CHAPTER 5

Estrogen, MSI and Lynch Syndrome-Associated Tumors

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Under review
ABSTRACT

Estrogens play a major role in the biology of hormonally responsive tissues but also in the normal physiology of various non-typical hormone responsive tissues. In disease, estrogens have been associated with tumor development, in particular with tumors such as breast, endometrium, ovary and prostate. In this paper we will review the molecular mechanisms by which estrogens are involved in cancer development, with a special focus in Lynch syndrome-related tumors. Further, we discuss the role estrogens might have on cell proliferation and apoptosis, how estrogens metabolites can induce DNA damage, and we discuss a possible connection between estrogens and changes in DNA (hypo- and hyper-) methylation. In this review we will also address the protective effect that high levels of estrogen have in MMR-related neoplasias.
INTRODUCTION

The most common types of cancer worldwide occur in hormonally responsive tissues, such as breast, endometrium, ovary and prostate. Tumors occurring in these tissues show strong associations with the exposure to exogenous or endogenous steroidal hormones.

Estrogens are a group of steroid compounds which are present in both men and women; however, their levels are significantly higher in women of reproductive age. There are three types of estrogens of which 17β-estradiol is the most potent one as has the highest affinity for its receptors. It is produced in high amounts in pre-menopausal women by the ovary. The second endogenous but less potent estrogen is estrone. It is produced from androstenedione, the immediate precursor of estrone. The third estrogen is estriol, a metabolite of estradiol. It is mainly produced by the placenta during pregnancy and is found in lower concentrations than estradiol and estrone in non-pregnant women (Chen et al., 2008).

Estrogens act through the estrogen receptors (ERs). ERs are ligand-activated transcription factors that have several domains that can bind estrogens and activate transcription of several estrogen-responsive genes (see Figure 1) (Notarnicola et al., 2001). There are two receptor isoforms, ERα and ERβ (Tsai & O’Malley, 1994; Hall et al, 2001). When estrogen binds to these receptors, the receptors dimerize, go to the nucleus and bind to specific DNA sequences, the consensus estrogen response elements (EREs) of ER-responsive genes (Klein-Hitpass et al., 1989).

The receptors may form ERα (αα) or ERβ (ββ) homodimers or ERαβ (αβ) heterodimers (Li X et al., 2004). The activation of ER is influenced by a set of different co-activators, enzymes, and co-repressors. These factors influence the assembly of the transcriptional complex and the subsequent transcription of the ER-responsive genes. This is called ‘the canonical pathway’ of ER.

A ‘non-canonical’ pathway of ER has also been described, in which genes are activated without having ERE-like sequences. This non-classical mechanism accounts for the transcriptional activation of approximately one-third of all estrogen responsive genes (Huang et al., 2004).
Alternative mechanisms without DNA binding have also been described. DNA binding proteins such as specificity protein 1 (SP1) are activated by the direct binding of ER (Velarde et al., 2007), for a schematic representation see Figures 1 and 3.

Estrogens play a major role in controlling the menstrual cycle, pregnancy, thus female reproduction. However, estrogens are not only important for the biology of hormonally responsive tissues; they also play an important role in bone strengthening and cholesterol metabolism, and have an influence on the central nervous system and the gastrointestinal physiology (Roy & Liehr, 1999; Nilsson & Gustafsson, 2001). On one hand ER signaling plays an important role in many normal physiological processes, on the other hand several studies have shown that estrogens and their metabolites are also involved in tumor development.

In this review we will address the different possible mechanisms by which estrogens can be involved in tumor development and in particular, we will focus on how the hormone can be involved in the development of Lynch syndrome-related neoplasias showing microsatellite instability.

**Figure 1.** Mechanisms of action of estrogens.
ESTROGEN AS A CARCINOGEN

The International Agency for Research on Cancer (IARC) recognized in 1987, for the first time, that elevated concentrations of estrogens lead to an increased risk of breast and uterine cancers (IARC, 1987). However, only in 2006, Russo & Russo described in vivo malignant transformation of human breast epithelial cells by estrogens (Russo & Russo, 2006). Salama et al. (2008) showed that catecholestrogens induce oxidative stress and malignant transformation of human endometrial glandular cells.

