory cytokines, may also cause epigenetic aberrations, which in turn result in changes in gene expression and subsequently other phenotypic changes such as increased cellular proliferation (Wu *et al*., 2008a) and perhaps some phenotypic changes.

Remarkably, developmental exposures to chemicals can result in aberrant methylation. Mice neonataly exposed to DES are reported to have demethylation of estrogen-responsive gene lactoferrin in their uteri, along with uterine tumor (Li *et al*., 1997; McLachlan *et al*., 2006). Neonatal exposure to DES can also lead to the hypomethylation nucleosomal binding protein 1 in mice (Tang *et al*., 2008). In mice exposed to DES *in utero*, Hoxa10 hypermethylation has been reported (Bromer *et al*., 2009). Nutritional factors and stress have also reported to alter DNA methylation during early life

(Li *et al*., 2003; Waterland and Jirtle, 2004; Weaver *et al*., 2004; Champagne *et al*., 2006; McGowan *et al*., 2009). Although it is still unclear as how much nutritional factors, stress and exposure to certain chemicals in early life and thus aberrant epigenetic changes that they may cause contribute to the risk of endometriosis, it should be noted that the concept of 'fetal origins of adult onset diseases' is fairly new, and research in this area can be quite challenging for obvious reasons. Nevertheless, the developmental origins of many chronic diseases such as type 2 diabetes have now been demonstrated epidemiologically (Barker, 2003). Incidentally, Missmer *et al*. (2004) reported that *in utero* exposure to DES nearly doubles the risk of developing endometriosis in women whereas low-birthweight increases the risk by 30%. Further research in area is sorely needed, not just for the sake of understanding of endometriosis pathogenesis but also because proper nutritional intervention may reverse the aberrant epigenetic changes (Dolinoy *et al*., 2006, 2007).

Therapeutic implications

Unlike DNA mutations or copy number changes, DNA methylation, histone and protein modifications are reversible. Hence, enzymes that regulate the epigenetic changes could be ideal targets for intervention by pharmacological means. From the above discussion on miRNA and epigenetics, it could be speculated that the use of HDACIs and/or DMAs could also rectify miRNA deregulation in endometriosis. Given the evidence that endometriosis may be an epigenetic disease, encouraging *in vitro* results on the use of HDAC inhibitors (HDACIs) as a potential therapeutics for endometriosis have been reported. Treatment of an endometrial stromal cell line with the HDACI, TSA resulted in decreased proliferation (Wu and Guo, 2006) and cell cycle arrest (Wu and Guo, 2008). The effect is likely through, perhaps in part, the up-regulation of PR-B by TSA (Wu and Guo, 2006), possibly through increased acetylation of histones in chromatins. Treatment of TSA also inhibited interleukin (IL)-1β-induced cyclooxygenase-2 (COX-2) expression (Wu and Guo, 2007). This is significant, since COX-2 overexpression has been observed in ectopic endometrium (Ota *et al*., 2001), found to correlate with endometriosis-associated pain (Matsuzaki *et al*., 2004; Buchweitz *et al*., 2006), and reported to be a biomarker for recurrence (Yuan *et al*., 2008). TSA treatment is also reported to up-regulate peroxisome proliferator-activated receptor gamma (PPARγ) expression in endometrial stromal cells (Wu and Guo, 2009). PPARγ agonists have been reported to inhibit vascular endothelial growth factor expression and angiogenesis in endometrial cells (Peeters *et al*., 2005), inhibit TNF-induced IL-8 production in endometriotic cells (Ohama *et al*., 2008), and repress ectopic implants in animal models of endometriosis (Lebovic *et al*., 2004; Aytan *et al*., 2007; Lebovic *et al*., 2007).

In two endometriotic cell lines, TSA treatment resulted in attenuated invasion and reactivated E-cadherin expression (Wu *et al*., 2007a). This appears to suggest that some cellular phenotypes of endometriotic cells, such as invasiveness, may be mediated epigenetically and, as such, could be tamed by epigenetic reprogramming through pharmaceutical means.

