Role of stromal-epithelial interactions in hormonal responses*

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Summary. Steroid sex hormones (17β-estradiol, testosterone, dihydrotestosterone, and progesterone) and aryl hydrocarbons such as the dioxins regulate epithelial proliferation and secretory protein production and differentiation in their respective target organs in male and female urogenital tracts and mammary glands. Recent evidence has demonstrated that stromal-epithelial interactions are critical for mediating the effects of these molecules on epithelial cells. Our results have indicated that estradiol, testosterone, progesterone, and dioxin regulate epithelial proliferation (stimulation or inhibition) via paracrine mechanisms requiring the appropriate receptor in the stroma. The androgen receptor (AR), estrogen receptor alpha (ERα), progesterone receptor (PR), or aryl hydrocarbon receptor (AhR) in the epithelium are neither necessary nor sufficient for the regulation of epithelial proliferation. Moreover, during prostatic development, signaling through the stromal AR is required to induce prostatic epithelial identity, ductal morphogenesis and glandular differentiation. Epithelial functional differentiation is regulated in the prostate, uterus, and vagina via AR (prostate) and ERα (uterus and vagina). In these organs both epithelial and stromal steroid receptors are required for steroidal regulation of certain aspects of epithelial differentiation such as epithelial secretory protein production in the uterus and epithelial cornification in the vagina and prostate (squamous metaplasia). The mechanistic basis of these stromal-epithelial interactions is poorly understood, but growth factors appear to be mediators of these cell-cell interactions.

Introduction

Organs of the male and female urogenital tracts and mammary gland consist of an epithelial parenchyma and associated fibromuscular stroma. In the adult uterus, the luminal epithelium, associated glands and stroma comprise the endometrium, which is in turn surrounded by the myometrium. In the adult prostate, sleeves of smooth muscle cells and fibroblasts surround the epithelial ducts. The development of organs composed of an epithelial parenchyma is dependent on reciprocal mesenchymal-epithelial interactions. Indeed, morphogenesis and differentiation of both the epithelium and mesenchyme are abortive if the epithelium and mesenchyme are grown separately (Marker et al., 2003). Paracrine signals from the mesenchyme initially induce and specify epithelial identity, e.g., whether the epithelium will differentiate as a uterus, vagina or prostate (Cunha et al., 1998). Second, the mesenchyme induces and specifies the morphological form or pattern of the epithelium, that is, whether the epithelium will form a simple vesicle (bladder), whether it will form a branched ductal network (prostate or mammary gland), whether it will form a large unbranched tube (vagina) or a tube with glandular evaginations (uterus). Third, during the course of epithelial morphogenesis the mesenchyme regulates epithelial proliferation in a highly coordinated fashion to generate specific epithelial patterns (Marker et al., 2003). Fourth, the mesenchyme specifies functional differentiation. This includes specifying the types of epithelial secretory proteins produced or the expression of specific cytoskeletal or membrane proteins (keratins and uro-
plakins) (Cunha et al., 1992; Hayward, 1992; Kurita et al., 2001a). Fifth, during development and in adulthood epithelial apoptosis is an important biological mechanism in which the occupancy status of the stromal androgen receptor (AR) in the prostate and stromal progesterone receptor (PR) in the uterus plays a key role in regulating epithelial apoptosis (Kurita et al., 2001b).

Tissue recombination experiments have revealed that interactions between the developing mesenchyme and epithelium are reciprocal. For example, if the epithelium of the uterus is ablated in vivo, the stromal cells no longer undergo decidualization in response to estrogen plus progesterone (Lejeune et al., 1981). This finding indicates that the epithelium is providing at least a permissive signal that allows the normal stromal response to hormone treatment. Subsequent work by Bigsby showed that, under progestosterone dominance, the epithelium elaborates factors that stimulate the underlying stroma to proliferate when estrogen is administered. Through the use of tissue recombinants uterine, but not vaginal, epithelium paired with uterine stroma allowed stromal cell proliferation in response to estradiol plus progesterone, indicating specificity in the epithelial signal that permitted the stromal proliferative response (Bigsby, 2002).

Since the development, growth, and function of male and female reproductive tracts are dependent upon reciprocal stromal-epithelial interactions in addition to being regulated by steroid sex hormones, it is reasonable to investigate hormone action in male and female reproductive tracts in the context of receptor-mediated events in which epithelial versus stromal receptors may play specific roles. This approach has been made possible with the availability of mutant mice null for the androgen receptor (AR), estrogen receptor alpha (ERα), estrogen receptor alpha (ERβ), progesterone receptor (PR), and aryl hydrocarbon receptor (AhR).

Investigation of AR null Tfm (testicular feminization) mice has revealed that development of the prostate and other male accessory sexual organs is dependent upon signaling through the AR (Ohno, 1979), that is, male AR null mutant mice fail to develop any masculine accessory sex organs. Conversely, embryonic development of the uterus and other female reproductive organs is normal in ERα knockout (αERKO) mice (Lubahn et al., 1993). However, postnatal differentiation, growth, and function of the uterus (and other estrogen target organs) during the juvenile period and adulthood are impaired in ERKOα mice (Couse and Korach, 1999). Investigation of PR knockout (PRKO) mice reveals normal embryonic development of the female genital tract, even though progesterone signaling through the PR is essential in the mature uterus during the estrous cycle and during the establishment and maintenance of pregnancy and lactogenesis of the mammary gland (Lydon et al., 1996; Lydon et al., 1995). Likewise, the AhR knockout (AhRKO) mouse provides a useful tool for producing tissue recombinants lacking AhR in one or more tissue compartment and for investigating the mechanism by which AhR agonists inhibit uterine epithelial proliferation (Lin et al., 2002). Thus, the profound phenotypes of these receptor mutant mice have provided the means for testing the relative roles of epithelial versus stromal steroid receptors in hormone action in male and female reproductive organs.

