CHAPTER

25

Steroid Receptors in the Uterus and Ovary

April K. Binder, Wipawee Winuthayanon, Sylvia C. Hewitt
Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NC, USA

John F. Couse
Taconic Farms, Albany Operations, Rensselaer, NY, USA

Kenneth S. Korach
Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, NC, USA

INTRODUCTION

The ovarian-derived sex steroid hormones dictate the uterine estrous or menstrual cycle in mammals and are therefore essential to the establishment and maintenance of pregnancy. The uterine response to the preovulatory rise in circulating estradiol (E2) is required to prepare the tissue for the forthcoming rise in progesterone (P) that accompanies ovulation and is critical to embryo implantation. This physiological coordination between the ovary and uterus is common to the large majority of mammals studied and the spectrum of actions and effects of the sex steroids in uterine tissue are mediated by their cognate NRs. Estrogen was first described almost 100 years ago as a substance that induced estrus. We now know from many studies over the past 50 plus years that its physiological activity is broader than simply inducing a secretory response in the reproductive tract—estrogen also affects multiple organs that previously were not considered estrogen responsive. The mechanistic theory for explaining the biological actions of steroid hormones came in 1960 when Elwood Jensen and colleagues first described the uptake and retention of labeled E2 in certain tissues that, because of this retention, became referred to as estrogen target tissues. The ovary and uterus were amongst the tissues showing this property. The hormone tissue retention concept was then shown to be reflective of other steroid hormones, exemplified by progesterone, glucocorticoid, and thyroid hormones. The Jensen and Gorski laboratories were then the first to show that the tissue retention of estrogen was due to the presence of specific proteins contained within those tissues, which became referred to as estrogen receptors (ER). Numerous research groups followed with studies showing that the receptor proteins were hormone specific and their presence was linked to some biological activity in certain tissues. Subsequent development some 20 years later of the first antibodies to the ER allowed for more direct detection and qualification of the protein levels in tissues under different physiological states or disease conditions. Although initially the dramatic physiological effects of estrogens on the mammalian uterus were appreciated and well described, later investigations began to elucidate the precise mechanisms and signaling pathways involved. Our understanding of steroid hormone action in the uterus has been greatly advanced by utilization of new technologies with the generation of knockout and knock-in models, including receptor or co-activator null, tissue and cell selective null, and mutant knock-in models in mice. These models are also used in combination with microarray, RNA-seq, and ChIP-seq methods that allow for comprehensive mapping of interaction of nuclear receptors (NRs) with chromatin and modulation of genomic response to steroids in uterine
tissues. Together, these models and techniques have led to better understanding of the molecular details of steroid receptor roles in biological processes.

Receptor-mediated actions of progestins, androgens, and estrogens are central to reproduction. Perhaps unique among the various categories of signaling pathways, the sex steroid hormones and their cognate receptors are surely mentioned, if not discussed in detail, in every chapter of this volume. The sex steroid hormones are integrated into every aspect of mammalian reproductive physiology in both sexes, including sexual maturation and development, gametogenesis, hypothalamic–pituitary control of gonadal function, sexual and maternal behavior, pregnancy, and lactation. Disruption of the signaling pathways for any one of the three gonadal steroids leads to reduced fecundity, if not infertility, due to aberrations in multiple organ systems. Therefore, a thorough understanding of the sex steroid receptors in terms of their expression in reproductive and endocrine tissues, their mechanism of action, their role in reproductive processes, and their interaction with nonsteroidal signaling pathways is instrumental to our ability to manage infertility and reproductive disease.

This chapter principally covers the signaling pathways for progestins, androgens, glucocorticoids, and estrogens, and the role of each in ovarian and uterine function in mammals. Because of the importance of the sex steroids and the likelihood that each is discussed in several other chapters of this volume, it is appropriate to first discuss the structure, mechanism of action, and signaling pathways for each of the sex steroid receptors. Sections then follow on the role of sex steroids in ovarian and uterine function. We chose to divide each of these sections according to the sex steroid receptor family and those mechanisms of receptor action that are most relevant to ovarian and uterine function. The section Sex Steroid Receptors and Ovarian Function discusses what is currently known concerning each particular sex steroid signaling pathway in terms of its expression pattern and distribution within the ovary, and its intraovarian role in granulosa cell proliferation and differentiation, thecal cell function, and ovulation. Because a separate chapter (Chapter 23) is dedicated to the corpus luteum, the role of the sex steroid receptors in luteal function is not covered in detail. The section Sex Steroid Receptors in Uterine Function discusses what is currently known concerning each sex steroid signaling pathway in terms of its expression pattern and distribution within the uterus and its role in uterine cell proliferation and differentiation. New since the previous editions of this volume is the development of tissue selective null or "knock-in" mouse models for each of the sex steroids. The study of each of these models has already and will continue to make enormous contributions to our understanding of the reproductive role of each receptor signaling pathway in toto. Therefore, we include a detailed description of the ovarian and uterine phenotypes for each of these models in their respective sections.

THE STEROID RECEPTORS

The steroid receptors are part of the family of nuclear receptors (NRs) that is ubiquitous throughout the animal kingdom. The members of the NR family fulfill a plethora of functions and are integral to the development and maintenance of multiple physiological systems. Continued discovery of new NRs has led to a considerable expansion of the NR family and demanded a system of categorization. NRs have been assigned to three classes.\(^1\) Class I includes the steroid receptors (ER, PR, AR, GR), which interact with high specificity to their steroid ligand as well as to DNA motifs comprised of inverted-repeat hexanucleotides separated by a 3-base pair (bp) spacer. The Nuclear Receptors Nomenclature Committee has developed an NR nomenclature scheme that is based largely on sequence homology and evolutionary comparisons and divides the over 60 NRs into seven subfamilies and a varied number of groups within each subfamily.\(^1\) The ERs (ER\(\alpha\) and ER\(\beta\)) are the sole members of the NR3A subgroup, ER\(\alpha\) and ER\(\beta\) being NR3A1 and NR3A2, respectively.\(^2\) The PR and AR are the third and fourth members of the NR3C subgroup that also includes the GR and the mineralocorticoid receptor.\(^4\) The official gene names for these steroid receptors are ESR1 and ESR2 for ER\(\alpha\) and ER\(\beta\), respectively, PGR for progesterone receptor, AR for androgen receptor, and GCR for glucocorticoid receptor. The general properties of the sex steroid receptor family and those mechanisms of receptor action that are most relevant to ovarian and reproductive tract function are outlined following.

Genes, mRNA, and Regulation

The genes encoding the sex steroid receptors exhibit a highly conserved structural organization. The human PR (PGR or NR3C3), AR (AR or NR3C4), ER\(\alpha\) (ESR1 or NR3A1), ER\(\beta\) (ESR2 or NR3A2), and GR (GCR or NR3C1) genes are each composed of eight coding exons, and vary in length from 40 kb (ER\(\beta\)) to >140 kb (ER\(\alpha\)).\(^5\) In each case, the N-terminal domain (NTD) of the receptor is usually encoded by a single exon, the two zinc fingers of the DNA binding domain, each encoded by separate exons (2 and 3), and the ligand binding domain (LBD), encoded by exons 4–8 (Figure 25.1).
Estrogen Receptor

The human \( ESR1 \) (ER\( \alpha \)) cDNA was first cloned in 1985\(^9-11\) and has since been isolated from numerous additional species.\(^{12,13}\) A second ER gene, termed \( ESR2 \) (ER\( \beta \)), was discovered in 1996 in rat\(^14\) and human\(^15\) tissues and has since been cloned from many species.\(^{13}\) Unlike the A and B forms of PR and AR, ER\( \alpha \) and ER\( \beta \) are not isoforms but rather distinct receptors encoded by two genes on different chromosomes. The ER\( \alpha \) proteins are 595 and 599 amino acids in length in human and mice, respectively, with an approximate molecular weight of 66 kDa (Figure 25.1).\(^8,16-18\) Multiple promoter and regulatory regions in the 5'-untranslated sequences of the human and rat \( ESR1 \) gene have been described, yet all possess the same single open reading frame.\(^8\) Numerous naturally occurring variants of the \( ESR1 \) mRNA in normal and neoplastic tissues of several species have been described, but the existence of corresponding proteins remains controversial.\(^{18}\)

The promoter region of the \( ESR1 \) gene has been relatively well characterized and indicates a complex regulation of expression.\(^8,19-21\) Cicatiello et al.\(^{22}\) demonstrated that the proximal 0.4 kb of the mouse \( Esr1 \) promoter is sufficient to provide for widespread but specific expression of a reporter construct in vivo. Regulatory elements that may provide for AP-1, Sp1, and ER autoregulation of the human \( ESR1 \) promoter have been described.\(^{23,24}\) Decreased \( ESR1 \) expression is linked to receptor-mediated actions of vitamin D\(^{25}\) and increased intracellular cAMP or mitogen-activated protein kinase activity.\(^{23}\) Increased methylation of the \( ESR1 \) promoter is implicated in reducing ER levels, especially in tumorigenic tissues.\(^{23}\)

The \( ESR2 \) genes of multiple species yield numerous transcripts that range from 1 to >9 kb in length, in contrast to the single predominant transcript of ~7 kb transcribed from the \( ESR1 \) gene.\(^{18}\) Initial descriptions of human and rodent ER\( \beta \) projected a protein of 485 amino acids. However, it is now apparent that translation of the \( ESR2 \) mRNA initiates upstream of these original open reading frames and yields a receptor of 549 amino acids in rodents and 530 amino acids in humans, each with an approximate molecular weight of 60–63 kDa (Figure 25.1).\(^8\) Therefore, ER\( \beta \) is slightly smaller than ER\( \alpha \), and most of this difference lies within the N-terminus. A number of

---

**FIGURE 25.1** The steroid family of receptors and endogenous ligands. (A) Schematic illustration of the structural organization of the sex steroid nuclear receptors. The more highly conserved C and E domains are depicted as open boxes and the less well-conserved A/B, D, and F domains as filled bars. The F domain is unique to the estrogen receptors (ER). The functions of the modular domains are indicated as are the two known transcriptional activation function domains, AF-1 and AF-2 (as well as AF-3 found only in PRB). The AF-1 domain is harbored in the A/B region and exhibits constitutive activity in vitro, whereas the AF-2 domain lies with the ligand binding (E) domain and is critical to ligand-induced receptor activation. Both domains synergize during ligand-activated receptor actions. NLS, nuclear localization signal. (B) Comparison of the human steroid receptors, the androgen receptor (AR), estrogen receptors \( \alpha \) (ER\( \alpha \)) and \( \beta \) (ER\( \beta \)), progesterone receptor B (PRB) and A (PRA) and the glucocorticoid receptor (GR). The amino acids that compose each domain of the human forms are indicated. For PRA, the numbers refer to the amino acid residues in reference to PRB.
variant transcripts of the *ESR2* gene have been described; however, unlike ERα, there is growing evidence that some of these variants may coexist with the wild-type (WT) receptor form in certain tissues (Figure 25.2). Portions of the mouse *Esr2* gene have been characterized and possess potential binding sites for numerous transcription factors and the initiation of transcription from at least two distinct untranslated exons.

**Progesterone Receptor**

Two distinct promoters in the *PGR* gene provide for the generation of two major PR isoforms, PRB (114 kDa) and PRA (94 kDa), in most species except rabbit, which possess PRB only. The two PR forms are identical in all regions except the NTD, which is truncated by 128–164 amino acids in PRA, depending on the species (Figure 25.1). Both PR isoforms are expressed at relatively equal levels in tissues, although differences in the ratio have been noted in certain tissues. A third isoform, termed PR-C, an N-terminally truncated form that lacks the full A/B domain and first zinc finger of the C domain, has been described, but its expression as a protein is controversial. The level of PR expression is modulated by ER-mediated effects of E2 in certain but not all tissues, and this is especially true in the female reproductive tract. Several naturally existing *PGR* variant transcripts harboring exon deletions or distinct 5′-untranslated sequences have been described but are not well characterized in terms of protein expression or functionality.

**Androgen Receptor**

The *AR* gene is located on the X chromosome, and therefore genetic males possess only a single copy. Two distinct start sites in the *AR* gene are used to produce two isoforms, AR-B (110 kDa) and AR-A (87 kDa), which differ only in the N-terminus (Figure 25.1). However, in contrast to the PR, the two known AR isoforms exhibit only subtle functional differences. A unique feature of the *AR* gene relative to...
its sex steroid receptor counterparts is the presence of polymorphic repeats of glutamine and glycine in the NTD, the former of which has been linked to certain chronic diseases and cancer in humans. There are binding sites for a wide array of transcription factors in the AR promoter, including Sp1, cAMP-response element binding (CREB) protein, and c-myc, suggesting complex tissue-specific regulation. Direct androgen-mediated autoregulation of AR expression has also been shown.

Glucocorticoid Receptor

Although not historically recognized for its roles in uterus and ovary, GR is ubiquitously expressed, and more recent studies have demonstrated important interactions between GR and sex steroid receptor mediated signaling. GR isoforms, are produced by alternate splicing leading to different c-terminal amino acids. GRα is the primary isoform, whereas GRβ is unable to bind GR ligands and can behave as a dominant negative inhibitor of GRα activity.

Receptor Structure

Common to all members of the NR family is a modular structure of domains, each of which harbors an autonomous function that is critical to total receptor action. The sex steroid receptors are composed of five functional modules, an N-terminal domain (NTD) or A/B domain, the DNA binding (C) domain, a hinge (D) region, and an LBD (E) (Figure 25.1). The ERs also possess a unique C-terminal F domain of unknown function. Our understanding of the NR functional domains and their importance to overall receptor activity is largely derived from the in vitro study of artificially generated mutant receptors and more recently from X-ray crystallography studies.

**NTD or A/B Domain**

The NTD or A/B domains of the NR family members greatly vary in length and share little homology among the steroid receptors, although some structural features are conserved (Figure 25.1). Crystallography studies of the steroid receptor NTD have been largely unsuccessful because this portion of the receptor fluctuates in aqueous solutions. However, evidence suggests that intramolecular interactions between the A/B and other receptor domains are likely to induce a more structured NTD. Current models of NR signaling incorporate the flexibility of intrinsically disordered (ID) regions of the receptor, including the NTD, into a mechanism of allosteric interaction and coordination of ligands, DNA motifs, and NR domain functions. The NTD of each of the sex steroid receptors harbors the transcriptional activation function-1 (AF-1) domain and provides for cell and promoter-specific activity of the receptor as well as a site for interaction with co-receptor proteins (Table 25.1). The AF-1 domain alone can confer constitutive transcriptional (i.e., ligand-independent) activity when linked to a heterologous NR DNA binding domain in vitro, but this function is largely overcome in the context of the whole receptor. Posttranslational modifications to the A/B domain can dramatically affect the overall behavior of the receptor and are thought to be an important mechanism for the modulation of AF-1 functions. Phosphorylation of the A/B domain is the most well-characterized posttranslational modification and occurs in all three sex steroid receptors via the actions of multiple intracellular signaling pathways, including mitogen-activated protein kinase pathways, the cAMP/protein kinase A (PKA) pathway, and cyclin-dependent kinases.

The extended N-terminal sequences that are unique to PRB (Figure 25.1) provide a third transcriptional AF-3 domain in this isoform that is lacking in PRA. This may allow for PRB-specific expression of certain P regulated functions.

### TABLE 25.1Steroid Receptor Co-Regulator Complexes

<table>
<thead>
<tr>
<th>Complex</th>
<th>Functions</th>
<th>Comments</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC1, SRC2, SRC3</td>
<td>Interact with Helix12 of agonist-bound NR, interact with SWI/SNF, histone modifiers</td>
<td>Made up of &gt;20 subunits, MED 1-31, arranged in 3 modules (head, middle, tail)</td>
<td>51,52</td>
</tr>
<tr>
<td>Mediator</td>
<td>“Bridges” NR and transcriptional “machinery” (RNA Pol II) to control transcription</td>
<td>Made up of 9+ subunits, examples include BRG1, BRM, BAF subunits</td>
<td>53,54</td>
</tr>
<tr>
<td>SWI/SNF</td>
<td>Regulate access to enhancer sequences via chromatin remodeling, ATPase activity</td>
<td>Acetyltransferase (HAT; e.g., p300/CPB), deacetylase (HDAC; e.g., NCoR), Methyl transferase (e.g., PMRT/CARM), de-methylase</td>
<td>55,56,57</td>
</tr>
<tr>
<td>Histone Modifiers</td>
<td>Modify histones to increase or decrease transcription</td>
<td>Structure made up of 20S catalytic core particles (CP), 19S regulatory particles (RP)</td>
<td>58,59</td>
</tr>
<tr>
<td>26S Proteasome</td>
<td>“Clears” transcriptional modulatory proteins to facilitate subsequent transcription, transcriptional termination</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. FEMALE REPRODUCTIVE SYSTEM
Steroid receptor
Hormone response elements (HRE)

Consensus half-sites

12345
ERE: GGTCAG
GRE: GAACA

Inverted palindromic HRE
with 3 bp spacer

12345 XXX 54321

(A) The DNA binding domain (C) of the sex steroid receptors
is highly conserved and composed of two zinc fingers and a C-terminal extension (CTE). The ERα DBD is shown. Each zinc finger is composed of four conserved cysteine residues that coordinate to chelate a single zinc ion. Helix 1 of the first zinc finger contains the P-box residues involved in the discrimination of the HRE. Residues in the second zinc finger (helix 2) form the D box that provides a dimerization interface. The CTE is critical

(B) Comparison of amino acids in P box region:
ERα, ERβ: CEGCKAFFKRSIQ
NERKI ERα: CAACKAFFKRSIQ
EAAE ERα: CEGCAAFFAESIQ
GR: CGSCKVFKRAVE
AR: CGSCKVFKRAAE
PR: CGSCKVFKRAME

Comparison of amino acids in CTE:
ERα: GMKGGIRKDRGGRMLKH
ERβ: GMVKCGSRERGCYRLVR
GR: GMNLEARKTKK--KIKGIQ
AR: GMTLGARKKLKGLNKLQE
PR: GMVLGGRKFKKFNKVRV

(C)
genes. In general, PRB is a stronger activator of transcription versus PRA when acting on a hormone response element (HRE)–driven gene construct in vitro. Furthermore, antagonist-bound PRB acts as a strong transcriptional activator in certain cell and promoter contexts, whereas antagonist-bound PRA is inactive.

The NTD of the AR is especially autologous in vitro and is likely more important to overall AR transactivation than the C-terminal AF-2 domain (described later). Furthermore, the first 30 residues of the AR NTD are highly conserved and important for interactions with the LBD that provide for agonist-induced stabilization of the receptor. Unique to the AR among the sex steroid receptors are a series of amino acid repeat sequences in the NTD, most notably the polymorphic tracts of glutamine and glycine, of which certain lengths are thought to be linked to various human diseases or cancer.

The greatest structural disparities between ERα and ERβ lie within the A/B domain, which is approximately 30 amino acids shorter in ERβ and exhibits only >20% homology. This divergence likely accounts for the many functional differences that have been revealed from comparative studies of the two ER forms. In general, ERβ tends to be a less effective transcriptional activator compared with ERα when acting in a classic estrogen response element (ERE)–driven mechanism in vitro. An ERα/ERβ chimera in which the A/B domain of ERβ has been replaced with that of ERα is able to activate transcription much better than the native ERβ. Furthermore, certain antagonists (e.g., tamoxifen) that exhibit no such activity with ERα exhibit no such activity with ERβ.

**DNA Binding or C Domain**

The C domain of the NR family members is that portion of the receptor that specifically functions to recognize and bind to the cis-acting enhancer sequences, or HREs, that are located within the regulatory regions of target genes. It is the most highly conserved (55–80%) region among the NR family members. The C domains of ERα and ERβ are practically identical (>95% homology) in most species and are therefore expected to exhibit a similar affinity for the same HREs. The functionality of the C domain is provided by a motif of two zinc fingers, each composed of four cysteine residues that chelate a single Zn²⁺ ion, and are always encoded by separate exons within the gene.

Crystallography studies indicate a highly conserved structure consisting of dual α-helices positioned perpendicular to each other. Amino acids in the C-terminal “knuckle” of the first zinc finger form the “P box” (proximal box) of the DNA binding domain and confer sequence specificity to the receptor; hence, the proximal zinc finger is often referred as forming the “recognition helix” (Figure 25.3). Amino acids at the N-terminal “knuckle” of the second zinc finger form the “D-box” (distal box) and are more specifically involved in differentiating the “spacer” sequence within the HRE as well as providing an interface for receptor dimerization.

The consensus HREs that sex steroid receptors bind are composed of two 6-bp palindromic sequences arranged as an inverted repeat and always separated by a 3-bp spacer (Figure 25.3). Because the P-box residues are identical among the AR, PR, and GR, these receptors bind a common consensus HRE consisting of two 6-bp palindromic half-sites (5′-PuG[GG/AA]-PuGAC) arranged as an inverted repeat and always separated by a 3-bp spacer (Figure 25.3). The consensus HRE bears the same arrangement but is composed of a unique palindromic half-site (5′-PuGGTCA) (Figure 25.3). The inverted-repeat arrangement of the palindromic sequences is unique to sex steroid receptor HREs and dictates that the receptors homodimerize in a “head-to-head” position when bound to DNA. Evidence indicates that the AR can uniquely dimerize in a “head-to-tail” fashion on an HRE of the monomer 5′-GGTCT. More recent structural analysis has revealed the importance of the 10-30 amino acid carboxy terminal extension (CTE) of the DBD in DNA interaction. Although this region is variable between steroid receptors, it is crucial for DNA binding, particularly for sequence selectivity of DNA binding, by extending the interaction surfaces between the receptor and the DNA.

**Hinge Region or D Domain**

Historically, the D domain was thought to primarily serve to connect the more highly conserved C and E domains of the receptor. The previously described CTE extends into the hinge region, which also harbors a nuclear localization signal, and influences cellular compartmentalization of receptors, as well as sites of posttranslation modifications. Current mechanisms suggest this nonconserved and intrinsically disordered (ID) domain is important for intramolecular allosteric
interactions involving the N-terminal and ligand binding domain (LBD)\(^{48}\) (described earlier in the section NTD or A/B Domain). This type of flexible structural interaction works to allow rapid response to diverse modulators governing changes in biological environments.\(^{48}\)

**LBD or E Domain**

The LBD or E domain of the steroid receptors is a highly structured multifunctional region that primarily serves to specifically bind ligand and provide for hormone-dependent transcriptional activity.\(^{12}\) An AF-2 domain located in the C-terminus of the E domain mediates this latter function. The AF-2 domain is subject to posttranslational modifications\(^{12}\) and is an especially strong activator of transcription in the ER and PR but is markedly weaker in the AR, where it is more involved in interactions with residues in the NTD.\(^{37,38}\) Also harbored within the E domain is a strong receptor dimerization interface, sites for interaction with heat shock proteins, and nuclear localization signals.\(^{12,48}\) Although there is minimal homology in the primary sequence of the LBD for the sex steroid receptors, comparative studies of the crystal structures of liganded and unliganded LBDs indicate a highly conserved structural arrangement. These structural studies indicate that the LBD is composed of 11 \(\alpha\)-helices (H1, and H3–H12) arranged in a three-layer \(\alpha\)-helical sandwich to create a hydrophobic ligand binding pocket near the C-terminus of the receptor.\(^{48}\) The resulting shape and volume of the ligand binding pocket is larger than necessary to accommodate the corresponding ligand, suggesting that key interactions between the ligand and specific receptor residues are more critical to conferring ligand specificity.\(^{61}\) Receptor binding to an agonist ligand leads to rearrangement of the LBD such that H11 is repositioned and H12 swings back toward the core of domain to form a “lid” over the binding pocket. This agonist-induced repositioning of H12 leads to the formation of a hydrophobic cleft, or “NR box”, by helices 3, 4, and 5 on the receptor surface, constituting the AF-2, which serves to recruit co-activators (Table 25.1) to the receptor complex. In contrast, receptor antagonists are unable to induce a similar repositioning of H12, leading to a receptor formation that is incompatible with co-activator recruitment and is therefore less likely to activate transcription. The LBDs of PRB and PRA are identical and provide for high affinity binding to P.\(^{12,50}\) The LBD of the AR in humans, rats, and mice is identical and provides for high affinity binding of two endogenous androgens, T and 5α-hydroxyT (DHT), the latter of which binds with much greater affinity.\(^{38,66}\) Numerous high affinity synthetic ligands for PR and AR have been developed, including the agonists R5020 and R1181, respectively (Table 25.2).\(^{12}\) The LBDs of ER\(\alpha\) and ER\(\beta\) exhibit less than 60% homology but bind the endogenous ligand, E2, with similar affinity (ER\(\alpha\), 0.1 nM; ER\(\beta\), 0.4 nM).\(^{8,12,17}\) Both ER forms also bind the synthetic estrogen diethylstilbestrol (DES) with relatively equal affinity.\(^{65}\) However, given the divergence in homology, it is not surprising that ER\(\alpha\) and ER\(\beta\) exhibit measurable differences in their affinity for other endogenous steroids and xenoestrogens.\(^{8,17}\) Natural and synthetic steroidal and nonsteroidal ER agonists and antagonists have been described, some of which show specificity for one or the other ER subtype (Table 25.2), illustrating differences between the LBDs of ER\(\alpha\) and ER\(\beta\), and provide for powerful pharmacological tools to discern the overall function of each ER (Table 25.2). The most widely used ER subtype selective ligands currently in use are propylypyrazole (PPT), an ER\(\alpha\) selective agonist, and diarylpropionitrte (DPN), an agonist showing preference, but not exclusive selectivity, towards ER\(\beta\).\(^{8}\)

**F Domain**

Among the sex steroid receptors, only ERs possess a well-defined F domain (Figure 25.1). This region is relatively unstructured and harbors little known function, although some data indicate a role in co-activator recruitment, dimerization, and receptor stability.\(^{61,62,68–70}\)

**Co-Regulatory Complexes**

All steroid receptors interact with co-regulatory molecules, co-activators, and co-repressors.\(^{51,71}\) The primary co-activator interaction for steroid receptors (SR) is with p160/SRC (steroid receptor co-activator) 1, 2, and 3.\(^{52,72,73}\) SRC1 (NCOA1), SRC2 (GRIP1, TIF2), and SRC3 (pCIP, RAC3, ACTR, TRAM, A1B1) interact with helix 12 of SRs via “LXXLL” motifs in their nuclear receptor interacting domain, which are leucine rich regions with “X” designating any amino acid.\(^{52}\) SRCs also contain activation domains that recruit secondary molecules such as p300, and a bHLH-PAS motif within the N-terminal region, which can interact with other transcription factors.\(^{52}\) The elegant complexity of co-activator composition and function has been increasingly revealed. Steroid receptors and SRCs function as a nexus interacting with massive multimeric complexes, such as the SWI/SNF chromatin remodeler, mediator complex, or proteasomes (Table 25.1).\(^{73}\) These interactions facilitate coordination of the specific functions necessary to allow appropriate gene and cell selective access to chromatin, via modifications of histones or members of co-regulatory complexes.\(^{74}\) In this way, co-activators dynamically mediate and coordinate processes necessary to accomplish transcription, including initiation, elongation, termination, and clearing or turnover of the transcriptional modulators.

**Receptor Mechanisms of Action**

Our understanding of the mechanisms by which steroid hormones and their cognate intracellular receptors influence cell function and behavior has expanded
profoundly since 1988 when Clark and Markaverich reviewed the field for the inaugural edition of this volume. Much of that earlier chapter remains contemporary in reference to general receptor biochemistry and ligand-dependent activation, which is now referred to as the “classical” or ligand-dependent direct-DNA binding model of receptor function (Figure 25.4). In the years since, numerous discoveries have been made that illuminate the complexity of sex steroid receptor signaling in cells and tissues, such as the discovery of additional receptor forms and variants (e.g., ERβ) and high-resolution crystallography of receptor domains. The entrée into the “omics” era has facilitated massive expansion of study of transcriptional regulation and chromatin remodeling. In addition, several alternative receptor signaling mechanisms that diverge from the classic model have become apparent, including “tethering” of the sex steroid receptor to heterologous DNA-bound transcription factors to provide for steroid regulation of genes that lack HRE sequences within their promoter (Figure 25.4); plasma membrane steroid signaling, often referred to as “nongenomic” steroid actions; and ligand-independent “cross talk” with intracellular and second messenger systems that provide for sex steroid receptor activation in the absence of the cognate steroid ligand (Figure 25.4). The existence of multiple receptor-mediated signaling pathways likely accounts for the refined control and plasticity of tissue responses to sex steroids. These modes of sex steroid receptor action as currently understood are discussed following.

Ligand-Dependent Actions: Direct or Classical

The classic model of steroid receptor action states that the receptor resides in the nucleus or cytoplasm but is sequestered in a multiprotein inhibitory complex in the absence of hormone (Figure 25.4). The lipophilic steroid ligands are able to freely diffuse across the plasma and nuclear membranes and bind their cognate receptor. The binding of ligand results in a
conformational change in the receptor, transforming it to an “activated” state that is now available for homodimerization, increased phosphorylation, and binding to an HRE within target gene promoters, and interaction with chromatin and transcriptional mediators. NRs seem to be preferentially recruited to open regions of chromatin. Studies using MCF7 breast cancer cells indicate that FoxA1 acts as a pioneering factor, providing accessible regions in the chromatin that recruit ERα. The ligand-HRE-bound receptor complex then engages co-activator molecules as described before, leading to modulation of transcription rates of responding genes. This classic steroid receptor mechanism is dependent on the functions of both AF-1 and AF-2 domains of the receptor, which synergize via the recruitment of co-activator proteins, most notably the p160 family members. Depending on the cell and target gene promoter context, the DNA-bound receptor complex may positively or negatively affect expression of the downstream target gene. Initially, study of NR-mediated gene regulation was carried out on a gene-by-gene basis using a handful of known hormone regulated transcripts, such as the ER target genes PS2, lactotransferrin, and PR or the AR target gene prostate specific antigen. Now, after numerous comprehensive analyses of hormonally regulated transcriptional profiles, using microarray and more recently RNA-seq, thousands of NR targets have been found in various cell lines and tissues.

In general, PRB can better or uniquely activate transcription versus PRA when acting on progesterone response element–regulated genes in various cell types in vitro due to the presence of a third AF-3 in the NTD of PRB. Furthermore, PRA can repress the transcriptional activities of PRB in certain cell and promoter contexts, suggesting that PRA may modulate P responsiveness in certain tissues. Interestingly, PRA can also repress the actions of other heterologous NRs, including the AR, ER, and GR. Depending on the cell and target gene promoter context, the DNA-bound receptor complex may positively or negatively affect expression of the downstream target gene. Initially, study of NR-mediated gene regulation was carried out on a gene-by-gene basis using a handful of known hormone regulated transcripts, such as the ER target genes PS2, lactotransferrin, and PR or the AR target gene prostate specific antigen. Now, after numerous comprehensive analyses of hormonally regulated transcriptional profiles, using microarray and more recently RNA-seq, thousands of NR targets have been found in various cell lines and tissues.

In general, PRB can better or uniquely activate transcription versus PRA when acting on progesterone response element–regulated genes in various cell types in vitro due to the presence of a third AF-3 in the NTD of PRB. Furthermore, PRA can repress the transcriptional activities of PRB in certain cell and promoter contexts, suggesting that PRA may modulate P responsiveness in certain tissues. Interestingly, PRA can also repress the actions of other heterologous NRs, including the AR, ER, and GR. The variant, PR-C, is primarily cytosolic and lacks the A/B domain and the 5’ portion of the C domain, and has been shown to bind P and enhance the actions of PRB and PRA, but the presence of encoded protein and a potential biological role remains controversial. Some evidence for a role in P signal dampening during labor has been described for PR-C. When acting via a classic ERE-driven mechanism in vitro, ERα homodimers and ERα/ERβ heterodimers tend to be stronger activators of transcription compared with ERβ homodimers. Corroborating in vivo evidence of differential regulation and heterodimer formation by the ER subtypes has been difficult to generate. However, a microarray study by Lindberg et al. using bone tissue from ovariectomized mice found that ERβ generally inhibits ERα-mediated gene expression, but in the absence of ERα, ERβ can partially provide some E2-stimulated gene expression. No evidence for ERα or ERβ preferential regulation of specific transcripts or promoters has been forthcoming; rather, it seems the

FIGURE 25.4 Ligand-dependent and ligand-independent nuclear receptor mechanisms. The direct “classic” model of sex steroid receptor (SR) action involves direct interaction between SR bound to hormone (triangles) and HRE; the tethered pathway utilizes indirect “tethering” of SR to genes via interactions with other transcription factors (TF). “Nongenomic” signaling is initiated by membrane-localized receptors modulating extranuclear second messenger (SM) signaling pathways. Ligand-independent responses occur as a result of transduction of membrane receptor signaling, such as growth factors, to nuclear SR. Source: Adapted with permission from Ref. 18.
important factor is which receptors are expressed in a responding tissue.

**Indirect/Tethered Actions (HRE Independent)**

Ligand-activated steroid receptors can stimulate the expression of genes that lack a conspicuous HRE within their promoter. This has been especially demonstrated for ERα84–86 but is also known to occur for PR.87 This mechanism of HRE-independent steroid receptor activation is postulated to involve a “tethering” of the ligand-activated receptor to transcription factors that are directly bound to DNA via their respective response elements (Figure 25.4). This mechanism involves a mediator component (e.g., p160) between ER and AP-1 versus direct interaction.85 Interestingly, ERβ is unable to enhance the actions of a DNA-bound AP-1 complex when bound to E2 but can do so in the presence of certain selective estrogen receptor modifiers (SERMs) such as tamoxifen, raloxifene, and ICI 164,384.86 A similar HRE-independent mechanism of sex steroid receptor regulation has been documented for genes that possess a GC-rich region or Sp1 binding site within the promoter, upon which the actions of a bound Sp1 complex can be enhanced by ERα.84 These mechanisms have primarily been demonstrated using in vitro cell culture models.

**Nongenomic Actions**

All models of sex steroid receptor action described thus far influence cellular phenotypes by actioning in the nucleus and modulating target gene expression. The required gene transcription and mRNA translation is a relatively slow process; microarray studies demonstrate that initial transcriptional responses occur beginning about 60 min after administering hormones. Therefore, the numerous examples described to date in which steroids affect cellular dynamics within seconds to minutes of exposure have remained difficult to explain within the context of the previous models. Rapid cellular changes such as increased intracellular calcium or cAMP or activation of kinase signaling cascades have been attributed to sex steroid exposure of varied cell types and tissues.88,89 Because these steroid effects do not involve direct steroid receptor activation of gene transcription, they are often collectively referred to as “nongenomic” pathways of steroid action. Indeed, several of the documented effects lack a conspicuous nuclear component or have even been demonstrated to occur in enucleated cells, yet others can ultimately affect gene expression. Therefore, rather than nongenomic, some have proposed these types of steroid actions to be loosely categorized as plasma membrane–associated steroid signaling events. Although there are numerous examples of putative membrane-associated steroid signaling effects in reproductive tissues and processes, a thorough description of these mechanisms is beyond the scope of this chapter.

An obstacle to our better understanding of membrane-associated effects of steroids is the lack of data on the nature of the cell-surface steroid “receptors”. Questions remain concerning whether the membrane-associated receptors are identical or variant forms of the sex steroid NR or instead distinct receptors altogether. P is known to rapidly induce maturation in Xenopus oocytes that are arrested in the G2 phase of meiosis I,90 without requiring protein synthesis. Most evidence indicates this process begins at plasma membrane progesterone binding sites that are unique from the classic nuclear forms of PR, called progesterone receptor membrane component 1 (PGRMC1),91 although cDNA homologs of the PR were cloned from Xenopus and found to be associated with P-induced oocyte maturation.89 In other studies, P is able to inhibit apoptosis of rat granulosa cells in vitro via a membrane-bound receptor that is immunoreactive to antisera directed against the LBD of the classic PR.89

Rapid effects of E2 have been described in a vast array of tissues, including a rapid activation of endothelial nitric oxide synthase in endothelial cells, potentiation of currents induced by the ion channel agonist kainite in hippocampal neurons, and influx of calcium in uterine endometrial cells.88,90 The evidence of an association between ERα and plasma membrane–bound components has increased, supporting the view that this mechanism may indeed account for certain rapid effects of E2.92,93 In this sense, the various nongenomic actions of E2 are often categorized according to their susceptibility to inhibition by classical ER antagonists (e.g., ICI type compounds).92 E2-induced activation of membrane ion channels, endothelial nitric oxide synthase, and mitogen-activated protein kinase kinase are all inhibited by ER antagonists and therefore likely involve membrane-associated ERα or ERβ.92 In contrast, examples of E2-induced PKA and protein kinase C (PKC) activation are not inhibited by ICI compounds and are therefore not likely to involve the nuclear ER form.92 A recent study utilized a mutated form of the mouse ERα engineered to remain sequestered outside the nucleus (ERαH2NES), regardless of estrogen ligand. This form of ER did not mediate transcriptional responses but maintained estrogen-induced MAPK phosphorylation.94 Targeting steroid receptors to the membrane involves palmitoylation, which is facilitated by HSP27.88 The palmitoylation promotes interaction with caveolin-1, which then results in localization of the receptor in membrane caveolin rafts. Another potential mediator of rapid membrane localized hormone response is the G protein-coupled receptor GPER (originally referred to as GPR30), which is activated by E2.95 GPER-null mice lack reproductive phenotypes,96 although effects on the degrees of uterine responses elicited by E2 have been observed with G15, a GPER selective antagonist, suggesting a potential role for GPER in modulating...
Ligand-Independent Actions: Membrane Receptor Cross Talk

We now have ample evidence that the sex steroid receptors can be activated via intracellular second messenger and signaling pathways, allowing for the induction of sex steroid target genes in the absence of steroid ligand, or the enhancement of steroid hormone signaling (Figure 25.4).29,103–105 Polypeptide growth factors are able to activate ERα-mediated gene expression via mitogen-activated protein kinase activation of ERα in the absence of E2 (Figure 25.4). Similarly, interleukin-6 stimulation of cells leads to increased AR-mediated gene expression.38,106 and cyclin-dependent kinases have been shown to activate PR-regulated gene expression.25 The intracellular signaling molecule cAMP, a common second messenger of G protein–coupled receptors and an activator of the PKA pathway, can stimulate increased PR-, AR-, and ER-mediated transcription of target genes in the absence of steroid ligand.29,103 A well-characterized example of ligand-independent cAMP activation of PR occurs after dopamine stimulation of dopaminergic neurons107 and is the mechanism by which sexual behavior is induced in female rodents. Likewise, growth factors are able to mimic the effects of E2 in the rodent uterus via E2 independent activation of ERβ.109,110 Most interesting are recent studies indicating that the MAP kinase protein ERK is co-recruited to chromatin with ERα.111 Ligand-independent activation of sex steroid receptors is believed to rely largely on cellular kinase pathways that alter the phosphorylation state of the receptor and/or its associated proteins (e.g., co-activators, heat shock proteins) (Figure 25.4). As in the classic ligand-dependent mechanism described earlier, specific receptor domains are critical to ligand-independent activation as well. ER activation by peptide growth factor–signaling pathways appears to be more dependent on AF-1 functions, whereas the effects of increased intracellular cAMP are postulated to depend on the AF-2 domain and do not require a functional AF-1.103

Receptor Antagonists

Structural analyses of the sex steroid receptors provide insight into the agonist/antagonist actions of endogenous or synthetic ligands (Table 25.2). Certain ligands possess “mixed” agonist/antagonist activity that is dependent on the receptor, cell, and promoter context, leading to the more descriptive terms of SERMs, selective PR modulator, and selective AR modulator. As described in the subsection Receptor Mechanisms of Action of the section Steroid Receptors, sex steroid receptor agonists interact with the LBD to generate an ordered array of 11 α-helices that is most conducive to receptor interaction with co-activator proteins and/or promotes disassociation of co-repressor proteins. In turn, antagonist ligands generally fail to generate the necessary changes in receptor structure that induce full transcriptional activity.38,112–114

Antiprogestins have a number of applications in reproductive medicine, including use as contraceptives due to their ability to prevent implantation or ovulation.115 The currently available PR antagonists are all competitive inhibitors of P binding to the PR LBD.115 Type I anti-progestins, such as ZK 98299 (onapristone), bind PR but fail to induce receptor phosphorylation and induce weak receptor binding to HREs.115,116 In contrast, type II anti-progestins, such as RU 486 (mifepristone, Table 25.2), bind PR and promote receptor phosphorylation, homodimerization, and binding of the receptor complex to an HRE, but are unable to activate transcription.50 Crystallography data indicate that RU 486 binding to PR induces an arrangement of helix 12 that is not conducive to binding of steroid receptor co-activators and may even promote recruitment of co-repressors such as NCoR.50 Type II anti-progestins do possess some PR agonist activity when acting in the context of the cAMP/PKA signaling pathway in certain cell types.50

The field of antiestrogens and SERMS has been an area of intense research because the potential clinical applications include treatments for fertility, cardiovascular disease, osteoporosis, cognitive function, postmenopausal symptoms, and breast and gynecological cancers.18 The best-characterized antiestrogens are competitive inhibitors of E2 binding to ER and have been classified into two major groups. The type I class of ER antagonists includes the triphenylethylene compounds, tamoxifen (Table 25.2), hydroxytamoxifen, and raloxifene and are characterized by mixed agonist/antagonist activity depending on the receptor, cell, and promoter context.18 Upon binding the ER, these compounds selectively inhibit AF-2 function but leave AF-1–mediated receptor functions intact, thus explaining the selective agonist activity. In contrast, the type II compounds are considered pure antagonists, the most well characterized being ICI 182,780 (Table 25.2), which is a 7α-substituted derivative of E2. This compound binds the ER but possesses extended side chains that prevent co-activator association with the NR box of helix 12 and may even promote interaction with co-repressors as well as increased receptor degradation,18 resulting in
SEX STEROID RECEPTORS IN UTERINE FUNCTION

Estrogen Receptor Signaling in Uterine Function

The ovarian-derived sex steroid hormones dictate the uterine estrous or menstrual cycle in mammals and are therefore essential to the establishment and maintenance of pregnancy. The uterine response to the preovulatory rise in circulating E2 is required to prepare the tissue for the forthcoming rise in P that accompanies ovulation and is critical to embryo implantation. This physiological coordination between the ovary and uterus is common to the large majority of mammals studied, and the spectrum of actions and effects of the sex steroids in uterine tissue are mediated by their cognate NRs. Estrogen was first described almost 100 years ago as a substance that induced estrus. Although initially the dramatic physiological effects of estrogens on the mammalian uterus have been appreciated and well described, later investigations begun to elucidate the precise mechanisms and signaling pathways involved in estrogen responses. Our understanding of steroid hormone action in the uterus has been greatly advanced by utilization of new technologies with the generation of knockout and knock-in models, including receptor or co-activator null, tissue and cell selective null, and mutant knock-in models in mice. These models are also used in combination with microarray, RNA-seq, and ChIP-seq methods that allow for comprehensive mapping of interaction of NRs with chromatin and modulation of genomic response to steroids in uterine tissues. Together, these models and techniques have led to better understanding of the molecular details of steroid receptor roles in biological processes. The following sections discuss what is currently known and hypothesized about sex steroid receptor actions in the mammalian uterus.