In a preliminary study, TSA has been found to inhibit the expression of SLIT2 (Zhao *et al*., unpublished data), a member of the SLIT family of secretory glycoproteins that are recently found to attract vascular endothelial cells *in vitro* and promote tumor-induced angiogenesis (Wang *et al*., 2003), and, more recently, found to be a constituent biomarker for recurrence of endometriosis (Shen *et al*., 2009).

There are indications that show HDACIs may be analgesic when treating endometriosis. The first such indication comes from the report that three HDACIs, TSA, suberic bishydroxamate and valproic acid (VPA), suppress spontaneous and oxytoxin-induced uterine contractility (Moynihan *et al*., 2008). It has been shown that women with endometriosis have aberrant uterine contractility during menses with increased frequency, amplitude and basal pressure tone as compared with those without (Bulletti *et al*., 2004). There is sign that in uterus from women with dysmenorrhea there is a lack of synchronization in fundal-cervical contraction (Kitlas *et al*., 2009). Incidentally, progesterone, a traditional drug for treating endometriosis-associated dysmenorrhea, can also inhibit myometrial contraction (Ruddock *et al*., 2008).

The *in vivo* data also appear to be encouraging. In mice with surgically induced endometriosis, treatment with TSA significantly reduced the average size of ectopic implants as compared with the controls (Lu *et al*., unpublished data). And this finding has been replicated in rats treated with VPA (Liu *et al*., unpublished data). More remarkably, it was found that induced endometriosis resulted in hyperalgesia or 'central sensitization' although TSA or VPA treatment significantly improved mice's or rats' perception of pain induced by noxious stimuli (Lu *et al*., unpublished data; Liu *et al*., unpublished data).

Taking advantage of an existing drug, VPA, that is an HDACI with known pharmacology, and the advantage that adenomyosis, once called endometriosis *interna*, can be diagnosed quite accurately by non-invasive imaging techniques and that adenomyosis shares with endometriosis many similarities, Liu and Guo tested VPA on three patients as a new therapeutics and found that it was well tolerated and, after 2 months of use, the pain symptoms was dramatically reduced (Liu and Guo, 2008). In addition, the uterus size was reduced by an average of one third. Results from more patients show that VPA can effectively alleviate adenomyosis-associated pain (X.S. Liu, personal communication).

The focus of current therapeutic approach has been so far confined to HDACIs and although there is no direct link to the reported aberrant methylation in endometriosis, the rationale is supported by the cross-talk between DNA methylation and histone modifications and the evidence that they work in concert to control gene expression (Fuks *et al*., 2000, 2005). It remains unclear as whether DNA methylation or histone modification is the primary signal by which gene expression is determined. Hence the change in histone modification may result in change in DNA methylation, and vice versa. The inhibition of histone deacetylation can result in DNA demethylation, as evidenced by the demethylation of E-cadherin as a result of HDACI treatment (Wu *et al*., 2007a).

The focus on HDACIs was also influenced by the *in vitro* observation that HDACI treatment appeared to be more potent than DMAs in suppression cellular proliferation (Y. Wu *et al*., unpublished data). In addition, it was influenced by some practical considerations: the two most widely used DMAs, azacytidine and decitabine, need to be administrated either intravenously or subcutaneously for several days (Silverman *et al*., 2002; Kantarjian *et al*., 2006). This route of administration may be acceptable to cancer patients but probably not to patients with endometriosis. Moreover, early positive results

of azacytidine use were overshadowed by profound and prolonged cytopenias and prohibitive gastrointestinal system toxicity (Saiki *et al*., 1978). Since drugs for treatment of endometriosis tacitly demand better side-effect profiles (Guo, 2008), side effects would be a legitimate concern. In contrast, one HDACI, VPA, was approved by the U.S. Food and Drugs Agency about 30 years ago with a well-known pharmacology and an excellent safety record. Hence, as a result of prioritization, the focus was placed on HDACIs, instead of DMAs.

There is indication that HDACIs appears to synergize with DMAs, resulting in more potent anti-proliferative effect than either used alone and more robust re-expression of methylation-silenced genes (Nie *et al*., unpublished data), as in cancer cells (Cameron *et al*., 1999). Clearly, future research should illuminate this further.