These and many other studies point towards a direct link between cancer initiation and estrogens. However, how estrogens contribute to cancer is still not totally clear. Several possible mechanisms have been put forward (see Figure 2):

1) Estrogens promote cell proliferation via ER mediated signaling, both through genomic and non genomic pathways, which promotes cell proliferation and growth, and reduces sensitivity to apoptosis. For instance, estrogens stimulated ERs which then can up-regulate Wnt11 expression, which causes the tumor to resist going into apoptosis (Katoh, 2003).

2) Another possibility is that estrogens promote signaling via cell membrane-related but ER-independent phosphorylation of target genes. Examples are the phosphorylation of AKT and ERK by estrogens in ER negative cells (Bouskine et al., 2008; Zhang et al., 2009).

3) Estrogens lead to the production of toxic species that are able to induce tumor development. Estrogens are converted to catecholestrogens by cytochrome P450-mediated hydroxylation. Catecholestrogens, specifically the 4-hydroxylated steroids, and their semiquinone and quinone reactive intermediates are considered carcinogenic (Liehr, 2000). Various types of damage are found associated with either estrogen quinones binding covalently to DNA or by free radical action. Aneuploidy, gene amplification, arrest of DNA replication as a result of estrogen–DNA adduction, single strand breaks, microsatellite instability, small insertions, and deletions are all examples of these types of DNA damage (Roy & Liehr, 1999; Liehr, 2001; Fernandez et al., 2006).

4) Estrogens are found associated with changes in the methylation status, both hypo- and hyper–methylation of DNA. An example of an estrogen-related
hypomethylated gene is \textit{PAX2} (Wu et al., 2005). As an example of estrogen-related hypermethylation gene is the estrogen receptor gene and \textit{MLH1} (Slattery et al., 2001; Campan et al., 2006). The last example is particularly interesting, since in this review we focus on MMR related neoplasia.

What is also believed to have a significant effect on cell growth and tumor formation is the balance between the ER$\alpha$ and ER$\beta$ isoforms (Matthews & Gustafsson, 2003). The activation of ER$\alpha$ is associated with increasing cell proliferation whereas ER$\beta$ promotes apoptosis. For instance in endometrial cells it was shown that a down-regulation of ER$\alpha$ results in a reduction of cell proliferation, an effect that was not seen when ER$\beta$ was blocked.

\textbf{ASSOCIATION BETWEEN LOW LEVELS OF ESTROGEN AND MSI IN LYNCH SYNDROME-ASSOCIATED TUMORS}

\textbf{Lynch syndrome-associated MSI tumors}

Several studies have showed that there is an association between estrogen exposure and the presence of microsatellite instability in tumors. Microsatellite instability (MSI) is a hallmark of tumors from patients with Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC). This inherited cancer syndrome is characterized by the development of colorectal cancer (CRC), endometrial cancer and various other cancers and is caused by a mutation in one of the mismatch repair (MMR) genes \textit{MSH2}, \textit{MLH1}, \textit{MSH6} or \textit{PMS2}. Colorectal cancer is the most common cancer found in Lynch syndrome: almost all patients develop colorectal cancer, followed by endometrium cancer which occurs in approximately 30\% of all female patients (for a review on this see Vasen et al. 2007). Although MSI is the major characteristic of Lynch syndrome patients, it is also found in 15-25\% of sporadic colorectal, endometrial and gastric (Boland et al., 1998). MSI is defined by the accumulation of insertions and/or deletions at short DNA repeats (microsatellites), leading to different lengths of the repeat. Accumulation of such mutations in coding mononucleotide repeats of genes with
important regulatory functions, e.g. tumor-suppressor genes, is thought to be a key event in the development of MSI tumors.