ER Expression in the Uterus

**RODENTS**

ERα is present in the female reproductive tract of rodents throughout late fetal and neonatal development, puberty, and adulthood. In mice, ERα transcript has been detected in the blastocyst, but immunoreactivity first appears in the mesenchymal cells of the fetal uterus as early as gestational day 15, whereas epithelial expression is delayed until the late fetal period but increases substantially on postnatal day 4 and peaks on day 16. The level of ERα transcripts in the murine uterus during neonatal development closely mirrors the patterns indicated by earlier immunohistochemical studies. There is reportedly no ERβ immunoreactivity in the neonatal mouse uterus, although transcripts are detected at a modest level. In adult mice, ERα transcripts and immunoreactivity are quite high in uterine epithelial cells before ovulation but decrease during pregnancy, whereas expression in the stroma continues. In contrast, ERβ expression is relatively low in uteri of virgin and pregnant mice. Adult rats exhibit a similar uterine ER expression pattern such that ERα transcripts and immunoreactivity are easily detectable in the epithelium, stroma, and myometrium, and levels peak during the proestrous period of increased circulating E2 levels. The evidence of ERβ expression in the rat uterus is conflicting; whereas ERβ expression has been reported to be in approximately 40% of rat uterine epithelial and stromal cells regardless of the estrous cycle stage, others report a total lack of detectable ERβ. These observations are plagued by the inconsistent quality of antibodies against ERβ. In ovariectomized mice, ERα and ERβ proteins were detected in the epithelial, stromal, and myometrial cells, indicating that removal of ovarian hormones alters the ER expression pattern. Although ERβ protein is present in the mouse uterus, there is overwhelming functional evidence that ERα is responsible for mediating the many effects of estrogens in the uterus. As described following, only ERα-null female mice exhibit a severely attenuated response to acute and chronic estrogen (e.g., E2 or DES) treatment in terms of uterine growth and altered gene expression, as well as exhibit impaired embryo implantation. Furthermore, categorical studies have shown that the toxic effects of neonatal estrogen exposure in the neonatal female mouse uterus are clearly mediated by ERα.

**DOMESTIC ANIMALS**

In the neonatal ovine uterus, ERα mRNA and protein levels are highest in the developing glandular structures; detectable but lower in the luminal epithelia, stroma, and vascular endothelial cells; and absent in the myometrium. In the adult ewe, ERα expression is detected...
In all uterine cell types and is highest on day 1 of the 16- to 18-day cycle, concurrent with peak plasma E2 levels.\textsuperscript{138,140} During days 1–6, when P levels rise and E2 declines, ER\textalpha expression decreases accordingly and does not begin to rise again until the end of the cycle nears on days 11–15.\textsuperscript{138,140} A similar pattern of uterine ER\textalpha expression is reported during the 21-day bovine cycle.\textsuperscript{141} In situ hybridization and immunohistochemical analyses indicate maximum ER\textalpha levels on days 1–3, especially in the glandular and stromal cells; these levels generally decrease by day 6, although cells comprising the deep glands maintain ER\textalpha expression.\textsuperscript{142} The observed cyclical changes in ER\textalpha expression in ovine and bovine uteri are consistent with steroid regulation of receptor levels. Indeed, E2 treatment of ovariectomized cows leads to increased ER\textalpha expression in the glands, stroma, and luminal epithelia, whereas P alone or in combination with E2 elicits a decrease in ER\textalpha levels.\textsuperscript{142}

**PRIMATES**

Early investigations in human uterine tissue found that levels of ER immunoreactivity or high-affinity E2 binding vary during the menstrual cycle such that peak expression occurs in the mid- to late proliferative phase and then decreases at ovulation and commencement of the secretory phase.\textsuperscript{143–145} This peak in uterine ER levels at the time of rising plasma E2 levels is similar to several other species and suggests that expression is autoregulated. However, maintenance of uterine ER expression in postmenopausal women suggests that nonsteroidal regulatory factors are also involved.\textsuperscript{145} The ovulatory decline in ER\textalpha levels among all uterine cell types is concurrent with the rise in circulating P levels and suggests that PR-mediated P actions may act to downregulate ER expression. Such PR-mediated decrease in ER\textalpha has been reported in experimental systems as well.\textsuperscript{146} A distinct gradient of ER levels among the functional components of the human uterus was also reported such that levels are highest in the fundus and progressively decrease in those tissues closer to the cervix.\textsuperscript{147,148}

The discovery of ER\textbeta forced a reevaluation of uterine ER expression because the early studies discussed previously did not use methods that adequately distinguished between the two ER forms. More recent studies definitively show that ER\textalpha is the predominant form in the human uterus and accounts for the increased receptor levels that occur during the proliferative phase.\textsuperscript{149} ER\textalpha transcripts and protein are especially high in glandular epithelial and stromal cells during the proliferative phase and exhibit a dramatic decrease among uterine tissues upon entering the secretory phase of the cycle.\textsuperscript{149} The levels of ER\textalpha transcripts and immunoreactivity in the uterine myometrium are much lower and exhibit little change during the menstrual cycle.\textsuperscript{149} Still, comparatively higher ER\textalpha levels are described in the subendometrial myometrium during the proliferative phase and are postulated to be involved in uterine peristaltic activity.\textsuperscript{149}

ER\textbeta expression in the human uterus is much lower relative to ER\textalpha but is generally present in the same cell types and exhibits comparative changes during the menstrual cycle.\textsuperscript{149,150} There is one report of increased ER\textbeta immunoreactivity in the uterine stroma during the secretory phase.\textsuperscript{150} ER\textbeta immunoreactivity is especially detectable in vascular smooth muscle cells and increases therein during the secretory phase, suggesting a role for this receptor form in vascularization.\textsuperscript{150} The human-specific ER\textbeta isoform, ER\textbeta cx, also called ER\textbeta 2 (Figure 25.2), is also detected in the uterus and is present along with full-length ER\textbeta (ER\textbeta 1) in the functional and basal layer endometrial glands.\textsuperscript{151} Interestingly, ER\textbeta cx immunoreactivity decreases in the basal layer glandular cells during the mid-secretory phase, whereas little change occurs in the level of full-length ER\textbeta,\textsuperscript{151} suggesting that differential expression of ER\textbeta isoforms over the course of the menstrual cycle may modify the cellular responses to E2.

Nonhuman primates exhibit uterine ER expression patterns similar to those described in humans. ER\textalpha immunoreactivity in baboon uteri is highest in endometrial glandular, stroma, and myometrial smooth muscle cells during the proliferative phase and decreases in all cell types upon commencement of the secretory phase.\textsuperscript{152} A similar ER\textalpha expression pattern in epithelial, stromal, and myometrial cells is reported in the cynomolgus monkey uterus.\textsuperscript{153} ER\textalpha immunoreactivity is also reported in the vasculature, including the spinal arteries, of the baboon uterus.\textsuperscript{152} Ovariectomy followed by exogenous E2 or E2 plus P treatments in baboons leads to changes in uterine ER expression that mirror the menstrual cycle, supporting the direct role of ovarian steroids in the regulation of uterine ER levels.\textsuperscript{152} Considerable levels of ER\textbeta transcripts are also detected in the epithelial, stromal, and myometrial cells of cynomolgus monkey uteri.\textsuperscript{154} In contrast, ER\textbeta immunoreactivity is reportedly low in normal uterine tissue from baboons, although higher levels are observed in diseased glands associated with endometriosis.\textsuperscript{155}

**Uterine Phenotypes in Mouse Models of Disrupted Estrogen Signaling**

The murine models of disrupted estrogen signaling have proven invaluable to experimental investigation of estrogen actions in the uterus and the contribution of each ER form to these functions (Table 25.3). In addition to the ER-null models are two independently derived lines of mice that lack the capacity to synthesize E2 due to disruption of the Cyp19 gene.\textsuperscript{131,156} Following, we will describe how these different mouse models have helped to delineate the role of ER in the uterus.
### TABLE 25.3 Uterine Phenotypes in Mice Null or Mutated for Sex Steroid Receptors and Signaling

<table>
<thead>
<tr>
<th>Mutated or Null for Sex Steroid Receptors and Signaling</th>
<th>Uterine Phenotypes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Esr1</strong>−/− (homozygous null alleles for ERα: αERKO and Ex3αERKO)</td>
<td>Normal uterine development but exhibits hypoplastic uteri Inensitive to the proliferative and differentiating effects of endogenous growth factors and exogenous E2 Implantation defect *Exhibit decidualization</td>
<td>135,157–161</td>
</tr>
<tr>
<td>NERKI+/− (one mutated allele of two-point mutation in ERα DBD and one WT allele)</td>
<td>Normal uterine development but exhibits hyperplastic uteri Hypersensitive to estrogen</td>
<td>132</td>
</tr>
<tr>
<td>KIKO (ERAA/−) (one mutated allele of two-point mutation in DNA binding domain of ERα and one ERαKO allele)</td>
<td>Normal uterine development</td>
<td>162,163</td>
</tr>
<tr>
<td>ERαEAAE/EAAE (homozygous animal of 4-point mutation of DBD ERα)</td>
<td>Normal uterine development but exhibits hypoplastic uteri Loss of E2-induced uterine transcripts</td>
<td>164</td>
</tr>
<tr>
<td>ERαAF-1 (deletion of amino acids 2–128 on ERα)</td>
<td>Normal uterine development and architecture Blunted E2 response</td>
<td>165,166</td>
</tr>
<tr>
<td>ERαAF-2 (deletion of amino acids 543–549 on ERα)</td>
<td>Normal uterine development but exhibits hypoplastic uteri Insensitive to E2 treatment</td>
<td>167</td>
</tr>
<tr>
<td>ENERKI (ERαG525L) (homozygous animal of 1-point mutation in LBD of ERα)</td>
<td>Normal uterine development but exhibits hypoplastic uteri Insensitive to E2 treatment IGFI induced slight uterine epithelial proliferation compared to control littermates (nonhomogenous pattern)</td>
<td>168</td>
</tr>
<tr>
<td>AF2ERKI/KI (homozygous knock-in of 2-point mutation in LBD of ERα)</td>
<td>Normal uterine development but exhibits hypoplastic uteri Insensitive to E2 treatment ER antagonists and partial agonist (ICI 182,780 and TAM) induced uterine epithelial proliferation Growth factor did not induce the uterine epithelial cell proliferation</td>
<td>169</td>
</tr>
<tr>
<td>Wnt7aCre+;Esr1f/f (uterine epithelial cell–specific deletion of ERα)</td>
<td>Normal uterine development Sensitive to E2- and growth factor–induced epithelial cell proliferation Selective loss of E2-target gene response Implantation defect</td>
<td>170</td>
</tr>
<tr>
<td><strong>Esr2</strong>−/− (homozygous null alleles for ERβ: βERKO, Ex3βERKO, and <strong>ERβL−/−L−</strong> )</td>
<td>Exhibit grossly normal uterine development and function Sensitive to E2 treatment Some <strong>Esr2</strong>−/− lines reported elevated uterine epithelial proliferation after E treatment compared to WT Some are completely sterile (due to ovarian phenotype)</td>
<td>158,171–173</td>
</tr>
<tr>
<td>αβERKO (homozygous null for both ERα and ERβ)</td>
<td>Normal uterine development but exhibit hypoplastic uteri, similar to those of <strong>Esr1</strong>−/− Insensitive to E2</td>
<td>158,174</td>
</tr>
<tr>
<td>Cyp19a1−/− (homozygous null aromatase: ArKO)</td>
<td>Normal uterine development but exhibits hypoplastic uteri Sensitive to E2-induced epithelial cell proliferation</td>
<td>131,156,175</td>
</tr>
<tr>
<td>Pgr−/− (homozygous null alleles for PRA and PRB: PRKO)</td>
<td>Exhibit grossly normal uterine development Implantation and decidualization defects Exhibit epithelial hyperplasia in response to E Loss of P-induced proliferative switch in uterine epithelial and stromal cells</td>
<td>176–178</td>
</tr>
</tbody>
</table>
### Mutated or Null for Sex Steroid Receptors and Signaling

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uterine Phenotypes</th>
<th>References</th>
</tr>
</thead>
</table>
| Wnt7a<sup>Cre</sup>;Pgr<sup>fl/−</sup> (uterine epithelial cell–specific deletion of PR) | Normal uterine development  
Loss of P-induced uterine epithelial P target gene transcription  
Abnormal proliferative switch of uterine epithelial and stromal cells  
Implantation and decidualization defect  
Infertile | 179 |
| PRKO and PR<sup>fl/fl</sup> (homozygous null alleles for PR) | Normal uterine development  
Abnormal epithelial proliferation in response to P  
Implantation and decidualization defect  
Infertile | 176 |
| PRA<sup>−/−</sup> (homozygous null alleles for PRA: PRAKO) | Normal uterine development  
Resemble PRKO phenotypes  
Infertile | 180 |
| PRB<sup>−/−</sup> (homozygous null alleles for PRB: PRBKO) | Normal uterine development  
Normal implantation and decidualization responses  
Do not exhibit epithelial hyperplasia in response to E2  
Normal fertility | 183,182 |
| AR<sup>−/−</sup> (homozygous null alleles for AR: ARKO) | Normal uterine development, function, and maintenance of pregnancy  
Slightly longer uterine horns compared to controls  
Slightly reduced uterine circumference  
Reduced uterine hypertrophic response to gonadotropins  
Increase placenta size (placentamegaly)  
Subfertile | 185,184 |
| AR<sup>−/−</sup> in-frame deletion of exon 3, loss of DNA binding activity | No overt uterine phenotype  
Subfertile | 185 |
| SPARKI (homozygous knock-in of mutated DBD of AR) | Normal female fertility, no uterine phenotype reported | 186 |

* Ex3αERKO females have a similar uterine phenotype to the original αERKO except for exhibiting a decidualization defect, which may be due to the splice variants in the original αERKO that retains ER activities.  
** ERβ<sup>−/−</sup> females are the only line of ERβ knockout animals that are reported to be completely sterile.

### MICE LACKING ERα

Unlike the AR, which is mutated in the naturally occurring Tfm rat and mouse, no known mutations of the ERα have been reported, thus the deletion of ERα was thought to be lethal. Similarly, only one male patient and one female patient with ERα mutation have been discovered. The male patient’s mutation results in severe truncation of the ERα protein due to a stop codon in the A/B domain. The female patient has a point mutation in her ERα LBD that decreases activity by reducing E binding more than 200-fold. ERα-null mice were the first experimentally generated steroid-receptor-null animal, reported in 1993, and preceded the discovery of ERβ in 1996. There are currently numerous reported lines of ERα-null mice and additional lines of mice with mutations in functional domains of ERα. Three separate lines of ERα-null mice were generated: the αERKO, first described by Lubahn et al. in 1993, the ERαKO (or Ex3αERKO), described by Dupont et al. in 2000 and by Hewitt in 2010, and ERα<sup>−/−</sup>, described by Antonson et al. in 2012. Homologous recombination was employed to either disrupt (αERKO) or completely excise (ERαKO and ERα<sup>−/−</sup>) exon 3 of the murine Esr1 (ERα) gene. The uterine estrogenic response in αERKO females differs from the latter two lines, as αERKO animals have a low level of truncated ERα protein produced from a splice variant, which preserves some residual biological functions, but all ERα-null female mice are infertile. Recently, an ERα-null rat has been derived using zinc finger nuclease (ZFN) genome editing. All phenotypes examined thus far were previously seen in the ERα-null mice, including infertility due to hypoplastic uteri, polycystic ovaries, and ovulation defects. Interestingly, the recently described female patient with homozygous ERα mutation also has cystic ovaries and a small uterus despite elevated circulating E.

### MICE LACKING ERβ

The first description of ERβ-null mice followed the discovery of ERβ by only two years. Prior to generation of the ERβ-null model, very little was known as to the role of ERβ in biological processes, making it difficult to predict possible phenotypes. Therefore, the ERβ-null mice have served a tremendous role in providing insight
into the importance of the newly discovered ER form to female fertility, and studies to date indicate ERβ plays a particularly important role in ovarian function. Four different lines of ERβ-null mice have been described. The βERKO was first described by Krege et al. in 1998,171 and the ERβKO, or Ex3βERKO, was described by Dupont et al. in 2000.158 Homologous recombination was employed in both lines to disrupt exon 3 of the murine Esr2 (ERβ) gene. As described to date, the reproductive, endocrine, and ovarian phenotypes of both lines are indistinguishable, with both exhibiting female subfertility. Then, in 2002, Shughrue et al. reported the third line of ERβKO animals, however, no uterine or ovarian phenotypes were reported.191 Recently, ERβKO51−/− animals, which contain LoxP sites flanking exon 3 of Esr2, were generated using the Cre/loxP recombination system.172 Interestingly, female mice from this recently described ERβKO51−/− colony were reported to be sterile due to an ovarian defect.

**MICE LACKING ERα AND β**

Much insight into the physiological roles of E2 has been gained from the study of mice lacking one or the other known ER forms. Still, conclusions drawn from these models are confounded by possible compensatory mechanisms provided by the opposite ER in each respective ERKO model. Furthermore, mice lacking only one ER form will lose possible ERα/ERβ heterodimer-mediated actions.192,193 Therefore, compound ER-null mice (i.e., αβERKO) represent an opportunity to elucidate potential compensatory and cooperative actions of ERα and ERβ. The two reported lines of compound ER-null mice are the αβERKO, described by Couse et al. in 1999,174 and the EREβKO, described by Dupont et al. in 2000.158 Both were generated by crossbreeding animals heterozygous for the respective individual ER-null mouse and, as described to date, exhibit comparable reproductive, endocrine, and ovarian phenotypes as discussed in the subsection *Mice Lacking ERα and β* within the section *Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling* of this chapter. Shughrue et al. generated an additional EREβKO line in 2002; however, their reproductive function was not described, as the study focused on ER-mediated estrogen responses in the brain.191

**MICE LACKING CYP19**

Estrogens are produced by aromatase cytochrome P450, the product of Cyp19 gene. Female mice with disruption of circulating estrogen production exhibit altered female reproduction.131,156,175 There are three animal models of Cyp19-null (called ArKO). Fisher et al. reported the first mouse line in 1998, which disrupted exon 9 of Cyp19 gene, as the region is a highly conserved region among all aromatases.156 Later, in 1998, Honda et al. reported a mouse line with targeted disruption of exons 1 and 2 of the Cyp19 gene.175 Toda et al. generated the most recent mouse line of Cyp19-null in 2001 with a targeted disruption of exon 9 of the Cyp19 gene.131 These ArKO female phenotypes are indistinguishable.131,156,175 Briefly, these mice are infertile due to ovarian dysfunction marked by cystic follicles and a failure to respond to exogenous gonadotropins. This phenotype is described in detail in the subsection *Mice lacking Cyp19* within the section *Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling* of this chapter.

**UTERINE PHENOTYPES IN GLOBAL ER- OR CYP19-NULL MICE**

Females within each respective model exhibit a similar phenotypic syndrome. Female mice lacking ERα or aromatase are infertile due to dysfunction of numerous physiological systems, including the ovary (see the section *Estrogen Receptor Signaling in Ovarian Function* and uterus, whereas ERβ-null females exhibit reduced or lost fecundity that is largely attributable to ovarian dysfunction. A level of caution is warranted when making phenotypic comparisons between the ER-null and Cyp19-null models because sensitivity to maternally derived estrogens may provide a more normal developmental environment during gestation in Cyp19-null mice, and sensitivity to dietary estrogens during adulthood is able to abate several phenotypes in Cyp19-null mice.194 The reported uterine phenotypes are summarized in *Table 25.3*.

All lines of ER-null females exhibit uteri that possess the expected tissue compartments, myometrium, endometrial stroma, and epithelium134,149,174 (Figure 25.5). However, in females lacking functional ERα or Cyp19, uteri are overtly hypoplastic and exhibit severely reduced weights relative to WT littermates,131,134,156,195 whereas ERβ-null females possess uteri that are grossly normal and responsive to ovarian-derived steroids134 (Figure 25.5). The uterine endometrium of ERα-null females is severely hypotrophic, poorly organized, and possesses a paucity of glandular structures (Figure 25.5(B)).159,196 The luminal and glandular epithelial cells in ERα-null uteri are severely immature with fewer glands and consistently exhibit a cuboidal morphology, versus the tall columnar morphology and basal location of the nucleus of an “estrogenized” epithelium in WT uteri (Figure 25.5(B)). Therefore, fetal, neonatal, and perinatal development of the female reproductive tract in mice is largely independent of ERα- and ERβ-mediated actions, but estrogen responsiveness and sexual maturation of the adult uterus are ablated after the loss of functional ERα.

The totality of the ERα-null phenotype and lack of any overt uterine abnormalities in ERβ-null females suggest that ERβ has little function in mediating estrogen actions in the uterus. Moreover, ERβ-null also demonstrated a similar uterine phenotype as ERα-null (Figure 25.5).197
Weihua et al. reported that ER\(\beta\)-null females exhibited a slightly aberrant uterine growth response after estrogen replacement; however, the uterine bioassay was conducted in immature intact, not ovariectomized adult, animals.\(^{133}\) In addition, Wada-Hiraike et al. showed that in immature females, loss of ER\(\beta\) leads to increased uterine epithelial proliferation induced by E2 compared to WT uteri.\(^{173}\) Although ER\(\beta\)-null females are subfertile, when pregnancies are established they are sustained to term,\(^{171}\) indicating uterine competence. More recent findings suggest that loss of ER\(\beta\) leads to complete sterility due to a defect in ovarian function,\(^{158,172}\) described in more detail in the subsection Mice Lacking ER\(\beta\) of the section Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling of this chapter.

**MICE WITH UTERINE-SPECIFIC DELETION OF ER\(\alpha\)**

Currently, only a single mouse model with tissue-specific deletion of ER\(\alpha\) in the uterus has been published. Our laboratory has described selective deletion of ER\(\alpha\), using the Cre/loxP recombination system, by crossing \(Wnt7a^{\text{Cre+}}\) with \(Esr1^{1/1}\) animals\(^{159}\) to generate mice with deletion of ER\(\alpha\) in uterine epithelial cells (\(Wnt7a^{\text{Cre+}};Esr1^{1/1}\)). The expression of ER\(\alpha\) in the uterine luminal and glandular epithelium of these animals was ablated, while the ER\(\alpha\) expression in the stromal cells and other uterine cells remains intact.\(^{170}\) The epithelial ER\(\alpha\) was ablated not only in the uterus in this mouse line\(^{170}\) but also in the oviduct (unpublished data). As expected, based on findings in the global ER\(\alpha\) knockouts, loss of uterine epithelial ER\(\alpha\) has no effect on female reproductive tract development. Uterine histological analysis showed a similar uterine morphology as wild-type control.\(^{170}\) The \(Wnt7a^{\text{Cre+}};Esr1^{1/1}\) uteri are sensitive to 24 h treatment of E2, as the uterine epithelial proliferation is preserved (Figure 25.6). However, \(Wnt7a^{\text{Cre+}};Esr1^{1/1}\) uteri lack a complete uterine response to E2, apparent following a 3-day uterine bioassay, which demonstrated a blunted growth response and increased apoptotic activity in \(Wnt7a^{\text{Cre+}};Esr1^{1/1}\) compared to the...
control uteri. Additionally, a lack of ERα expression in the uterine epithelial cells contributes to complete infertility, partly due to an implantation defect. This suggests that uterine epithelial ERα is dispensable for early uterine proliferative responses but crucial for complete biological responses induced by E2, as well as for embryo implantation.

**MICE WITH MUTATED DNA BINDING DOMAINS OF ERα**

The generation of mouse lines with mutation in the specific domains of ERα allows us to dissect the biological functionality of individual domains of the receptor in vivo. To date, there are two mouse lines with mutations that are designed to disrupt the DNA binding function of the ERα that have been “knocked-in” (KI) at the Esr1 gene locus. The first line was generated by replacing critical P-box amino acids E207 and G208 with alanines (Figure 25.3(B)). This line was named “Nongenomic ER knock-in” (NERKI), as these mutations were intended to restrict ERα signaling to the nongenomic and tethered mechanisms (Figure 25.4). Female NERKI+/- animals that have one mutated allele and one WT allele were infertile, exhibiting a highly novel hyperplastic uterine phenotype, so NERKI+/- males were crossed with ERα-null heterozygous (WT/KO) females to produce mice with one NERKI mutated allele and one deleted Esr1 allele, called ERα KIKO or ERαAA/- as described by O’Brien et al. in 2006. The second line of DNA binding domain knock-in animals possessed mutation of four amino acids in the first zinc finger of the Esr1 gene, substituting Y at position 201 with E, and in the critical P-box (Figure 25.3(B)), K at position 210 with A, K at position 214 with A, and R at position 215 with E (Figure 25.3(B)) as described by Ahlbory-Dieker et al. in 2009 (called ERαEAAE/EAAE).164

The NERKI+/− females have normal uterine development but exhibit hyperplastic uterine and are hypersensitive to estrogen. This suggests that disruption of the ERα binding to ERE while maintaining the presence of a normal WT allele leads to an aberrant overstimulated uterine response to estrogen. Interestingly, these NERKI+/− are infertile and exhibit a uterine abnormality of enlarged hyperplastic endometrial glands despite possessing normal levels of circulating sex steroids.132

ERαAA/- females have normal uterine development. Initially, O’Brien et al. reported that ERαAA/- females, with mutation of the DNA binding domain, maintained proliferative responses—induced by E2. However, in subsequent studies, no uterine proliferation was observed. Ahlbory-Dieker et al. showed that, unlike the NERKI+/-, females heterozygous for the ERαEAAE mutation are fertile. The homozygous ERαEAAE/EAAE females have normal reproductive tract development, but uteri are severely hypoplastic (Figure 25.7(A)), similar to global ERα-null uteri. Additionally, ERαEAAE/EAAE uteri do not respond to E treatment, as the estrogen-regulated genes (such as Pgr, Cdkn1a, and Igf1) failed to be upregulated in ERαEAAE/EAAE compared to control uteri. The females from these two mouse lines with point mutations in the DNA binding domain of ERα are infertile. Thus the physiological function of the DNA binding domain of ERα is crucial for female reproduction.
25. STEROID RECEPTORS IN THE UTERUS AND OVARY

4. FEMALE REPRODUCTIVE SYSTEM

MICE WITH MUTATED AF-1 OR AF-2 DOMAINS OF ERα

As discussed in the section Receptor Structure, AF-1 and AF-2 are important for ER transcriptional activity (Figure 25.1). A single mouse line with amino acids 2–128 deleted from exon 1 of Esr1, which removes the AF domain, called ERαAF-10, was described by Billon-Gales et al. in 2009. There are three reported mouse lines with mutation in AF-2 domain of ERα. In the first line, the animals have a single point mutation in ERα of G at position 525 to L in the ligand binding domain (LBD), and this model was described by Sinkevicius et al. in 2008 (called “Estrogen-nonresponsive ERα Knock-in or ENERKI” or ERαG525L). Billon-Gales et al. generated a second line of LBD mutation animals in 2011, with amino acids 543–549 deleted from the LBD of ERα, removing helix 12 and thus AF-2 functionality (called ERαAF-20). One month after the second line was published, Arao et al. reported the third line of animals with mutations within LDB of ERα with two point mutations of L at position 543 and 544 to A (called AF2ERK1/1 animals). All females from the ERαAF-10, ERαG525L, ERαAF-20, and AF2ERK1/1 mouse lines are sterile.

ERαAF-10 females exhibited minimal uterine wet weight gain compared to ER+/+ uteri after treatment with E2 pellets for two consecutive weeks (Figure 25.7(B)), while ERαAF-20 females did not respond (Figure 25.7(C)). This indicates that the ERαAF-2 functional domain contributes to minimal uterine weight increase induced by E2 in the absence of AF-1. Both lines of AF-2-mutated animals (ERαG525L and AF2ERK1/1) display severely hypoplastic uteri (Figure 25.7(D,E)) and lack uterine growth response to E2 treatment. Interestingly, uterine wet weight can be increased by using the synthetic ERα agonist PPT (Table 25.2) in ERαG525L.
or by using the ER antagonists ICI 182,780 or tamoxifen (Table 25.2) in AF2ERki/ki females. The ability of the antagonists to mediate responses seems to be due to a unique conformation of the LBD of the AF2ER that leads to AF-1-dependent transcriptional activity. 

Arao et al. also demonstrated that the uterine response to ICI or tamoxifen includes increased DNA synthesis in the uterine epithelial cells of AF2ERki/ki. The growth factor IGF1 (insulin-like growth factor 1) induced minimal uterine epithelial proliferation in ERαC525L, and was ineffective in AF2ERki/ki uteri. Together, these findings indicated that both AF-1 and AF-2 activation domains of ERα contribute to a normal regulation of uterine growth and reproductive functions. As the AF domains mediate ER-co-regulator interaction (Table 25.1), this emphasizes the importance of effective ERα co-activator protein recruitment for successful uterine E2 response. Similarly, mice lacking sufficient SRC-1 co-activator (Src1−/−) exhibit measurably diminished uterine response to E2.

**Uterine Phenotypes in Mouse Models with Mutations in Transcriptional Proteins that Affect ER Function**

**MICE WITH UTERINE-SPECIFIC DELETION OF COUP-TFII**

Chicken ovalbumin upstream transcription factor II (COUP-TFII), encoded from the Nr2f2 gene, is a transcription factor, belonging to nuclear receptor superfamily. COUP-TFII is expressed in uterine stromal cells and is crucial for female reproduction. The tissue-specific deletion of COUP-TFII (COUP-TFIIf/d) in the uterus using PgrCre+ crossed with COUP-TFIIf/d animals demonstrated that COUP-TFIIf/d females exhibited normal uterine development but were completely infertile, in part, due to lack of implantation. Kurihara et al. also showed an enhanced estrogentic response in the epithelial cells of COUP-TFIIf/d uteri, as E-responsive genes lactotransferrin (Ltf) and mucin 1 (Muc1) were elevated during the receptive window, which suggested that COUP-TFII modulates uterine epithelial ERα activity during pregnancy. Inhibition of ERα activity using ER antagonist (ICI 182,789) partially rescued implantation in COUP-TFIIf/d females and corrected the expression level of Ltf and Muc1. These results suggest COUP-TFII plays an essential role in regulating uterine ERα activity during early pregnancy in rodents.

**MICE WITH UTERINE-SPECIFIC DELETION OF REA**

Repressor of estrogen receptor activity (REA) is an ER co-regulator that represses ER activity both in vitro and in vivo. In Chinese hamster ovarian (CHO) cells, REA is shown to be a direct co-regulator of ERα and ERβ, but not PR or RAR. In 2005, Park et al. generated a mouse line with a targeted deletion of REA exon sequence encoding amino acids 12–201 (heterozygous REA or REA+/−). Gross uterine morphology of REA+/− appeared normal compared to control littermates. However, the REA+/− females exhibited hypersensitivity to E2 treatment, as uterine weight and luminal epithelial cell height and proliferation were increased compared to E2-treated control uteri. In addition, E2-induced uterine genes (such as C3 and Ltf) were also elevated in E2-treated REA+/− compared to E2-treated control uteri.

Due to the embryonic lethality of REA−/−, uterine deletion of REA was generated using PgrCre+ crossed with REAf/d, producing REAf/d (heterozygous deletion) and REAd/d (homozygous deletion) animal models. Uterine epithelial cell hyperproliferation after E2 treatment was seen in REAf/d, similar to the E2 response of REA+/− uterus. The enhanced uterine weight increase by E2 in REAf/d females is in part due to increased levels of the aquaporin water transport gene (Aqp4). The levels of Aqp3 and Aqp5 induced by E2 treatment were similar between control and REAf/d uteri, but higher levels of Aqp4 were reached in REAf/d than control uteri after E treatment. REAf/d females were subfertile, while REAd/d females were completely infertile. No ovulation defect was found in REAf/d females, rather REAf/d females showed altered uterine growth and maturation, as well as implantation, decidualization, and placentation defects. Aberrant apoptosis is observed in REAf/d uteri, as reflected by drastic increases in both TUNEL and active caspase-3 levels in REAf/d uteri compared to wild-type uteri. These findings suggest that REA regulates proper ER responsiveness in vivo and is crucial for ER-mediated female uterine function.

**MICE WITH UTERINE-SPECIFIC DELETION OF MSX1 AND MSX2**

Mammalian homeobox genes, Msx1 and Msx2, are crucial for organogenesis and embryo development. Nallasamy et al. reported that the selective ablation of either Msx1 (PgrCre+;Msxf1f/f, called Msx1f/d) or Msx2 (PgrCre+;Msx2f/f, called Msx2f/d) in the uterus lead to subfertility in females, however, deletion of both Msx1 and Msx2 in the uterus (Msxf1f/d;Msx2f/d), resulted in complete infertility. This indicates that Msx1 and Msx2 functions can partially compensate for each other. The female infertility in Msxf1f/d;Msx2f/d females is in part due to an implantation defect. Deletion of Msx1 and Msx2 in the uterus causes uterine hypersensitivity to estrogen due to an increase in uterine ERα activity. Increased ER phosphorylation in uterine epithelial and subepithelial stromal cells, resulting from high fibroblast growth factor (FGF) expression, were observed. Female mice with uterine Msx1 and Msx2 deletion exhibited not only an altered ERα activity but also showed aberrant expression of Wnt/β-catenin signaling molecules. This indicates that homeobox genes Msx1 and Msx2 in the uterus are mediators of physiological ERα activity, which subsequently play pivotal roles in establishing successful pregnancy.
MICE WITH UTERINE-SPECIFIC DELETION OF NCOA6

Nuclear receptor co-activator-6 (NCOA-6) is an ERα co-activator. Kawagoe et al. reported that NCOA-6 regulates uterine ER activity by using selective deletion of Ncoa6 in the uterus (PgrCre;Ncoa6f/f, called Ncoa6<sup>d/d</sup>).<sup>211</sup> Ovulation, fertilization rates, and blastocyst development are all normal in Ncoa6<sup>d/d</sup> females; however, embryo implantation fails. The implantation defect is in part due to an increased level of the ERα co-activator, SRC3, in the uterine epithelial cells of Ncoa6<sup>d/d</sup>, which causes elevated uterine epithelial ERα activity compared to wild-type uteri. Moreover, a diminished PR activity, particularly during the implantation period, was observed. Implantation was partially rescued and the level of MUC1 was corrected in uterine luminal epithelia by treating Ncoa6<sup>d/d</sup> females with the ER antagonist ICI 182 780.<sup>211</sup> These findings suggest that loss of uterine NCOA-6 causes implantation failure due to an aberrant ER-estrogen hypersensitivity.

MICE WITH UTERINE-SPECIFIC DELETION OF MIG6

Mitogen-inducible gene 6 (Mig-6, encoded from the Errfi1 gene) is a downstream target of P-PR and SRC1 action in the uterus.<sup>108</sup> However, uterine-specific deletion of Mig-6 (Mig-6<sup>d/d</sup> or PgrCre;Mig-6f/f) demonstrated altered ERα signaling resulting in uterine hyperplasia.<sup>108</sup> Mig-6<sup>d/d</sup> uteri are enlarged, with weights approximately 3-fold more than wild-type (Mig-6<sup>f/f</sup>) uteri. Immunohistochemical analysis demonstrated that Mig-6<sup>d/d</sup> uteri exhibit hyperproliferation of the endometrium, with increased phospho-H3 staining, as a result of increased ERα expression and phosphorylation.<sup>108</sup> The ERα targeted genes, lactoferrin (Lf), chloride channel calcium–activated 3 (Clca3), and complement component 3 (C3) were also elevated in Mig-6<sup>d/d</sup> uteri in the presence of both E and P; however, the PR-targeted genes amphiregulin (Areg) and follistatin (Fst) remained comparable to wild-type uteri. Ovariectomized Mig-6<sup>d/d</sup> females that were treated with E for 3 months exhibited endometrial cancer. However, co-treatment with E and P attenuated the pathology in the uterus of E treated Mig-6<sup>d/d</sup>, although the uterine hyperplasia was still observed in Mig-6<sup>d/d</sup> females. Additionally, the expression level of MIG-6 in women with endometrioid carcinoma was elevated compared to normal endometrium.<sup>108</sup> These findings together suggest that Mig-6, as a tumor suppressor in both rodents and human, plays a crucial role in uterine growth in response to E in part by mediating the protective action of P.<sup>108</sup>

MICE WITH UTERINE-SPECIFIC DELETION OF HAND2

Heart and neural crest derivatives expressed 2 (Hand2) is regulated by P in the uterus, as protein expression of HAND2 is induced in the uterine stromal cells after P treatment.<sup>212</sup> PRKO uteri showed loss of Hand2 expression, indicating the direct regulation of Hand2 by PR.<sup>212</sup> Selective ablation of Hand2 in the uterus (Hand2<sup>d/d</sup>) using the PgrCre;Hand2<sup>f/f</sup> animal model demonstrated an elevated uterine ER activity.<sup>212</sup> Loss of uterine Hand2 expression in the stromal cells leads to aberrant fibroblast growth factor receptor (FGFR) signaling, which subsequently induced ERK signaling and ERα activity in the uterine epithelium (Figure 25.8), resulting in complete infertility. The infertility in Hand2<sup>d/d</sup> females is in part due to an implantation defect as a result of enhanced uterine epithelial ERα activity.

**FIGURE 25.8** Schematic representation of paracrine and autocrine mechanisms of uterine proliferation in response to hormones. E2 stimulation of proliferation of the uterine epithelium requires the presence of functional ERα in the underlying stroma independent from epithelium, indicating that E2/ERα actions in the stroma induce the secretion of paracrine factors that then act on the epithelium to stimulate proliferation. Progesterone (P) acts through epithelial and stromal PRs to inhibit the proliferative response of the epithelium to E, while inducing proliferation of the underlying stroma. BM, basement membrane. (For detail see text.)
activity during the implantation period. This suggests that Hand2 expression in the uterus, orchestrated by an action of PR, mediates a proper physiological ERα activity (Figure 25.8) in response to ovarian hormones, leading to successful pregnancy establishment.