An understanding of the mechanisms by which androgens, estrogens, progestins and dioxins elicit effects in reproductive tissues is important for the following reasons: a) the critical role of these hormones in normal differentiation and function; b) their involvement in the etiology and progression of various pathologies; c) potential effects of environmental exposure to estrogenic, androgenic, anti-estrogenic and anti-androgenic compounds as well as AhR agonists; and d) the increased use of drugs which modulate the signaling systems for androgens, estrogens, and progestins. Evidence indicates that interactions between the stroma and epithelium in adult organs or between mesenchyme, the undifferentiated embryonic/fetal precursor of stroma, and the embryonic epithelium are critical for mediating the effects of estrogenic, androgenic, and prostational agents. Indeed, many effects of these hormones on male and female reproductive tract epithelium are mediated totally or in part through the appropriate receptors in the stroma (Cooke et al., 1998; Cunha et al., 1998). In this review we focus on the stromal-epithelial interactions involved in normal and abnormal hormonal responses in male and female hormone target organs. Stromal-epithelial interactions are reciprocal, so the role of the epithelium in regulating stroma will also be considered.

**Model systems for studying the mechanism of the effects of hormones and dioxins on reproductive epithelium**

Genetic engineering of mice in which a specific gene has been inactivated or “knocked out” provides a versatile and powerful tool for examining the role of specific genes and their corresponding proteins. In the field of steroid receptor biology, the spontaneous AR-null Tfm mouse has been critical in our understanding the role of the AR in masculine development (Ohno, 1979). The development of the αERKO mouse (Lubahn et al., 1993), in which the ERα gene has been rendered non-functional by gene targeting, has defined the phenotypic and functional consequences of an absence of ERα (Couse and Korach, 1999). Similarly, PRKO and
AhRKO mice have provided similar tools to explore the role of the PR and AhR in various aspects of reproductive development and function (Lydon et al., 1995, 1996; Schmidt et al., 1996).

Over the years we have used an experimental system that utilizes tissues from steroid receptor and aryl hydrocarbon receptor knockout mice to study the mechanism of androgen, estrogen, progesterone, and dioxin action in male and female genital tracts and the mammary gland. The crucial feature of this system involves enzymatically separating and recombining the epithelium (E) and stroma (S) from a receptor knockout (KO) mouse with that of the wild-type (wt) mouse, which expresses a receptor (Fig. 1). This method makes it possible to experimentally control the hormone receptor status of both the stroma and epithelium by preparing tissue recombinations that lack a receptor in both stromal and epithelial compartments (KO-S+KO-E), express a receptor in either the epithelium or stroma (KO-S+wt-E and wt-S+KO-E), or express a receptor in both the epithelium and stroma (wt-S+wt-E). These tissue recombinants are then grafted into host animals, and their responsiveness to the test hormone examined. By analyzing the effects of a lack of stromal and/or epithelial receptors on epithelial hormonal response such as epithelial mitogenesis or secretory protein production, the role of steroid receptors in each tissue compartment can be definitively determined.

The effect of androgens on embryonic mouse mammary gland development

Klaus Kratochwil was the first to carry out tissue recombination experiments on hormone action in his study of androgen-induced regression of the embryonic mammary rudiment (Kratochwil and Schwartz, 1976). Embryonic murine mammary buds develop in both male and female fetuses, but regress in males. In most mouse strains fetal testicular androgens elicit condensation of the mesenchyme around male mammary buds and trigger destruction of the epithelial rudiment through reciprocal interactions between the epithelium and mesenchyme (Kratochwil, 1987). The embryonic mouse mammary gland is initially unresponsive to testosterone at E11 and E12, becomes responsive to testosterone at E13, and completes regression by E16 (Kra-tochwil, 1977). Acquisition of sensitivity to testosterone correlates temporally with the expression of the AR in the mammary mesenchyme at 12 days of gestation (Kratochwil and Schwartz, 1976) when the mammary epithelial bud becomes surrounded by a population of AR-positive mesenchymal cells (Heuberger et al., 1982). The presence of mesenchymal AR and the corresponding absence of epithelial AR suggests that the embryonic mammary bud is destroyed by testosterone-activated mesenchymal cells.
rather than by direct hormone action.

The apposition of AR-positive mesenchymal cells around the embryonic mammary epithelial bud suggests that either the mammary epithelium attracts preexisting AR-positive mesenchymal cells or that the epithelium induces AR expression in the surrounding mesenchyme. These possibilities were tested in tissue recombination studies in which the AR-negative inter-bud mesenchyme (dermis) was associated with the embryonic mammary, lung, or salivary gland epithelium. The resulting tissue recombinations were grown in vitro and assessed for AR expression. The mammary bud epithelium consistently induced AR in the inter-bud mesenchyme (which is normally AR-negative), and thus the mammary buds became surrounded by an AR-positive mesenchyme. This induction of mesenchymal AR only occurred when mammary epithelium was used and not when epithelia of the salivary gland or lung were combined with inter-bud mesenchyme (Durnberger and Kratochwil, 1980). Thus, the mammary epithelium specifically induced the expression of ARs in adjacent mesenchymal cells.

The question of whether testosterone elicited mammary epithelial regression by direct action on the epithelium or by paracrine action mediated by mesenchymal AR was answered by analysis of tissue recombinants composed of mammary gland epithelium and mesenchyme of wt and AR null Tfm (testicular feminization, Tfm) mice. Tfm mice have a point mutation in the gene encoding the AR (He et al., 1991), fail to express a functional AR, and consequently are insensitive to androgens. Accordingly, endogenous or exogenous androgens cannot elicit regression of mammary glands of embryonic male Tfm mice. Durnberger and Kratochwil analyzed the four possible mammary tissue recombinations composed of wt and Tfm epithelium (E) and mesenchyme (M). Testosterone elicited destruction of the mammary epithelium only in tissue recombinations prepared with wt (AR+) mesenchyme (wt-M+wt-E and wt-M+Tfm-E), and not when AR negative Tfm mesenchyme was used (Tfm-M+Tfm-E and Tfm-M+wt-E) (Durnberger and Kratochwil, 1980) (Fig. 2). These data conclusively demonstrated that androgens elicit embryonic mammary epithelial regression via the AR-positive mesenchyme, which in response to androgens is induced to condense around the epithelium, thus triggering epithelial regression (Durnberger and Kratochwil, 1980).

