Epigenetic setting for long-term expression of estrogen receptor α and androgen receptor in cells

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Abstract

Epigenetic regulation of the nuclear estrogen and androgen receptors, ER and AR, constitutes the molecular basis for the long-lasting effects of sex steroids on gene expression in cells. The effects prevail at hundreds of gene loci in the proximity of estrogen- and androgen-responsive elements and many more such loci through intra- and even inter-chromosomal level regulation. Such a memory system should be active in a flexible manner during the early development of vertebrates, and later replaced to establish more stable marks on genomic DNA. In mammals, DNA methylation is utilized as a very stable mark for silencing of the ERα and AR isoform expression during cancer cell and normal brain development. The factors affecting the DNA methylation of the ERα and AR genes in cells include estrogen and androgen. Since testosterone induces brain masculinization through its aromatization to estradiol in a narrow time window of the perinatal stage in rodents, the autoregulation of estrogen receptors, especially the predominant form of ERα, at the level of DNA methylation to set up the "cell memory" affecting the sexually differentiated status of brain function has been attracting increasing attention. The alternative usage of the androgen-AR system for brain masculinization and estrogenic regulation of AR expression in some species imply that the DNA methylation pattern of the AR gene can be established by closely related but different systems for sex steroid-induced phenomena, including brain masculinization.

Key words: estrogen receptor, androgen receptor, DNA methylation, brain masculinization, autoregulation, cell memory

Introduction

In mammals, sex-dependent compositions of the cells in the brains and the nature of many patterns of behaviors cannot be attributed directly to genetic differences. Rather, an important feature is that, in rodents for example, endocrine disturbance at the fetal and/or postnatal stages irreversibly changes behaviors such as lordosis (in females) and mounting (in males) that occur after the pubertal stage and are normally correlated with the genetic sex. In some cases, lordosis can even be observed in males, and mounting in females (Sodersten, 1978). In such phenomena, two classes of sex steroid hormones produced largely in gonadal tissues, estrogen and androgen, play many important physiological and pathological roles in a sex-dependent manner. In this review, I summarize the present understanding of the cellular impacts of the "genomic" effects of sex steroids, in particular the effect of estrogen through its interaction with its predominant nuclear receptor, ERα. Associated theoretical epigenetic pathways triggered by estrogen and androgen and potentially leading to the differential expression of mammalian behaviors will be featured.

Sex Steroid Hormones: Epigenetic Regulators of Brain Masculinization in Rodents?

In general, estradiol (E2) and testosterone (T) circulate predominantly in adult females and males of vertebrates, respectively. Therefore, one could readily assume that these hormones set up the molecular basis to establish the endocrine circumstances and thereby influence the expression of sex-dependent behavior. For example, removal of

the ovaries, the major source of E2, after the pubertal stage strongly deactivates the hypothalamus-pituitary-gonadal (HPG) axis, thereby disturbing the pulsatile- and surge-mode of luteinizing hormone secretion in rodents and many other mammals including sheep, goat, cow and pig (Bronson, 1981; Goodman, 1978; Kraeling et al., 1998; McCarthy and Swanson, 1976; Mori et al., 1987; Webb et al., 1981). In parallel, the hypothalamus-pituitary-adrenal (HPA) axis is affected in ovariectomized animals (Seale et al., 2004). Since these unregulated states are largely rescued by exogenous treatment with E2 (Christian et al., 2005), estrogen has been focused on as an important trigger for sexual reproduction and many sex-dependent behaviors. A similar situation is true for males, in that castration of the testes, the major source of T, perturbs the HPG and HPA axes, and T supplementation restores many sex-dependent behaviors (Putnam et al., 2003). These losses and recoveries occur on a day- or week-scale (Lindzey et al., 1998), and drastic changes of cell composition do not seem to be associated with these experimental events. In contrast, cell composition is drastically affected during the developmental stages. In fact, E2 triggers the cell fate specification at the perinatal stage in rodent brains (Schwarz and McCarthy, 2008). For example, the medial preoptic area (MPOA) and bed nucleus stria terminalis (BNST), which are famous sexually dimorphic brain nuclei, show fewer apoptotic cells after estradiol benzoate (EB) or testosterone propionate (TP) treatment at the perinatal stage in rodents (Chung et al., 2000; Hsu et al., 2001). Cell proliferation and migration can also be associated with estrogen signals (McCarthy, 2008). Paradoxically, cell death is increased in a subset of sexually dimorphic nuclei by estrogen signals. The anteroventral periventricular nucleus

is the best example so far in which TUNEL-positive cells are increased after neonatal treatment with EB or TP in male and female rats (Arai et al., 1994; Arai et al., 1996). Changes of the survival and migration of the cells by the presence or absence of estrogen could explain the phenomenon of "imprinting" of the brain at the critical period, also known as brain masculinization (Cooke et al., 1998; McCarthy, 2008). Since the nuclear estrogen receptor ERα can contribute to the molecular complex of histone-interacting proteins and histone-modifying enzymes (Heinzel et al., 1997), the highly ordered chromatin structure can be differentially established and somehow fixed long-term in ERα-positive cells. In this way, the estrogen-ERα complexes could change particular cell characteristics rather than causing cells to die, proliferate, or migrate in some cases. To decipher these complex ways of "imprinting" in specific brain regions, it would be simplest to start by first dissecting the mechanisms involved at the cellular level.

