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REVIEW

Role of melatonin in the epigenetic regulation of breast cancer

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Abstract The oncostatic properties of melatonin as they directly or indirectly involve epigenetic mechanisms of cancer are reviewed with a special focus on breast cancer. Five lines of evidence suggest that melatonin works via epigenetic processes: (1) melatonin influences transcriptional activity of nuclear receptors (ERα, GR and RAR) involved in the regulation of breast cancer cell growth; (2) melatonin down-regulates the expression of genes responsible for the local synthesis or activation of estrogens including aromatase, an effect which may be mediated by methylation of the *CYP19* gene or deacetylation of *CYP19* histones; (3) melatonin inhibits telomerase activity and expression induced by either natural estrogens or xenoestrogens; (4) melatonin modulates the cell cycle through the inhibition of cyclin D1 expression; (5) melatonin influences circadian rhythm disturbances dependent on alterations of the light/dark cycle (i.e., light at night) with the subsequent deregulation of *PER2* which acts as a tumor suppressor gene.

Keywords Melatonin · Breast cancer · Epigenetic mechanisms · Nuclear receptors · Aromatase · Telomerase · Cell cycle · Light at night · Clock genes

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Introduction. Cancer: genetic and epigenetic mechanisms

The term epigenetics describes the study of heritable alterations in gene expression that occur in the absence of changes in genome sequence. This can be contrasted with genetics, which deals with the transmission of information based on differences in DNA sequence. The traditional view that gene and environment interactions control disease susceptibility can now be expanded to include epigenetic reprogramming as a key determinant in the origin of human disease [1]. The classic concept of carcinogenesis describes a multistage process consisting of three distinct phases: initiation, promotion, and progression. Mutations are clearly implicated in initiation. Most mutations are sporadic and their prevalence increases with age. Other mutations are inherited. Regardless, a mutation may result in activation of oncogenes or inactivation of tumor suppressor genes [2].

Gene silencing or inactivation of a tumor suppressor gene may be not only due to a mutation, but also a result of a translocation or inhibition of transcription. Inactivation of genes that regulate cell proliferation and death is a critical part of the neoplastic process. One important mechanism that may lead to inhibition of transcription is gene silencing through epigenetic alterations such as acquisition of promoter methylation and changes in chromatin structure. Activation of oncogenes may also be a result of epigenetic changes through post-translational modifications in histone acetylation or DNA methylation [3].

The epigenome (the overall epigenetic state of a cell) can be transmitted from parent to daughter cell maintaining a specific epigenotype within cell lineages. Thus, the phenotype is a result of the genotype (the specific DNA sequence) and the epigenotype. The genotype must exist in

a particular chromatin configuration, the epigenotype, which allows a secondary level of fine control over gene expression. The epigenotype shows far greater plasticity than the genotype, and it has been speculated that epigenetic errors could be a major contributor to human diseases [4]. Epigenotype is generally accepted to be less stable than the genetic system and more sensitive to environmental [5], nutritional [6] and chemical toxicants which behave as endocrine disruptors [7, 8] and which may contribute to the development of abnormal phenotypes. For example, dietary supplements such as folate or several vitamins that affect the activity of methylating enzymes can be important in the incidence of colon cancer [9, 10]. A methyl-deficient diet has been shown to induce liver cancer associated with enhanced expression of *c-ras, c-myc* or *c-fos* oncogenes [10, 11]. Epigenetic memory of cells can be passed onto subsequent generations and can transfer the perturbed epigenome upon unaffected or normal genetic sequences. In contrast to gene mutations, epigenetic changes occur at a higher frequency than genetic changes, are limited at defined regions of a gene, and are reversible upon pharmacological treatment [12].

Since the genome contains information in two forms, genetic and epigenetic, initial studies focused on human cancers and rapidly revealed that most of human cancers are related to epigenetic aberrations (i.e., epimutations) [3] including epigenetic silencing of tumor suppressor genes because of hypermethylation and oncogenes because of demethylation [13, 14]. To date, numerous tumor suppressor genes have been found to undergo hypermethylation in cancer [15, 16]. Such epimutations rarely appear in healthy tissue indicating that epigenetic therapies may have high tumor specificity. Currently, two DNA methyl transferases (DNMT) inhibitors received US Food and Drug Administration approval for the treatment of myelodysplastic syndrome. 5-azacytidine (Vidaza®) and its derivative decitabine (Dacogen®) are now being marketed [17] and several presently available drugs are under extensive clinical investigation [18].

DNA methylation and histone acetylation as the best characterized epigenetic markers

Our current understanding of epigenetic gene regulation involves basically two classes of molecular mechanisms: DNA methylation and histone modifications [19, 20]. A variety of enzymes are involved in this process including most importantly DNA methyltransferases (DNMTs), histone deacetylases (HDACs) and histone acetyl transferases (HATs) [21].

DNMTs add a methyl group to the cytosine ring to form methyl cytosine. This modification takes place only on a cytosine that precedes a guanosine in the DNA sequence, called the CpG dinucleotide. GpG sites are not randomly distributed in the genome. The GpG-reach regions (known as GpG islands) are mainly present on the promoter region of genes [22, 23]. These CpG islands in gene promoter regions are usually unmethylated in normal cells and allow for active gene transcription. In cancer cells, CpG islands that are normally unmethylated may become methylated, resulting in silencing of important genes, such as inactivation of tumor suppressor genes. At the same time, CpG dinucleotides in other regions may become unmethylated, leading to diminished transcriptional repression of normally silenced genes such as oncogenes [22, 23].