Androgenic effects on prostatic development are mediated via ARs through mesenchymal-epithelial interactions. During fetal development ARs are initially detected solely in the urogenital sinus mesenchyme prior to and during prostatic bud formation. ARs are undetectable in developing prostatic buds suggesting that mesenchymal (but not epithelial) AR play a critical role in the early phases of prostatic development (Cooke et al., 1991; Takeda et al., 1991). To reveal the respective roles of epithelial versus mesenchymal ARs in prostatic development, prostatic tissue recombinants were prepared with urogenital sinus mesenchyme (M) and urogenital sinus epithelium (E) from AR-positive wt and AR-deficient Tfm mice (Fig. 3) (Cunha et al., 1987). As expected, tissue recombinants composed of Tfm-M+Tfm-E did not form a prostate even in the presence of androgens. As a positive control, wt-M+wt-E epithelium tissue recombinants formed a prostate in response to androgens. Tfm-M+wt-E tissue recombinants did not undergo prostatic development in the presence of androgens. This suggested a critical role of mesenchymal AR in prostatic development, which was confirmed in the reciprocal wt-M+Tfm-epithelium tissue recombinant in which AR-deficient Tfm epithelium underwent prostatic development in association with AR-positive wt urogenital sinus mesenchyme. Significantly, in wt-M+Tfm-epithelium tissue recombinants the AR-deficient Tfm epithelium underwent androgen-dependent ductal morphogenesis, epithelial proliferation and columnar cytodifferentiation, thus forming a glandular epithelium resembling the prostate (Cunha and
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Lung, 1978) (Fig. 3). Epithelial proliferation was examined specifically in wt-M+Tfm-epithelium tissue recombinants and was shown to be induced by testosterone to levels comparable to that in wt-M+wt-epithelium tissue recombinants. For estrogen target organs, a proliferative response to estradiol was analyzed in uterine and vaginal tissue recombinants prepared with wild-type (ERα-positive) and aERKO (ERα-negative) epithelium and mesenchyme. Results given in the table indicate (see figure) that testosterone and estradiol stimulate epithelial proliferation via paracrine influences downstream of the binding of hormones (testosterone or estradiol) to mesenchymal/stromal hormone receptors (HR) as appropriate.

**Fig. 3.** Stimulation of epithelial proliferation in androgen target organs (prostate) and estrogen target organs (uterus and vagina). For the prostate, a proliferative response to testosterone was analyzed in tissue recombinants composed of wild-type (AR-positive) and Tfm (AR-negative) epithelium and mesenchyme. For estrogen target organs, a proliferative response to estradiol was analyzed in uterine and vaginal tissue recombinants prepared with wild-type (ERα-positive) and aERKO (ERα-negative) epithelium and mesenchyme. Results given in the table indicate (see figure) that testosterone and estradiol stimulate epithelial proliferation via paracrine influences downstream of the binding of hormones (testosterone or estradiol) to mesenchymal/stromal hormone receptors (HR) as appropriate.

**Fig. 4.** Stimulation of mammary ductal growth by estradiol. Mammary tissue recombinants were prepared with wild-type (ERα-positive) and aERKO (ERα-negative) mammary epithelium and fat pad. Results given in the table indicate (see figure) that estradiol stimulates mammary ductal growth via paracrine influences downstream of the binding of estradiol to ERα in the fat pad (stroma).

**Fig. 5.** Stimulation of mammary ductal growth by estradiol. Mammary tissue recombinants were prepared with wild-type (ERα-positive) and aERKO (ERα-negative) mammary epithelium and fat pad. Results given in the table indicate (see figure) that estradiol stimulates mammary ductal growth via paracrine influences downstream of the binding of estradiol to ERα in the fat pad (stroma).

**Estradiol induces epithelial proliferation indirectly through stromal ERα in the mouse reproductive tract**

Once the aERKO mouse became available, the tissue recombination system was used to determine the respective roles of stromal versus epithelial ERα in estradiol-induced uterine epithelial proliferation. The original aERKO mouse engineered by Lubahn et al. (Lubahn et al., 1993) has been shown to be able to produce truncated forms of ERα (Dupont et al., 2000) and in a strict sense may not merit the designation of truly ERα null. Nonetheless, this original aERKO mouse is profoundly insensitive to estrogen stimulation, and a more recently engineered aERKO mouse completely devoid of ERα has revealed few additional phenotypes (Dupont et al., 2000). Accordingly, uteri from the original aERKO mice and wt mice were separated into epithelial and stromal components, and the following tissue recombinations were prepared: wt-S+wt-E; wt-S+aERKO-E; aERKO-S+wt-E and aERKO-S+aERKO-E. Tissue recombinants were transplanted under renal capsules of intact adult female nude mice and grown for 1 month. Then all hosts were ovariectomized.
To examine the effects of estradiol on epithelial proliferation in uterine tissue recombinants, seven days after ovariectomy, half of the hosts were treated with estradiol while the other half were given an oil vehicle. The epithelial labeling index with 3H-thymidine or Ki-67 was then determined. These studies demonstrated that an ER- negative epithelium can synthesize DNA and proliferate in response to estradiol when associated with an ER- positive stroma (Cooke et al., 1997). The epithelial labeling index in tissue recombinants composed of wt-S+wt-E and wt-S+ERKO-E increased several fold (and comparably) by estradiol treatment (Fig. 3). In contrast, αERKO-S+wt-E and αERKO-S+αERKO-E uterine tissue recombinants failed to respond to estradiol and had low epithelial labeling following estradiol and oil treatment (Fig. 3). Thus, estradiol did not stimulate epithelial proliferation in tissue recombinants lacking the stromal ERα, even when the epithelial ERα was present. Taken together, these results indicate that epithelial ERα was neither necessary nor sufficient to mediate the proliferative response to estradiol, while the stromal ERα was obligatory. Comparable results were obtained in vaginal tissue recombinants in which the