**Vaginal and Cervical Phenotypes in Mouse Models of Disrupted Estrogen Signaling**

The vaginal mucosa is composed of an epithelium and underlying stroma, possesses significant levels of ER, and is highly sensitive to estrogens. Endogenous or exogenous estrogens induce stromal differentiation, epithelial proliferation, and epithelial keratin synthesis to produce the stratified layer of cornified cells that lines the vaginal lumen. These changes in the vaginal mucosa are used experimentally to estimate the level of circulating gonadal steroids and approximate the estrous cycle phase. Despite exposure to elevated endogenous E2 levels, the vaginal tissue of ERα-null females lacks any indications of estrogenization (Figure 25.5(B)). Exogenous administration of E2 or DES also elicits no discernible vaginal response in ERα-null females. In contrast, the vaginal mucosa of ERβ-null females undergoes the normal cyclic changes as dictated by ovarian sex steroids (Figure 25.5(B)). An effect on the vaginal mucosa similar to that observed in ERα-null females is produced in rodents after prolonged ovariectomy or exposure to ER antagonists. Therefore, estrogenization of the vaginal mucosa in rodents, which is critical to mating, is ERα dependent. Using a human papilloma virus (HPV) transgenic model of cervical cancer, Chung et al. demonstrated that estrogen promotes development of HPV-induced cervical cancer through ERα, as αERKO HPV-transgenic animals were protected from developing disease.

**Progesterone Receptor Expression in the Uterus**

In the murine uterus, PR transcript and protein levels are low on days 1–2 of pregnancy with the highest expression occurring in the epithelia and subepithelial stroma. PR levels rise in these same tissues on days 3 and 4 of pregnancy, leading up to implantation. Receptor-mediated E2 actions are a primary regulator of PR expression in the rodent uterus, but these actions are complex and specific to the different uterine compartments. Ovariectomy leads to increased PR levels in the uterine luminal epithelium that can be reduced upon exogenous E2 treatments, indicating E2 acts to repress PR expression in this uterine compartment. Recombination experiments of stromal and epithelial tissues similar to those described earlier, as well as studies using epithelial selective ERα deletion, indicate this to be a paracrine-mediated effect that requires functional ERα in the stroma. Simultaneous with causing decreased PR expression in the epithelium, E2 treatments lead to increased PR levels in the uterine stroma of ovariectomized mice. This is an ERα dependent regulation, as PR is found only in epithelial cells, regardless of E2 treatment in αERKO. Additionally, epithelial ERα is dispensable for this response as increased PR expression in the stromal cells is observed in the absence of epithelial ERα. These observations have been largely confirmed in studies using a PR-lacZ reporter mouse that represents Pgr gene promoter activity as β-galactosidase activity in situ. In these studies, β-galactosidase activity representative of PR expression is observed in luminal and glandular epithelium after ovariectomy and decreased after estrogen treatment. P treatments also lead to decreased PR-lacZ expression in all cell types.

In the adult ovine and bovine uterus, PR expression is highest during the early proliferative phase and present in all uterine cell types but is most abundant in the stroma and myometrium. PR levels decline as the cycle progresses to the secretory phase, initially in the stroma and myometrial cells. In the human uterus, PR immunoreactivity is increased in all cell types during the proliferative phase but measurable lower than ER levels. During the secretory phase, PR levels are maintained in the stroma and myometrium but decrease in the glandular epithelium such that levels are eventually undetectable. Studies using PR isoform selective antibodies have shown that the decrease in PR levels that occurs in the glandular epithelium during the mid-secretory phase is largely representative of PRA, leading to an increased PRB/PRA ratio in these cells. In stroma, where fewer cells expressed PR relative to glandular epithelium, PRA is more abundant. During the late secretory phase, PRA levels in the stroma decrease but remain detectable, whereas stromal PRA expression decreases occur earlier and are significantly diminished by the late secretory phase. The predominance of PRA in the glandular epithelium during the early secretory phase may serve to inhibit further estrogen-mediated proliferation, as the uteri in PRA-null mice exhibit an exaggerated proliferative response to estrogens. As P levels rise during the mid-secretory phase, PRB is concentrated in nuclear foci, suggesting active transcriptional activity. A similar finding of focal-concentrated PRB is reported in endometrial cancer cells in postmenopausal women, suggesting that inappropriate PRB activity may be associated with uterine cancer. The pattern of PR expression in uteri of nonhuman primates is similar to that described in humans. In female baboons, PR levels are increased during the proliferative phase and then decrease accordingly during the secretory phase.

**Uterine Phenotypes in Mouse Models with Disrupted Progestosterone Signaling**

**MICE LACKING PR**

There are several reported lines of PR-null mice. Mice lacking both nuclear PR isoforms (PRKO) were first
described by Lydon et al. in 1995 and were generated via targeted disruption of exon 1 of the murine Pgr gene.\textsuperscript{176} In 2006, Hashimoto-Partyka et al. generated the PR (Pgr\textsuperscript{1/4})-targeted mutation in exon 1 of PR using Ella-Cre promoter, which expressed Cre recombinase in the pre-implantation embryo in order to create a deletion of exon 1 and loss of PR protein.\textsuperscript{177} In 2010, Fernandez-Valdivia et al. described a mouse line that had a deletion of PR exon 2 and loss of PR protein using Pgr\textsuperscript{1/4} and ZP3-Cre promoter expressing mice (called PR\textsuperscript{d/d}).\textsuperscript{178}

Mice lacking only PRA (PRAKO)\textsuperscript{180} or PRB (PRBKO)\textsuperscript{182,227} were generated via a Cre/loxP-based gene targeting strategy that mutated the respective start codons for each isoform while preserving the translational reading frame of the other. Comparative studies in all models have greatly furthered our understanding of the divergent functions for the two PR isoforms. Recently, Franco et al. generated an epithelial cell specific deletion of both PRA and PRB in the uterus (called Wnt7a-Cre\textsuperscript{+}PRf)\textsuperscript{228} using Wnt7a-Cre expressing promoter crossed with Pgr\textsuperscript{f/f} animals.\textsuperscript{179}

PR-null mice exhibit normally developed uteri that possess the expected uterine architecture (Table 25.3).\textsuperscript{181,182} During the secretory phase of the murine estrous cycle, decreasing circulating levels of E2 concurrent with rising P cause a shift in uterine proliferation from the epithelial to stroma cells.\textsuperscript{228} Estrogen, through ER\textalpha, is known to stimulate uterine epithelial cell proliferation in adult mouse uteri;\textsuperscript{125,139,229} and P exhibits the antiproliferative effect on the action of E2 (Figure 25.8).\textsuperscript{230,231} Therefore, PR-mediated P actions negatively modulate E2-induced proliferation of the uterine epithelium.\textsuperscript{228} In addition, ovariectomized PR-null females treated with daily injections of E2 and P for 3 weeks exhibit abnormally large fluid-filled uteri that are characterized by a thickened uterine wall due to extensive extracellular edema, hyperproliferation of the glandular epithelia resulting in a disordered cellular arrangement, and acute inflammation of the endometrium.\textsuperscript{176} The hyperestrrogenic response in PR-null uteri is similar to that observed in ovariectomized mice after prolonged exposure to unopposed estrogens\textsuperscript{229} and provides strong support of the modulatory actions of PR in the uterus.\textsuperscript{176} Similar experiments in isofrom-specific PR-null mice indicate this phenotype is reproduced in PRA-null mice only, whereas PRB-null mice exhibit a normal uterine response, indicating that PRA is primarily responsible for negatively modulating the uterine response to E2.\textsuperscript{180–182}

**Uterine Phenotypes in Mouse Model with Mutations in Transcriptional Proteins That Mediate PR Function**

**MICE WITH UTERINE-SPECIFIC DELETION OF P160 (SRCs)**

The SRCs mediate P-dependent action in the uterus.\textsuperscript{232} Immunohistochemical analysis in the uteri of pregnant mice demonstrated the spatiotemporal expression pattern of SRC1 and SRC2 in the luminal epithelial, stromal as well as secondary decidual zone. The uterine expression pattern of SRC1 and SRC2 was similar to the pattern of PR expression during pregnancy.\textsuperscript{232} In uteri from ovariectomized animals, the majority of SRC1 and SRC2 was expressed in the luminal and glandular epithelium, much like PR expression.\textsuperscript{232} Both E and P, either alone or administered together, increased the expression of SRC1 and SRC2 in the uterine stromal cells.\textsuperscript{232}

There are several reports describing SRC-null mice and resulting effects on female reproductive functions.\textsuperscript{201,232–234} Global disruption of the Src1 gene demonstrated that both males and females were fertile, however, females exhibited a blunted response to ovarian hormones.\textsuperscript{201} Treatment of Src1\textsuperscript{−/−} females with E2 for 3 consecutive days resulted in less uterine weight increase than in WT, similarly, artificial induction of decidualization resulted in less stromal differentiation of Src1\textsuperscript{−/−} uteri.\textsuperscript{201} Due to a lethality of Src2 global knockout,\textsuperscript{235} uterine-specific SRC2 deletion (Src2\textsuperscript{d/d}) was generated using Pgr\textsuperscript{Cre} crossed with Src2\textsuperscript{f/f} animals.\textsuperscript{233} Src2\textsuperscript{d/d} females were infertile, in part, due to an implantation and decidualization defect.\textsuperscript{232,233,236} E2-induced uterine epithelial cell proliferation was preserved in Src2\textsuperscript{d/d} uteri.\textsuperscript{233} The decidualization markers such as Bmp2, Ptgs2 (Cox-2), and Fst were significantly decreased in Src2\textsuperscript{d/d} when compared to wild-type uteri.\textsuperscript{231} A complete absence of decidual response was observed in Src2\textsuperscript{d/d} uteri in a Src1\textsuperscript{−/−} background.\textsuperscript{253} However, Src3\textsuperscript{−/−} females had no overt uterine phenotype.\textsuperscript{232} These findings indicate that of the three SRCs, SRC2 is most crucial for P-dependent uterine function.

**MICE WITH UTERINE-SPECIFIC DELETION OF COUP-TFII**

COUP-TFII regulates uterine ER function during early pregnancy as mentioned earlier. Additionally, the COUP-TFII\textsuperscript{d/d} animal model also demonstrated that COUP-TFII is a downstream effector for PR-mediated decidualization.\textsuperscript{202} Loss of uterine COUP-TFII resulted in a defect in decidualization, as reflected by lack of expression of decidual cell markers (WNT4 and BMP2).\textsuperscript{203} Treatment of COUP-TFII\textsuperscript{d/d} females with ICI 182,780 restores the expression of WNT4 and BMP2 in the decidual cells.\textsuperscript{203} Together, the findings from uterine-specific deletion of COUP-TFII suggest that COUP-TFII regulates P signaling during implantation and decidualization by controlling uterine ER activity.

**MICE WITH UTERINE-SPECIFIC DELETION OF REA**

As discussed earlier, REA is crucial for uterine responses to ovarian hormones and establishment of pregnancy. Using REA\textsuperscript{d/d} animals also demonstrated that loss of uterine REA led to aberrant PR function, which resulted in a decidualization defect.\textsuperscript{207} The decidual cell
markers, Wnt4 and Bmp2, were not increased in REA<sup>d/d</sup> uteri after artificial decidualization. The defect in decidualization is due to loss of PR expression after uterine REA ablation. These findings indicate that uterine REA expression is not only crucial for ER activity but also for PR function to establish and maintain pregnancy.

**MICE WITH UTERINE-SPECIFIC DELETION OF FKBP5s**

FK506 Binding Proteins 4 (FKBP4; encoded by Fkbp52 gene) and 5 (FKBP5; encoded by Fkbp51 gene) are immunophillin family co-chaperones that interact with PR in the absence of the ligands. Fkbp4 is expressed in the luminal and glandular epithelial cells during implantation, whereas a minimal level was detected in the uterine stromal cells, similar to PR expression. During the decidual response, Fkbp4 was highly expressed in both primary and secondary decidual zones. Although the expression of Fkbp5 also showed a similar pattern, the level of expression was much lower than that of Fkbp4. The global knockout of FKBP5 was fertile and showed no impairment of uterine functions. However, the global knockout of FKBP4 caused complete female infertility due to implantation and decidualization defects, suggesting that FKBP4 not only binds to PR but is crucial for PR action during pregnancy.

**Uterine Response to Estradiol and Progesterone**

**Changes in Physiology**

Estrogens stimulate a complex process of epithelial proliferation and differentiation in the sexually mature uterus that leads to the formation of a multilayered secretory endometrium. This effect is critical to provide a suitable intrauterine environment for the establishment and maintenance of pregnancy. Early studies in neonatal and prepubertal rodents found that both the uterine stroma and epithelium proliferate in response to estrogens (Figure 25.9); however, estrogen-induced mitogenesis in uteri of sexually mature rodents is limited to the epithelium. Therefore, sexual maturation of the rodent uterus is not simply marked by the presence of ER or an estrogen response but is rather the acquired capacity to undergo synchronized phases of proliferation and differentiation asdictated by the ovarian-derived sex steroids. The lack of estrogen-induced uterine epithelial proliferation in ERα-null uteri indicates the essential role of ERα in this process (Figure 25.6). Although ERα is present in both epithelial and stromal compartments, tissue recombination studies and study of the uterine epithelial-specific ERα null indicate the proliferative epithelial response to estrogens is indirect and dependent on stromal ERα actions (Figure 25.6 and a working model in Figure 25.8).

Treatment of ovariectomized mice with estrogens (e.g., E2 or DES) has long served as an experimental model to mimic the uterine events that occur during the estrous phase of the rodent cycle or immediately after the preovulatory E2 surge. Morphological and biochemical changes occur in the rodent uterus after estrogen stimulation following an established biphasic temporal pattern (Table 25.4). Estrogen-stimulated changes in the rodent uterus that occur early, within the first 6 h after treatment, include increases in nuclear ER occupancy, water imbibition, vascular permeability and hyperemia, prostaglandin release, glucose metabolism, eosinophil infiltration, gene expression (e.g., c-fos), and lipid and protein synthesis (Table 25.4). Recent ERα ChIP-Seq profiles from uterine tissues showed that the receptor occupies chromatin sites and that E2 treatment increases ERα recruitment. These processes are then accompanied by a delayed response that peaks after 24–72 h and includes dramatic increases in RNA and DNA synthesis, epithelial proliferation, and differentiation toward a more columnar secretory phenotype, dramatic increases in uterine weight, and continued gene expression (e.g., lactotransferrin) (Table 25.4). Ovariectomized mice exhibit a three- to four-fold increase in uterine weight after three daily treatments with E2 or DES, whereas no such response is observed in the uteri of ERα-null females. The early phase effects of water imbibition and hyperemia as well as the late-phase effects of increased DNA synthesis and epithelial proliferation are absent in ERα-null uteri (Figure 25.6). Interestingly, females that are heterozygous for the Esr1 gene...
and stromal cells. 245,246 ICI 182,786 (ER antagonist) strongly inhibited E2-induced Cebpb transcript in the uterus, suggesting an ER-dependent expression of C/EBPβ. 247 In addition, loss of epithelial ERα in the uterus did not alter E2-induced Cebpb expression, indicating that Cebpb expression is independent of epithelial ER. 170 This points to the action of estrogen through ERα as the major mediator of C/EBPβ expression in the uterus. Indeed, the deletion of C/EBPβ (C/EBPβ−/−) leads to a lack of the E-induced uterine proliferative response 245 as reflected by the absence of mitotic activity, S-phase activity, and an increase in apoptotic activity in the uterine epithelial cells. 246 In addition to a blunted uterine growth response to hormones, the C/EBPβ−/− females also exhibit complete infertility 247 due to implantation and decidualization defects. 245

Pan et al. demonstrated that the uterine expression of minichromosome maintenance proteins (MCMs), a complex required for DNA synthesis initiation, is induced after E2 treatment, specifically MCM2 and MCM3 248 (Figure 25.9). MCM2 activity is crucial and required for DNA synthesis in the uterine epithelial cells. 249 The DNA replication of uterine epithelial cells induced by E2 is attenuated by P action, which will be discussed further in a later section.

**Estrogen–Growth Factor Cross Talk in the Uterus**

The autocrine and paracrine actions of polypeptide growth factors are an integral component of the uterine response to estrogens. The uterine response to E2 is modulated by stromal factors, such as IGF1, that are induced by E2 and then impact epithelial responses. 250 Igf1 transcript is increased with concomitant decrease of Igfbp3 242 and activation of the IGF1 receptor and downstream effectors following E2 treatment. 251 Igf1 transcript is increased in both stromal and epithelial compartments of the uterus by E2, with greater signal apparent in the stroma (Figure 25.10(A)). 252 Igf1 has been demonstrated to play an essential role in the uterine growth response, as Igf1−/− null mice lack a full uterine proliferative response, and more specifically, lack G2/M progression of the epithelial cells following E2 stimulation. 253 Additionally, transgenic mice overexpressing Igfbp1, which sequesters, and therefore decreases, the amount of available IGF1, have an attenuated uterine response to E2. 254 Uterine response is restored by transplanting Igf1KO uterine tissue into a WT host 255 which demonstrated the paracrine effect of the host Igf1. Further, E2 treatment results in the activation of downstream mediators of Igf1 signaling, including the Igf1 receptor, IRS1, 251 AKT, and inhibition of GSK3β, 252 leading to nuclear translocation of CCND1 and epithelial proliferation. Additionally, picropodophyllin (PPP), an IGFR inhibitor, blunted the proliferative effect of E2. This effect of PPP on E2-stimulated uterine proliferation could be reversed, however, by

---

**TABLE 25.4 Biphasic Response of the Rodent Uterus to Estrogens**

<table>
<thead>
<tr>
<th><strong>EARLY UTEROTROPIC RESPONSES (WITHIN 6H)</strong></th>
<th><strong>LATE UTEROTROPIC RESPONSES (WITHIN 24H)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear localization of estrogen receptor/recruitment to chromatin</td>
<td>Second peak of nuclear localization of estrogen receptor</td>
</tr>
<tr>
<td>Activation of receptor-tyrosine kinase pathways</td>
<td>Changes in gene expression (induction/repression of “late” genes)</td>
</tr>
<tr>
<td>Changes in gene expression (induction/repression of “early” genes)</td>
<td>Increased protein synthesis</td>
</tr>
<tr>
<td>Increased vascular permeability</td>
<td>Increased DNA synthesis</td>
</tr>
<tr>
<td>Water imbibition</td>
<td>Epithelial proliferation in “waves”</td>
</tr>
<tr>
<td>Hyperemia</td>
<td>Cellular hypertrophy</td>
</tr>
<tr>
<td>Eosinophil infiltration</td>
<td>Overall increase in uterine dry weight</td>
</tr>
<tr>
<td>Albumin accumulation</td>
<td>Calcium influx</td>
</tr>
<tr>
<td>Increased electrolytes</td>
<td>Increased lipid synthesis</td>
</tr>
<tr>
<td>Lysozyme labilization</td>
<td>Increased cyclic nucleotides, prostaglandins, and associated enzyme activity</td>
</tr>
<tr>
<td>Increased cyclic nucleotides, prostaglandins, and associated enzyme activity</td>
<td>Increased glucose metabolism and associated enzyme activity</td>
</tr>
<tr>
<td>Increased glucose metabolism and associated enzyme activity</td>
<td>Calcium influx</td>
</tr>
<tr>
<td>Calcium influx</td>
<td>Increased protein synthesis</td>
</tr>
<tr>
<td>Increased lipid synthesis</td>
<td>Increased vascular permeability</td>
</tr>
</tbody>
</table>

Disruption possesses approximately one-half the normal level of ERα in the uterus, but their response to estrogen treatment is comparable with wild-type females. The total lack of response to estrogens in ERα-null uteri as well as a lack of late biological response in epithelial ERα knockout uteri provide strong evidence that ERα is required to mediate the full biochemical and biological uterine response to estrogens. 159,170

**Molecular Mechanisms of Estrogen-Induced Uterine Proliferation**

Numerous studies have elucidated the molecular mechanisms of E2-induced uterine epithelial cell proliferative responses in animal models. For example, the transcription factor CCAAT enhancer binding protein beta (C/EBPβ) is involved in hormone-induced uterine proliferation. 243 Maximum uterine expression of C/EBPβ is induced 1 h after E2 treatment in both epithelial and stromal cells. 245,246 ICI 182,786 (ER antagonist) strongly inhibited E2-induced Cebpb transcript in the uterus, suggesting an ER-dependent expression of C/EBPβ. 247 In addition, loss of epithelial ERα in the uterus did not alter E2-induced Cebpb expression, indicating that Cebpb expression is independent of epithelial ER. 170 This points to the action of estrogen through ERα as the major mediator of C/EBPβ expression in the uterus. Indeed, the deletion of C/EBPβ (C/EBPβ−/−) leads to a lack of the E-induced uterine proliferative response 245 as reflected by the absence of mitotic activity, S-phase activity, and an increase in apoptotic activity in the uterine epithelial cells. 246 In addition to a blunted uterine growth response to hormones, the C/EBPβ−/− females also exhibit complete infertility 247 due to implantation and decidualization defects. 245

Pan et al. demonstrated that the uterine expression of minichromosome maintenance proteins (MCMs), a complex required for DNA synthesis initiation, is induced after E2 treatment, specifically MCM2 and MCM3 248 (Figure 25.9). MCM2 activity is crucial and required for DNA synthesis in the uterine epithelial cells. 249 The DNA replication of uterine epithelial cells induced by E2 is attenuated by P action, which will be discussed further in a later section.

**Estrogen–Growth Factor Cross Talk in the Uterus**

The autocrine and paracrine actions of polypeptide growth factors are an integral component of the uterine response to estrogens. The uterine response to E2 is modulated by stromal factors, such as IGF1, that are induced by E2 and then impact epithelial responses. 250 Igf1 transcript is increased with concomitant decrease of Igfbp3 242 and activation of the IGF1 receptor and downstream effectors following E2 treatment. 251 Igf1 transcript is increased in both stromal and epithelial compartments of the uterus by E2, with greater signal apparent in the stroma (Figure 25.10(A)). 252 Igf1 has been demonstrated to play an essential role in the uterine growth response, as Igf1−/− null mice lack a full uterine proliferative response, and more specifically, lack G2/M progression of the epithelial cells following E2 stimulation. 253 Additionally, transgenic mice overexpressing Igfbp1, which sequesters, and therefore decreases, the amount of available IGF1, have an attenuated uterine response to E2. 254 Uterine response is restored by transplanting Igf1KO uterine tissue into a WT host, 255 which demonstrated the paracrine effect of the host Igf1. Further, E2 treatment results in the activation of downstream mediators of Igf1 signaling, including the Igf1 receptor, IRS1, 251 AKT, and inhibition of GSK3β, 252 leading to nuclear translocation of CCND1 and epithelial proliferation. Additionally, picropodophyllin (PPP), an IGFR inhibitor, blunted the proliferative effect of E2. This effect of PPP on E2-stimulated uterine proliferation could be reversed, however, by
co-treatment with PPP and the GSK3β inhibitor, SB415286 (Figure 25.10(B)). These findings elegantly illustrate a direct role for IGF1R-initiated signaling through AKT and GSK3β on uterine response to E2.

In addition, IGF1 or EGF treatment of ovariectomized wild-type mice elicits a pattern of global gene expression similar to that induced by E2, although some estrogen-specific genes are revealed. IGF1 treatment was shown to increase the expression of an ERE-driven luciferase reporter gene in transgenic mice, providing the first in vivo evidence of E2-independent ER activation by growth factor. ER-null mice provide an excellent in vivo model for the study of ER–growth factor “cross talk” mechanisms in the uterus. ERα-null uteri express wild-type levels of functional EGFR but are unresponsive to the mitogenic actions of EGF, confirming the interaction of these two signaling systems. However, not all EGF responses are lacking in the uteri of αERKO females, as upregulation of the c-fos gene by this growth factor remains intact. Similar studies have demonstrated that the uterine response to IGF1 is compromised in ERα-null females. Cunha and colleagues used the ERα-null mice in a series of tissue recombination experiments to further demonstrate interaction between ER and growth factor signaling in the murine reproductive tract. In these studies, uterine stoma and epithelium are enzymatically disassociated from prepubertal mice and recombined with corresponding tissue from animals of different treatments or genotypes. The resulting chimeric stromal-epithelial unit is then implanted under the kidney capsule of an ovariectomized nude mouse that is then treated with various hormonal combinations. A caveat to these studies is that neonatal tissues are used and may not accurately reflect the uterine physiology of sexually mature females. Nonetheless, these methods have been effectively used to demonstrate that estrogen-induced proliferation of uterine and vaginal epithelium requires functional ERα in the underlying stroma only, whereas estrogen-induced increases in secretory products (e.g., LTF, complement C3, keratins) in the uterine or vaginal epithelium requires functional ERα in both uterine compartments. These findings have now been confirmed in sexually mature intact uterine tissue using uterine epithelial cell–selective ERα-null mice. In addition, treatment of uterine epithelial ERα-null females with IGF1 or EGF mimics the uterine epithelial cell DNA synthesis stimulated by E2 (Figure 25.11). These studies strongly support a paracrine mechanism of estrogen-mediated epithelial proliferation that requires ERα in the stroma (working model in Figures 25.8 and 25.11). Interestingly, microarray studies indicate that the growth factor–induced genomic response is intact in ERα-null uteri, although epithelial proliferation is absent, suggesting a greater complexity to the estrogen–growth factor signaling “cross talk” than was originally perceived. GF signaling is thought to mainly utilize AF-1 via MAPK phosphorylation sites in the N-terminus. Thus is was expected that IGF1 or EGF treatment of AF2ERKI/KI mice would lead to uterine growth, however, no response was seen, indicating functional AF-2 is also involved in this mechanism.

**Antiproliferative Functions of PR in the Endometrium**

Tong and Pollard demonstrated that P inhibits E-induced uterine proliferation via the inhibition of DNA synthesis and PCNA expression (a component of DNA polymerase gamma, which is required for S-phase entry). Additionally, Ray and Pollard demonstrated that Kruppel-like transcription factor 4 (KLF4) expression is induced whereas KLF15 is suppressed by E2 treatment in the uterine epithelial cells. Conversely, co-treatment with P and E decreased KLF4 expression and increased KLF15 in the uterus. In addition, overexpression of uterine KLF15 using an adenovirus leads to a lack of E2-induced uterine epithelial proliferative
response due to the loss of MCM2 expression, demonstrating that KLF15, through the action of P, inhibits E2-induced uterine epithelial DNA synthesis by inhibition of MCM2 expression. Moreover, P also diminished E2 induction of Cyclin D1 nuclear translocalization and expression of Cyclin A, which consequently decreased the phosphorylation of pRb and p107. Together, these findings described the mechanism by which P inhibits cell cycle activity induced by E2 in the uterine cells (see Figure 25.8).

P not only inhibits the uterine epithelial proliferation but also contributes to an induction of stromal cell proliferation in the presence of E2. Recombination experiments using uterine tissues from PR-null mice demonstrate that the antiproliferative functions of P in the uterine epithelium are paracrine mediated and require PRA in the stroma (Figure 25.12). However, selective deletion of PR in the uterine epithelial cells (Wnt7a-Cre;PR−/− animal model) demonstrated that epithelial PR expression is dispensable for E2-induced uterine weight increase and induction of stromal cell proliferation but required for the P-mediated inhibition of epithelial cell proliferative response induced by E2. Wnt7a-Cre;PR−/− animals exhibit an aberrant uterine expression of MCM3 and Cyclin D1, which are important mediators of E2-induced uterine epithelial proliferative responses.

Moreover, P treatment also inhibits the pro-inflammatory activity induced by estrogen. The inflammatory response observed in PR-null uteri after prolonged estrogen/P treatment is extensive and consists of “marked” infiltration of polymorphonuclear leukocytes into the endometrial stroma, mucosal epithelium, and uterine luminal fluid. In vitro studies have shown that P inhibits the expression of chemotactic cytokines for neutrophil and lymphocyte infiltration and may reduce prostaglandin E levels in uterine decidual and chorionic tissue during early pregnancy. Therefore, PR-mediated P actions may be critical to suppressing the uterine inflammatory response that may accompany embryo implantation.

**Roles of PR in the Rodent Uterus during Pregnancy**

PR-mediated P actions in the uterus are critical to preparing the uterine endometrium for pregnancy. Embryo implantation is a highly complex process that requires synchronized cooperation between the blastocyst and uterine endometrium (see Chapter 38). Circulating E2 levels peak at ovulation and elicit a cascade of proliferation and differentiation in the luminal and glandular epithelium of the uterus, including induction of PR expression in the endometrial stroma and myometrium. Postovulatory increases in circulating P then
cause decidualization, a complex process that involves massive proliferation and differentiation of the endometrial stroma along with localized increases in vascular permeability and edema.\textsuperscript{34,268,269} This process involves the synthesis and interaction of numerous hormones and signaling pathways, including prolactin, cytokines, prostaglandins, and extracellular matrix components.\textsuperscript{268,269} The result is a remarkable swelling of the uterine stroma that is thought to be necessary for implantation by forcing the uterus to close down on the blastocyst.\textsuperscript{268,269} The final stage of apposition is a grasping of the blastocyst and ultimate attachment to the uterine wall, a process thought to be dependent on secondary rises in ovarian-derived E\textsubscript{2}.\textsuperscript{268,269} Therefore, preparation of the uterus for blastocyst implantation is dependent on the multifunctional and sometimes opposing effects of E\textsubscript{2} and P. In addition, PR-null models suggest that PRA is crucial not only for implantation but also the decidualization process as female mice lacking only PRA fail to exhibit a uterine decidual response, illustrating the importance of PRA for establishing successful pregnancy.\textsuperscript{176,178,180} Recently, Franco et al. demonstrated that the epithelial PR is responsible for establishing successful pregnancy as the deletion of uterine epithelial PR contributes to an impaired embryo attachment, implantation, and decidual response.\textsuperscript{179} Details of implantation and decidualization mechanisms are discussed in Chapter 38.

**Maintenance of Progesterone Action in the Absence of ER**

The \textit{Pgr} gene is a well-described target of estrogen-induced expression via the classic model of ER action, especially in the uterus.\textsuperscript{32,33} The lack of E\textsubscript{2}-induced increases in \textit{Pgr} expression in ER\textalpha-null uteri confirms the regulatory dependence on ER\textalpha action.\textsuperscript{189} Therefore, it was hypothesized that disruption of the ER\textalpha gene may subsequently result in abnormally low levels of PR in ER\textalpha-null uteri and thereby render this tissue refractory to P as well. However, assays for \textit{Pgr} expression and P binding in ER\textalpha-null uteri indicate PR levels are only reduced by approximately half.\textsuperscript{110,161,189} Furthermore, a greater proportion of PR is nuclear localized in ER\textalpha-null uteri (\textapprox 25\%) relative to wild-type (\textapprox 5\%).\textsuperscript{161} Western blots indicate no difference in the relative levels of PRA and PRB between genotypes, with PRA consistently present in greater amounts in both.\textsuperscript{161} Therefore, a loss of ER\textalpha action in the uterus has not led to a complete lack of PR or to altered and preferential transcription from one of the two \textit{Pgr} gene promoters. Furthermore, several P-dependent actions are preserved in ER\textalpha-null uteri, including P-induced expression of amphiregulin and calcitonin and the uterine decidual response,\textsuperscript{161} but not embryo attachment or implantation.\textsuperscript{133}

The postovulatory nadir in circulating E\textsubscript{2} levels has long been known to be critical to uterine decidualization in mice.\textsuperscript{270} Studies have shown that the ER antagonist ICI 182,780 prevents artificially induced uterine decidualization in wild-type mice, indicating involvement of ER signaling.\textsuperscript{161} Therefore, it is surprising that ER\textalpha-null mice exhibit a uterine response when exposed to an artificial model of hormonal and mechanical induction of uterine decidualization.\textsuperscript{161,271} However, this may partly be due to the residual ER\textalpha protein produced by the splice variant found in the uterus of the \textalpha-ERKO line made by targeted disruption.\textsuperscript{189} Nevertheless, these findings suggest that an altered “organization” of the uterine tissue or compensatory pathways might provide for hormonally driven uterine decidualization in ER\textalpha-null mice. Alternatively, some genes detected at the time of implantation, including the gap junction protein connexin 26,\textsuperscript{272} and the cytokine leukemia inhibitory factor,\textsuperscript{135} are regulated by dual pathways. One involves estrogen-stimulated ER\textalpha, and the ability to induce via this mechanism is lost in the \textalpha-ERKO. The second pathway is initiated by decidualization-associated signals and is ER\textalpha independent and retained in the \textalpha-ERKO. This indicates a redundancy of regulatory mechanisms that allows retention of gene regulation in the \textalpha-ERKO and accounts for the ER\textalpha-independent uterine decidualization response observed in ER\textalpha-null mice. Recent studies demonstrated that de novo E\textsubscript{2} synthesis occurs locally during decidualization, especially on day 6 and 7 of pregnancy in mice,\textsuperscript{273} indicating an important role for local E\textsubscript{2} synthesis for establishment of successful pregnancy.

**Androgen Receptor Signaling in Uterine Function**

**AR Expression in the Uterus**

AR is present in uterine tissues of multiple species,\textsuperscript{130,274–278} although the function of androgen signaling in the uterus remains unclear. In rodents, ARs are present in all uterine cell types but most highly expressed in the myometrium, where expression may be positively regulated by estrogens.\textsuperscript{276,279} In humans, ARs are also detected in the myometrial and endometrial uterine tissues, and levels increase during the proliferative phase.\textsuperscript{280}

**MICE LACKING AR**

There are several reported lines of AR-null as well as mice with knock-in of mutated forms of AR (reviewed in Refs 281,282). \textit{Tfm} mice were first described in 1970 and are a naturally existing androgen-resistant mutant\textsuperscript{283} with an inactivating mutation of the \textit{Ar} gene.\textsuperscript{284,285} Comparable inactivation mutations of the \textit{Ar} gene and resulting phenotypes are well described in rats and humans.\textsuperscript{296} Because the \textit{Ar} gene is located on the X chromosome and \textit{Tfm} males are infertile, it is impossible to breed for XX female mice that are homozygous for the
mutation. Lyon and Glenister overcame this challenge more than 30 years ago by utilizing embryo aggregation to generate a limited number of Tfm chimeric males that were fertile and carried germ cells harboring the Ar mutation. More recently, AR-null female mice were generated via a Cre/loxP targeting scheme that allows for tissue and temporal specific disruption of the Ar gene and therefore the generation of fertile male carriers of the targeted Ar allele. Exon 2 of the murine Ar gene was targeted for deletion in nearly all lines that have been described in the literature. Additionally, one line of mice with in-frame deletion of exon 3 of the Ar gene has been generated, however, the uterine phenotype of this line has not been described. The exon 2 AR-null females have normal uterine development, although the uterine circumference is smaller than that of wild-type uteri (Table 25.3). Hu et al. demonstrated that the AR-null uteri had increased uterine horn length, but smaller uterine diameter, as a result of decreased endometrial and myometrial areas during diestrus, when compared to wild-type uteri. These AR-null females were subfertile, due to impaired folliculogenesis (discussed later in the section Ovarian Phenotypes in Mouse Models of Disrupted Androgen Signaling). Still, studies to date indicate that AR-null females exhibit reproductive phenotypes that are quite similar to those originally described in Tfm/Tfm females.

Recently, a mouse line was developed in which mutation of the AR DNA binding domain alters the ability of AR to bind to selective androgen response elements. This mouse line was generated by Schauwaers et al. with a targeted mutation of 12 amino acids in the second zinc-finger of the DNA binding domain at the exon 3 of AR (called SPARKI). The heterozygous or homozygous SPARKI females do not have an overt phenotype and have normal fertility indicating selective AR DNA binding is not critical for female reproductive organ development and function.

Treatment of hypophysectomized rats with DHT is known to cause increased uterine weight in rodents, indicating that ligand-dependent AR actions can affect uterine responses. Several studies also showed that DHT induced uterine weight increase in αERKO animals. Upregulation of AR expression is observed in αERKO uteri after DHT treatment, suggesting that DHT exerts its action in the uterus through AR in the absence of ERα. In addition, AR agonists increased myometrial thickness in the uteri of ovariectomized rats but inhibited estrogen-induced epithelial proliferation. Similar observations were made in female mice, which exhibited not only uterine epithelial cell proliferation but also myometrial smooth muscle cell proliferation after DHT treatment. AR-null female mice exhibit relatively normal uteri that are somewhat hypoplastic, although this phenotype may be more representative of decreased E2 synthesis in the ovaries rather than a role for AR in maintaining uterine weight (Table 25.3). Furthermore, AR-null females are able to establish and maintain pregnancies to term. Microarray studies have indicated that DHT leads to a pattern of uterine gene regulation that is remarkably similar to that elicited by estrogens but less robust, with 86% of the DHT response consisting of a subset of estrogen responses. The fold response of the overlapping genes was in general more robust after estrogen treatment versus DHT. Thus, despite differences in biological outcomes, the global genomic patterns elicited by estrogens and nonaromatizable androgens largely overlap. Genes noted included those involved in metabolism, tissue growth and remodeling, transcription, protein synthesis and processing, and signal transduction. Suppression of AR expression in human endometrial stromal cells (HESCs) using siRNA led to a decrease in decidual cell proliferation and differentiation. These findings suggest that AR plays a minimal role for uterine growth and differentiation but is not essential for uterine development.

Glucocorticoid Receptor Signaling in Uterine Function

**GR Expression in the Uterus**

The uterus is not considered a classic target tissue of glucocorticoid action, although GRs are present throughout the cell types of the rodent uterus, including uterine natural killer cells (uNK cells). Homozygous GR-null animals generated via homologous recombination in embryonic stem cells die at birth due to respiratory failure. Therefore, the generation of tissue-specific GR deletion in female reproductive tissues is crucial for studying the role of GR in reproductive functions.

**Uterine Functions of GR**

The limited experimental data available indicate that GR-mediated glucocorticoid actions are present in the uterus. Treatment with the GR agonist, dexamethasone, did not increase the weight of immature rat uterus compared to E. However, dexamethasone inhibits the uterine weight increase induced by estrogen in the rodent uteri. Surprisingly, stimulation of the GR signaling pathway by dexamethasone treatment in immature rats elicits a pattern of gene expression that is remarkably similar to, but not as robust as, estrogen. However, the molecular mechanism by which dexamethasone inhibits uterotrophic action of estrogen remains unclear. A recent study reported that E-induced human uterine leiomyoma cell proliferation is suppressed by GR activation.
leucine zipper) is crucial for immune-related function of glucocorticoid activity.\textsuperscript{300} Whirledge and Cidlowski also demonstrated that E suppressed \textit{Gilz} gene expression induced by dexamethasone in human uterine epithelial (EC1) cells.\textsuperscript{300} This suggests that estrogen and glucocorticoid exert interplay between immune-responsive functions in the uterus.

GR activity not only exhibits inhibitory effects on estrogenic action in the uterus, but may also play important roles in the decidual response, as it is known that GR is expressed in uterine natural killer (uNK) cells,\textsuperscript{296} and that the uNK cells play important roles for establishment and maintenance of successful pregnancy (reviewed in Ref. 301). However, the role of GR in uNK cell activity in the uterus remains unclear.

**MicroRNAs in Uterine Reproductive Functions**

MicroRNA (miRNA) are very small RNA molecules (18–24 nt) that are transcribed as larger primary RNAs (priRNA), either within introns of encoding transcripts or from miRNA encoding promoters. priRNA is processed to a shorter stem and loop pre-miRNA form by the activity of the DGCR8/DROSHA microprocessor complex. The pre-miRNA is then exported from the nucleus by exportin 5, to the cytoplasm where DICER cleaves the loop, leaving miRNA, which then functions to interact with mRNA, targeting it for degradation or repressing translation.\textsuperscript{302,303} miRNA is particularly critical to female reproductive tract function, as mouse models with deletion of DICER in these tissues lose fertility. Estrogen has been demonstrated to regulate levels of miRNA in uterine tissue.\textsuperscript{304,305}

The biological roles of Dicer are crucial for a normal development of limbs,\textsuperscript{306} muscle,\textsuperscript{307} and lung.\textsuperscript{308} In addition, recent findings demonstrated that expression of Dicer is not only critical for female germ line biological functions\textsuperscript{300} but also for embryo implantation.\textsuperscript{310} Although miRNAs are expressed in the female reproductive tract, their precise physiological function remains unclear. To study the role of dicer and miRNAs during female reproduction, several studies used \textit{Dicer}\textsuperscript{1/\textminus} crossed with \textit{Amhr2}\textsuperscript{Cre/+} animals to generate a specific deletion of dicer in the mesenchymal layer of female reproductive tract (called \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+}).\textsuperscript{311–313} Female mice with a lack of Dicer1 expression in the mesenchyme exhibit a complete infertility.\textsuperscript{311–313} Loss of Dicer1 has no effect on uterine development as the uterine epithelial cells, glands, stroma, and myometrium are present in \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} females.\textsuperscript{311,313} Uterine horns of \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} females are shorter and hypoplastic when compared to control (\textit{Dicer}\textsuperscript{1/\textminus}) uteri.\textsuperscript{311,313} Additionally, the \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} uteri respond normally to an artificial decidual stimulation.\textsuperscript{312} An ovarian defect was observed as increased apoptosis within the granulosa cells of \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} compared to wild-type ovaries.\textsuperscript{312} Moreover, \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} females exhibit an aberrant oviductal morphology with enlarged and fluid-filled cystic oviducts.\textsuperscript{311–313} No embryos were found in \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} uteri, but were discovered to be retained within the oviduct.\textsuperscript{312,313} The oviducts from \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} females have an augmented inflammatory response seen as an increase in recruitment of lymphocytes and macrophages into the oviductal tissues.\textsuperscript{311} Moreover, \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} females are also defective in embryo transport as transferred blue beads were mostly retained in the \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} oviduct, whereas in WT females the beads moved through the oviduct and were found to be in the uterine lumen.\textsuperscript{313} A lack of a proper embryo transport in \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} appears to be due to a uterotubal junction defect, resulting in retrograde uterine flow into the oviduct.\textsuperscript{313} Additionally, the expression of Wnt/\textbeta-catenin signaling molecules was altered in the \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} oviduct.\textsuperscript{311,313} A recent finding from a uterine-specific Dicer deletion using \textit{Pgr}\textsuperscript{Cre/+} and \textit{Dicer}\textsuperscript{1/\textminus} animals demonstrated that the females are infertile.\textsuperscript{314} Loss of uterine dicer leads to uterine developmental defects, including absence of glandular epithelium and increased apoptosis in the stromal cells.\textsuperscript{314} Stromal cell proliferation could not be induced in \textit{Dicer}\textsuperscript{1/\textminus};\textit{Pgr}\textsuperscript{Cre/+} uteri treated with estrogen and P.\textsuperscript{314} A decidualization defect was also found in \textit{Dicer}\textsuperscript{1/\textminus};\textit{Pgr}\textsuperscript{Cre/+}, indicating loss of uterine response to P.\textsuperscript{314} Additionally, loss of uterine dicer expression also leads to a dysregulation in Wnt/\textbeta-catenin signaling molecules, similar to the findings reported in \textit{Dicer}\textsuperscript{1/\textminus};\textit{Amhr2}\textsuperscript{Cre/+} females. These findings together suggest that miRNAs, produced by Dicer processing, play pivotal roles in female reproductive functions needed for establishing successful pregnancy.