DNA methylation is mediated by several proteins. As noted, DNMTs add methyl groups to the cytosines in CpG dinucleotides. Three active DNMTs have been recognized in humans and are designated DNMT1, DNMT3a, and DNMT3b. Each DNMT may have a specific role in the methylation process, or may act in association with another methyltransferase [19].

DNA methylation occurs in a complex chromatin network and can be influenced by several modifications in the structure of histones [24]. Chromatin is a nucleoprotein complex consisting of a basic repeating unit known as the nucleosome. A single nucleosome contains two turns of DNA wrapped around a core histone octamer comprising the histones H2A, H2B, H3 and H4. Nucleosomes represent the first level of compaction in chromatin, restricting access to enzymes involved in DNA metabolism. In addition to these basic components, linker histones and a variety of non-histone proteins are incorporated to generate a fully functional genome within a higher-order chromatin structure.

Indeed, the transcriptional status of all genes (silent, repressed or active) is determined by its chromatin environment and many molecular responses to toxicants involve alterations in gene expression that are elicited via changes in the chromatin structure of target genes [4]. The steady state level of histone acetylation is regulated by the HATs and HDACs. HATs are responsible for the addition of acetyl groups that stabilize open chromatin structures, while the HDACs deacetylate histones, and are thus responsible for resetting chromatin into a close conformation. Open chromatin is transcriptionally active, whereas condensed chromatin is transcriptionally inactive. Reduced or abnormal histone acetylation has been found in many types of cancer including breast cancer [23–25].

Major epigenetic alterations in breast cancer

Hypermetylation of CpG-island promoter can influence genes involved in DNA repair, cell cycle control,

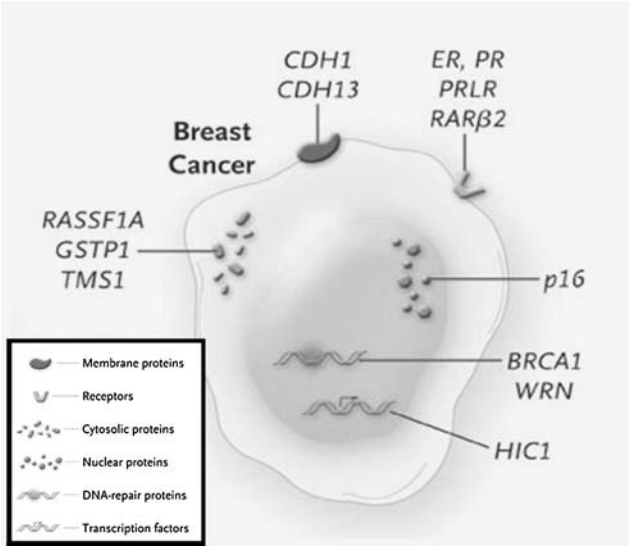

Fig. 1 Hypermetylation of CpG islands in promoter regions of genes involved in breast cancer. Modified from Esteller [25]

apoptosis, angiogenesis, cell-to-cell interactions, etc, all of which are involved in cancer development [25] (Fig. 1). In reference to breast cancer, hypermetylation of CpG islands occurs in DNA repair genes including *BRCA1*, genes encoding BRCA1 binding protein (*SRBC*), tumor suppressor genes like *CDH1*, *CDH13*, genes involved in cell adherence and the metastasic process (*CDH1-E-cadherin, CDH13-H-cadherin*), cell cycle inhibitor genes ($p16^{ink4a}$), genes coding tyrosine kinases (*SYK*) or metabolic enzymes (*GSTP1*), genes coding estrogen, progesterone and prolactin receptors (*ER, PR PRLR*), proapoptotic genes (*TMS1*) and histone/protein metyltransferase (RIZ1), as well as global genomic hypomethylation. Collectively, these changes constitute the epigenetic aberrations [25, 26].

Melatonin and epigenetics of breast cancer

Breast cancer is a complex disease that results from a multi-stage process involving deregulation of a number of different signaling cascades. It is also the most frequent non-skin cancer to affect women worldwide, and remains a major public health burden. As previously summarized, in breast cancer multiple genes are methylated compared to non-cancerous tissue. DNA methylation, acquired over time, leads to silencing of genes that are critical in several pathways. For example, genes involved in growth (estrogen receptor, progesterone receptor) metastasis (*TIMPs*), and limitless replicative potential (*cyclin D, pl6, BRCA1*) can be altered in breast cancers [12, 26]. Methylation of CpG islands within the specific promoter region usually leads to the silencing of gene expression, as is frequently observed in tumor-suppressor genes in tumors [27]. In contrast, the global hypomethylation seen in a number of cancers, such as breast and cervical cancer, is related to an increase in the grade of malignancy [28]. A growing number of findings indicate aberrant gene expression in breast cancer cells that, in some cases has been associated with alterations in the expression, the activity or/and the recruitment of HATs and HDACs. For example, the functional activity of ERα is an important promoter of human breast cancer, and is regulated by acetylation. The exact role of ERα acetylation and the deacetylation may be involved in both hormone-sensitive and hormone-insensitive breast cancer [29].