Until now, most studies on molecular mechanisms governing the epigenetic setting have focused on histone modifications and DNA methylation mainly occurring at the CG dinucleotide in animals. Histone modifications are commonly utilized in a wide range of species, including single cell organisms (Jenuwein and Allis, 2001). On the other hand, the overall DNA methylation level differs depending on the species. For example, only trace amounts of methylcytosine can be found in *Drosophila melanogaster* (Kunert et al., 2003; Lyko et al., 2000). In mammals, DNA methylation is a fundamental mechanism that differentiates the gene expression pattern in the brain (Imamura et al., 2001; Jones and Takai, 2001). Indeed, mutations in genes associated

with DNA methylation have frequently been shown to lead to many defects in neural systems. For example, mutations in MECP2, a methylcytosine interacting protein, is known to cause Rett syndrome (Amir et al., 1999). Mutations in a *de novo* DNA methyltransferase (DNMT), DNMT3B, lead to ICF syndrome (Hansen et al., 1999; Xu et al., 1999). Both of these syndromes show some characteristics of neuronal disorders. The DNA methylation system is also known to be critical for genomic imprinting (Heard et al., 1999), transposon silencing (Bird and Wolffe, 1999; Walsh and Bestor, 1999), chromatin stability (Eden et al., 2003), and tissue-dependent gene expression (Shiota et al., 2002). In mammals, DNA methylation imposes restraints on the pluripotency because once the patterns are established during development they can be maintained through cell division (Sharif et al., 2007). Conversely, some fishes, which contain much lower DNA methylation activity (Cross et al., 1991), are found to easily and reversibly change their sex status according to the environmental context (Grober and Sunobe, 1996). These facts support the idea that sex-dependent patterns of behaviors are established through epigenetic processes. The sex-dependent patterns of mammalian behaviors could be acquired through highly irreversible processes during development by exposure to sex steroid hormones. In particular, it could be hypothesized that the long-term effects of the sex steroids at the developmental stage on behaviors after puberty are somehow marked at the genome level.

Priming Effect of Sex Steroid Hormones on Gene Transcription in Cells

The biogenesis of E2 from T is accomplished by the catalytic action of

aromatase P450. Although ERα is the predominant nuclear receptor for estrogen, ERβ is known to be another nuclear receptor (Couse and Korach, 1999). So far, AR is the only known receptor for androgen. In addition to these nuclear receptors, ER-X and two G-protein-coupled receptors, GPR30 and Gq-mER, bind to estrogen to transduce the estrogen signals rapidly to adjust the cellular status (Funakoshi et al., 2006; Qiu et al., 2003; Qiu et al., 2006; Revankar et al., 2005; Toran-Allerand, 2005). The differential presence of various forms of estrogen and androgen receptors in cells makes it difficult to understand the whole picture of sex steroid signaling. Although membrane-bound receptors might play some roles in gene regulation, it is simplest to first consider only the role of nuclear receptors in the epigenetic effects of estrogen and androgen.

Early work on the Xenopus vitellogenin gene identified a minimal estrogen responsive element (ERE) core sequence composed of two 6-base asymmetrical elements separated by three spacer nucleotides: 5'-GGTCAnnnTGACC-3' (Klein-Hitpass et al., 1986). A similar but not identical structure has been found for the androgen responsive element (ARE). Most AREs conform to a consensus sequence composed of two 6-base asymmetrical elements separated by three spacer nucleotides: 5'-AGAACAnnnTGTTCT-3' (http://www.genome.jp/htbin/www_bfind?transfac). Occupancy of ERE and ARE by the steroid-nuclear receptor complex can lead to the acute upregulation of the physically associated gene. There are many examples of acute upregulation by the estrogen-ER complex together with other nuclear transcription factors (Gruber et al., 2004). Conversely, removal of steroid hormones also causes an acute decrease of gene transcription. However, there are examples in which

supplementation of steroid hormones to ERα-positive breast cells gradually upregulates the cell cycle, followed by DNA methylation, histone modification, and microRNA expression changes (Kovalchuk et al., 2007). These occurrences are associated with transformation into hyperplastic states such as those in carcinomas and metastatic cells. Another report showed that, on disruption of ERα signaling by small interfering RNA, polycomb repressors and histone deacetylases (HDACs) are recruited to initiate stable repression of the progesterone receptor (PR) gene, a known ERα target, in breast cancer cells (Leu et al., 2004). In these cells, ERα repression is accompanied by PR mRNA disappearance one day later. This event is also accompanied one week later by DNA methylation of the PR promoter, leaving a stable mark that can be inherited by cancer cell progeny. Reestablishing ER signaling alone is not sufficient to reactivate the PR gene, rather, reactivation of the PR gene also requires DNA demethylation. The removal of the nuclear estrogen signal induces progressive DNA methylation of multiple ERα targets in breast cancer genomes (Leu et al., 2004). Considering this kind of long-term effect of sex steroid hormones on setting the target gene expression status, it would be important to see the precise timeline of epigenetic alterations occurring in cells.