**Changes in Uterine Gene Expression**

The dramatic physiological changes that occur in the uterus in response to steroid hormones are presumably the ultimate effects of equally dramatic changes in gene expression among the uterine cells. It is unlikely that the E2–ER complex is directly involved in mediating the whole genomic response in the uterus but more plausibly serves to stimulate a cascade of downstream signaling pathways that act to amplify the estrogen action. However, early investigations of the genomic response to estrogens in the rodent uterus discovered a handful of genes that are directly regulated via the classic ER mode of action, including \textit{Pgr} and \textit{Ltf}. The uteri of \textit{Esra1}\textsuperscript{null} females fail to exhibit estrogen-induced increases in \textit{Pgr} and \textit{Ltf} expression,\textsuperscript{314} indicating the importance of ER\textalpha to this response. Furthermore, estrogen-stimulated increases in PR in the rodent uterus are localized to
the stromal and myometrial compartments, whereas increased Ltf is limited to luminal and glandular epithelium, indicating that ERα functions are critical to induced gene expression in multiple uterine cell types. Further complexity of estrogen action in the uterus is illustrated by the simultaneous induction of PR expression in the myometrium and stroma while eliciting a decrease in PR levels in the luminal epithelium. Similarly, the c-fos gene is rapidly induced by estrogen in the uterus, and this is ERα dependent.

Microarray analysis of gene expression has significantly advanced understanding of genomic response of the rodent uterus to E2. Numerous studies have used microarray techniques to map the global gene expression patterns after estrogen exposure in the uterus and largely demonstrate that the biphasic uterine response to estrogens, so well characterized by physiological indicators discussed earlier (Table 25.4), is mirrored by the global changes in gene expression (Figure 25.13). The clearly defined patterns of early and late response genes found in mouse uterine tissues are completely lacking in ERα-null uteri. The identified genes fall into functional groupings, including signal transduction, gene transcription, metabolism, protein synthesis and processing, immune function, and cell cycle. Surprisingly, the expression levels of a striking number of genes are repressed by estrogen in the mouse uterus, and these effects were either absent in ERα-null uteri or relieved by co-treatment with ER antagonists, indicating that ERα is also actively involved in this process.

Comparative gene array analyses have also been conducted on human endometrial tissues during the proliferative and secretory phases of the menstrual cycle and have revealed gene expression patterns that are similar to those described in rodents.

Microarray analyses have also been used to map the uterine response to P and have identified numerous target genes, including those involved in immune function, metabolism, growth factor regulation, signal transduction, and extra- and intracellular structure. These observations have identified numerous signals and mechanisms important for normal uterine function and involved in uterine cancer (Table 25.5).

Microarray approaches have been utilized to investigate the molecular responses to xenoestrogens, such as the industrial chemical bisphenol A (BPA) and the pesticide metabolite 2,2-bis(p-Hydroxyphenyl)-1,1,1-trichloroethane (HPTE). Microarray transcript profiles reveal that both xenoestrogens induce responses that are highly correlated to E2 response early (2h), but less correlated later (24h), similar to a pattern seen with a weak estrogen such as estriol. Accordingly, BPA and HPTE, like estriol, are unable to induce uterine growth to the same degree as E2.

### TABLE 25.5 Steroid Receptor Uterine Target Genes from Microarray Studies

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Targets</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>Ihh, Hoxa10, FoxO1, Bmp2, FKBP52, Cebpb, Wnt pathway, Calbindin 9k, Ppar, Lox 12/15,Mig-6, Ihh, Klk5, Klk6, Bcl2, Fst</td>
<td>232,245,326,328-331</td>
</tr>
<tr>
<td>E</td>
<td>cell cycle regulators CcnB1+2, Cdc2a, p21, Mad2l2, Cdc25c, Gadd45g, DNA replication licensing MCM2, Aqp, thioredoxin pathway, Mig-6, Wnt signaling, Cyr61, RAMP3, Aqp5, Bop1/Scx, Muc1, Inhbb,</td>
<td>199,242,248,332-334</td>
</tr>
<tr>
<td>T</td>
<td>ATM/Gadd45g pathway</td>
<td>335</td>
</tr>
<tr>
<td>Dex</td>
<td>Ad1, Mgl1, Yht6, Tis11, Id3, Gilz</td>
<td>40,300</td>
</tr>
</tbody>
</table>

**FIGURE 25.13** Top: Schematic representation of uterine biphasic responses that occur following a single injection of E into an ovariectomized mouse (see also Table 25.4). **Bottom**: Transcriptional profile of uterine RNA, fold changes of transcripts in comparison of vehicle-treated animals at indicated times after E injection. White and black boxes highlight transcripts that typify early and late phases, respectively.
Whole transcriptome analyses are now routinely incorporated into studies of disruptions in signaling pathways underlying uterine phenotypes of mouse models such as those described in Table 25.3. Thus, microarray comparisons have now become just one of many tools employed for investigation of uterine functions. The data are routinely deposited in the publically accessible site GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/). Table 25.5 lists examples of steroid-regulated uterine transcripts discovered in microarray studies.

**Chip-seq**

More recently, technologies to facilitate evaluation of sites of transcription factor interaction with chromatin (by enriching a DNA binding protein, such as ERα, that has been cross-linked in situ to chromatin, with immunoprecipitation [chromatin immunoprecipitation or ChIP], followed by hybridizing the associated DNA to a chip tiled with promoter region sequences [ChIP-Chip] or by “next generation” massively parallel sequencing [ChIP-seq]) have been developed and widely utilized to study sites of nuclear receptor interaction (Figure 25.14). Initial studies focused on ERα binding in MCF7 breast cancer cells, and several similar studies followed, which are summarized and compared in several review articles, and reported that most sites were distal from transcriptional start sites (TSS) or were in intronic regions, rather than adjacent to TSS, as models of NR regulation of target transcripts had hypothesized. These comprehensive maps of cis-acting transcriptional regulators have been dubbed “cistromes”. The initial ERα cistrome–associated sequences were evaluated for enrichment of transcription factor motifs, and confirmed binding to the experimentally defined “ERE” sequence. In the case of the MCF7 cells, enrichment of motifs for forkhead binding factors (Fox) was apparent. Owing to the abundant expression of the FoxA1 member of the Fox family, a potential role for FoxA1 in estrogen response was pursued with an arsenal of bioinformatic, Next Gen sequencing, and biological studies that demonstrated the ability of FoxA1 to “pioneer”, and thus make accessible, regions of the chromatin that were subsequently targeted by ERα.

Most ERα cistromes reported have utilized in vitro cultured cell models, although ERα profiles from mouse liver tissue have been obtained. A ChIP-seq study by Hewitt et al. examining ERα binding sites in mouse uterine tissue indicated that, much like the MCF7 breast cancer study, most ERα sites were not proximal to TSS. Subsequent comprehensive ChIP-Chip or ChIP-seq studies have been published for the PR in mouse uterus, and T47D breast cancer and leiomyoma cells and for the AR in mouse epididymis skeletal muscle myoblasts, ZR-75-1 breast cancer cells, and LnCAP prostate cancer cells. GR binding has been evaluated in mouse liver tissue, mouse epithelial cells, and A549 lung cancer cells. We have learned that NRs bind to thousands of sites within the cellular chromatin, and that not all potential HREs in every cell demonstrate cognate NR binding. Rather, it is apparent that chromatin exhibits “pre-opened” regions destined to recruit NR. For ER in MCF7, FoxA1 establishes ER and AR accessible regions; for other cells or other NRs this function might be mediated by other “pioneers”, such as API for GR. The accessible chromatin regions are co-localized within nuclear “hubs” that seem to optimize frequency of interaction with NR. ChIP-seq is also used to locate other molecules involved in chromatin remodeling and transcriptional regulation, and to examine activating or repressive histone modifications or “marks”. These maps of relative locations and dynamics of NR and chromatin components greatly enhance our understanding of hormone response mechanisms.

**SEX STEROID RECEPTORS AND OVARIAN FUNCTION**

The functions of the sex steroids and their cognate receptors in ovarian function are especially complex and multifaceted. P, T, and E2 are all synthesized and secreted by the ovary during folliculogenesis and act via both extraovarian (i.e., endocrine) and intraovarian (i.e., para-/autocrine) pathways to profoundly influence all aspects of ovarian function. The endocrine actions of the sex steroids in the hypothalamic–pituitary axis are critical to the regulation of gonadotropin secretion and the ovarian cycle and are described in more detail in other chapters of this book. In turn, our appreciation of the extent and importance of the para-/autocrine actions of sex steroids within the ovary has increased substantially over the past years. Herein, we specifically review the literature concerning the expression of the sex steroid receptors (Table 25.6) in the mammalian ovary and employ the described null and mutated mouse models that have been developed as a platform to focus on recent revelations concerning the intraovarian roles of steroid signaling in ovarian function. A caveat to experimental testing of steroid action and steroid receptor studies in a tissue such as the ovary is that the organ is also producing the steroid being studied. Androgen, estrogen, and progesterone are all synthesized by the ovary; therefore, removal and replacement studies to analyze response are impossible. Thus use of mutant or null mice allows evaluation of the phenotype reflecting loss of functional interactions between steroids and their receptors.
FIGURE 25.14  (A) Schematic (Source: Modified with permission from Ref. 337), shows method to evaluate the “cistrome” of a steroid receptor or other chromatin interacting factors using ChIP-Chip or ChIP-seq. Cells or tissues are treated with a chemical, often formaldehyde, to cross-link DNA binding proteins to DNA. Then, chromatin (DNA and attached proteins) is isolated and fragmented with sonication or another method to break the chromatin into small pieces (approx. 500 bp). The protein of interest is immunoprecipitated with an antibody to enrich DNA fragments bound to the protein. Cross-linking is reversed to allow the enriched DNA to be purified. For ChIP–chip, DNA is amplified and labeled and hybridized to a chip spotted with promoter regions. For ChIP-seq, the DNA is sequenced using massively parallel (Deep) sequencing. The sequence “reads” are mapped onto known genomic regions, resulting in peaks indicating regions of binding of the immunoprecipitated DNA binding protein. Both methods are normalized in comparison to “input” chromatin that has not been immunoprecipitated.

(B) A screenshot showing an example from ERα and RNA polymerase II cistromes in the vicinity of the Fos transcript from a mouse uterine dataset is shown.
Estrogen Receptor Signaling in Ovarian Function

Estrogen Receptor Expression in the Ovary

There is a plethora of evidence for ER expression in somatic cell types of the mammalian ovary, and numerous reviews describe the importance of ERα and ERβ in ovarian function. As early as 1969, Stumpf demonstrated the specific uptake of [3H]-E2 in rat ovaries using dry-mount autoradiography. Richards later demonstrated that the predominance of high-affinity E2 binding sites in the rat ovary is found in the granulosa cell component and that these levels are regulated by estrogen and gonadotropins. Successful cloning of the individual ER genes from multiple species and continued development of better ER-specific immunoglobulin allow for more detailed characterization of ERα and ERβ expression and regulation in the mammalian ovary. It should be pointed out that immunodetection and expression patterns of steroid receptors are dependent on the quality of the antibodies used. Such antibodies often vary between studies of ovarian tissue as described following and may explain both species and tissue differences.

ER expression patterns are well conserved among mammalian species, although distinct differences are apparent in late stage follicles. A high level of ERβ expression in granulosa cells of growing follicles is conserved among rodents, large animals, and primates. ERα expression is exclusive to thecal/interstitial cells in the stroma of the ovary in rats and mice (Table 25.6, mice) but this compartmental expression pattern does not hold true in hamsters, domestic animals, monkeys, or humans (Figure 25.15). In fact, current data indicate that granulosa cells of preovulatory follicles in large animals and primates express comparable levels of both ERα and ERβ, and, in humans, ERα may predominate. Similarly, thecal cells in large animal and human ovaries possess both ER forms. These data force us to consider a greater potential role for ERα/ERβ heterodimers in the ovaries of large animals and primates that may not occur in rodents. The more restricted expression pattern of ERα and ERβ in rodent ovaries does not exclude possible cooperative action between the two receptors, as suggested by the unique ovarian phenotypes in compound ER-null mice compared to each single knockout ovarian phenotype (Table 25.7). Although much has been learned concerning ER localization, it remains difficult to delineate which of the incongruent findings among species are truly representative of divergent expression patterns or are more attributable to disparities in techniques and immunoreagents. This is further confounded by evidence that levels of ER mRNA do not always correspond with the levels of immunoreactive protein within the ovarian compartments. The following section reviews the literature concerning the expression patterns of ERα and ERβ in the ovaries of rodents, domestic animals, and primates, and the changes in expression patterns that occur during folliculogenesis. Altered expression has been implicated in diseases affecting ovarian function (including polycystic ovarian syndrome and ovarian cancer); however, this will not be addressed.

Estrogen Receptor α

RODENTS

ERα is localized to the ovarian interstitial/stroma, thecal cells of growing follicles, and surface epithelium in rat and mouse ovaries. This expression pattern is not evident in hamsters, where both receptors are detectable to varied degrees throughout the different somatic cell types of the ovary. In the fetal rat, ERα expression is detectable shortly after the first indications of gonadal differentiation. Postnatal rat and mouse ovaries exhibit ERα mRNA shortly after birth, but levels remain relatively constant; whereas ERβ levels rise substantially during this period. Immunoreactivity for ERα in neonatal rat ovaries indicates ERα expression is limited to thecal/intertitial cells and the ovarian surface epithelia and absent in granulosa cells and oocytes. A similar pattern of ERα expression occurs in fetal hamster ovaries, which exhibit immunoreactivity as early as gestational day 14 and significant increases thereafter during the neonatal period. Yang et al. report that a noticeable increase in ERα immunoreactivity occurs in the granulosa cells and oocytes of primordial follicles during neonatal days 8–15.

In adult rats and mice, ERα expression continues to be exclusively localized to thecal cells of growing follicles, interstitial/stromal cells, and the ovarian surface epithelium (Figure 25.15). Electrophoresis mobility shift assays (EMSA) of nuclear extracts from rat granulosa cells indicate that virtually all of the

### TABLE 25.6 Localization of Steroid Receptor Expression in the Mouse Ovary

<table>
<thead>
<tr>
<th>Steroid Receptor</th>
<th>Granulosa</th>
<th>Theca</th>
<th>Oocyte</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>ERα</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>ERβ</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PRA</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>PRB</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>AR</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>GR</td>
<td>+++</td>
<td>++</td>
<td>–</td>
<td>ND</td>
</tr>
<tr>
<td>Aromatase</td>
<td>+++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**ND:** Not determined.

**–:** Not expressed.

**+/-,** +,** ++,** +++:** Intensity of signal indicated by number of + symbols.
### Table 25.7: Ovarian Phenotypes in E Signaling Mutant and Null Mouse Models

<table>
<thead>
<tr>
<th>Mutated or Null for Sex Steroid Signaling</th>
<th>Ovarian Phenotypes</th>
<th>Hormone Levels</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Esr1</em>&lt;sup&gt;−/−&lt;/sup&gt; (homozygous null alleles for ER&lt;sub&gt;α&lt;/sub&gt; animal)</td>
<td>Anovulatory and infertile</td>
<td>Elevated T, E2 and LH</td>
<td>157–160</td>
</tr>
<tr>
<td></td>
<td>Hemorrhagic and cystic pathology</td>
<td>Normal FSH &amp; P</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No CLs present in ovarian sections</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased expression of steroidogenic enzymes</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reduced response or failure to respond to exogenous gonadotropins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NERKI&lt;sup&gt;+/−&lt;/sup&gt; (one mutated allele with 2-point mutation in DNA binding zinc finger of ER&lt;sub&gt;α&lt;/sub&gt; and one WT allele)</td>
<td>Anovulatory and infertile</td>
<td>Normal LH, FSH and E2</td>
<td>132</td>
</tr>
<tr>
<td></td>
<td>No plugs observed in NERKI females after superovulation and natural mating</td>
<td>Reduced P</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Superovulation was able to partially restore ovulation in NERKI mice, however also increased hemorrhagic follicles in the ovaries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>KIKO (ER&lt;sup&gt;AA/−&lt;/sup&gt;) (one mutated allele of 2-point mutation in DNA binding domain of ER&lt;sub&gt;α&lt;/sub&gt; and one ERαKO allele)</td>
<td>Anovulatory and infertile</td>
<td>Normal E2 and P</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>No CLs present in ovarian sections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ENERKI (ERα&lt;sup&gt;G525L&lt;/sup&gt;) (homozygous animal of 1-point mutation in LBD of ER&lt;sub&gt;α&lt;/sub&gt;)</td>
<td>Anovulatory</td>
<td>Elevated serum E2, T and LH</td>
<td>168</td>
</tr>
<tr>
<td></td>
<td>Hemorrhagic and cystic ovarian pathology</td>
<td>Normal FSH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased number of atretic antral follicles and no CLs</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperplastic theca cells in response to LH (data not shown)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ER&lt;sup&gt;AAE/EAAB&lt;/sup&gt; (homozygous animal of 4-point mutation of DBD ER&lt;sub&gt;α&lt;/sub&gt;)</td>
<td>Infertile</td>
<td>Not reported</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>Hemorrhagic ovaries</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AF2ER&lt;sup&gt;KI/KI&lt;/sup&gt; (homozygous animal of 2-point mutation in LBD of ER&lt;sub&gt;α&lt;/sub&gt;)</td>
<td>Anovulatory and infertile</td>
<td>Elevated serum LH and E2</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>Hemorrhagic and cystic ovarian pathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No CLs present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cyp17cre:ERα&lt;sup&gt;lox/lox&lt;/sup&gt; (Theca cell specific ER&lt;sub&gt;α&lt;/sub&gt; knockout)</td>
<td>Fertility normal in young mice, but 6 month old animals have reduced fertility and longer estrous cycle</td>
<td>Elevated T at both 2 &amp; 6 months</td>
<td>361,362</td>
</tr>
<tr>
<td></td>
<td>Normal FSH</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decreased LH at 2 months with further decrease at 6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>ER&lt;sub&gt;β&lt;/sub&gt;STL−/L− females are the only line of ER&lt;sub&gt;β&lt;/sub&gt; knockout animals that are sterile.</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ER<sub>α</sub>AF-1<sup>0</sup>** | Ovarian phenotype not reported | Unknown | 165 |
**ER<sub>α</sub>AF-2<sup>0</sup>** | Ovarian phenotype not reported | Unknown | 167 |
**αβERKO** | Anovulatory and infertile | Elevated LH and T | 158,174 |
|                                           | No CLs and few large follicles | Normal FSH and P | |
|                                           | Ovarian transdifferentiation to Sertoli-like cells that express Sox9 | | |
|                                           | Altered expression of steroidogenic enzymes | | |

**Cyp19α1<sup>−/−</sup>** | Anovulatory and infertile | No E2 | 131,136,175,194,364,365 |
|                                           | Hemorrhagic and cystic pathology | Elevated LH, FSH and T | | |
|                                           | No CLs present in ovarian sections | | |
|                                           | Poor response to exogenous gonadotropins, although treatment with E2 in addition to gonadotropins improves ovulatory response | | |
|                                           | Ovarian transdifferentiation to Sertoli-like cells that express Sox9 | | |
specific ERE-bound complexes are supershifted by anti-ERβ but not anti-ERα immunoglobulin, providing further support that rat granulosa cells possess very little ERα. Western blot analyses of whole cell or nuclear extracts from isolated rat granulosa cells do indicate a low level of ERα protein of 61 kDa, and use of laser capture microdissection (LCM) shows some expression of ERα mRNA in granulosa cells (Figure 25.15). Unlike ERβ, ERα expression remains relatively constant throughout the rat estrous cycle. Furthermore, no change in ERα levels is detected in granulosa cells cultured in FSH/T over a period of 72h; however, a 24-h treatment with forskolin induces a marked increase in ERα immunoreactivity in the nuclei. ERα localization in the adult hamster ovary is somewhat divergent from that in rats and mice. Yang et al. report moderate clear ERα immunoreactivity in thecal/interstitial cells, but also appreciable immunoreactive granulosa cells of small preantral follicles. In the granulosa cells of antral follicles, ERα immunoreactivity is strongest among those in proximity to the forming antrum. FSH treatment (twice a day for 2 consecutive days) or a single injection of E2 elicits a significant induction of ERα expression in both thecal and granulosa cells in hypophysectomized hamsters. Still, Western blot analyses of whole ovarian homogenates from hamsters indicate an ERβ:ERα ratio of 14:1 during the follicular phase, indicating that ERβ predominates. However, the rapid decline in ERβ and concurrent increase in ERα that occurs just prior to and shortly after the gonadotropin surge adjusts this ratio to 2:1, suggesting that ERβ plays a more predominant role in follicular growth and ERα is more important during luteinization in the hamster ovary.

**DOMESTIC ANIMALS**

In fetal bovine ovaries, ERα was shown to be localized in all cell types involved with follicle development, including granulosa cells, oocytes, and epithelial cells throughout development, with expression becoming

![Diagram](image_url)

**FIGURE 25.15** (A) Immunohistochemistry for ERα and ERβ in adult mouse ovary. Immunohistochemistry for ERα (top) indicates specific nuclear immunoreactivity in thecal/interstitial cells and thecal cells (TC) in and around a preantral follicle. Granulosa cells lack any measurable immunoreactivity for ERα. The staining around the outer surface of the oocyte is nonspecific and not representative of ERα immunoreactivity. Immunohistochemistry for ERβ (bottom) indicates specific nuclear immunoreactivity in the granulosa cells (GC) throughout a preantral follicle. Thecal cells (TC) lack any measurable immunoreactivity for ERβ, but the surrounding thecal/interstitial cells exhibit some cytoplasmic staining. Immunohistochemistry was done by Dr. Madhabananda Sar (see Ref. 366 for protocol). (B) Pure populations of granulosa and theca cells were isolated from large preovulatory follicles using laser capture microdissection (LCM), and RNA was reverse transcribed and real time PCR was performed using primers specific for ERα and ERβ.
more localized to thecal and some stromal cells during the later stages of development. In adult bovine ovaries, ERα immunoreactivity is relatively high in thecal cells of secondary and tertiary follicles, substantially weaker in granulosa cells of tertiary follicles, and totally absent in primordial, primary, and secondary follicles. In agreement, Barisha et al. found ERα transcripts are detectable in both granulosa and thecal cell fractions, but levels are substantially higher in the latter cell type. Furthermore, thecal ERα expression appears to be differentially regulated as it is almost four-fold higher in follicles of 20–180 mm versus those <0.5 mm, whereas ERβ expression in thecal cells remains constant among follicles of different sizes. ERα expression in granulosa cells, although much lower relative to theca, also increased with advanced follicle size. In porcine ovaries, ERα immunoreactivity is detected in both theca interna and granulosa cells, but only in large follicles; and even then ERα immunoreactivity is considerably moderate relative to ERβ. In ovine ovaries, ERα mRNA and immunoreactivity is detected in both cell types but predominates in granulosa cells and is particularly high in cumulus oophorus cells of small antral follicles.

PRIMATES

The first reported study of ERα immunoreactivity in the rhesus or cynomolgus monkey ovaries found that ERα was undetectable in all ovarian cell types except the surface epithelia, regardless of menstrual stage. However, a later study in baboon ovaries using the same antibody found nuclear ERα immunoreactivity in 30–40% of the granulosa cells of healthy antral follicles, and detectable but less intense staining in granulosa cells of preantral follicles. This same study found the stroma, interstitial, and thecal cells to be largely unlabeled. Pau et al. produced similar findings of ERα expression in the granulosa cells of rhesus monkey ovaries by in situ hybridization.

High-affinity E2 binding sites in normal human ovarian tissue were first described several decades ago, but these techniques were not able to differentiate between the two ER forms. Iwai et al. used anti-ERα antisera similar to that in the nonhuman primate studies to demonstrate immunoreactivity in the granulosa cells of antral and preovulatory follicles in human ovary but a total absence in primordial, preantral follicles, atretic follicles, and thecal/interstitial cells. Pelletier and El-Alfy used a different anti-human ERα antisera and reported results that are in contrast to those of Iwai et al. i.e., a total lack of ERα immunoreactivity in granulosa cells but clear nuclear staining of theca interna cells, interstitial gland cells, and ovarian surface epithelia. A possible explanation for this discrepancy may be a lack of large antral follicles in samples evaluated by Pelletier and El-Alfy since both studies agree that ERα is not detectable in the granulosa cells of less mature follicles. In support of this explanation is a report by Taylor and Al-Azzawi in which ERα immunoreactivity is illustrated in the granulosa cells of what is clearly a large antral follicle when using antisera raised against the bovine ERα. Indeed, Saunders et al. described ERα immunoreactivity that is limited to granulosa cells of large antral follicles and undetectable in smaller follicles in the human ovary. Also, in agreement with Pelletier and El-Alfy, Saunders et al. found ERα immunoreactivity in theca cells of preantral and antral follicles, and ovarian surface epithelia. ERα transcripts are detectable in human nonluteinized granulosa cells and granulosa-luteal cells collected at the time of oocyte retrieval during in vitro fertilization (IVF) procedures. In fact, Jakimiuk et al. found ERα mRNA and protein levels are two- to four-fold higher in granulosa versus thecal cells of small antral follicles in human ovaries. Furthermore, ERα protein levels remain elevated in granulosa cells but decrease in thecal cells of dominant follicles. In luteinized human granulosa cells, Chiang et al. found ERα levels to remain relatively constant over a period of 10 days in culture but always much lower than ERα mRNA levels. Recent reports in human ovarian samples collected during elective hysterectomy demonstrate that ERα is expressed in granulosa cells of antral follicles, with weak to no positive immunohistochemical staining in luteinized granulosa cells supporting previous data.

Estrogen Receptor β

In the nonpregnant ovaries of most mammalian species, ERβ is clearly the predominant ER form and is most often exclusively localized to granulosa cells of follicles from the primary to preovulatory stage. This expression pattern is documented in the ovaries of rats, mice, hamsters, cows, sheep, pigs, nonhuman primates, and humans. Rodents

In developing rat ovaries, ERβ expression is detectable shortly after the first indications of gonadal differentiation on gestational day 14, and levels increase thereafter during prenatal development. A second, more robust increase in ERβ expression occurs during days 10–15 of neonatal development in rat ovaries and is detectable as early as day 15. ERβ and immunoreactivity is largely localized to granulosa cells of growing follicles and notably absent in primordial follicles and oocytes. Developing mouse ovaries exhibit a comparable pattern ERβ expression. In contrast, ERα is limited to thecal and interstitial cells and exhibits little change in levels with increased age in developing rat and mouse ovaries. The hamster ovary exhibits a fairly similar ontogeny as ERβ is detectable as early
as gestational day 13 and increases thereafter to peak on postnatal day 10, throughout which ERβ immunoreactivity is predominantly localized to granulosa cells but detectable in some interstitial cells and oocytes.\textsuperscript{377}

In the ovaries of prepubertal and adult rats and mice, ERβ immunoreactivity is nearly exclusive to the nuclei of granulosa cells of healthy follicles at all advanced stages of folliculogenesis\textsuperscript{324,129,130,366–370,374,402} and is notably decreased or absent in atretic follicles\textsuperscript{129,366} (Figure 25.15). Primordial follicles, thecal cells, oocytes, ovarian surface epithelium, and luteal cells lack ERβ immunoreactivity in adult rat and mouse ovaries;\textsuperscript{129,130,366–369,374,402} although some cytoplasmic staining in the latter cell type is reported when using certain antisera.\textsuperscript{129,366} Granulosa cell specific expression of ERβ in the rat ovary was reproduced using three separate anti-rat ERβ antisera, two raised against residues 467–485 and a third raised against residues 54–71.\textsuperscript{369} Furthermore, independent studies using in situ hybridization produced results congruent with the immunohistochemical findings of strict localization of ERβ mRNA to granulosa cells of healthy growing follicles,\textsuperscript{14,129,375,401} although Bao et al. reported scattered but specific hybridization for ERβ mRNA in thecal cells of healthy medium and large follicles in the rat ovary.\textsuperscript{400}

A study by Saunders et al.\textsuperscript{370} stands in contrast to those just discussed. In this study, strong nuclear immunoreactivity is described throughout the different somatic cell types of the rat ovary, including thecal, interstitial, and luteal cells, as well granulosa cells of maturing follicles when using an anti-rat ERβ antiserum raised against residues 196–213. Yang et al. reported similar findings of ERβ immunoreactivity in thecal/interstitial cell preparations from hamster ovary using different ERβ-specific antisera.\textsuperscript{374} Still, the results of Saunders et al. are problematic because they include reports of substantial ERβ immunoreactivity in certain reproductive tissues of the rat, such as the oviduct and uterus,\textsuperscript{370} that are otherwise thought to possess relatively low or null levels of ERβ mRNA and immunoreactivity. Two obvious differences between the immunohistochemical study of Saunders et al.\textsuperscript{370} and others are the aforementioned use of different antisera as well as different tissue fixative.

The previous discrepant findings notwithstanding, ERβ is clearly the predominant ER form present in granulosa cells of healthy growing follicle in the rodent ovaries. Recent isolation of pure granulosa and theca cells from mouse ovaries using laser capture microdissection demonstrates that ERβ mRNA expression is solely in granulosa cells (Figure 25.15). Any specific distribution of ERβ throughout the subpopulations of granulosa cells of growing follicles has been difficult to ascertain. In general, ERβ immunoreactivity is continuous and strong among the mural cells and relatively uniform among those layers closer to the antrum and oocyte.\textsuperscript{356,367,369} However, not all granulosa cells of healthy growing follicles in the rodent ovary express ERβ, as negative cells are randomly distributed throughout the follicle.\textsuperscript{366,367}

Data from Western blots of nuclear extracts from whole ovaries or isolated granulosa cells support the immunohistochemical data that ERβ is the predominant receptor form in rodent granulosa cells.\textsuperscript{367,371} Electrophoresis mobility shift assays in which whole cell extracts from ovaries or isolated granulosa cells of rats provide further evidence of the predominance of ERβ.\textsuperscript{367,371,410} Sharma et al. found that antisera raised against residues 54–71 of rat ERβ produces the optimum results on Western blot and detects four immunoreactive bands of 58/52 to 46/44 kDa in size, the 58 kDa protein presumably being full-length ERβ (ERβ1 in Figure 25.2).\textsuperscript{371} Antiseras raised against the AF-2 (residues 182–485) domain of rat ERβ detects a single specific protein of 60 kDa in whole-cell extracts from both rat and mouse ovaries that comigrates with the putative full-length ERβ.\textsuperscript{367} Choi et al.\textsuperscript{402} and Hiroi et al.\textsuperscript{368} produced comparable findings of immunoreactive proteins at 60 and 55 kDa in rat and mouse ovarian extracts when using either a monoclonal antibody mapped to residues 272–285 of human ERβ or antiseras raised against C-terminal residues 467–485 of rat ERβ, respectively. In the hamster, a single ERβ-immunoreactive protein of 54 kDa is detected.\textsuperscript{374} To date, ERβ antibodies are not as specific as those manufactured for ERα, and produce bands in the ERβ-null tissues similar to those observed in WT tissues (unpublished data from Korach laboratory) such that further characterization of ERβ localization is challenging.

Several different ERβ isoforms are present in rodent ovaries, notably ERβ2 (Figure 25.2), ERβ-Δ3, and ERβ2-Δ3, the latter being a compound form of the two former variants. ERβ-Δ3 is an exon 3 deletion that results in a receptor lacking the C-terminal zinc-finger of the DNA binding domain and therefore unable to bind an ERE.\textsuperscript{193} ERβ2 is an especially interesting variant because it possesses an insert of 18 amino acids in the N-terminal region of the LBD (Figure 25.2) that causes a 35-fold reduction in affinity for E2 and a 1000-fold decrease in E2-induced transactivation activity in vitro.\textsuperscript{193} This isoform is not found in human ovary (Figure 25.2). Pettersson et al.\textsuperscript{193} speculate that ERβ2 may be especially attuned to mediating E2 actions within healthy growing follicles where intrafollicular E2 levels greatly exceed that required to activate wild-type ER forms. Differential RT-PCR indicates a 1:1 ratio of ERβ1:ERβ2 mRNA in the ovary of adult\textsuperscript{371,410,411} and neonatal rats,\textsuperscript{323} while transcripts encoding ERβ1-Δ3 or ERβ2-Δ3 variants are detectable but at substantially lower levels.\textsuperscript{410,411} Pettersson et al. demonstrated that FLAG-tagged clones of ERβ1 and ERβ2 expressed in 293T cells co-migrate at approximately 60 kDa\textsuperscript{411} suggesting that resolution of the two isoforms by Western blot may be difficult. However, several studies illustrate
two distinct ERβ-specific bands appear on Western blots from rats and mouse ovarian extracts. Furthermore, O’Brien et al. fractionated rat granulosa cell preparations in a 7.5% SDS-PAGE from which immunoreactive protein bands presumed to be ERβ1 (60 kDa) and ERβ2 (62 kDa) were resolved; they further remarked that an equal ratio of ERβ1:ERβ2 transcripts does not correlate to actual protein amount by Western blot, as much greater amounts of ERβ1 protein are present. In retrospect, previous [3H]-E2 binding data also supports a predominance of ERβ1 versus ERβ2 in rat granulosa cells. Because recombinant ERβ1 and ERβ2 differentially bind E2 with affinities of 0.14 and 5.1 nM, respectively, one would expect differentiation of the two forms when assessed by Scatchard plot analysis. Therefore, the earlier studies using [3H]-E2 and more recent studies using [125I]-17α-iodovinyl-11β-methoxyE2 that report a single, saturable, high-affinity binding factor with a Kd = 0.4 nM in rat ovarian or granulosa cell extracts are congruent with ERβ1 as the predominant form present. Similar data of a single E2 binding component with a Kd = 1–1.4 nM in hamster ovaries is also reported.

The majority of studies on the regulation of β expression during folliculogenesis focus on the rat ovary. While some report high but relatively constant levels of ERβ mRNA leading up to proestrus, more detailed studies indicate a substantial increase in ERβ expression that peaks in medium sized (275–450 mm) follicles possessing clearly defined antrum. Most studies agree on the precipitous decline in ERβ levels that occur shortly after the ovulatory gonadotropin surge and the low levels that remain during estrus in rats and hamsters (Figure 25.16). In retrospect, it is evident that the decreasing effect of the gonadotropin surge on ERβ levels was first indicated by Richards in 1975, in which a 75% decrease in high-affinity E2 binding sites in granulosa cells of hypophysectomized, FSH/E2-primed rats was found to occur following a single LH treatment. This effect of LH on ERβ expression has been reproduced in vivo in gonadotropin-primed rats and mice in which a single hCG injection led to a rapid decrease in ERβ mRNA and protein levels of more than 50% within 6–9 h (Figure 25.16). Similarly, granulosa cells isolated from pregnant mares serum gonadotropin (PMSG)-stimulated rats and exposed to hCG in vitro exhibit a comparable loss of ERβ protein when assessed by western blot or EMSA.

Therefore, induction of the LH-signaling pathway in differentiated granulosa cells is the primary stimulus for rapidly decreased ERβ expression. Evidence of a direct role of LH comes from findings that only preovulatory follicles that co-express LH receptor exhibit a decline in ERβ expression; while smaller, LH-receptor negative follicles maintain ERβ expression as late as 24 h post-hCG exposure. The inhibitory effect of LH on ERβ

**FIGURE 25.16 Regulation of ERβ expression in adult rat ovaries during the estrous cycle or after exogenous gonadotropin treatments.** (A) Quantification of gene expression from Northern blot analysis (not shown) for ERα and ERβ in adult rat ovaries during the estrous cycle. Band intensities were measured on a phosphorimager and normalized to an S16 internal control for each time point. mRNA levels are shown relative to the level at E1100 (set to 1.0). Sera from animals were used to determine LH concentrations; the onset of the LH surge was observed at 1600 h of proestrus, and the peak was observed at 1800 h of proestrus. Annotation at bottom indicates the estrous cycle stage and hour of tissue collection: E, estrous; M, metestrous; D, diestrous; P, proestrous. (Source: Reproduced with permission from Ref. 380.) (B) Reverse transcriptase polymerase chain reaction was used to quantify ERβ mRNA levels in the ovaries of immature rats after treatment with PMSG alone or PMSG for 48 h followed by hCG. The ratio of ERβ/S16 of control rats with no hormonal treatment was set to 1.0. Shown are the mean ± SE (n = 4). (Source: Reproduced with permission from Ref. 380.) (C) ERβ immunoreactivity in rat ovaries treated with vehicle (Veh), PMSG for 48 h, or PMSG for 48 h followed by hCG. ERβ immunoreactivity is detected in granulosa cells of small and large antral follicles in Veh PMSG animals. Stars indicate the location of antral in antral follicles. PMSG-treated rats were injected with an ovulatory dose of hCG and ovaries isolated after 3, 9, 12, or 24 h. The expression of ERβ protein in granulosa cells 9 h after hCG is reduced in large antral follicles (left), greatly reduced in preovulatory follicles (center), but does not change in small antral follicles (right). Similar expression is observed 12 h after hCG administration. One day (24 h) after hCG treatment, ERβ expression is not detected in corpora lutea (small arrowheads) but is highly expressed in preantral (right) and small antral follicles. Magnification 250×. (Source: Reproduced with permission from Ref. 367.)
expression can be reproduced by exposing differentiated granulosa cells to either forskolin or TPA, activators of the protein kinase-A and protein kinase-C pathways, respectively, and thought to mimic the effects of LH stimulation. Furthermore, forskolin or TPA treatment reproduce the same temporal pattern of decreased ERβ expression that follows LH or hCG exposure, suggesting a common mechanism. Guo et al. demonstrated both activators and mimics of the LH-signaling pathway elicit the rapid decline in granulosa cell ERβ levels by decreasing the stability of ERβ transcripts rather than via a repression of gene expression.

In contrast to the peri-ovulatory decrease in ERβ expression that occurs, there is little known about the positive regulation of ERβ expression in rat granulosa cells. Induction of folliculogenesis by PMSG or FSH in hypophysectomized rats leads to a significant rise in E2 binding among granulosa cells, presumably representative of ERβ, but this is likely due more to an increased number of granulosa cell population rather than direct gonadotropin-stimulated ERβ expression. The presence of ERβ in the granulosa cells of primary follicles, considered to be insensitive to direct gonadotropin stimulation, supports a minor role for FSH in ERβ expression. Furthermore, ERβ levels exhibit little change in immature rat or mouse ovaries 48h after a single injection with PMSG and no reports exist of altered ERβ expression in the ovaries of mice null for FSH signaling. Sharma et al. found that ERβ protein levels in gonadotropin-primed rat granulosa cells evaluated are highest if assessed after isolation and decrease steadily to undetectable levels within 72h in culture, even in the presence of FSH and T. However, this pattern was not totally reproduced at the level of ERβ mRNA, which drops by only 55% when cultured for the first 24h in the absence of hormones but stabilizes upon the addition of FSH and T, suggesting that FSH may be more important to the maintenance rather than stimulation of granulosa cell ERβ expression. In contrast to the previous findings, preovulatory rat granulosa cells allowed to lose ERβ expression following long-term (6 day) culture exhibit a significant rise in levels 48h after exposure to forskolin, a direct PKA activator and thought to mimic FSH signaling. Interestingly, FSH may have a greater positive influence on ERβ expression in the hamster ovary where a substantial increase in expression in preantral and antral follicles is observed in hypophysectomized females following 1–2 days of treatment with ovine FSH and ovaries of neonatal hamsters in which prenatal FSH action is inhibited exhibit significant decreases in ERβ expression.

