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Are Estrogens of Import to Primate/Human Ovarian Folliculogenesis?*

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ABSTRACT

The notion that estrogens play a meaningful role in ovarian folliculogenesis stems from a large body of *in vitro* and *in vivo* experiments carried out in certain rodent models, (*e.g.*, rats) wherein the stimulatory role of estrogen on granulosa cell growth and differentiation is undisputed. However, evidence derived from these polyovulatory species may not be readily generalizable to the monoovulatory subhuman primates, let alone the human. Only recently, significant observations on the ovarian role(s) of estrogen have been reported for the primate/human. It is thus the objective of this communication to review the evidence for and against a role for estrogens in primate/human ovarian follicular development with an emphasis toward the application of the concepts so developed to contemporary reproductive physiology and to the practice of reproductive medicine. The role(s) of estrogens will be examined not only by analyzing the physiological evidence to the effect that these hormones control ovarian function and follicular growth, but also by summarizing the molecular evidence for the existence and distribution of the cognate receptors. (*Endocrine Reviews* **22:** 389–424, 2001)

I. Introduction

THE NOTION that estrogens play a meaningful role in ovarian folliculogenesis stems from a large body of *in vitro* and *in vivo* experiments carried out in certain rodent models (*e.g.*, rats) wherein the stimulatory role of estrogen on granulosa cell growth and differentiation is undisputed (1, 2). However, evidence derived from these polyovulatory species may not be readily generalizable to the monoovulatory subhuman primates, let alone the human. Only recently, significant observations on the ovarian role(s) of estrogen have been reported for the primate/human. It is thus the objective of this communication to review the evidence for and against a role for estrogens in primate/human ovarian

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follicular development with an emphasis toward the application of the concepts so developed to contemporary reproductive physiology and to the practice of reproductive medicine.

In this regard, the evidence relevant to the human will be compared to that of other species, to be presented in the first part of this review. The role(s) of estrogens will be examined not only by analyzing the physiological evidence to the effect that these hormones control ovarian cell function and follicular growth, but also by summarizing the molecular evidence for the existence and distribution of the cognate receptors. Based on cumulative data in several species, an operational model appears to be emerging, in large part due to the discovery of a new estrogen receptor (ER) type (*i.e.*, ERβ), the identification of human examples of specific mutations in enzymes of the steroidogenic pathway, as well as the generation of specific null mice mutants for the ERα, ERβ, and the aromatase genes.

It must be pointed out, however, that even though this review will question the role of estrogens in primate/human follicular development, one must remain cognizant of the unassailable extraovarian roles of estrogens in these species. Clearly, estrogens play a vital role in supporting the growth and differentiation of the Mullerian (3) and mammary (4, 5) complexes. Of equal import is the role played by estrogens in the synchronization of ovarian follicular development with the midcycle gonadotropin surge (6) and the preparation of the uterus for implantation (7–9). Somewhat less well characterized are the "nonreproductive" effects of estrogenic substances, which may include effects on the cardiovascular (10, 11) and skeletal (12, 13) systems. Intriguing research has also been conducted to describe the role of estrogens in the central nervous system wherein it appears that estrogens may play a physiological role in the modulation of mood, cognition, and behavior (14, 15).

II. The Nonprimate Ovary as a Site of Estrogen Reception (Table 1)

For the most part, it has been assumed that the ovarian actions of estrogens are mediated via classical nuclear ERs. Specific ovarian estrogen binding (documented by way of radioligand assays) was first shown to exist in the ovaries of intact or hypophysectomized immature rats (16–20). Hormone binding studies by several investigators also confirmed the presence of ERs in the ovaries of intact immature hamsters, rabbits, guinea pigs, and mice (17, 21, 22). Autoradiographic studies by Stumpf (23) demonstrated the preferential localization of silver grains over nuclei of granulosa

TABLE 1. The nonprimate ovary as a site of estrogen reception

Author/year	Species	Developmental stage	Imaging technology	Processing technology	ERα status	ERβ status
Kuiper *et al.* (1996)	Rat	Mature	ISH	N/A	Stroma	GC
Byers *et al.* (1997)	Rat	Immature Mature	ISH	FF	Low level throughout the ovary	GC CL
Saunders *et al.* (1997)	Rat	Mature	IHC	Paraffin	No signal detected	GC CL
Shughrue *et al.* (1998)	Rat	Mature	ISH	FF	GC Stroma	GC TC CL
Pelletier *et al.* (1999)	Rat	Mature	ISH/IHC	FF Paraffin	TC/GE Stroma	GC
Sar and Welsch (1999)	Rat	Neonatal (1 day) Postnatal (5–10 days) Immature (21–23 days) Mature	IHC	FF Paraffin	TC Stroma GE	GC
Fitzpatrick *et al.* (1999)	Rat	(17β Estradiol-primed) Immature	IHC	N/A	TC Stroma	GC
Mowa and Iwanaga (2000)	Rat	Mature	ISH	FF	TC Stroma GE	GC TC Stroma
Mowa and Iwanaga (2000)	Rat	Fetal (from 12 days) Postnatal (1–24 days)	ISH	FF	TC Stroma GE	GC TC Stroma
Wang *et al.* (2000)	Rat	Mature	ISH/IHC	Paraffin	GC/TC Stroma GE/Oocytes	GC CL GE (faint)
Bao *et al.* (2000)	Rat	Mature	ISH	FF	TC Stroma	GC TC
Fitzpatrick *et al.* (1999)	Mouse	(PMSG-primed) immature	IHC	N/A	TC Stroma	GC
Jefferson *et al.* (2000)	Mouse	Fetal Postnatal (5 day) Immature (26 day) Fetal (from 12 days) Postnatal (1–24 days)	IHC	Paraffin	TC Stroma	GC
Rosenfeld *et al.* (1999)	Cow	Mature	ISH/IHC	FF/paraffin	TC	GC/TC

FF, Fresh-frozen; GC, granulosa cell; CL, corpus luteum; ISH, *in situ* hybridization; TC, theca cell; N/A, not applicable/available; IHC, immunohistochemistry; GE, germinal epithelium.

cells after *in vivo* administration of [^{3}H]17β-estradiol, thereby suggesting that the site of action of estrogen in the rat ovarian follicle is the granulosa cell. Subsequent cellular localization studies confirmed the rat granulosa cell as a site of estrogen reception (16). More recently, Arakawa *et al.* (20) documented (using radioligand receptor assays) the presence of ERs in antral follicles of rats.

Although conventional radioligand receptor assays localized ERs to the granulosa cells of several species (see above), molecular probing of the mouse granulosa cell gave rise to some uncertainty as to whether or not the murine granulosa cell is a site of "classical" estrogen reception. Specifically, Hillier *et al.* (24) detected a 1.5-kb ERα-hybridizing mRNA in the mouse ovary much smaller than the classical 6.5-kb ERα mRNA. This mRNA species hybridized to probes specific for the steroid receptor binding regions of the mouse ERα (domains E and F) and was enhanced in granulosa cells *vs.* residual ovarian tissues. Wu *et al.* (25), in turn, reported ERα transcripts in the mouse oocyte as detected by RT-PCR.

Clemens and Richards (26) provided preliminary documentation of ERα transcripts in rat granulosa cells. Specifically, primers were designed to amplify a 517-bp fragment from exon 3 to exon 5 of the rat ER. After reverse transcription of granulosa cell RNA, PCR was performed in the presence of labeled nucleotides, thereby allowing the identification and quantification of the 517-bp product and the splice variants involving exons 3–5. Subject to these limitations, no significant ERα splice variants were detected. *In vivo* and *in vitro* studies revealed the granulosa cell ERα transcripts to be down-regulated by 17β-estradiol and even further reduced by the acute effects of the LH surge. As such, these findings provide a documentation of the existence of hormonally responsive ERα transcripts in rat granulosa cells.

While the ERα gene has been recognized since first cloned from the human breast cancer cell line MCF-7 in 1985 (27), the ERβ cDNA was only isolated in 1996. The initial tissue sources of ERβ transcripts were the rat prostate and ovary (28) and the human testis (29). After the cloning of ERβ in rats and humans (28–46), the same was accomplished for mice (47, 48), sheep (49), cows (50), and nonhuman primates (51). Ovarian expression studies of the ERβ gene have since been accomplished in rats (52–54), mice (55, 56), monkeys (57, 58), humans (59, 60), and, more recently, cows (50).

In rodent reproductive tissues, the ERβ gene has been shown to be predominantly expressed in the ovary (61–63). ERα transcripts, in turn, appear to predominate in the uterus, cervix, and vagina (53, 54, 64). Localization of ERβ transcripts to the rat ovary was convincingly demonstrated by both *in situ* hybridization and RT-PCR (28, 29, 56, 65–71). Although ERβ transcripts predominate in rat ovaries (68), both the ERα and ERβ genes have been shown to be expressed in the ovaries albeit mostly in distinct cellular compartments (54, 62, 65).

The expression of the ERα gene in the mouse ovary dates back to fetal life, while ERβ gene expression appears to be initiated at birth. The latter was shown to increase with age (55, 63), possibly due to the onset of follicular maturation. In the rat ovary, ERβ gene expression was apparent by fetal day 14, a sharp increase being noted during the first week of neonatal life (53).

In rat ovaries, ERβ proved highly localized to granulosa cells of healthy follicles, from the primary to the preovulatory stage (54, 62, 72) (Figs. 1 and 2). ERβ transcripts were also expressed in theca cells although more weakly when compared with their expression in granulosa cells (65). Some studies have described scattered ERβ gene expression in the ovarian stroma (65). The primordial follicle, the oocyte, and the germinal epithelium do not appear to be sites of ERβ expression (73).

ERα and ERβ transcripts do not display a complete nonoverlapping pattern of expression in the rat ovary (73). The ERα gene is expressed in granulosa cells albeit less abundantly (64, 66). When compared with the ERβ gene, the ERα gene is expressed mainly in theca and stroma cells (53, 54), as well as in the germinal epithelium (53, 54, 64). Primordial follicles, oocytes, and corpora lutea (54, 61) do not seem to express ERα transcripts (73).

Some studies suggested that ERβ gene expression in the rodent ovary may be under LH/hCG control (67). Indeed, preovulatory follicles, as well as newly formed corpora lutea of mice, express lower levels of ERβ transcripts, a phenomenon possibly due to the preovulatory LH surge (70) (Fig. 3). The ability of LH/hCG to down-regulate ERβ gene expression has also been evident in studies using cultured rat granulosa cells (67). Unlike the ERβ gene, ovarian ERα gene expression proved consistently uniform throughout the rat ovarian cycle. The pregnant rat ovary is a site of ERα reception (54, 69).

The precise role(s) of both ERs in rodent ovarian function has yet to be fully understood. The strong signals corresponding to ERβ mRNA in the mice/rats ovary, in contrast to relatively weak expression of ERα mRNA, may imply that the intraovarian effects of estrogen are primarily mediated by ERβ.

In summary, the ability of estrogens to influence the above mentioned activities in the rat ovary is due to the action of two receptors (α and β) described to date. The rodent granulosa cells contain predominantly, if not exclusively, the β-subtype, whereas the ERα subtype was detected mainly in the theca layer and in the interstitium. Although ERα and ERβ are not expressed in the rat ovary in a complete nonoverlapping pattern, the predominance of specific subtypes in different cellular compartments within the ovary suggests that each ER subtype may be responsible for distinct downstream activities in the cell types in which they are expressed.

Although not thoroughly characterized, porcine ovarian follicles express high-affinity nuclear estrogen binding sites in classical equilibrium binding assays with *in vitro* dissociation constants of approximately 0.6 to 1.1 nM (74, 75). There is little or no knowledge of specific subtype (α or β) expression and differential regulation of ERs in porcine theca or granulosa cells. However, estrogen exerts consistent actions on selected facets of follicle development and/or cytodifferentiation (see below).

III. The Nonprimate Ovary as a Site of Estrogen Action

A. The rat (Table 2) and mouse

In 1940, Richard I. Pencharz (76) reported on the ability of systemically administered diethylstilbestrol (DES) to pro-

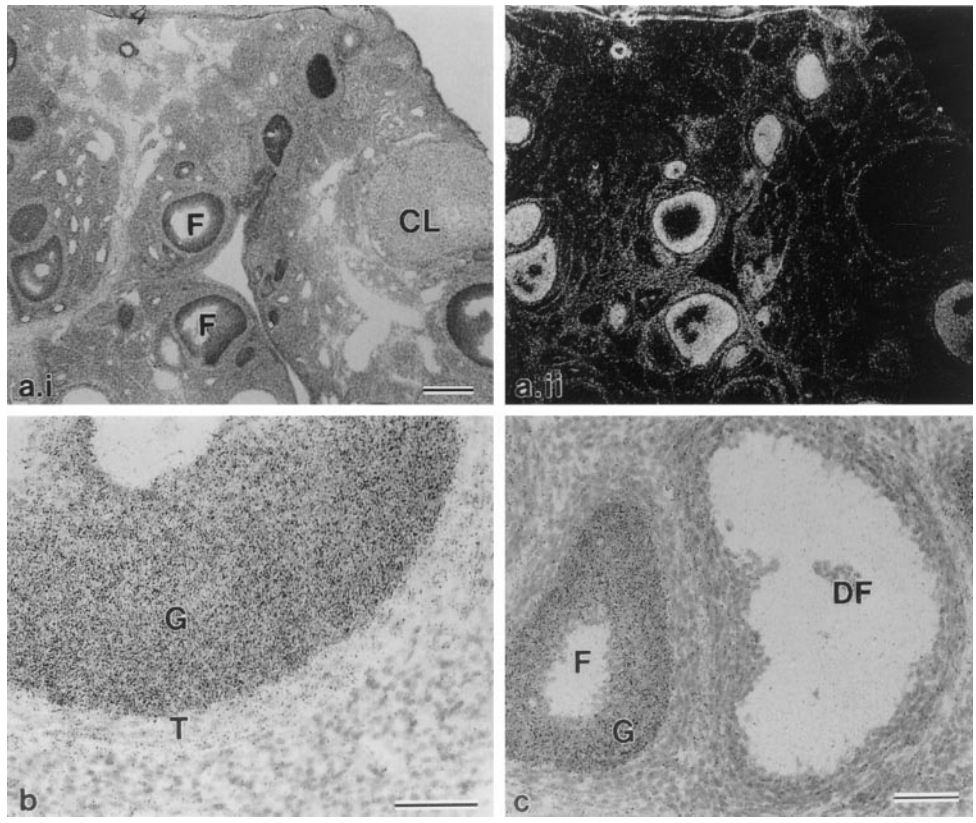

FIG. 1. Localization of ERβ mRNA in adult rat ovary during dioestrus. Bright- (a.i) and dark-field (a.ii) images show intense signal for ERβ mRNA in the granulosa of ovarian follicles (F) at different stages of development, but negative signals in the corpus luteum (CL). b, Pronounced expression of ERβ mRNA in granulosa cells (G) and moderate expression in theca cells (T) of healthy follicles. c, Expression of mRNA is not detectable in the granulosa cells of degenerating follicles (DF). *Bar* = 200 μm (a.i), 50 μm (b and c). [Reproduced with permission from Mowa and Iwanaga: *J Endocrinol* 165:59–66, 2000 (54). © the Society for Endocrinology.]

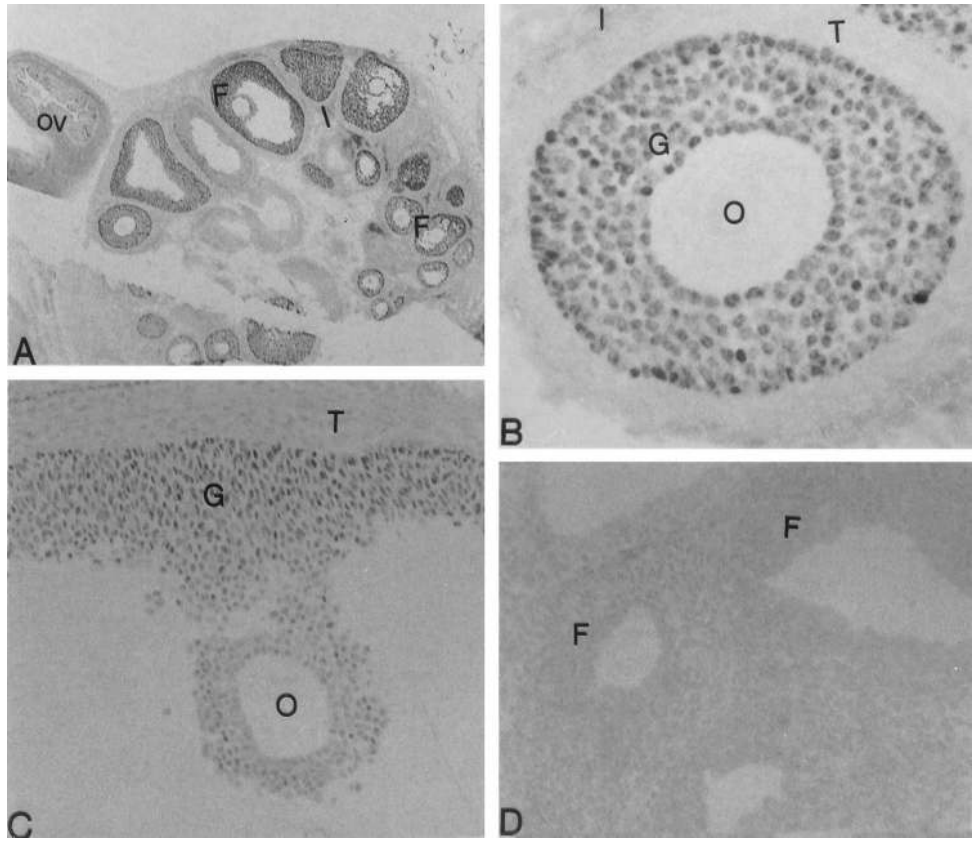

FIG. 2. Localization of ERβ in 23-day-old (A and B) and 60-day-old (C and D) rat ovary. Frozen sections of rat ovary were incubated with PAI P310 ERβ antibody (A–C) or preadsorbed ERβ antibody with the peptide immunogen (D). Note the immunostaining in follicles (F), specifically in nuclei of granulosa cells (G; panels B and C) and the lack of staining in theca cells (T), interstitial cells (I), and oocytes (O). No staining was observed when peptide-adsorbed antibody was used (panel D). Counterstained with hematoxylin; magnification, ×110 (A), ×800 (B), and ×440 (C and D). [Reproduced with permission from Sar and Welsch: *Endocrinology* 140:963–971, 1999 (73). © The Endocrine Society.]