Melatonin is a secretory product of the pineal gland discovered just 50 years ago [30]. The number of physiological functions attributed to this indoleamine is so diverse than the adjective "pleiotropic" is frequently used to refer to the nature of its actions. Among melatonin's properties is its ability to inhibit the growth of mammary cancer cells both in vitro and in vivo; this suppressive effect is especially apparent in reference to ER positive tumors [31, 32]. Cohen et al. [33] proposed in 1978 a possible relationship between pineal function and mammary carcinogenesis by considering the possible antiestrogenic actions of melatonin. This hypothesis has since been examined in numerous epidemiologic and experimental studies. To the epidemiologic studies belongs the demonstration of a decreased nocturnal plasma melatonin peak in patients with ER positive breast cancer [34] as well as the assessment of low risk of breast cancer among blind women [35, 36] and the inverse association between breast cancer incidence and degree of visual impairment [37]. In the visually impaired individuals, the total or partial suppression of the light input would be expected to result in increased circulating melatonin levels which could explain the low incidence of tumours. More recently, it has been demonstrated, in a prospective case-control study in nurses, that higher melatonin levels, as measured in first morning urine, are associated with a lower risk of breast cancer [38]. On the contrary, the high incidence of breast cancer among women exposed to light during night, such as shift workers [39], and those exposed to low frequency electromagnetic fields [40], could be explained by the reduced melatonin synthesis under these environmental conditions. The epidemiologic evidence is reinforced by the experimental demonstration that low intensity light exposure during nocturnal darkness, with the subsequent reduction of melatonin synthesis and secretion, enhanced the growth of previously induced rat mammary tumors [41] or MCF-7 cell xenografts in nude mice [42]. The experimental studies, carried out with different animal models of mammary carcinogenesis and breast cancer cell lines have confirmed that melatonin, in vivo, reduces the incidence and growth of chemically induced, or spontaneous mammary tumors in rodents, whereas in vitro, at concentrations corresponding

to the physiological levels present in human blood during the night (1 nM), melatonin inhibits proliferation, increases expression of p53 and reduces the invasiveness of the estrogen-responsive MCF-7 human breast cancer cells [31, 32, 43–46].

In the next subsequent sections the oncostatic properties of melatonin directly or indirectly related with epigenetic mechanisms of cancer will be reviewed with a special focus on breast cancer.

Nuclear receptors (NRs) and co-regulators

Members of the NRs or ligand-dependent transcription factors play a multitude of essential roles in development, homeostasis, reproduction, and immune function [47]. NRs regulate transcription by several mechanisms and can both activate and inhibit gene expression [48]. The NRs include steroidal transcription factors such as the estrogen (ER) glucocorticoid (GR), thyroid hormone receptor (TR), liver X receptor (LXR), frasenoid X receptor (FXR), vitamin D receptor (VDR), retinoid acid receptor (RAR), retinoid X receptor (RXR) and peroxisome proliferators-activated receptors (PPARs) [49, 50].

Co-regulators (co-repressors and co-activators), those that interact directly with NRs, exist in large steady-state complexes with multiple secondary co-coregulator partners. Each component may possess multiple enzymatic capabilities such as acetyltransferase, deacetylase methyltransferase ultimately making these complexes versatile enzymatic machines for regulating gene expression. The activity of co-regulators is directly affected by its methylation, acetylation, or other modifications of their status, forming a posttranslational modification code. This code then controls the co-regulator's transcriptional activity and transcription factor preferences [51].

Co-regulator-mediated modulation of gene expression targets histones in the chromatin that surround genes [52] generated by a ''histone code'' in virtually all parts of DNA [53]. Thereby, co-regulators lead to a variety of biological responses that are distinct from targeting histones and further epigenetic modification of DNA by altering the methylation levels. In this manner, co-regulators can be differentially coded, allowing for an extremely large degree of combinational complexity. It is estimated that $\sim$25,000 human genes exist, of which differential splicing introduces more diversity, yielding $\sim$125,000 different coding transcripts and potential proteins [54]. Co-regulators are "master regulators" responsible for establishing homeostasis for cells, tissues, and metabolism and development throughout the life. There is no doubt that co-regulator dysfunction is not restricted to rare genetic conditions, but instead is involved in numerous human diseases [53, 54].

Half of the NRs are so-called "orphan" receptors because the identity of their ligand, if any, is unknown. The definition of orphan receptors is a loose and paradoxical one because, by definition, orphan receptors are receptors for which no ligand is known. The term "receptor" itself implies that a physiological ligand should exist, even though there is still no consensus in the field as to whether this will be true for all orphan NRs. Because the absence of proof is not the proof of absence, it is extremely difficult to demonstrate that a given orphan NR truly has no endogenous ligand. Complicating the issue is the fact that once a natural ligand has been discovered for an orphan NR, the receptor is no longer considered to be an orphan, despite the fact that it may retain structural and functional features more similar to the other orphan NRs than to the classic SR and TR. Two prime examples are the PPARs and RXRs, which were discovered as orphan NRs, but which are now clearly considered to be liganded receptors, although the precise identity of their physiological, endogenous ligands is somewhat controversial [49]. Some of receptors belonging to RXRs are no longer orphaned. Evidence of a genomic action of melatonin via nuclear RZR/ROR receptors was initially suggested in 1994 [55]. Subsequent studies have detected the nuclear melatonin receptor (NMRs; RXRs) mRNA transcripts by using in situ hybridization neuronal tissue [56–59] including pineal gland [60] and many other tissues as well [59, 61–63].