The existing evidence of E2 regulation of ERβ expression is conflicting. Treatment of hypophysectomized rats with E2 for 3–4 days results in steady and dramatic increases in E2 binding sites in granulosa cells, but once again this is more likely due to an increased granulosa cell population. However, Tonetta et al. demonstrated that FSH-induced increases in E2 binding sites in granulosa cells of hypophysectomized rats are blocked by co-administration of an ER antagonist, suggesting an autoregulatory element for ER expression. In contrast, Kim and Greenwald found little change in the number of E2 binding sites in hamster ovaries following E2 or DES treatment, although animals were exposed for only 1–2 days, and therefore significant gains in granulosa cell number may not have been achieved. Drummond et al. reported that 1–4 days of treatment with DES has no discernible effect on the levels of ERβ1 or ERβ2 transcripts in immature rat ovaries. Yang et al. found that hypophysectomized hamsters exhibit an almost sixfold increase in ERβ expression as detected by immunohistochemistry 24h after a single injection of 0.1mg E2-valerate. Furthermore, rat granulosa cells isolated from E2-primed versus untreated animals exhibit a four- to six-fold higher basal estrogenic activity on an ERE-luciferase reporter construct, suggesting that individual cellular levels of ERβ are increased by E2 treatment. Also, isolated rat granulosa cells exhibit an increased nuclear intensity for ERβ immunoreactivity 1.5h after E2 exposure that is maintained for 24h but totally lost by 48h. Still, the failure of granulosa cells to totally sustain ERβ expression when maintained in FSH and T, and therefore capable of synthesizing endogenous E2, is puzzling. It is plausible that E2 regulation of ERβ expression in granulosa cells may require the actions of ERα in thecal cells, which cannot be mimicked when granulosa cells are isolated in culture. Preservation of ERβ expression in preantral follicles of ERα-null ovaries argues against this hypothesis. Recent work in KGN cells suggest that GIOT-4, a cofactor regulated by FSH, may act to increase ERβ expression as well as work in combination with ERβ to regulate target genes in follicles. Although this work was done primarily in KGN ovarian cells, it was also reported that GIOT-4 and ERβ are co-localized in growing follicles providing support for the co-regulation. The expression of GIOT-4 has not been examined in in vitro-cultured granulosa cells. Therefore, although the signaling factors that may be most important to inducing ERβ expression remain unknown, it is clear that the level of ERβ is highly dependent upon the state of granulosa cell differentiation as cells from primary, growing, and preovulatory follicles exhibit divergent expression patterns and mechanisms of regulation.

DOMESTIC ANIMALS

Descriptions of ERβ expression in the ovaries of domestic animals are limited but generally indicate patterns that are congruent with rodent ovaries. In the fetal bovine ovary, ERβ is localized in the cell types associated with follicle growth, predominantly in pregranulosa...
and granulosa cells. In early development, epithelial cells show expression; however, this becomes punctate as follicles begin to develop. In adult bovine ovaries, Rosenfeld et al. found that ERβ mRNA and immunoreactivity is restricted to granulosa cells of small and large antral follicles with no detectable levels in thecal cells. A follow-up study further demonstrated substantial ERβ immunoreactivity in cumulus oophorus and antral granulosa cells relative to mural granulosa, as well as mentioned significant ERβ expression in oocytes, the latter finding being common only to hamsers. In contrast to the immunohistochemical data, Walther et al. and Berisha et al. found ERβ transcripts are detectable in both granulosa and thecal cells. In ovine ovaries, ERβ is highly expressed in granulosa cells of growing follicles, and levels exhibit little change over the course of the estrous cycle. However, Jansen et al. report via in situ hybridization that ERβ mRNA is greatest in follicles ≤3 mm and declines in larger follicles during the early follicular phase. Both reports describe low but detectable ERβ expression in thecal cells of growing follicles in the sheep ovary, as well as in ovarian surface epithelia. In porcine ovaries, ERβ is almost equally expressed among the granulosa and theca interna of medium and large follicles, as well appreciable detection in the oocyte and ovarian surface epithelia. Like the rodent, atretic follicles in porcine ovaries exhibit reduced ERβ expression. LaVoie et al. found that ERβ mRNA levels in whole ovarian lysates exhibit little change during the porcine estrous cycle and are relatively equal when comparing follicles of 1–5 mm in size.

An interesting commonality among bovine, ovine, and porcine ovaries is the presence of a truncated ERβ isoform lacking most of the ligand binding domain, termed ERβΔLBD in the cow and ERβ-Δ5 in sheep and pig (Figure 25.2). In all three species, ERβ-Δ5 is a deletion of exon 5 and encodes a receptor isoform that is truncated at the carboxyl terminus when comparing follicles of 1–5 mm in size. ERβ expression in porcine ovaries is the presence of a truncated ERβ isoform that is produced by two specific ERβ protein bands at 55 and 63 kDa. Western blots of baboon ovarian homogenates indicate two specific ERβ protein bands at 55 and 63 kDa. An extensive immunohistochemical study in marmoset ovaries using antisera raised against human ERβ found extensive immunoreactivity in granulosa cells of primary through to mid- and late follicular stage follicles, including those with a large antrum, but a clear lack of staining in atretic follicles. No specific pattern of ERβ immunoreactivity was apparent among subpopulations of granulosa cells within any one follicle. In contrast to baboon ovaries but in agreement with cynomolgus monkey ovaries, thecal cells of large growing follicles as well as the ovarian surface epithelia in marmoset ovaries possess appreciable levels of ERβ immunoreactivity.

High-affinity E2 binding sites in normal human ovarian tissue were described several decades ago. Al-Timimi et al. found 45 of 89 normal ovaries from premenopausal women to possess detectable E2 binding sites; whereas all of the (n = 10) postmenopausal ovaries assayed were devoid of detectable binding. Vierikko et al. reported high-affinity E2 binding in a similar percentage of premenopausal ovaries but did find 67% of postmenopausal samples were also positive, although the levels of E2 binding in both are notably lower than that for P. The advent of better reagents has allowed for differentiation of the two ER forms within human ovaries. ERβ expression was first detected in human ovary by Northern blot analysis. Follow-up studies using RNase-protection assays or RT-PCR indicate a relatively equal ratio of ERα:ERβ transcripts in mixed-cell homogenates from ovaries of pre- and postmenopausal women but exclusively ERβ transcripts in luteinized granulosa cells isolated from women undergoing IVE.

Three reports describing immunohistochemical localization of ERβ in human ovaries produced comparable findings of specific immunoreactivity among granulosa cells of follicles from primary to late antral stage, and specific, but considerably lower, levels in thecal cells of preantral and antral follicles. Furthermore, all three demonstrate substantial ERβ immunoreactivity in human ovarian surface epithelia. Hillier et al. also found hybridization to granulosa cells of follicles at all different stages of development, as well as substantial labeling in theca interna and ovarian surface epithelia. Similar findings are described by Pau et al. in granulosa cells of rhesus monkey ovary. In the baboon ovary, Pepe et al. found ERβ immunoreactivity is abundant in granulosa cells of follicles at all stages, including large antral follicles but notably lacking in thecal cells. Western blots of baboon ovarian homogenates indicate two specific ERβ protein bands at 55 and 63 kDa. An extensive immunohistochemical study in marmoset ovaries using antisera raised against human ERβ found extensive immunoreactivity in granulosa cells of primary through to mid- and late follicular stage follicles, including those with a large antrum, but a clear lack of staining in atretic follicles. No specific pattern of ERβ immunoreactivity was apparent among subpopulations of granulosa cells within any one follicle. In contrast to baboon ovaries but in agreement with cynomolgus monkey ovaries, thecal cells of large growing follicles as well as the ovarian surface epithelia in marmoset ovaries possess appreciable levels of ERβ immunoreactivity.

High-affinity E2 binding sites in normal human ovarian tissue were described several decades ago. Al-Timimi et al. found 45 of 89 normal ovaries from premenopausal women to possess detectable E2 binding sites; whereas all of the (n = 10) postmenopausal ovaries assayed were devoid of detectable binding. Vierikko et al. reported high-affinity E2 binding in a similar percentage of premenopausal ovaries but did find 67% of postmenopausal samples were also positive, although the levels of E2 binding in both are notably lower than that for P. The advent of better reagents has allowed for differentiation of the two ER forms within human ovaries. ERβ expression was first detected in human ovary by Northern blot analysis. Follow-up studies using RNase-protection assays or RT-PCR indicate a relatively equal ratio of ERα:ERβ transcripts in mixed-cell homogenates from ovaries of pre- and postmenopausal women but exclusively ERβ transcripts in luteinized granulosa cells isolated from women undergoing IVE.

Three reports describing immunohistochemical localization of ERβ in human ovaries produced comparable findings of specific immunoreactivity among granulosa cells of follicles from primary to late antral stage, and specific, but considerably lower, levels in thecal cells of preantral and antral follicles. Furthermore, all three demonstrate substantial ERβ immunoreactivity in human ovarian surface epithelia. Hillier et al. also found
ERβ transcripts are detectable in primary cultures of human ovarian surface epithelial cells, however, similar evaluations in cell lines derived from human ovarian surface epithelium indicate no detectable ERβ mRNA. Recent examination of human ovaries found that ERβ1 and ERβ2 are expressed in granulosa-luteal cells as well as endothelial cells at all stages of the luteal phase. This study also used in vitro luteinized granulosa cells, treated with hCG or E2. Treatment of the luteinized granulosa cells with hCG reduced expression of ERα, ERβ1, and ERβ2 by approximately 50%, while treatment with E2 reduced expression of ERα and ERβ1 with no significant change in ERβ2, suggesting that human granulosa cells may respond to LH by reducing ERβ in a manner similar to that found in rodent ovaries.

Studies to date indicate that human tissues express a unique C-terminal variant of ERβ termed ERβcx or human ERβ2. Transcripts encoding ERβcx are detectable in multiple human tissues but most especially in ovary, testis, thymus, and spleen and are often at levels equal to or greater than wild-type ERβ. Immunohistochemistry employing ERβcx-specific antisera indicate the variant is nuclear localized in granulosa cells of early follicles. The physiological function of ERβcx remains unclear. It is unable to bind E2, but in vitro studies indicate the isoform may preferentially heterodimerize with ERα and act as a dominant negative modulator of ERα action. Rodents also possess an ERβ variant (ERβ2) that poorly binds E2, but this receptor form does not inhibit wild-type ERα or ERβ activity. Furthermore, categorical studies have shown that the rodent ERβ2 variant is not detected in human tissues.

**Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling**

**MICE LACKING ERα**

Several lines of ERα-null mice have been characterized as listed in Table 25.7. Neonatal and prepubertal ERα-null mice exhibit relatively normal ovaries except for signs of premature folliculogenesis as indicated by the sporadic presence of large antral follicles. Adult ERα-null females are anovulatory and hence infertile, and exhibit ovaries that possess normal pre- and small antral-stage follicles, multiple hemorrhagic cysts, absence of corpora lutea, reduced number of interstitial glandular cells, and levels of Cyp17 and Hsd17b1 are reduced to normal after reduction of gonadotropin levels. ERα-null oocytes exhibit increased expression of Cyp17 and Cyp19, both of which are secondary to the loss of ERα actions in the hypothalamic pituitary axis. ERα-null mouse ovaries are characterized by several large, hemorrhagic, and cystic follicles; a sparse number of follicles at the early stages of proliferation; and a lack of corpora lutea.

![FIGURE 25.17 Ovarian phenotypes in ER-null mice. (A–C) Shown are cross-sections from representative adult ovaries of wild-type (A), ERβ-null (B), and ERα-null (C) female mice. Wild-type and ERβ-null ovaries each exhibit all stages of folliculogenesis except corpora lutea, although large antral follicles are sparse in the ERβ-null ovary. In contrast, ERα-null ovaries are characterized by several large, hemorrhagic, and cystic follicles; a sparse number of follicles at the early stages of proliferation; and a lack of corpora lutea. (D) Cross-section of a representative ERα-null ovary after prolonged treatment with a GnRH antagonist. Circulating LH levels were reduced in ERβ-null females by treatment with a GnRH antagonist (60 μg Antide) every 48 h from the age of 28–53 days. Ovaries were collected within 24 h of the final treatment. The characteristic ovarian phenotypes of ERα-null mice (shown in C) are prevented by reducing circulating LH levels, indicating these more dramatic phenotypes are secondary to the loss of ERα actions in the hypothalamic pituitary axis that are necessary to maintain proper LH levels and not due to the loss of ERα within the ovary. (Source: Reproduced from Ref. 415.) (E) Ribonuclelease protection assays for the steroidogenic enzymes in adult wild-type (WT) and ERα-null ovaries treated either with vehicle (V) or a GnRH antagonist (A) every 48 h for 12 days (as described above). ERα-null ovaries exhibit increased expression of Cyp17 and Cyp19, both of which are reduced to normal after reduction of gonadotropin levels. ERα-null ovaries also uniquely express Hsd17b3, a Leydig cell–specific enzyme, and the expression is dependent on gonadotropin stimulation as it is ablated after the reduction of circulating LH levels with GnRH-antagonist treatments. All samples are normalized to β-actin (Actb) mRNA levels. Source: Reproduced with permission from Ref. 431.**
and mast cell infiltration in the interstitium (Figure 25.17) (Table 25.7). The cystic and hemorrhagic follicles likely originate from antral follicles that fail to ovulate due to acyclicity (i.e., lack of an LH surge) and therefore become atretic and accumulate within the ovary. They are characterized by a mural granulosa cell layer of one to several cells thick surrounding an enormous fluid-filled antrum containing blood and immune cells; a degenerating ovum if visible at all; elevated FSH-receptor and LH-receptor expression in the granulosa cell layer, and a hypertrophied theca (Figure 25.17) that also exhibits elevated LH-receptor levels. Therefore, ERα is not required for the recruitment and early growth of follicles or the induction of gonadotropin receptors in thecal and granulosa cells, but is vital to the later stages of folliculogenesis in the mouse ovary.

ERα-null (αERKO) females exhibit a severely disrupted reproductive hormonal milieu, which in toto represents the cause and effect of the overt ovarian phenotypes (Table 25.7). Plasma LH level in αERKO females is elevated three to eight-fold relative to their wild-type littermates, while FSH levels remain normal. This is interesting in light of the fact that ovarioectomy of normal WT mice leads to elevation of circulating FSH and suggests that the αERKO differs from ovariecstomized WT females. Therefore, the ovarian phenotypes of thecal hypertrophy; elevated steroidogenic enzyme expression, and increased sex steroid synthesis in αERKO females are congruent with hypergonadotropic-hypergonadism. A synopsis of evidence that supports acyclicity and chronically elevated LH as a primary cause of the other ovarian phenotypes in ERα-null females includes: (1) the cystic and hemorrhagic follicles appear after the onset of puberty (approximately 40 days of age), (2) the cystic follicles and elevated steroidogenesis (Figure 25.17) are prevented when plasma LH levels are reduced to normal via treatments with a GnRH antagonist, (3) transgenic mice possessing elevated LH but functional ERα exhibit a comparable ovarian phenotype that is rescued by periodic ovulatory doses of exogenous hCG to induce luteinization, and (4) immature αERKO females successfully ovulate, albeit with a reduced number of oocytes compared to wild-type mice, and form some corpora lutea when treated with exogenous gonadotropins prior to the onset of the cystic phenotype. Others propose that aberrantly high intraovarian histamine levels due to infiltration of mast cells in the interstitium may also contribute to cyst formation in ERα-null ovaries. Therefore, the previous findings indicate that a primary role of ERα in murine ovarian function may be extraglandular, i.e., as an essential mediator of the endocrine actions of E2 in the hypothalamic–pituitary axis that are critical to gonadotropin regulation. Indeed, prolonged treatment of female rodents with antiestrogens that cross the blood–brain barrier (e.g., ZM-189,154, EM-800) and produce an αERKO-like gonadotropin profile lead to a similar ovarian phenotype; whereas treatments with tamoxifen, a receptor antagonist that does not alter gonadotropin secretion generates no such effects in the ovary.

A prominent phenotype in αERKO ovaries may be indicative of an intraovarian role for ERα is their elevated capacity to synthesize androgens. Relative to their wild-type littermates, αERKO females possess plasma levels of androstenedione and T that are increased 3 and 40-fold, respectively. Indeed, LH is the primary stimulus of thecal androgen synthesis and plasma LH and thecal cell LH-receptor levels are both significantly increased in αERKO females (as discussed previously). Therefore, it is not totally unexpected that αERKO ovaries exhibit increased expression of the enzymes necessary for androgen synthesis, most notably a remarkable increase of Cyp17 expression, the enzyme necessary for the final step of androstenedione synthesis (Figure 25.17). However, the elevated plasma androgens found in αERKO females is likely not due solely to LH-mediated hyperstimulation of the theca. E2 from granulosa cells is proposed to mediate an intraovarian short feedback loop upon thecal cells to negatively modulate androgen synthesis during the later stages of folliculogenesis by primarily targeting CYP17 activity. Given that ERα is the dominant ER form expressed in rodent thecal cells, the elevated Cyp17 expression found in αERKO ovaries suggests ERα mediates this effect of E2 on androgen synthesis. Additional support for a specific ERα-mediated effect on thecal cell steroidogenesis comes from our findings that chronically elevated LH in wild-type and ERβ-null females leads to a much more moderate increase in Cyp17 expression and androgen synthesis relative to αERKO females. Furthermore, individually cultured ERα-null follicles in vitro secrete substantially more androgens relative to similarly propagated wild-type follicles even though the level of gonadotropin stimulation is held constant. Therefore, it may be concluded that ERα is paramount to maintaining proper androgen synthesis in rodent females via: (1) endocrine actions in the hypothalamic–pituitary axis that negatively modulate LH secretion, and (2) intraovarian actions on thecal cells to negatively modulate Cyp17 expression.

The granulosa cell–specific enzymes necessary for E2 synthesis, Cyp19 and Hsd17b1, are also expressed at elevated levels in αERKO ovaries. As a result, plasma E2 levels are typically increased almost 10-fold relative to wild-type littermates. It is likely that the plasma androstenedione levels discussed earlier would be even greater in αERKO females if ovarian aromatase activity was not also equally elevated and providing for efficient conversion. Although these findings in αERKO females suggest that ERα may also negatively modulate
E2 synthesis in granulosa cells, there is little precedent for this hypothesis. The chronically elevated plasma LH in αERKO females is also not likely to positively influence granulosa cell E2 synthesis as most evidence indicates that LH stimulation of granulosa cells leads to decreased aromatase activity.414–416 Furthermore, individually cultured ERα-null follicles in vitro continue to exhibit heightened E2 synthesis relative to similarly propagated wild-type follicles in an environment of controlled FSH and LH stimulation.417 Instead, increased aromatase activity in ERα-null ovaries is likely due to the positive actions of estrogens on FSH-induced granulosa cell steroidogenesis414,418–419 which are presumably mediated by ERβ and therefore remain intact in ERα-null ovaries. Androgens have also been shown to augment FSH induction of E2 synthesis420,421 and therefore the elevated androstenedione and T levels characteristic of αERKO females may also contribute to increased E2 synthesis in granulosa cells.

The several attempts to induce ovulation of ERα-null females collectively indicate an age-dependent effect of the loss of ERα. Schomberg et al. report that 4-month-old αERKO females do not successfully ovulate following exogenous treatments with PMSG and hCG, although this conclusion was based solely on the absence of corpora lutea rather than actual oocyte numbers.422 Two later studies focused on younger αERKO females (3–5 weeks) and produced comparable results of successful ovulation (oocytes in the oviduct) and formation of functional corpora lutea, although the oocyte yield was reduced compared to age-matched wild-type females.423 Oocytes harvested from ERα-null females successfully undergo in vitro fertilization, indicating that ERα may not be important to oocyte function.424 In contrast, Dupont et al. report that the other line of ERα-null females (ERαKO) fail to ovulate or exhibit corpora lutea following exogenous gonadotropin treatments even at 21–25 days of age.425 Studies in immature αERKO females of pure C57BL6 background (versus the mixed background of earlier studies) found that these mice can elicit a response to gonadotropin-induced ovulation.426 This discrepancy in vivo ovulatory success between the αERKO and ERαKO mice may be due to differences in genetic strain, the considerable disparity in the doses of gonadotropin used, or the small sample size in the Dupont et al. (n = 3). In further support of a minor role for ERα in ovulation, Emmen et al. demonstrated that individual ERα-null follicles propagated and induced to ovulate in vitro behave no differently than similarly cultured wild-type follicles.427

The discrepancies in the ovulatory response described herein may also be due to the splice variant of ERα expressed in the αERKO that was able to respond to exogenous gonadotropins (albeit reduced number [~15 oocytes] compared to wild-type [~41 oocytes]).428 These mice were found to have expressed a splice variant in some tissues, and to circumvent this, a new ERα-null mouse model was made by Cre/loxP-mediated recombination (Ex3αERKO).429 The ovarian phenotype and hormonal milieu of these mice is similar to that observed in the αERKO mice.430,431 However, the ability of these animals to respond to exogenous gonadotropins has not been fully studied to date. Preliminary data suggest reduced response to exogenous gonadotropins, as less than 50% ovulate and those that do respond ovulate a significantly lower number of oocytes (preliminary data Korach laboratory); however, further work is necessary to confirm this finding and fully characterize the ovulatory response of the Ex3αERKO mice.

Recently, a woman with homozygous mutation of ERα leading to severe E resistance was reported.432 It is interesting to note that, similar to the ERα null mouse lines, she had cystic ovaries and elevated E, but unlike the mouse models, her LH and T levels were normal or nominally elevated. This is perplexing in light of the mechanisms discussed previously proposing the key role of LH elevation in causing the hemorrhagic cystic ovarian phenotype, and suggests notable interspecies differences in the mechanisms underlying these processes.

MICE WITH OVARIAN-SPECIFIC DELETION OF ERα

In rodent species, ERα is expressed predominately in theca cells, while ERβ is expressed in granulosa cells. Global loss of ERα in mice leads to a severe ovarian phenotype with large hemorrhagic and cystic phenotype due increased serum LH from loss of negative feedback in the hypothalamic–pituitary axis. While examination of ovarian function in these global ERα-null mice has provided important data on the necessity of ERα for ovarian function, the models make it difficult to delineate the importance of ERα specifically in the theca cells of the ovary. To circumvent this, Bridges et al. generated a mouse model with Cre-recombinase under control of the Cyp17 promoter so that it is expressed specifically in theca cells in the female mouse, and when crossed with a ERαf/f mouse deletes expression of ERα in theca cells in the ovary.433 Further characterization of this mouse and ovarian function in the absence of ERα demonstrated that these mice prematurely lose fertility.434

Theca cell–specific ERα knockout (thESr1KO) mice did not have an altered estrous cycle, fertility as measured by the number of offspring born, or ovarian response to exogenous gonadotropins (superovulation) in animals aged 2 months; however, these measures were significantly reduced in thESr1KO females at 6 months of age (Table 25.7).435 In response to exogenous gonadotropins the thESr1KO animals release similar numbers of oocytes to WT at 2 months of age (27 in WT versus 22 in thESr1KO); however, by 6 months there are significant reductions in oocytes released (22 in WT versus 6 in
thEsr1KO) and the ovaries show an increase in the number of cystic/hemorrhagic follicles. Additionally, the oocytes collected from the thEsr1KO ampulla appeared to be more degenerated than WT oocytes, however, further studies are required to examine the viability of oocytes collected from thEsr1KO mice. The age-related reduction in fertility coincides with an increase in the length of estrous cycle these animals have at 6 months of age, demonstrating that ERα is important for maintenance of fertility, but its expression in thecal cells is dispensable for normal ovarian response in younger mice.

Global ERα-null mice have increased serum LH, which contributes to the cystic and hemorrhagic phenotype presumably due to loss of negative feedback in the hypothalamic–pituitary axis. Loss of ERα specifically in the theca cell layer leads to a reduction of serum LH in mice at 2 months of age, and a further reduction at 6 months. This reduction in LH in the thEsr1KO animals was not due to reduced gonadotropes in the anterior pituitary, as there was actually an increase in the number of gonadotrope cells. Moreover, no difference is seen in the amount of LH stored in these cells as examined by immunofluorescence. This confirms that the excess LH in the global ERα-null models is due to loss of ER in the hypothalamic–pituitary axis, and suggests that ERs expression in the ovary may be important for positive feedback and/or normal LH secretory patterns, although this needs to be tested experimentally.

Previous work has suggested that androgen synthesis is negatively regulated by estrogen through paracrine signaling actions, which can be examined in the thEsr1KO mouse model. Examination of serum hormone levels at both 2 and 6 months shows that T is increased, confirming that ERα is necessary for maintenance of normal T levels. Examination of the expression of several steroidogenic enzymes found that Cyp17 expression was increased at both 2 and 6 months following PMSG and hCG stimulation, while Cyp19 expression was not significantly different between WT and thEsr1KO animals. While expression of Cyp17 is increased, the protein concentration of CYP17 was also increased in thEsr1KO ovaries in both aged animals and younger mice. CYP17 levels were also examined via immunohistochemistry during diestrus, when CYP17 activity and expression should be minimal, and increased CYP17 was observed in interstitial cells in the thEsr1KO ovary compared to wild-type ovaries. The collective data support the notion that ERα signaling is necessary to support proper androgen synthesis in the ovary.

MICE WITH MUTATED DNA BINDING DOMAINS OF ERα

As discussed earlier in this chapter, the ERs may act as transcription factors on certain genes in the absence of a classical ERE within the regulatory sequences. This alternative mechanism of ER action, often referred to as “tethering”, is thought to occur via interaction with transcription factors that are in fact bound directly to DNA (Figure 25.4). To develop an in vivo model for the study of ERs signaling via ERE-independent pathways, Jakacka et al. generated mice that possess a mutant form of the zinc-finger of the ERα DBD. Because this mutation was incorporated into the endogenous Esr1 gene (i.e., a “knock-in”), transcriptional expression of the mutant ERα is presumably no different than that of an undisrupted Esr1 gene. Female animals that are heterozygous for the ERα mutation, termed NERKI+/- or ERαAA/+ mice, are infertile and exhibit distinct ovarian phenotypes characterized by follicles of all stages of growth but no corpora lutea and lipid-filled cells throughout the stroma (Table 25.7). The latter phenotype is similar to that of female mice lacking functional steroid acute regulatory protein (STAR) and correlates with reduced STAR expression in NERKI+/- ovaries. Upon stimulation with exogenous gonadotropins, NERKI+/- ovaries exhibit multiple large hemorrhagic follicles similar to those found in ERα-null ovaries. NERKI+/- females exhibit normal plasma gonadotropin and E2 levels and proper expression of steroidogenic enzymes in the ovary. Still, induced ovulation in NERKI+/- females is only partially successful in causing some oocytes to be released and the formation of corpora lutea, as several preovulatory follicles fail to rupture and become hemorrhagic.

Without further data it is difficult to determine the exact cause of the NERKI+/- ovarian phenotypes. Jakacka et al. postulate that the mutant ERα may act as a dominant-negative form of ER, however, the mutant ERα does not exhibit this property in vitro. Furthermore, prolactin (Prl) is a known ERE-regulated gene and exhibits no change in expression in NERKI+/- females. An imbalance between ERE-dependent and -independent pathways or tissue-specific inhibitory effects of the mutant ERα may also explain some of the resulting phenotypes.

To further explore the role of the DNA binding domain in ERα function in the ovary, both the ERαAA/- (also called “KIKO”) and ERαAAE mouse models (described in the section Mice with Mutated DNA Binding Domains of ERα) were developed, both expressing only ERα that is unable to bind directly to ERE DNA motifs. KIKO were found to be anovulatory and infertile (Table 25.7). Further characterization of the ovaries of these mice found follicles at most stages of development, however, there was a lack of corpora lutea in the ovary sections. The lack of corpora lutea and estrous cycle in these mice suggests that they do not ovulate, however, the ability of these mice to respond to exogenous gonadotropins was not examined. These animals have some of the ovarian phenotypes identified in the NERKI+/-,
demonstrating that the DNA binding activity of ERα is important for normal ovarian function. ERαEAAE/EAAE females are infertile and have a hemorrhagic and cystic ovarian pathology (Table 25.7) similar to the ovarian phenotypes observed in the other models that carry a mutation in the DNA binding domain. While further characterization of the ovarian phenotype in these animals is necessary to determine the exact cause of the ovarian phenotypes, the data to date suggest that DNA binding is an essential activity of ERα in normal ovarian function.

The KIKO animals have normal levels of E2 and P (Table 25.7) and hormone levels were not examined in the ERαEAAE/EAAE mouse model although ovarian phenotypes were similar between the two mouse models, suggesting that regulation of serum steroid levels are not dependent on ERα DNA binding activities. Furthermore, the role of the DNA binding domain in ERα in tissues other than the ovary (such as the pituitary) could contribute to some of the observed phenotypes. Tissue-specific knock-in mutations to examine the role of direct DNA binding of ERα in ovarian function experiments could provide more mechanistic properties of the role of ERα, however, these experiments have not been done to date.

MICE WITH MUTATED AF-1 OR AF-2 DOMAINS OF ERα

While the models presented to date discuss the role of the DNA binding domain of ERα, there are other important domains of the nuclear receptor that may also contribute to the role of ERα in the ovary. To examine this, two different mouse models were developed with mutations in the ligand binding domain of ERα. In the first model, a single point mutation was made in the LBD (Figure 25.1) where a leucine was substituted in place of residue 525 where normally a glycine resides. This mutation alters the binding pocket, preventing normal ligand binding due to the increased side chain present in leucine compared to the single methyl group present in glycine. The knock-in mouse model ERα(G525L), hereafter referred to as ENERKI, is anovulatory with a hemorrhagic and cystic ovarian pathology (Table 25.7). The animals were found to have an increased number of atretic antral follicles, which presumably became the hemorrhagic/cystic follicles observed due to lack of rupture. Furthermore, these animals did not have any corpora lutea present, similar to the ERα suggesting that they do not ovulate, demonstrating the importance of ligand binding ERα for normal ovarian function.

The AF-1 and AF-2 domains of ERα are important for ER function as demonstrated in vitro reporter assays. Mouse models were developed with each AF domain deleted, and uterine function was determined as described earlier in this chapter; however, the ovarian phenotype or hormone levels of these mice were not reported (Table 25.7). To examine the importance of the AF-2 domain, a knock-in mutation in the AF-2 domain of ERα was made such that E2 is no longer able to transactivate E-dependent transcription. This two-point mutation in the AF-2 domain was introduced into a knock-in mouse referred to as AF2ERKI/KI. The mice are anovulatory and infertile (Table 25.7), similar to the global αERKO knockout mouse models developed to date. The ovaries of these mice also resemble the ovaries in ERα-null mouse models, in that they are hemorrhagic and cystic and lack presence of corpora lutea. Furthermore, the endocrine milieu of these mice is similar to the global ERα-null animals; the AF2ERKI/KI mice have elevated serum LH and E2 (Table 25.7) presumably due to lack of negative feedback in the hypothalamic-pituitary axis. The ovarian functions of other mice with AF-1 or AF-2 disruptions (ERαAF-1-3 and ERαAF-2-3) were not reported (Table 25.7). These models suggest that the ligand binding domain of ERα is required for normal ovarian function; however, the results differ slightly from the tissue-specific ERα knockout mouse described earlier. Therefore, further work using a tissue-specific knock-in mouse model would be useful in determining the mechanistic role of the different domains of ERα in the ovary.

MICE LACKING ERβ

Several facets of ovarian physiology thought to be dependent on the paracrine actions of E2 are clearly maintained in ERα-null and ERα-mutated ovaries, including granulosa cell proliferation, LH-receptor and aromatase expression, antrum formation, and the attenuation of atresia. The preservation of these estrogen actions in ERα-null ovaries strongly suggests their dependence on ERβ, which continues to be expressed in αERKO granulosa cells. Neonatal ovaries from ERβ-null mice exhibit no gross abnormalities, however, there are some differences in the expression of extracellular matrix proteins at this time of development. Adult ERβ-null ovaries possess all stages of folliculogenesis, a slight but perceptible increase in atretic follicles, and a paucity of corpora lutea (Table 25.7; Figure 25.17). Cheng et al. found that among 5-month-old ERβ-null females, less than 30% exhibit corpora lutea compared to 100% incidence in wild-type littermates. Furthermore, when corpora lutea are present in ERβ-null ovaries, there are rarely more than two versus upwards of seven in wild-type females. Continuous mating studies with ERβ-null females indicate a severe impairment in fecundity, but there is discernible variability in this phenotype among age-matched animals. Consistent among βERKO pregnancies is reduced litter size of approximately one-third relative to wild-type.
However, whereas some βERKO females exhibit the expected number of pregnancies over a 4-month period, others become pregnant only once while still others exhibit total infertility.171,172 This variability in fecundity among ERβ-null females remains puzzling but is reported in both independently generated lines.158,171 Given that the ERβ-null lines represent distinct targeting schemes for the Esr2 gene on different genetic backgrounds, the observed variability in fertility is likely related to the loss of ERβ functions. A similar variability in oocyte yield is found when immature ERβ-null females are induced to ovulate by exogenous gonadotropin treatments.158,171,172,432 A third ERβ-null mouse model was recently developed in which all mice were found to be infertile and unable to respond to exogenous gonadotropins.172 These animals also had a significant reduction in the number of corpora lutea present in the ovaries of the ERβ-null mice.172 While subfertile versus infertile phenotypes are observed in the different ERβ-null mouse lines, it should be noted that although some mice did produce pups and respond to exogenous gonadotropins, the number was significantly lower than that observed in WT mice, demonstrating the importance of ERβ in normal ovarian function. Therefore, the observed sub/infertility in ERβ-null females appears to originate from disrupted ovarian function that is best characterized to date as infrequent and inefficient spontaneous ovulation. A lack of evidence indicating embryo resorption during gestation in mated βERKO females171 rules out an extragranulosa contribution, although such phenotypes have not been categorically studied.

Histological evaluation of ERβ-null ovaries following induced ovulation reveals multiple preovulatory follicles possessing underdeveloped antrum and minimal cumulus expansion.158,171,172,432 Krege et al.171 report obvious corpora lutea in immature βERKO ovaries 20h after hCG treatment, but Dupont et al. remark that no luteal cells were observed in similarly treated ERβKO ovaries that did not ovulate.158 However, immature βERKO females do indeed exhibit functional corpora lutea following induced ovulation, as indicated by histological evaluation of the ovaries and a substantial rise in plasma P levels following induced ovulation.432 Luteinization and increased P synthesis is also observed when individually propagated ERβ-null follicles are exposed to an hCG bolus in vitro.440 Furthermore, luteinization occurs in unruptured preovulatory follicles both in vivo and in vitro,134,171,440 resulting in “trapped” oocytes similar to those observed in other null mouse models.176,452

ERβ-null females exhibit a relatively normal reproductive endocrine milieu.451 Basal gonadotropin levels are within the range of control values,432 although one report remarks that plasma LH levels are slightly increased in βERKO females.453 This notwithstanding, ERβ-null females clearly do not exhibit the ovarian phenotypes that are associated with chronically elevated LH.415,433 Therefore, ERα is clearly the principal receptor involved in mediating the negative feedback effects of E2 on gonadotropin secretion from the hypothalamic–pituitary axis. Sex steroid hormone levels in ERβ-null females are also comparable to wild-type littermates.431 Additionally, the overtly estrogenized uterine and vaginal tissues observed in ERβ-null females indicate sufficient ovarian estrogen synthesis in the absence of functional ERβ.134 This preservation of normal E2 levels in βERKO females is surprising given the evidence that estrogens are necessary to maximize FSH stimulation of aromatase activity in granulosa cells.442,444–447

Recent studies into the role of ERβ in normal ovulatory function have found that ERβ is necessary in granulosa cells for normal response to both gonadotropins FSH and LH. Granulosa cells isolated from ERβ-null ovaries and grown in culture had reduced levels of cAMP in response to FSH stimulation compared to granulosa cells isolated from WT ovaries.454 The reduced signaling response to FSH was also observed in granulosa cells from in vitro–stimulated ovaries, and whole follicles grown in vitro.454,455 The reduced cAMP accumulation after FSH stimulation coincided with a reduction of LH-receptor (officially Lhcgr) mRNA accumulation.455 This suggests that granulosa cells in ERβ-null ovaries are unable to stimulate increased LH-receptor expression, which could contribute to the inability of these cells to respond to LH intracellular signaling components,463,455 and ultimately ovulate. Still, βERKO females clearly do not exhibit a normal ovarian cycle, as evidenced by vaginal smears indicating animals in persistent estrous and the paucity of corpora lutea in the ovary. This suggests the βERKO female mounts a preovulatory rise in E2 that is insufficient to induce the gonadotropin surge from the hypothalamic–pituitary axis, and/or a diminished ability of the βERKO ovary to respond to the LH surge. As mentioned before, androgens also augment FSH-induction of E2 synthesis,442,448,449 and provide for compensatory actions in ERβ-null granulosa cells.

MICE LACKING ER α AND β

Congruent with the loss of ERα, αβERKO females are infertile and do not spontaneously ovulate.136,174 Adult αβERKO ovaries possess structures appropriate to the normal ovary, including primordial and growing follicles, although the latter possess an underdeveloped antrum, reduced granulosa number and a thin poorly structured theca (Table 25.7).158,174 Cystic follicles that are somewhat characteristic of those found in ERα-null ovaries are also present but are not as large or as hemorrhagic in αβERKO ovaries and are more apt to exhibit only a thin layer of granulosa cells.158,174 We have used mice with chronically elevated expression of LH from a transgene (α-LHβCTP mice) to show that the formation
of hemorrhagic and cystic follicles in the mouse ovary are due to ovarian acyclicity compounded by chronically elevated plasma LH in the presence of normal FSH levels. The occurrence of hemorrhagic follicles in α-LHβCTP transgenic ERα-null mice and the absence of hemorrhagic follicles in α-LHβCTP transgenic ERβ-null mice demonstrates a role of ERβ in development of the cysts. Therefore, the absence of hemorrhagic and cystic follicles in αβERKO ovaries indicates an important intraovarian role for ERβ in this pathology. A recent compound αβERKO mouse model that does not express the splice variant for ERα has elevated plasma LH and hemorrhagic and cystic ovarian phenotype observed in the ERα-null mice. The discrepancy observed in these mice suggests that the presence of the splice variant, genetic background, or mode of knockout in the mouse models may contribute to the ovarian pathology observed. Further studies are necessary to determine the role of ERα or ERβ in development of hemorrhagic follicles within the ovary.

A most remarkable feature of adult αβERKO ovaries is the presence of seminiferous tubule-like structures that often occupy large portions of the gonad but are conspicuously absent in ERα- or ERβ-null ovaries (Figure 25.18). Morphological observations indicate these structures are the “ghosts” of atretic follicles as they possess an intact basal lamina, partial layers of granulosa cells, and an invariably degenerating oocyte (Figure 25.18). Furthermore, these structures are postpubertal as Couse et al. report no such structures in αβERKO ovaries at 10 days of age and Dupont et al. concur in another line of ERαβKO ovary as late as 23 days of age. The defining characteristic of the “ghost” follicles that led to their initial description as testis-like seminiferous tubules is the overt presence of Sertoli-like cells in the lumen (Figure 25.18), including: (1) alignment with the basal lamina of the follicle wall, (2) a tripartite nucleolus, (3) numerous veil-like cytoplasmic processes extending inward toward the lumen, (4) ectoplasmic specializations that are unique to Sertoli cells in prepubertal testis, and (5) immunoreactivity for Müllerian-inhibiting substance and sulfated glycoprotein-2.

