—Review—

# Sex steroid hormones and related substances involved in primordial follicle activation

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***Abstract:*** Primordial follicles maintain dormancy in the ovary and only a small number of them are activated towards ovulation every day. Several signaling pathways in oocytes and the surrounding flattened pre-granulosa cells have been shown to play crucial roles in primordial follicle activations and sex steroid hormones are also known to affect it. Intrinsic estrogen, estradiol, has been reported to suppress primordial follicle formation and the later primordial follicle activation in rodent neonatal ovaries. Conversely, some phytoestrogens and endocrine disruptors which possess intrinsic estrogen-like biological activity with different binding affinities to estrogen receptors, can activate primordial follicles. Testosterone and dehydroepiandrosterone have been used in fertility treatments in the expectation that they would activate primordial follicle, although evidence of their efficacy is inconclusive. Progesterone suppresses primordial follicle formation and the later primordial follicle activation in rodent neonatal ovaries. Synthetic progestins possess the ability to bind to steroid hormone receptors other than the progesterone receptor. Thus, progestins may regulate primordial follicle activation through other sex hormone receptors. It may be possible to regulate primordial follicle activation by sex steroid hormones in the future. However, it is not yet clear which pathway mediates the effect of these hormones on primordial follicle activation, and this will need to be studied in the future.

***Key words:*** Androgen, Estrogen, Primordial follicle activation, Progestogen

## Introduction

Ovaries play roles in the supply of oocytes and sex steroid hormones in reproduction [1, 2]. These roles can continue over three decades in humans as oocytes are stocked as dormant primordial follicles, which provide females with a constant stream of growing follicles. In the ovary, the majority of primordial follicles maintain dormancy while a small number undergo gradual development towards ovulation. This initiation of follicle growth is defined by primordial follicle activation. Primordial follicle activation is thought to proceed when the primordial-to-primary transition can be noted morphologically, when flattened granulosa cells proliferate and become cuboidal. However, the real process of primordial follicle activation is bidirectional and involves both the differentiation of flattened granulosa cells and the robust growth of dormant oocytes. Several signaling pathways in oocytes and flattened granulosa cells have roles in the regulation of primordial follicle activation. Several oocyte-specific transcription factors such as the phosphatidylinositol 3 kinase (PI3 K) pathway [3, 4], the mechanistic target of the rapamycin complex 1 (mTORC1) pathway [5, 6], growth differentiation factor (GDF) 9 [7, 8], and transforming growth factor (TGF) $\beta$ [9], as well as granulosa cell secreted factors such as Kit ligand (stem cell factor) and mTORC1 have been shown to regulate the activation of dormant primordial follicles [10, 11].

Sex steroid hormones have also been studied with regard to their effects on primordial follicle activation [12–14] and androgens are already being used clinically with the expectation of fertility promotion [15–17].

Moreover, phytoestrogens and endocrine-disrupting chemicals, which possess intrinsic estrogen-like biological activity, have been reported to have effects on the stockpile of oocytes and fertility [18–21].

In this article, the effect of sex steroid hormones, including synthetic hormone compounds, phytoestrogens and endocrine-disrupting chemicals on primordial follicle activation is reviewed and discussed in order to provide insight into the fertility of women. The detailed molecular mechanisms of primordial follicle activation [1, 2] and

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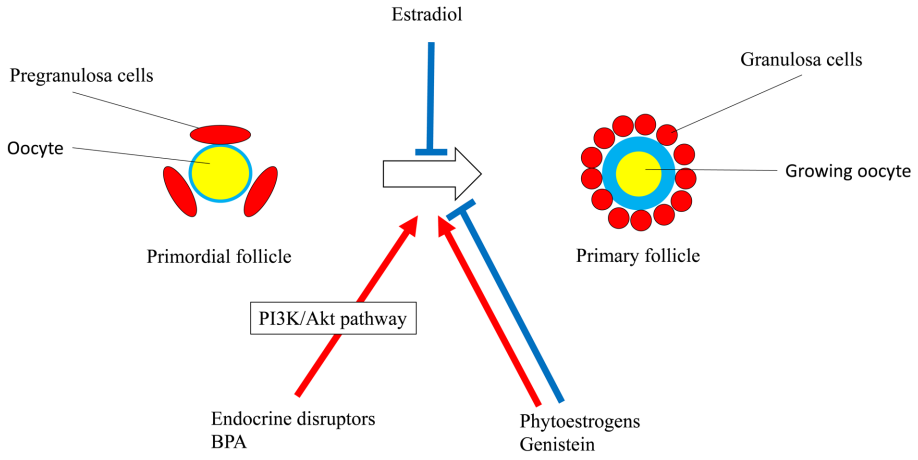