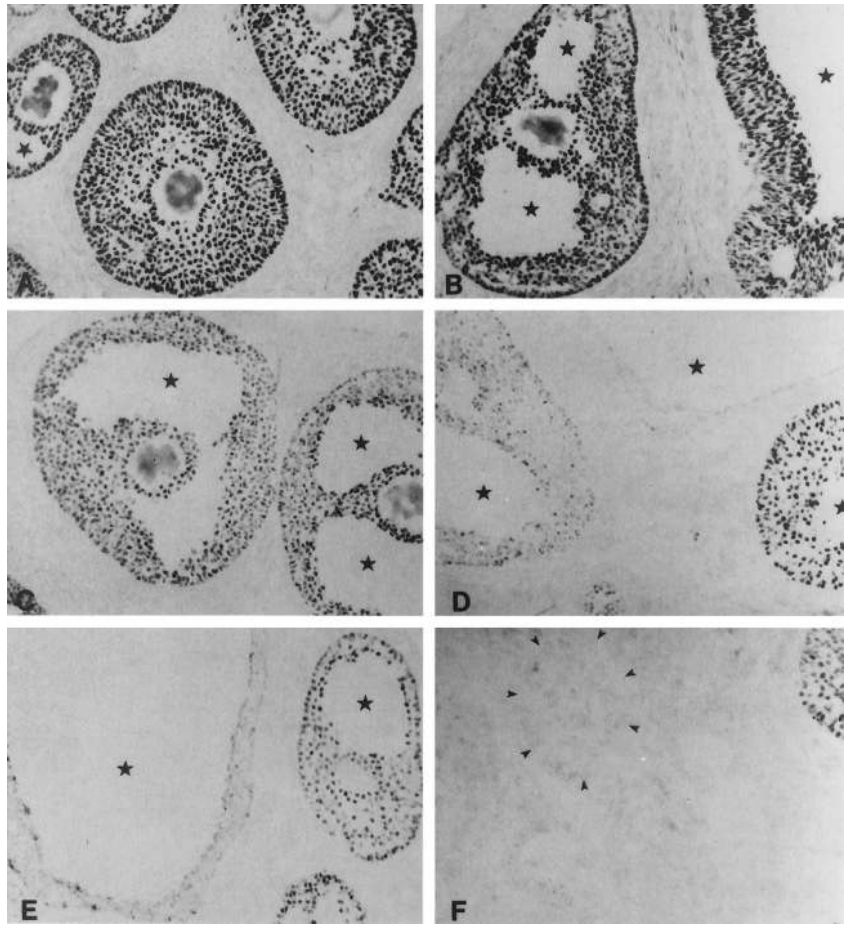

FIG. 3. Effect of hCG on protein ERβ expression using immunocytochemistry. Ovary sections from rats were incubated with an ERβ antiserum. ERβ protein was detected in granulosa cells of small and large antral follicles in vehicle-treated (A) and PMSG-treated (B) animals. *Star* indicates the location of antrum in antral follicles. PMSG-treated rats were injected with an ovulatory dose of hCG and ovaries were isolated after 3 h (C), 9 h (D), 12 h (E), or 24 h (F). The expression of ERβ protein in granulosa cells 9 h after hCG was reduced in large antral follicles (*left*, D), greatly reduced in Graafian follicles (*center*, D) but did not change in small antral (*right*, D) follicles. Similar expression was observed 12 h after hCG administration (E). One day (24 h) after hCG treatment, expression was not detected in corpora lutea (*small arrowheads*, F) but highly expressed in preantral (*right*, F) and small antral follicles. Magnification: A–F, ×250. [Reproduced with permission from S. L. Fitzpatrick *et al.*: *Endocrinology* 140:2581–2591, 1999 (70). © The Endocrine Society.]

duce considerable ovarian enlargement even after hypophysectomy. Moreover, the implantation of DES at the time of hypophysectomy not only maintained ovarian weight but also rendered the ovaries more responsive to exogenous gonadotropins. Indeed, a striking ovarian enlargement occurred in DES-primed animals, which were subsequently injected with CG (*i.e.*, Antuitrin "S"). The ovaries of DES-treated animals consisted of healthy, predominantly solid (*i.e.*, preantral), medium-sized follicles packed tightly at the expense of markedly reduced interstitial tissue. DES-primed/CG-treated animals, in turn, displayed large antral follicles, many corpora lutea, and, in two instances, hemorrhagic follicles.

Similar results were reported that very year by Williams (77), followed in turn by several other contributions (78–90) that largely confirmed and, to some degree, extended the original observations of Pencharz (*e.g.*, establishing dose-response relationship and/or examining the role of estrogens other than DES). Ingram, who also administered estrogen to hypophysectomized rats, either by using 1 mg/day of DES (91) or 5 μg/day of estradiol dipropionate (92), in both studies for 5 days, noted the same number of follicles less than 300 μm in diameter, but a considerable increase in the number of medium sized follicles (>300 μm) (Fig. 4). These observations led Ingram to conclude that the trophic effect of estrogen is attributable to its ability to retard the loss of developing follicles in the hypophysectomized rat. More recently, Goldenberg and associates (93) sequentially administered DES (in total doses ranging from 0–4 mg for either 2 or 4 days) and FSH (a total dose of either 0, 50, 100, or 200 μg in 6 sc injections over 72 h), revealing that an early effect of treatment with DES is to increase DNA synthesis by granulosa cells. Moreover, treatment with DES produced an increase in the growth rates of follicles larger than 200–300 μm in diameter while decreasing the rate of atresia. Qualitatively comparable results were reported in several other contributions (16, 94–96). That estrogens may, in fact, be antiatretic has most recently been supported by the demonstration of their ability to inhibit ovarian granulosa cell apoptosis (97). In this study, a time-dependent increase in ovarian DNA fragmentation was observed when hypophysectomized rats with subcutaneous capsules of DES had these implants removed. This effect was shown to be prevented by treatment with either estradiol benzoate (3 mg/day) or DES (0.5 mg/every 12 h). The above notwithstanding, evidence to the contrary has been put forth (98), which illustrates the controversial nature of this issue.

Above and beyond their impact on follicular growth and atresia, estrogens have also been shown to affect cytodifferentiation, either by themselves as it is in the modulation of follicular intercellular gap junctions (99, 100), ER content (16), adenylate cyclase activity (101, 102), and cell cycle activator cyclin D2 (103), or as a result of synergy with FSH, as in FSH binding (104), LH binding (101, 104, 105), aromatase activity

TABLE 2. The nonprimate ovary as a site of estrogen action

Author/year	Species	Estrogen	Endpoints
Pencharz (1940)	Immature HPX rat	DES and E_2 dipropionate implants	Ovarian weight
Williams (1940)	Immature HPX rat	DES, SILASTIC implant, 8–13 days	Ovarian weight
Simpson *et al.* (1941)	Immature HPX rat	DES, 1 mg/daily/8 days	Ovarian weight/number of medium-sized follicles
Paesi *et al.* (1952)	Immature intact and HPX rat	E_2 benzoate, 0.002–100 μg/daily/7 days	Ovarian weight
deWit *et al.* (1953)	Immature HPX rat	E_2 benzoate, 100 mg/daily/ 1-3-6 days	Number of medium-sized follicles
Payne and Hellbaum (1955)	Immature HPX rat	DES, 0.005–1 mg/daily/4 days	Ovarian weight and histology
Payne and Runser (1958)	Immature HPX rat	DES, 1 mg/daily/4 days	Ovarian responsiveness to gonadotropins
Payne *et al.* (1959)	Immature HPX rat	DES, 1 mg/daily/4 days	Dose-response curve of ovarian responsiveness to gonadotropins
Payne and Runser (1959)	Immature HPX rat	DES, 1 mg/daily/4 days	Ovarian responsiveness to gonadotropins
Ingram (1959)	Immature rat	E_2 dipropionate, 5 μg/daily/5 days	Ovarian weight/number of medium-sized follicles
Ingram (1959)	Mature HPX rat	DES, 1 mg/daily/5 days	Ovarian weight/number of medium-sized follicles
Meyer and Bradbury (1960)	Immature HPX rat	DES, 0.002–2 mg/daily/2–3 days	Ovarian responsiveness to gonadotropins
Bradbury (1961)	Immature HPX rat	E_2, 0.5–1.0 mg/ovarian surface	Ovarian weight Ovarian responsiveness to gonadotropins
Goldenberg *et al.* (1972)	Immature HPX rat	DES, 0–1 mg/daily/6 days	Granulosa cell proliferation Ovarian responsiveness to gonadotropins
Merk *et al.* (1972)	Immature HPX rat	DES, 0.5–1 mg/daily/6 days	Follicular intercellular gap junction
Harman *et al.* (1975)	Immature HPX rat	DES, 0.1 mg/daily/4 days	Anti-atretic effect on preantral follicles
Richards *et al.* (1976)	Immature HPX rat	E_2, 2 mg/daily/4 days	FSH and LH receptor at the level of the granulosa cell
Rao *et al.* (1978)	Immature HPX rat	E_2, 2 mg/daily/1–4 days	Ovarian cellular proliferation
Richards *et al.* (1979)	Immature HPX rat	E_2, 1.5 mg/daily/3 days	Adenylate cyclase activity in granulosa cells
Jonassen *et al.* (1982)	Immature intact and HPX rat	E_2, 1.5 mg/daily/3 or 4 days	Modulation of adenylate cyclase activity in granulosa cells
Adashi and Hsueh (1982)	Immature HPX rat	DES SILASTIC implant, 4–6 days	Ovarian aromatase activity response to FSH
Kessel *et al.* (1985)	Immature HPX rat	DES SILASTIC implant, 4–6 days	LH receptor at the level of granulosa cell
Sadrkhanloo *et al.* (1987)	Immature HPX rat	DES SILASTIC implant, 4–7 days	Ovarian follicle atresia
Billig *et al.* (1993)	Immature HPX rat	DES E_2 benzoate	Ovarian granulosa cell apoptosis

HPX, Hypophysectomy; E_2, estradiol; DES, diethylstilbestrol.

(106, 107), progestin biosynthesis (108), and A-kinase content (109).

Mice responded to DES [but less so to 17β-estradiol cyclopenthylpropionate (ECP)] with an increase in the number of small or large preantral follicles (22), but without an increase in ovarian weight. Apart from the latter, in the few reports on the effects of estrogen in immature mice, both stimulatory and inhibitory effects on follicular development were noted (110, 111). However, since these observations were made in pituitary-intact mice, it is difficult to conclude that estrogens may have been acting directly at the level of the ovary. Fortunately, and more recently, Wang and Greenwald demonstrated unequivocally the ability of ECP, 10, 50, or 250 μg daily for 1–4 days, to stimulate the growth of preantral and antral follicles in the hypophysectomized mouse, an effect associated with a delay of follicular atresia (112). Moreover, estrogens were shown to synergize with FSH in the enhancement of follicular proliferation and differentiation as well as in the attenuation of follicular atresia (112). Taken together, it would appear that the mouse, similar to the rat, is estrogen-responsive. The precise reason(s) underlying the apparent discrepancy with earlier work remains uncertain.

B. The hamster

Although the direct effect of estrogen on rat ovarian follicular development is well established, the role of estrogen in ovarian folliculogenesis may be a species-specific phenomenon. Thus, extrapolation of the rat data to other species may not be feasible. Indeed, hypophysectomized adult hamsters, injected with estradiol benzoate, 10 μg daily for 3 days, exhibited neither increased ovarian weight nor enhanced effects of FSH on follicular development (113). Thus, hypophysectomized (or intact) hamsters may not respond to estrogen by increasing the number of large preantral follicles as reported in the rat. Similar results were obtained for the intact or hypophysectomized immature hamster (17). It is possible that for the immature and adult hamster, unlike the rat, estrogens do not play a major role in the recruitment of large ovarian preantral follicles. In this same context, Hutz *et al.* (21) set out to observe the response of granulosa cells from gonadotropin-primed hamsters to treatment with DES or 17β-estradiol under *in vitro* circumstances. Interestingly the application of DES inhibited the accumulation of estrogen regardless of the presence or absence of FSH in the culture medium. In contrast, the combination of 17β-estradiol plus

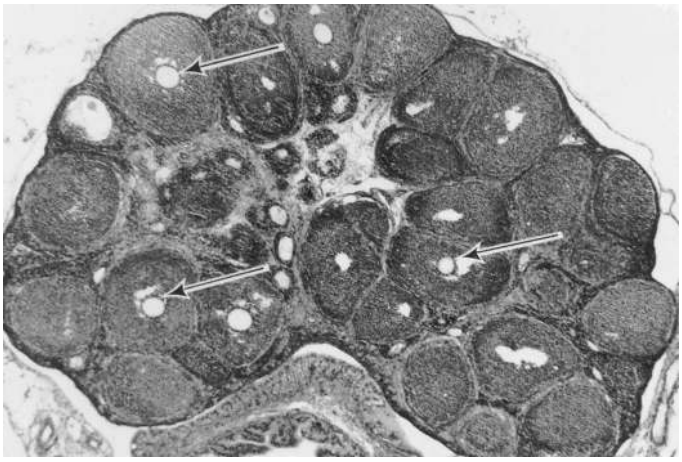

FIG. 4. Photomicrograph of an ovary from the immature hypophysectomized DES-treated rat. Cross-section of the entire ovary showing a near-uniform population of late secondary (type 5a and 5b) and early tertiary (type 6) follicles. The type 6 exhibits cavitation (*arrows*) or beginning antrum formation. Magnification, ×29. [Modified from R. Sadrkhanloo *et al.*: *Endocrinology* 120:146–155, 1987 (98). © The Endocrine Society.]

FSH augmented the accumulation of progesterone, which clearly argues that estrogen is facilitating granulosa cell differentiation. These findings were interpreted to mean that estrogens can be nonstimulatory or inhibitory to the function of hamster granulosa cells *in vitro* in parallel to effects shown *in vivo*.

Since the role of estrogens in hamster ovarian folliculogenesis is uncertain, comparisons between hamster and other animal models as well as conclusions on the effect of estrogen on ovarian follicular development are hindered by the paucity of available data.

C. The rabbit and guinea pig

Immature rabbits treated with ECP or DES for 3 days failed to increase ovarian weight but did increase the number of small or large preantral follicles (22). In contrast, estrogen-treated guinea pigs displayed a significant increase in ovarian weight, a phenomenon attributable to an increase in the number of large antral follicles (22).

D. The pig

Experimental studies support a thesis of multipotential actions of estrogen on follicular growth and cytodifferentiation in the pig (74, 114–119). However, since much of this knowledge is based on descriptive *in vitro* studies, which may involve risk, interpretative complications due to 1) the presence of serum (120, 121), 2) failure to distinguish atretic from healthy follicles, 3) assessment of a limited degree of estrogen's interactions with other regulators of follicle function, and (4) focus on growth and cytodifferentiative features of the granulosa, but not theca cell, it is difficult to make definitive assertions on the role of estrogen in the swine ovary.

Whereas *in vivo* estrogen administration in the (hypophysectomized) rat clearly supports follicular and ovarian growth, this inference is confounded in the pig by well documented granulosa cell-proliferative responses at least *in vitro* to insulin-like growth factor I (IGF-I), the production of which by granulosa cells can be driven by GH alone or estrogen and FSH combined (122–125). Moreover, IGF-I or estrogen each is capable (alone or in concert with FSH) of augmenting indices of *in vitro* granulosa cell proliferation in the pig, *e.g.*, inducing proliferating-cell nuclear antigen (PCNA) expression and increasing tritiated thymidine incorporation or cellular DNA content (126, 127).

The full physiological *in vivo* role(s) of estrogen in modulating ovarian cellular growth will be challenging to unmask in swine, since multiple intrafollicular factors also appear to control granulosa cell growth, and their possible interactions with estrogen have not yet been explored, *e.g.*, inhibitory [tumor necrosis factor-α (TNFα) (126)], or stimulatory [relaxin (128), transforming growth factor-β (TGFβ) (129), endothelin-1 (129), epidermal growth factor (EGF) (127), cAMP analogs (127), and T_4 (130)].

Nonetheless, because the majority of correlative studies document an inverse relationship between intrafollicular estradiol concentrations and one or more cytological measures of atresia (*e.g.*, Ref. 131), several important considerations arise, namely whether 1) estrogen is obligatory to maintain healthy late-follicle development; or, conversely, 2) failure of follicular development, predicated on whatever mechanistic grounds, is heralded by loss of estrogen-synthesizing capacity. Both views are supported, but not proven, by the concomitant waning of estrogen synthesis and other biosynthetic functions in early follicular atresia in the pig, *e.g.*, inhibin production or FSH-stimulated cAMP accumulation (131). Moreover, aromatase activity in the pig is controlled by multiple factors other than FSH acting alone [*e.g.*, inhibitory: cytokines, such as interleukin-6 (IL-6) (132), PRL (133), extracellular purines (134), insulin-like growth factor binding protein-3 (IGFBP-3) (135), and high concentrations of LH (136); or stimulatory: IGF-I or EGF (137)].

In contrast to the sparse evidence for follicle (or granulosa cell) growth-promoting actions of estrogen in the pig (above), significant trophic actions of estrogen on multiple endpoints of granulosa cell cytodifferentiation are well recognized. For example, estradiol acts trophically *in vitro* as a potent biological amplifier of PRL, FSH, and IGF-I or IGF-II's stimulation of progesterone production by (immature) porcine granulosa cells in monolayer sparsely serum-supplemented or serum-free first-passage cultures (74, 118, 119, 138–151). The mechanisms underlying such singular and synergistic actions of estradiol include enhanced low density-lipoprotein (LDL)-receptor-expression, increased cholesterol uptake and utilization (152), augmented cytochrome $P450_{scc}$ enzymic activity (148, 150, 152), increased conversion of pregnenolone to progesterone, heightened *de novo* synthesis of cellular cholesterol via 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (146), and augmented steroidogenic acute regulatory protein expression, without evident inhibition of progesterone's metabolism to 5α-dihydroprogesterone (74). These *in vitro* trophic actions of estradiol occur over 36–48 h or longer without any measurable proliferative effects. Rather, inhibition of porcine granulosa cell proliferation with a noncompetitive (suicide) inhibitor of polyamine biosynthesis amplifies the daily rate of *in vitro* progesterone-

expressed biosynthesis per granulosa cell (153), suggesting an inverse relationship between granulosa cell proliferation and cytodifferentiation (154).

Estrogen's modulation of progesterone biosynthesis by pig granulosa cells is bipotential in a time-dependent sense (74, 75, 118, 119, 138–140). Short-term (2–18 h) exposure to estradiol consistently inhibits progesterone biosynthesis, apparently by directly antagonizing pregnenolone's conversion to progesterone via 3β-hydroxysteroid dehydrogenase (74, 153). This direct action is mimicked by a nonmetabolizable estrogen, moxestrol, and is not antagonized by the antiestrogen, keoxifene (144). Since inhibition can be reproduced in broken cell (microsomal) preparations, it is likely that direct steric inhibition of enzyme catalysis occurs. The mechanisms underlying delayed (34–36 h) escape from this acute inhibition are not yet known (74, 118, 119, 138, 139). However, an analogously rapid inhibition of steroidogenesis by estrogen is described in pig theca cells (155–157). Such steroidogenic autoinhibitory actions of estrogen may be relevant as the preovulatory LH surge unfolds, when intrafollicular 17β-estradiol concentrations approach or exceed 1 μg/ml (74, 158), thereby (inferentially) limiting a premature elevation of progesterone concentrations before corpus luteum formation and/or thereby restraining the preovulatory (theca cell-derived) androgen surge.