Fig. 5. Progesterone inhibition of estrogen-induced uterine epithelial proliferation. Uterine tissue recombinants were prepared with wild-type (PR-positive) and PRKO (PR-negative) epithelium and stroma as indicated in the table (upper left). The immunohistochemical images are tissue recombinants composed of A) wild-type stroma (wtS) + wild-type epithelium (wtE), B) prko stroma (prkoS) + prko epithelium (prkoE), C) wild-type stroma (wtS) + prko epithelium (prkoE), and D) prko stroma (prkoS) + wild-type epithelium (wtE). The tissue recombinants were treated with estradiol plus progesterone as described (Kurita, 1998). Epithelial labeling with 3H-thymidine (black nuclei) has been inhibited by progesterone in A (the wtS+wtE positive control) and in C (the wtS+ prkoE recombinant), but not in B (the prkoS+prko negative control) or in D (the prkoS+wtE recombinant). Staining in A–D is for the progesterone receptor (brown) with hematoxilyn counterstain. The cartoon (right) indicates that estradiol (E) stimulates uterine epithelial proliferation via paracrine influences. Estrogen-induced uterine epithelial proliferation is inhibited by progesterone (P) actions mediated via PR in the stroma. There are several mechanistic scenarios: 1) Progesterone may act within the stroma cell to prevent elaboration of the estrogen-induced trophic factor, 2) Progesterone may induce the synthesis and secretion of a factor that can neutralize the estrogen-induced trophic factor in the extracellular compartment, 3) Progesterone may induce the synthesis and secretion of a paracrine factor (such as TGFβ) that upon reception by the epithelial cell inhibits uterine epithelial proliferation.
proliferative effect of estradiol was mediated by stromal and not epithelial ERα (Fig. 3) (Buchanan et al., 1998a).

Recent work by Bigsby et al. has confirmed that stromal ERα alone is sufficient for maximal epithelial proliferation in response to estradiol, in that wt-S+αERKO-E tissue recombinants expressed epithelial proliferation equal to that seen in wt-S+wt-E tissue recombinants (Bigsby et al., 2004). However, these investigators reported that epithelial proliferation in αERKO-S+wt-E tissue recombinants from hosts given estradiol was somewhat increased compared with either αERKO-S+wt-E tissue recombinants from oil-treated hosts or αERKO-S+αERKO-E tissue recombinants from either E2- or oil-treated hosts. However, the epithelial proliferative response to estradiol in the αERKO-S+wt-E tissue recombinants was far less than the epithelial proliferation seen in either wt-S+αERKO-E or wt-S+wt-E tissue recombinants in the same response. The stroma used in these studies was obtained from neonatal mice, and then the tissue recombinants were grown for 8-9 weeks, as opposed to the 5-week growth period used with the grafts in our studies. Bigsby et al. suggest that this longer growth period may result in the epithelium becoming more mature and differentiated, and that this may allow a partial proliferative response in the epithelium mediated directly through ERα in that tissue (Bigsby et al., 2004). Therefore, although Cooke et al. (1997) and Bigsby et al. (2004) show that stromal ERα is both necessary and sufficient to mediate the full epithelial mitogenic response to estradiol, direct epithelial effects of estradiol may be capable of inducing limited epithelial proliferation in fully mature uterine epithelium even in the absence of ERα in the stroma (Bigsby et al., 2004; Cooke et al., 1997).

Mammary ductal growth in the mouse is mediated via ERα in the stroma

Mammary glands of adult αERKO mice are rudimentary, and alveoli do not form even though embryonic development of the mammary gland is normal (Bocchinfuso and Korach, 1997). The lack of ductal outgrowth at puberty in αERKO mice could result from an absence of ERα signaling either in the stroma (fat pad), epithelium, or both. To resolve the respective roles of epithelial versus stromal ERα in mammary ductal growth, the four possible tissue recombinants were constructed with an epithelium (E) and fat pad (FP) from neonatal wt and ERd knock-out (KO) mice: wt-FP+wt-E, wt-FP+αERKO-E, αERKO-FP+αERKO-E, and αERKO-FP+wt-E (Cunha et al., 1997). Tissue recombinants were grown as subrenal capsule grafts in nude mice. Impaired ductal growth was observed when an αERKO fat pad was used (αERKO-FP+αERKO-E and αERKO-FP+wt-E) (Fig. 4). Extensive ductal growth occurred when a wt fat pad was used (wt-FP+wt-E and wt-FP+αERKO-E). These studies demonstrated that the estrogenic stimulation of ductal growth is a paracrine event mediated by ERα-positive stromal cells. In adulthood it appears that the ERα requirement for ductal growth is slightly different. In these experiments, mammary epithelial cells were isolated from adult αERKO mice and wt mice and were injected into syngeneic epithelial-free mammary
fat pads of 3-wk-old female aERKO or wt mice. These studies revealed that both stromal and epithelial ERα are required for maximum mammary ductal growth (Mueller et al., 2002), similar to that in the uterine study described above (Biggsby et al., 2004). Presumably, ERα-mediated ductal morphogenesis and alveolar development may involve the induction of estrogen-responsive genes (such as PR) within the mammary gland and perhaps also in peripheral endocrine tissues that contribute to mammary gland development and function. Incidentally, mammary ductal growth is normal in ERβ knockout (βERKO) mice (Krege et al., 1998).