Since the identification of a canonical ERE, several computational approaches have been undertaken to identify ERα target genes at the genome-wide level (Bajic et al., 2003; Bourdeau et al., 2004). For example, in excess of 70,000 putative EREs have been found in the human genome, over 17,000 of which are located within 15 kb of the transcription start sites of genes (Bourdeau et al., 2004). Six hundred sixty of these are conserved between the mouse and human genomes, and a fraction of these

have been confirmed experimentally to function as genuine ERα-interacting sites. In addition, imperfect EREs compared with the consensus sequence frequently show ERα binding activity (Gruber et al., 2004). Very recently, identifying long-distance chromatin interactions with ERα has been attempted at the genome-wide level (Fullwood et al., 2009). Using a combinatorial technique of chromatin immunoprecipitation and ligation-mediated PCR, called ChIA-PET, DNA regions physically nearby the ERα-bound regions have been extensively sequenced. The results using E2-treated MCF-7 cells showed 1451 intra-chromosomal and, surprisingly, 15 inter-chromosomal overlapping clusters. Each of these more than one thousand clusters contains several genes, many of which showed coordinated upregulation of their transcription by E2 treatment. The regions close to the ERα-bound regions showed a tendency to rapidly constitute active chromatin structures reminiscent of the active gene transcription in response to E2. Even the genes in these clusters located relatively far from the ERα-bound region also seemed responsive to E2, resuming transcription two days later on average. Such a tendency was not seen for the genes outside of the clusters. Therefore, the time lag of transcription alteration between the immediate-early and other genes raises the interesting possibility that genes in a cluster constitute an intra-chromosomal loop structure for a commonly regulated epigenetic setting (Fig. 1). In this model, a specific gene cluster could be organized to restrict the epigenetic effects of the estrogen-ERα complex within a loop to strengthen the coordination of the transcription, and this structure would isolate this effect to prevent leaky transcription of the genes located outside of the loop. The differential ERα-triggered epigenetic setting

depending on cell type may account for the tissue- and sex-dependent differences of cell fates resulting from various degrees of responsiveness to estrogen.

Cell-Dependent Autoregulatory Loops of Sex-Steroid Receptor Genes

Estrogen signaling frequently affects the ERα transcription in ERα-positive cells. That is, ERα-positive cells can sensitize or desensitize their estrogen signaling pathways in a cell-intrinsic manner. For example, a low dose of E2, given to ovariectomized animals to mimic the preovulatory estrogen surges, acutely enhanced ERα as well as PR gene expression in specific uterine cells (Ing and Tornesi, 1997). These promoters were also modulated by E2 in estrogen-responsive breast cancer cell lines (Donaghue et al., 1999; Saceda et al., 1988). In the case of T47D, ZR-75, and EFM-19 breast cancer cells, E2 increased ERα expression. In contrast, the ERα promoter was downregulated by E2 in MCF-7 breast cancer cells, in which E2 reduced the receptor expression. Therefore, ERα regulation by estrogen may strongly differ in different cells. The kinetics of ERα mRNA and protein expression in MCF-7 cells were investigated after acute treatment with E2 (Saceda et al., 1988). The data have suggested that E2 downregulates ERα mRNA by inhibition of ERα gene transcription at early times and by a posttranscriptional effect on receptor mRNA at later times. Actually, three promoters have been identified for human ERα. The use of the three promoters was examined in ER-positive breast cancer cell lines, cell lines derived from other malignancies, and some normal tissues (Donaghue et al., 1999). Many estrogen-responsive breast cancer cells used all three promoters. Cell lines derived from

other malignancies and other normal tissues that express lower levels of ERα showed more selective promoter usage. This raised the possibility that the level of expression of ERα is determined by the number of promoters used, rather than the selective use of specific promoters. However, the number of known alternative ERα promoters is still growing in the human, mouse and rat (Kos et al., 2001; Wilson et al., 2008), and therefore, the possibility that the selective usage of specific promoters accounts for the tissue- or cell-specific ERα expression cannot be ruled out.

In rodents, ERα mRNA is expressed in several brain regions, including the MPOA (Shughrue et al., 1992). In male mice, a high density of ERα was found in a small number of hypothalamic cells of the MPOA, arcuate, and ventromedial nuclei (Agarwal et al., 2000). A low or medium density of ERα was observed in cells of the lateral preoptic area, supraoptic nucleus, BNST, and in the central, medial and anterior cortical amygdaloid nuclei. Estrogens are believed to downregulate their own receptors in most rodent brain regions, because ovariectomy and subsequent EB supplementation very frequently increase and decrease the ERα mRNA level, respectively (Hamada et al., 2005; Lauber et al., 1991; Shughrue et al., 1992). This general tendency was also assessed with aromatase knockout (ArKO) mice in which conversion of T to E2 is impaired (Agarwal et al., 2000). The number of cells containing ERα protein was significantly increased in the MPOA of the ArKO male mice. Similarly, male rats treated with an aromatase inhibitor escaped from the inhibitory effect of T on ERα expression in many brain regions, suggesting that T functions through its aromatization to E2 for the downregulation of ERα expression (Clancy and Michael, 1994). Thus,

estrogen can downregulate ERα in brain cells.