Potential detrimental effects of epigenetic therapies and possible ways to circumvent them

Since global hypomethylation is a notable feature of cancer and is reported to cause genomic instability (Eden *et al*., 2003; Gaudet *et al*., 2003), there may be a legitimate concern as whether the use of DMAs and/or HDACIs in treating endometriosis would increase the risk for cancer. After all, endometriosis is not a fatal disease even if left untreated, hence the demand for better safety and side-effect profiles is higher than anti-cancer drugs (Guo, 2008).

Several studies have shown that only a small percentage (0.2−3%) of silenced genes are up-regulated by DMA treatment in cancer cells (Suzuki *et al*., 2002; Heller *et al*., 2008) and in normal fibroblast cell lines the number of genes affected is even lower (0.4%) (Liang *et al*., 2002). Similarly, HDACIs also up-regulate a small subset of genes (0.4−2%) and are quite specific in their activation and repression of distinct genes (Van Lint *et al*., 1996; Heller *et al*., 2008). Although the percentage of affected genes is generally small, it is still possible that these affected genes may be important enough in causing unacceptable side-effects, even though such data are lacking as of now. In addition, it has been shown that the withdrawal of methyltransferase inhibitors (such as DMAs) is followed by a rapid return of methylation (Bodden-Heidrich *et al*., 1999), suggesting that achieving a long-lasting epigenetic reprogramming may require continued drug treatment.

Even with these concerns, it should be noted that two DMAs, 5-azacitidine and 5-aza-2′-deoxycitidine (decitabine), appear to be well-tolerated, and no significant demethylation of repetitive elements or any indication of secondary malignancies was found (Yang *et al*., 2003). In fact, chromosomal abnormalities were even found to be reversed in 31% of patients with myelodysplastic syndrome (MDS) who took decitabine (Lubbert *et al*., 2001). Therefore, the two drugs have now been approved in the US for treating MDS and are the only agents known to improve the natural history of MDS (Garcia-Manero *et al*., 2008).

There is also a concern that VPA use may increase the risk of polycystic ovarian syndrome (Chandrareddy and Muneyyirci-Delale, 2008). Yet from the very same study based on which the concern was raised, the authors actually stated explicitly that '[n]one of the tested AEDs influenced 3βHSDII or P450c17 activities at concentrations normally used in AED therapy' (Fluck *et al*., 2005) ('AEDs' stands for antiepileptic drugs, including VPA; italics mine). As Paracelsus, considered to be the father of modern toxicology, said, *Sola dosis facit venenum* (only dose makes the poison). Hence the extrapolation of the observation under the high-dosage to the situation of low-dosage should be made with extreme caution.

Just as the dose, the duration of medication, perhaps to a lesser degree, can also make a difference. The two studies based on which the concern was raised actually examined women taking VPA for a period of time much longer than 3 months as we used. The women in one study (Bofinger *et al*., 2001) had taken VPA for ≥2 years whereas in the other (McIntyre *et al*., 2003) the average duration of taking VPA is 28 months. Even if VPA is proved to have an unfavorable risk to benefit ratio, it is still premature to throw the baby out with the bathwater, since VPA is just one of many HDACIs and some novel HDACIs may still hold the promise of being more efficacious whereas having less side effects.

Besides dose and treatment duration, one promising way to improve drug safety and to minimize side-effects is to use the local route for administration, namely the drug-containing intrauterine system (IUS). There is indication that levonorgestrel-releasing IUS (LNG-IUS) appears to be efficacious in treating endometriosis, adenomyosis and their associated pain (Bahamondes *et al*., 2007). But little work has been done in this area.

Regardless, the long-term safety of HDACIs and/or DMAs, when used to treat endometriosis, should be carefully evaluated. Fortunately, such data may come from cancer clinical trials.

Diagnostic and prognostic implications

Besides providing novel targets for drug therapy, epigenetic aberrations, once identified, may also provide promising prospects for diagnostic and/or prognostic purposes. One attractive approach is the identification of DNA methylation markers, which can be used for many specimens, such as menstrual blood.

Any biomarker, in order to be clinical useful, should ideally have high specificity and sensitivity. In addition, it should be easily detectable in specimens procured through minimally invasive manner. DNA methylation biomarkers appear to fit the latter requirement quite well.