In the unligated state, PPARs associate with a multicomponent complex containing co-repressors with HDAC activity [64]. The nuclear co-repressor complex inhibits transcription by deacetylating histones [65]. Upon ligand binding, PPARs recruit co-activators that lead to histone acetylase activity and initiate a sequence of events that induces gene transcription processes [66, 67]. RXR can regulate transcription in a heterodimeric complex and generally does not involve gene transcription. Through its role as a required heterodimeric partner, RXRs control the function of many other NRs, thus integrating a unique transcriptional network dependent on RXR responses [68, 69]. RXR forms heterodimers with virtually all NRs including GR, ER, TR, PPAR, VDR, LXR and FXR. Both in vitro and in vivo approaches have revealed that NRs require RXR as a heterodimerization partner for their function. NRs can activate transcription as monomers and/or dimers with the RXR. Ligand-activated NRs dissociate from co-repressors and recruit co-activator proteins, which promote transcriptional activation [54, 70–72].

Regarding nuclear and epigenetic involvement in breast cancer in the context of melatonin, it is remarkable that transcriptional activity of ERα, GR and RAR receptors, which are involved in the regulation of breast cancer cell growth [73], are modulated by melatonin [74]. In the same context, PPARγ agonists significantly inhibit breast cancer

growth [75] and RXR agonists potentiate the antiproliferative and apoptotic effects of PPARγ agonists [76].

Eck-Enriquez et al. [77] demonstrated that MCF-7 cells treated sequentially with melatonin and *all-trans*-retinoic acid (*at*RA) resulted in enhanced sensitivity to the apoptotic effects of *at*RA, which did not appear to be due to increased expression of the RAR, but rather to enhanced transcriptional activity.

Sharma et al. [78] provide direct evidence of epigenetic actions for melatonin including NRs, co-regulators and histone acetylating enzymes. In this study, melatonin significantly increased mRNA expression for various HDAC isoforms and increased histone H3 acetylation in neural stem cell lines; also DNMT inhibitory actions of melatonin may suggest an epigenetic regulation at NR/co-regulator level rather than selective enzymatic inhibition or activation. The apparent nuclear harmony possibly through heterodimerization of melatonin-liganded RXR may open new avenues in both the pathogenesis of breast cancer and therapeutic advantages of melatonin in combination with certain NRs agonists and epigenetic modifiers.

Estrogens (E) play an important role in the development of breast cancer. They act on nuclear ER receptors and induce the expression of E-dependent genes. Furthermore, prolonged exposure to estradiol (E_2), which exerts effects on epithelial cells either directly or via stromal cells, allows cells to propagate heritable changes including DNA methylation [26]. Thus, for more than a century, suppression of estrogenic actions has been a therapeutic tool in breast cancer therapy. Melatonin counteracts the effects of E_2 and xenoestrogens on breast cancer cell proliferation, invasiveness and telomerase activity [46, 79–82], augments the sensitivity of MCF-7 to other anti-estrogens such as tamoxifen [43], and down-regulates the expression of proteins, growth factors, and proto-oncogens regulated by estrogens [45, 83]. These antiestrogenic effects depend on the interaction of melatonin with the ERα. The nature of this interaction has been investigated for recent years. Melatonin decreases the expression of ERα and inhibits the binding of the E_2-ER complex to the estrogen response element on DNA [84–86]. These effects depend on melatonin binding to specific melatonin (MT1) membrane receptors [87–91], also found in human breast tissue, both normal and tumorous [92]. The role of calmodulin as a mediator of the interactions of melatonin has been recently demonstrated [93, 94]. Interestingly, whereas melatonin is a specific inhibitor of E_2-induced ERα-mediated transcriptional activation, it does not inhibit transactivation of ERβ which does not interact with calmodulin [95]. The loss of ER expression indicates a poor prognosis for a significant number of breast cancer patients [26]. Recent results show that p53 up-regulates ERα genes expression and pRb2/p130 has also an important role in the transcriptional regulation of the ERα promoters [26, 96, 97]. These findings suggest that both p53 and pRb2/p130 may be targets for the development of novel therapeutic strategies in the treatment of ER-negative breast tumors, by re-establishing ER expression in ER-negative breast cancer [26]. The combination of demethylating agents and HDAC inhibitors has also been demonstrated to be synergic in the re-expression of ERα in the ER negative breast cancer cells [26].

Breast cancer incidence among women with gross cystic breast disease (GCBD) is to 2–7 times higher than that of the general population although the mechanism for this increase remains unexplained. Burch et al. [98] investigated 142 breast cyst fluid (BCF) samples were collected from 93 women with GCBD [98]. They observed the lowest growth index was among BCF samples with elevated concentrations of both melatonin and estrogens when added to MCF-7 cell line medium suggesting a beneficial coexistence of estrogens and melatonin, possibly at the NR level.

Approximately one third of breast cancers do not express the ERα. These tumors exhibit a greater aggressiveness and do not respond to endocrine therapy with estrogens as a target. The loss of ER expression is the result of the hypermethylation of CpG islands within the ERa promoter [26]. Treatment of ER-negative human breast cancer cells with methyltranferase or histone deacetylase inhibitors leads to a partial demetylation of the ER CpG and re-expression of ER mRNA as well as synthesis of functional ER proteins [26, 99].