E. Possible interactions of estrogen with other putative ovarian regulators

Whereas estrogen is the primary focus above, its critical interactions with other known and putative intrafollicular regulators will be essential to unravel the complete understanding of the ovarian follicular development in the pig and other species (130, 159, 160). For example, a reciprocal relationship emerges between growth (cell proliferation) and cytodifferentiation in response to certain modulators, *e.g.*, endothelin-1 (129, 161, 162), polyamines (153), and TNFα (126). This observation, while not universal (IGF-I tends to promote both granulosa cell proliferation and differentiation) illustrates the potential complexity inherent in *in vivo* interpretations of estrogen's acting in combination with other potent regulators of folliculogenesis, such as the intraovarian IGF-I system (145). Estrogen-growth factor interactions are of particular interest, when folliculogenesis is viewed as a continuum of changing cellular commitment to replication *vs.* cytodifferentiation.

How estrogen interacts, if at all, with specific cell-surface adhesive proteins, such as connexin, laminin, or integrin, in their presumptive maintenance of commitment to granulosa cell lineage (120, 122, 163) is likely of significance in the maturing follicle, as well as possibly in the earliest stages of preantral follicle formation (164, 165).

Estrogen's presumptive modulation of the calcium-calmodulin, C-kinase, and A-kinase effector pathways in granulosa and theca cells will also require further study (125, 140, 141, 166–184). In this light, it will be critical to establish whether, when, and how estrogen participates in rescuing follicles from early or incipient atresia (131, 185). Differential hybridization of pig granulosa and theca cell mRNAs collected in untreated *vs.* estrogen-treated conditions, as accomplished for FSH recently (186), could be one step toward addressing this difficult issue. *In vitro* whole-follicle cultures also may be useful in this context (187).

Given the pivotal role inferred to date for the IGF-I and IGF-II (and their associated binding proteins) systems in follicle development, exactly how estrogen impacts the multifaceted actions of IGF-I and IGF-II in the pig will require further clarification (135, 149–152, 181, 182, 188–191). In addition, estradiol's interactions with FSH receptor-mediated cytodifferentiative actions on granulosa cells should be evaluated further, given the unequivocally central role of this gonadotropin in the follicle development in this and other species (190).

Further knowledge of how estradiol modulates intrafollicular production and actions of the multiple cytokines and activin/inhibin glycoproteins (and the structurally unrelated follistatin) may also help better elucidate estrogen's role in folliculogenesis in the pig. Although of interest for other investigative purposes, the recent development of pig granulosa cell clonal lines is not likely to be so rewarding in dissecting the foregoing broader physiological issues (192). Lastly, we suggest that the potent luteotropic effects of estrogen in the pig, albeit well supported by available data in this species, require better mechanistic understanding (193–197). The foregoing queries will eventually also need to be addressed with respect to the nature and mechanisms of estrogen's modulation of theca and/or interstitial cell function in the ovary. The latter proposition assumes our thesis that folliculogenesis progresses under the dual orchestration of the intrafollicular and perifollicular milieus, which likely jointly coordinate follicle development.

IV. Lessons Learned from the Estrogen-Resistant Model-Estrogen Receptor Null Mutants (ERKOs)

The first and only known case of clinical estrogen insensitivity in man was reported by Smith *et al.* (198). This male patient displayed no detectable response to estrogen administration due to an inactivating mutation of the ERα gene. The patient was tall and normally masculinized. However, estrogens proved to be of import for bone maturation and for mineralization. Although no examples of estrogen resistance exist in the primate/human female, the recent flurry of activity in this area might indicate that such an occurrence is not an impossibility. Clearly, once such an individual is identified, significant lessons could be learned with respect to the relative importance of estrogens to ovarian follicular maturation in the human female.

Short of such primate/human models, note must be made of the recent works reporting the generation null mutants for the ERα (199–205) and ERβ (206) genes by way of gene targeting (homologous recombination) technologies (207). Compound null mutants were also generated. A detailed account of this work was recently offered (207). Therefore, only a brief discussion of the relevant points will appear here.

ER$\alpha-/-$ mice survived to adulthood featuring a normal gross phenotype. However, null-mutant female mice proved infertile, displaying hypoplastic uteri and hyperemic ovaries with no detectable corpora lutea (205). Histological sections

of ovaries from homozygous null mutants revealed no defects in germ cell formation or migration. Further, developmental progression through the primordial, primary, and antral follicle stages appeared normal. However, functional maturation to preovulatory follicles was arrested resulting in atresia or in "anovulatory follicles," which in many cases formed large, hemorrhagic cysts containing few, if any, granulosa cells. Stated differently, ovarian follicles of adult ER$\alpha-/-$ mice may progress to a "Graafian" state albeit in the face of abnormal stratification of granulosa cells, some areas of the follicle being surrounded by multiple layers of cells, other regions featuring a single layer of squamous-appearing cells (205, 208) (Fig. 5). Estrogen actions, such as the attenuation of apoptosis or the amplification of the LH receptor content in granulosa cells of antral follicles, appear preserved, probably due to mediation by the ERβ receptor.

The phenotype of the ER$\alpha-/-$ mice is probably attributable, in part, to chronic exposure to abnormally high levels of LH (207). Support for this hypothesis can be drawn from several studies. Prolonged treatment with antiestrogens, which possess the ability to cross the blood brain barrier and therefore produce chronically elevated levels of LH, have produced a similar ovarian phenotype (209–211). Similarly, targeted transgenic overexpression of the LHβ subunit gene resulted in increased serum levels of LH and an identical ovarian phenotype (212, 213). In addition, prolonged treatment of the ER$\alpha-/-$ mice with a GnRH antagonist reduced serum LH levels and prevented the cystic ovarian phenotype (214).

However, the role of LH in the evolution of the above phenotype can be questioned, given two other null mutant models in which the hemorrhagic cyst phenotype is absent (in the face of chronically elevated LH levels). For example, the null mutant for the FSHβ-subunit gene (215), otherwise replete with LH, proves the importance of FSH in follicular cyst formation. Similarly, the null mutant for the P450-aromatase gene (216), characterized by high circulating levels of both LH and FSH, but absent estradiol, points out a role for estradiol in the genesis of the (hemorrhagic) cyst phenotype.

To test whether ER_α is required for ovulation and corpus luteum formation, gonadotropins were used to superovulate immature $ER_\alpha-/-$ mice and wild-type siblings (208, 214). Gonadotropin therapy resulted in ovulation in both ER$\alpha-/-$ and wild-type mice. However, fewer gonadotropin-treated null mutants ovulated. In addition, ER$\alpha-/-$ mice yielded significantly less oocytes. Surprisingly, ovulated/ruptured ovarian follicles of null mutants developed into corpora lutea of apparent normal morphology boasting 3-fold the concentrations of serum progesterone as compared with controls. However, adult $ER_\alpha-/-$ mice could not be induced to ovulate, probably due to the elevated circulating levels of LH and the development of hemorrhagic ovarian cysts (200).

Null mutants for the ERβ gene survive to adulthood and exhibit a phenotype distinct from that of their ER$\alpha-/-$ counterparts. Unlike ER$\alpha-/-$ mice, the ER$\beta-/-$ female mouse proved fertile, albeit with apparently less frequent ovulation (206). Consequently, fewer corpora lutea were apparent, the litter size being smaller than in wild-type mice. Not unlike the ER$\alpha-/-$ mice, gonadotropin-treated ER$\beta-/-$ mice responded with fewer oocytes released than their wild-type counterparts (206).

Histological analysis of ovaries of ER$\beta-/-$ mice was mostly normal, except for an increased number of early atretic follicles and the sparse presence of corpora lutea, suggesting arrested folliculogenesis. Superovulation disclosed a reduced ovulatory capacity of ER$\beta-/-$ mice as compared with their wild-type counterparts. The histology of the ovaries from superovulated ER$\beta-/-$ mice revealed the presence of numerous unruptured preovulatory follicles, indicating deficiency in the response to the gonadotropin surge (hCG). The relative deficit of spontaneous ovulation in the ER$\beta-/-$ mice may be due to diminished up-regulation of ovarian progesterone receptor (PR) levels by gonadotropins, or an alteration in gonadotropin synthesis or secretion (207, 217). Dupont *et al.* (218) described a similar phenotype for ERβ null mutant, but their data reveal that only half of the superovulated female ERβ null mutants ovulate, in contrast to the study of Krege *et al.* (206) who reported that 80% of the superovulated animals ovulated.

Recently, mice lacking both ERα and ERβ were generated (219). ER$\alpha\beta-/-$ mice exhibited normal reproductive tract development but proved infertile. Ovaries of adult ER$\alpha\beta-/-$

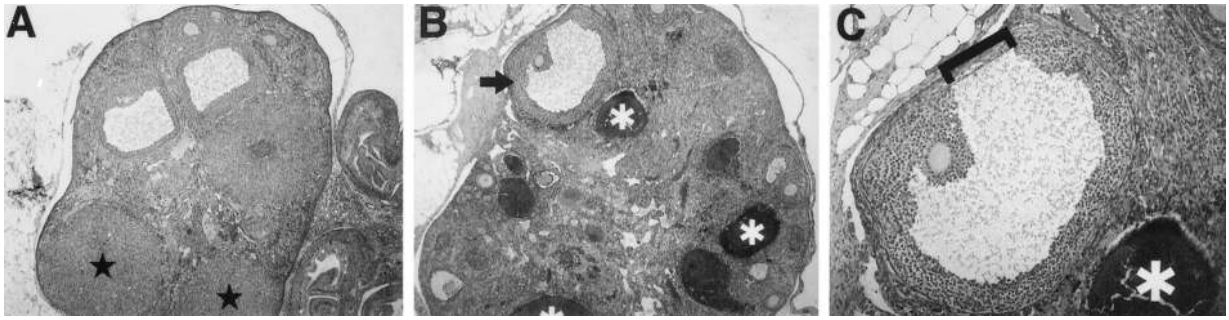

FIG. 5. Histological examination of adult wild-type (WT) and ERαKO ovaries. A, Histological examination of normal adult WT ovaries revealed many corpora lutea (CL) (*stars*). *Magnification bar* = 500 μm. B, Ovaries from ERαKO female mice contained many hemorrhagic ovarian cysts (*asterisks*). However, occasional Graafian ovarian follicles (*arrow*) were present. *Magnification bar* = 500 μm. C, Higher magnification of Graafian ovarian follicle from adult ERαKO ovary depicted in panel B reveals that there was differentiation of granulosa cells with cumulus cells surrounding the ovary and follicular antral fluid in the central portion of the ovarian follicle. However, there was abnormal stratification of granulosa cells, with a portion of the follicle lined by a single layer of cells (*bracketed area*). *Magnification bar* = 200 μm. [Reproduced with permission from C. S. Rosenfeld *et al.*: *Biol Reprod* 62: 599–605, 2000 (208). © Society for the Study of Reproduction.]

mice exhibited follicle transdifferentiation to structures resembling seminiferous tubules of the testis, including Sertoli-like cells expressing Mullerian inhibiting substance, sulfated glycopropein-2 and *Sox9* (biochemical markers of Sertoli cell differentiation). In some follicles, a recognizable but degenerating oocyte was present, whereas others featured no evidence of germ cells. These findings indicate that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary. Interestingly, serum LH levels were higher than those observed in ER$\alpha-/-$ mice, suggesting that both ERs are required for estradiol-mediated regulation of LH secretion (219).

In summary, these findings suggest that prenatal reproductive tract development in the mouse can occur in the absence of ERα or ERβ, an observation consistent with traditional theory. However, reproductive function is undoubtedly affected. Since, ovarian folliculogenesis, ovulation, and corpus luteum formation can occur in the absence of ER_α or ERβ, albeit less effectively than in wild-type mice, it might be suggested that neither one of the known ERs is essential, but that each may play a facilitatory role in ovarian follicular development and maturation.

V. Lessons Learned from the Targeted Disruption of the CYP-19 (Aromatase) Gene-Aromatase Null Mutant (ArKO)

The formation of estrogens from C_{19} steroids is catalyzed by aromatase cytochrome P450, the product of Cyp 19 gene. Null mutant mice for aromatase gene (ArKO) were generated (216), thereby affording the opportunity to examine the role of estrogen in the follicular development in the mouse ovary. Upon reaching sexual maturity, female ArKO mice, born phenotypically normal, develop a male body habitus with excessive internal fat deposition, underdeveloped uteri, and ovaries lacking corpora lutea. The clitoral glands, mammary glands, and gonadal fat pads enlarge. Histological evaluation of the ovaries reveal the presence of many large follicles filled with granulosa cells and evidence of antrum formation, but no corpora lutea. As expected, testosterone and LH levels are markedly elevated. The high testosterone levels presumably reflect stimulation of the theca interna cells by LH (216, 220–230). Although estrogen was not a prerequisite for the reinitiation of follicle growth (from the point of primordial follicle up to the antral follicle stage), note was made of a block of follicular development and absent corpora lutea. The ovarian phenotype degenerated with age upon the appearance of hemorrhagic cystic follicles and the loss of secondary and antral follicles coincident with the infiltration of macrophages and with stromal hyperplasia (228, 231). In summary, the ArKO female mouse is infertile, as a consequence of disrupted folliculogenesis and failure to ovulate.

VI. The Primate/Human Ovary as a Site of Estrogen Reception

Essential to any discussion of a local paracrine/autocrine role for estrogens in primate/human ovarian physiology must be a review of the evidence relevant to the possibility that the primate/human ovary may be a site of estrogen reception.

A. Molecular probing (Table 3)

Billiar *et al.* (232), relying on Northern blot analysis, made use of a cDNA probe corresponding to the 1.8-kb open reading frame of the ERα expressed by the MCF-7 human breast cancer cell line (232). A 7-kb ERα mRNA species was detected

TABLE 3. The primate/human ovary as a site of estrogen reception—molecular probing

Author/year	Species	Developmental stage	Imaging technology	ERα status	ERβ status
Billiar *et al.* (1992)	Baboon	Mature	NB	Whole ovary (+)	NA
Chandrasekher *et al.* (1994)	Cynomolgus/rhesus	Mature	RT-PCR	Whole ovary (+) GE (+) CL (−) GC (−) GC* (−)	NA
Pau *et al.* (1998)	Rhesus	Mature	RT-PCR ISH	Whole ovary (+) GC (+)	Whole ovary (+) GC (+)
Chaffin *et al.* (1999)	Rhesus	Mature	RT-PCR	GC (+) GC* (+)	GC (+) GC* (+)
Pelletier *et al.* (1999)	Cynomolgus	Mature	ISH	NA	GC (+) TC (+) GE (+) CL (+)
Wu *et al.* (1993)	Human	Mature	RT-PCR/SB	Whole ovary (+) Oocyte (+) GC* (−)	NA
Hurst *et al.* (1995)	Human	Mature	RT-PCR/SB	GC* (+)	NA
Revelli *et al.* (1996)	Human	Mature	ISH	GC (+) TC (+) CL (+) Stroma (+) oocyte (−)	NA
Brandenberger *et al.* (1997)	Human	Fetus	RT-PCR	Whole ovary (+)	Whole ovary (+)
Enmark *et al.* (1997)	Human	Mature	ISH RT-PCR	NA	Stroma (+) GC* (+)
Brandenberger *et al.* (1998)	Human	Mature	RT-PCR/SB	Whole ovary (+)	Whole ovary (+) GC* (+)
Chiang *et al.* (2000)	Human	Mature	RT-PCR NB	GC* (+)	GC* (+)

ISH, *In situ* hybridization; GC, granulosa cell; (+), positive result; NB, Northern blot analysis; GC*, luteinized granulosa cell; (−), negative result; SB, Southern blot analysis; CL, corpus luteum; TC, theca cell; N/A, not applicable/available; GE, germinal epithelium.

when using a poly(A)$^+$-enriched mRNA fraction representing whole ovarian material from two animals. Unfortunately, the use of whole ovarian material all but precluded the localization of ERα mRNA to discrete follicular or perifollicular components. Consequently, one cannot rule out that the positive signal reflects germinal epithelial contamination.

Wu *et al.* (233), in turn, employing the RT-PCR technique, examined unfertilized human oocytes, cumulus-oocyte complexes (COC), whole ovarian tissue, and isolated granulosa cells. Total ovarian homogenates were secured from two patients undergoing surgery for benign gynecological conditions. Oocytes, COC, and granulosa cells were obtained from patients undergoing transvaginal follicular aspiration in the course of *in vitro* fertilization-related procedures. The relevant DNA was digested with RNase-free DNase to minimize genomic DNA contamination. Reverse-transcribed RNA was amplified over 30 cycles with specific oligonucleotide primers defining a 263-bp cDNA fragment corresponding to the entire DNA binding domain of the human ERα and which crossed two introns. The identity of the resultant products was confirmed by sizing and Southern blot analysis. Using this approach, the authors were able to demonstrate ERα transcripts in whole ovarian material. Similarly, both oocytes and COCs were found to be ERα-positive. However, when isolated granulosa cells or cumulus masses devoid of oocytes were examined, both were judged to be ERα-negative. As such, these data suggest that the human oocyte, but not the human cumulus granulosa cell, is a site of estrogen reception. These observations do not exclude the possibility that noncumulus (*i.e.*, membranous) granulosa cells may also be ERα-positive. The interpretation of the oocyte/COC data must also take into account the fact that the oocytes in question failed to fertilize *in vitro* and thus may not be representative. The authors do state, however, that all oocytes were judged to be mature (as determined by the breakdown of the germinal vesicle and the extrusion of the first polar body within 48 h after aspiration) and that the apparent failure to fertilize was most likely due to a severe male factor. It also remains possible that the ERα positivity may be the result of contamination of the COC samples with nonovarian ERα-positive material as a result of transvaginal ovarian follicular aspiration (*e.g.*, vaginal mucosa or ovarian germinal epithelium).

More recent reports by Hurst *et al.* (234, 235), using RT-PCR technology applied to highly luteinized human "granulosa cell" mRNA, provide data in apparent conflict with those reported by Wu *et al.* (233). To identify the possibility of genomic DNA contamination, the oligonucleotide primers were so designed as to correspond to base pairs 570–852 in the B and C domains of the human ERα cDNA, a stretch of nucleotides known to span intron 1. Southern blotting of the amplified products with a ^{32}P-labeled ERα mRNA probe confirmed the existence of ER transcripts (Fig. 6). The data suggest the existence of ERα transcripts in follicular aspirates consisting largely of luteinized granulosa cells. However, since the granulosa cells studied were obtained via transvaginal needle aspiration of ovarian follicles in the course of oocyte retrieval in conjunction with *in vitro* fertilization procedures, the signal detected may correspond to cells other than granulosa cells known to be present in follicular aspirates (*e.g.*, representatives of the white blood cells series). Consequently, there exists the strong possibility of contamination by vaginal tissues or by ovarian germinal epithelium, which may be ERα-positive, thereby reducing the level of confidence in the conclusions from this study.