Since menstrual blood contains the same DNA (and thus methylation status) as that from endometrial cells, and since the endometrium from women with endometriosis is somewhat different from that of women without (Vinatier *et al*., 2000), menstrual blood could be a valuable, abundant, non-invasive and convenient source for detection of methylation changes, as reported (Fiegl *et al*., 2004). A recent preliminary study using menstrual blood provides the evidence that the frequency of ERβ hypermethylation in women with endometriosis is significantly lower than that in women without endometriosis (Shen *et al*., unpublished data). This seems to echo the result by Xue *et al*. (2007a) that ERβ is hypomethylated in endometriosis.

Of course, it is unclear as of now as whether the DNA methylation markers based on menstrual blood are of any use for early diagnosis of endometriosis. It is also unclear as whether they would be of value for differential diagnosis of endometriosis, which could be more challenging. Much more work is warranted.

DNA methylation markers may also prove to be useful for prognostic purposes. The preliminary results by Shen *et al*. seem to suggest

that PR-B promoter hypermethylation found in tissue samples harvested at the time of surgery may be a biomarker for recurrence (Shen *et al*., unpublished data), which is consistent with the published findings (Wu *et al*., 2006b; Shen *et al*., 2008). In any case, very little has been published in this area, even though it is an area that is likely to be clinically most useful and could bring tangible results to better patient care. The identification of patients with high-risk of recurrence should accord for further intervention. On the other hand, patients with low-risk of recurrence may be advised not to take any medication, which often have side effects.

Genetic versus epigenetic

There is a prevailing view that endometriosis is a polygenic disease and, as such, genetic polymorphisms that predispose women to endometriosis can be identified through linkage or association studies. Once identified, one can hope to better understand the pathophysiology of endometriosis, with promises to develop new diagnostic tools and therapeutics. Yet despite many publications, seemingly little headway has been made in elucidating the specific genetic factors that have a major impact on the risk of developing endometriosis (Di and Guo, 2007). Few, if any, positive findings from genetic association studies have been replicated (Altmuller *et al*., 2001; Hirschhorn *et al*., 2002; Chanock *et al*., 2007; Kavvoura *et al*., 2007), and those who tried to replicate previously reported positive findings often end up with negative results. Not surprisingly, three meta-analyses on association of endometriosis and some genetic polymorphisms coding for dioxin detoxification enzymes, sex steroid biosynthesis and their receptors found no evidence of association (Guo, 2005a, b, 2006a) even though meta-analyses is known to have upward biases in risk estimates, especially the 'winner's curse' of first reports (Weiss *et al*., 2008). A recent review on this topic found 'a strikingly large amount of conflicting results' and concluded that 'polymorphisms may have a limited value in assessing possible development of endometriosis' (Falconer *et al*., 2007).

Although conceptually the identification of endometriosis susceptibility genes is rather straightforward and the genotyping technology is fast and affordable, the presumption that major susceptibility genes exist for endometriosis still needs to be carefully scrutinized. With the current view that complex diseases emerge from molecular networks that are modulated by complex genetic loci and environmental and/or life-style factors (Ghazalpour *et al*., 2006; Keller *et al*., 2008), such a presumption may seem somewhat over-simplified.

A recent assessment of achievements in identifying complex disease genes indicates that most genetic loci identified to be associated with the disease risk confer only miniscule relative risks, ranging from 1.02 to 1.50 (Kraft and Hunter, 2009). Even when genetic polymorphisms that are associated with a modest increase in risk are combined, they generally have a low discriminatory and predictive ability (Janssens and van Duijn, 2008). For human height, a trait that is known to be mostly hereditary, it is calculated that approximately 93 000 single nucleotide polymorphisms are required to explain 80% of the population variation (Goldstein, 2009). This nearly astronomical number certainly is not going to inspire any enthusiasm for conducting large-scale gene hunting projects, and seriously questions their value in genetic screening, genetic testing, and the possibility of developing gene-derived therapy (Maher, 2008; Wade, 2009).