Stromal progesterone receptors mediate the inhibitory effects of progesterone on estrogen-induced uterine epithelial proliferation

Uterine epithelial proliferation is regulated by estradiol and progesterone, whose levels fluctuate through estrous and menstrual cycles. Estradiol induces uterine epithelial proliferation, while progesterone promotes functional differentiation and inhibits epithelial proliferation. In rodents, Martin et al demonstrated that pretreatment with progesterone inhibits estrogen-induced uterine epithelial proliferation (Martin et al., 1973). Progesterone elicits its effects through the PR, which is expressed in epithelial, stromal and myometrial cells of the uterus (Ohta et al., 1993). While it is logical that progesterone would inhibit uterine epithelial proliferation by acting through epithelial PR, the presence of stromal PR raises the possibility that progesterone may inhibit estrogen-induced uterine epithelial proliferation through it. Tissue recombinants composed of uterine tissues from PRKO and wt mice were used to resolve this issue (Kurita et al., 1998). In this way, we produced the following tissue recombinants in which PR was present or absent in epithelium, stroma, or both: wt-S+wt-E, PRKO-S+PRKO-E, wt-S+PRKO-E, and PRKO-S+wt-E. Tissue recombinants were grown as grafts in female nude mice for 4 weeks, and then all hosts were ovariectomized. Two weeks following ovariectomy, hosts received estradiol with or without progesterone. Eighteen hours after the last hormone injection, epithelial labeling index was determined using 3H-thymidine autoradiography. In response to estradiol, all four types of tissue recombinants (wt-S+wt-E, PRKO-S+PRKO-E, wt-S+PRKO-E, and PRKO-S+wt-E) showed a several-fold increase in the epithelial labeling index compared with the oil-treated group (Fig. 5). In tissue recombinants prepared with PR-positive wt stroma (wt-S+wt-E, wt-S+PRKO-E), estradiol-induced epithelial proliferation was profoundly inhibited by progesterone, even in the wt-S+PRKO-E tissue recombinants, which lacked epithelial PR. This indicated a key role for stromal PR in the inhibition of uterine epithelial proliferation (Fig. 5). Conversely, in tissue recombinants prepared with PRKO stroma (PRKO-S+PRKO-E and PRKO-S+wt-E), progesterone did not inhibit estrogen-induced epithelial proliferation. Thus, epithelial proliferation remained elevated to levels comparable to those of estradiol-treated tissue recombinants even in PRKO-S+wt-E grafts, which contained epithelial PR. These results demonstrate that stromal PR is both essential and sufficient to mediate the inhibitory effect of progesterone on estrogen-induced uterine epithelial proliferation (Kurita et al., 1998). Moreover, epithelial PR is neither necessary nor sufficient for the progesterone inhibition of estrogen-induced epithelial proliferation in the mouse uterine epithelium.

Stromal aryl hydrocarbon receptors mediate the inhibitory effects of dioxin on estrogen-induced uterine epithelial proliferation

The prototypical AhR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is the most environmentally ubiquitous dioxin, and a variety of studies have indicated that TCDD can have potent anti-estrogenic effects on the rodent uterus in vivo and on the proliferation of MCF-7 cells in vitro (Gallo et al., 1986; Gierthy et al., 1993; Holcomb and Safe, 1994). Since E2 induction of uterine epithelial proliferation is induced through stromal ERα (Cooke et al., 1997), we determined whether TCDD could inhibit processes such as estradiol-induced uterine epithelial proliferation through AhR in the epithelium and/or stroma. To determine through which tissue compartment(s) TCDD acted to inhibit the E2-induced uterine epithelial proliferation, we employed a tissue separation-recombination methodology using uterine tissues from wt and AhRKO mice. Our initial results indicated that both uterine epithelium and stroma expressed AhR (Buchanan et al., 2000). Tissue recombinants consisting of a wild-type uterine stroma and wild-type (wt-S+wt-E) uterine epithelium showed a marked decrease in epithelial proliferation in response to estradiol plus TCDD compared with similar tissue recombinants grown in hosts receiving estradiol alone (Fig. 6). This inhibitory response was not seen in AhRKO-S+AhRKO-E tissue recombinants. Likewise, the anti-estrogenic effects of TCDD were not seen in tissue recombinants composed of an AhRKO uterine stroma plus a wt uterine epithelium, suggesting that the anti-estrogenic effects of TCDD were mediated through a stromal AhR. When uterine stroma from AhRKO mice was recombined with wt uterine epithelium in which...
TCDD failed to inhibit estradiol-induced uterine epithelial proliferation (Buchanan et al., 2000). Thus, TCDD acts through a stromal AhR to inhibit estradiol-stimulated uterine epithelial proliferation, while no epithelial AhR is involved. A possible explanation for these results is that the liganded AhR may alter the normal stromal response to ERα activation in this tissue to diminish the production of the putative stromal signal that induces epithelial proliferation in response to estradiol.