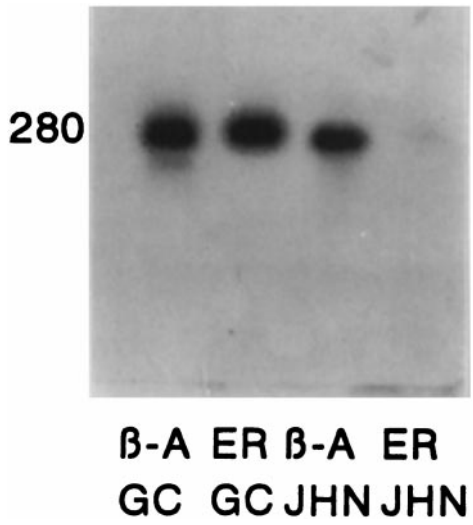

FIG. 6. Agarose gel demonstrating the amplification of the 282-bp ERα fragment in human granulosa cells (GC), but not in the negative control human glioblastoma cell line, JHN J889H. The β-actin (b-A) fragment is amplified from both cell types. [Reproduced with permission from B. S. Hurst *et al.* : *J Clin Endocrinol Metab* 80:229–232, 1995 (234). © The Endocrine Society.]

The technique of RT-PCR of ER_α mRNA has also been applied to rhesus monkey ovaries (236). Before collection of tissue, each animal was stimulated with 60 IU purified human urinary FSH (Metrodin, Serono, Norwell, MA) during menstrual cycle days 1–6 followed by 60 IU of a purified mixture of human urinary FSH and LH (Pergonal, Serono) for days 7–9. Animals were divided into groups, one of which received an ovulatory stimulus of hCG 1000 IU im on day 10. Oocytes and granulosa cells were collected by follicular aspiration of follicles at laparotomy. A Percoll gradient was used to enrich the granulosa cell sample after oocyte removal. Samples were dissected to yield germinal epithelium-enriched material. Corpora lutea were obtained by luteectomy from early, mid, and late luteal phase animals (as judged by days post-LH surge). Total RNA was isolated from uterine myometrium (designed to serve as a positive control), spleen (designed to serve as a negative control), whole ovary, germinal (surface) epithelium- enriched cortical and medullary compartments of the ovary, granulosa cells of preovulatory follicles before and after an ovulatory stimulus, and corpora lutea from the early, mid, and late luteal phase of the menstrual cycle. Primers were chosen to bracket a 360-bp sequence corresponding to the human ERα steroid-binding domain and to span an area containing an intron to identify the possibility of genomic contamination. Amplified products of the expected size for an ER were obtained from myometrial RNA and no product was obtained from spleen. ERα mRNA was detected in whole ovary and in germinal epithelium-enriched cortical compartments, with a barely visible product occasionally observed in medullary compartments of the ovary. ERα mRNA was not detected in any

corpora lutea, throughout the luteal phase or in granulosa cells obtained by follicular aspiration before or after an ovulatory stimulus. Sequence analysis of the ERα product revealed 99% homology to the cDNA for the hormone-binding region of human ERα. The apparent absence of ERα mRNA in various ovarian compartments was taken to suggest a lesser, if any, role for estrogen in the primate ovarian life cycle. In agreement with immunohistochemical studies (56), no ERα mRNA was detected in ovarian tissues other than the germinal epithelium. In fact, although whole ovarian material proved ERα mRNA positive, specimens devoid of germinal epithelium proved ERα mRNA negative. The intriguing finding of the possible presence of medullary ovarian ERα transcripts remains to be explained. It is possible that this signal originates in oocytes or migratory lymphocytes assuming the stromal cells themselves are ERα negative.

Revelli *et al.* (237) also undertook to evaluate the expression of ER transcripts in the ovaries of 25 healthy eumenorrheic women. Ovarian biopsies were taken in different phases of the menstrual cycle during laparotomy or operative laparoscopy performed for extraovarian benign diseases. Using *in situ* hybridization targeted at ERα transcripts, Revelli and associates documented ERα transcripts in 17.1% of primordial follicles. The proportions of preantral and antral follicles positive for ERα transcripts were 30.7 and 37.5%, respectively. No more than 25% of theca cells proved ERα transcript-positive. Active corpora lutea stained positive for ERα transcripts in 50% of the cases. Corpora albicantes always stained negative. In all subjects, the stroma surrounding both follicles and corpora lutea contained several fibroblast-like cells that stained positive for ERα transcripts. Oocytes and blood vessels stained negative in all cases.

Brandenberger *et al.* (238) compared the expression profiles of ERα and of ERβ transcripts in the midgestational human fetus by semiquantitative RT-PCR. ERα was detected in the human fetal ovary. However, the relative amounts of ERα transcripts in the human fetal ovary were substantially reduced as compared with the levels observed in the uterus. In contrast, significant amounts of ERβ transcripts were present in fetal ovaries. In relative terms, ERβ transcripts were far more prominent than their ER_{α} counterparts. The presence of ERβ transcripts in ovarian granulosa cells of adult women was also convincingly demonstrated (239). Similar amounts of ERα and ERβ mRNA were reported for normal ovaries in all age groups from 33 to 75 yr (59). Luteinized granulosa cells also expressed a significant level of ERβ mRNA (59).

Note is also made of the contribution of Pau *et al.* (240), who were able to observe (by means of RT-PCR and *in situ* hybridization) weak to moderate signals of ERβ and strong signals of ERα mRNA within the granulosa layer of primate ovarian follicles. Similarly, whole ovarian mRNA yielded appropriate amplicons corresponding to ERα and ERβ after RT-PCR.

Pelletier *et al.* (57), in an effort to clarify the expression of ERβ in the reproductive organs of primates, performed *in situ* hybridization studies in ovaries of adult female Cynomolgus monkeys (*Macaca fascicularis*). ERβ mRNA localized to granulosa cells of follicles at different stages of development, including small growing and secondary (antral) follicles. Primordial follicles, however, were devoid of ERβ expression. The theca interna cells were also strongly labeled. It was not possible to identify any preovulatory follicle. The corpora lutea appeared to be a site of ERβ gene expression, whereas interstitial cells were consistently negative. A strong autoradiographic reaction obtained for the ovarian capsule, *i.e.*, the surface epithelium and the stroma cells.

Mention must also be made of studies on the regulation of ERα and ERβ transcripts in the superovulated primate follicle (241). ERα transcripts did not change whereas ERβ transcripts decreased 12–36 h after the ovulatory stimulus. Steroid ablation reduced ERα transcripts 12 h after hCG, an effect partially reversible by progestin replacement. ERβ transcripts were unaffected by steroids. These data demonstrate hCG-initiated, steroid-dependent (ERα) and -independent (ERβ) expression of receptor transcripts in primate granulosa cells during the periovulatory interval. Differences in patterns of expression may relate to the possible diverse roles of steroid hormones in periovulatory events.

Recent *in vitro* studies on ERα and ERβ gene expression in human granulosa-lutein cells have improved the understanding of the hormonal regulation of ERs in the ovary (242). Although transcripts and protein are identified for both ERα and ERβ in human granulosa cell cultures, ERα is expressed at a lower level than ERβ. Basal expression studies indicated that ERα mRNA levels remained unchanged, whereas ERβ mRNA levels increased with time in culture. Treatment with hCG significantly attenuated the expression of both receptors. Similarly, treatment with GnRH led to inhibition of the ERα and ERβ transcripts and proteins. These results suggest that ERβ more than ERα may play a dynamic role in regulating corpus luteum formation in the human.

It has been observed that both human ER genes give rise to a significant number of mRNA isoforms, which, in turn, exhibit differential expression among the tissues in which they reside (41, 243). In human ovaries, the C and F mRNA isoforms of the ERα gene appear to be the major forms detected among six human mRNA isoforms, A–F. Moore *et al.* (41), in evaluating the expression of the five mRNA isoforms for the human (h) ERβ gene (hERβ 1–5), was able to identify hERβ-1 and -2 as most abundant in the normal ovary, whereas hERβ-4 could not be detected (41). In addition, a novel human estrogen receptor β isoform, ERβcx, has been identified: one that is truncated at the C-terminal region but possesses an extra 26 amino acids due to alternative splicing. The ERβcx transcript is expressed in the adult human ovary (244). Interestingly, ERβcx displays no ligand binding ability and fails to form any shifted complex in gel shift assays. Moreover, in a transient expression assay, ERβcx shows no ligand-dependent transactivation ability of a basal promoter and also cannot interact with a cofactor, TIF1α, in the presence or absence of 17β-estradiol. ERβcx preferentially forms a heterodimer with ERα rather than with ERβ, inhibiting DNA binding by ERα. Thus, ERβcx may potentially inhibit ERα-mediated estrogen action.

In summary, the nonhuman primate ovary is a site of expression of ERα transcripts, mainly at the level of the germinal epithelium (236). The granulosa cell layer may also be a site of ERα gene expression (240). The adult human ovary, in turn, may express ERα transcripts at the level of the

oocyte (233) and the luteinized granulosa cell (234) and, as recently documented, the germinal epithelium (60). Revelli *et al.* (237), however, failed to document ERα transcripts at the level of the human oocyte; instead, they reported ERα transcript positivity in a portion of follicular granulosa cells and in a minority of theca cells. The status of the oocyte notwithstanding, it would appear that the adult human ovary is a site of ERα expression. ERα transcript positivity was also noted in 50% of active corpora lutea (237). The fetal human ovary is a site of ERα and ERβ gene expression wherein the ERβ transcripts are more abundant (238). Recent probing establishes the presence of ERβ transcripts in the adult human ovary at the level of the granulosa cell (59, 239) and the germinal epithelium (60). In a recent study, both ERα and ERβ transcripts colocalized to the human corpus luteum (245). In the nonprimate ovary, ERβ transcripts are consistently expressed in granulosa cells of growing follicles as well as in the theca cell layer and germinal epithelium (57). Given the recent discovery of the inhibitory version of an ER, *i.e.*, ERβcx (244), it is reasonable to presume that the net estrogenic impact at the level of the human ovary may be determined by the relative expression and interaction of the various ER subtypes.

B. Immunohistochemical studies (Table 4)

The first reported attempt to identify ERs in the primate ovary was performed by Hild-Petito *et al.* (246) using adult rhesus (*Macaca mulatta*) and cynomolgus (*Macaca fascicularis*) monkeys. Ovaries were collected from adult animals in the early, mid, and late follicular phase as well as in the luteal phase of the menstrual cycle (n = 3–6 ovaries per stage). Specific monoclonal antibodies, directed against the human ERα (H222 and D75), were employed (247, 248). Surprisingly, immunoreactive ER was only localized to the ovarian germinal epithelium, an observation applicable to all stages of the cycle (Fig. 7). Indeed, all the other structures in the ovary were immunonegative, regardless of the phase of menstrual cycle. In contrast, progesterone receptors (PRs) were readily demonstrated using similar techniques in many of the (ER-negative) ovarian tissues studied. As such, these findings lend further credence to the experimental conclusions by arguing against the possibility that the apparent paucity of ER reflects technical shortcomings.

Although the precise reason(s) underlying the apparent absence of primate ovarian follicular ER remains unknown, it is possible, as the authors recognized, that the limited sensitivity of the technique might be a factor. Specifically, the receptor may exist at levels below the threshold of detection of the immunohistochemical technique employed. Alternatively, the primate ovarian ER may exist in a form that did not react with the specific monoclonal human ER-directed antibody used. However, the latter possibility appears unlikely since the technique employed capably localized ER to the monkey ovarian germinal epithelium and the rabbit corpus luteum, as well as to the monkey cervix and oviduct. The preceding arguments notwithstanding, the relative importance of a second ER species (ERβ) in the ovary must now be considered (see below).

A similar immunohistochemical study was performed by Iwai *et al.* (249) using human ovarian material collected at the

TABLE 4. The primate/human ovary as a site of estrogen reception—immunohistochemistry

Author/year	Species	Developmental stage	Antibody	Immunoreactivity
Hild-Petito *et al.* (1988)	Rhesus/cynomolgus	Mature	H222/D75	ER (+) in GE, all others structures negative
Billiar *et al.* (1992)	Baboon	Mature	H222/D75	ER (+) in GC of AF, TC (+), Stroma (−)
Hutz *et al.* (1993)	Rhesus	Mature	H222	ER (+) in GC of AF, TC (+), GE (+), Interstitium (+)
Saunders *et al.* (2000)	Marmoset	Mature	ERα = NCL-ER-6F11 ERβ = P3/P4/P7	ERα = GC (+) (M/L follicles) TC (+) GE (+) ERβ = GC (+) (S/M/L follicles) TC (+) GE (+)
Iwai *et al.* (1990)	Human	Mature	H222	ER (+) in GC of AF/DF CL (−) GC* (−) GE (NA)
Horie *et al.* (1992)	Human	Mature	H222	ER (+) in GC of AF/DF GC* (+) GE (NA)
Suzuki *et al.* (1994)	Human	Mature	EDR_{1d5}	ER (+) in GC of AF/DF TC (−) Stroma (−) CL (−) Oocytes (−)
Revelli *et al.* (1996)	Human	Mature	NA	ER = GC (+) (S/M/L follicles) TC (+), Stroma (+), CL (+)
Taylor and Al-Azzawi (2000)	Human	Mature	ERα = anti-bovine ERα (05–394) ERβ = anti-rat ERβ (06–629)	ERα = GC (+) TC (−) CL (−) ERβ = GC (+) (S/M/L follicles) TC (+), CL (+)
Saunders *et al.* (2000)	Human	Mature	ERα = NCL-ER-6F11 ERβ = P3/P4/P7	ERα = GC (+) (M/L follicles) TC (+) GE (+) ERβ = GC (+) (S/M/L follicles) TC (+) GE (+)
Pelletier and El-Alfy (2000)	Human	Mature	ERα = HC-20 ERβ = directed to a SP (aa 46–63 of ERβ)	ERα = TC (+) GE (+) interstitium (+) ERβ = GC (+) (S/M/L follicles) GE (+), interstitium (+)

ER, estrogen receptor; ERα, estrogen receptor subtype α; GE, germinal epithelium; GC, granulosa cell; AF, antral follicle; CL, corpus luteum; TC, theca cell; DF, dominant follicle; N/A, not applicable/available; ERβ, estrogen receptor subtype β; (+), positive result; (−), negative result; SP, synthetic peptide; GC*, luteinized granulosa cell; S, small; M, medium; L, large; aa, amino acid.

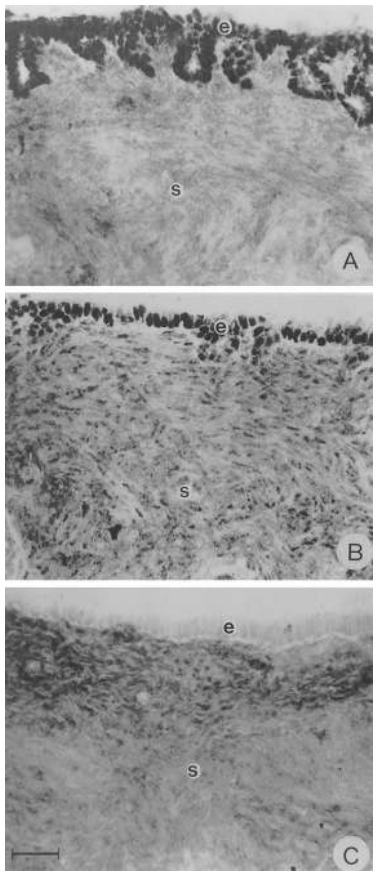

FIG. 7. Frozen sections of ovarian cortex from one monkey during the early follicular phase of the menstrual cycle. Germinal epithelium (e) exhibited intense nuclear staining when the antibodies against ER(A) or PR(B) were used. When the first antibody was replaced with antibody to antigen B of timothy grass pollen (C), the nuclear reaction product was absent. Sections were counterstained with hematoxylin. Magnification, ×320. *Bar* is 31 μm. [Reproduced with permission from S. Hild-Petito *et al.*: *Endocrinology* 123:2896–2905, 1988 (246). © The Endocrine Society.]

time of laparotomy. Subjects studied were in various stages (follicular and luteal) of the ovarian cycle. Immunohistochemical staining involved the use of a commercially available hERα-directed monoclonal antibody (H222). For the majority of the ovarian samples studied, the results were in agreement with those reported by Hild-Petito *et al*. (246) in that most tissues examined were ER negative. For example, granulosa cells of primordial and preantral follicles as well as stroma and theca cells from all cycle phases were ER negative. In contrast, midfollicular phase antral follicles contained many granulosa cells, which stained weakly to moderately positive for ER. Moreover, by the late follicular phase (pre-LH surge), the dominant preovulatory follicle contained granulosa cells, which stained intensely positive for ER. Interestingly, two dominant follicles taken from women at the time of the LH surge (LH = 73.8 and 63.4 mIU/ml, respectively) and judged to be immediately preovulatory were only faintly ER positive. All luteal phase cells were ER negative including newly formed corpora lutea secured on menstrual days 15 and 16. When nondominant follicles were examined, only faint ER immunoreactivity of granulosa cells was noted during the preovulatory period. However, when examined at the time of the LH surge, granulosa cells of nondominant follicles were judged intensely ER positive. Similarly, granulosa cells taken from nondominant follicles of corpora lutea-bearing ovaries were only faintly ER positive. All atretic follicles at all stages of the ovarian cycle were ER negative. Interestingly, however, the authors do not comment on the status of the germinal epithelium. Of some note is the apparent rapid disappearance of ER positivity after the LH surge. This phenomenon may correspond to the previously documented postovulatory decrease in the ER content of rat (16, 26, 66, 67) and rabbit (250) granulosa cells in response to high levels of LH.

In assessing the validity of this study (249), note must be made of the fact that the fidelity of the technique was confirmed by demonstrating ER staining of control tissues (samples of endometrium and commercially supplied ER-positive tissues). However, negative controls were not used. It is unclear whether the apparent differences between the preceding two studies, in which the same ER_{α} antibody (H222) was used, reflect species specificity or technical differences. Therefore, at the least, early follicular development would seem to occur independent of classical ER-mediated mechanisms.

A subsequent study yielded similar results (251). As part of an investigation of the immunohistochemical localization of the androgen receptor (AR) in the human ovary throughout the menstrual cycle, ovarian samples were also stained for ER and PRs. Using the same methodology as in the previous study, the authors were able to demonstrate immunoreactive ER in granulosa cells of dominant follicles. Here again, a decrease was noted in the degree of positive staining of dominant follicles after the LH surge. Importantly, however, all primordial and primary follicles proved ER negative by this technique as were all luteal and stromal tissues. The authors do not comment on the status of the germinal epithelium or of nondominant follicular material.