**Fig. 1.** Estrogens and their related substances involved in primordial follicle activation. Phytoestrogens such as BPA affects primordial follicle activation with a possibility that the action may be opposite by the exposure amount. Endocrine disruptors may activate primordial follicles through the activation of PI3 K/Akt pathway although estradiol may suppress this activation.

their clinical applications such as *in vitro* activation (IVA) is reviewed in other articles [22, 23].

## Estrogen's Involvement in Primordial Follicle Activation

Estrogen (s) act by binding to estrogen receptors (ERs), localized within the nucleus and/or on the cell membrane [24, 25]. Approximately 90–95% of human ERs are localized within the cell nucleus with a few located on the plasma membrane (membrane ERs; mERs). mERs activate cell signaling via modulation of intracellular signaling cascades. Nuclear ERs act as transcriptional factors and are further subdivided into two types, ERα and ER*β* [26, 27]. These two receptors, encoded by two distinct genes, are localized within different tissues [28, 29]. ERα is mainly expressed in the uterus and breast, and ER*β* is expressed in a variety of organs in mice and humans [27, 30].

mERs include two nuclear estrogen receptors (ERα, ER*β*), which are located on the plasma membrane, as well as membrane specific receptors, G protein coupled receptor 30 (GPR 30, also called GPER), ERx, ER-X, and Gq-mER [24, 25, 31]. These membrane receptors mediate the non-genomic action of estrogen. Estradiol binds GPR30, resulting in intracellular calcium mobilization and synthesis of phosphatidylinositol (3, 4, 5)-trisphosphate (PI3) in the nucleus in humans [32, 33].

ER-X shows sequence homology with ERα and ER*β* and activates the MAPK/ERK pathway in frogs and rodents [34]. ERx and Gq-mER are members of the membrane estrogen receptor family and little is known about these receptors.

ERα is expressed at higher levels in granulosa cells of growing follicles than ER*β*, while ERα is expressed in thecal and interstitial cells in humans [35, 36]. Primordial, primary and some secondary follicles express mRNAs corresponding to ERα and *β*. Both ERα and *β* are localized in oocytes in rodents and humans [35, 36].

Theca cells and oocytes possessed significant levels of GPR30 in rodents and humans [37, 38], and the expression of GPR30 is greater in mature follicles than in primordial follicles [37–39].

The effects of estrogens and the related substances on primordial follicle activation are shown in Fig. 1. The aromatase knockout ($Ar^{-/-}$) mouse, which does not synthesize endogenous estrogens, has relatively larger oocyte within primordial follicles as well as increased GDF9 mRNA expression compared to the wild-type (WT) mouse [12]. Treating $Ar^{-/-}$ mice with estradiol led to a reduction in oocyte diameter, indicating that estradiol may suppress the growth of oocytes during primordial follicle activation. However, no differences were observed between $Ar^{-/-}$ and WT mice in the expressions of Müllerian-inhibiting substance (MIS, anti-Müllerian hormone), Wilms tumor 1 (WT1) protein or proliferating cell nuclear antigen (PCNA) in primordial and primary follicles. Treatment of $Ar^{-/-}$ mice with estradiol has no effect on early follicle numbers. Thus, it appears that estradiol has a role in suppressing the differentiation and growth of oocytes

at the primordial follicle stage, while not affecting pregranulosa cell differentiation of primordial follicles, resulting in no difference seen in the rate of primordial follicle transition to primary follicle.

When newborn rat ovaries were cultured for 7 days, the rate of primordial follicle transition to the primary follicle was found to be 3 times greater *in vitro* than *in vivo* [40]. Addition of estradiol to the medium significantly reduced the rate of primordial to primary follicle transition. Approximately 30% of follicles underwent primordial to primary follicle transition in estradiol treated ovaries compared to 60% in control ovaries. Therefore, estradiol treatment resulted in greater numbers of primordial follicles and reduced the number of primary follicles. However, estradiol had no effect on the primordial to primary follicle transition in ovaries collected at postnatal day 4, suggesting estradiol delay germ cell nest breakdown, or its effects are restricted to the initial stages of primordial follicle pool formation.