Regulation of epithelial differentiation in the female reproduction tract

Estradiol is a regulator of uterine epithelial secretory proteins such as lactotransferrin (Pentecost et al., 1988) and complement component C3 (Sundstrom et al., 1989). The inability of αERKO mice lacking ERα to induce lactotransferrin in response to estradiol treatment indicates that ERα is essential for this process (Couse et al., 1995). The relative role of epithelial versus stromal ERα in the induction of
uterine secretory proteins and their mRNAs was examined using the same four types of uterine tissue recombinants used in our earlier studies of estradiol-induced epithelial proliferation: wt-S+wt-E, wt-S+αERKO-E, αERKO-S+αERKO-E, αERKO-S+wt-E (Buchanan et al., 1998b). These were grown in intact female nude mice, which were subsequently ovariectomized and injected daily with oil or estradiol for up to 3 days. Grafts were recovered, and lactoferrin and C3 were assessed at the protein and mRNA levels. The results demonstrated that lactoferrin protein or mRNA induction by estradiol required both stromal and epithelial ERα (wt S wt E)(Fig. 7). The absence of ERα in either tissue compartment abolished the induction of lactoferrin or its mRNA. Similar results were obtained with C3, demonstrating the dependence of estradiol-induced secretory protein production on the simultaneous presence of both the stromal and epithelial ERα. This dependence on both stromal and epithelial ERα contrasts dramatically with estradiol-induced epithelial mitogenesis, which requires only stromal ERα. The obligatory role for epithelial ERα in mediating the normal estradiol-induced production of uterine epithelial secretory products is the first known function attributed to epithelial ERα in vivo. Furthermore, the demonstration that both stromal and epithelial ERα are necessary for the production of uterine epithelial secretory proteins represents the first time any epithelial response to estradiol has been shown to simultaneously require ERα in both the stromal and epithelial compartments.

Comparable results were obtained for vaginal epithelium, which responds to estradiol by undergoing cornification, a response that involves both proliferative and differentiative events. Through analysis of the same four types of vaginal tissue recombinants used earlier, we concluded that the full manifestation of vaginal cornification involves epithelial proliferation, which is mediated via stromal ERα and expression of proteins associated with cornification (keratin 10, involucrin, etc.) whose expression is mediated via the epithelial ERα (Fig. 8) (Buchanan et al., 1998a; Kurita et al., 2001a). Thus, the emerging pattern is that epithelial proliferation is mediated via stromal receptors, and the functional differentiation of the epithelium simultaneously requires epithelial receptors.

Another differentiation marker of uterine and vaginal epithelia is the PR, which is one of the most well studied estrogen-regulated genes. In most cells/tissues, PR is up-regulated by estrogen. Accordingly, in vaginal epithelial cells in vivo, PR is undetectable in ovariectomized mice and strongly expressed following estrogen treatment (Kurita et al., 2000a). In contrast, mouse uterine epithelium expresses high levels of PR in the absence of estrogen, and, surprisingly, estrogen down-regulates PR in mouse uterine epithelium (Murakami et al., 1990; Tibbetts et al., 1998).

This unusual expression pattern of PR is unique for uterine epithelium of rats and mice. Predictably, PR is highly expressed in uterine epithelium of αERKO mice and is not down-regulated by estrogen (Kurita et al., 2000a). In vaginal epithelium of αERKO mice PR is low or undetectable with or without estrogen treatment. Thus, regulation of PR via ERα signaling is completely opposite between uterine and vaginal epithelia in the mouse. Estrogen up-regulates PR in vaginal epithelium and down-regulates PR in uterine epithelium. It is puzzling how ERα can have opposite effects (activation and suppression) on PR in the uterus and vagina. We have demonstrated fundamental differences in the cellular mode of estrogen regulation of epithelial PR in the uterus and vagina through analysis of tissue recombinants composed of vaginal and uterine epithelia (E) and stroma (S) from wt and αERKO mice: wt-S+wt-E, wt-S+αERKO-E, αERKO-S+αERKO-E, αERKO-S+wt-E. These tissue recombinants were grown for 4 weeks under the renal capsule of female hosts, which were then ovariectomized. PR was undetectable or very low in both vaginal epithelium and stroma in all 4 types of vaginal tissue recombinants in ovariectomized hosts treated with oil only. PR was strongly expressed in both the epithelium and stroma of wt-S+wt-S vaginal tissue recombinants treated with estradiol but was undetectable in αERKO-S+αERKO-E vaginal tissue recombinants (Fig. 9) (Kurita et al., 2000a). In wt-S+αERKO-E and αERKO-S+wt-E tissue recombinants, E2-treatment respectively induced PR only in the wt-S or wt-E, respectively. Therefore, PR expression is directly regulated by estrogen in vivo in vaginal stroma and epithelium through ERs in the responding tissue.

In contrast to the vagina, all four types of uterine tissue recombinants (wt-S+wt-E, wt-S+αERKO-E, αERKO-S+αERKO-E, αERKO-S+wt-E) strongly express PR in epithelia of the oil-treated group. Epithelial PR was down-regulated by E2 in uterine tissue recombinants prepared with wt-S (wt-S+wt-E and wt-S+αERKO-E). Even in the absence of functional epithelial ERα in uterine wt-S+αERKO-E tissue recombinants, estradiol dramatically reduced the PR level in the epithelium (Fig. 10). Conversely, estradiol did not down-regulate epithelial PR in uterine tissue recombinants prepared with αERKO-S (αERKO-S+wt-E and αERKO-S+αERKO-E) despite the expression of epithelial ERα in the αERKO-S+wt-E tissue recombinants. These results definitively demonstrate that ERα in uterine stroma are essential for the down-regulation of uterine epithelial PR induced by estradiol treatment. ERα in uterine epithelium are neither necessary nor sufficient for an estrogen-induced down-regulation of uterine epithelial PR. Thus, uterine epithelial PR expression is regulated by estradiol through a paracrine mechanism, which is mediated via stromal ERαs (Kurita et al., 2000a).
This differential pattern of regulation by PR in uterine and vaginal epithelia is the result of uterine and vaginal stromal induction during development (Kurita et al., 2001a). Both the uterus and the upper vagina (Müllerian vagina) develop from the Müllerian duct. Tissue recombination experiments have shown that neonatal uterine and vaginal epithelia can be induced by the stroma to change their differentiation pattern during the first week after birth (Cunha, 1976; Kurita et al., 2001a). We have recently determined that the p63 transcription factor is the identity switch for uterine and vaginal epithelial differentiation (Kurita and Cunha, 2001; Kurita et al., 2004). When p63 is induced by a vaginal stroma (VgS) in uterine Müllerian duct epithelial (UtE) cells, vaginal differentiation occurs (Table 1). When vaginal Mullerian duct epithelial cells (VgE) are grown in association with a uterine stroma (UtS, UtS+VgE recombinants), p63 is not induced and uterine differentiation occurs. Accordingly, the Mullerian vagina of...
p63 knockout mice differentiates into uterine epithelium and expresses a high level of PR in the absence of E2 (uterine pattern of PR regulation) (Kurita et al., 2004).