That same year Billiar *et al.* (232) reported on their studies using baboon (*Papio anubis*) ovaries obtained from five nonpregnant adult animals in the mid or late follicular phase of the menstrual cycle. The methodology employed to detect ER was similar to that used by Hild-Petito *et al.* (246), *i.e.*, immunohistochemical staining with ERα-directed monoclonal antibodies D75 and H222. Immunohistochemical staining of baboon ovaries revealed ER positivity for nuclei of approximately 30–40% of granulosa cells from ostensibly healthy antral follicles. In contrast, presumptively healthy preantral and atretic graafian follicles exhibited relatively low levels of positive labeling. Stromal and interstitial cells were uniformly ER negative. However, rare thecal nuclei were ER positive.

By 1993, Hutz *et al.* (252) reported on an autoradiographic and immunohistochemical study designed to detect ER in the rhesus monkey (*Macaca mulatta*). Here, six animals were studied of which three were normally cycling and in the luteal phase as judged by the presence of a corpus luteum. For liquid emulsion autoradiography, sections were incubated in the presence of 11.4 pM of [2,4,6,7-^{3}H(N)]-17β-estradiol. The authors report significant binding to the corpus luteum as well as less intense binding to an antral follicle.

However, the latter observation may be compromised by the fact that similar intensity did not appear to differ from that observed in nonspecific controls (*i.e.*, section incubated with unlabeled DES). Overall, signals were considered saturable and were inhibited by coincubation with unlabeled estrogen agonists or antagonists. Immunohistochemical staining was carried out with the human ERα-directed monoclonal antibody H222 at a concentration of 0.5–2.0 μg/ml. Here, however, incubation with a second goat antirat IgG antibody and a peroxidase-amplifying system was employed with an aim to increase signal intensity. The authors report significant positivity for the germinal epithelium as well as for the granulosa cells of antral follicles, interstitial cells, and thecalutein cells. The use of positive or negative control tissues was not reported.

More recently, Suzuki *et al.* (253) set out to correlate the expression of immunoreactive ER, PRs, and ARs with the expression of steroidogenic enzymes in the human ovary. Fifty specimens of apparently normal human ovaries from women of reproductive age were removed in the course of surgery for uterine or cervical carcinoma. The phase of the menstrual cycle was determined by a combined classification system comprised of histological endometrial dating, serum 17β-estradiol, and serum progesterone concentration. Using human ERα-directed monoclonal antibody ER1D5, positive immunoreactivity was observed only in granulosa cells from aromatase-positive antral or preovulatory follicles (n = 2). Importantly, the ability of this antibody to recognize the ERβ variant could not be established at the time and remains uncertain at the time of this writing. Subject to these limitations, primordial, primary, and preantral follicles as well as all stromal/thecal tissue, corpora lutea, and degenerating follicles were negative.

Revelli *et al.* (237) have undertaken to analyze the expression of immunoreactive ERs in the ovary of 25 healthy eumenorrheic women. Specific monoclonal antibodies, directed against the human ERα, were employed in the course of the immunohistochemical evaluation. Granulosa cells stained positively for ER. The proportions of preantral and antral follicles with ER-positive granulosa cells were at 23.1 and 37.5%, respectively. For theca cells, no more than 25% stained positive for ER. Active corpora lutea stained positive for ER in 50% of cases. Corpora albicantes always stained negative. In all subjects, the stroma surrounding both follicles and corpora lutea contained several fibroblast-like cells that stained positive for ER. Oocytes and blood vessels stained negative in all cases.

The identification of a second intracellular ER in 1996 (29), ERβ, has stimulated interest in the role of this newly recognized receptor in ovarian physiology. In rodents, after all, several studies have suggested that the ERβ transcripts predominate in the ovary (61, 64). Moreover, granulosa cells proved to be the major cellular compartment that is home to ER_β transcripts within the ovary (61, 73).

Despite these striking findings on the expression of ERβ in the rat ovary, only few data are available on the pattern of expression of this ER subtype in the primate ovary. Taylor and Al-Azzawi (254) may have provided the first relevant results on the expression of the ERα and ERβ proteins in normal adult human ovaries. ERβ localized to cell nuclei of multiple ovarian cell types, including granulosa cells of small, medium, and large follicles, theca cells, and corpora lutea. ERα, in turn, was only weakly expressed in the nuclei of granulosa cells, but was not expressed in the theca cells or in corpora lutea.

Duffy *et al.*(255), performing Western Blot analysis to evaluate the expression of the ERα and ERβ proteins in the Rhesus monkey corpus luteum, observed that ERβ displayed peak of expression at the mid-late luteal phase and declining levels by the late luteal phase. Unlike ERβ, the inconsistent detection of ERα protein by Western blot suggests that the levels of ERα in the primate corpus luteum are very low.

Saunders *et al.* (58), studying immunoreactivity of both nuclear ERs in the ovaries of human and monkeys (*Callthrix jacchus*) (Fig. 8), used three antibodies directed against different peptide segments of the human ERβ protein. The specificity of these antibodies was validated by performing Western blot analysis. Importantly, the antibodies in question were able to bind recombinant human ERβ, but were unable to do the same for human ERα. The pattern of expression of both receptors in the marmoset was mirrored by that of the human ovaries. Immunoreactive ERα was noted in the nuclei of granulosa cells of medium and large follicles. Small follicles (one or two layers of granulosa cell) proved negative. Given medium-sized follicles, ERα protein expression appeared weaker than noted for ERβ. In contrast, a clearly defined layer of granulosa cells adjacent to the basal lamina proved ERα positive when examined in antral follicles. Unlike ERα, immunoreactive ERβ was identified in nuclei of granulosa cells as early as in small follicles (one or two layers of granulosa cell) as well as in medium-sized and large antral follicles. Not unlike ERα, ERβ revealed a striking pattern of expression within antral follicles wherein the protein appeared to be mainly expressed close to the basal lamina. Expression for both receptors was noted in theca cells surrounding preantral and antral follicles as well as in the germline epithelium.

Pelletier and El-Alfy (256), assessing the cellular localization of ERs in human reproductive organs, identified ERβ immunoreactivity in the nuclei of granulosa cells of growing follicles (all stages, from primary to mature follicles), in the interstitial gland and in the ovarian surface epithelium. Nuclear staining for ERα was noted for the theca, interstitial gland, and the ovarian surface epithelium.

Recent *in vitro* studies on ERα and ERβ gene expression in human granulosa-lutein cells have improved the understanding of the hormonal regulation of ERs in the human ovary (242). Using Western blot analysis, both receptors were identified in cultured human granulosa cells. Treatment of the latter with hCG and GnRH led to down-regulation of the ERα and ERβ proteins. These results suggest that ERβ and ERα may be involved in the regulation of corpus luteum formation in the human (242).

In summary, then, the nonhuman primate ovary is a site of expression of immunoreactive ER at the level of the germinal epithelium (246, 252) and the granulosa cells of healthy antral follicles (232, 252). Both ER subtypes appear to be expressed at the level of granulosa cell, theca cell, and germinal epithelium in nonhuman primates. However, ERα immunoreactivity, unlike ERβ, is restricted to granulosa cells of

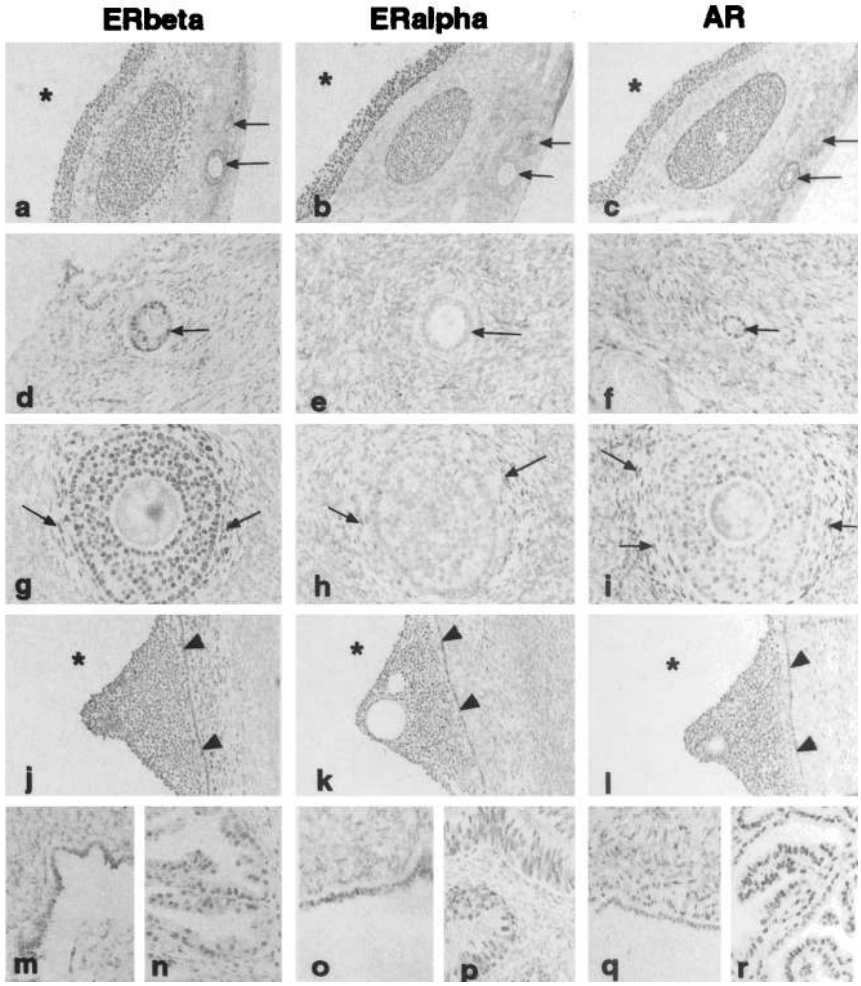

FIG. 8. Comparative expression of ERβ, ERα, and androgen receptor (AR) proteins in marmoset and human follicles. Sections were incubated with antibodies directed against hERβ (anti-P4; a, d, g, j, m, and n), hERα (b, e, h, k, o, and p), or AR (c, f, i, l, q, and r). Panels a–c are closely adjacent sections of the same ovary taken from a marmoset at the late follicular phase of the cycle; note the preantral (*arrows*) and large antral follicles (*). In human ovaries, ERβ and AR proteins were detected in follicles with one or two layers of granulosa cells (d and f), but these cells lacked immunodetectable ERα (e). In addition, ERβ and AR are present in granulosa and theca cells (*arrows*) in a preantral follicle (g and i) and an approximately 10-mm antral follicle (*; j and l). Expression of ERα could also be detected in the theca surrounding both follicles and in the granulosa cell nuclei of the large antral follicle (k). In the preantral follicle (h) containing multiple layers of granulosa cells, a few cells had weak immunopositive staining for ERα. In surface epithelial cells, ERβ (m), ERα (o), and AR (q) were detected. Positive control tissues were as follows: marmoset prostate, ERβ (n) and AR (r); and marmoset uterus, ER (p).

antral follicles (58). The human ovary, in turn, may be a site of expression of immunoreactive ER at the level of the healthy antral granulosa cell and particularly the preovulatory granulosa cell from "dominant" follicles (237, 249, 252, 253). Granulosa cells of nondominant follicles may also be ER-positive at the time of the LH surge (249, 252). As for nonhuman primates, immunoreactive ERα and ERβ were identified in granulosa cells, theca cells, and the ovarian surface epithelium (58, 254, 256). Active corpora lutea stain positive for both ERs (254). The human oocyte was noted as negative (237). Clearly, additional immunohistochemical studies are required using specific antisera for both ERs to establish the relative preponderance of ERα and ERβ receptor proteins in the ovary. Careful delineation of the cross-reactivity of the antibodies employed appears essential.

VII. Is an Estrogen-Free (or at Least Poor) Intrafollicular Environment Compatible with Follicular Development, Ovulation, and Corpus Luteum Formation?

A. Follicular "expansion" vs. follicular "growth": an important conceptual distinction

In the following text, evidence will be displayed that follicular "expansion," oocyte maturation, fertilization, and, at least, initial embryonic cleavage are possible in a local microenvironment variably devoid of estrogenic support. Special note, however, must be made of the use of the term follicular "expansion" as distinct from the term follicular "development" or "growth." The reasons for these distinctions draw on the realization that sonographic evidence of antral formation cannot be equated with the cytodifferentiative and mitotic events associated with "true" follicular maturation, since sonography technology, although a great tool, is a nonmechanistic, nonfunctional method of evaluation of ovarian follicular cell differentiation and function. As such, the following studies suggest that suboptimally primed follicles may fail to respond to the subsequent ovulatory trigger. Consequently, follicular "expansion" in the face of a substantial hypoestrogenic environment may not be compatible with a successful ovulatory cascade. Indeed, the simple accumulation of follicular fluid and hence the formation of a sonographically detectable antrum does not necessarily imply appropriate granulosa cell proliferation and differentiation, nor does it attest to the corresponding alterations occurring at the level of the theca-interstitial cell compartment. Consequently, in the interest of conservatism as well as accuracy, the term follicular "expansion" is best used to emphasize that the conclusions rely heavily on sonographic technology.

B. Lessons learned from the 17α-hydroxylase/17–20 lyase deficiency

To further elucidate the role of estrogen in primate/human folliculogenesis, use can also be made of "experiments of nature" *i.e.*, women afflicted with inborn errors of metabolism that prevent the biosynthesis of estrogens. Of special interest is the example of the 17α-hydroxylase/17–20 lyase deficiency form of congenital adrenal hyperplasia (257), a defect associated with marked impairment of glucocorticoid, androgen, and estrogen biosynthesis. Although the women in question suffer from hypergonadotropic hypogonadism and sexual infantilism, early reports noted the presence of many primary and secondary follicles in ovarian material (258). In fact, many of the patients displayed bilateral multicystic ovaries at the time of laparotomy (259–261). Although substantial follicular atresia has been noted, follicular development up to and including the antral stage has been observed. However, preovulatory follicles are not identified and frequent atretic small graafian follicles are seen (259).

Of particular relevance to this review is a patient afflicted with virtually complete 17α-hydroxylase/17–20 lyase deficiency who, despite castrate levels of estrogens, underwent an apparently successful induction of ovulation associated with progressive follicular "expansion." Oocyte retrieval, *in vitro* fertilization, and early embryonic cleavage followed suit (262). Before treatment, the patient displayed undetectable plasma 17β-estradiol levels ($E_2 < 20$pg/ml). Of concern however, is the observation that the circulating gonadotropin levels (FSH and LH levels of 28.5 and 9.7 IU/liter, respectively) were lower than might be expected in a patient with this condition. Conceivably, this may represent a partial enzyme deficiency. Vaginal sonography revealed multiple ovarian follicle-like structures (8–26 mm). Subsequent treatment was carried out against the backdrop of adrenal suppression with prednisone designed to ensure blood pressure control as well as to decrease the circulating levels of adrenal-derived progesterone, the potentially adverse impact of which on endometrial (and perhaps follicular) maturation was projected. Controlled ovarian stimulation entailed GnRH-mediated pituitary down-regulation upon which "pure" urinary hFSH was superimposed for 14 days at increasing doses of up to 300 IU/day. Ovulation was triggered with hCG (Fig. 9). Follicular development was monitored by vaginal sonography.

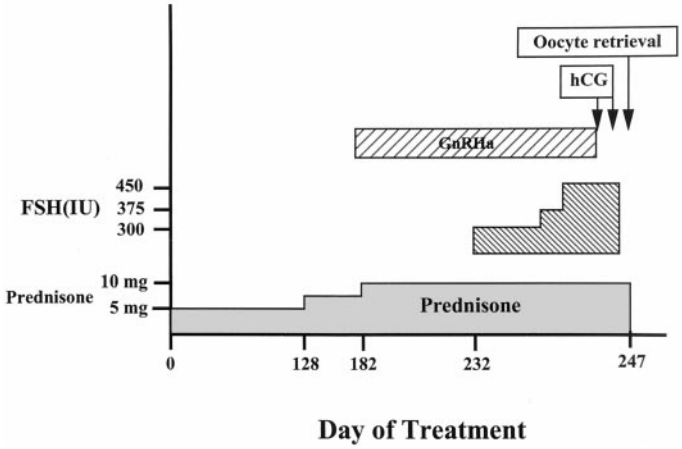

FIG. 9. Superovulation paradigm was applied to a female subject with virtually complete 17α hydroxylase/17–20 lyase deficiency. [Derived from (262).]

Throughout ovulation induction, serum 17β-estradiol levels as well as serum androstenedione levels remained undetectable. As expected, the circulating levels of LH also remained quite low at 3.5 IU/liter. The circulating levels of FSH, however, increased progressively in keeping with the exogenously provided hFSH injections (Fig. 10). Despite the backdrop of profound hypoestrogenism, sonographic monitoring revealed progressive follicular "expansion," the largest follicle exceeding 18 mm in diameter (Fig. 11). Several follicles were noted to develop. Overall, three apparently antral follicles were aspirated, each yielding an oocyte surrounded by a corona/cumulus complex. Two of the three oocytes fertilized and cleaved (albeit suboptimally) to the seven-cell stage in 60–75 h after aspiration, only to be arrested thereafter.

Strikingly, follicular fluid 17β-estradiol concentrations in the patient under study were 103–104 times lower than those

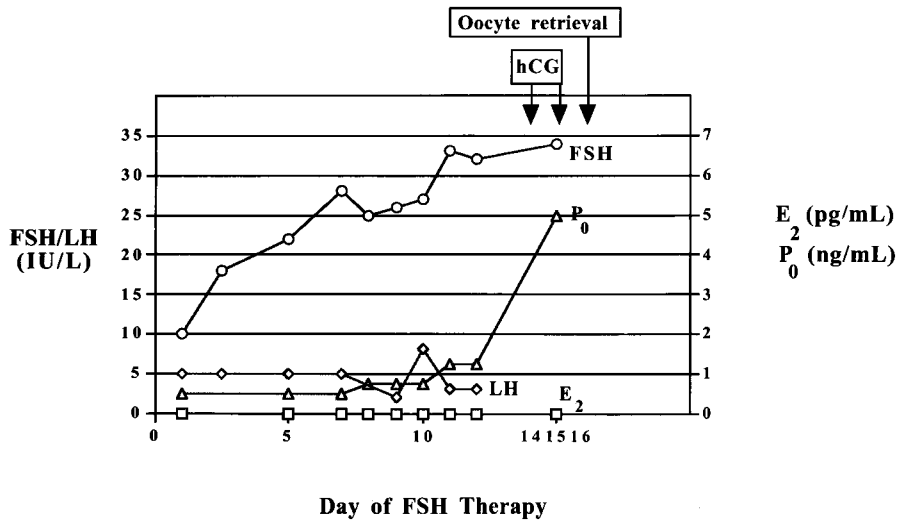

FIG. 10. Circulating levels of estradiol (E_2), progesterone (Po), LH, and FSH in the course of superovulation as applied to a female subject with virtually complete 17α-hydroxylase/17–20 lyase deficiency. [Derived from (262).]