These results indicate that estradiol might suppress primordial follicle formation and the later primordial follicle activation. Thus, its effect may be stage specific and not essential to the completion of primordial follicle activation.

Ovarian-derived estrogens are not the only compounds that activate ERs. Plant compounds such as phytoestrogens have intrinsic estrogen-like biological activity, mainly due to the presence of a phenolic A ring, a crucial factor for receptor binding [41, 42]. Soy protein contains phytoestrogens such as isoflavones, genistein and daidzein. Phytoestrogens are believed to signal predominantly via ER*β*. Genistein, in particular, has a 20-fold higher binding affinity for ER*β* than ERα [43].

Genistein exposure has been reported to alter early follicular development in rodents *in vivo* [18]. Genistein exposure of 50 mg/kg decreased the numbers of healthy primordial, primary and secondary follicles, and increased the number of antral follicles in 18-day-old Wistar rats, suggesting that genistein administration augments primordial follicle activation and accelerates follicle development. However, in the same study genistein increased the number of atretic secondary and antral follicles, indicating that the addition of genistein may not produce viable oocytes.

Another study reported that 160 mg/kg of genistein administration increased the number of primordial follicles and decreased antral follicle numbers in 3-month-old Sprague-Dawley rats, indicating that genistein suppresses primordial follicle activation and reduces the growing follicle pool [19]. The use of rat strains, the animal's ages or genistein dosage might explain the differences between these two studies.

Endocrine disruptors are a byproduct synthesized during the production of chemical substances, and they interfere with endocrine (or hormone) systems even at relatively low concentrations [44–46]. Endocrine disruptors can cause reproductive disorders, birth defects and other developmental disorders, and hormone disruptors can impair any system in the body that is regulated by hormones [44–46].

It is known that everyone is exposed to endocrine disruptors in daily life, as they have been detected in low concentrations in numerous products. Chemicals commonly detected include dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCB's), bisphenol A (BPA) et cetera [44–49]. BPA is discussed below, as a representative endocrine disruptor.

BPA has been reported to disrupt neonatal follicular development in rodents. Administration of BPA (20 mg/kg) to neonatal stage rats from postnatal day 1 (PND1) to PND7 resulted in fewer primordial follicles and increased the numbers of growing follicles [20]. Oocyte survival and apoptosis did not differ between BPA-treated and control mice. Primordial and recruited follicles showed relatively higher p27 levels, whereas ER*β* and cellular proliferation increased in recruited follicles. ERα-positive primary follicles increased in BPA treated mouse ovaries compared to controls. These results suggested that BPA reduces the primordial follicle pool by primordial follicle activation during the neonatal stage.

The number of primordial follicles was reduced, and the number of primary follicles increased in 4-day-old mouse ovaries cultured with BPA for 10 days [21]. The concentration of BPA used (0.1 *μ*M) was equivalent to the physiological concentration in children's blood. The reduction in primordial follicles was due to increased follicle activation but not apoptosis, as determined by immunohistochemistry for Ki-67 and active caspase-3. BPA treatment induced upregulation of the PI3 K/Akt pathway, which was reversed by concomitant treatment with PI3 K inhibitor. The BPA-induced primordial follicle activation involved the PI3 K/Akt pathway.

The phosphatase and tensin homologue (PTEN) is a negative regulator of PI3 K [1, 2]. BPA is known to induce proliferation in breast cancer cell lines through dysregulation of the PTEN/serine/threonine kinase/p53 axis [50]. Administration of BPA downregulated PTEN expression and induced excessive activation of primordial follicles in the ovaries of 6-week-old female CD-1 mice [51]. This effect was partially reversed by PTEN overexpression through gene transfection using an adenoviral vector. Thus, it appears that BPA induces excessive primordial