**Estrogens and MSI**

Notarnicola et al. (2001) described a significant association between MSI and ER status in colorectal tumors. Interestingly, they verified that the MSI tumors showed low levels of ER expression. Moreover, the withdrawal of estrogens also resulted in an increasing risk of MSI CRC tumors. An interesting hypothesis was made by Breivik et al. (1997) by linking estrogens to gender differences in CRC through a mechanism involving MSI.

The presence of low levels of ERs can be linked to hypermethylation of the estrogen receptors (Slattery et al., 2001). However, the mechanism linking estrogens to ER methylation and to MSI is not yet known.

Since ER inactivation is due to hypermethylation in 90% of colon cancers (Issa et al., 1994), it was hypothesized that estrogens affect DNA methylation in general (Slattery et al., 2001). In fact, it was suggested that estrogens might be key factors in the development of the CpG island methylator phenotype (the CIMP phenotype) (Baylin & Herman, 2000; Newcomb et al., 2007), a phenotype commonly present in many different types of tumor. In CIMP tumors, hypermethylation of promoter regions of regulatory genes, such as tumor suppressor genes, is a general feature. This CIMP phenotype might be the missing link between estrogens and the hyper-methylation of the ERs and the MSI phenotype, as MSI in the sporadic tumors is mostly caused by hypermethylation of the MLH1 promoter.

Besides global hypermethylation (CIMP phenotype), global hypomethylation of introns and coding sequences of genes is also observed in tumors. In CRC, 35%-60% of the cases show reduction of methylation (Shen et al., 2009). An example of a gene that is hypomethylated in estrogen-responsive tumors is PAX2 (Wu et al., 2005). This is corroborated by *in vitro* and animal studies that showed that estrogens lead to a lower DNA methylation of specific genes and that they are able to restore protective methylation patterns (Newcomb et al., 2007).
Estrogens, Mismatch Repair and cancer

Slattery et al. (2001) raised the idea that at least one of the major MMR genes is estrogen-responsive and that loss of estrogen results in loss of DNA mismatch repair capacity. Wada-Hiraike and colleagues (2005) reported a direct interaction between ER and the MMR gene, MSH2, from immunoprecipitations and pull down assays. In fact the authors suggested that MSH2 is a potent co-activator of ERα. An interesting possibility arose from this study: common co-activators of ER and even ER itself might have a functional role in DNA MMR (Wada-Hiraike et al., 2005). Miyamoto et al. (2006) demonstrated that cells under a high-level estrogen environment have increased levels of both MLH1 and MSH2 proteins. Moreover, it was observed by the same authors that MLH1 and MSH2 expression is up-regulated and activity MMR increased by estradiol treatment mediated by the ER pathway (Miyamoto et al., 2006). The higher MMR activity would ideally compensate the replication errors occurring at a highly proliferative stage. Lower MMR activity would then also explain the occurrence of endometrial carcinogenesis.
in a less proliferative stage, when the estrogens levels are low, as in postmenopausal women. These data support all the association between estrogens and MMR, suggesting an estrogen-mediated transcriptional activation of the MMR complex protein.

In summary, both studies show a positive correlation between estrogens and MMR activity. So high levels of estrogen give rise to higher cell proliferation and the system seems to protect itself against DNA damage by activating the MMR system. Thus estrogens may initially protect against cancer by activating the MMR system. However, when the MMR system is deregulated, for instance, by hypermethylation of the MLH1 gene, this protective mechanism is lost. It is also interesting to note that tumors of the endometrium are seen mostly in postmenopausal women, women who have low levels of estrogens.

**ESTROGEN AND ENDOMETRIAL CANCER**

Endometrial cancers (EC) can be divided in two classes, an estrogen-associated type and a non-estrogen-associated type. The first group, to which all MSI-high endometrium tumors belong, is called type I EC. These tumors are found in women with long-term unopposed exposure to estrogens, which might be caused by nulliparity, early menarche, late menopause or the use of estrogen replacement therapy. Obesity is also considered a major risk factor, as adipose tissue gives rise to a higher estrogen concentration (Salama *et al.*, 2008). About 80% of all endometrial carcinomas are estrogen-associated carcinomas (Amant *et al.*, 2005; Sherman, 2000).