However, ERα regulation by estrogen seems to differ according to the brain region. For example, EB was shown to decrease immunostaining intensity for ERα in the ventrolateral hypothalamus and BNST, but not in the periventricular preoptic area or medial amygdala of female rats (DonCarlos et al., 1995; Lauber et al., 1991). Moreover, EB treatment fails to significantly downregulate ERα mRNA levels in male rats, in contrast to the downregulation in female rats (Lauber et al., 1991).

DNA Methylation of the ERα Promoter

To reconcile the mutually opposite effects of estrogen on ERα expression, namely, its upregulation and downregulation according to the cell type, it is necessary to think of the other factors that modulate the quantity of ERα mRNA. It should be noted that long-term deprivation of estrogen in the culture media of ERα-positive breast cancer cells can generate ERα-negative subclones that are completely insensitive to estrogen for their cell growth (Pink and Jordan, 1996). This phenomenon strongly suggests the involvement of an epigenetic mechanism to preset the local chromatin structure for the basal ERα expression. In this context, formation of transcriptional repression complexes including DNMT, HDAC and/or methyl-CpG binding protein is emerging as an important mechanism in silencing a variety of methylated tissue-specific and imprinted genes (Imamura et al., 2001). In fact, methylation of the ERα CpG island is associated with loss of ERα expression in human breast cancer cells (Ottaviano et al., 1994). Treatment of ERα-negative human breast cancer cells with the DNMT1 inhibitor

5-aza-2'-deoxycytidine (5-aza-dC) leads to ERα mRNA and protein re-expression (Ferguson et al., 1995). Also, the HDAC inhibitor trichostatin A (TSA) could induce ERα transcripts (Yang et al., 2000). In addition, the combination of 5-aza-dC and TSA induced a synergistic increase in ERα transcripts, occurring concomitantly with markedly reduced soluble DNMT1 expression and activity, partial demethylation of the ERα CpG island, and increased acetylation of histones H3 and H4. These data suggest that the activities of both DNMT1 and HDAC are key regulators of methylation-mediated ERα gene silencing.

It has been demonstrated that thousands of CpG islands show tissue- or cell-dependent patterns of DNA methylation (Shiota et al., 2002). Therefore, distinct DNA methylation patterns in the respective cells can establish or fix cellular phenotypes. On the one hand, each differentiated cell maintains its DNA methylation pattern (Shiota and Yanagimachi, 2002). On the other hand, dynamic DNA methylation changes occur during development, and cell differentiation is always associated with DNA methylation and demethylation, forming cell-specific patterns (Ohgane et al., 2002). DNA demethylation occurs through either passive mechanisms by inhibiting the DNMT1 or through active enzymatic reactions. Active demethylation has been observed in many cells but the mechanisms involved are relatively unknown (Collas, 1998; Fremont et al., 1997; Imamura et al., 2004; Jost et al., 1997; Jost and Jost, 1994; Jost et al., 1995; Jost et al., 1999; Kim et al., 2009; Ma et al., 2009). Although long-term maintenance of DNA methylation patterns is a prerequisite for an animal's life, the overall methylcytosine content gradually decreases in parallel with aging processes. Since gene

body regions account for only a small percentage of the genome, gain or loss of the overall methylcytosine content might largely affect “bulk” sequences such as transposable elements constituting heterochromatin structure to maintain chromosomal stability. In line with this tendency, most cancer cells show a hypomethylated status at the transposable elements (Baylin et al., 1998). Paradoxically, an overall decrease of DNA methylation content is associated with local induction of the DNA methylation at multiple gene loci (Baylin et al., 1998). Considering that the circulating and local contents of sex steroid hormones dynamically change according to the developmental and aging context, DNA methylation changes related to ERα expression could occur in various brain cells, including neurons, in a spatiotemporal manner.

In fact, recent studies have shown that the DNA methylation status of ERα in physiologically normal cells differs during development depending on the cell type. In the mouse cortex, ERα mRNA expression is high early in postnatal development but declines starting at postnatal day (P) 10 and is virtually absent in the adult cortex. Several regions of the ERα promoter displayed a significant increase in methylation at P18 and P25 compared with P4 (Westberry et al., 2010). In the mouse cortex, DNMT3A (the de novo DNMT) peaked at P10 and was decreased by P25. DNMT1 increased across development and stayed high in the adult cortex. A chromatin immunoprecipitation assay showed a correlation between association of MECP2 with the ERα promoter and the increase in DNA methylation and decrease in ERα expression after P10 (Westberry et al., 2010).