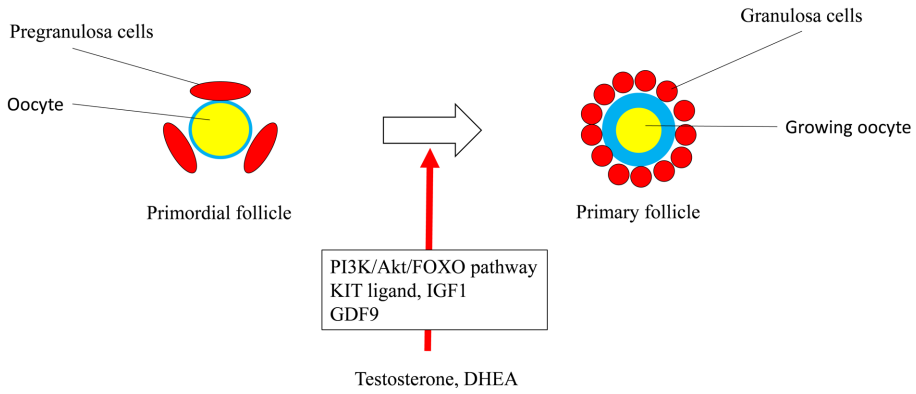


**Fig. 2.** Androgens in primordial follicle activation. Androgens, testosterone and DHEA, promote primordial follicle activation through activation of PI3 K/Akt/FOXO pathway and/or increased stimulation of KIT ligand, IGF1 and GDF9.

follicle activation mainly through suppression of PTEN.

As noted above, BPA is known as a substance impacting reproduction in rodents. The effects of BPA exposure on human ovarian reserve have also been studied. A prospective study of women with infertility and polycystic ovarian disease reported that high levels of BPA in urine were associated with low antral follicle counts [52, 53]. The association between urinary BPA concentrations and ovarian response in women undergoing IVF has been also investigated. BPA was detected in the urine of the majority of women undergoing IVF and was inversely associated with the number of oocytes retrieved and peak estradiol levels [52]. One of the reasons offered to explain these results was earlier follicular depletion due to accelerated primordial follicle activation induced by BPA [52].

Taken together, phytoestrogens and endocrine disruptors may activate primordial follicles in rodents, and endocrine disruptors may also activate depletion of the primordial follicle pool in humans, whereas intrinsic estrogen, estradiol, suppresses primordial follicle pool formation and the later primordial follicle activation in neonatal rats. Although the intracellular actions of estrogens through a variety of ERs are gradually being elucidated, the precise mechanisms of the modulation of primordial follicle activation by estrogens are not yet clearly understood.

## Androgens' Involvement in Primordial Follicle Activation

Androgen (s) act by binding to androgen receptors (ARs) [54]. Two isoforms of ARs (A and B) have been identified as nuclear AR [55]. AR-A is formed by *in vitro* proteolysis of AR-B. Nuclear ARs act as transcriptional factors [54]. Similar to estrogen receptors, ARs are also localized on cell membrane [56]. Cell surface receptors, membrane ARs (mARs), rapidly alter cell signaling via modulation of intracellular signaling cascades [56, 57]. The mARs include ZIP9 and GPRC6A. ARs are expressed in all cells forming follicles, including oocyte, granulosa and theca cells [58]. In bovine, non-human primates and humans, ARs are expressed in connective tissue and cortical stroma [58–60]. In rodents, primates and humans, an increase in the expression of AR was detected in granulosa cells from primary follicles and it peaked at the antral stage [58–60]. The existence and roles of ZIP9 and GPRC6A in mammalian ovaries remains unknown [61].

The crucial role of granulosa cell ARs has been demonstrated using the phenotype of the AR knockout mice. This phenotype is subfertile, with reduced follicle progression, fewer ovulations, and a smaller litter size [58].

The effects of androgens on primordial follicle activation are shown in Fig. 2. Testosterone rapidly increased the intra-oocyte PI3 K/Akt/ Forkhead box (FOXO) 3 pathway in mouse primordial follicles, with a 2-fold increase in the ratio of primary to primordial follicles compared to control mice [14]. The number of primary follicles increased via the phosphatidylinositol 3-kinase/Akt pathway after 10 days of culture with testosterone. Androgen-induced FOXO3 activation and translocation of the FOXO3 protein from the oocyte nuclei to the cytoplasm may be a crucial step for primordial follicle activation. Testosterone is also capable of down-regulating GDF9 expression via its receptor. Androgen exposure resulted in excess early

follicular development by inducing FOXO3 translocation in oocytes and follicular arrest due to down-regulating GDF9 expression.

The number of primary follicles significantly increased in female rhesus monkeys, following treatment with testosterone or dihydrotestosterone (DHT) for 3–10 days, compared to controls. Androgen treatment resulted in a 3-fold increase in the expression of insulin-like growth factor I (IGF-I) mRNA and a 5-fold increase in IGF-I receptor mRNA in primordial follicle oocytes. The effect of DHT was identical to testosterone, indicating that its effects are androgen receptor-mediated. These findings suggest that androgens promote primordial follicle activation and that oocyte-derived IGF-I plays a role in this activation process [13, 62].