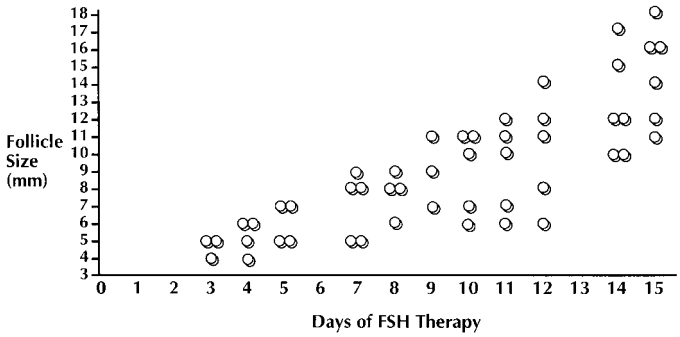

FIG. 11. Sonographically determined ovarian follicular size in the course of superovulation as applied to a female subject with virtually complete 17α-hydroxylase/17–20 lyase deficiency. [Derived from (262).]

encountered in control follicles (30, 22, and 272 pg/ml *vs.* 0.36–1.96 μg/ml). Intrafollicular testosterone levels were similarly reduced 100–1,000 times relative to those encountered in controls. To eliminate possible non-17β-estradiol estrogenic substances in the follicular fluid, the authors set out to assay the "total estrogen-active" materials using a recently developed RRA (263). The latter is based on the competitive inhibition of binding of radiolabeled 17β-estradiol to rat uterine receptors. Using this approach, the authors confirmed the intrafollicular environment of the index subject to be extremely hypoestrogenic with follicular fluid values of 0.15 and 0.23 nmol/liter (normal range 1,140–2,400) of ER binding equivalent (EBE), a level similar to that documented in the serum of castrate women (264).

Cultured granulosa cells derived from the index patient were able to survive, grow, and proliferate *in vitro* despite the virtual absence of estrogenic support (264). When compared with granulosa cells from normal controls, those from the affected patient produced, as expected, higher levels of progesterone but lower levels of testosterone, androstenedione, and 17α-hydroxyprogesterone. Basal 17β-estradiol production by the relevant granulosa cells was also significantly lower relative to controls (0.11 *vs.* 3.67 pg/10^5 cells) as was the total content of estrogenic substances by EBE (0.14 *vs.* 4 pg/10^5 cells). Similar differences were noted after *in vitro* supplementation of granulosa cells with exogenous testosterone or androstenedione. Specifically, the addition of 10^{-7} M testosterone or androstenedione led to an approximately 100-fold increase in 17β-estradiol production by control and affected granulosa cells, thereby suggesting the presence of significant endogenous aromatase activity in the face of an extreme hypoestrogenic environment.

In summary, then, markedly reduced to nonexistent intrafollicular and circulating concentrations of 17β-estradiol are compatible with follicular "expansion," retrievable and fertilizable oocytes, as well as with cleavable and apparently transferable embryos. However, the actual viability of the embryos remains to be demonstrated.

C. Lessons learned from the 3β-hydroxysteroid dehydrogenase (3β-HSD) deficiency

To further examine the role of estrogen in primate/human folliculogenesis, Zelinski-Wooten *et al.* subjected a group of cycling female Rhesus monkeys to ovulation induction with hFSH (Metrodin, 30 IU im twice daily, days 1–6 beginning with menstruation) and hFSH + hLH (Pergonal 30 IU im twice daily beginning on day 7), in the absence or presence of the 3β-HSD inhibitor trilostane (TRL) given on days 1–8 of the menstrual cycle (265). Follicular development was monitored by transabdominal sonography, ovulation being triggered with hCG (Profasi, 1,000 IU im on day 8). Oocytes were obtained by follicular aspiration 34 h after hCG administration.

As a measure of the utility of TRL, the authors assessed the circulating levels of pregnenolone, the immediate precursor/substrate of 3β-HSD. The circulating levels of pregnenolone were shown to be substantially, enhanced (66-fold) in TRL-treated primates in keeping with an apparently effective blockade of 3β-HSD activity. Importantly, treatment with TRL led to a reduction in serum 17β-estradiol levels to 7% of that of control animals throughout the follicular phase. Despite this dramatic reduction in 17β-estradiol levels, neither the total number of large antral follicles per animal (17 ± 1 *vs.* 18 ± 2) nor their size distribution differed significantly from TRL-untreated controls. Furthermore, the overall maturation pattern of collected oocytes (atretic, prophase I, metaphase I, or metaphase II) was not altered by TRL treatment. Note was made, however, of a reduction in the percentage of metaphase II oocytes that were successfully fertilized (15 *vs.* 65%). Moreover, metaphase I oocytes that required more than 8 h to complete meiosis *in vitro* failed to fertilize in three of four animals receiving TRL relative to controls (31%). Taken together, these observations suggest that follicular development and the completion of meiosis may be unaffected by the low estrogen levels but that cytoplasmic oocyte maturation and/or function could be unfavorably affected. Indeed, the acquisition of oocyte competence for fertilization may require adequate amounts of intrafollicular steroids.

In summary, then, markedly reduced circulating concentrations of 17β-estradiol are compatible with seemingly normal follicular "expansion" as well as retrievable oocytes of seemingly normal quantity and maturity. However, fertilization rates appear to be adversely affected. Consideration must also be given to the possibility that TRL-treated animals may display nonspecific side effects relative to the therapeutic agent independent of its 3β-HSD-inhibitory activity. Clearly, no evidence exists to either support or negate such a possibility. However, complete interpretations of the findings of this study must include consideration of this issue. Consideration must also be given to the realization that low circulating levels of estrogen need not be equated with low intrafollicular concentrations of estrogen. This important qualification was pointed out by Mannaerts and associates (266) in their studies of the immature hypophysectomized rat. Under those circumstances, treatment with recombinant FSH led to significant follicular "expansion" in the face of moderately reduced circulating levels of 17β-estradiol. However, under these very same circumstances, systemic therapy proved capable of promoting an increase in the intraovarian 17β-estradiol content, a phenomenon possibly causally related to follicular "growth" and to increased ovarian weight.

D. Lessons learned from aromatase deficiency

In yet another experimental model, the aromatase inhibitor 1,4,6-androstatrien-3,17-dione (ATD) was used as a means to inhibit estrogen production during gonadotropin-mediated ovarian stimulation (267). Specifically, cycling female rhesus monkeys (n = 6) were treated beginning at menses with a regimen of 30 IU hFSH im twice daily given on days 1–6 followed by 30 IU hFSH and 30 IU hLH for 3 days. On day 10, hCG (10,000 IU im) was given to induce preovulatory follicular maturation. In addition, four animals received twice daily oral (1.0–1.25 g) ATD (Steraloids, Wilton, NH), an aromatase inhibitor. Follicles were aspirated at laparoscopy 27 h after hCG, the resultant oocytes being subjected to fertilization *in vitro*. The follicular and hormonal response to stimulation as well as the numbers and function of the oocytes obtained were evaluated.

As expected, animals treated with ATD displayed a drastic reduction in serum 17β-estradiol levels to 37% of that of controls within 8 h of ATD treatment and to 16% of control by the day of hCG injection. In turn, the circulating levels of androstenedione rose. Despite the drastic reduction in the circulating levels of 17β-estradiol and the increase in the circulating levels of androgens, the overall number of large antral follicles (16 $\pm$ 3 for controls and 20 $\pm$ 3 for ATD-treated) and their size distribution (as assessed by ultrasonography) proved comparable for control and ATD-treated animals. Similarly, no difference was noted in the number of oocytes collected or in the proportion of oocytes reinitiating meiosis (MI at the time of collection). In contrast, ATD-treated animals displayed a marked increase (31 *vs.* 11%) in the proportion of prophase I oocytes. Moreover, ATD-treated oocytes displayed retarded *in vivo* completion of maturation to MII (4% *vs.* 26%). Interestingly, the latter retardation was not observed *in vitro*. Furthermore, two of the four ATD-treated animals yielded oocytes that were morphologically abnormal. Finally, oocytes from ATD-treated animals displayed significantly reduced rates of fertilization (9% *vs.* 25%) as compared with controls. However, the cleavage rate after successful fertilization was similar for ATD-treated *vs.* ATD-untreated controls. *In vitro* cultures of granulosa cells collected at the time of oocyte aspiration revealed equivalent 24-h progesterone production in treated and control animals.

Overall, these observations suggest that the acute reduction in the circulating levels of 17β-estradiol during the terminal stage of gonadotropin-induced stimulation had little effect upon follicular recruitment and "expansion." However, an apparent detrimental effect upon gametogenic function may, in fact, exist. In this respect, these observations differ from those made in the aromatase-inhibited rat for which a reduction was noted in the number of follicles that completed maturation and were able to ovulate *in vivo* (267). In part, however, this latter difference may reflect the timing and duration of aromatase inhibition.

More recently, Selvaraj *et al.* (268, 269) and Shetty *et al.* (270) reported on a related experimental paradigm designed to examine the effects of blocking estrogen biosynthesis during the follicular phase on follicular maturation in the adult female bonnet monkey. The experimental design called for the administration of the aromatase inhibitor CGS 16949A by Alzet mini-pump from day 3 of the menstrual cycle. This approach resulted in 53% and 70% reduction in the basal and surge levels of 17β-estradiol, respectively. However, no obvious effect was noted on follicular maturation, ovulation, and luteal function as assessed by serum hormone profiles as well as by laparotomy. Moreover, the concurrent administration of FSH and an aromatase inhibitor resulted in the suppression of the FSH-induced increase in the circulating levels of 17β-estradiol (by 100%). Still, no effect was noted on either the number of follicles developed or their size relative to control. Granulosa and theca cells, removed on day 9 of the treatment cycle, proved responsive to gonadotropins under *in vitro* circumstances, disclosing no evidence to the effect that cellular development and maturation of follicular cells were significantly affected.

It may be important to note, however, that the above mentioned experimental paradigms, not unlike some of the preceding paradigms, represent a hypoestrogenic but by no means an estrogen-free circumstance. Above and beyond this qualification, interpretation of the findings is further confounded by the difficulties in distinguishing between the impact of decreased circulating levels of 17β-estradiol and those attributable to increased circulating levels of androgens. Last, but not least, consideration must be also given to the possibility that ATD-treated animals may display nonspecific side effects relative to the therapeutic agent independent of its estrogen-inhibitory activity. Although there is little evidence to either support or negate the latter possibility, complete interpretation of the findings of this study must include consideration of this issue. Moreover, whether abrogation of oocyte nuclear maturation after aromatase inhibition *in vivo* is due to androgen excess and/or a reduction of 17β-estradiol remains to be determined.

More recently, an extreme example of complete aromatase deficiency in the adult human female was described (271, 272). Specifically, two mutations were detected in the CYP 19 gene in an 18-yr-old 46 XX female afflicted with ambiguous external genitalia, primary amenorrhea, sexual infantilism, and multicystic ovaries. Evaluated at birth for ambiguous external genitalia, the patient was judged to be afflicted with a nonadrenal form of female pseudohermaphroditism. The fetal masculinization could, in retrospect, be ascribed to defective placental conversion of C19 steroids to estrogens leading to increased levels of C19 steroids, which can be converted to testosterone peripherally. At 17 months of age, normal internal female genital structures were identified at laparotomy. The ovaries at that time were grossly and microscopically normal. At puberty, the clitoris had progressively enlarged to 4 $\times$ 2 cm, and pubic and axillary hair were Tanner stage III. Basal plasma testosterone levels were elevated at 95 ng/dl, androstenedione levels were 185 ng/dl, and plasma estrone and 17β-estradiol levels were undetectable. ACTH and dexamethasone tests indicated a nonadrenal source of testosterone and androstenedione. Plasma FSH and LH hormone levels were markedly elevated. Quantification of urinary steroids by gas chromatography-mass spectrometry indicated normal levels of C19 and C21 steroids but very low levels of estrone and 17β-estradiol. Sonography and magnetic resonance imaging showed multiple bilateral 4 to

6 cm ovarian cysts. Thus, the pubertal failure and the development of multicystic ovaries at the normal age of puberty may be attributed to aromatase deficiency and the consequent elevation of FSH and LH levels. Estrogen treatment resulted in a decrease in plasma gonadotropins, breast development, a prepubertal growth spurt, menarche, and regression of the ovarian cysts.

Most recently, Morishima *et al.* (273) reported on the aromatase deficiency in a 28-yr-old 46 XX proband followed since infancy. This woman, afflicted with ambiguous genitalia at birth, went on to develop progressive signs of virilization at puberty with no evidence of estrogen action. In addition, note was made of hypergonadotropic hypogonadism, multicystic ovaries on pelvic sonography, and tall stature. Physical examination disclosed Tanner stage 2 pubic hair, facial comedones and acne, but no breast development. The bone age proved marginally retarded. The basal concentrations of plasma testosterone, androstenedione, and 17α-hydroxyprogesterone, were elevated whereas plasma 17β-estradiol was low. Cyst fluid from the multicystic ovaries displayed a strikingly abnormal ratio of androstenedione and testosterone to 17β-estradiol and estrone. Hormone replacement therapy led to breast development, menses, resolution of ovarian cysts, and suppression of the elevated FSH and LH values. Analysis of genomic DNA in transformed lymphoblasts indicated a single mutation in exon IX of the CYP19 gene associated with marked reduction of aromatase activity (0.2% of the aromatase activity of the wild-type enzyme). Around age 13, the patient underwent abdominal exploration revealing several multiloculated cystic masses in both ovaries, the largest of which measured 8 $\times$ 6 cm. Follicular cyst fluid displayed very low concentrations of 17β-estradiol and estrone ($<$ 1/1500 th of the normal value). Biopsy specimens of the cystic masses revealed both stages of involuting follicles, excessive atresia of follicles, increased thickness with more collagen in the tunica, and dense fibrotic subcortical stroma. The pathology was judged consistent with multicystic ovaries.

It is clear, once again, that follicular "expansion" is possible in the virtually complete absence of estrogens, presumably under the influence of elevated gonadotropins. Although these findings support the thesis that follicular antrum formation is, in fact, possible in the face of complete estrogen deficiency, the health of such follicles and their oocytes is subject to question. Yet another more recent case, reported by Mullis *et al.* (274), proved confirmatory. The relevant literature was recently summarized by Bulun (275).

E. Lessons learned from the intensely hypogonadotropic model

A relevant clinical study was performed by Couzinet *et al.* (276) involving women with surgically induced panhypopituitarism (n = 6) or congenital isolated gonadotropin deficiency (n = 4). All subjects had previously been hospitalized so as to establish the absence or presence of pulsatile LH secretion. All subjects displayed no detectable LH pulsation. Plasma LH and FSH levels proved to be $<$2.5 mIU/ml, with none of the patients responding with gonadotropin increments to iv GnRH administration. It is this population, which was subjected to a cross-over study, performed with partially purified urinary hFSH + hLH (hMG-Inductor, Searle, Paris, France) and highly purified urinary hFSH (Fertiline, Searle, 0.8% endogenous LH activity). A mouse Leydig cell *in vitro* bioassay was used to assess the LH activity of the purified hFSH preparation revealing bioactivity of 0.064 IU/ampoule (*i.e.*, LH content 0.09%).

Patients were treated with 225 IU hFSH/day im for 10 days, hCG being given 24 h after the last hFSH dose. After a 3-month waiting period the patients were switched to the hMG arm of the study. Importantly, given treatment with hFSH, plasma LH levels remained undetectable, as did urinary LH excretion. The mean basal serum 17β-estradiol level, 11 pg/ml, increased slowly to a day 10 (preovulatory) peak of 207 pg/ml (Fig. 12). The latter was judged significantly lower when compared with levels reached in both normally cycling women and in the hypogonadotropic women treated with hMG. Serum estrone levels remained low, rising from 14 to 82 pg/ml, while circulating androstenedione and testosterone levels were not significantly increased (androstenedione rose from 20 to 40 ng/dl; testosterone rose from 20 to 30 ng/dl). Ovulation was presumed to have occurred in six of nine women treated with gonadotropins who received hCG, an inference made from the post-hCG plasma levels of 17β-estradiol and progesterone.

It must be pointed out, however, that none of the subjects

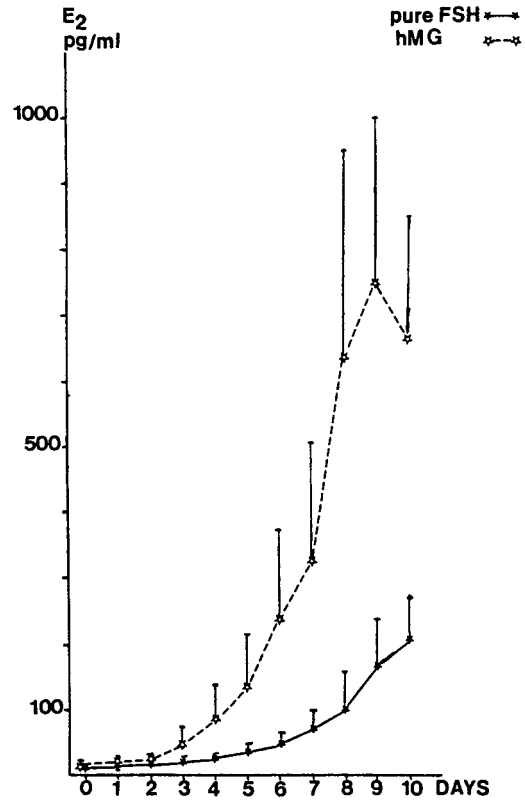

FIG. 12. Circulating levels of 17β-estradiol (E_2) as assessed by RIA over a 10-day period during which ovulation induction was carried out either with highly purified urinary hFSH (pure FSH) or partially purified urinary hFSH + hLH (hMG). [Reproduced with permission from B. Couzinet *et al.*: *J Clin Endocrinol Metab* 66:552–556, 1988 (276). © The Endocrine Society.]

under study were LH free. Indeed, some of the subjects displayed minute levels of LH in extracted and concentrated urinary samples. Moreover, there was a finite, albeit minute, amount of exogenous LH activity even in the purified FSH preparation. This LH "contamination" notwithstanding, 17β-estradiol levels were significantly lower than normally encountered. Even though follicular "expansion" was demonstrated by ultrasound in all women regardless of the plasma estrogen level, note was made of the fact that the circulating levels of 17β-estradiol at the time of hCG administration were lower in the three women who did not ovulate as compared with their ovulatory counterparts (18, 34, and 114 *vs.* a mean of 207 pg/ml). As such, these observations might indicate that the health and functional capacity of the follicle might be related to the intrafollicular level of estrogens.