In the uterine epithelium, progesterone antagonizes estradiol-induced gene expression. Both the induction of lactoferrin and repression of PR by estradiol are inhibited by progesterone in the uterine epithelium. Tissue recombination studies using uterine tissues from PRKO and wt mice have confirmed the involvement of stromal PR in the antagonism by progesterone of estradiol induction of PR and lactoferrin in uterine epithelia. Progesterone regulation of epithelial PR was studied in wt-S+wt-E and PRKO-S+wt-E tissue recombinants (Fig. 11). Estradiol down-regulated epithelial PR in both types of tissue recombinants, but epithelial PR remained elevated following estradiol plus progesterone treatment only in wt-S+wt-E tissue recombinants. PR was not regulated by progesterone in PRKO-S+wt-E tissue recombinants even though PR was expressed in the epithelium. Thus, progesterone requires stromal PR to inhibit estradiol-induced down-regulation of epithelial PR (Kurita et al., 2000b). Epithelial PR is not sufficient in itself.

The inhibitory effect of progesterone on uterine lactoferrin expression was also studied in the four types of uterine tissue recombinants (wt-S+wt-E, PRKO-S+wt-E, wt-S+PRKO-E and PRKO-S+PRKO-E). Estradiol induced lactoferrin in all types of uterine tissue recombinants irrespective of the PR status of the epithelium and stroma, presumably by estradiol action via ER $\alpha$ present in both the epithelium and stroma. Progesterone blocked estradiol-induced lactoferrin expression only in wt-S+wt-E tissue recombinants in which PR was present in both the stroma and epithelium (Fig. 12). Progesterone failed to block E2-induced lactoferrin expression in wt-S+PRKO-E, PRKO-S+wt-E and PRKO-S+PRKO-E tissue recombinants. Thus, both epithelial and stromal PR are essential for the full antagonism of progesterone on an E2-induced lactoferrin expression (Kurita et al., 2000b).

### Table 1. Uterine and vaginal epithelial features are induced and determined by the mesenchyme with which the epithelium is associated during development.

<table>
<thead>
<tr>
<th>Stroma</th>
<th>Epithelium</th>
<th>Differentiation</th>
<th>p63</th>
<th>Keratin14</th>
<th>Involucrin (E2)</th>
<th>PR oil</th>
<th>E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>uterus</td>
<td>uterus</td>
<td>uterus</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>vagina</td>
<td>vagina</td>
<td>vagina</td>
<td>+</td>
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<td>–</td>
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</tr>
<tr>
<td>uterus</td>
<td>vagina</td>
<td>uterus</td>
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</tr>
<tr>
<td>vagina</td>
<td>uterus</td>
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<td>+</td>
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</tbody>
</table>

From Cunha (1976); Kurita et al. (2001a, 2004)

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**Induction of prostatic squamous metaplasia requires both stromal and epithelial ER$\alpha$**

Estrogenic effects on the prostate are complex and involve both direct and indirect actions. Squamous metaplasia is a direct effect of estrogen on the prostate induced by long-term exposure to high levels of exogenous or endogenous estrogen (Risbridger et al., 2001a). Prostatic squamous metaplasia is usually reversible following removal of the estrogenic stimulus. Squamous metaplasia of the prostatic epithelium is characterized by the total replacement of the columnar secretory epithelium by layers of stratified squamous cells (Risbridger et al., 2001a). Estrogenic induction of prostatic squamous metaplasia is mediated through ER$\alpha$ signaling. The prostate has two estrogen receptor subtypes, ER$\alpha$ and ER$\beta$ (Lubahn et al., 1993; Kuiper et al., 1996). ER$\alpha$ and ER$\beta$ are predominantly expressed in the prostatic stroma and epithelium, respectively (Lau et al., 1998; Prins et al., 1998; Chang and Prins, 1999). The roles of ER$\alpha$ and ER$\beta$ in prostatic squamous metaplasia have been studied through use of aERKO and $\beta$ERKO mice (Risbridger et al., 2001a). In our studies, DES induced prostatic squamous metaplasia in wt and $\beta$ERKO mice, but not in a ERKO mice (Risbridger et al., 2001b). Thus, signaling through an ER$\alpha$ is required for the induction of prostatic squamous metaplasia. The respective role of epithelial versus stromal ER$\alpha$ in the induction of prostatic squamous metaplasia was examined by analyzing prostatic tissue recombinants constructed with a prostatic stroma (S) and epithelium (E) from wt or aERKO mice: wt-S+wt-E, a ERKO-S+ aERKO-E, wt-S+aERKO-E, and aERKO-S+wt-E. All tissue recombinants were grown under the renal capsules of intact male nude mice for four weeks to allow prostatic development to proceed. The hosts were...
then treated with subcutaneous pellets containing the synthetic estrogen, diethylstilbestrol (DES), for three weeks. Squamous metaplasia was only observed in wt-S+wt-E tissue recombinants and not in ṦERKO-S+_halSError, wt-S_绦ERKO-E, ṦERKO-S+wt-E tissue recombinants (Fig. 13). These results revealed the importance of stromal-epithelial interactions and a requirement of both epithelial and stromal ERα to elicit estrogen-induced prostatic squamous metaplasia. It should be noted that prostatic squamous metaplasia is similar in many ways to vaginal cornification. Both conditions involve epithelial proliferation as well as epithelial keratinization. Presumably, the development of prostatic squamous metaplasia involves the stimulation of epithelial proliferation mediated by stromal ERα and an epithelial squamous differentiation mediated by epithelial ERαs.