External stimuli also seem to affect the establishment of the methylation

pattern of the ERα promoter. Maternal care of rat pups can lead to long-term effects affecting the life-long response to stress in the offspring (Liu et al., 1997). Mothers that have high rates of licking and grooming behavior have offspring with a more modest response to stress. Adult offspring of mothers that exhibit high licking and grooming activity have increased expression of ERα mRNA in the MPOA, and this increased expression is associated with less methylation at the ERα promoter, while the ERα promoter in offspring from low licking and grooming mothers is hypermethylated (Francis et al., 1999). In addition, variations in the rates of licking and grooming are inherited. Mothers with high rates of licking and grooming activity have pups that later exhibit similar behavior when they become mothers (Francis et al., 1999).

A Model for the Estrogen Effect on ERα mRNA Expression

With the analogy of brain learning, cells with the potential to transcribe ERα may memorize the surrounding estrogen availability for the expression of specialized cell function. In line with this idea, during >8 months of deprivation of E2 from the culture media, breast cancer cells with a low level of ERα protein gradually lose the ability of ERα transcription (Pink and Jordan, 1996). After 2-month resupplementation of E2 in the media, these cells occasionally recover ERα expression competence. Such cells show steady ERα upregulation by E2 treatment. On the other hand, different cells originating from the same organ but with relatively higher levels of ERα mRNA might be affected by the possible destabilization activity of an E2-ERα complex that has been proved to bind to the coding sequence of ERα mRNA (Kaneko et al., 1993). This would

be possible if an excess amount of E2-ERα complex that could not enter into the nucleus due to physical or biochemical blockage remained located in the cytoplasm. Based on the several lines of information described above, a schematic representation of possible ERα-triggered alteration of the cell status is shown in Fig. 2.

Phases A and B: Starting from the low expression status of ERα at the early stage, cells show the capacity to incorporate the extracellular estrogen signal and try to amplify this signal by upregulation of ERα. This phase could be in a learning state of the cell in terms of establishing intra- and inter-chromosomal structures through the interaction of increased E2-ERα complex with widespread ERE in the genome for the large-scale epigenetic setting triggered by E2. After a considerable level of ERα is reached, cells behave to maintain the estrogen signal to keep the equilibrium state, which is reminiscent of the homeostatic state.

Phase C: During aging, overall DNA methylation and other chromatin modification activities gradually decrease in cells, while gene loci including ERα are targeted by local DNA methylation activity. In parallel, circulating E2 also decreases. This decrease within a certain time range is further memorized in cells to accelerate the DNA methylation of the ERα promoter, which is also frequently seen in ERα-negative cancer cells.

Phase D: If cells do not have the capacity or chance to incorporate the estrogen signals, a concomitant increase of general DNA methylation activity specifically or non-specifically closes the chromatin structure at the ERα *cis*-regulatory regions by DNA methylation and/or histone modifications such as methylation at lysine

9 of histone H3. Once cells learn the expression downregulation via DNA methylation and other epigenetic modifications, such cells would rarely express ERα, thereby greatly reducing the possibility of its expression except after abnormal treatment such as long-term exposure to estrogen.

The model shown here has been made based largely on cancer cell studies. Since the estrogen regulation system varies widely, it would be valuable to test several simple models of the phases described above. Of course, mechanisms mediating epigenetic alterations may differ in postmitotic brain cells and cancer cells of different origins. Nonetheless, it should be noted that acquiring the unmethylated status of the ERα promoter by either passive mechanisms (lack of faithful replication of DNA methylation patterns) or active epigenetic mechanisms (enzymatic mechanisms) is prerequisite for sensing the estrogen signal in postmitotic cells as well as cancer cells. Even in cells where estrogen can function to acutely downregulate the ERα transcription as represented by phase B, the ERα promoter is expected to be in unmethylated status. In other words, estrogen-sensing cells have learned to establish the unmethylated status in the ERα promoter. On the other hand, once DNA methylation occurs at the ERα promoter during the course of development or aging as represented by phases C and D, it seems very hard to remove this methylation. Accordingly, defining the cells represented by the phase A would greatly accelerate clarification of the mechanism of brain masculinization.