Note must also be made of a case study by Shoot *et al.* (277) of a patient with congenital isolated gonadotropin deficiency. The purpose of this phase I study was to assess the pharmacokinetics and safety of a recombinant hFSH preparation. Conception was not attempted. The recombinant form of human FSH (recFSH) is, by definition, devoid of LH activity, and therefore an excellent tool for the investigation of the effects of truly pure FSH. An *in vitro* mouse Leydig cell testosterone bioassay revealed recFSH to possess intrinsic LH bioactivity of less than 0.025 mIU LH/IU FSH as compared with 2.4 mIU LH/IU FSH activity for partially purified urinary FSH (278). Baseline serum studies confirmed the patient's diagnosis by revealing FSH levels of 1.2 IU/liter and LH levels of 0.37 IU/liter (Fig. 13). The corresponding circulating 17β-estradiol levels were 17 pg/ml. Interestingly, baseline ultrasound examination revealed numerous ovarian follicles <4 mm in diameter even before ovarian stimulation was begun. Treatment consisted of the administration of recombinant hFSH (75 IU/day im during the first week followed by 150 IU/day im during the second week). On treatment day 13, gonadotropins were discontinued according to protocol when the leading follicle reached 14 mm in diameter. Six days later, ultrasound examination revealed multiple ovarian follicles (six of which were 12–18 mm in diameter), the three leading ones being subjected to aspiration. hCG (10,000 IU) was then administered intraperitoneally, and hormonal and follicular sonographic monitoring was continued for 3 additional weeks. For comparison purposes, follicular fluid was obtained by aspiration of dominant and nondominant follicles from 23 normally cycling women.

As expected, treatment with the recombinant hFSH led to an increase in the circulating levels of FSH to 8.5 IU/liter, serum LH levels remaining low at 0.09 to 0.38 IU/liter (Fig. 13). Serum 17β-estradiol levels (Fig. 14) peaked at only 64 pg/ml on day 15 (day of maximal follicular size), endometrial thickness increasing by only 2 mm during treatment (Fig. 13). Not unexpectedly, no oocytes were obtained at the time of follicular aspiration. Follicular fluid LH and FSH levels were undetectable. Similarly, follicular fluid 17β-estradiol levels were limited to an average of 1.48 ng/ml (Fig. 14). Such intrafollicular 17β-estradiol concentrations contrast sharply with those encountered in unstimulated controls, *i.e.*, 3.965 ng/ml for large follicles.

As such, this study demonstrates that follicular "expansion" is possible in the face of an 17β-estradiol-poor (but not 17β-estradiol-free) intrafollicular microenvironment. The above notwithstanding, the preceding study does not shed light on the ovulatory capacity of "expanded"/17β-estradiol-poor follicles, nor is any insight derived as to the functional adequacy of the oocytes in question.

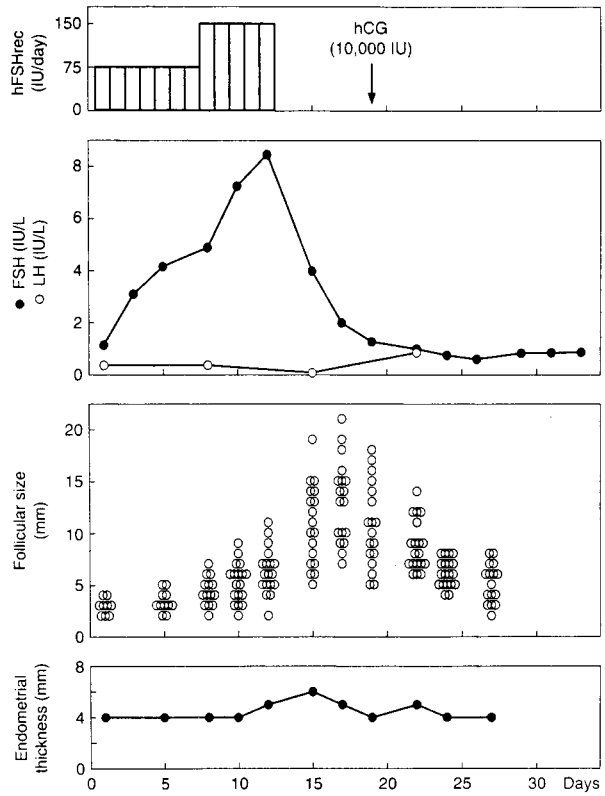

FIG. 13. Recombinant hFSH and hCG dose regimen administered to a patient with isolated gonadotropin deficiency. Serum FSH and LH levels are shown in the *upper two panels*. Diameters of separate follicles as determined by vaginal sonography for both ovaries, and sonographic estimation of endometrial thickness are shown in the *lower two panels*. [Reproduced with permission from D.C.J.M. Schoot *et al.*: *J Clin Endocrinol Metab* 74:1471–1473, 1992 (277). © The Endocrine Society.]

More recently, Shoham *et al.* (279) reported on two volunteer women afflicted with hypogonadotropic hypogonadism who underwent successful follicular "expansion" with recombinant hFSH as part of an open phase I clinical trial study designed to assess the safety and pharmacokinetics of a recFSH preparation. Both patients were diagnosed with isolated hypogonadotropic hypogonadism with FSH and LH levels of less than 1.5 IU/liter. The treatment protocol and response are demonstrated in Fig. 9. Both patients refrained from exogenous estrogen therapy for a period of 30 days before the study. Stimulation with recombinant hFSH (Organon 32489 with specific activity 15 IU/mg protein) was continued until a follicle of 14 mm was detected by transvaginal ultrasound. Since neither patient desired pregnancy, hCG was not given. Gonadotropins were administered in a stepwise fashion. Patients received 75 IU hFSH for cycle days 1–7 followed by 150 IU for days 8–14, followed by 225 IU for days 15–17. One patient received an additional 4 days of treatment at the 225 IU/day dosage. Final steady-state FSH

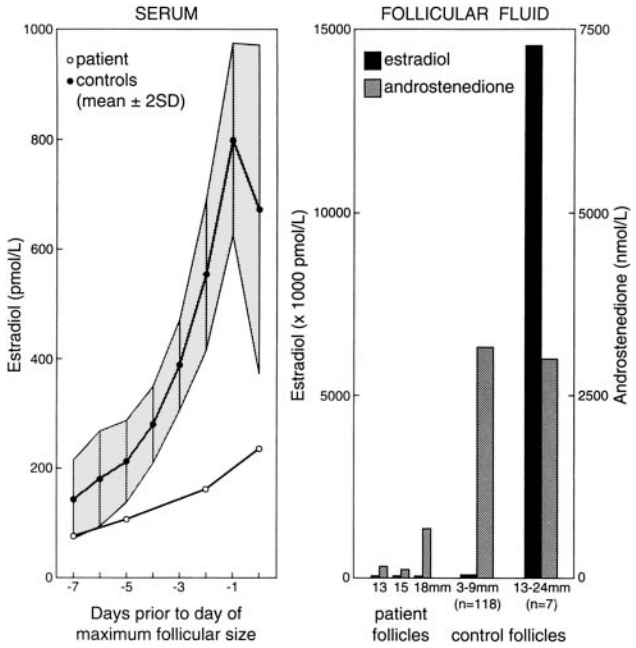

FIG. 14. *Left panel,* Circulating levels of estradiol as measured in a patient with isolated gonadotropin deficiency after the administration of recombinant hFSH before the day on which ovarian follicles reach their maximum size (day 17). As a reference, the circulating levels of daily estradiol (mean ± 2 SD) are shown for seven normally cycling women up to the day of the LH peak. *Right panel,* Intrafollicular concentrations of 17β-estradiol and androstenedione in three separate follicles (13, 15, and 18 mm diameter) obtained from a patient with isolated gonadotropin deficiency after the administration of recombinant hFSH. Median 17β-estradiol and androstenedione concentrations in small (3–9 mm) follicles (n = 118), and large (13–24 mm) follicles (n = 7) are shown. [Reproduced with permission from D.C.J.M. Schoot *et al.*: *J Clin Endocrinol Metab* 74:1471–1473, 1992 (277). © The Endocrine Society.]

levels were 11.8 and 10.1 IU/liter. Mean serum LH levels for the two patients did not differ from those at baseline (0.11 IU/liter *vs.* 0.15 IU/liter). Similarly, serum androstenedione and testosterone levels also did not significantly increase from baseline. Neither patient displayed detectable serum levels of progesterone at any point in the stimulation protocol. No change in endometrial thickness was observed in either patient during the study period. Each patient demonstrated a small, but finite, increase in serum 17β-estradiol levels to 21 and 38 pg/ml, respectively.

At the conclusion of the stimulation period, the first patient displayed three ovarian follicles as detected by ultrasound ranging from 11–17 mm. The second patient displayed six follicles ranging from 8–17 mm. It must be noted that a single follicle (13 and 15 mm) was detected in each of the patients before stimulation. As such, these observations and ones reported earlier (280) further support the notion that follicular "expansion" can occur in a strikingly hypoestrogenic environment.

Schoot *et al.* (281) also reported on the ovarian response of intensely hypogonadotropic women to recombinant hFSH. Some of the seven women under study had previously been subjected to hypophysectomy. Others suffered from isolated gonadotropin deficiency or from hypogonadotropic hypogonadism of the Kalmann type. Circulating serum FSH and LH levels in these subjects were 0.25 and 0.06 IU/liter, respectively. The study protocol called for the injection of recombinant hFSH for 3 weeks, the dose being increased from 75, to 150, to 225 IU/day in weekly increments. Whereas FSH concentrations were increased as expected, serum LH concentrations remained unchanged. Interestingly, two of the subjects in question displayed absent follicular development, but a labile rise in the circulating levels of immunoreactive inhibin. These patients were deemed to suffer from ovarian failure or the resistant ovary syndrome, prompting their exclusion from the study protocol. The remaining five subjects displayed circulating immunoreactive inhibin concentrations compatible with levels encountered during the normal late follicular phase. In contrast, circulating 17β-estradiol levels only increased to a mean of 21 pg/ml the range being 5–57 pg/ml. Importantly, sonographic follicular "expansion" to preovulatory size was clearly apparent despite the limited 17β-estradiol response. As such, these findings are compatible with the concept that follicular recruitment and "expansion" are possible in the face of minimal circulating estrogen concentrations. Comparable findings were reported by Balasch *et al.* (282).

Akin to the above studies are several others wherein intact subjects were first pretreated with a GnRH agonist to downregulate pituitary gonadotropin secretion only to be stimulated thereafter with either (crudely purified) hFSH/hLH, partially purified urinary FSH preparations, or rec FSH (281). Although this paradigm is a theoretically valid hypoestrogenic model and may provide for interesting data, one must keep in mind that current GnRH agonists may not effect complete pituitary gonadotropin suppression (283). Moreover, even modest concentrations of serum LH are compatible with normal ovarian follicular development (283, 284). Clearly, partially purified urinary FSH preparations are not without some residual LH activity.

Another drawback related to the conclusions that may be extracted from the above mentioned hypoestrogenic model is the fact that the human ovary is the site of expression of genes for GnRH and its receptor (285–287). It is even possible that the human ovary possesses an intrinsic GnRH axis, although a full understanding of the role of this axis within the human ovary is yet to be realized (286). Thus, the administration of pharmacological doses of GnRH agonists (or antagonists) might directly perturb ovarian physiology that cannot be underestimated. Consequently, FSH-primed/GnRH agonist-treated subjects may not constitute a truly representative model of an estrogen-free environment.

GnRH antagonists, in turn, may produce more complete suppression of pituitary gonadotropin levels and may theoretically eliminate the residual LH activity that confounds studies with GnRH agonists (288). While ovulation induction with rechFSH in intact GnRH antagonist-pretreated animals is theoretically an excellent hypoestrogenic model, even this paradigm represents, at best, an estrogen-attenuated circumstance. Here, cynomolgus monkeys were treated with the GnRH antagonist antide (Nal-Lys-GnRH, 3 mg/kg/day) for 20 days beginning in the midluteal phase. After 10 days of treatment with the antagonist, animals were randomized to treatment with either (289) rechFSH (10 IU/day; n = 3), rechFSH (20 IU/day; n = 3), or hFSH +hLH (20 IU/day; n =

3). At the lower dosage, the rechFSH failed to stimulate either follicular growth (as judged by ultrasonography) or 17β-estradiol production. At the higher dosage of rechFSH, however, multiple follicular development was documented (by ultrasonography), in the face of relatively low circulating levels of 17β-estradiol (257 ± 53 pg/ml on the day of follicular aspiration). Animals treated with both FSH and LH displayed equivalent numbers and sizes of follicles in the face of significantly higher circulating 17β-estradiol levels. Follicular fluid 17β-estradiol levels were consistent with the serum levels. These observations were taken to mean that in this relatively LH-deficient primate model, FSH alone is capable of stimulating ovarian follicular growth despite markedly reduced 17β-estradiol production.

In a related publication, Zelinski-Wooten *et al.* (290) evaluated the role of recombinant human FSH with and without recombinant LH after 90 days of GnRH antagonist (Antide) treatment in macaques. Multiple follicular growth required a longer interval after recombinant FSH than recombinant FSH plus recombinant LH, but the total number of follicles per animal did not differ between groups. The circulating levels of 17β-estradiol were 4-fold lower in recombinant FSH-treated animals as compared with those subjected to combination therapy. In contrast, more oocytes completed meiosis to metaphase II and fertilized after recombinant FSH therapy as compared with combination therapy. Follicular growth and maturation in LH-deficient macaques occurred with FSH alone, thereby confirming the role of FSH in follicular expansion. Interestingly, however, the apparent higher fertilization rates associated with the FSH alone stimulation argues for a possible deleterious effect of LH in this regard, a notion currently under active investigation.

F. Impact of estrogen deficiency on oocytic and early embryonic development

Several of the studies cited above appear to indicate that an estrogen-poor or estrogen-free intraovarian environment in the primate may adversely affect oocyte quality and early embryonic development (265). These findings could be in keeping with the apparent presence of ERα transcripts in human oocytes (233). In this context, an experimentally-induced 3β-HSD deficiency in the primate model was associated with a marked reduction in the fertilization rate (265). Likewise, systemically induced aromatase blockade was associated with a detrimental effect on oocyte function (267). Similar conclusions were also reached by Couzinet *et al.* (276) whose findings suggested that the health and functional capacity of the follicle might be related to the intrafollicular level of estrogens.

To assess the quality of FSH-primed/estrogen-free follicles, Wang and Greenwald (291) undertook to treat adult hypophysectomized mice with recombinant hFSH (4 μg/day) twice a day for 3 days, beginning 12 days after hypophysectomy. Concurrent with the last FSH injection, hCG was injected to induce ovulation. Animals were mated and killed 1–4 days later. Interestingly, treatment with FSH alone produced large preovulatory follicles. The ovulation rate proved virtually normal for hCG-triggered mice (90% as distinct from 100%). However, successful mating was noted in only 45% of the hCG-triggered mice, fertilization being limited to 56% of the oocytes so generated. The significantly decreased percentage of successful mating is striking. With a 1:1 ratio of mating females and males, the fertilization rate should be about 90% since the ovulation rate is 100% in hCG-triggered groups. Moreover, only 5% of ovulated eggs developed to four-cell stages *in vivo* by day 3 after hCG triggering. Comparable results were secured *in vitro*. Indeed, embryos formed consequent to hCG triggering displayed significant meiotic arrest manifested by the observation that only 22% (rather than 80%) of two-cell embryos were converted to blastocysts by 96 h (rather than 72 h) of culture. Eleven percent of one-cell embryos divided to the four-cell stage. Although hCG triggering resulted in a virtually immediate increase in the secretion of progesterone, a post-hCG increase in 17β-estradiol levels was not noted until day 2. Thus, in hypophysectomized adult mice, FSH alone induces the growth of follicles to preovulatory stages under estrogen-free circumstances. Subsequent ovulation induced by hCG results in limited fertilization and limited development of preimplantation embryos *in vivo* and *in vitro*. These abnormalities could be due to the insufficiency of follicular steroids, the immaturity of the oocytes, and the presumably abnormal oviductal environment.

VIII. The Primate/Human Ovary as a Site of Estrogen Action

Unlike the nonprimate, far less information is available on the direct effects of estrogen on the primate/human ovary. In large measure, the relative paucity of information as it relates to the primate reflects the complexity and expense associated with this experimental model. The inherent limitations imposed on human investigation account, if only in part, for the limited amount of information pertaining to the human ovary. The above notwithstanding, this section attempts to summarize key observations relative to the primate/human ovary secured under either *in vitro* or *in vivo* circumstances.

A particularly elegant model is represented by the immature cynomolgus macaque paradigm. In a series of studies reported by Koering *et al.* (292), use was made of this hypogonadotropic model to assess the impact of systemic estrogen therapy on ovarian function. Given the presumed quiescence of the hypothalamic-pituitary axis, the effects of estrogen on the ovary could be deemed direct in nature. In an elegant cross-over experimental design in which each animal served as its own control, animals were treated with 20 mg/kg/day of DES for 14 days. One ovary each was removed before and after treatment and numbers of follicles were counted and compared. Examination of the ovaries revealed that after treatment with DES 1) numbers of primordial follicles were unchanged, 2) preantral follicles decreased in number, 3) antral follicle numbers decreased slightly, and 4) numbers of early atretic follicles remained unchanged. This was in sharp contrast to the many well documented studies carried out in hypophysectomized immature rats wherein treatment with DES caused a clear stimulation of follicular development. The finding of no increase

in atresia rates suggests that DES treatment arrested the development of antral follicles, thereby diminishing developmentally associated atresia. Similar observations were reported in a follow-up publication (293).

A significant focused line of studies, summarized by Hutz *et al.* (294) made use of the adult cycling rhesus monkey (*Macaca mulatta*). In this model, treatment with estrogens led to atresia of the dominant follicle. Specifically, Silastic 17β-estradiol-containing capsules were inserted subcutaneously for 24–48 h. Treatments that resulted in peripheral 17β-estradiol plasma levels in excess of 300 pg/ml for greater than 24 h were associated with irreversible atresia of the dominant follicle. Unfortunately, this treatment regimen may also be associated with suppression of the circulating levels of FSH followed by a rise in gonadotropins with removal of the capsules. Thus, it is possible that a central effect (*i.e.*, withdrawal of FSH support) is the cause of the observed follicular atresia.

In an effort to address the limitations of the *in vivo* paradigm, Hutz and associates (295) assessed whether 17β-estradiol in amounts similar to those found in monkey follicular fluid directly alters *in vitro* progesterone accumulation by granulosa cells aspirated from the follicles of cycling rhesus monkeys. Follicular contents were aspirated from three to five animals on each of days 8–13 of the cycle. Granulosa cells were incubated with zero or 2–200 ng/ml of 17β-estradiol and the cultures maintained for 72 h. These experiments revealed that 17β-estradiol, at concentrations found in follicular fluid, can inhibit progesterone output by monkey granulosa cells, thereby supporting a direct effect and by inference independence from a central phenomenon.