Granulosa and Sertoli cells fulfill analogous gametogenic roles as “nurse” cells in the ovary and testis, respectively, and are postulated to derive from a common embryiological precursor cell during early gonadal
The origin of the Sertoli-like cells in αβERKO ovaries remains a perplexing question. Do they originate from a bi-potent precursor population present in the αβERKO ovary at birth, or are they the result of granulosa cell transdifferentiation following oocyte death and follicle atresia? A report of extra-follicular Sertoli cells in the ERαβKO ovary supports the existence of an embryonal precursor cell population, yet similar interstitial cell populations are not found in ovaries from the other αβERKO. In turn, the following findings that are common to both lines support a pathway of granulosa cell transdifferentiation: (1) the Sertoli-like cells are not apparent prior to 30 days of age, (2) the spherical shape of the “tubules” suggested by two-dimensional histology suggests they originate from a once-healthy follicle in which only the basement membrane remains, and (3) the appearance of Sertoli cells is strongly correlated with oocyte death and follicle atresia. Furthermore, Sox9 is highly expressed in both αβERKO and ERαβKO ovaries and localized to the Sertoli-like cells. SOX9 is an Sry-related transcription factor that is critical to normal Sertoli cell differentiation during testis development in rodents and humans. Transgenic overexpression of Sox9 leads to phenotypic male gonadal development in XX mice and therefore acts “downstream” of the Y-linked male-determining gene, Sry. In strong favor of a pathway of granulosa cell transdifferentiation, Sox9 expression in ERαβKO ovaries is restricted to the granulosa cells of atretic follicles and precedes the overt appearance of Sertoli-like cells but is not detectable in the granulosa cells of primordial follicles. Furthermore, those cells expressing Sox9 are initially present with the confines of the follicle “ghosts” and appear in the intrafollicular spaces of the ovary only after the phenotype has progressed to an advanced stage, supporting a follicular (i.e., granulosa) origin.

Similar phenotypes of “sex-reversal” are reported in fetal rodent ovaries following: (1) transplantation of the fetal ovary to an adult host, (2) in vitro exposure to purified Müllerian inhibiting substance (MIS), (3) transgenic MIS overexpression in vivo, (4) transgenic Sox9 overexpression in vivo, and (5) following targeted disruption of the Wnt4 gene in mice. Although the αβERKO ovarian phenotype shares a number of morphological similarities with the previous findings, including aberrant expression of the MIS, SGP-2 and Sox9 genes, a remarkable distinction in the αβERKO ovary is the postnatal onset of the phenotype. The previous descriptions of ovarian sex reversal are reported to occur or even require fetal ovarian tissue. Therefore, the observed “sex-reversal” adult αβERKO ovaries is the first to be described in the postnatal mouse gonad, indicating that the potential of female ovarian somatic cells to redifferate into Sertoli-like cells may be present throughout life in the mouse. Recently, postnatal reprogramming of ovaries to testis with cells that are positive for SOX9 expression and cells resembling Sertoli cells were reported in adult mice lacking FOXL2. This provided a second postnatal model for this ovarian transdifferentiation, and suggested that FOXL2 is an important transcription factor necessary for granulosa cell differentiation and ovarian function. Interestingly, the Ex3αβERKO mouse model shows loss of Foxl2/FOXL2 expression followed by increased Sox9/FOX9 expression and appearance of Sertoli-like cells in the ovary of adult mice.

A causal link between the loss of all ER function and postnatal morphological ovarian sex reversal is unclear. Dupont et al. report that female mice possessing only one functional ERα allele but lacking functional ERβ also exhibit the αβERKO ovarian phenotype, suggesting a gene-dosage effect for ERα. The majority of documented cases of similar phenotypes in the mammalian ovary are preceded by massive germ cell loss. The remarkable oocyte attrition that occurs in the αβERKO ovaries suggests a similar mechanism occurs in this model; yet it remains unclear if the progressive loss of germ cells in αβERKO ovaries illustrates the loss of critical intraovarian estrogen signaling that is perhaps dependent upon ERα/ERβ cooperation. A role of direct estrogen/ER actions in gonadal differentiation is supported by reports of sex reversal in turtles and whiptail lizards following developmental exposure to aromatase inhibitors. The mouse Sox9 promoter lacks an obvious ERE but does possess two consensus binding sites for GATA-1, a transcription factor expressed in Sertoli cells during differentiation of mouse testis but repressed by germ cells in adult testis. Furthermore, ERα can repress the transcriptional activities of GATA-1, suggesting that aberrant Sox9 expression leading to Sertoli-cell differentiation in αβERKO ovaries may be due to increased GATA-1 activity following the loss of ERα and other potential oocyte-derived inhibitory factors. As a similar phenotype is observed in FOXL2 knockout ovaries, it has been hypothesized that Foxl2/FOXL2 is regulated by ERE-dependent transcription and loss of this regulation contributes to the Sertoli-cell phenotype in the ovaries of knockout mice. This does not account for the lack of transdifferentiation during ovarian development or in neonatal animals, which suggests that this “sex-reversal” or transdifferentiation phenotype is a complex trait that requires aberrant regulation/expression of multiple genes. Current studies are underway with the Ex3αβERKO mouse model to look at ovarian gene expression and identify possible candidate genes that may contribute to the granulosa cell transdifferentiation observed.

Female αβERKO animals exhibit a gonadotropin profile that is similar to αERKO females, although plasma LH levels are noticeably higher. Therefore, the hormonal
milleau in αβERKO females may impact the ovarian “sex reversal” observed.\textsuperscript{174,431} Still, transgenic mice possessing chronically elevated plasma LH also display a progressive loss of oocytes but lack any evidence of ovarian “sex reversal.”\textsuperscript{435,439,475} Interestingly, αβERKO ovaries do not exhibit αERKO-like increases in ovarian aromatase or E2 synthesis.\textsuperscript{431} This finding provides further support that elevated LH and precursor substrates, combined with the positive actions of ERβ, lead to enhanced E2 synthesis in ERα-null ovaries—a scenario that would be disrupted in αβERKO ovaries. The dramatic loss of germ cells in the αβERKO ovary may also impact gonadotropin responsiveness of granulosa cells as oocyte-derived factors are known to positively influence steroidogenesis.\textsuperscript{476} Further studies are necessary to determine how germ cell loss contributes to the observed phenotype.

MICE LACKING CYP19

In the ovary, CYP19, or aromatase, is expressed exclusively in the granulosa cells (Table 25.6). Initial reports of Cyp19-null ovaries indicated no phenotypic difference from WT, suggesting estrogen-independent signaling mechanisms were involved in eliminating the phenotypes of the ERKO mice.\textsuperscript{477} It was not until Cyp19-null mice were fed a soy-free diet that phenotypes appeared. These observations were some of the first to directly demonstrate dietary estrogen contamination in feed. Therefore, to study the phenotypes associated with loss of estrogens, Cyp19 null are kept on soy-free diets. Therefore, to study the phenotypes associated with loss of estrogens, Cyp19 null are kept on soy-free diets. Under these conditions, Cyp19-null mice have ovaries with all stages of folliculogenesis, including multiple large antral follicles but no corpora lutea at 10–14 weeks of age (Table 25.7).\textsuperscript{131,156,364} At 21 weeks of age, Cyp19-null ovaries continue to exhibit all stages of folliculogenesis, but larger follicles possess a reduced number of granulosa cells and become enlarged, cystic, and hemorrhagic, similar to ERα-null follicles.\textsuperscript{64} By 1 year of age, Cyp19-null ovaries are characterized by a paucity of normal follicles, an increased number of cystic and hemorrhagic follicles, significant collagen deposition, and massive macrophage infiltration into the interstitium (Table 25.7).\textsuperscript{64} Several findings indicate infertility among Cyp19-null females due to an inability to spontaneously ovulate, including: (1) absence of corpora lutea at all ages,\textsuperscript{131,156,364} (2) lack of pregnancies during continuous mating,\textsuperscript{131} and (3) total failure to ovulate following exogenous gonadotropin treatments.\textsuperscript{131} Recently, it has been demonstrated that co-treatment of Cyp19-null mice with E2 and PMSG followed by hCG can rescue the anovulatory phenotype,\textsuperscript{365} demonstrating the necessity of E2 signaling in addition to the gonadotropins for normal ovulatory function.

Surprisingly, initial descriptions of the ovarian phenotypes in Cyp19-null females did not include remarks of Sertoli-like cells similar to those found in αβERKO females. However, Britt et al. later demonstrated that mutant females maintained on a diet free of phytoestrogens exhibit a clearly exacerbated ovarian phenotype by 6 weeks of age that included hemorrhagic and cystic follicles, seminiferous tubule–like structures possessing Sertoli-like cells, and an almost 40-fold increase in Sox9 expression.\textsuperscript{194} By 16 weeks of age, the tubule-like structures and Sertoli cells occupy up to 80% of the ovary and are strikingly similar to those found in αβERKO ovaries in both morphology and gene expression.\textsuperscript{194} Additional supporting evidence of Sertoli-like cells in Cyp19-null ovaries is documentation of Sertoli cell-specific ectoplasmic specializations and immunoreactivity for Espin, an important component of Sertoli cell junctions in testis.\textsuperscript{194} Recent microarray analysis has identified 450 genes that are differentially expressed in Cyp19-null ovaries compared to wild-type ovaries, with 291 showing increased expression and 159 showing reduced expression.\textsuperscript{478} Several of these genes have been implicated in testis development, however, further studies and confirmation are necessary to determine potential candidates that may contribute to ovarian transdifferentiation. Interestingly, a similar phenotype of “sex reversal” has not been described in the ovaries of a second line of Cyp19-null females.\textsuperscript{131}

As expected, the reproductive hormonal milieu in female Cyp19-null mice is severely disrupted. Plasma E2 levels\textsuperscript{131} and ovarian aromatase activity\textsuperscript{156} are below the level of detection, supporting the existence of a single aromatase-encoding gene in mice. In turn, plasma T levels in Cyp19-null females are 10-fold that found in wild-type littermates.\textsuperscript{131,156} Increased plasma androgens in Cyp19-null females are likely the combined effect of an accumulation of androgen precursors that occurs in the absence of aromatization to E2, and increased synthesis due to hyperstimulation of the ovarian theca by five-fold increased plasma LH.\textsuperscript{156,364} Thus, increased plasma LH and androgens are characteristic of mice lacking ERα-mediated estrogen actions following the loss of receptor (ERα-null animals) or ligand (Cyp19-null animals). However, one striking difference between Cyp19-null and ERα-null females is the four- to six-fold increase in plasma FSH that is unique to the former.\textsuperscript{64} Interestingly, Cyp19-null females exhibit the above abnormal hormone milieu regardless of phytoestrogen content in the diet,\textsuperscript{194} suggesting that dietary phytoestrogens may attenuate some but not all of the effects that follow the loss of endogenous E2 synthesis.

Studies have indeed shown that Cyp19-null mice still possess functional ERα signaling.\textsuperscript{479} Tonic E2 replacement therapy in Cyp19-null females over a period of 21 days leads to restored gonadotropin homeostasis, improved follicular development in the ovary, a reduced presence of Sertoli-like cells and Sox9 expression, and restoration of ovulation and corpora lutea formation.\textsuperscript{479,480} In

4. FEMALE REPRODUCTIVE SYSTEM
a similar study, Toda et al. report that E2 administration to Cyp19-null females every fourth day for 4 weeks also ameliorates the ovarian phenotypes but did not restore ovulation.\textsuperscript{131} Ovulation was partially restored when the mice were treated with E2 in addition to exogenous gonadotropins,\textsuperscript{365} although oocyte numbers were still significantly lower than those observed in WT mice. Furthermore, treatment of the Cyp19-null mice with a GnRH antagonist and E2 replacement was able to restore normal gonadotropin levels, while treatment with an antiandrogen had no effect. This demonstrates that loss of E2 in these animals contributes to the ovarian phenotype observed and also indicates that an indirect effect from excess androgens does not directly contribute to the excess gonadotropins, hemorrhagic follicles, or Sertoli-like cells present in the Cyp19-null mice.\textsuperscript{481}

\textbf{Intraovarian Roles of Estradiol in Ovarian Function}

The importance of the extraovarian or endocrine roles of estrogen signaling in ovarian function has long been recognized and is the precedent for steroid-based oral contraceptives.\textsuperscript{482,483} In contrast, the significance of estrogen signaling within the ovary is not well understood due to the inherent difficulties associated with the study of a particular hormone’s action within the very tissue from which it is synthesized and secreted. Past in vivo investigations employing ER antagonists\textsuperscript{217–219,484} or aromatase inhibitors\textsuperscript{485–486} to inhibit hormone receptor actions within the ovary have provided informative but often equivocal findings\textsuperscript{217–219} due to the following limitations: (1) the enormous levels of endogenous E2 in the ovary are difficult to overcome by pharmacological administration of a receptor antagonist, (2) the enormous levels of aromatase activity in the ovary are difficult to overcome by pharmacological administration of an enzyme inhibitor, (3) the effects of a pharmacological ER antagonist due to actions directly in the ovary versus actions in the hypothalamus or pituitary are difficult to discern, (4) the well-characterized agonists (e.g., E2 and DES) comparably activate both ER\textalpha{} and ER\textbeta{}, (5) early antagonists were not selective for the two known ERs, and (6) the two ERs may respond differently to agonist or antagonist ligands. The generation of ER \textalpha{} and Cyp19-null mice, and development of improved ER-selective ligands or SERMs\textsuperscript{63,487,488} have contributed to better understanding over the years.

The abundance of data on the localization of ER\textalpha{} and ER\textbeta{} within rodent follicles allows for a simplified model of estrogen actions within the ovary. For example, given that ER\textalpha{} predominates in thecal cells, does it mediate the inhibitory effects of E2 on LH-stimulated androgen synthesis? In turn, as ER\textbeta{} clearly is the predominant receptor in granulosa cells, is it responsible for mediating the known estrogen augmentation of FSH actions? This section employs the contributions from studies of the pertinent null- or mutated-mouse models as a platform to discuss the current view on intraovarian estrogen/ER signaling.

\textbf{GRANULOSA CELL PROLIFERATION}

A number of studies in immature hypophysectomized rodents have demonstrated that DES or E2 treatments over 2–4 days leads to a marked increase in ovarian weight\textsuperscript{489} a significant rise in the number of medium-sized preantral follicles and increased DNA synthesis in both thecal and granulosa cells,\textsuperscript{422,490–492} indicating a direct and gonadotropin-independent effect of E2 on the ovary. Similar data have been reported following DES treatment of intact immature rats.\textsuperscript{493} However, there is equally convincing data that sequential treatment of immature hypophysectomized rodents with DES followed by FSH results in a synergistic response in terms of increased ovarian weight and DNA synthesis, and elicits several major indices of granulosa cell differentiation, i.e., antrum formation and acquisition of LH-receptor expression, that are not observed with either hormone alone.\textsuperscript{359,490,491,494} Furthermore, co-administration of an antiestrogen (cis-clomiphene) inhibits FSH-induced increases in ovarian weight and follicular growth in hypophysectomized rats, suggesting that ER-mediated effects are necessary for a full response to gonadotropin.\textsuperscript{495} The synergistic actions of E2 on FSH-induced granulosa cell proliferation have been replicated in vitro,\textsuperscript{496} although others report that FSH-mediated growth of isolated murine follicles is not affected by anti-E2 antisera or ER antagonist (ICI 182,780).\textsuperscript{497} The capacity of E2 to enhance the granulosa cell response to FSH is not believed to rely on estrogen-induced increases in FSH-receptor levels, although some evidence indicates such an effect may play a greater role in mice versus rats.\textsuperscript{359,492,494} These differences will be easier to study with the recently described ER\textalpha{}-null rat,\textsuperscript{190} which can be used to compare the roles of ERs in rat versus mouse ovarian functions. Still, pretreatment of hypophysectomized rats with antiestrogen (CI628) inhibits FSH-induced increases in granulosa cell FSH receptor levels.\textsuperscript{418}

It is generally agreed that the synergistic response of granulosa cells to estrogens and discovery of ER\textbeta{} and its substantial expression in the granulosa cells of growing follicles and absence in atretic or luteinized follicles strongly suggests that the trophic actions of estrogens are direct and ER\textbeta{} mediated. Support-
17β-E2 in hypophysectomized rats; whereas the ERα-selective agonist 3,17-dihydroxy-19-nor-17α-pregna-1,3,5 (10)-triene-21,16α-lactone has little effect. 488 However, although these data indicate that E2/ERβ actions may indeed enhance FSH-induced granulosa cell proliferation, several findings question the overall importance of this intraovarian role. First, ERβ-null ovaries exhibit a relatively normal number of growing preantral follicles with no obvious reduction in the granulosa cell population. 158,171,498 Furthermore, immature ERβ-null females treated with PMSG exhibit the expected increase in the number of small and large preantral follicles. 158,440 In addition, granulosa cell tumors, due to the loss of inhibin-α (Inha) in mice continue to proliferate regardless of the presence or absence of functional ERβ. 499 It is possible that ERα may provide some compensatory actions as ERβ-null follicles show increased expression of ERα mRNA. 555 Furthermore, Cyp19-null mice are presumably devoid of ERβ-dependent ER action but exhibit ovaries possessing all stages of follicle growth, each with a normal complement of granulosa cells. 364 Therefore, the data suggest that although estrogen-induced granulosa cell proliferation is likely mediated by ERβ, it is not obligatory to gonadotropin-induced follicle growth in rodent ovaries. 

ERβ may facilitate gonadotropin-induced granulosa cell proliferation through the induction of cyclin-D2 (Ccnd2), which enhances progression of the cell cycle from the G0 to S phase. Targeted disruption of the Ccnd2 in mice indicates this cyclin protein is obligatory to granulosa cell proliferation as Ccnd2-null ovaries lack follicles beyond the small preantral stage. 500 Furthermore, the arrest in folliculogenesis observed in Ccnd2-null mice is not rescued by exogenous FSH treatment in vivo or in vitro, indicating the critical downstream role of cyclin-D2 in gonadotropin-mediated granulosa cell proliferation. 500 In rats, Ccnd2 expression is localized to the granulosa cells of early growing follicles and is significantly induced by PMSG or FSH treatments. 501 However, E2 also induces Ccnd2 expression in rat granulosa cells, and although the response is less rapid relative to that elicited by FSH, the overall induction by E2 is higher and better sustained over 24h. 501 Furthermore, E2 elicits a concomitant decrease in the expression of Cdkn1b (p27), a cyclin-dependent kinase inhibitor. 501 E2 induction of Ccnd2 in undifferentiated rat granulosa cells in vitro is inhibited by the ER antagonist ICI 164,384, indicating this to be an ER-mediated event, presumably ERβ. 501 Therefore, parallel pathways of FSH and E2 converge to induce and maintain Ccnd2 expression in granulosa cells, thereby forcing cell cycle progression and subsequent proliferation. 501 Interestingly, adult ERβ-null ovaries possess normal granulosa cell expression of Ccnd2 502 as well as exhibit the expected induction following PMSG exposure, 502 further supporting the hypothesis that FSH and E2 work in parallel. Therefore, these observations and the absence of any gross deficiency in granulosa cells in ERβ-null ovaries suggests that FSH may play a greater role in inducing Ccnd2 expression and granulosa cell proliferation. Interestingly, E2 specifically induces Ccnd2 expression in keratinocytes via the cAMP/PKA signaling pathway that leads to activation of CREB, 502 the same intracellular pathway employed by FSH signaling in granulosa cells. These findings suggest that E2 may also function within the FSH signaling pathway to induce Ccnd2 in granulosa cells.

A second mechanism by which E2 may amplify FSH-induced granulosa cell proliferation is via interactions with the ovarian IGF1 axis. Like Ccnd2-null mice, IGF1-null mice exhibit a total failure of follicles to progress beyond the small preantral stage. 503,504 This arrest in folliculogenesis in IGF1-null ovaries is primarily due to a >50% reduction in FSH receptor levels in the granulosa cells, severely hampering their response to FSH. 504 However, Kadakia et al. demonstrated that IGF1-null granulosa cells are also refractory to E2-induced proliferation and exhibit a 50% lower rate of DNA synthesis and decreased Ccnd2 induction following E2 treatment in vivo. 505 The poor response of IGF1-null granulosa cells to E2 may be due to a considerable reduction in ERβ levels as IGF1 is reportedly necessary for the maintenance of ERβ expression in granulosa cells in vitro. 506 There are currently no reports describing the levels of ERβ expression in the ovaries of IGF1–null mice. Interestingly, Richards et al. demonstrated that E2 significantly upregulates the expression of several components of the IGF1 signaling pathway in rat granulosa cells, including the IGF1 receptor-β subunit, the glucose transporter, Glut-1, and the forkhead family member, FKHR (Foxo1). 507 These findings suggest that E2 may enhance IGF1 actions in granulosa cells by regulating targets important for cellular energy flow, glucose metabolism, and cell survival. 508 In summary, IGF1 actions are clearly necessary to provide for sufficient FSH signaling in granulosa cells, which in turn, is necessary for the induction of aromatase activity and E2 synthesis, thereby providing ligand for ERβ to enhance the effects of IGF1, hence forming an autocrine regulatory pathway to promote granulosa cell proliferation. 506 The extent to which these findings may translate to the human ovary remains to be determined since IGFI, rather than IGF1, is the predominant and gonadotropin-regulated IGF form in the human ovary. 507

The previous discussion provides a compelling argument that the mitogenic actions of E2 on granulosa cells are direct and ERβ mediated. However, thecal cell ER actions may also be involved. For example, ERα in the thecal cells may respond to granulosa cell–derived E2 by inducing the synthesis and secretion of a paracrine-acting growth factor(s) that then becomes the principal stimulus for granulosa cell proliferation. Indeed, Dorrington and...
colleagues have proposed that thecal-derived transforming growth factor-β (TGFβ) is estrogen induced and stimulates granulosa cell proliferation. Similar examples of growth factor–mediated cooperative actions between theca and granulosa cells are postulated to regulate follicle steroid production. Still, ERα-null follicles do not exhibit any gross deficits in granulosa cell number and possess a normal number of preovulatory follicles following gonadotropin stimulation. It is plausible that a dramatic effect on granulosa cell proliferation may only become apparent following the loss of both putative estrogen pathways, i.e., the direct (ERβ-mediated) and indirect (ERα-mediated) actions—hence explaining the unique somatic cell phenotypes observed in the ovaries of compound ER-null mice and older Cyp19-null mice on a soy-free diet in absence of estrogen ligand.

**GRANULOSA CELL DIFFERENTIATION**

The process of granulosa cell differentiation that occurs during progression from a preantral to preovulatory follicle has been an area of intense research. Fully differentiated preovulatory follicles in mammalian ovaries are distinctly characterized by: (1) a large fluid-filled antrum, (2) acquisition of LH responsiveness, (3) significant increases in aromatase (CYP19) activity, and (4) increased inhibin synthesis. Although FSH is the primary stimulus for both granulosa cell proliferation and differentiation, these two processes appear to be independent as the undersized follicles in Ccnd2-null ovaries still develop an antrum, possess sufficient aromatase activity, and are responsive to exogenous LH stimulation. Therefore, granulosa cell proliferation and differentiation are separate gonadotropin-induced events during folliculogenesis.

The absolute requirement of FSH signaling in the process of granulosa cell differentiation is illustrated by the lack of antral follicles, aromatase and E2 synthesis, and granulosa cell LH receptor in mice null for FSH action. Igf1-null follicles exhibit a similar failure to differentiate; this is postulated to be due to a lack of sufficient FSH-receptor expression. However, numerous studies in rat ovaries have demonstrated that FSH induction of antrum formation, aromatase expression and activity, and LH-receptor expression require E2 for maximum effect. FSH acts via a classic heterotrimeric G protein–coupled receptor pathway that may activate multiple intracellular second messenger systems, the most well characterized being activation of adenylyl cyclase, which leads to intracellular accumulation of adenosine 3',5'-monophosphate (cAMP) and activation of protein kinase A (PKA). The long-recognized synergism between E2 and FSH on granulosa cell differentiation is believed to be due to the capacity of E2 to both amplify the level of FSH-stimulated intracellular cAMP and augment the downstream actions of cAMP itself. The mechanisms involved remain poorly understood and are unlikely to involve estrogen-induced increases of FSH receptor levels but appear more likely to rely on E2’s capacity to positively modulate the granulosa cell adenylyl cyclase system.

ER-mediated estrogen activation of the adenylyl cyclase system and subsequent cAMP-regulated gene expression has been demonstrated in uterus and breast cancer cells. Furthermore, in vitro and in vivo studies indicate that ERα or ERβ can activate ERE-dependent gene transcription via the cAMP/PKA signaling pathway in the absence of E2. This pathway involves the CREB protein, which is also known to be critical to FSH induction of gene expression. Therefore, given the existing evidence that E2 is required to maximize the FSH response, combined with the known cooperative activity between the ER and PKA signaling pathways, it may be reasonable to expect that a loss of ER action in the ovary would cause severe deficits in granulosa cell differentiation. Indeed, the ovarian phenotypes in ERβ-null females in terms of antrum formation, aromatase expression, and LH responsiveness collectively represent an attenuated response to FSH-induced granulosa cell differentiation in the absence of ERβ. Additionally, ERβ-null granulosa cells and follicles have a reduced response to FSH marked by reduced cAMP accumulation in vivo and in vitro. The reduction in cAMP can be overcome when cells are stimulated with forskolin to activate cAMP accumulation, demonstrating that altered cAMP/PKA signaling contributes to the reduced granulosa cell differentiation observed in ERβ-null ovaries and suggests that maximal activation requires cross talk between FSH and ER-mediated signaling pathways. Possible downstream mediators were recently identified in a microarray study, providing key targets to further study in the future.

**ANTRUM FORMATION**

Goldenberg et al. first demonstrated that both FSH and estrogen are required for full antrum formation in preovulatory follicles of hypophysectomized rat ovaries. Wang and Greenwald produced similar data in hypophysectomized mice, reporting that FSH plus E2 leads to an average of >70 large antral follicles per ovary versus an average of six or none when treated with FSH or E2 alone, respectively. The requirement of FSH plus E2 for antrum formation holds true when rat preantral follicles are grown in vitro. Others have shown that exposure to aromatase inhibitors during gonadotropin-induced folliculogenesis also reduces the number of healthy antral follicles in rats, although contradictory findings are reported when follicles are
similarly exposed in vitro.\textsuperscript{523} Independent evaluations of ovaries from three separate lines of ER\( \beta \)-null mice indicate a paucity of follicles with fully developed antra, even following standard gonadotropin stimulation.\textsuperscript{158,172,440} These data strongly suggest that the vital role of E2 in FSH-induced antrum formation is mediated by ER\( \beta \). A role for ER\( \alpha \) in antrum formation cannot be excluded but is unlikely given the hallmark phenotype in ER\( \alpha \)-null ovaries is the invariable presence of severely oversized antral follicles, lending to their description as “cystic”.\textsuperscript{157–159,373,415} Furthermore, a role of aberrant LH action cannot be discounted, as mice overexpressing a mutant LH receptor have large cystic and hemorrhagic follicles similar to those observed in ER\( \alpha \)-null mice.\textsuperscript{524} While it is possible to hypothesize that ER\( \beta \) may contribute to the formation of exaggerated antrum due to hyperstimulation of the receptor, the data supporting this idea are not clear. For example, mice that lack both ER forms (\( \alpha \)-ERKO),\textsuperscript{158,171} or transgenic \( \beta \)-ERKO females that have elevated LH\textsuperscript{439} do not have large cystic follicles, although a new line of compound \( \alpha \)-ERKO mice (Ex3\( \alpha \)-ERKO) do have large cystic follicles\textsuperscript{456} as do the Cyp19-null mice.\textsuperscript{480} This contradictory data suggests that the mechanism behind antrum formation may not be due solely to overstimulation of ER\( \beta \), and multiple mechanisms may exist. Furthermore, mice lacking ER\( \alpha \) specifically in the theca cells develop cystic follicles as they age in response to excess gonadotropins, which is not observed in younger mice,\textsuperscript{362} suggesting a more complex mechanism contributes to antrum formation.

As mentioned, FSH alone will induce the formation of small antra in growing follicles whereas E2 has no such effect in vivo and in vitro, but both hormones are required for full antrum development.\textsuperscript{490,492,522} Compared to the total failure of antrum formation among follicles in the murine models of disrupted FSH signaling,\textsuperscript{420,503,512,513,525} similarly sized follicles in ER\( \beta \)-null ovaries exhibit an initiation of antrum formation but fail to develop to a full preovulatory stage.\textsuperscript{158} Quantitative analyses of immature ER\( \beta \)-null ovaries 48h after PMSG stimulation indicate an increased number of small antral follicles but fewer large (preovulatory) follicles relative to wild-type females, suggesting an arrest in antrum formation.\textsuperscript{140} Likewise, a large percentage of ER\( \beta \)-null follicles fail to reach maximum size when grown in culture with FSH and E2.\textsuperscript{440} These follicles also fail to accumulate maximal cAMP levels after FSH stimulation,\textsuperscript{455} suggesting that multiple signaling pathways may contribute to antrum formation. These data support the role of FSH actions in the initiation of antrum formation, but in the absence of ER\( \beta \)-mediated E2 augmentation, maximum antrum development is prohibited.

Because so little is known about the physiology of antrum formation, the role of ER\( \beta \)-mediated estrogen actions is speculative. It has long been proposed that antrum development follows gonadotropin-stimulated increases in granulosa cell proteoglycan secretion, which act to increase osmotic pressure within the follicle and cause the influx of water from the ovarian vasculature.\textsuperscript{526,527} McConnell et al. report that water movement into mouse follicles in vitro occurs via a transcellular pathway and is likely mediated by aquaporins-7, -8, or -9 on the surface of granulosa cells.\textsuperscript{528} Although there is currently no evidence of estrogen regulation of aquaporin expression in the ovary, E2 is known to influence fluid transport in the uterus via regulation of aquaporins -1,\textsuperscript{332} -2, and -3,\textsuperscript{529} and 5 and 8.\textsuperscript{242,530} Furthermore, ER\( \alpha \) is principally involved in the regulation of Na\(^+\) and water transport across the efferent ductules in the rodent testis,\textsuperscript{531} suggesting ER\( \alpha \) may play a similar role in follicles. Antrum formation may not rely totally on hydromechanical mechanisms but is likely to require cell–cell interactions among the granulosa cell layer. Gore-Langton and Daniel demonstrated in vitro that rat preantral follicles lacking an intact basement membrane and therefore unable to provide a sealed environment exhibit a reorganization of granulosa cells that is unmistakably antrum-like in response to FSH plus E2, supporting a role for cell–cell interaction during antrum formation.\textsuperscript{522} A lack of antral follicles in the ovaries of mice lacking the gap junction component connexin-37 (Cx37) further supports a need for cell–cell interactions among granulosa cells.\textsuperscript{532} E2 is known to dramatically increase the number of gap junctions between granulosa cells,\textsuperscript{533} as well as regulate ovarian expression of certain component proteins.\textsuperscript{534–536} Additionally, the extracellular matrix may be important to antrum formation. Mice lacking ER\( \beta \) exhibit altered expression of several extracellular matrix proteins, including increased expression of Collagen 11a1 and Nidogen 2 at both the mRNA and protein level in both prepubertal and adult ovaries,\textsuperscript{451,500} which may alter the follicle composition and contribute to loss of antrum in these mice. Future studies of a potential regulatory role for E2 in the expression of the aquaporins, connexin, and extracellular matrix proteins may reveal a precise role for ER\( \beta \) in antrum formation.

**AROMATASE AND ESTRADIOL SYNTHESIS**

Acquisition of aromatase activity and E2 synthesis is a hallmark of healthy preovulatory follicles in mammalian ovaries. In monotocous species, including women, 90% of the circulating E2 is estimated to originate from the dominant follicle in the ovary. Aromatase (CYP19) expression in rat, mouse, bovine, marmoset, and human ovaries, via activation of the gonad-specific promoter II,\textsuperscript{537} is exclusive to and highest in the granulosa cells of fully differentiated healthy follicles (Table 25.6).\textsuperscript{400,538–540} Likewise, E2 levels are consistently highest in the follicular fluid of healthy preovulatory, and substantially
lower in overtly atretic, follicles. The endocrine actions of ovarian-derived E2 are critical to preparing the female reproductive tract for a forthcoming pregnancy and priming the hypothalamic–pituitary axis for generation of the gonadotropin surge. However, there is equally abundant evidence that E2 acts in a paracrine or autocrine manner to augment FSH stimulation of aromatase activity in granulosa cells and thereby positively influences its own rate of synthesis (Figure 25.19). This pattern of CYP19 expression described herein strongly resembles the profile of ERβ during folliculogenesis, suggesting that the E2-induced enhancement of aromatase activity in granulosa cells is ERβ mediated.440

FIGURE 25.19 Synergistic action of sex steroids and FSH in the induction of Cyp19 expression and aromatase activity in rodent granulosa cells. (A) Shown is the effect of in vitro treatment with the synthetic estrogen DES on FSH-induced aromatase activity in granulosa cells isolated from immature hypophysectomized rats. Granulosa cells were cultured for 3 days in the presence or absence of FSH (10ng/ml) with or without increasing concentrations of DES. Three days later, the cells were washed with medium and reincubated for a 5-h test interval in medium supplemented with androstenedione. The accumulation of estrogen (as measured by radioimmunoassay) during this test period is taken as a measure of the level of aromatase activity. C, controls. (Source: Reproduced with permission from Ref. 445.) (B, C) Shown is the effect of FSH and sex steroids on Cyp19 expression and aromatase activity in cultured granulosa cells. Granulosa cells were isolated from 26-day-old rats that had been untreated (unprimed) or treated with E2 (1.5mg/day) for 3 days (E-primed). Cells were incubated in serum-free medium with 5α-dihydroT (DHT) at 20nM, FSH (50ng/ml), or FSH in combination with DHT (20nM), T (20nM), or E2 (20nM). Cells were also incubated in medium containing 5% fetal bovine serum (FBS), with FSH alone (50ng/ml), or in combination with T (20nM). After 48h in culture, cells were collected for RNA extraction and evaluation of Cyp19 expression by Northern Blot analysis (B) or for measurement of aromatase activity (C). Source: Reproduced with permission from Ref. 442.
FSH is clearly the principal stimulus for the acquisition of aromatase activity in granulosa cells. Once again, female mice lacking FSH (Fshb-null mice) exhibit phenotypes that are consistent with a severe reduction in circulating estrogens, including uterine hypoplasia and elevated plasma levels of LH. Furthermore, the ovaries of Fshb-null mice exhibit a six-fold reduction in Cyp19 transcripts, although these data are not corroborated in Fshr-null mice. FSH regulation of Cyp19 expression in granulosa cells is believed to require the actions of multiple transcription factors, including steroidogenic factor-1 (SF-1) and β-catenin, CREB protein, GATA-4 and CBP, the former three of which possess cognate response elements within the rodent and human CYP19 promoter II.

The mechanism by which E2 augments FSH stimulation of aromatase activity or Cyp19 expression in granulosa cells is unclear but shown to occur in isolated granulosa cell cultures, ruling out the influence of a thalamic-derived factor and supporting a direct role for ERβ. Coadministration of FSH and an ER antagonist (tamoxifen) to granulosa cells either in vivo or in vitro inhibits the expected induction of aromatase activity, although others report conflicting data using a different antagonist (ICI 182,780) in whole follicle culture experiments. More recently, Emmen et al. and Rodriguez et al. found that ERβ-null follicles grown in vitro do indeed secrete significantly less E2 relative to wild-type follicles. The absence of a consensus ERE within the rat or human Cyp19 promoter II makes it unlikely that the effect of E2 is via a direct ERβ mechanism (Figure 25.4). However, ERβ may interact with the cAMP/PKA pathway, SF-1, CREB, GATA-4, CBP, or other unknown transcription factors that act to promote Cyp19 expression. Indeed, ERβ has been shown to associate with both CREB and CBP in heterologous in vitro systems. The ERs are also known to influence GATA-directed gene expression via direct interactions with multiple members of the GATA family of transcription factors. Therefore, there is ample evidence to support ER interaction with the multitude of transcription factors known to influence Cyp19 expression, but the exact nature by which ERβ is involved requires further study.

**ACQUISITION OF LUTEINIZING HORMONE RECEPTOR**

In contrast to the constitutive expression of LH receptor in thecal cells, granulosa cells express LH receptor only in follicles in the late preovulatory stage. This limited expression of LH receptor to the granulosa cells of healthy preovulatory follicles provides for an intraovarian mechanism that ensures only those follicles that are suitable for ovulation acquire the capacity to respond to the LH surge. As with the other markers of follicle differentiation discussed previously, FSH is the primary stimulus of LH-receptor (Lhcgr) expression in preovulatory granulosa cells. However, FSH exposure alone leads to a minimal increase of LH-receptor levels in the ovaries of hypophysectomized rats, and then only after 4 days of treatment; whereas pretreatment of animals with E2 for 4 days prior to FSH exposure leads to an enormous induction of LH-receptor levels that are limited to the granulosa cells of preovulatory follicles (Figure 25.20). This requirement for FSH plus E2 and the temporal pattern of LH-receptor expression has been reproduced in rat granulosa cells in vitro, with maximum levels reached after 48–72 h. Lhcgr mRNA levels mirror the LH-receptor protein levels and exhibit a comparable FSH/E2 regulation in rat granulosa cells. Further confirmation that estrogens are required to augment FSH-induced LH-receptor expression in granulosa cells comes from studies that have collectively shown the following: (1) only estrogens (e.g., E2, diethylstilbestrol) or substrates for aromatization (e.g., androstenedione, T, estrone) are effective, (2) nonaromatizable androgens (e.g., DHT) and P have little effect (Figure 25.20), (3) coculture with androgen receptors, or 17β-E2-specific antisera block FSH induction of LH receptor, and hCG-induced ovulation, and (4) Lhcgr levels are decreased five-fold in the ovaries of Cyp19-null mice. The poor ovulatory response of ERβ-null ovaries to an ovulatory dose of the LH analog, hCG, strongly suggests that ERβ mediates the synergistic actions of E2 during FSH induction of the Lhcgr gene in granulosa cells. In contrast, the granulosa cells of large antral follicles in Eρα-null ovaries exhibit abnormally high levels of LH receptor, even after becoming enlarged and cystic.

The complexity of the Lhcgr promoter is illustrated by the differential regulation between thecal and preovulatory granulosa cells. Not surprisingly, FSH induction of LH-receptor expression in granulosa cells occurs via the cAMP/PKA pathway. However, the proximal promoter of the Lhcgr gene in both humans and rats lacks a consensus cAMP response element (CRE) similar to those that confer FSH regulation of the Cyp19 promoter II. Studies indicate that a GC-rich region possessing a cluster of Sp1 binding sites and an ERE half site contributes to FSH and 8-bromo-cAMP induction of the rat Lhcgr promoter in vitro. Furthermore, multiple nonconsensus cAMP-response elements within this GC-rich region of the rat Lhcgr promoter provide a platform for assembling an undefined complex of nuclear proteins from granulosa cell extracts that confer cAMP induction. With such limited data concerning Lhcgr regulation, it is difficult to speculate where the actions of ERβ may impact LH responsiveness in granulosa cells. ERβ-null granulosa cells have reduced cAMP accumulation after FSH signaling and also show reduced Lhcgr accumulation compared to wild-type granulosa cells.
The reduced expression suggests it may be downstream of cAMP, and in vitro follicle culture studies found increased Lhcgr expression when follicles were treated with forskolin to induce cAMP accumulation.\textsuperscript{455}

As discussed before, E2 enhances the level and action of FSH-stimulated increases in intracellular cAMP to maximize Cyp19 expression, and it is likely that a similar mechanism is employed for induction of LH-receptor levels. However, there are notable differences in the E2/FSH regulation of Cyp19 versus Lhcgr expression. FSH induction of Lhcgr expression in preovulatory granulosa cells is augmented by estrogens only,\textsuperscript{515,548,549} whereas nonaromatizable androgens or estrogens act through their respective receptor pathways to enhance FSH induction of Cyp19 expression.\textsuperscript{442} This divergence in regulation is critical since androgen augmentation of FSH on Cyp19 expression allows for FSH/AR-mediated initiation of E2 synthesis in preantral follicles shortly after thecal cells begin synthesizing aromatizable precursors; whereas the specificity for ER/E2 actions in the FSH induction of the Lhcgr gene provides that LH receptors are acquired only by those follicles that have sufficient E2 synthesis, and are hence healthy and suitable for ovulation. Secondly, Farookhi and Desjardins\textsuperscript{549} demonstrated that FSH induction of Lhcgr but not Cyp19 expression is lost when estrogen-pretreated granulosa cells are dispersed in culture by EGTA-disruption of cell–cell contacts.\textsuperscript{549} A requirement for intact cell–cell contacts for E2/FSH-mediated Lhcgr expression may ensure that atretic follicles, which often exhibit a fragmented and less compact granulosa cell population, do not acquire the capacity to respond to an LH surge and ovulate an unhealthy oocyte.