Breast cancer and aromatase enzyme complex: epigenetic involvement and role of melatonin

In the postmenopausal period, estrogens are synthesized in mammary tissue by the enzyme complex known as aromatase encoded by the *CYP19* gene, from the androgenic precursors of adrenal origin (Fig. 2). Therefore aromatase is crucial, especially for postmenopausal breast cancer development as well as endometrial, ovarian and uterine disorders [100]. Breast tumors from postmenopausal women maintain a high estrogen content, even though the plasma concentrations of estradiol decrease to very low values [101]. One pathway for in situ synthesis involves the conversion of androstenedione to estrone/estradiol, catalyzed by aromatase and it may play a role in postmenopausal breast cancer development [102]. Aromatase is sufficient to maintain preneoplastic changes without circulating estrogens in breast tissue [103]. Other enzymes involved in the local biotransformation of esteroids are the reluctant isoforms of 17beta-hydroxysteroid dehydrogenases (17β-HSD) which catalyze the conversion of the

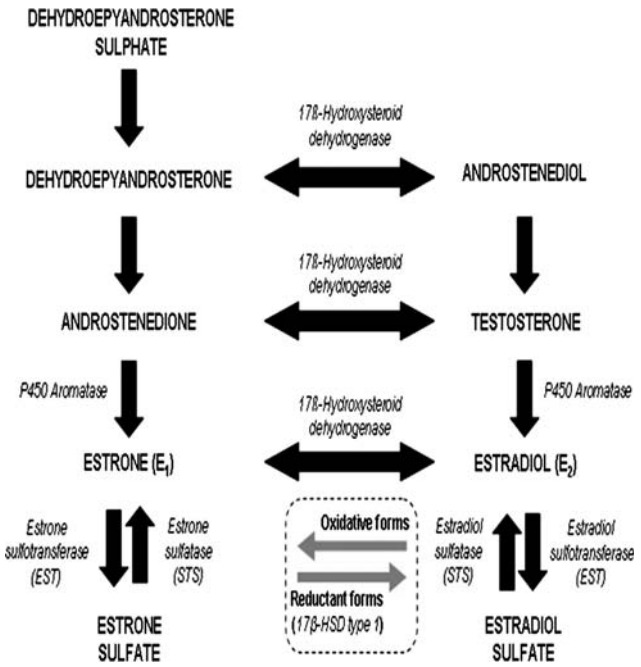

Fig. 2 Pathways of interconversion of steroids and enzymes involved. From Gonzalez et al. [110]

relatively weak estrone (E_1), androstenedione and 5-androstenedione into the more potent estradiol (E_2), testosterone and 5-dihydrotestosterone, whereas the oxidative isoforms catalyze the formation of steroids of low activity, thus exerting a protective role in different tissues including the mammary gland [104, 105]. Finally, estrogen sulfatases (STS) convert estrogen sulfates into E_1 and E_2 and estrogen sulfotransferases (EST) catalyze the conversation of estrogens into their sulfates, thus providing protection from excessive estrogenic effects [104, 105] .

Sanchez-Barcelo et al. [106, 107] recently demonstrate that melatonin modulates local estrogen biosynthesis by reducing aromatase expression and activity in MCF-7 human breast cancer cells as well as in glioma cells, and enhances the antiaromatase activity of aminoglutethimide [108]. In vivo, melatonin inhibits the growth of DMBA-induced mammary tumors by limiting the tumoral aromatase activity and the local synthesis of estrogens [109]. Melatonin, at physiological (1 nM) concentrations, also reduces the synthesis of biologically active estrogens in MCF7 cells, through the inhibition of STS and 17βHSD1 and the stimulation of EST, the enzyme responsible for the formation of the biologically inactive estrogen sulfates [110]. Although the exact mechanism as to how melatonin down-regulates aromatase expression at the transcriptional level in MCF-7 cells is unknown, recent evidence suggests epigenetic involvement. Izawa et al. [111] reported that the up-regulation of aromatase gene in endometrial cells may be ascribed to a disorder of methylation status within CpG islands in aromatase genes. When endometrial cells were treated with 5-aza-deoxycytidine (a DNMT inhibitor), aromatase mRNA expression was markedly enhanced depending on the same proximal promoters as those in endometrial cells [111]. They also found that treatment with a DNMT inhibitor enhanced aromatase expression in the eutopic endometrium [111]. If melatonin has epigenetic actions on the aromatase gene, melatonin causes methylation of the *CYP19* gene or deacetylation of *CYP19* histones and leads to gene silencing remains unknown.

The crucial transcription factor nuclear factor-κB (NF-κB) is involved in *CYP19* activation by inducing several pro-inflammatory molecules including tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), cyclooxygenase (COX-2) and prostaglandin E2 (PGE2) [112]. Positive correlations have been detected between TNF-α, COX-2 and aromatase expression in human breast cancer tissue [113]. In mice genetically engineered to overexpress COX-2 in the mammary gland, increased levels of PGE2 and aromatase were found [114]. Subaramaiah et al. [115] reported that p300, a co-regulator which has HAT activity, is important in the regulation of *CYP19* leading to increased aromatase expression via this pathway.

Of interest is that melatonin acts on every molecule in this pathway through its nuclear actions. As recently documented by Esposito et al. among others [116–118], melatonin suppresses NF-κB binding to DNA via a NRs cross-talk and decreases TNF-α, iNOS, COX-2 and PGE2 levels. Furthermore, Deng et al. [119] revealed that melatonin inhibits p300 HAT activity and abrogated p300-augmented COX-2 and iNOS expression. Melatonin suppresses macrophage COX-2 and iNOS synthase expression by inhibiting p52 acetylation and binding to DNA. This experiment suggests that melatonin causes deacetylation and leads to iNOS and COX-2 gene silencing. One mechanism by which PPARγ and RXR heterodimer inhibit breast cancer growth is also by means of the same pathway [76]. Fan et al. [120] reported that activation of PPARγ and RXR receptor inhibits aromatase transcription via NF-κB. Although not confirmed, PPARγ agonists and melatonin may act through in this pathway as well as suggesting that melatonin may be a "gene silencer" through modulation of histone acetylation and DNA methylation.