Further complexity was introduced by the observations of Harlow *et al.* (296) whose *in vitro* studies made use of the common marmoset monkey. Specifically, granulosa cell cultures from reproductively suppressed monkeys were cultured for 48 h in the absence or presence of hFSH, with or without various sex steroids. Expectedly, treatment with hFSH produced dose-dependent stimulation of granulosa cell steroidogenesis. As was previously documented in the rodent, the concurrent addition of testosterone produced substantial amplification of the hFSH effect. Similar amplification was noted for 5α-dihydrotestosterone, a nonaromatizable androgen, but not for 17β-estradiol, thereby suggesting specific androgenic synergism with FSH. These studies were taken to mean that androgen may play a local role in the regulation of FSH-stimulated granulosa cell function during follicular development in primates. Moreover, these findings suggested that unlike the rodent (42–49), estrogen is incapable of modulating FSH hormonal action as it relates to the cytodifferentiation in the primate granulosa cell. However, as demonstrated by Shaw and Hodges (297), the above conclusions must be tempered by the recognition that the findings are highly contingent upon the experimental design in question. Indeed, given an identical experimental model, prior 17β-estradiol priming was associated with an increase in FSH and cAMP-supported steroidogenesis. Moreover, concurrent pretreatment with tamoxifen produced complete abrogation of this estrogenic effect. Taken together, these observations suggest that subject to the experimental design of the study, estrogen may either prove stimulatory to FSH-supported granulosa cell steroidogenesis or else prove inert.

To further distinguish between the latter possibilities, Shaw and associates (298) undertook to reinvestigate the role of estrogen and IGF-I in the modulation of FSH action in cultured marmoset granulosa cells. Animals underwent oophorectomy in the follicular phase, and all follicles ≥0.5 mm were dissected and granulosa cells harvested. Granulosa cells were cultured in the presence of increasing concentrations of IGF-I (1–30 ng/ml), with and without 17β-estradiol (10^{-7} M for 48 h) up to a maximum of 6 days. While IGF-I was able to increase progesterone production after ≥4 days of culture, the addition of 17β-estradiol was without effect. This is in contrast to aromatase activity for which the addition of an 17β-estradiol/IGF-I combination was associated with a 13- to 18-fold increase after 2 days of culture. Results were corrected for cell number to control for any potential effects of 17β-estradiol on granulosa cell proliferation. Taken together, these observations suggest yet another set of select circumstances wherein estrogen may be stimulatory to marmoset granulosa cell steroidogenesis. The apparent divergent actions of estrogen in the context of the mammalian ovary were elegantly summarized by Hutz (299), whose observations provide the additional benefit of a comparison with an avian paradigm.

Taken together, the preceding observations, partially reviewed elsewhere (300), clearly document the primate antral follicle as a site of estrogen action. However, no uniform pattern appears to emerge in that the outcome was often highly contingent upon the experimental paradigm under study. The relatively limited nature of these conclusions is further compounded by the recognition of the paucity of data as they relate to the human paradigm. Clearly then, additional studies would be required to clarify the precise role estrogens play in the modulation of the function (as opposed to growth) of the somatic ovarian cell in both the primate and human ovary.

IX. Estrogen Reception and Action: The Nonclassical Alternative(s)

When determining the status of "classical" ovarian ERs, consideration must be given to the possible involvement of estrogens (if any) by way of alternative (and admittedly hypothetical) mechanism(s). Traditional theory has held that steroids function via binding to nuclear receptors with subsequent activation of gene transcription and protein synthesis. However, the possibility does exist that estrogens act via nonclassical mechanisms in the ovary. Such a model might involve nonclassical nuclear ERs, novel forms of nonnuclear (possibly membranous) ERs, other "orphan" receptors of the steroid/thyroid receptor superfamily, or non-receptor-mediated events.

In addition to the conventional hormone-dependent regulation of the activity of members of the steroid/thyroid receptor family, some studies demonstrated substantial interaction between signal transduction pathways and steroid receptors. In some cases, the modulation of kinase/phosphatase activity in cells caused the activation of steroid re-

ceptors in the absence of hormone. Nuclear receptors have been reported to be activated by different signaling pathways, including those stimulated by the neurotransmitter dopamine (301, 302), growth factors such as EGF (303–305), transforming growth factor-α (TGF-α) (303), and IGF-I (303, 306). The so-called ligand-independent mechanism of steroid receptor activation has been described for different steroid receptors, such as for progesterone (307), androgen (308), and ERs (309). Although a complete understanding of the molecular mechanisms underneath the ligand-independent activation of steroid receptors is yet to be uncovered, in the case of the ER there is evidence that altered receptor phosphorylation may play a role in this process (310, 311).

Recent evidence from diverse lines of investigation has suggested that novel forms of steroid hormone receptors may exist and act in heretofore unrecognized mechanisms. In support of this possibility, Kudolo *et al.* demonstrated an additional class of low-affinity cytosolic ERs in rat granulosa cells (312–314). Importantly, this receptor could not be demonstrated in the nucleus. It has also been suggested that there exists a family of membrane-associated rapidly acting steroid receptors, which function independently of protein synthesis. Indeed, membrane-associated receptors have been suggested for aldosterone (315, 316), glucocorticoids (317–321), androstenedione (322), testosterone (323, 324), and progesterone (325–334) in human and nonhuman cells, including the *Xenopus* oocyte. These receptors have been demonstrated to mediate rapid nongenomic effects (*in vitro*), which may be transduced via guanine nucleotide-binding protein(s) or ion channels. Similar receptors have been localized to the membrane of amphibian and mammalian neurons and may be the route of action of "neurosteroids" (*i.e.*, steroids that are produced and act locally in the CNS), which appear to have a rapid onset of action (335–340).

Special attention must also be given to the work of Pietras and Szego (341–343), who observed that estrogen may have a rapid membrane-mediated action in the rat endometrial cell. Specifically, estrogen administration proved capable of inducing luminal morphological changes in microvilli within 30 sec (344). Further studies by this group have partially isolated ostensible "estrogen receptors" from the hepatocyte plasma membrane (343). Cardiovascular examples were also described (345).

In this context, it has been recognized that the effects of estrogen on the cardiovascular system may be produced through either the classical (genomic) activation of transcription factors or newly identified rapid (nongenomic) direct actions on the vasculature (346–348). Animal and human studies have disclosed that physiological levels of estrogen can rapidly cause vasodilation (346). Reports in human subjects support that this effect is largely mediated by activation of endothelin nitric oxide synthase (eNOS) (349, 350). Indeed, it appears that ERα mediates the short-term effects of estrogen on eNOS activity (351, 352). In cells lacking ERα and eNOS, the acute response of eNOS to estradiol may be achieved by cotransfection of cDNA for those two proteins (351).

Note should be made of the contribution of Morley *et al.* (353), who investigated the effects of steroids on the intracellular calcium ionic concentration in chicken granulosa cells obtained from the two largest preovulatory follicles of laying hens. The results indicate that estrogens almost instantaneously trigger the release of calcium from intracellular stores, an effect that may be mediated through phosphoinositide breakdown. The striking rapidity of this estrogen-induced internal calcium mobilization is consistent with the activation of a cell surface receptor, which is different from the conventional nuclear ER.

As is true for the granulosa cell, one must not exclude the possibility of membrane-mediated nongenomic estrogenic effects at the level of the oocyte. In this respect, the demonstration of rapid 17β-estradiol-induced alteration in intracytoplasmic calcium economy (354) strongly supports such a possibility. Specifically, it has been suggested that local intrafollicular estrogen may directly and nonclassically (*i.e.*, nongenomically) affect the developmental potential of human oocytes. Tesarik and Mendoza (354) observed 17β-estradiol-mediated modulation of intracellular calcium oscillations at the time of germinal vesicle breakdown (GVB), in a process analogous to that noted in amphibians. Human oocytes were aspirated in conjunction with micromanipulation-assisted fertilization procedures. Those oocytes that had not undergone GVB, and which did not display signs of degeneration, were loaded with the calcium indicator dye fluo-3. Relative changes in the intracellular concentration of calcium were visualized via confocal scanning microscopy of emitted fluorescence. No spontaneous calcium fluctuations were observed in control oocytes incubated in the absence of estrogen. In contrast, oocytes incubated in the presence of 17β-estradiol (1 μmol/liter) displayed a transient increase in calcium followed by a series of secondary calcium oscillations lasting 1–6 h (Fig. 15). These calcium oscillations were observed in both intact and zona-free oocytes, yet occurred with a quicker onset in zona-free cases. The triggering of calcium oscillations was specific to estrogens and was not observed after the administration of androstenedione or progesterone. The site of 17β-estradiol action was apparently the oocyte plasma membrane in that membrane-impermeant estradiol-17β-estradiol conjugated to BSA was as effective a stimulus as free 17β-estradiol. A suggestion was also made as to an effect of 17β-estradiol on the developmental potential of the oocyte. A subgroup of oocytes were incubated *in vitro* in an effort to promote maturation to the MII stage. Although 17β-estradiol supplementation had no effect on the percent of oocytes that matured *in vitro*, those oocytes that were matured in the presence of 17β-estradiol were more likely to develop two pronuclei and to cleave at least once (following *in vitro* insemination with spermatozoa) than oocytes incubated in the absence of 17β-estradiol. This observation suggests a potential effect of estrogen upon early oocytic or embryonic development.

A study by Razandi *et al.* (355) demonstrated membrane and nuclear-associated estrogen binding sites after the transfection of Chinese hamster ovary (CHO) cells with either ERα or ERβ cDNA. The nuclear estrogen-binding component was attributed to nuclear localization of both ERα and ERβ. Competitive binding studies of the nuclear and membrane fractions revealed very similar dissociation constants. Estrogen binding to ERα and ERβ in CHO cells resulted in the rapid activation of Gaq and Gas proteins as well as induction of

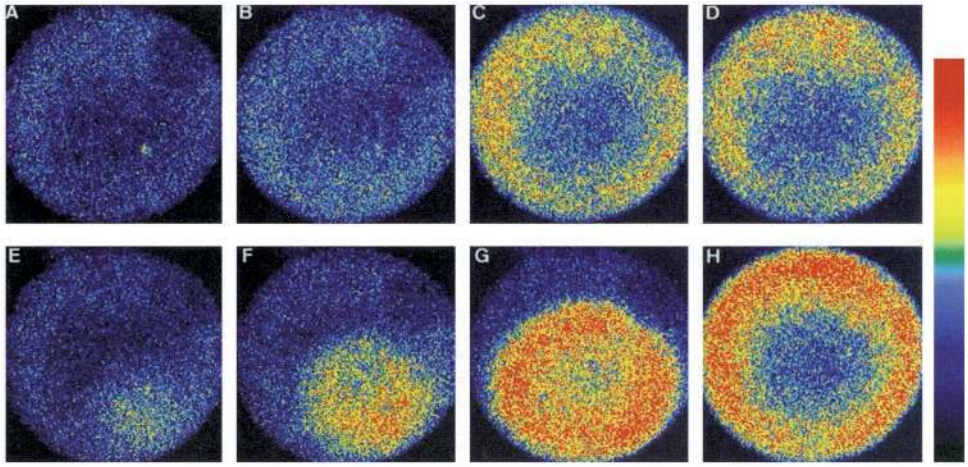

FIG. 15. Spatial dynamics of intracellular free calcium concentration ([Ca^{2+}]i) elevations induced by estradiol in a maturing oocyte. The [Ca^{2+}]i changes are in pseudocolor according to the *scale bar*, where the lowest values are coded *black*. A–D, Rising phase of initial [Ca^{2+}]i increase after estradiol addition to a zona-free oocyte. The time interval between successive images displayed in this series is 4 sec (every fourth image from a series of recordings taken at 1-sec intervals is shown). E–H, Rising phase of the first of the series of secondary [Ca^{2+}]i elevations after the initial estradiol-induced [Ca^{2+}]i increase. The time interval between successive images displayed in this series is 1 sec. [Reproduced from J. Tesarik and C. Mendonza: *J Clin Endocrinol Metab* 80:1438–1443, 1992 (354). © The Endocrine Society.]

inositol phosphate production and adenylate cyclase activity. The induction of these signaling molecules subsequently led to the activation of the mitogen-activated protein kinase ERK. c-Jun N-terminal kinase activity was stimulated by estrogen in cells expressing the ERβ isoform. This study suggests that plasma membrane and nuclear forms of the ER arise from a single transcript and that the membrane-associated receptors are linked to downstream signaling entities.

X. Summary

It was the purpose of this review to critically evaluate the role of estrogen in primate/human follicular development as compared with other species. This section attempts to answer some of the key questions raised:

1. Is the primate/human granulosa cell a site of estrogen reception? Given what is currently known, the answer to this question is an affirmative one. On the one hand, molecular probing failed to detect classical ERα in granulosa cells of human origin (233) or in granulosa cells of primate origin (236). Another study (234), however, suggested the presence of ERα transcripts in luteinized human granulosa cells. Some of the above studies contrast, however, with immunohistochemical studies wherein both the primate (246) and human (237, 249, 251, 253) granulosa cells have been noted to be ER positive, particularly at midcycle and in dominant follicular structures. It is possible, since the primate/human granulosa cell is also a site of ERβ expression, that the immunoreactivity noted in some of the preceding studies constitutes cross-reactivity with ERα-directed antisera. Yet another primate study, however, failed to note immunoreactive ER in granulosa cells (252). The precise reasons underlying the apparent discrepancies between the various studies remain uncertain at this time. The fetal human ovary is a site of ERα and ERβ gene expression, the ERβ transcripts being more abundant (238). Recent probing establishes the presence of ERβ transcripts in the adult human ovary and the adult granulosa cell (59, 239). Note must also be made of the newly discovered ERβcx the potential role of which in inhibiting ERα-mediated estrogen action must be taken into account (244). Finally, consideration must also be given to the possibility that estrogen may be acting at the level of the granulosa cell by means other than classical nuclear ERs. The convincing demonstration of an apparent nongenomic action of estrogen at the level of the chicken granulosa cells (rapid release of intracellular calcium) supports such a possibility (353). Thus, on balance, estrogen action at the level of the primary/human ovary is undoubtedly representative of the net impact generated by the relative contribution of the various ER subtypes at the genomic or nongenomic levels.

2. Is the primate/human oocyte a site of estrogen reception? Given what is currently known, the answer to this question is uncertain. Indeed, a single study wherein molecular probing (PCR) was employed (233) detected ERα transcripts in the human oocyte. Revelli *et al.* (237), however, failed to document ERα transcripts at the level of the human oocyte. No immunoreactive data are available to confirm or negate these observations. No information exists at this time as to the possibility that the human oocyte is a site of ERβ gene expression. Given the apparent import of estrogen to normal cytoplasmic gametogenic maturation (112, 199, 267, 269, 354), it is possible that classical oocytic ERs may subserve a meaningful functional role. However, as is true for the granulosa cell, one must not exclude the possibility of membrane-mediated nongenomic estrogenic effects. In this respect, the demonstration of rapid 17β-estradiol-induced alteration in intraoocytic calcium economy strongly supports such a possibility (354).

3. Is an estrogen-free/poor intrafollicular environment compatible with follicular development, ovulation, and

corpus luteum formation in the primate/human? To the extent that follicular "development" entails antrum formation and therefore sonographically detectable "expansion," the answer is an affirmative one. Indeed, in a manner not unlike that observed in the rodent, an estrogen-free/poor environment is compatible with FSH-induced increments in ovarian size, weight, and antral volume. Thus, the antrum-forming property of FSH, long suspected and herein confirmed once again, is perhaps best illustrated by an environment that may possibly be estrogen free (or at least estrogen poor), *i.e.*, the case of the aromatase-deficient woman (271–275). However, it is not immediately apparent that follicular "expansion" needs to be equated with true optimal follicular development, the ingredients of which must include enhanced granulosa cell division and differentiation as well as gametogenic maturation. In contrast, however, the very process of follicular rupture and the process of corpus luteum formation appear to proceed unperturbed, despite the fact that the antecedent follicular phase was characterized by an estrogen-free/poor intrafollicular environment (265, 267).

4. Is an estrogen-free/poor intrafollicular environment compatible with gametogenic maturation in primates/humans? The answer to this question is probably a negative one. Indeed, a number of primate studies appear to indicate that an estrogen-free/poor intrafollicular environment is associated with marked decrements in the rates of meiotic maturation and fertilization (82, 265). Data derived from rodent models further suggest a compromise in early embryonic development (291).

XI. Directions for Future Research

Future research must settle the question of whether or not the primate/human granulosa cell or oocyte are, in fact, sites of estrogen reception. Studies of granulosa cells and of oocyte ER mRNA expression need be repeated in human/primate models. In this context, strong emphasis could be placed on surgically derived human ovarian material for which the confounding variable of vaginal mucosal contamination is a nonissue. This material could be subjected to intense immunohistochemical staining (using variant-specific antibodies) and to careful molecular probing using a battery of complementary and therefore confirmatory techniques. In addition, a significant effort must be invested in the probing for ERβ transcripts and in the detection of the corresponding protein by means of specific antisera. In so doing, one should be able to establish whether or not the primate/human ovary is in fact a site of estrogen reception and to establish the relevant preponderance of the relevant ER subtypes.

More dynamic *in vitro* circumstances may be employed to address the same issues. For example, the development of long-term human granulosa cell lines may prove helpful in this context. However, the maintenance of the differentiated phenotype by the latter, ideally representative of the different phases of follicular development, is not attainable at this time. Moreover, long-term cultures of follicles *in vitro*, an emerging technology, may also be put to good use under circumstances that ensure either the complete suppression of aromatase or the antisense-mediated blockade of the corresponding transcripts. Such an approach may well provide substantial insight into the relative role of estrogens in folliculogenesis and in the maturation of the oocyte. Reliance on follicular material from ERα or ERβ knockout mouse models may prove indispensable.

In this context, continued *in vivo* studies of the hypogonadotropic state may prove useful in conjunction with the employment of recombinant FSH preparations. Specifically, GnRH antagonist-treated subjects may be assessed for their estrogen-independent follicular potential. Still, it must be emphasized that such experimental settings may fall short of perfection, thereby failing to yield unequivocal results. Further evaluation will also have to be undertaken in settings wherein the intraovarian concentrations of 17β-estradiol are approaching or indeed zero (*e.g.*, aromatase deficiency). It must be pointed out, however, that circulating estrogen deficiency cannot be equated with intraovarian estrogen deficiency (266). Hence, it is conceivable that some intraovarian estrogen formation may be possible even in the complete absence of LH (266), a poorly understood phenomenon possibly attributable to extant intraovarian or circulating androgenic substrates.

Finally, one must keep an open mind as to the possibility that ovarian estrogen action is mediated, in part, through a nongenomic mechanism. With rapid advances in membrane and ion channel physiology, there is every reason to believe that new insight will be derived in this evolving area.

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