Interestingly, the profile of estrogens and metabolites present in these tumors seems to play an important role in the mechanism leading to endometrial cancer. Different rates of the different possible metabolites of estrogens are associated with different effects on the endometrium, and thus carry different risks of developing endometrial cancer (Takahashi *et al.*, 2004).
Tamoxifen, estrogen and endometrial cancer

Besides estrogens, tamoxifen can also be associated with cancer development, in particular endometrial cancer. Tamoxifen is a drug used as adjuvant treatment of ER-positive breast cancer. It acts as an estrogen antagonist in those tumors, reducing tumor growth. However, it was shown that it acts as an estrogen agonist in other tissues such as bone, where it prevents osteoporosis (Howell et al., 2004; Smith & O’Malley, 2004). In the endometrium it induces cell proliferation. An increased risk of developing EC is reported for postmenopausal women undergoing tamoxifen therapy (Polin & Ascher, 2008). The working mechanism of tamoxifen is binding of the compound to ERs and inducing a tamoxifen-specific signaling (see figure 3). This signaling probably depends on the concentration of estrogen (e.g. menstrual status of the patient), the ratio between ER\(\alpha\) and ER\(\beta\), and on the expression of the co-activators and co-repressors, all of which might be tissue-specific.

**Figure 3.** Estrogen and tamoxifen signaling; different factors affecting the signaling pathways of both ligands.
ESTROGENS AND COLON CANCER

Expression of ERs has also been demonstrated in non-hormonally responsive tissues, such as the gastrointestinal mucosa and its associated tumors, suggesting that estrogens also have a role in these tissues that were previously not thought of as hormone-responsive tissues (Potter 1995; Grodstein et al., 1999; Slattery et al., 2001; D’Errico and Moschetta, 2008).

All findings in colorectal cancer support the hypothesis that high estrogen levels can have a protective effect in specific phases of life. These findings have been used as an explanation for the gender bias observed in CRC incidence, with women having a lower incidence of the disease than men. Hormonal changes associated with pregnancy (McMichael and Potter, 1980), and hormone replacement therapy (HRT) have been associated with lower risk of CRC (Potter, 1995; Peipins et al., 1997; Chen et al., 1998; Kadiyska et al., 2007).

Most likely this protective effect of estrogens in CRC largely depends on the ratios of receptor α and β. ERβ is the predominant ER isoform in colon tissue and probably the responsible isoform for estrogen transcriptional effects (Foley et al., 2000; Jassam et al., 2005; Kennelly et al., 2008). This situation is quite different in breast and endometrium, where ERα is the main isoform present. It has, however, been suggested that ERβ modulates the function of ERα, and that an increased ratio of ERα/ERβ is associated with a progression from a healthy to carcinoma state in those tissues (Jazaeri et al., 2001).

ESTROGENS AND KNOWN CANCER-RELATED PATHWAYS

Estrogens can activate proteins other than the ERs. For instance they have been reported to activate the protein kinase A pathway (Fu & Simoncini, 2008), by binding to the G protein–coupled receptor GPR30. Moreover, both insulin-like growth factor 1 (IGF-1) receptor signaling and EGF receptor signaling can be activated by estrogens (Song et al., 2007). Binding of estrogen to these growth receptors leads to dimerization of the receptor, and activation of their kinase activity. Phosphorylation of these proteins leads to activation of downstream
signaling pathways, such as the MAPK and PI3K/Atk pathways. The activation of PI3K/Akt pathway has been observed in ER-positive human breast cancer cells (Castoria et al., 2001; Marquez & Pietras, 2001; Sun et al., 2001; Duan et al., 2002; Razandi et al., 2004; Lee et al., 2005), in rat and mouse endometrial epithelial cells (Dery et al., 2003; Chen et al., 2005), and in human endometrial cells during the proliferative phase (Guzeloglu Kayisli et al., 2004). Activation of PI3K/Akt has been associated with cell survival in a variety of cancers (Castoria et al., 2001; Lee et al., 2005).