Telomere length, telomerase and melatonin

Telomeres, the physical ends of linear chromosomes, play an important role in maintaining the chromosome integrity and stability as well as in DNA replication [121]. During the cell division cycle, these structures are progressively shortened since DNA polymerase cannot replicate the terminal end of chromosomes. To overcome this loss of DNA,

telomerase, a multi-component ribonucleoprotein, replenishes telomeric DNA by synthesizing the G-strand, whereas the C-strand is synthesized by the normal DNA replication machinery [121]. Human telomerase is basically composed of: a catalytic subunit (hTERT: human telomerase reverse transcriptase) which, by using the chromosome end as a primer, drives the synthesis of the G-reach strand of telomeric DNA with a RNA subunit acting as a template (hTR: human telomerase RNA) [121]. The hTERT subunit is the rate-limiting determinant of telomerase enzyme activity [121].

Activation of telomerase plays an important role in human cancer development, providing the mechanism for an unlimited neoplastic cell division capacity [121]. Telomerase activity is observed in 85–90% of all cancers, whereas it is absent in most differentiated tissues [122]. In relation to breast cancer, telomerase activation is a relatively earlier event in the carcinogenic process [123], and the expression of hTERT closely correlates with telomerase activity thus serving as an indicator of telomerase activation [124].

Telomerase activity is also under control of epigenetic regulation [125, 126]. Increasing evidence indicates that chromatin modifications are important regulators of mammalian telomeres. The suppression of epigenetic regulators such as DNA- and histone-methyltransferases induces the loss of the control of telomeric length with the subsequent telomere elongation [125–127]. Guilleret and Benhattar [128] reported that 5-azacytidine (a DNMT inhibitor) reduces hTERT expression, telomerase activity and shortens telomeres in a human cervical adenocarcinoma cell line. Interestingly, hTERT is likely to be regulated by methylation, and hypermethylation of hTERT promoter is likely to be necessary for its expression [128]. This preliminary finding has recently been confirmed by Renaud et al. [129]. They revealed that hTERT expression is induced when the hTERT CpG island is sufficiently hypermethylated.

Similarly, melatonin inhibits telomerase activity in MCF-7 breast cancer cells and decreases mRNA levels of hTERT, as well as hTR [80]. Furthermore, melatonin inhibits the hTERT expression induced by either natural estrogens (17β-estradiol) or metalloestrogens (cadmium) in MCF-7 human breast cancer cells [81, 82]. Interestingly, this action of melatonin resembles that of DNMT inhibitors [130] e.g., tea polyphenol (−)-epigallocatechin-3-O-gallate (EGCG) [131], a compound that reduces cellular proliferation and induces apoptosis of MCF-7 cells [132]. Like melatonin, EGCG significantly decreases hTERT mRNA with the down-regulation of hTERT gene expression in MCF-7 cells seemingly being largely due to epigenetic alterations [132]. Interestingly, Leon-Blanco et al. [133] observed a significant decrease in the RNA levels of hTERT after treatment with an GP 52608 (an agonist of melatonin nuclear receptors), while treatment with S 20098 (an agonist of the melatonin membrane receptors) had opposite effects. This study strongly indicates that nuclear and/or epigenetic, but not membrane receptor-mediated actions of melatonin, are responsible for the decrement in the RNA levels of hTERT in the MCF-7 cell line. Furthermore, EGCG down-regulates ERs function in MCF-7 cells [134] in a similar manner to that of melatonin's modulation of ERs in these cells [32, 135].

The link between histone deacetylation and telomerase activity has been studied in normal as well tumor cells. Mukhopadhyay et al. [136] demonstrated that trichostatin A (TSA), a HDAC inhibitor, induces hTERT expression and activates telomerase activity in normal lung cells compared to non-small-cell lung cancer, and that hTERT is directly correlated to histone acetylation in normal cells. However, in prostate cancer cells, TSA treatment decreases hTERT expression and telomerase activity [137]. The increase in hTERT expression following TSA treatment has also been reported in other cancer cell lines including Ha1-IM, SiHa and Hela cells [138]. It is likely that, in cancer cells, histones of telomerase are already acetylated and active. Therefore, inhibition of HDAC may either have no effect [136] or may augment telomerase activation [138].

To reduce telomerase activity in cancer cells but not in normal cells, inhibition of DNA methylation, as obtained in the melatonin and EGCG studies, seems feasible, since telomerase histones are already acetylated in cancer cells. Moreover, several reports indicate that DNMT inhibitors (e.g., EGCG) reactivate some methylation-silenced tumor suppressor genes in human colon cancer HT-29 cells, prostate cancer PC3 cells and esophageal cancer KYSE 150 cells [139].

Cyclin d1 connection in the pathogenesis of breast cancer

Class D cyclins (D1, D2 and D3), each of which binds cyclin-dependent kinase, associates with, and regulates activity of, transcription factors, co-activators and co-repressors that govern histone acetylation and chromatin remodeling proteins. It is cyclin D1 over-expression that is predominantly associated with tumorigenesis and cellular metastases. In human cancer, over-expression of cyclin D1 is one of the most commonly observed alterations in the transit through the G phase of the cell cycle. In a large majority of breast cancer cases, cyclin D1 is over-expressed and its levels correlate with a negative prognosis [140].

Cyclin D1 forms physical associations with more than 30 transcription factors or transcriptional co-regulators.