Differential Impact of AR Expression Setting Depending on Cells and Species

In many androgen target tissues, androgens promote downregulation of AR mRNA levels (Hackenberg et al., 1992; Krongrad et al., 1991; Quarmby et al., 1990; Shan et al., 1990; Wolf et al., 1993). The amount of AR mRNA has been shown to increase with androgen withdrawal and to decrease below control levels after androgen stimulation in rat ventral prostate, coagulating gland, epididymis, seminal vesicle, kidney, and brain, and in several prostate cancer cell lines. In the case of the rat ventral prostate, AR mRNA increased within 24 h after castration and remained elevated for 4 days (Quarmby et al., 1990). TP treatment beginning 24 h after this castration reduced ventral prostate AR mRNA within 8 h. In this case, E2 administration after castration had no significant effect on prostatic AR mRNA. Although the general tendency is androgen-mediated downregulation of AR, androgenic upregulation of AR mRNA has also been observed in a few tissues (Antonio et al., 1999; Gonzalez-Cadavid et al., 1993; Kerr et al., 1995; Khetawat et al., 2000; Nastiuk and Clayton, 1994; Wiren et al., 1997), which is reminiscent of the diversified autoregulation of ERα by estrogen. Administration of a non-aromatizable androgen, dihydrotestosterone (DHT), to castrated male rats has been shown to upregulate AR levels in the bulbocavernosus and levator ani muscles (Antonio et al., 1999). In humans, megakaryocyte and erythroleukemia AR expression is upregulated dose-dependently by T (up to 10 nmol/L), but downregulated by a much higher level of T (100 nmo/L) (Khetawat et al., 2000). Treatment of osteoblastic cells with DHT increased AR mRNA steadily in a time- and dose-dependent fashion (Wiren et al., 1997). Reporter assays with the proximal 5'-flanking region of the human AR promoter reproduced this effect of DHT on RNA

expression (Wiren et al., 1997).

In the brain, AR mRNA expression occurs predominantly in the hypothalamus. The highest density of AR mRNA is localized in the central part of the MPOA and the principal portion of the BNST (Handa et al., 1996). Castration of adult male rats causes an increase in AR mRNA density in both brain areas. Therefore, the negative regulation of the expression of AR by androgen seems to be largely restricted to the predominant area of AR function. However, a situation similar to that of the differential autoregulation of ERα together with estrogen could again be observed for AR. In fact, comparable levels of AR mRNA can be found in specific cells of the hippocampus. CA1 pyramidal cells are AR-positive and form the major signal output of the hippocampal trisynaptic circuit. A significant decrease occurs in the AR mRNA content of the hippocampus in rats after castration or in intact male rats after daily injections of the AR antagonist flutamide (Kerr et al., 1995).

It has been proposed that methylation of CpG sites in the AR promoter may influence the long-term but reversible inactivation of transcription of the AR gene in androgen-independent metastatic prostate cancers (Jarrard et al., 1998; Kinoshita et al., 2000). Normal prostate epithelial cell strains showed no DNA methylation of the AR gene. In contrast, increased methylation was seen in the AR expression-negative cell lines Du145, DuPro, TSU-PR1, and PPC1, as well as in normal female breast and ovarian tissues. Exposure of AR-negative prostate cancer cell lines to 5-aza-dC induced the reexpression of AR mRNA in AR expression-negative cell lines (Jarrard et al., 1998).

Interestingly, in the male and female mouse brain cortex, methylation of a few CG sites in the AR core promoter has been shown to be increased by TP, but decreased by E2 (Kumar and Thakur, 2004a). These sex steroid hormones concomitantly affected DNase I accessibility to the AR core promoter (Kumar and Thakur, 2004b), suggesting that setting of the chromatin structure on the AR promoter occurs with ERα and AR. In fact, in the adult male rat, androgen and estrogen act synergistically in the regulation of male reproductive behaviors (Baum, 1979; Feder et al., 1974; Morali et al., 1977). In the MPOA and BNST of the adult male rat, AR and ERα mRNAs have been found to be distributed in unique but overlapping patterns (Handa et al., 1996). In this case, treatment of castrated adult males with DHT reversed the effects of castration on AR mRNA in both the short- and long-term castrated animals, but had no effect on ERα mRNA in either of these brain nuclei, whereas EB treatment increased AR mRNA in the long-term castrates only and decreased ERα mRNA in both long- and short-term castrates. This suggests a complex regulation of AR in specific brain regions. So far, little information is available about the DNA methylation pattern on the AR as well as the ERα gene after the short- and long-term androgen exposure or removal. Nonetheless, AR has been shown to regulate the masculinization of the mouse brain (Sato et al., 2004). Furthermore, several lines of pharmacological evidence have suggested that AR is the predominant target for androgen-triggered brain masculinization in some species. In rhesus monkeys, prenatal administration of the nonaromatizable androgen DHT to females caused coital masculinization as readily as T (Pomerantz et al., 1986). In a carnivore, the ferret, neonatal exposure to T, but not its metabolites E2 or DHT, caused

coital masculinization (Baum, 1976; Baum et al., 1983; Baum et al., 1982). Therefore, in addition to the setting of the chromatin modifications on ERα, that on AR by androgen and estrogen could cause comparable impacts on brain masculinization.

Concluding Remarks

Nuclear estrogen and androgen signaling pathways are very complex. Diversified autoregulatory loops of ERα or AR caused by its direct interaction with estrogen or androgen further make it difficult to understand the whole picture of sex steroid signaling. Epigenetic modifications, especially DNA methylation of the ERα and AR gene regions, could partially explain the highly irreversible changes of cell responsiveness to sex steroid hormones in mammals. Local epigenetic engineering of ERα and AR, if it can be achieved, could be used to determine the exact degree of the contribution of DNA methylation to cell fate specification during development and aging in future studies (Imamura et al., 2004). This would be especially important for making the next breakthrough in understanding the irreversibility of sex-dependent behaviors.