FIGURE 25.20 Synergistic action of E2 and FSH in the induction of LH receptor in rodent granulosa cells. (A) Induction of Lhcgr (LH/CG-R) mRNA in granulosa cells of hypophysectomized rats after treatment with FSH and/or E2. Animals were untreated (H) or injected with 1.5 mg E2/day for 3 days (HE), 1 mg FSH twice daily for 2 days (HF), or a sequential combination of E then FSH (HEF). Animals were euthanized after treatment and granulosa cells isolated from the ovaries for total RNA extraction. Top: Northern blot of 20\,\mu\text{g} RNA/lane probed for Lhcgr (LH/CG-R) mRNA. (PO GC: pro-ovulatory granulosa cell RNA) (Source: Reproduced with permission from Ref. 550.) (B) Effect of antiestrogens on the induction of LH-receptor levels by FSH, forskolin, or 8-bromo-cAMP. Granulosa cells were isolated from immature rats implanted with DES pellets for 4 days. 2 \times 10^5 cells were cultured for 48h with FSH (100 ng), forskolin (10\,\mu M), or 8-bromo-cAMP (5\,nM), with or without E2, 10^{-8}\,\text{M} in the absence or presence of antiestrogens, keoxifene (K, 1\,\mu M), or tamoxifen (T, 1\,\mu M). Media were then removed and cells were analyzed for LH-receptor content by radiolabeled binding assay. The first bar on the left side of the figure is FSH (A), forskolin (B), or 8-bromo-cAMP (C) alone. Control cells bound less than 10 pg hCG/2 \times 10^5 cells, and the data are not shown. Source: Reproduced with permission from Ref. 514."

Like granulosa cells, follicular thecal cells also undergo a process of differentiation during progression from a preantral to preovulatory follicle.\textsuperscript{561} Fully differentiated thecal cells are best characterized by their unique capacity to convert cholesterol to C_{19} steroids, primarily androstenedione.\textsuperscript{561} A principal element of the two-cell, two-gonadotropin paradigm of steroidogenesis in maturing follicles (Figure 25.21) is that thecal cells exclusively possess the 17\beta-hydroxylase:C_{17,20}-lyase activity necessary for synthesizing aromatizable precursors; whereas the specificity for ER/E2 actions in the FSH induction of the Lhcgr gene provides that LH receptors are acquired only by those follicles that have sufficient E2 synthesis, and are hence healthy and suitable for ovulation. Secondly, Farookhi and Desjardins\textsuperscript{549} demonstrated that FSH induction of Lhcgr but not Cyp19 expression is lost when estrogen-pretreated granulosa cells are dispersed in culture by EGTA-disruption of cell–cell contacts.\textsuperscript{549} A requirement for intact cell–cell contacts for E2/FSH-mediated Lhcgr expression may ensure that atretic follicles, which often exhibit a fragmented and less compact granulosa cell population, do not acquire the capacity to respond to an LH surge and ovulate an unhealthy oocyte.

ESTRADIOL MEDIATED NEGATIVE FEEDBACK ON THECAL CELL FUNCTION

Like granulosa cells, follicular thecal cells also undergo a process of differentiation during progression from a preantral to preovulatory follicle.\textsuperscript{561} Fully differentiated thecal cells are best characterized by their unique capacity to convert cholesterol to C_{19} steroids, primarily androstenedione.\textsuperscript{561} A principal element of the two-cell, two-gonadotropin paradigm of steroidogenesis in maturing follicles (Figure 25.21) is that thecal cells exclusively possess the 17\beta-hydroxylase:C_{17,20}-lyase activity necessary for synthesizing aromatizable precursors; whereas the specificity for ER/E2 actions in the FSH induction of the Lhcgr gene provides that LH receptors are acquired only by those follicles that have sufficient E2 synthesis, and are hence healthy and suitable for ovulation. Secondly, Farookhi and Desjardins\textsuperscript{549} demonstrated that FSH induction of Lhcgr but not Cyp19 expression is lost when estrogen-pretreated granulosa cells are dispersed in culture by EGTA-disruption of cell–cell contacts.\textsuperscript{549} A requirement for intact cell–cell contacts for E2/FSH-mediated Lhcgr expression may ensure that atretic follicles, which often exhibit a fragmented and less compact granulosa cell population, do not acquire the capacity to respond to an LH surge and ovulate an unhealthy oocyte.
is effectively illustrated by the 12-fold reduction in Cyp17 expression in ovaries of LH-receptor (Lhcgr)-null mice, as well as the five-fold increase in expression in the ovaries of transgenic mice that possess chronically elevated LH levels. Likewise, chronically elevated plasma LH leads to a comparable increase in Cyp17 expression and androstenedione synthesis in both ERα-null and ERβ-null ovaries and cultured follicles, indicating that neither ER is obligatory to the positive regulation of thecal cell steroidogenesis.

Therefore, androgens are necessary to serve as both a stimulus of and substrate for aromatase activity in granulosa cells. However, a proper androgen:estrogen ratio is critical as an intrafollicular milieu of increased androgens is strongly associated with atresia. It is generally believed that an intraovarian mechanism exists to negatively modulate thecal cell androgen synthesis so that levels do not surpass the aromatase capacity of the granulosa cell layer. Indeed, defects in this intraovarian mechanism are postulated to be an underlying cause of the excess thecal cell androgen synthesis that is a hallmark of polycystic ovarian syndrome (PCOS) in women. There is ample evidence that granulosa cell-derived E2 may mediate a feedback loop on thecal cells to decrease further androgen synthesis in thecal cells however, the mechanism remains unclear. Estrogens have been shown to inhibit CYP17 activity in both thecal and Leydig cell preparations while having no effect on activities of the upstream steroidogenic enzymes or via direct inhibition of enzymatic activity. Indeed, estrogens can compete with androstenedione for CYP17 enzymatic activity; however, this effect could not be reproduced in ovarian lysates in vitro. Additional studies in dispersed rat ovarian cells found that E2 can compete with androstenedione for CYP17 enzymatic activity; however, this effect could not be reproduced in ovarian lysates in vitro.
activity that occurs just prior to ovulation in rats, and that E2 significantly reduces Cyp17 expression in the testes of rats and fish. Our findings that ERα-null ovaries exhibit increased Cyp17 expression and enzymatic activity despite a milieu of significantly elevated E2 also supports a mechanism of ERα-mediated suppression of Cyp17 transcription in theca cells versus direct inhibition of CYP17 activity. Furthermore, Taniguchi demonstrated that in vitro–cultured ERα-null follicles have increased Cyp17 expression similar to that observed in WT follicles cultured in the presence of an aromatase inhibitor. Treatment of ERα-null follicles with E2 or an ERα-specific agonist as well as an aromatase inhibitor reduced Cyp17 expression to that observed in wild-type vehicle treated follicles. Therefore, the excess androgen synthesis observed in ERα-null ovaries is due to the loss of ERα-mediated suppression of Cyp17 expression, indicating the existence of intraovarian feedback actions of E2 that are mediated by ERα and critical to maintaining homeostasis of androgen levels in the female mouse.

OVULATION

E2 clearly facilitates the acquisition of several gonadotropin-dependent effects during folliculogenesis, including: (1) the proper cellular organization of the follicle, e.g., antrum and cumulus oocyte complex; (2) the necessary enzymatic activity, e.g., E2 synthesis; and (3) the essential receptor signaling pathways; e.g., LH receptor. All of these characteristics define a healthy differentiated follicle and are necessary for a proper response to the gonadotropin surge and expulsion of a competent oocyte. However, a need for estrogen signaling in the ovulating follicle or following the postgonadotropin surge remains unclear. Selvaraj et al. found that coadministration of immature rats with PMSG and an aromatase inhibitor (Fadrozole) leads to a reduced number of healthy antral follicles that culminates with a severely reduced ovulatory response to a bolus of hCG. These data support the intrafollicular role of E2 in preparing competent preovulatory follicles for ovulation. However, in an additional experiment, Selvaraj et al. once again treated immature rats with PMSG but delayed administration of the aromatase inhibitor by 40 h such that exposure occurred just 6 h prior to hCG induced ovulation. These animals exhibit an equally poor response in terms of follicular rupture and recoverable oocytes from the oviduct; and this effect is abated by coadministration of E2 just prior to induced ovulation. These data in turn suggest that E2 does facilitate the ovulatory process. However, inhibition of E2 synthesis is known to cause severe intraovarian and intrafollicular accumulations of P and androgens that could also lead to a poor ovulatory response. In contrast to the above in vivo findings, Hu et al. reports that isolated mouse follicles grown exposed to an aromatase inhibitor in vitro exhibit a normal ovulatory response to hCG, including maturation of the cumulus–oocyte complex. Furthermore, detrimental effects of aromatase inhibition such as those reported in the rat ovary are not observed in similarly treated hamsters, rabbits, or monkeys.

Multiple investigations of the ovulatory capacity of ER-null and CYP19-null mice have been conducted. As described before, ERα-null females are anovulatory throughout life and gonadotropin-induced ovulation is unsuccessful in ERα-null females at 4 months of age when the ovarian cystic phenotype is advanced. However, immature (≤28 days of age) ERα-null (αERKO) females do respond to induced ovulation and yield recoverable oocytes in the oviduct at an average yield of 14.5 oocytes/ERα-null female versus 40.6 oocytes/wild-type female. Similar data has been reported in ERα-null females as old as 5 weeks of age. Interestingly, Dupont et al. report that immature females from their ERα-null (ERαKO) line exhibit a total failure to ovulate in response to exogenous gonadotropin treatments, although this study involved only three females. Preliminary studies suggest Ex3etERKO mice have a significantly reduced ovulation response, where less than 50% of mice ovulate in response to exogenous gonadotropins, with few oocytes produced from those that do ovulate (Korach laboratory, unpublished data). These observations suggest that the response observed previously was possibly due to the presence of residual ERα splice variant expressed in the αERKO mice, and thus further studies are underway to confirm these preliminary studies.

The cause of the reduced ovulatory response to exogenous gonadotropins in ERα-null females is unclear and suggests a facilitatory role for ERα in follicular rupture. However, the expected induction of several genes that are critical to follicle rupture, such as P receptor, prostaglandin-synthase-2, occurs in gonadotropin-primed ERα-null ovaries shortly after hCG treatment. Furthermore, ERα-null ovaries form functional corpora lutea following induced ovulation, indicating that granulosa cells of ERα-null preovulatory follicles possess the capacity to respond to LH. Therefore, the reduced ovulatory response in ERα-null ovaries is more likely attributable to premature increases in endogenous plasma LH and subsequent ovarian androgen synthesis. Although immature ERα-null ovaries do not yet manifest the overt morphological effects of LH-hyperstimulation, e.g., cystic follicles, they do exhibit elevated LH-receptor levels. Cumulative damage in the ovary from chronic LH hyperstimulation likely causes the age-related reduction in gonadotropin-induced ovulatory capacity in ERα-null females discussed previously. Indeed, Armstrong and colleagues demonstrated in rats that treatment with PMSG preparations possessing increased LH activity lead to a reduced ovulatory response to subsequent hCG stimulation and that this effect is likely due to inappropriate high stimulation of androgen synthesis during follicle maturation. Studies showing ERα-null follicles allowed to differentiate in vitro under controlled hormonal
stimulation exhibit a rate of hCG-induced rupture that is comparable to similarly cultured wild-type follicles further indicating that any role for ERα in the ovulatory process is minimal. The ERα was found to be necessary for ovulatory response in aging animals, as thEsr1KO mice had normal ovulatory response at 2 months that was severely dampened by the time the mice were 6 months old. The premature loss of fertility in these mice suggests that ERα is important for normal ovulatory function; however, the precise mechanism has yet to be elucidated.

The loss of functional ERβ clearly leads to ovarian defects that are inhibitory to ovulation. Both lines of ERβ-null females exhibit severely reduced fertility due to ovarian defects; however, unlike ERα-null females, ERβ-null animals do spontaneously ovulate. Crege et al. found that immature ERβ-null (βERKO) females exhibit a severely reduced oocyte yield of 6 oocytes/female versus 34 oocytes/female among age-matched wild-types following gonadotropin-induced ovulation. Dupont et al. report a similar average yield in immature ERβKO females (17.6 versus 37 oocytes/female), even when excluding those females that do not ovulate at all. A third line of ERβ-null mice was found to be unresponsive to exogenous gonadotropins, and no animals were able to ovulate (n = 19). Collectively, even though some animals are able to respond with some oocytes ovulated, the models demonstrate the necessity of ERβ for optimal ovulatory response.

Immature ERβ-null ovaries collected 20 h after hCG-induced ovulation reveal multiple preovulatory follicles that retain their oocyte and fail to undergo expansion of the cumulus–oocyte complex. Furthermore, hCG induction of prostaglandin-synthase 2 and PR, two events that are critical to follicular rupture, are significantly compromised in immature βERKO ovaries. ERβ-null follicles induced to ovulate in vitro exhibit an equally poor rate of rupture and gene induction following hCG exposure. The sum of in vivo and in vitro data from ERβ-null mice strongly indicates an inability to fully respond to an endogenous LH surge or an exogenous bolus of hCG. This is likely due to insufficient acquisition of LH-receptor expression among granulosa cells of ERβ-null preovulatory follicles. Indeed, Clemens et al. found that the ER antagonist ICI 164,384 inhibits FSH-induced differentiation of rat granulosa cells in vitro, as illustrated by a severely compromised induction of PR expression following stimulation of the cAMP/ PKA signaling pathway. However, if exposure to the ER antagonist is delayed until the time of ovulatory stimulation, little effect is observed, further supporting a role for ERβ in granulosa cell differentiation and LH-receptor expression rather than during ovulation. ERβ-null follicles cultured in vitro also have a reduced ovulatory response and reduced cAMP accumulations after FSH stimulation. Interestingly, treatment of these follicles with forskolin to increase cAMP levels was able to increase the ovulation of these follicles in vitro, suggesting that ERβ is necessary for maximal ovulatory response downstream of gonadotropins, although ERβ is not directly contributing to follicle rupture.

Evidence of any cooperative action between ERα and ERβ in ovulation may be derived from studies of the compound ER-null and CYP19-null mice. The single report of induced ovulation in immature ERα/ERβ-null (ERαβKO) females describes a total failure of ovulation. Not surprisingly, ovaries from these females exhibit underdeveloped preovulatory follicles that failed to exhibit cumulus–oocyte complex expansion or luteinization, indicating an attenuated response to hCG similar to that in ERβ-null ovaries. Investigations in both lines of Cyp19-null females produced comparable results of a total failure to ovulate and luteinize. Interestingly, Huynh et al. remarked that cumulus–oocyte complexes lacking oocytes were recovered from the oviducts of 4-week-old Cyp19-null females following induced ovulation, indicating that some elements of follicle rupture may occur after degradation of the oocyte. The more severe ovulatory phenotypes in compound ER-null and Cyp19-null females suggest that the intraovarian functions of both ERα and ERβ are necessary for ovulation but not necessarily for the process of follicular rupture. Recent studies in Cyp19-null mice suggest that treatment with E2 as well as exogenous gonadotropins could stimulate some ovulation, suggesting the importance of the steroid and gonadotropin hormones in regulating ovulation.

ERα-MEDIATED REPRESSION OF LEYDIG CELL DEVELOPMENT IN THE OVARY

As discussed in earlier sections, female mice lacking ERα action due either to the loss of receptor (αERKO, thEsr1KO, and αβERKO) or ligand (Cyp19-null) exhibit abnormally high levels of plasma T. For example, the average plasma T level in adult αERKO and αβERKO females is 5.5 ng/ml and 2.1 ng/ml, respectively, versus 0.14 ng/ml in wild-type females. In females of the two existing Cyp19-null lines, Fisher et al. report average plasma T levels in mutant females to be 2.3 ng/ml versus 0.24 ng/ml in wild-type, while Toda et al. report 1.4 ng/ml in the mutant females versus 0.13 ng/ml in wild-type. These levels of plasma T in ERα-null and Cyp19-null females surpass the lower limits characteristic of normal male mice (2–10 ng/ml) and are obviously very uncharacteristic of normal females. The mouse ovary possesses the enzymatic machinery necessary for T synthesis, although it is not a major product. The two-cell, two-gonadotropin paradigm of ovarian steroidogenesis postulates that thecal cell–derived androstenedione serves as a substrate for two sequential reactions in granulosa cells, CYP19-mediated aromatization to estrone followed by a reduction to E2 by 17β-hydroxysteroid dehydrogenase type I.
The murine form of 17β-HSD 1 is reported to efficiently reduce androstenedione to T as well as estrone to E2; whereas the human form may not possess this activity. Therefore, the accumulation of androstenedione that occurs in ERα-null and Cyp19-null ovaries due to LH hyperstimulation of the theca in both, and the absence of aromatization in the latter provides for sufficient substrate for 17β-HSD 1 conversion to T. However, further investigations have discovered that ERα-null and Cyp19-null ovaries uniquely possess substantial levels of 17β-hydroxysteroid dehydrogenase type III (Hsd17b3), a Leydig cell–specific enzyme in testes that specifically functions to reduce androstenedione to T. Remarkably, the level of Hsd17b3 expression in αERKO, αβERKO, and Cyp19-null ovaries is comparable to that found in testes of wild-type males. Therefore, the abnormally high capacity of ERα-null and Cyp19-null ovaries to synthesize T may be attributed to the accumulation of androstenedione and its conversion to T by 17β-HSD 3 rather than 17β-HSD 1. Hsd17b3 expression in Leydig cells is primarily LH regulated. The ectopic expression in ERα-null ovaries is also dependent on LH hyperstimulation as expression is eradicaded following prolonged treatment of animals with a GnRH antagonist. In ERα-null females, Hsd17b3 expression is limited to the ovarian interstitium and possibly the theca. However, it is unclear whether these cells are Leydig-like and suggestive of an “organizational” defect in ERα-null ovaries or are thecal/interstitial type cells within which the loss of ERα leads to loss of Hsd17b3 expression, i.e., an “activational” defect. Evidence to support the latter hypothesis includes: (1) testes from ERα-null males also exhibit elevated Hsd17b3 expression and T synthesis; (2) female mice possessing chronically elevated LH but intact ERα signaling do not exhibit ovarian Hsd17b3 expression; and (3) prolonged E2 therapy in Cyp19-null females eradicates Hsd17b3 expression. In turn, there exists supporting evidence for the former hypothesis that a defect during ovarian differentiation may occur in the absence of ERα. Thecal and Leydig cells are believed to derive from a common bi-potent embryological precursor cell during gonadal differentiation. In Cyp19-null ovaries, Britt et al. have described the presence of cells resembling the mature Leydig cells of testes, in that they possess a tubulovesicular arrangement of mitochondrial cristae, an annular nucleolus, and an abundance of smooth endoplasmic reticulum in whorl-like formations. These Leydig cell–like ultrastructural features were also observed in ERα-null ovaries. These Leydig-like cells in Cyp19-null ovaries are located in the interstitial regions and often proximal to the basement membrane of atretic follicles that exhibit Sertoli-like cells in the follicle lumen. Indeed, E2 is a potent inhibitor of Leydig cell proliferation in vitro and in vivo, and E2 replacement in Cyp19-null females reduces the presence of Leydig-like cells in the ovaries. Therefore, ERα may be critical to promoting thecal cell differentiation and inhibiting the Leydig cell phenotype during ovarian differentiation. A similar phenotype of Leydig cell differentiation, Hsd17b3 expression, and “masculinization” of the ovary and reproductive tract is reported in female mice lacking Wnt4 function.

**DISRUPTED ESTROGEN SIGNALING IN HUMANS**

There is one case of a human female that is autosomal recessive for an inactivating mutation of the ESR1 (ERα) but no patients with ESR2 (ERβ) mutation reported. There are cases of human females that are autosomal recessive for inactivating mutations of the CYP19 gene and therefore lack the ability to synthesize E2. The CYP19 mutated females present with androgen-induced pseudohermaphroditism at birth, and amenarche, polycystic ovaries, and an absence of female secondary sex characteristics at puberty. Three reports describe elevated plasma FSH and LH levels, leading to hypergonadism that manifests as increased plasma androgens and virilization. The female with Esr1 mutation has polycystic ovaries and elevated E2. The ovarian pathology exhibited is somewhat similar to that observed in ERα-null females and compatible with a diagnosis of PCOS. To date there are no indications of “sex reversal” in terms of male cell types or gene expression in the ovaries of CYP19-null human females. Estrogen and P replacement effectively alleviates all of the above phenotypes.

Polymorphisms of the human ESR1 gene have been linked to breast cancer, cardiovascular disease, osteoporosis cognitive function, and multiple reproductive anomalies. A polymorphic (TA)n repeat within the ESR1 promoter region has been linked to premature ovarian failure but has no effect on plasma levels of E2, sex-hormone binding globulin (SHBG), T, or FSH in premenopausal women during the follicular phase. Two PvuII single nucleotide polymorphisms (SNP) in the ERS1 gene have been described to date. The first of these is a glutamine-to-glycine transformation at amino acid 400 and has received little investigative attention. The second is an anonymous SNP located in intron 1, 400 bp upstream of exon 2, and is found in approximately 30% of women. Although the intronic ESR1 PvuII SNP does not affect ERα receptor levels it may be associated with: (1) poor performance in women undergoing in vitro fertilization; (2) increased plasma E2 and androstenedione levels; and (3) late onset of menarche and/or menopause.

Two SNP of the ESR2 gene have been described to date, an Rsal SNP leads to a silent nucleotide change in exon 5, and an AluI SNP occurs in the 3-untranslated region of exon 8. Two SNP of the ESR2 gene are more
prone to exhibit ovulatory defects, menstrual disorders, and elevated LH; while women that are homozygous for both exhibit more severe idiopathic ovulatory defects.616

**Progesterone Receptor Signaling in Ovarian Function**

**PR Expression in the Ovary**

The first report of saturable, high-affinity P binding sites in the rat ovary is that of Schreiber and colleagues in 1979617,618 and has since been followed by similar descriptions in human,389–391,619 and cow619,620 ovaries. Later immunohistochemical studies primarily localized ovarian PR expression to the theca of large preovulatory follicles, the surface epithelia, and stromal/interstitium, and found this expression pattern is well conserved among species, including mice,621,622 pig623 monkeys,386,624 and humans.392,625,626 Numerous studies in species ranging from rodents to large domestic animals and primates agree that granulosa cells possess minimal PR expression throughout folliculogenesis except for the period 2–4 h after the preovulatory gonadotropin surge, whereupon an enormous induction of PR expression occurs in preovulatory granulosa cells and peaks just prior to follicle rupture.386,392,415,621–624,627–630 (Figure 25.22). This dramatic increase in PR expression in the granulosa cells of ovulatory follicles is absolutely essential to follicle rupture.182,631 This induction of PR expression in ovulating follicles is quite transient and lasts less than 12 h in rodent ovaries415,621,628 but is maintained throughout luteinization and corpus luteum formation in porcine623 and primate386,392,624 ovaries.

The regulation of ovarian PR expression prior to the preovulatory surge is poorly characterized. PMSG treatment of immature rats leads to a four-fold increase in cytochrome P binding sites in immature rats leads to a four-fold increase in cytochrome P binding sites (Figure 25.22). This dramatic increase in PR expression in the granulosa cells of ovulatory follicles is absolutely essential to follicle rupture.182,631 This induction of PR expression in ovulating follicles is quite transient and lasts less than 12 h in rodent ovaries415,621,628 but is maintained throughout luteinization and corpus luteum formation in porcine623 and primate386,392,624 ovaries.

The regulation of ovarian PR expression prior to the preovulatory surge is poorly characterized. PMSG treatment of immature rats leads to a four-fold increase in cytosolic P binding sites632 and a modest two-fold increase in Pgr mRNA levels628 after 48 h, indicating that FSH or subsequent E2 signaling may stimulate these basal levels of PR expression. In contrast, the mechanisms underlying the dramatic induction of PR expression in preovulatory granulosa following the gonadotropin surge are relatively well characterized. Over the course of the estrous cycle in rats, Pgr mRNA levels are increased 30-fold during a 4 h period (1800–2200 h) on the evening of proestrus and then return to baseline shortly after (2400 h).628 The increased expression is almost exclusive to the mural granulosa cells of healthy preovulatory follicles in rats,628,633 indicating that only fully differentiated granulosa cells possess the capacity for such rapid induction of the Pgr gene (Figure 25.22). Although PR expression climaxes within hours after plasma E2 and LH levels climax, the following evidence indicates LH is most directly involved: (1) expression peaks just 2 h after plasma LH levels climax versus >8 h after peak plasma E2 levels,628 (2) hCG treatment 48 h after PMSG exposure leads to a comparable increase in PR expression, whereas PMSG and concomitant increases in E2 have a minimal effect,415,621,628 (3) treatment of hypophysectomized or immature rats, or differentiated rat granulosa cells with E2 has no marked effect on ovarian PR expression,422,634 (4) inhibition of the LH surge by pentobarbital abates the increase in PR expression despite having no effect on the rise in plasma E2 levels,628 (5) LH but not E2 elicits a dramatic PR induction in differentiated granulosa cells from rat634,635 or porcine629 ovaries in vitro, (6) an ER antagonist (ICI 164,384) does not inhibit forskolin-induced PR expression in differentiated rat granulosa cells,422 (7) the putative ERE-like region of the rat Pgr promoter does not bind ERα or ERβ in ovarian extracts,579 and (8) ER (mainly ERβ) levels are rapidly decreasing at the time of rising PR expression in preovulatory granulosa cells.

The above data provide a convincing argument that the rapid and transient induction of PR expression in preovulatory granulosa cells is directed by the LH surge. Clearly only granulosa cells of preovulatory follicles in vivo or fully differentiated granulosa cells in vitro possess the capacity to respond to the LH surge and increase PR expression, presumably due to their unique acquisition of LH receptor.579,634 Indeed, granulosa cells isolated from estrogen-primed immature rats require a 48-h exposure to physiological concentrations of FSH and T to acquire LH induction of PR expression.579 LH-mediated induction of the intracellular cAMP/protein kinase-A pathway is likely the principal stimulus for increased PR expression in preovulatory granulosa cells as large doses of FSH, forskolin, or (Bu)2cAMP also elicit a comparable response in rat ovaries in vivo634 and differentiated rat634,635 or porcine629 granulosa cells in vitro. However, GnRH, PMA, or EGF exposure of differentiated rat granulosa cells in vitro elicits a comparable induction and temporal pattern of PR expression, suggesting that stimulation of protein kinase C or tyrosine kinase signaling cascades may also be involved.635 In fact, Sriraman et al. report that forskolin and PMA synergize to induce increased PR expression in undifferentiated

**FIGURE 25.22 Regulation of PR expression in mouse ovaries and granulosa cells during gonadotropin-induced ovulation.** (A, B) Immunohistochemistry for PR in the ovary of a PMSG-primed mouse 4 h after hCG treatment, illustrating the dramatic induction of PR immunoreactivity in the granulosa cells of preovulatory follicles (PoF) but not surrounding preantral follicles. (B, higher magnification of A.)
Hormone Levels

Ovarian Phenotypes

PRA: PRAKO)

PRA and PRB: PRKO)

The mechanisms by which LH activation of the PKA pathway leads to increased PR expression in preovulatory granulosa cells remain unclear. The Pgr promoter is complex, consisting of distal and proximal regions and able to give rise to multiple transcripts. Initial studies concluded that an estrogen-response element-like region (ERE3) within the proximal rat Pgr promoter is important to LH induction, but later studies that better modeled the structure of the rat Pgr promoter indicated these sequences are dispensable. Sriraman et al. demonstrated that two Sp1/Sp3 binding sites within the proximal promoter of the mouse Pgr gene bind Sp1/Sp3 and are essential to forskolin/PMA-induced expression. Furthermore, LH induction of Pgr also involves MAPK activation as mice lacking ERK1/2 in granulosa cells do not induce expression of Pgr in response to an hCG ovulatory signal. While these mice show reduced expression, the direct targets of ERK1/2 remain unknown at this time. Recent evidence in porcine granulosa suggests that androgens may regulate the direct targets of ERK1/2.

Differential expression of PRA and PRB among thecal and granulosa cells throughout the stages of folliculogenesis and shows less variation over the course of the estrous cycle. The enormous induction of PR expression that occurs in preovulatory granulosa cells on the evening of proestrus is primarily representative of PRA, resulting in a 2:1 ratio of PRA:PRB in these cells. In turn, the more moderate LH-induced increases in PR expression in thecal cells is predominantly PRB. During the metestrous stage and rising P synthesis that follow ovulation, PRB is the sole isoform in thecal cells of preantral and antral follicles. The differential expression of PRA and PRB suggests that specific roles exist for each isoform.

Ovarian Phenotypes in Mouse Models of Disrupted Progesterone Signaling

Several mouse lines have been developed that are null for both PR forms and exhibit grossly normal ovaries but are anovulatory and therefore infertile (Table 25.8). Furthermore, gonadotropin-induced ovulation of PR-null females is unable to rescue the anovulatory phenotype as no oocytes could be recovered following treatment.

<table>
<thead>
<tr>
<th>Mutated or Null for Sex Steroid Signaling</th>
<th>Ovarian Phenotypes</th>
<th>Hormone Levels</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgr−/− (homozygous null alleles for PRA and PRB: PRKO)</td>
<td>Anovulatory and infertile</td>
<td>Elevated LH</td>
<td>176–178</td>
</tr>
<tr>
<td></td>
<td>Failure to respond to superovulation—large follicles present in ovary with trapped oocytes</td>
<td>Normal FSH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>No follicle rupture</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No CL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRA−/− (homozygous null alleles for PRA: PRAKO)</td>
<td>Anovulatory and infertile</td>
<td>Not reported</td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>Reduced to no response to superovulation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Large follicles present with trapped oocytes similar to PRKO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>No follicle rupture</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRB−/− (homozygous null alleles for PRB: PRBK0)</td>
<td>Fertile</td>
<td>Not reported</td>
<td>182,227</td>
</tr>
<tr>
<td></td>
<td>Ovaries appear normal</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Respond to superovulation similar to wild-type</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Instead, PR-null ovaries following induced ovulation exhibit multiple large, preovulatory follicles, each possessing a healthy, competent oocyte but a total failure to rupture176,178 (Figure 25.23). The follicular cells of the unruptured preovulatory follicles undergo luteinization and form functional corpora lutea within the expected time frame, suggesting that some elements of an LH response are intact176,178,182,633 (Figure 25.23). Similar experiments in isoform-specific PR-null females indicate a more severe phenotype following the loss of PRA relative to PRB, as females lacking PRA ovulate an average of 9 oocytes/female versus 32 oocytes/female among wild-type littermates; whereas PRB females exhibit no deficits in oocyte yield.180,182,227 This finding that the PRA isoform is more critical to ovulation180 is congruent with the above studies of Gava et al. that demonstrate this isoform is much more dramatically induced by the LH surge in preovulatory granulosa cells. This also supports the finding that PRA in the presence of ligand regulates more genes in granulosa cells in vitro compared to cells expressing PRB.639 Still, the absolute failure of ovulation even after gonadotropin induction in females lacking both PR forms indicates a level of cooperation between PRA and PRB that is vital to follicular rupture.180 Recently, a mouse model was developed to use for tissue-specific deletion of PR,177 however to date only global knockout models have been published that show a similar phenotype as previously reported.176,178

**Intraovarian Role of Progesterone in Ovarian Function**

**FOLLICLE GROWTH AND DIFFERENTIATION**

Although immunohistochemical studies indicate that both PR isoforms are expressed at basal levels throughout theca176 and granulosa cells,622 there is little evidence that P action profoundly influences follicle growth and differentiation in the rodent ovary. Administration of P or synthetic progestins to immature or hypophysectomized rats has no obvious effect in the ovary in terms of estrogen receptor levels338,422 or gonadotropin/estrogen-induced follicle growth and maturation.338,640 In addition, P has no measurable influence on FSH-induced granulosa cell proliferation186; or growth and differentiation of whole follicles497 in vitro. Perhaps most indicative of a minor role for P signaling during folliculogenesis prior to ovulation is the lack of any overt phenotype in follicle growth, maturation, or steroidogenesis in the ovaries of PR-null mice (Table 25.8).176–178,641 Indeed, Robker et al. demonstrated that the expected FSH- or PMSG-induced increases in Cyp19 and Lhcgr expression in mature follicles occurs in PR-null ovaries.633

**OVULATION**

P expression in the ovary is relatively low throughout folliculogenesis except during the 4–6 h period immediately after the ovulatory gonadotropin surge, during which an enormous induction of P expression occurs in granulosa cells of ovulating follicles628,634,635 and is synchronized with an equally acute increase in ovarian P synthesis. These phenomena alone are indicative of an important role for PR-mediated P signaling during ovulation. Experimental evidence of a critical role for P in ovulation comes from studies in which ovulation is blocked when peri-ovulatory ovaries or follicles are exposed to: (1) anti-P antisera,642 (2) inhibitors of P synthesis,643–645 or (3) PR antagonists.621,646,647 Finally, the
25. STEROID RECEPTORS IN THE UTERUS AND OVARY

1164

4. FEMALE REPRODUCTIVE SYSTEM

anovulatory phenotype of PR-null mice discussed before provides definitive evidence that PR actions are required in the mammalian ovary during the period just prior to ovulation.\(^{176-178,180}\) PR-null females exhibit multiple large, preovulatory but unruptured follicles following induced ovulation.\(^ {176}\) Therefore, follicle growth and differentiation are unaffected by the loss of PR functions as PR-null follicles exhibit fully formed antra and increased Cyp19 and Lhcgr expression following FSH or PMSG treatment.\(^ {176,633}\) Furthermore, although follicle rupture is impaired, PR-null ovaries exhibit several indications that other LH- or hCG-induced responses are intact, including: (1) rapidly decreased Cyp19 expression,\(^ {633}\) (2) dramatic induction of Ptgs2 expression,\(^ {633}\) (3) expansion of the cumulus–oocyte complex,\(^ {176}\) and (4) luteinization and formation of functional corpora lutea.\(^ {182,633}\) These findings in PR-null females are congruent with earlier studies using P synthesis inhibitors\(^ {645,648}\) or PR antagonists,\(^ {646}\) which also report that ovulatory levels of prostaglandins and steroids are largely unaffected by inhibition of P signaling.

The previous data clearly indicate that PR actions are highly specific and critical for follicle rupture. Studies have discovered that PR-null ovaries fail to exhibit LH-induced expression of several proteases, including ADAMTS-1, ADAM8, and cathepsin-L, which are postulated to be involved in degradation of the follicle wall and extracellular matrix that is necessary for oocyte extrusion (Figure 25.24). ADAMTS-1 (Adams1) and ADAM8 are members of the A disintegrin and metalloproteinase family of proteases and are dramatically increased in granulosa cells of preovulatory follicles 12 h after hCG treatment, after peak PR expression and coinciding with a period of follicular rupture.\(^ {644,649}\) A similar pattern of peri-ovulatory ADAMTS-1 expression is documented in preovulatory follicles of porcine,\(^ {650}\) equine,\(^ {630}\) and primate\(^ {651}\) ovaries. Cathepsin-L (Ctsl) is also significantly increased following hCG exposure in granulosa cells of preovulatory follicles and also peaks 12 h after treatment.\(^ {633,652}\) PR-null females,\(^ {633}\) as well as wild-type female rats exposed to a P synthesis inhibitor near the time of hCG treatment\(^ {644,653}\) fail to exhibit hCG-stimulated increases in Adams-1 expression, indicating this effect of LH is primarily dependent on PR-mediated P action. ADAMTS-1-null female mice exhibit a severely compromised ovulation and a phenotype of large, preovulatory but unruptured, follicles following gonadotropin-induced ovulation.\(^ {654,655}\)

Interestingly, PRA-null mice exhibit a severe ovulatory phenotype similar to total PR-null females but possess normal LH-stimulated induction of Adams-1 and Ctsl during induced ovulation,\(^ {182}\) suggesting possible compensatory actions by PRB but also questioning the importance of ADAMTS-1 and cathepsin-L to follicle rupture. ADAM8 was recently shown to be regulated specifically by PRA in reporter assays, while PRB was unable to regulate the promoter of ADAM8 in vitro.\(^ {649}\) This could explain why PRA-null mice show LH-stimulated induction of Adams-1 and Ctsl yet are unable to ovulate, although further studies are needed to confirm this.

Several other genes have been identified downstream of PR and suggested to be important in follicle rupture and ultimately ovulation. Since ovulation is discussed more fully in another chapter (Chapter 22) of this volume, we will not provide in-depth description, although

FIGURE 25.24 Ovarian expression of ADAMTS-1 in PR-null ovaries during gonadotropin-induced ovulation. Immature heterozygous (PR+/-) and homozygous PR-null (PRKO) mice were left untreated or injected with PMSG and euthanized 46 h later, or were treated with PMSG for 46 h followed by hCG and euthanized 7, 12, and 24 h later. Reverse transcriptase polymerase chain reaction analysis of total RNA from whole ovaries indicates that ADAMTS-1 expression is dramatically increased by hCG in PMSG-primed mice, but this induction is lacking in PR-null ovaries, indicating dependence on PR function. ADAMTS-1 expression was normalized to expression of ribosomal protein L19. Source: Reproduced with permission from Ref. 633.
we will briefly describe what is known to date. These include SNAP25, synaptosomal-associated protein 25, an LH-regulated gene that has decreased expression in PR-null mice but not Pigs2 mice.656 Furthermore, Snap25 was PR regulated in in vitro reporter assays, and the PR antagonist RU486 was able to inhibit this regulation. SNAP25 acts to alter cytokine and chemokine secretion in granulosa cells, which may act to facilitate follicle rupture.656 Endothelin 2 (Edn2) was identified to be an LH-regulated gene with undetectable levels in PR-null mice that plays a role in follicle rupture.657,658 Recent studies have shown that Edn2 is regulated by PRA,639 and studies in rats have shown that it acts to stimulate smooth muscle contraction and follicle constriction.659 EDN2 provides the mechanical actions that help to facilitate follicle rupture and successful ovulation. This is supported by its regulation specifically by PRA, which is essential for ovulation, whereas loss of PRB doesn’t reduce ovulatory response as described previously.

Numerous transcription factors have also been identified as PR targets, and knockout mouse models have demonstrated that these factors are necessary for ovulation in mice. Hypoxia inducible factor 1 (Hif1) is induced by FSH in rat granulosa cells cultured in vitro,660,661 and have also been found to be regulated by PR.662 Furthermore, the expression of several HIF family members, including Hif1a, Hif2a, and Hif1b, are reduced in PR-null ovaries,662,663 suggesting an important role in follicle rupture. To further explore this possibility, pharmacological inhibitor of HIF activity was shown to block ovulation and inhibit follicle rupture,662 demonstrating the importance of HIF activity in regulating ovulation. Another nuclear receptor, PPARG, is also necessary for follicle rupture as demonstrated by targeted deletion in granulosa cells in mice that had no response to exogenous gonadotropins.664 The action of PPARG was found to be mediated by several PR target genes, including Il6 and Edn2,664 demonstrating that several targets are regulated downstream of PR and LH signaling to coordinate follicle rupture and ovulation.

Androgen Receptor Signaling in Ovarian Function

**AR Expression in the Ovary**

AR expression has been documented in the ovaries of multiple species, including mouse,373,450 rat,130,665–668 pig,669,670 sheep,671 cow,672 monkey,274,395,673–675 and human.395,625,676–679 The ovarian pattern of AR expression is well conserved among species, with levels being detectable throughout the stages of folliculogenesis except primordial follicles, with the highest expression in the granulosa cells of small preantral follicles, detectable expression in thecal/interstitial cells, and little to no expression in luteal cells (Table 25.6).

In the ovaries of multiple species, Ar/AR expression among growing follicles is inversely correlated with the extent of granulosa cell differentiation (Figure 25.25). A mechanism to decrease AR levels during follicle maturation is consistent with the need to reduce sensitivity to intrafollicular androgens, which rise to levels sufficient for aromatization to E2, as activating AR ligands are detrimental to the health of preovulatory follicles.680 In the ovaries of gonadotropin-stimulated rats, Testsuka and Hillier found that large antral follicles (>400 μm) exhibit a 2.75-fold higher level of Cyp19 expression relative to small follicles (<200 μm) but a 51% decrease in Ar expression.665 In rat ovaries, Ar expression is first apparent in early postnatal development in follicles of the preantral stage.681 In adult ovaries, decreasing AR immunoreactivity occurs during follicle differentiation; a decrease is first apparent in mural granulosa cells and then progresses in those cells closest to the antrum.682 Interestingly, cells composing the cumulus–oocyte complex of preovulatory follicles are believed to be the last to differentiate682,683 and maintain Ar expression throughout.667

This gradient of AR expression suggests that AR signaling correlates with the differentiation state of granulosa cells and follicle development.

In primate and human ovaries, AR expression is also highest in the granulosa cells of small growing follicles, yet evidence of an inverse correlation with follicle differentiation is conflicting.625,674,675,678,684 Hillier et al. found AR immunoreactivity in the marmoset ovary is most abundant in granulosa cells of healthy pre- to small antral follicles and low or absent in preovulatory follicles of late follicular stage.674 In contrast, minimal differences in granulosa cell AR expression between preantral and large antral follicles are reported in rhesus monkey ovaries.675 Furthermore, healthy follicles at late stages of maturation in human ovaries are described to possess significant AR immunoreactivity.625,678,679 A recent report has suggested that AR expression is highest in small antral follicles and decreases as the follicle matures,685 suggesting that message levels may not correspond with AR protein levels in human follicles.