The identification of genetic polymorphisms that predispose women to endometriosis is usually through two means: one is linkage studies, and the other, association studies. Linkage studies aim to identify genes responsible for the phenotype of interest through DNA markers physically linked with the putative genes in pedigree data (Ott, 1999). The association studies, on the other hand, attempt to identify correlations between genetic variants and phenotypic differences on a population scale, often through the detection of differential genotypic frequencies in cases and controls (Cardon and Bell, 2001). The success or failure of both approaches hinges critically on several implicit yet important assumptions that are often taken for granted. These assumptions include, but not restricted to: (i) There exist susceptibility genes that predispose women to endometriosis; (ii) Genotype, as determined by DNA extracted from peripheral blood, completely determines the gene expression level, regardless which tissues or organs; (iii) The hereditary materials are written in DNA sequences and thus are impervious to life-style or environmental influences.

The validity of the first assumption has been questioned by Di and Guo (2007). The second assumption has not been scrutinized closely so far and emerging observations appear to cast serious doubts on its validity. First, there are about 210 distinct cell types in humans, each with different functions even though all apparently having an identical genome. For example, pancreatic islet cells produce insulin whereas other types of cells do not. It turns out that one important way to regulate which gene is expressed or not is through epigenetic mechanisms. In insulin-producing pancreatic islet cells, the insulin gene displays histone modifications characteristic of active genes but in cells that do not produce insulin these modifications are absent and in fact show modifications characteristic of inactive genes (Mutskov *et al*., 2007). Second, that genotype, a combination of two copies of a gene each coming from a parent, determines gene expression levels may have many exceptions. X chromosome inactivation and genomic imprinting aside, one recent genome-wide study of allele-specific transcription of about 4000 genes shows that over 300 of them were subject to random monoallelic expression (Gimelbrant *et al*., 2007). The majority of these showed biallelic expression along with clonal expansion. Conceivably, this should result in macroscopic patches of tissues within an organ, generating mosaic gene expression patterns within a tissue. Incidentally, all three ways to regulate gene expression, i.e. X chromosome inactivation, genomic imprinting and monoallelic expression, are based on epigenetic regulation mechanisms. Imprinted loci are less prone to dietary and dynamic changes as compared with CpG islands elsewhere, since imprints, unlike DNA methylation, are initiated during gametogenesis and are inherited by mature gametes and then transmitted to embryos (Ferguson-Smith and Surani, 2001).

The counterexamples to the last assumption are also numerous and accumulating. Cigarette smoke has been shown to demethylate the oncogene synuclein-γ in lung tissues via the down-regulation of DNMT3B (Liu *et al*., 2007). More remarkably, maternal care during infancy has been shown to be associated with methylation of ERα in rats, and have a permanent impact on offspring's hypothalamic-pituitary-adrenal responses to stress, cognitive ability in tests of hippocampal function and reproductive behavior (Liu *et al*., 1997, 2000; Champagne *et al*., 2003, 2006, Szyf *et al*., 2005). In humans, childhood abuse is found to be associated with glucocorticoid receptor methylation and expression (McGowan *et al*., 2009).

Thus, it becomes increasingly evident that the way the information is distributed along chromosomes is far more complex than previously thought (Pearson, 2006). The focus on sequence variation and its impact on disease risk could somehow distract our attention from other possible and perhaps more important causes, such as life-style (e.g. delayed child-bearing), and epigenetic aberration such as aberrant methylations (Robertson and Wolffe, 2000), histone acetylation (Huang *et al*., 2003), and other chromatin remodeling mechanisms (Mutskov *et al*., 2007).

Given the wide spectrum of symptomology in endometriosis, it is unlikely one or few polymorphisms would account for all causes of endometriosis and for its variable age at onset. If susceptibility-conferring DNA variants do exist, they are either likely to be responsible for only a small portion of endometriosis, as in the case of BRCA1 to breast cancer, or are individually of small marginal importance and of high frequency, and are characterized by extensive heterogeneity. The small, likely marginal, individual effect of each of the variants will be difficult to justify for genotype-based interventions that are typically costly, difficult to execute, and uncertain in outcome (Baird, 2001; Cooper and Psaty, 2003). Hence, for majority of endometriosis cases, epigenetic aberrations are very likely the main culprit.