Several NRs, including GR, ERα, and PPARγ, bind directly to cyclin D1 and both basal and ligand-dependent transactivation of NRs is regulated by cyclin D1 [141]. Not surprisingly, cyclin D1 is a co-activator of ERα transcription and a co-repressor of GR and PPARγ [142]. A key event for the anti-proliferative effects of anti-estrogens appears to be the down-regulation of cyclin D1 [143].

Santos et al. [144] reported that the repression of cyclin D1 expression by HDAC inhibitors (butyrate and SAHA) was stronger than that found with a pure anti-estrogen (ICI 182.780). This study also revealed that HDAC inhibitors down-regulate ER levels and transcription of ER target genes in breast cancer cells. Apart from similar effects of HDAC inhibitors including downregulation of ER levels and transcription of ER target genes in breast cancer cells, melatonin has been shown to inhibit transcription of cyclin D1 expression supporting the epigenetic efficacy of melatonin on hormone sensitive breast [145] and prostate cancer [146]. Several years ago, it was reported that melatonin significantly increases the duration of the cell cycle of human breast cancer cells [147].

Epigenetic actions of melatonin via the circadian system

There is increasing interest in the possible role of environmental factors that can alter normal endocrine function, often referred to as 'endocrine disruptors', in the etiology of cancer. Of particular interest is the potential influence of exposure to light-at-night (LAN), and sleep disruption as occurs in night shift workers, on endocrine function and the regulation of hormones, e.g., melatonin, that are important in the etiology of some types of cancer [148]. It is important take into consideration the role of light as the main zeitgeber of the circadian system and that circadian rhythm disruptions may be epigenetic causes of cancer [149]. Persons who engage in night-shift work are subjected to the influence of both factors, and exhibit altered hormone profiles, most importantly decreased melatonin production and a disrupted melatonin rhythm [38]; these changes could increase the risk of hormone-related diseases, including breast and prostate cancer (see Section "Melatonin and epigenetics of breast cancer" of this article). A recent meta-analysis [150], and on-going studies in Denmark and Seattle have recently found a strong relation between night-shift and increased breast cancer [151, 152]. Moreover, melatonin-depleted blood from postmenopausal women exposed to LAN stimulates growth of rat hepatomas and MCF-7 xenografts in comparison to the effects of night-collected blood with normally elevated melatonin [149]. It has also been shown that MCF-7 cell proliferation was greater in presence of sera obtained from breast cancer women than with sera from healthy women [153], although the authors did not find differences in the melatonin concentrations between the two sera, probably because melatonin levels were only measured in morning samples. Interestingly, the serum concentration of melatonin in breast cancer women decreased after mastectomy, thus suggesting that the presence of the tumor could induce changes in the secretory pattern of this hormone, as was previously described for PRL and GH.

The mammalian clock machinery includes, among others, three period proteins (PER1, PER2, and PER3), two cryptochromes (CRY1 and CRY2), CLOCK and two BMAL proteins. In mammals, the central elements of the circadian machinery, CLOCK and BMAL1, heterodimerize and involve transcriptional/translational feedback loops with negative and positive limbs [154]. Recent mammalian clock gene studies uncovered molecular clocks in many brain regions and peripheral tissues synchronized by the master clock [155]. Currently, it is thought that transcription of *PER* and *CRY* genes are driven by accumulating CLOCK-BMAL1 heterodimers. At the same time, PER2 up-regulates the levels of BMAL1 mRNA leading to the formation of CLOCK-BMAL1 heterodimers, which drive *PER2* and *CRY2* transcription and restart the cycle [156].

Doi et al. [157] reported that the CLOCK is a HAT suggesting a strong epigenetic involvement. Hirayama et al. [158] revealed that CLOCK also acetylates its own partner BMAL1 and CLOCK-mediated acetylation contributes in multiple ways to the time-dependent regulation of circadian physiology. The compelling links that exist between the circadian cycle and metabolism suggest that the HAT function of CLOCK may be controlled by the redox state of cells and as well as by the light/dark cycle [159].

At present, the mechanism by which the circadian clock influences tumor growth is not fully understood. Recently, the circadian clock gene *PER2*, which helps to synchronize mammalian organisms with environmental light, has been reported to function as a tumor suppressor gene. Fu et al. [160] observed the development of spontaneous lymphomas and teratomas in *PER2* knockout mice at only 6 months of age. Thirty per cent of mutant mice died before the age of 16 months. Disruption of the *PER2* gene in mice abolishes the response of all core circadian genes to gamma radiation whereas in wild-type mice, clock genes are induced rapidly, suggesting they may be involved in DNA damage response. Furthermore, a number of cell cycle and checkpoint proteins were deregulated in these *PER2* mutant mice including cyclin D1. Experimental evidence demonstrates the importance of circadian clock genes, in particular *PER2*, as regulators of the cell cycle and, therefore, cancer progression. Based on the role of *PER2* in cancer development and the clear epidemiologic connection between circadian disruption (e.g., LAN and

night-shift work) and the risk of breast cancer development in women, Xiang et al. [161] clearly demonstrated the tumor suppressive nature of *PER2* as evinced by inhibition of cell growth, induction of apoptosis, reduced colony formation and growth in MCF-7 cells. Gery et al. [162] reported that *PER2* links the circadian cycle to the ERα signaling network. While suppression of *PER2* stabilizes ERα, binding enhances ERα degradation. *PER2* itself was found to be estrogen inducible in these cells, suggesting a feedback mechanism to attenuate stimulation by estrogen. In addition, over-expression of *PER2* in breast cancer cells leads to significant growth inhibition, loss of clonogenic ability and apoptosis.