CO-ACTIVATORS AND CO-REPRESSORS OF ER PATHWAY

ER-mediated transcriptional regulation depends on the recruitment of co-activators and components of the RNA polymerase II transcription complex, which enhances target gene transcription (Klinge, 2000). Thus, the cellular availability of co-activators and co-repressors is an important determinant in the biological response to both steroid hormone agonists and antagonists in ER responsive tissues (Edwards, 2000).

Many such co-activators and repressors contribute to ER-mediated transcription events. ER-dependent gene transcription frequently depends on the presence of FOXA1. FOXA1 is expressed in the mammary gland, liver, pancreas, bladder, prostate, lung, and colon. Recently, Carroll et al. demonstrated that FOXA1 is required for optimal expression of nearly 50% of ERα-regulated genes and estrogen-induced proliferation, by enhancing binding of ERα to its target genes (Laganière et al., 2005; Carrol et al., 2005; Carrol & Brown, 2006).

In colon, binding of estrogens to ERα induces a cancer promoting response, whereas binding to ERα seems to exert a protective action (Weyant et al., 2001). Reasons for this can reside not only in the different expression patterns of ERα and ERβ in vivo but also their need to interact with cellular transcription cofactors which are not functionally equivalent and ubiquitously expressed in all cells (McDonnell & Norris, 2002), highlighting the importance of the ER-co-activators in colon cancer.

In endometrium, it has been suggested that co-regulators of ER are involved in tumor progression. It has been proposed that p160 steroid receptor cofactor
(SRC) can modulate ER activity and that overexpression of another member of SRC family, AIB1 (Amplified in Breast Cancer 1) in endometrial carcinoma lead to ER increased action and consequent progression to malignancy (Balmer et al., 2006). Moreover, recently we identified mutations in NRIP1 in MSI-H endometrium tumors in 35% of the investigated tumors (Ferreira et al., unpublished data). NRIP1 is a co-repressor of ER signaling. Our data convincingly show the importance of ER cofactors in the development of the tumor type.

**GENERAL CONCLUSIONS**

Estrogens are essential for maintaining of several tissues in humans, but they also play an important role in the carcinogenic process of many different tumor types. Although we know fairly well how estrogen signals, it is still not totally clear how estrogen exerts different effects in different tissues. The differences in the effects of estrogen mentioned in this review in the endometrium and colon may be partly explained by the different physiological roles these organs: the endometrium belongs to the reproductive system, and is a main target of sex hormones, whereas the colon belongs to the digestive system, which is less influenced by sex hormones. Moreover, the regulation of estrogen-responsive genes is different among tissues and is, for example, dependent on the mechanism of action of the ligand or the distribution of ER isoforms alpha and beta and their dimerization (Castiglione et al., 2008). Also the co-activator and co-repressor molecules might exist in different combinations or concentrations among tissues and lead to different results.

Furthermore, several ER and non-ER related pathways are now known to activate or be activated by estrogens. These pathways might be affected differently in distinct tissues and therefore have a broad spectrum of influence in tumor formation. Therefore the networks they form with estrogen should not be forgotten, even in tumors occurring in less hormonally-dependent organs.

There is still a lot to learn about the possible connection between microsatellite instability (MSI) and estrogens. Estrogens may have a protective effect, which is likely lost after the MMR system has somehow been modulated
(mutated). On the other hand estrogens can, by there carcinogenic effect, directly (by mutations) or indirectly (by methylation) inactivate the MMR pathway which results in MSI. MSI can subsequently result in mutations in cofactors or ER signaling which will modulate ER regulated transcription. Clearly the MMR system is a (direct or indirect) target of estrogens, making genes involved in the estrogen pathway potential candidates to be studied in MMR-deficient tumors. These findings might also prove useful in the design of novel therapies for such tumors.
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