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# Tissue interactions and estrogenic response during human female fetal reproductive tract development

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ABSTRACT

The role of tissue interactions was explored to determine whether epithelial differentiation within the developing human reproductive tract is induced and specified by mesenchyme in tissue recombinants composed of mouse vaginal mesenchyme + human uterine tubal epithelium (mVgM+hTubE). The tissue recombinants were grown in DES-treated ovariectomized athymic mice. After 2–4 weeks of in vivo growth, several vaginal specific features were expressed in the human tubal epithelium. The mesenchyme-induced effects included morphological change as well as expression of several immunohistochemical markers. Although the mesenchyme-induced shift in vaginal differentiation in the human tubal epithelium was not complete, the partial induction of vaginal markers in human tubal epithelium verifies the importance of mesenchymal-epithelial interactions in development of the human female reproductive tract.

In a separate experiment, DES-induction of uterine epithelial progesterone receptor (PGR) and estrogen receptor 1 (ESR1) was explored in tissue recombinants composed of wild-type or *Esr1*KO mouse uterine mesenchyme + human fetal uterine epithelium (wt UtM+hUtE and *Esr1*KO UtM+hUtE). The rationale of this experiment was to determine whether DES-induction of PGR and ESR1 is mediated directly via epithelial ESR1 or indirectly (paracrine mechanism) via mesenchymal ESR1. DES-induction of uterine epithelial ESR1 and PGR in *Esr1*KO UtM+hUtE tissue recombinants (devoid of mesenchymal ESR1) formally eliminates the paracrine mechanism and demonstrates that DES induction of human uterine epithelial ESR1 and PGR is directly mediated via epithelial ESR1.

## 1. Introduction

The tacit, but usually unproven, assumption inherent in animal models is that they are reflective of human biology. This approach is generally useful, even though substantial differences exist between human and animal anatomy, development and pathology. One field for which animal/human pathology is particularly congruent is the effects of exogenous estrogens on the developing female reproductive tract. Administration of the potent synthetic estrogen, diethylstilbestrol (DES), to pregnant women from the 1940s to the 1970s resulted in a broad spectrum of estrogen-induced malformations of the uterine tubes, uterine corpus, cervix and vagina that include T-shaped uterotubal junctions, malformed incompetent cervix, abnormally shaped endometrial cavity, vaginal adenosis as well as clear cell vaginal adenocarcinoma (Jefferies et al., 1984; Rennell, 1979; Stillman, 1982; Titus-Ernstoff et al., 2010; Herbst et al., 1971, 1975; Robboy et al., 1977, 1984, 2018; Hoover et al., 2011). An immense animal literature preceded/confirmed the effects of exogenous estrogens on female reproductive tract development. In addition, animal studies have provided a molecular underpinning for the teratogenic effects of exogenous estrogens on urogenital development (Herbst and Bern, 1981; Bern and Talamantes, 1981; Bern et al., 1984; McLachlan et al., 1975, 2001; McLachlan, 1981; Newbold et al., 1983; Newbold and MaLachlan, 1985; Newbold, 1995, 2004, 2008; McLachlan and Newbold, 1996; Kurita et al., 2004; Kurita, 2011; Laronda et al., 2012, 2013). The animal literature on this topic is replete with

*Abbreviations:* ESR1, Estrogen receptor alpha; DES, diethylstilbestrol; PGR, progesterone receptor

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estrogen-induce vaginal, cervical, uterine and tubal (oviductal) anomalies including cervicovaginal adenosis. While animal models are useful and relevant to many aspects of human biology/pathology, they are frequently the basis of governmental policy designed to protect human health. Accordingly, whenever possible it is important to establish by experimental means the relevance and predictability of animal studies to human biology/pathology.

Even though mouse-human similarities are now appreciated in estrogen-induced anomalies in the female reproductive tract, the molecular mechanisms that lead to human malformations remain enigmatic, despite some clues from animal studies (McLachlan and Newbold, 1996; Kurita et al., 2004; Kurita, 2011; Laronda et al., 2012, 2013; Terakawa et al., 2016). Direct experimentation on xenografts of human fetal female reproductive tracts treated with DES have provided important insights into the genesis of human malformations and have provided essential bio-endpoints of estrogenic endocrine disruptors. In this regard, we pioneered xenograft methods in 1982 in which human fetal female reproductive tracts were grown in athymic mouse hosts treated with DES and other hormonally active agents (Cunha et al., 1987b, 1987a; Robboy et al., 1982; Taguchi et al., 1983). Unfortunately molecular and immunohistochemical advances, not yet discovered, prevented exploration of biological mechanisms in our earlier studies. More recently, we have revisited human female reproductive tract development in a series of three papers that included a compendium of differentiation markers and how DES administration affects them in vivo (Cunha et al., 2017a, 2017b; Robboy et al., 2017).