The curse of heterogeneity can be a blessing for epigenetic research since the hallmark of epigenetic effects on gene transcription is the variable expression of a gene in an isogenic population (Whitelaw and Martin, 2001). Since transgenerational epigenetic inheritance (Morgante *et al*., 1999; Rakyan *et al*., 2003) and epigenetic inheritance of environmentally induced phenotypes (Weaver *et al*., 2004; Anway *et al*., 2005; Szyf *et al*., 2005) have been discovered recently in mammals, including humans (Suter *et al*., 2004; Hitchins *et al*., 2005), it is likely that epigenetic aberrations may be responsible for most cases of endometriosis and for its familial aggregation.

Several lines of evidence support this notion. First, the highest estimated monozygote (MZ) concordance in affection status in endometriosis, even with a small sample size and thus potentially biased, is 75% (Moen, 1994). Concordance (rate) is the probability that a pair of individuals will both have a certain characteristic, given that one of the pair has the characteristic. The discordance in affection in MZ may well be attributable to the age-dependent divergence in the epigenomes in the MZ twins (Fraga *et al*., 2005). Second, endometriosis displays remissions, relapses, and, in some mild or superficial endometriosis, even full recovery without any intervention (Hoshiai *et al*., 1993; Koninckx, 1994). This may be difficult to explain under the assumption of susceptibility-conferring DNA variants, but could be easily explained by the reversal of epigenetic changes. Third, the variable age at onset in endometriosis, although difficult to explain in genetics context, could be well explained in the epigenetics context. This is because methylation status undergoes age-dependent change (Boggi *et al*., 2001; Bennett-Baker *et al*., 2003; Issa, 2003), which, in turn, may affect gene expression levels. This age-dependent change in methylation status as well as in gene expression patterns could account for variable age of onset of endometriosis.

One recent study on rats provides evidence that epigenetics may play a role in transgenerational transmission of phenotypes. It is reported that in two groups of isogenic rats, one had surgically induced endometriosis whereas the other received only a sham surgery, the two groups showed different fecundity and also oocyte quality in rats themselves as well as their descendents (Stilley *et al*., 2009). Since these rats are genetically identical their offspring also have similar genetic background yet the infliction with endometriosis or not appears to result in difference in oocyte quality in their offspring.

Epigenome in endometrium can be shaped by environmental and life-style factors

Although sometimes it is tempting to dichotomize causes for endometriosis as either genetic or environmental, the truth may be that both genes and environmental factors are likely involved in causing endometriosis. It is also worth pointing out that environmental, or life-styles, factors can actually impact on genome, not necessarily in causing changes in DNA sequences but, rather, in shaping epigenotypes or the epigenome, which modulates gene expression, leading to the phenotype as we see it.

The best example came from a study of methylation patterns in two genes (CSX and CSX6) that are not expressed in endometrium (Kim *et al*., 2005). When a cell divides into two daughter cells, the methylation pattern at each CpG site may undergo stochastic changes due to random replication errors (molecular clock hypothesis). As a person ages, the stochastic methylations increase as a function of age (Issa, 1999, 2003). Hence, methylation potentially records the tempo and pace of cell divisions that occur during somatic cell tree expansion (Tanaka *et al*., 2003).

In endometrium, the driving force for cell divisions is estrogens (Pike *et al*., 2004). Various factors, such as pregnancy, birth, lactation, smoking and obesity or not, can affect global and local estrogen levels. The varying estrogen levels would thus affect the tempo and pace of cell divisions in endometrium, hence the life history of a woman's endometrium can be recorded, rather surreptitiously, within the epigenomes due to replication errors which serves as a molecular clock. As shown by Kim *et al*. (2005), the methylation patterns in two genes remarkably reflect a woman's age, reproductive history, and her body mass index. It is thus conceivable that the epigenomes of those genes involved in the pathogenesis of endometriosis are likely modifiable by some environmental or life-style factors yet to be identified, elevating or lowering the propensity to endometriosis. This seems to epitomize Theodosius Dobzhansky's rather prescient saying, 'Heredity is not a status but a process'. It is doubtful that the genome can be similarly modified in this manner.