In pigs, primordial to primary follicle transition occurs during late pregnancy. Injecting pregnant pigs on gestation days 83 (GD90 group) or 101 (GD108 group) with 50 mg/kg of flutamide, an anti-androgen, increased the ratio of primordial to primary follicles, compared to controls. The levels of c-Kit and KL mRNA expression were diminished in the GD90 group. Insulin like growth factor 1 (IGF1) mRNA expression increased in both groups, whereas IGF1 receptor (IGFR) mRNA expression decreased. AR signaling promoted primordial follicle activation via KL and c-Kit as well as IGF1 and IGF1R [63].

These results suggest that androgens might augment primordial follicle to primary follicle transition via upregulation of several pathways.

In clinical practice, the oral ingestion of dehydroepiandrosterone (DHEA) and dermal testosterone patches have been used prior to oocyte retrieval during IVF.

DHEA is an endogenous steroid, produced in ovarian theca cells and the adrenal cortex. It promotes follicular development and granulosa cell proliferation by increasing the intraovarian androgen concentration [64, 65]. Recently, patients with diminished ovarian reserve or diagnosed as poor responders have been treated with DHEA [64, 66–68].

A randomized, double-blinded, placebo-controlled trial revealed the antral follicle count (AFC) was larger after patients had ingested DHEA for 12 or 20 weeks, although no significant differences in serum levels of MIS and FSH were observed [15]. The levels of serum testosterone, DHEA sulfate and estradiol were significantly higher in the DHEA group.

On the other hand, another randomized, double-blind, placebo-controlled study showed that ingestion of DHEA for 12 weeks prior to IVF treatment in patients with normal ovarian reserve led to significantly higher serum and follicular DHEA-sulfate and testosterone levels [64]. However, there were no significant differences in AFC, MIS and FSH levels nor in ovarian response to ovarian stimulation and outcomes following IVF treatment.

The Cochrane Database of Systematic Reviews published the results of a meta-analysis on the efficacy of DHEA and testosterone treatments for patients diagnosed as poor responders who were treated using standard IVF protocols [69]. A significance relationship was found between pretreatment with DHEA and higher live birth rates or ongoing pregnancy rates (OR 1.88, 95% CI 1.30 to 2.71). However, when trials with performance bias were excluded, the results were no longer significant (OR 1.50, 95% CI 0.88 to 2.56). Pretreatment with testosterone also showed a relationship with higher live birth rates (OR 2.60, 95% CI 1.30 to 5.20). However, when trials with performance bias were excluded, similarly, the results were not significant (OR 2.00, 95% CI 0.17 to 23.49).

Pretreatment with DHEA or testosterone may improve live birth rates for women undergoing IVF treatment and poor responders. Although the evidence is inconclusive with respect to the effect of androgen usage on the clinical outcome, DHEA treatment is easy and inexpensive. A recent international survey revealed that 26% of IVF clinicians used DHEA as an adjuvant therapy with IVF treatment protocols for women with diminished ovarian reserve or who were poor responders [17].

Taken together, supplementation with DHEA or testosterone may improve the clinical outcomes of poor responders during IVF treatment, however, their use is still controversial. It is possible that they temporarily accelerate the activation of primordial follicles. Nevertheless, the efficacy of clinical androgen usage needs further study.

## Progestogen Involvement in Primordial Follicle Activation

Progestogens mainly act through binding to progesterone receptors (PRs) although progestin, synthetic progestogen, binds to other steroid hormone receptors including ER, AR, glucocorticoid receptor and mineralocorticoid receptor, which act as agonists and/or antagonists. Similar to ERs, PRs are localized not only in the nucleus but also on the cell membrane. Nuclear PRs act as transcriptional factors and can be further subdivided into three types, PRA, PRB and PRC [70–72]. Both PRA and PRB have been shown to be transcribed by the same gene. After post-transcriptional modification, truncated PRB becomes PRA [73]. PRC is the shortest isoform and has no transcriptional activity [71, 74]. Membrane PRs (mPRs) have also been identified in humans, as well as