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Figure Legends

Figure 1. ERα target gene regulation by chromatin setting through ERE-ERα interactions.

In this model, E2 alters the chromatin structure by E2-ERα association with the estrogen responsive element (ERE). On the ERE-ERα complex, several cofactors are recruited to concordantly establish the highly ordered chromatin structure. The E2 effect is exerted on many genes within an ERE-containing cluster. Genes proximal to the ERE tend to show rapid upregulation whereas expression of distal genes occurs later on. The ERE effect could be restricted by insulators to prevent changes of the expression of genes located outside of the cluster.

Figure 2. Cells with differential ERα expression setting by estrogen and DNA methylation during development and senescence.

Panel A indicates a cell in which E2 starts being incorporated at early developmental stages, such as the perinatal stage of mice corresponding to the critical period of brain masculinization. ERα could enter into the nucleus to upregulate the ERα mRNA expression. After a number of cycles of synergistic increase of ERα by an increase of circulating E2, E2-ERα association with ERα mRNA occurs in the cytoplasm. This kind of blockage and other biochemical signals could prevent the entry of E2-ERα into the nucleus, as shown in panel B. These negative regulations of ERα mRNA expression allow the stable existence of ERα in a cell. Panel C represents an aged cell in which

overall DNA methylation activities, represented by hexagons, are diminishing. Decrease of the circulating E2 is accompanied by senescence. Long-term decrease of E2 and the resultant reduced content of ERα could be sensed by the cell, which would as a result change the DNA methylation status of the ERα promoter from hypomethylated (open lollipops) to hypermethylated (filled lollipops) according to the lack of need for ERα even if the overall DNA methylation level were lowered. In contrast to the senescence stage, developmental stage cells contain much higher DNA methylation activities which react with genes as well as heterochromatin regions. If a cell is not exposed to E2, there is no chance of ERα expression, leading to long-term repression by DNA methylation and other modifications reminiscent of the closed chromatin structure, as shown in panel D.

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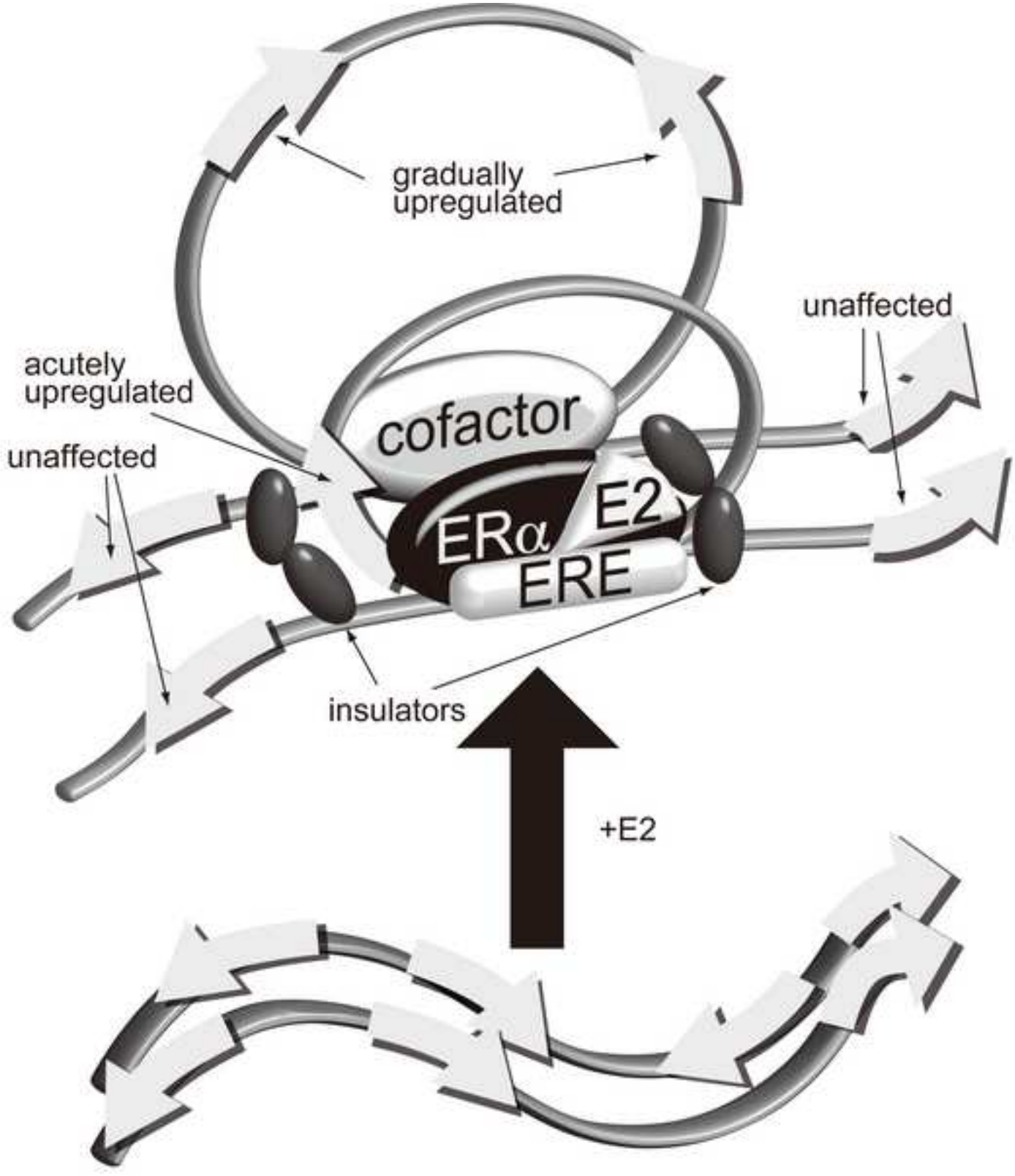