Studies carried out over 40 years ago established that uterine and vaginal mesenchyme induces and specifies epithelial differentiation (Cunha, 1976; Kurita, 2010, 2011; Kurita et al., 2001, 2005). Accordingly, vaginal mesenchyme instructively induces uterine epithelium to undergo vaginal epithelial differentiation (VgM+UtE⇝vaginal differentiation), and uterine mesenchyme instructively induces vaginal epithelium to undergo uterine epithelial differentiation (UtM +VgE⇝uterine differentiation). These inductive effects involve both morphological as well as molecular effects on the target epithelium. During vaginal development in mice, inductive cues from vaginal mesenchyme elicit epithelial expression of ΔN p63 (an isoform of p63) in Müllerian epithelium, which specifies vaginal squamous epithelial differentiation (Kurita and Cunha, 2001; Kurita et al., 2004, 2005; Terakawa et al., 2016). p63 is a member of the p53 family of transcription factors. Likewise, immunohistochemical detection of ΔNp63 in fetal and adult human vaginal epithelium suggests a similar role of ΔNp63 in human vaginal epithelial differentiation (Kurita et al., 2005; Fritsch et al., 2012, 2013; Cunha et al., 2017a). The current paper explores the role of mesenchymal-epithelial interactions in a tissue recombinant model consisting of mouse vaginal mesenchyme + human fetal uterine tube epithelium (mVgM+hTubE). The rationale for this particular experimental model is that expression of several differentiation markers is vastly different in vaginal versus tubal epithelium.

In both human and mouse female reproductive organs, estrogen receptor 1 (ESR1; also known as estrogen receptor α) is the dominant receptor for estrogen (Matsuzaki et al., 1999; Dupont et al., 2000). Earlier, we detailed the ontogeny of ESR1 during human fetal uterine development (Cunha et al., 2017a), demonstrating that ESR1 is first expressed in mesenchymal cells of human uterine corpus. Indeed, ESR1-immunoreactivity in uterine epithelial cells is rarely seen before the 21st gestational week, when endogenous estrogen levels are elevated (Oakey, 1970), thus suggesting that uterine epithelial ESR1 may be estrogen induced. Analysis of human fetal uterine xenografts treated with DES has verified this prediction (Cunha et al., 2017b). However, given that prior to DES treatment, ESR1 was detected in uterine mesenchyme and not epithelium, there are two potential mechanisms of DES induction of uterine epithelial ESR1: (a) DES may induce epithelial ESR1 directly via epithelial ESR1 whose expression is below the sensitivity of immunohistochemistry. (b) Alternatively, DES may induce epithelial ESR1 indirectly via mesenchymal ESR1 (paracrine mechanism). The same question is relevant to DES induction of epithelial progesterone receptor (PGR) in the developing human female reproductive tract (Cunha et al., 2017b).

The goal of the current paper based on our prior work (Cunha et al., 2017a; Robboy et al., 2017) is to use xenograft models (a) to determine the role of mesenchymal-epithelial interactions in epithelial differentiation during human female reproductive tract development, and (b) to determine whether estrogen regulates human uterine epithelial ESR1 and PGR via direct or paracrine mechanisms using tissue recombinants composed of human fetal uterine epithelium combined with mouse uterine mesenchyme derived from wild-type or *Esr1* knockout (*Esr1*KO) mice.

## 2. Materials and methods

### 2.1. General comments

The Committee on Human Research at UCSF (IRB# 12–08813) approved the collection of human fetal specimens devoid of patient identifiers after elective termination of pregnancy. Fetal age was estimated using heel-toe length (Drey et al., 2005). Gender was determined by Wolffian and Müllerian duct morphology as previously described (Robboy et al., 2017). Female internal genitalia were identified and isolated from the abortus specimen using a dissecting microscope. For this study 10 human fetal specimens were used at 8, 9, 10, 12, 13, 14, and 18 weeks of gestation.

### 2.2. Response of human fetal grafts of uterine corpus to DES in vivo

Intact human fetal reproductive tracts containing the uterine tube, uterine corpus, uterine cervix and vagina were grown for 4 weeks under the renal capsule of untreated and DES-treated (20 mg DES subcutaneous pellet) of ovariectomized female athymic mice as described previously (Cunha et al., 2017b). Histology and immunohistochemistry for ESR1 and PGR were performed on tissue sections as described below.

### 2.3. Preparation of heterotypic tissue recombinants

Tissue recombinant studies included: (a) mouse vaginal mesenchyme + human uterine tubal epithelium (mVgM+hTubE) and (b) wild-type or *Esr1*KO mouse uterine mesenchyme + human fetal uterine epithelium (wt UtM+hUtE and *Esr1*KO UtM+hUtE). For mVgM +hTubE tissue recombinants, mouse vaginal mesenchyme was isolated from 3-day-old neonatal mice and the tube epithelium was derived from 12 to 13 week specimens. To explore regulation of human uterine epithelial ESR1 and PGR, uterine mesenchyme was isolated from 5-day-old wild-type and *Esr1*KO neonatal mice and the human uterine epithelium was derived from 10 to 12 week specimens. Heterozygous male and female *Esr1*KO mice, a gift from Drs. Pierre Chambon and Andrée Krust, were bred to produce the *Esr1*KO neonatal mice, which were genotyped as described previously (Dupont et al., 2000). Tissue recombinant and xenografting methods have been described previously (Cunha, 1976; Cunha and Baskin, 2016). For mVgM+hTubE, wt UtM +hUtE and *Esr1*KO UtM+hUtE tissue recombinants, all hosts were ovariectomized at the time of grafting and were treated with a 20 mg DES pellet or were untreated (sham). For hosts bearing mVgM+hTubE tissue recombinants, the rationale for treating the hosts with DES was to promote stratified squamous vaginal differentiation. In response to DES grafts of human fetal uterine tube remain simple columnar, while grafts of human fetal vagina differentiate a thick glycogenated stratified epithelium (Cunha et al., 2017b). Thus, should vaginal differentiation be elicited in human tubal epithelium by mouse vaginal mesenchyme, epithelial differentiation should be distinctive. After 2 or 4 weeks of growth under the renal capsules, the tissue recombinants were