Since consistent evidence reveals that *PER2* is a circadian tumor suppressor gene, existence of this gene is welcome. However, its circadian nature suggests that *PER2* should fluctuate throughout a 24 h period depending on melatonin levels and the light-dark cycle. Boivin et al. [163] reported that circadian clock genes do oscillate in human peripheral blood mononuclear cells. Peak clock gene expression was observed mostly during the usual time of activity and light exposure. Transcript levels of *PER1-3* were found to correlate positively and significantly with the level of plasma melatonin. Peak clock gene expression followed the highest melatonin concentration by a maximum of 9 h [163]. Cajochen et al. [164] developed a non-invasive method to measure and quantify human circadian *PER2* gene expression in oral mucosal samples and found that *PER2* gene expression is both light and melatonin dependent. Consistent with this, evening exposure to blue light had a strong stimulatory effect on expression of *PER2* in humans [164]. Because of this, the circadian rhythmicity of PER2 gene is perturbed and may not function as a tumor suppressor gene.

The visible light spectrum covers a wavelength range from approximately 400 nM (violet) to 700 nM (red); blue light has a wavelength close to the lower visible range (around 500 nM). From the studies of Provencio et al. [165], it is known that circadian photoreception depends on the ganglion cell layer of the retina. In these cells, photopigments different than opsins have been described as involved in this non-visual pathway (the so-called "non-image-forming system") which carries photoperiodic information to the suprachiasmatic nuclei of the hypothalamus. These photopigments are melanopsins [165] and chryptocrhomes [166, 167] and they respond to a narrow band of blue wavelengths. Thus, the human circadian system is sensitive to non-visual effects of ocular light at short wavelengths [168], and maximal response to light for melatonin suppression [169, 170] and circadian phase shifting [171] is obtained between 446 and 483 nm (violet-blue). Acute (early) effect of light on melatonin, alertness, thermoregulation, and heart rate is blue-shifted, such that short wavelength of light at 460 nm induces a greater melatonin suppressive, alerting, hyperthermic and tachycardic effect than light at 550 nm [164, 172].

As a consequence, under a physiologic light-dark cycle, peak melatonin level is followed by *PER* expression and higher PER levels persevere until dusk [173]. Night-shift work and LAN as well as several indoor instruments, e.g., personal computers and TV can further prolong *PER* gene expression. Use of TV and/or computers in the evening is endemic in Western societies, and they emit a blue light mimicking "circadian high noon". Watching TV at night was recently shown to be associated with lower urinary melatonin metabolite concentrations [174]. Finally, Chen et al. [175] also found, in 55 cases of breast cancer, deregulated expression of the three *PER* genes in more than 95% of the breast cancer cells in comparison with the nearby non-cancer cells. More recently Gery et al. [162] demonstrated that *PER2* links the circadian cycle to the ERα signaling network. Thus, suppression of PER2 levels leads to ERα stabilization whereas its over-expression induces ERα degradation. Furthermore, PER2 itself is estrogen inducible in breast cancer cells; this fact suggesting a feedback mechanism which attenuates the stimulatory effects of the estrogens [162]. Interestingly, the *PER* gene deregulation is not caused by genetic mutations but by methylation of the *PER1* and *PER2* promoter [175], i.e., by epigenetic mechanisms. The methylation status of *PER2* has a strong correlation with *c-erB2* expression [175], and the over-expression of this oncogene may result in a more aggressive tumor phenotype. Whether methylation of *PER2* influences the c-erB2 expression, or vice versa, needs to be clarified [175].

Concluding remarks

Environmental factors are among the major determinants of epigenetic changes related to carcinogenesis. In regard to this, there is an obvious epidemiologic connection between the frequency of breast cancer and LAN and/or night-shift strongly relate to reduced melatonin production and a disrupted melatonin rhythm. Furthermore, the context of the current review, we speculate that postmenopausally estrogen usage increases breast cancer risk since its antagonist, melatonin, is physiologically reduced due to aging. In other words, the postmenopausal period may mimic night-shift work or LAN; thus high estrogen levels in the presence of attenuated circulating melatonin concentrations increases the likelihood of breast cancer. The studies carried out on breast cyst fluid from women with gross cystic breast disease demonstrated an inverse relationship between its concentration of melatonin and estrogen and its ability to induce proliferation of MCF-7

breast cancer cells. This fact suggests a beneficial co-existence between estrogen and melatonin possibly at the NRs level. These observations coupled with those coming from a variety of laboratories demonstrated the oncostatic properties of melatonin expose the interaction of melatonin with the estrogen signaling pathway as potentially one of the main mechanisms involved in development of breast cancer. The interaction with other signaling pathways has also been proven. Thus, MCF-7 cells treated sequentially with melatonin and *at*RA enhanced the apoptotic effects of *at*RA, which did not appear to be due to increased expression of the RAR, but rather to enhanced transcriptional activity. Similar effects were also obtained with PPARγ and melatonin.

The oncostatic effects of melatonin that are of a direct epigenetic nature have been demonstrated in studies unrelated to cancer cell proliferation. These studies reported that melatonin significantly elevates mRNA expression for various HDAC isoforms and significantly increases histone H3 acetylation in neural stem cells. This apparently dual function of melatonin as reported in this study as well as the DNMT inhibitory action of melatonin suggests an epigenetic regulation at the NR/co-regulator level rather than selective enzymatic inhibition or activation. The apparent nuclear harmony possibly through heterodimerization of melatonin-liganded RXR may open new avenues in both the pathogenesis of breast cancer and therapeutic advantages of melatonin in combination with certain NRs agonists and epigenetic modifiers.

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