**Stromal-epithelial interactions in the induction of cervicovaginal adenosis**

While estrogens are important physiologic signaling molecules in the female genital tract, exogenous estrogens administered during development can have both teratogenic and carcinogenic consequences in animals and humans. For example, DES was prescribed for pregnant women to prevent miscarriage, and women exposed to DES in utero (DES daughters) exhibit genital tract abnormalities includ-
ing cervicovaginal adenosis, which is characterized by the presence of columnar epithelia in the cervix and/or vagina (Robboy et al., 1981). DES daughters are at risk to develop clear-cell adenocarcinoma of the vagina (Herbst et al., 1971), and adenosis is thought to be its precursor (Robboy et al., 1981). Perinatal exposure of mice to DES induces a spectrum of reproductive tract lesions similar to those observed in humans (Forsberg, 1976; McLachlan et al., 1980; Plapinger and Bern, 1979). The mechanism of DES-induced cervicovaginal adenosis is not well understood, except for the fact that ERα is essential for the development of cervicovaginal adenosis induced by neonatal DES-exposure (Couse et al., 2001).

p63 is an identity switch for the differentiation of Müllerian duct epithelium into the squamous vaginal epithelium (Kurita et al., 2004). p63 is a homologue of the p53 tumor suppressor gene (Yang et al., 1998). During the course of differentiation, p63 is initially undetectable in the embryonic Müllerian duct. The middle portion of the Müllerian duct, destined to become the uterine epithelium, remains p63-negative. The lower portion of the Müllerian duct, destined to become the stratified squamous cervicovaginal epithelium, expresses p63 from about 18 days of gestation and thereafter. In mice treated neonatally with DES, the normal expression of a continuous layer of p63-positive basal epithelial cells was greatly inhibited in the Müllerian vagina and the cervix. This was manifested at day 5 as large gaps in the p63-positive basal epithelial layer in the fornix and common cervical canal, the primary site for the development of cervicovaginal adenosis (Forsberg and Kalland, 1981). A few days after the last DES injection on day 5, most of the gaps in the p63-positive basal epithelial layer in the fornix and common cervical canal were filled-in with p63-positive cells, but small patches of p63-negative epithelial cells remained in the fornix and common cervical canal as adenotic lesions. Thus, DES impaired the normal ontogeny of p63 in the developing cervicovaginal epithelium leading to foci of p63-negative glandular tissue in the cervix and vagina, adenosis (Kurita et al., 2004).

DES induces vaginal adenosis via ERα. Thus, DES disruption of p63 expression was never seen in the cervicovaginal epithelium of αERKO mice, i.e., the p63-positive
basal epithelial layer developed normally in DES-treated aERKO mice. DES action on p63 in the developing cervicovaginal epithelium therefore requires ERα. In the neonatal cervix and Mullerian vagina, ERα is highly expressed in both epithelial and mesenchymal cells (Kurita et al., 2001a). Consequently, DES action may be elicited via signaling through ERα in either epithelial and/or mesenchymal cells. To determine whether DES disrupts the induction of p63 and thus induces adenosis via ERα in epithelial or mesenchymal cells, the four types of tissue recombinants were constructed with a uterine epithelium (UtE) and vaginal mesenchyme (VgM) from aERKO and wt mice (wt-VgM+ wt-UtE, wt-VgM+aERKO-UtE, aERKO-VgM+wt-UtE and aERKO-VgM+aERKO-UtE) and grafted into ovariectomized female nude mice. In untreated hosts, the vaginal mesenchyme induced a p63-positive squamous basal epithelial layer in an originally p63-negative uterine epithelium in all four types of tissue recombinants (Fig. 14), which is indicative of normal cervicovaginal epithelial differentiation. When hosts were treated with DES, squamous p63-positive basal cells were not detected in the tissue recombinants prepared with a wt-uterine epithelium (wt-VgM+ wt-UtE and aERKO-VgM+wt-UtE), indicating that DES had inhibited normal cervicovaginal differentiation by direct action on epithelial ERα. In contrast, when an aERKO uterine epithelium was used to construct the tissue recombinants (wt-VgM+ aERKO-UtE and aERKO-VgM+aERKO-UtE), a normal p63-positive squamous basal epithelial layer developed even when the hosts were treated with DES. These results demonstrate that DES acts via the epithelial ERα to the inhibit induction of p63 and thus to induce adenosis in cervicovaginal epithelium. DES action via the mesenchymal ERα does not inhibit p63 expression, and thus the normal squamous differentiation of cervicovaginal epithelium occurs (Kurita et al., 2004).

**Conclusions**

The importance of the interaction of the stroma or its embryonic/fetal precursor, mesenchyme, with epithelia in the differentiation, growth, and morphogenesis of many organs has been well documented. The data presented here emphasize that stromal-epithelial interactions also play a critical role in the hormonal response of epithelial cells of male and female reproductive tracts and mammary glands. A complete understanding of the actions of hormones on their respective target organs requires a determination of the mechanism by which the stroma and epithelium normally communicate with each other and how this pattern of communication is altered by hormonal binding to stromal versus epithelial receptors. The experimental systems described here utilize tissue from gene knockout mice in conjunction with tissue separation/recombination and provide potentially valuable tools to investigate the respective roles of epithelial versus stromal hormone receptors, which is critical for understanding of the mechanisms by which hormones regulate epithelial growth, differentiation, and function. Finally, the paracrine mediators that are involved in these regulatory cell-cell interactions need to be elucidated. A variety of growth factors have been implicated as paracrine mediators.

**References**