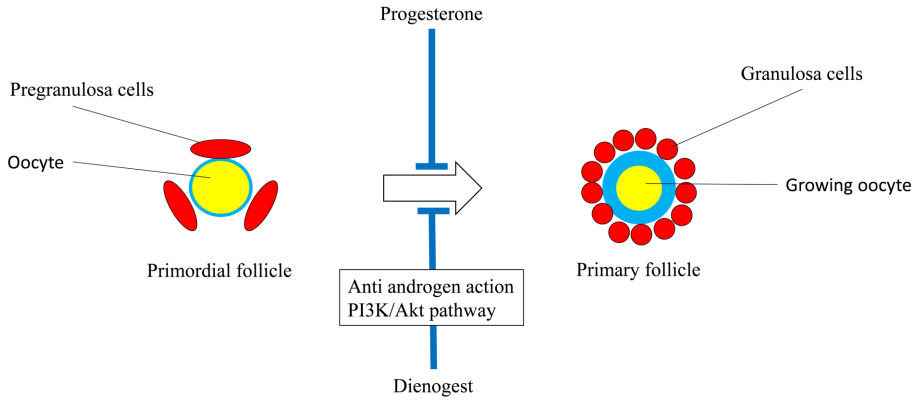


**Fig. 3.** Progestogens including synthetic progestin involved in primordial follicle activation. Synthetic progestins bind to steroid hormone receptors other than the androgen receptor, resulting in promoting or suppressing primordial follicle activation through a variety of the sex hormone receptors, although progesterone suppresses the primordial follicle activation. Diengest suppresses primoradial follicle activation through suppressed PI3 K/Akt pathway by anti-androgen actions.

mERs, and three are known: mPRα, mPR$\beta$ and mPR$\gamma$ [75, 76]. PR membrane component 1 (PGRMC1) is another mediator of progesterone action [77].

Granulosa cells do not express PRA or PRB in developing follicles of mice, rats, monkeys and humans, although both PRA and PRB are expressed in preovulatory follicle granulosa cells [78, 79]. There is no evidence of the presence of these receptors on oocytes [80, 81]. PGRMC1 is highly expressed in granulosa cells of developing follicles and luteal cells. The presence of PGRMC1 has been shown in germinal vesicle (GV) and metaphase II (MII) human oocytes [82].

The effects of progestogen on primordial follicle activation are shown in Fig. 3. When newborn rat ovaries are cultured in the presence of progesterone, the numbers of primordial follicles increase, whilst primary follicle numbers decrease. These results indicate that progesterone suppresses primordial follicle pool formation during germ cell nest breakdown and the later activation of primordial follicles, although the effects may be restricted to the stages during or just after the primordial follicle pool is formed in neonate [40]. Our group recently showed that a fourth-generation progestin possessed anti-androgen action. Dienogest was administered to female ICR mice (100 days old) for 60 days [83]. The mice treated with dienogest had more offspring and larger litter sizes than control mice. Greater numbers of primordial follicles were detected in mice ovaries at 4 and 80 days after the final dienogest administration. Higher serum levels of MIS were also detected 80 days after the final dienogest administration. The ratio of primary to primordial follicles decreased in 3-day-old newborn ovaries that had been cultured for 4 days with dienogest (10–7, 10–6 and 10–5 mol/L). These results indicate that dienogest suppresses the activation of primordial follicles during its administration and preserves the primordial follicle stockpile and subsequent fertility of mice.

Taken together, progesterone suppresses primordial follicle pool formation and the later primordial follicle activation, although the effect may be restricted to a specific stage. Synthetic progesterone may modify the activation of primordial follicles by binding to steroid receptors other than PR. However, further study is needed to prove this.

## Conclusion

Sex steroid hormones may have effects on primordial follicle activation, which influences oocyte stockpile at the reproductive age. Phytoestrogens and endocrine-disrupting chemicals need to be avoided, as they may have detrimental effects on reproduction. On the other hand, testosterone and DHEA have been used in clinical practice with the expectation that they would activate primordial follicles, although evidence of their efficacy is not conclusive. Synthetic progestins may act diversely on the regulation of primordial follicle activation because of their abilities to bind to steroid hormone receptors other than PR. It may be possible to regulate primordial follicles activation by sex steroid hormones in the future. However, it is not yet clear which pathway mediates the effect of these hormones on primordial follicle activation, and further study is required.

## Conflict of Interest

None of the authors have an interest to declare.

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