Conclusions and future perspectives

Emerging data now provide evidence that endometriosis is an epigenetic disorder, in the sense that epigenetics plays a definite role in the pathogenesis and pathophysiology of endometriosis. This is characterized, at least in part, by aberrant methylation and very recently by deregulation of miRNA expression in eutopic as well as ectopic endometrium. Published data also have shown that HDACIs have great potential as therapeutics for endometriosis. In addition, DNA methylation based as well as miRNA-based biomarkers may hold potential in diagnosis and predicting recurrence risks.

It has become abundantly clear that chromatin, once considered just a structural scaffold allowing the packaging of DNA, is actually a dynamic and key regulatory element of gene expression and actively

participates in several cellular processes such as mitosis and differentiation (Wolffe, 2001). DNA cytosine methylation also is now considered at the 'fifth base' (Lister and Ecker, 2009). Research in the last two decades shows that the changes in the fifth base, and the chromatin structure are dynamic and are functions of changes in environment and lifestyle. In view of this, the notion that genetic polymorphisms can accurately determine a woman's risk of developing endometriosis should be carefully scrutinized.

As a multifactorial disease, endometriosis involves both hormonal and immunological aberrations. Incidentally, epigenetics now has been shown to play roles in both hormonal and immunological aberrations. Although the possibility that certain susceptibility genes may be responsible for increased risk of endometriosis in perhaps a small portion of women, many aspects of endometriosis can be explained from an epigenetics perspective.

As the circle of knowledge increases in size, we are also faced with even more unknowns. What are other aberrant methylations in endometriosis? Are there any miRNAs that are aberrantly methylated in endometriosis? If so, can they be demethylated, resulting in increased expression? What are their target genes? Besides aberrant DNA methylation, are there any associated aberrant histone modifications, and, if any, what are they? For a specific gene such as PR-B, which proteins are involved in gene-silencing due to hypermethylation? Which proteins are involved in gene reactivation? Is there any coordination between DNA methylation and histone modification? Besides constant and continued proinflammation, is there any other mechanism that cause aberrant methylation? Is there any way to slow down the process or even halt it? Is there any way to permanently reverse it without causing collateral or unwanted damage?

For treatment, are HDACIs and/or DMAs truly efficacious in treating endometriosis? What are their side-effects, especially long-term? Since a sizable proportion of women with endometriosis may wish to reproduce, do these compounds have any negative effect on pregnancy and offspring? If there are side effects, is there any way to minimize them? Would miRNA inhibitors be therapeutically useful? What are their risk-benefit profiles?

These questions can only be addressed in future research. As reviewed here, endometriosis is clearly a disease involving epigenetic processes. Yet given the potential roles of DNA methylation, histone modifications, miRNA and various forms of protein PTMs, along with presumably interconnecting relationships among them and possible temporal and spatial dynamics, the resultant molecular mechanisms underlying the pathogenesis of endometriosis must be extremely complex. On top of this, endometriosis, as a disease entity, may well be pathophysiologically heterogeneous, as evidenced by two distinct classes of subtypes as classified by gene expression profiling (Wu *et al*., 2006a). This heterogeneity may possibly demand different treatment modalities. These, in conjunction with the recent setbacks in clinical trials of endometriosis (Guo *et al*., 2009), remind us that new biological discovery may not immediately lead to better patient care, which will require the collective effort by basic science, translational and clinical investigators. The identification of biomarkers for diagnosis and for predicting recurrence may also be more easily said then done, as seen in cancer (Ransohoff, 2004, 2008). The identification of DNA methylation biomarkers for diagnosing early endometriosis and for recurrence may also be very challenging. Yet the benefits may also be great.

Regardless, endometriosis epigenetics is a bourgeoning field, and may transform our understanding of the pathogenesis and pathophysiology of endometriosis, opening new avenues for diagnosis, treatment and prognostic prediction. So far we have only scratched the surface of this subject. With more research, we may come closer to prevent or at least treat this unrelentingly painful disease that is endometriosis.

Acknowledgement

The author would like to thank the three anonymous reviewers and an associate editor for their helpful comments.

Funding

The Pujian Project and grant 074119517 from the Shanghai Science and Technology Commission, grant 30872759 from the National Science Foundation of China, and a grant from the State Key Laboratory of Neurobiology, Shanghai Medical College, Fudan University.

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Submitted on May 22, 2009; resubmitted on July 22, 2009; accepted on July 30, 2009