Figure 1

5-Figure(s)
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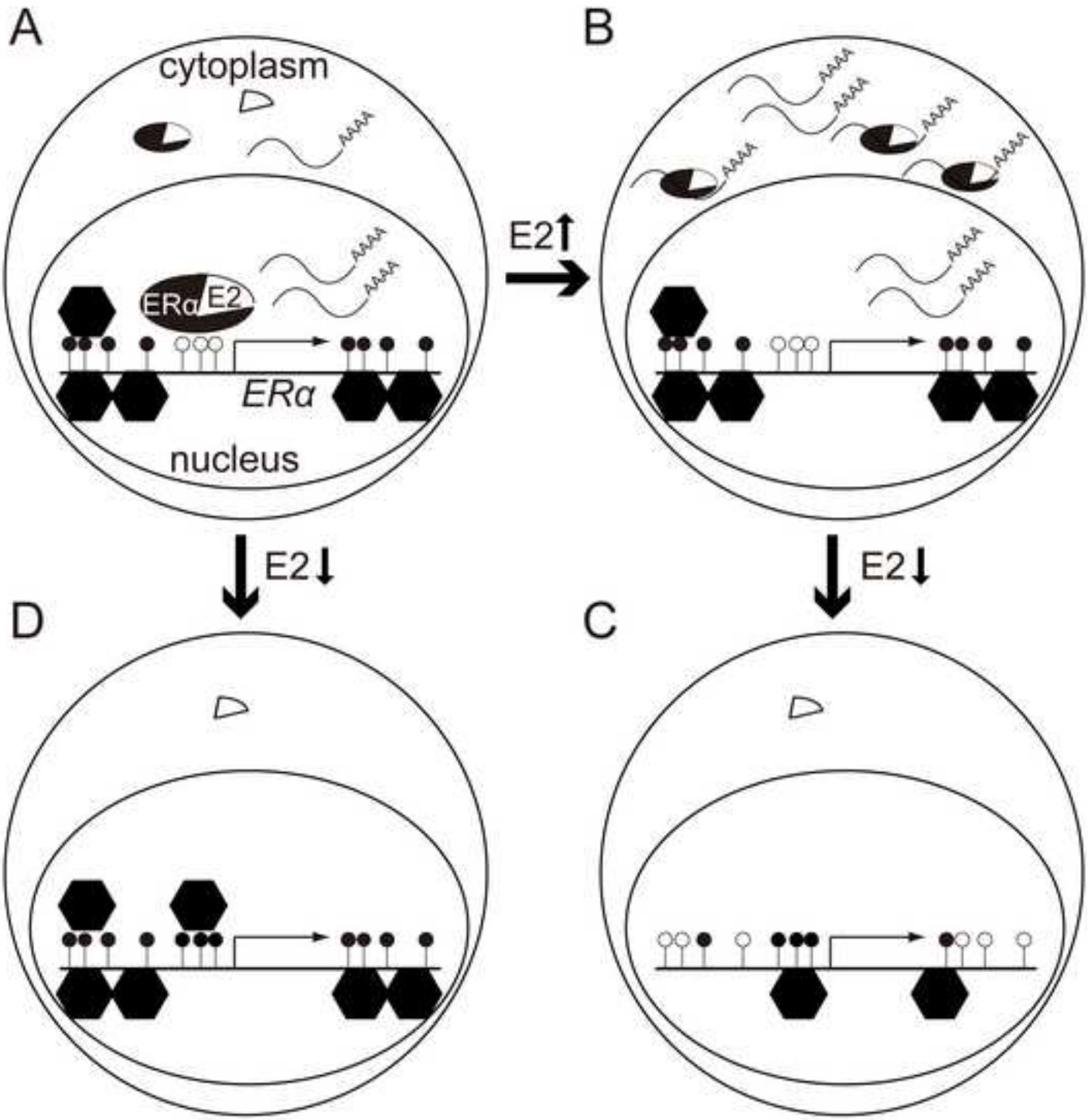

Figure 2