The regulatory factors for AR expression in the ovary are poorly understood. Small preantral follicles in rats maintain high AR levels after hypophysectomy indicating that gonadotropins are not required to induce AR expression.668 In fact, most evidence indicates that FSH or PMSG-induced differentiation of granulosa cells is the primary cause of reduced AR expression; Campo et al. demonstrated that PMSG treatment of rats leads to the replacement of high-affinity, low-capacity androgen binding sites by nonsaturable, low-affinity binding sites in the ovary.686 Similarly, immature rats treated with recombinant FSH over 48 h exhibit a 65% reduction in ovarian Ar mRNA levels and a further decrease when LH is included.668 However, neither FSH nor (BR)-cAMP
affect Ar mRNA levels in cultured rat granulosa cells in vitro. This suggests that a paracrine interaction with theca and/or stromal cells is necessary for reduced Ar expression observed in granulosa cells. Interestingly, DHT also elicits a 20% reduction in Ar expression in rat granulosa cells in vitro that is prevented by co-treatment with FSH. In contrast, T reportedly has little effect on AR expression in rhesus monkey ovaries. There is increasing evidence that E2 may play a role in decreasing granulosa cell AR levels during follicle differentiation. Like DHT, E2 exposure of rat granulosa cells also leads to a 20% reduction in Ar transcripts, but this effect is unabated by FSH. Furthermore, granulosa cells retrieved from untreated hypophysectomized rats respond well to DHT plus FSH and exhibit increased Cyp19 expression accordingly; but the effect of DHT is

FIGURE 25.25 Relationship between expression of androgen receptor and aromatase (CYP19) during folliculogenesis. (A) Expression of androgen receptor (Ar) and aromatase (Cyp19) expression in granulosa cells of small (~200 μm), medium (200–400 μm), and large (>400 μm) follicles from immature rats after treatment with PMSG. Expression was quantified by RNase-protection assay and normalized to 18S rRNA (not shown); data are expressed as percentage of control values (SEM of three separate trials, each consisting of 8–10 animals). a, b: P <0.05; a, c: P <0.01 (by ANOVA with the Newman–Keuls test). (Source: Reproduced with permission from Ref. 491.) (B) Hypothetical model of androgen utilization during folliculogenesis. As follicular development progresses, thecal androgen production gradually increases. During the early stages of follicular differentiation, androgens act via androgen receptor (AR) to enhance FSH-induced differentiation, including the stimulation of Cyp19 expression. During the final stages of follicular development, androgens primarily serve as a substrate for CYP19-mediated E2 synthesis under stimulation by FSH and LH. This differential regulation of AR and CYP19 may be important in shifting androgen utilization from action to metabolism, thereby ensuring a healthy transition of a follicle from the early maturation to full maturation stage. Source: Reproduced with permission from Ref. 683.
lost in granulosa cells isolated from E2-primed hypophysectomized rats, suggesting that estrogen pretreatment decreases AR expression. Indeed, mature follicles in ERβ-null ovaries are reported to possess aberrantly high AR expression relative to similarly staged follicles in wild-type ovaries, supporting the idea that ER may act to decrease Ar expression. While the hormonal regulation of Ar expression is unclear, recent reports suggest that a member of the orphan nuclear receptor family, Nur77, is required for maximal Ar expression in murine ovaries. Nur77 regulates several steroidogenic enzymes in the ovary, including those involved in androgen synthesis, and recent reports demonstrate 20% reduction in Ar expression in the granulosa cells from Nur77-null mice. Dia and colleagues were able to show that NUR77 bound directly to the promoter regulatory region of the Ar gene in mouse granulosa cells, providing insight into one of the transcriptional factors necessary for maximal Ar expression in the ovary.

**Ovarian Phenotypes in Mouse Models of Disrupted Androgen Signaling**

**MICE LACKING AR**

*Tfm* mice were first described in 1970 and are a naturally existing androgen-resistant mutant due to an inactivating mutation of the *Ar* gene. With the advent of gene targeting techniques, comparable inactivation mutations of the *Ar* gene and resulting phenotypes have been described in rats and humans. *Tfm/*Tfm female mice are fertile but exhibit a noticeably shortened reproductive life span that becomes apparent after approximately four litters compared to control (Tfm/X or X/X) females; however, individual litter sizes were not different. Histological evaluation of ovaries from the *Tfm/*Tfm breeding females indicated a sparse number of healthy follicles and a hypertrophied interstitium, both illustrative of premature ovarian failure (POF). The difficulties in generating *Tfm/*Tfm female mice limited more extensive studies.

Several global AR-null mouse models have been generated via a Cre/loxP targeting scheme that allows for tissue and temporal specific disruption of the *Ar* gene and therefore the generation of fertile male carriers of the targeted *Ar* allele (Table 25.9). In one study, global AR-null females made through deletion of exon 1 also causes a POF phenotype where the number of offspring decreases over time comitant with a decrease in the number of healthy follicles, suggesting defects in folliculogenesis. By 32 weeks of age, 40% of knockout females are infertile, similar to that observed in *Tfm/*Tfm females and the exon 2–deleted AR-null mice, and at 40 weeks of age all of the mice are infertile. Knockout of AR in exon 1 also causes a POF phenotype whereby the number of offspring decreases over time concomitant with a decrease in the number of healthy follicles, suggesting defects in folliculogenesis. By 32 weeks of age, 40% of knockout females are infertile, similar to that observed in *Tfm/*Tfm females and the exon 2–deleted AR-null mice, and at 40 weeks of age all of the mice are infertile. Several genes implicated in oocyte-granulosa cell communication were found to be increased, including kit ligand (*Kitl*). Kitl is a downstream target of AR as demonstrated by induction of *Kitl* expression following DHT treatment, while cotreatment with DHT and the antiandrogen flutamide attenuated the increased *Kitl* expression. This report suggests that AR signaling has
### TABLE 25.9  Ovarian Phenotypes in AR-Mutant or Null Mouse Models

<table>
<thead>
<tr>
<th>Mutated or Null for Sex Steroid Signaling</th>
<th>Ovarian Phenotypes</th>
<th>Hormone Levels</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tfm/Tim mice</td>
<td>Fertile but have a reduced number of pups/litter as they age Shortened reproductive life span</td>
<td>Not reported</td>
<td>283</td>
</tr>
<tr>
<td>AR−/− (exon 2)</td>
<td>Reduced fertility and shortened reproductive life span Adults exhibit grossly normal ovaries with reduced number of CL Abnormal estrous cycle Immature females have reduced response to gonadotropin-induced ovulation</td>
<td>Reduced P Other hormones not reported</td>
<td>183,290</td>
</tr>
<tr>
<td>AR−/− (exon 1)</td>
<td>Reduced fertility and POF phenotype where animals have a shortened reproductive life span Increased number of atretic follicles and reduced number of CL Altered ovarian gene expression in immature and adult animals</td>
<td>Normal serum E2, P, T, LH, and FSH</td>
<td>690</td>
</tr>
<tr>
<td>AR−/− (inframe deletion of exon 3, loss of DNA binding activity)</td>
<td>Delayed production of first litter Decreased pups/litter and reduced number of CL AR−/− animals had age-dependent reduction in pups/litter Increased number of unhealthy antral follicles Normal response to exogenous gonadotropins, but a reduced number of oocytes ovulated during natural mating</td>
<td>Normal serum E2, T, LH and FSH Increased intraovarian T in 10–12 week old mice</td>
<td>185</td>
</tr>
<tr>
<td>AMHCre+;Arfl/−;Arfl/+ (exon 3 floxed, loss of DNA binding activity in large preantral to antral follicles)</td>
<td>Subfertile due to reduced number of litters and age dependent decrease in total number of pups born Decreased fertility over time with a cumulative decrease in pups born per dam 3-month-old animals have increased large preantral and antral follicle numbers 6-month-old animals have increased length of estrous cycle Reduced cumulus expansion and oocyte/embryo viability</td>
<td>Normal serum LH and FSH</td>
<td>691</td>
</tr>
<tr>
<td>Amhr2Cre+;Arfl/− (exon 2 floxed) GCARcKO</td>
<td>Reduced fertility Altered follicle progression and development 8–9 week mice: normal estrous cycle, reduced pups/litter, reduced number of oocytes from natural mating, however, no differences observed when treated with exogenous gonadotropins 24 weeks: increased length of estrous cycle, reduced number of pups born per dam, reduced number of oocytes from natural mating and treatment with endogenous gonadotropins</td>
<td>Not reported</td>
<td>692</td>
</tr>
<tr>
<td>Gdf9Cre+;Arfl/− (exon 2 floxed, oocyte specific loss of AR)</td>
<td>Normal female phenotype except androgens were unable to promote oocyte maturation in vitro</td>
<td>Not reported</td>
<td>692</td>
</tr>
<tr>
<td>SPARKI (homozygous KI of mutated DBD of AR)</td>
<td>Normal female phenotype</td>
<td>Not reported</td>
<td>186</td>
</tr>
</tbody>
</table>

an important role in the oocyte-granulosa cell regulatory loop, although future work is needed to identify potential mechanisms of action.

These knockout mouse models demonstrated an important role for AR in normal ovarian function and suggest that loss of AR contributes to POF (Table 25.9). Both AR-null models lack all AR protein due to a truncated message and premature stop codon. A third AR-null mouse model was developed by Walters et al. where an in-frame deletion of exon 3 was made that led to production of an AR protein lacking the second zinc finger and is therefore truncated and unable to bind DNA. Mutations in this region of the AR gene have been identified in human patients with androgen insensitivity syndrome (AIS), demonstrating the importance of binding DNA to AR activities in normal physiological state. This mouse model allows for analysis of the direct effects of classical genomic AR signaling in the ovary compared to the AR-null models that lack all AR protein. Deletion of the DNA binding domain in female mice led to a subfertile phenotype where the females homozygous for the deletion had less offspring per litter compared to both heterozygous and WT females. Interestingly, the AR(exon 3)-null females had reduced numbers of CL similar to other AR-null mice, demonstrating the importance for intact AR signaling for this process.
Furthermore, these mice also had an age-dependent reduction in fecundity similar to the other AR-null models, and females heterozygous for the AR(exon3)-null deletion also showed an age-dependent reduction in offspring born at 6 months of age, suggesting a dosage effect. An increase was observed in the number of unhealthy antral follicles, although AR(exon3)-null mice did not have significant changes in overall follicle development even in animals aged 10 months in contradiction to reports in AR-null ovaries with reduced follicle numbers. While these models have indicated some differences in the role of AR in normal folliculogenesis, all demonstrate an increase in the number of atretic follicles, demonstrating the importance of AR signaling in maintaining ovarian health over time. AR-null mice have reduced response to gonadotropin-induced ovulation and examination of the AR(exon3)-null animals found that they respond to exogenous gonadotropin stimulation with similar numbers of oocytes ovulated; however, a decrease was observed in oocytes ovulated when examined after natural mating. This suggests that AR is necessary for normal ovulatory response; however, excess gonadotropins are able to override this defect in the presence of a truncated AR protein (lacking the entire DNA binding domain) compared to ovaries that are void of all AR protein. Mutation of the DNA binding domain that alters the ability of AR to bind to selective androgen response elements in the SPARKI mouse model presented by Schauwaers et al. did not lead to a female reproductive defect, suggesting that the ovary does not require selective AR binding to response elements as compared to the male SPARKI mice that have a reproductive phenotype and will not be further discussed herein.

The identification of genes necessary for oocyte-granulosa communication in the ovary as being AR targets provides a possible mechanism that is aberrant in AR-null ovaries, but further studies are necessary to confirm this difference and explore the pathways involved. Loss of the DNA binding domain in AR did not affect circulating hormone levels, as the mice had normal serum levels of E2, T, LH, and FSH; however, an increase in intraovarian T levels was observed in mice aged 10–12 weeks, suggesting alterations in the steroid environment locally may have paracrine effects on ovarian function that was not measured in other AR-null models.

**MICE WITH OVARIAN-SPECIFIC DELETION OF AR**

The global AR knockout mouse models described herein have offered many insights into the important role of AR in female reproduction, specifically in the ovary. However, these models lack AR in all tissues, including all parts of the hypothalamic–pituitary–gonadal axis, which could contribute to the ovarian phenotype observed. To circumvent this, two conditional knockout mouse models have been developed to remove AR specifically in granulosa cells of the ovary. These models provide a unique way to explore the role(s) of AR in female fertility and ovarian function.

Sen and Hammes used Amhr2-cre to delete exon 2 of AR in the ovary and found that the animals had reduced numbers of offspring born to young dams (2.8 pups in KO versus 7 in WT mice), similar to that observed in the global knockout animals. In these granulosa cell AR conditional knockout (GCARcKO) animals, altered follicle progression was observed in animals aged 9 weeks as well as in animals aged 24 weeks (Table 25.9), supporting observations in global knockout animals, where numbers of corpora lutea were reduced and atretic follicles were increased. These patterns persisted in older animals, which also showed an increased length of estrous cycle at 24 weeks of age compared to mice aged 9 weeks old that had a normal estrous cycle.

Several other differences were observed in animals at the two ages examined (9 weeks versus 24 weeks), including differences in ovulation. Mice aged 9 weeks had a reduced number of oocytes ovulated during natural mating, but treatment with exogenous gonadotropins was able to circumvent this defect and cause normal numbers of oocytes to be ovulated from these animals. As the animals aged, however, the ability of exogenous gonadotropins to override this defect was lost, and reduced oocyte numbers were observed from natural mating and superovulation paradigms. Failure of the ovary to respond to exogenous gonadotropins concurrent with a reduction in the total number of offspring born over a long-term fertility study (19 in KO versus 119 in WT mice) supports the POF phenotype observed in the global AR-knockout models.

The GCARcKO presented by Sen and Hammes was made using the Amhr2-cre mouse, which has been found to be active in tissues other than granulosa cells including the pituitary, which may complicate the findings. While reduced expression of Ar was not reported in the hypothalamus or the pituitary, the heterogeneous population of cells present could mask a reduction in gonadotrope cells that are required for normal female fertility. To eliminate this possibility and explore the classical role of AR in large preantral to antral follicles, Walters et al. used the Amh-cre mouse model to delete exon 3 in the ovary. Amh is the ligand for Amhr2 and is expressed specifically in large preantral to antral follicles during folliculogenesis demonstrated by crossing the Amh-cre mouse to R26R (ROSA) reporter mice. This mouse expresses β-galactosidase activity where Cre recombinase is expressed, and the X-Gal blue stain can be used to localize cells expressing the β-gal indicator within tissues. The Amh-cre mouse was crossed with exon3 floxed AR mouse that creates a truncated protein lacking the DNA binding domain. The use of this
mouse AR to be deleted in a temporal pattern and to examine whether DNA binding activities are necessary during this stage of folliculogenensis for normal ovarian function.

The $\text{AMH}^\text{Cre+} \text{Ar(exon3)}^{+/+}$ females are subfertile, and while the number of offspring born per litter was not significantly altered, an age-dependent decrease in the total number of pups born was observed$^{185}$ similar to the POF phenotype observed in other AR-null models (Table 25.9).$^{183,185,283,690,692}$ This provides evidence that AR binding to direct AR target genes in large preantral follicles is necessary for normal ovarian function over time. The $\text{AMH}^\text{Cre+} \text{Ar(exon3)}^{+/+}$ females also have an increased length of estrous cycle at the age of 6 months that is not observed in younger mice,$^{691}$ supporting the POF phenotype observed and the importance of AR in maintaining normal ovarian health. Altered follicle progression was observed in these mice, and at 3 months of age fewer large preantral and small antral follicles were observed; however, this difference was not noted in older animals.$^{691}$ While no difference is observed in follicle progression during folliculogenensis in $\text{AMH}^\text{Cre+} \text{Ar(exon3)}^{+/+}$ females aged 6 months, a significant increase in unhealthy follicles was observed,$^{691}$ supporting the notation that AR signaling is necessary for normal ovarian health and prevention of POF in the ovary.

Communication between granulosa cells and the oocyte are necessary for oocyte health and viability, and disruption of this communication can lead to infertility. Animals lacking functional AR protein have altered expression of several genes implicated in this communication network, including the AR-dependent gene $\text{Kitl}$. Pharmacological studies have suggested that T can induce mouse$^{697,698}$ and porcine$^{389,699,700}$ oocyte maturation presumably through classical AR signaling in vitro. To directly examine the role of AR expression in oocytes, a conditional knockout mouse model was developed using an oocyte-selective $\text{Gdf9-cre}$ crossed with exon 2–floxed AR.$^{692}$ These mice had normal fertility and no overt ovarian phenotype compared to WT controls, demonstrating that AR expression within oocytes is not necessary for female fertility, while granulosa cell specific expression of the AR nuclear receptor is necessary in vivo.$^{692}$ Interestingly, when oocytes were removed from immature unprimed females and grown in vitro, DHT was unable to induce oocyte maturation in the absence of AR expression in oocytes.$^{692}$ However, P-mediated oocyte maturation was not affected by the loss of AR in vitro, demonstrating that these oocytes could still mature, albeit not through an androgen-mediated mechanism. This suggests that AR expression in oocytes is necessary for in vitro maturation in the absence of granulosa cells, while in vivo expression of AR in the granulosa cells is sufficient for oocyte maturation, possibly due to P-mediated mechanisms.

Loss of AR in granulosa cells contributes to altered ovarian function including a POF phenotype and loss of ability of the ovary to ovulate oocytes through both natural mating or superovulation depending on the age the animals were tested.$^{183,185,692}$ While differences were observed depending on the AR-null mouse model examined and the ovulation paradigm presented, the data provide evidence that AR is necessary for normal ovulatory function of the ovary. Interestingly, a decrease was observed in the number of oocytes naturally ovulated by one granulosa cell–specific AR-null model,$^{692}$ while no differences were observed in a second model (Table 25.9).$^{691}$ These differences may be due to the timing of AR deletion or the different flox animals used (i.e., exon 2 versus exon 3). Further study was done on oocytes ovulated from natural mating in the $\text{AMH}^\text{Cre+} \text{Ar(exon3)}^{+/+}$ females to see if these oocytes could be fertilized and progress through early embryo development in vitro.$^{691}$ While the number of oocytes ovulated was not different, the number of fertilized oocytes was reduced (38.6% in KO versus 94.5% in WT), as was the number of the embryos able to progress to the two-cell stage of embryo development (35.7% in KO versus 89.5% in WT).$^{691}$ This supports the necessity of AR expression in granulosa cells to promote oocyte maturation and development, and indicates that DHT-mediated oocyte maturation could be mediated through expression of AR in granulosa cells. It also provides insight into the inability of some oocytes to respond to in vitro fertilization, which has implications in human health in cases where some women fail to respond to assisted reproductive technologies. The variety of AR-null mouse models to date provides evidence of the importance of AR signaling in normal ovarian development, female fertility, and follicle health.

**Intraovarian Role of Androgen in Ovarian Function**

The intraovarian roles of androgens can be categorized into three distinct functions: (1) as substrates for E2 synthesis, (2) as an enhancer of follicle differentiation, and (3) as a stage-specific inhibitor of follicle growth.$^{683}$ The role of thecal-derived androgens, most notably androstenedione, as substrates for E2 synthesis in granulosa cells is obviously critical given the profound importance of the latter hormone to reproductive function. While ovarian steroidogenesis is essential for function of the ovary and female fertility, this topic will not be covered here; instead we focus on the additional intraovarian actions of androgens, which include their role as activating ligands for AR-mediated effects.

**GRANULOSA CELL PROLIFERATION**

The follicular response to androgens is dependent upon the stage of growth and differentiation. In preantral,
undifferentiated follicles that are unable to synthesize E2, androgens promote gonadotropin-induced granulosa cell proliferation and maturation. In large, differentiated follicles that have acquired aromatase activity, E2 assumes the role of enhancing FSH-induced granulosa cell proliferation and differentiation, and androgens in excess of that required for conversion to E2 are detrimental to follicle health, leading to atresia. This paradox of androgen action during follicle growth and differentiation explains the contradictory results when comparing experimental studies.

Several studies in hypophysectomized rats have shown that coadministration of T inhibits estrogen or gonadotropin-induced granulosa cell proliferation and increased ovarian weight, and promotes degeneration in more mature follicles. In contrast, Armstrong and Papkoff found aromatizable androgens (e.g., T or androstenedione) enhance the promotional effects of FSH in the ovaries of hypophysectomized rats but that the nonaromatizable androgen DHT is inhibitory. Similar treatment with hCG instead of T has a comparable effect on gonadotropin or E2-induced follicle growth in hypophysectomized rats, but is prevented by AR-antagonists, indicating the detrimental effects of hCG are due to stimulation of thecal cell androgen synthesis. However, Bley et al. found that granulosa cells from preantral follicles of estrogen-primed rats proliferate at comparable rates in response to FSH plus E2 or DHT in vitro and that the promotional effects of the latter steroid are blocked by the antiandrogen hydroxyflutamide. Investigations toward the mechanism by which androgens inhibit granulosa cell proliferation indicate that DHT exposure to estrogen-primed rats prior to granulosa cell isolation causes a blunted response to forskolin-induced cyclin-D2 (CyclinD2) expression in vitro, leading to cell-cycle arrest and reduced proliferation. DHT is able to exert its inhibitory effect on Ccnd2 expression through AMP-activated protein kinase (AMPK) activation, which in turn inhibits phosphorylation of MAPK and ultimately impacts granulosa cell proliferation downstream of FSH. AMPK has a role in inhibiting cell proliferation, and pharmacological activation of the kinase can lead to cell cycle arrest, while blocking AMPK activity prior to treatment with DHT was able to overcome the inhibitory effect normally observed, providing insights into the mechanism of action of DHT inhibition on cell cycle progression in estrogen-primed rat granulosa cells. This work also highlights how androgens are able to be stimulatory during early stages of folliculogenesis and then inhibitory during the latter stages when estrogen signaling is the main driver of follicle development.

The stage-specific effects of androgens during folliculogenesis described herein may be more apparent in rat ovaries relative to other species. In hypophysectomized mice, Wang and Greenwald found that T or DHT, in combination with FSH, induces greater levels of DNA synthesis in antral follicles than FSH alone; a similar response is not observed in small- and medium-sized follicles. In preantral mouse follicles grown in vitro, antiandrogen antisera reduce FSH-stimulated growth and DNA synthesis; however, these data must be interpreted with caution since antiandrogen antisera may also remove androgens from the pool of substrates for E2 synthesis. Additional evidence that androgens may promote granulosa cell proliferation in mice comes from the findings of Burns et al. that the AR-antagonist flutamide reduces the growth rate of granulosa cell tumors in animals lacking functional inhibins. In vitro follicle culture studies demonstrated the importance of AR signaling in folliculogenesis, where treating follicles with antiandrogenic compounds reduced follicle growth during the preantral stage of folliculogenesis, demonstrating the importance of androgens in early follicle growth.

In cumulus cells of large antral follicles from porcine ovaries, DHT augments FSH or IGF1-induced proliferation, and this effect is inhibited by flutamide. Rhesus monkey ovaries exhibit a strong correlation between AR expression and cell proliferation and exhibit an increased number of follicles of all stages except large antral following T or DHT treatments. Yang and Fortune reported that T was able to stimulate early follicle maturation in bovine follicles. The collective data support the notion that androgens are important in granulosa cell proliferation during early stages, but at latter stages the effect appears to be species dependent.

**Granulosa Cell Differentiation**

Fully differentiated preovulatory follicles in mammalian ovaries are distinctly characterized by a large fluid-filled antrum, significantly increased aromatase (CYP19) activity, and acquisition of LH responsiveness. The absolute requirement of FSH signaling in this process is illustrated by the absence of all three phenotypes in mice null for FSH action. E2 is also required for full FSH-induced follicle differentiation. However, there is substantial evidence that androgens may be equally efficient as E2 in augmenting certain responses to FSH, most especially the induction of aromatase activity and antrum formation. Both T and DHT enhance FSH induction of aromatase activity in hypophysectomized rat ovaries or isolated granulosa cells in vitro in a dose-dependent fashion. Furthermore, pharmacological inhibition of ovarian androgen synthesis in rats simultaneously reduces aromatase activity that can be restored by exogenous T.

Fitzpatrick et al. found that T and DHT to a lesser extent, significantly enhances FSH induction of Cyp19 expression and aromatase activity in granulosa cells from
untreated hypophysectomized rats\(^442\) (Figure 25.19). This finding was supported by work done in vitro cultured granulosa cells isolated from rats where T was able to increase Cyp19 and P450scc expression. This regulation was found to be dependent on liver receptor homolog-1 (Lrh1) expression that is induced by T and not DHT through AR binding to Lrh1 promoter regulatory region\(^715\) and provides evidence that T, and not DHT, affects the expression of genes necessary for estrogen production during folliculogenes. Furthermore, Lrh1 expression is also induced by FSH in granulosa cells,\(^715\) providing a potential mechanism of action for synergy necessary for differentiation of granulosa cells in the ovary.

Interestingly, this effect is lost in granulosa cells isolated from estrogen-primed hypophysectomized rats,\(^442\) supporting the hypothesis that early growing follicles are more sensitive to androgen action and that estrogens downregulate AR expression as part of the process of follicle differentiation. Similar data has been produced in mice. For example, AR antagonists are able to inhibit FSH-induced E2 synthesis in individually cultured murine follicles if included at the beginning of the culture period prior to any indications of differentiation.\(^716\) Similarly, E2 output by large antral murine follicles in vitro is unaffected by DHT.\(^902\) In marmoset ovaries, granulosa cells isolated from small (0.5–1 mm) follicles are highly responsive to the enhancing effects of T or DHT on FSH-induced aromatase activity, but this response is lost in granulosa cells from larger (>2 mm) follicles whereupon DHT exposure becomes inhibitory.\(^717,718\) In contrast, 3 or 10 days of T treatment in rhesus monkeys has no effect on ovarian CYP19 expression.\(^684\)

The majority of data indicate that healthy preantral follicles are responsive to T and require the steroid for the initiation of Cyp19 expression by FSH in granulosa cells. Since androgen synthesis in thecal cells precedes the acquisition of aromatase activity in the accompanying granulosa cells, this mechanism of androgen/FSH synergism allows for more efficient induction of Cyp19 expression than would occur with FSH alone. Once the granulosa cells acquire sufficient aromatase activity, thecal-derived androgens become more important as substrates and ER-mediated E2 actions continue the role of synergizing with FSH to promote follicle differentiation (Figure 25.25). Hence, the hallmark of a healthy preovulatory follicle in rodent ovaries may be the presence of substantial aromatase activity and E2 output, and decreased AR expression and androgen sensitivity.

The mechanism by which androgens augment FSH action on granulosa cells is unclear but may differ from that hypothesized for E2. Whereas ER-mediated E2 actions are believed to enhance the amount and effectiveness of FSH-stimulated intracellular cAMP without obvious changes in FSH-receptor levels, evidence suggests that androgens may act at a site prior to adenyl cyclase. For example, neither T nor DHT synergize with (Bu)\(_2\)-cAMP to induce aromatase activity in rat granulosa cells\(^719\) whereas E2 does.\(^442,720\) Instead, AR-mediated androgen actions may largely act by increasing the level of FSH receptor in granulosa cells of preantral follicles. T can also restore the losses in FSH receptor and responsiveness that occur in rat granulosa cells cultured without gonadotropins, and this effect is inhibited by AR antagonists.\(^719\) Evidence that androgens increase granulosa FSH-receptor levels comes from reports that ovaries of 10-day-old and prepubertal AR-null females exhibit significantly reduced Fshr mRNA levels, even 48 h after PMSG treatment in the latter age group.\(^183\) Similarly, Weil et al. found that 3 or 10 days of T treatment in adult rhesus monkeys increases FSH-receptor expression in follicles of all stages except primary.\(^684\) In human small antral follicles cultured in vitro, a positive correlation was observed in AR expression, androgen concentrations in follicular fluid, and FSHR expression\(^723\) in small follicles demonstrating an association with AR and androgens in immature granulosa cells, and suggests that in human ovaries this association is important for normal follicle development.

AR-mediated androgen actions may also be involved in antrum formation. Murray et al. found DHT potentiates suboptimal doses of FSH to stimulate increased follicle diameter of murine follicles, and this promotional effect is inhibited by AR antagonists.\(^716\) T or DHT are also reported to be almost as effective as E2 in augmenting FSH-induced increases in the number of antral follicles in the ovaries of hypophysectomized mice.\(^492\) However, the ovaries of global AR-null mice do not show differences in the number of antral follicles,\(^183,185,690\) while in the granulosa-specific AR-null mice a reduction in the number of antral follicles was observed at all ages,\(^692\) or only in younger mice (3 months).\(^691\) The conflicting reports of antral formation in AR-null mice demonstrate the complexity of this developmental process in the ovary. Therefore, FSH-dependent antrum formation may be enhanced by either receptor-mediated androgen or estrogen actions, congruent with the process being part of the transition period during which follicles become less sensitive to androgens and more dependent on estrogens.

Not all FSH-dependent processes during follicle differentiation are enhanced by androgens. LH-receptor expression and LH responsiveness by granulosa cells occurs only in preovulatory follicles during the final stages of folliculogenesis, just prior to the LH surge. Unlike the above processes of follicle differentiation, FSH stimulation of LH-receptor expression in preovulatory granulosa cells is facilitated by estrogen or aromatizable androgens only\(^515,548,549\) while DHT has no effect or may even be inhibitory.\(^515,549\) These data

---

4. FEMALE REPRODUCTIVE SYSTEM
are consistent with the mutually exclusive pattern of AR and LH receptor in preovulatory follicles of rodent ovaries, as AR expression is limited to the cumulus cells\(^6^{67}\) while LH receptor is predominantly localized to mural and antral granulosa cells.\(^7^{22–24}\) In addition, in vivo studies demonstrating that AR antagonists have no effect on LH-receptor expression in the ovaries of FSH-stimulated, DES-primed hypophysectomized rats suggest that AR-mediated actions are not required to promote or maintain LH-receptor expression in preovulatory follicles.\(^7^{25}\) The specific requirement of ER-mediated E2 actions to augment FSH-stimulated LH-receptor expression in preovulatory granulosa cells provides a mechanism by which only those follicles that acquire sufficient aromatase activity and are suitable to ovulate acquire the capacity to respond to the LH surge.

**THECAL CELL STEROIDOGENESIS**

There are few studies on the role of androgen signaling in thecal cell function and steroidogenesis despite these cells being the primary source of androgen synthesis. Several nonsteroidal paracrine factors are known to modulate thecal cell steroidogenesis and have been more thoroughly studied.\(^4^{76,566}\) The limited available evidence suggests that AR-mediated androgen actions may negatively regulate thecal cell steroidogenesis, thereby forming an autocrine regulatory feedback loop. Mahesh and colleagues have shown in enriched thecal/interstitial cell cultures from rats that AR activation by nonaromatizable agonists attenuates hCG or hCG/IFG-1–stimulated increases in androstenedione synthesis by 32% and 40%, respectively, via selective inhibition of Cyp17 expression.\(^7^{26}\) Similar experiments demonstrated that AR-antagonists enhance hCG-induced androstenedione synthesis, further indicating a receptor-mediated effect of androgens on thecal cell steroidogenesis.\(^7^{26}\) The majority of recent studies published to date focus on the transcriptional regulation of genes involved in androgen biosynthesis in theca cells,\(^6^{87,688}\) but not on the autocrine action of the synthesized androgen(s) within the cells. In fact, a recent review entitled “Theca: The forgotten cell of the ovarian follicle” described the various factors that are known to regulate steroidogenesis and androgen production,\(^7^{27}\) which also highlights the need for more focused experiments looking at theca cell functions of AR. The use of conditional knockout mouse models to explore the role of AR specifically in theca cells could also shine light on this important question. Therefore, ER-mediated E2 actions (see the section Estrogen Receptor Signaling in Ovarian Function) and AR-mediated androgen actions may both contribute to maintaining homeostasis of androgen synthesis in the thecal cells of developing follicles.

**OVULATION**

Over the past decade there have been few studies aimed at determining a role for androgen signaling in follicle rupture and ovulation, the majority of these from the AR-null mouse models (Table 25.9). This is especially surprising since Mori et al. demonstrated in 1977 that inhibition of androgen action at the time of ovulation is detrimental to follicle rupture in the rat.\(^7^{28}\) These studies employed anti-T and anti-P antisera during induced ovulation in immature or hypophysectomized rats to demonstrate that: (1) acute treatment with anti-T antisera at the time or shortly after hCG-induced ovulation leads to a dose-dependent reduction in the number of ovulated oocytes, and (2) inhibition of ovulation by acute treatment with an anti-P antisera at the time of hCG treatment is rescued by concurrent treatment with T or DHT but not E2.\(^7^{28}\) In a similar study, Peluso et al. demonstrated that treatment of immature rats with the AR antagonists, cyproterone acetate or flutamide, 3h prior to hCG-induced ovulation drastically reduces the number of ovulated oocytes and prevents expansion of the cumulus–oocyte complex.\(^7^{29}\) Similar findings are reported in mice.\(^7^{30}\) The development of AR-null mouse models has provided some insight into the role of androgen signaling in ovulation. An ovulatory defect in AR-null mice is suggested by the presence of a normal number of preovulatory follicles but few corpora lutea in the ovaries,\(^4^{83}\) as well as reduced circulating P levels.\(^6^{89}\) Furthermore, gonadotropin-induced ovulation of immature AR-null females indicates a severely reduced response in terms of recoverable oocytes in the oviducts, and may be attributable to their failure to exhibit hCG-induced expression of P receptor, hyaluronyl synthetase-2, and tumor necrosis factor-\(\alpha\) stimulated gene 6.\(^6^{83}\) Reduced ovulatory rates were also observed in another AR-null mouse model lacking the DNA binding domain after normal mating.\(^1^{85}\) However ovulation rates after treatment with exogenous gonadotropins were normal. A similar finding was observed in granulosa cell–specific AR-null mouse model where oocytes collected after natural mating are reduced but animals respond to exogenous gonadotropins. Interestingly, when these animals were 6 months of age, they didn’t ovulate at all.\(^6^{92}\) These mouse models suggest that AR plays a role in normal ovulation, but stimulation with excess gonadotropins can overcome these deficiencies only in young mice. Further work to study the role of androgen signaling in other mammalian species including primates and humans could provide insight into possible fertility treatments.

**FOLLICLE ATRESIA**

Follicle atresia in the mammalian ovary has been thoroughly described in several reviews.\(^5^{65,680,731}\) Follicular atresia is a complex hormonally driven process.
involving both putative “survival” factors, e.g., insulin-like growth factor-1, epidermal growth factor, basic fibroblast growth factor, E2, and activin; and putative atretogenic factors, e.g., tumor necrosis factor-α, GnRH, and androgens. The respective action of each of these factors on atresia is dependent on the follicular stage, for example, epidermal growth factor and basic fibroblast growth factor begin to act early on primordial stage follicles, whereas insulin-like growth factor-1 affects early antral follicles.

Circumstantial evidence of the actions of androgen includes the numerous observations that atretic follicles consistently exhibit an increased androgen:estrogen ratio within the intrafollicular fluid. Supporting experimental evidence includes reports that low doses of hCG (to induce endogenous androgen production), T, or DHT following 4 days of E2 treatment in hypophysectomized rats lead to increased numbers of atretic follicles within 24 h. The detrimental effects of hCG or exogenous androgens are inhibited by co-treatment with anti-T antisera or AR antagonists, indicating the involvement of androgen receptor in this process. Interestingly, large (>150 μm) follicles are more susceptible to the atretogenic effects of androgens than small (<150 μm) follicles, indicating that androgens are largely atretogenic in late-stage follicles. In similar experiments on hypophysectomized rats, Billig et al. demonstrated that the follicular atresia that follows the withdrawal of 2 days of continuous estrogen (DES) treatment is enhanced by subsequent T exposure but prevented by E2. Conversely, Flaws et al. show follicular atresia induced by the endocrine-disrupting chemical methoxychlor.

Additionally, AR-null mouse models also have increased atretic follicles, demonstrating that AR signaling plays a role in follicle atresia; however, further studies are needed to understand the mechanism.

Glucocorticoid Receptor Signaling in Ovarian Function

While the ovary is not considered a classic target tissue of glucocorticoids, altered glucocorticoid production can affect reproduction and fertility throughout the HPG axis. Within the ovary, glucocorticoid signaling through GR can modulate steroidogenesis/gametogenesis and will briefly be discussed here. GR is expressed in the rat ovary, and examination of whole ovary expression shows that levels do not change upon treatment with exogenous gonadotropins. Further examination of GR expression in isolated granulosa cells shows no significant alterations in expression within these cells, although GR expression appears to be elevated in corpora lutea.

Glucocorticoid signaling in the ovary acts to inhibit LH action and steroid biosynthesis during folliculogenesis. Treatment of granulosa cells with cortisol or dexamethasone was able to inhibit FSH-stimulated increases in aromatase activity in vitro, while P production was increased compared to control-treated cells. This finding suggests that GR signaling may affect steroidogenesis of the ovarian steroids in a differential manner. Furthermore, this finding suggests that GR signaling in the ovary is important for P function and ovulation. Dexamethasone was also found to stimulate FSH-mediated increases in P production in porcine granulosa cells, suggesting a key role for GR signaling during late stages of ovulation and for corpora lutea function.

The expression of GR in granulosa cells does not change significantly during early follicle development; however, the treatment of cells with agonist shows differential activity depending on the stage of differentiation of the cells. To circumvent the action of glucocorticoids, expression of two metabolizing enzymes has been found to have stage-specific expression in the ovary. The expression of the type I and type II 11β-hydroxysteroid dehydrogenase (11HSD 1 and 11HSD 2) modulates the action of glucocorticoids by regulating their concentrations within tissues. This is important in the ovary during folliculogenesis since glucocorticoids are not synthesized within the tissue itself. During FSH-stimulated follicle growth, 11Hsd1 is expressed and acts to suppress glucocorticoid action, then as the granulosa cells become luteinized after LH stimulation, 11Hsd2 expression is increased. This acts to increase the action of glucocorticoid action on the ovary during luteinization and formation of the corpora lutea, which also shows an increase in GR expression, demonstrating the importance of GR signaling during this process. Interestingly, expression of 11Hsd2 is reduced during luteal regression, demonstrating the importance of GR activity specifically in the corpora lutea. The evidence to date suggests that GR signaling is important in ovarian function, although further work is needed to define the precise activity and necessity of this signaling mechanism specifically in the ovary.

SUMMARY

The salient aspects of the previous discussion are summarized in a model of sex steroid and steroid receptor action during folliculogenesis. Although certain postulated mechanisms and pathways portrayed in this model are supported by substantial evidence, other aspects are much more speculative and remain to be demonstrated. Regardless, the evidence is solid that the receptor-mediated steroid pathways exist in the ovary and their intra-ovarian functions are critical to female fertility.
CONCLUSION

Within this fairly extensive review we have endeavored to describe as comprehensively as possible what is currently known regarding the important functions of estrogen, progesterone, androgen, and glucocorticoid receptors in ovarian and uterine tissues. Clearly, an abundance of data has been obtained that has greatly advanced our understanding of the roles of these receptors since early descriptions more than five decades ago. Nevertheless, many open questions remain for further elaboration. At the level of the steroid receptors themselves, questions remain regarding how the receptors mediate the spectrum of responses, and what roles tissue and gene specificity of distinct activation domains on the receptors play. Additionally, better understanding of the interactions of receptors with chromatin, which are often quite far from regulated transcripts, and how the interactions lead to changes in transcription, are challenging questions to approach. We can hope to learn more about temporal patterns of changes in the organization of nuclear structures initiated by steroid receptors. At the whole animal level, what remains is to piece together advances gleaned from diverse models and methods into a coordinated description of the coordinated signaling between receptors for different steroids in cells and tissues that culminate in an optimal environment for fertilization, implantation, gestation, and parturition.

References


4. FEMALE REPRODUCTIVE SYSTEM
REFERENCES

REFERENCES


4. FEMALE REPRODUCTIVE SYSTEM
REFERENCES


25. STEROID RECEPTORS IN THE UTERUS AND OVARY


4. FEMALE REPRODUCTIVE SYSTEM
REFERENCES


435. Nilson JH, Abbad RA, Keri RA, Quirk CC. Chronic hypersecretion of luteinizing hormone in transgenic mice disrupts both ovarian and pituitary function, with some effects modified by the genetic background. Recent Prog Horm Res 2000;55:69–89; discussion 91.


4. FEMALE REPRODUCTIVE SYSTEM
REFERENCES
25. STEROID RECEPTORS IN THE UTERUS AND OVARY


4. FEMALE REPRODUCTIVE SYSTEM
REFERENCES


4. FEMALE REPRODUCTIVE SYSTEM


4. FEMALE REPRODUCTIVE SYSTEM
4. FEMALE REPRODUCTIVE SYSTEM

25. STEROID RECEPTORS IN THE UTERUS AND OVARY


