Roles of p63 in the diethylstilbestrol-induced cervicovaginal adenosis

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Summary

Women exposed to diethylstilbestrol (DES) in utero develop abnormalities, including cervicovaginal adenosis that can lead to cancer. We report that transient disruption of developmental signals by DES permanently changes expression of p63, thereby altering the developmental fate of Müllerian duct epithelium. The cell fate of Müllerian epithelium to be columnar (uterine) or squamous (cervicovaginal) is determined by mesenchymal induction during the perinatal period. Cervicovaginal mesenchyme induced p63 in Müllerian duct epithelium and subsequent squamous differentiation. In *p63*$^{-/-}$ mice, cervicovaginal epithelium differentiated into uterine epithelium. Thus, p63 is an identity switch for Müllerian duct epithelium to be cervicovaginal versus uterine. P63 was also essential for uterine squamous metaplasia induced by DES-exposure. DES-exposure from postnatal day 1 to 5 inhibited induction of p63 in cervicovaginal epithelium via epithelial ERα. The inhibitory effect of DES was transient, and most cervicovaginal epithelial cells recovered expression of p63 by 2 days after discontinuation of DES-treatment. However, some cervicovaginal epithelial cells failed to express p63, remained columnar and persisted into adulthood as adenosis.

Key words: Columnar-squamous transformation, Müllerian duct, Endocrine disruptor, Uterus, Estrogen receptor α

Introduction

Diethylstilbestrol (DES) is a synthetic estrogen that was prescribed to prevent miscarriage in pregnant women. It has been estimated that two to four million individuals were exposed to DES during pregnancy from 1946 to 1971 (Giusti et al., 1995). Women exposed to DES in utero (DES daughters) exhibit genital tract abnormalities, including cervicovaginal adenosis, that are characterized as the development of columnar epithelium in the cervix and/or vagina (Robboy et al., 1981). DES daughters are at risk of developing cervicovaginal clear-cell adenocarcinoma (Herbst et al., 1971), and cervicovaginal adenosis is thought to be the precursor of adenocarcinoma (Robboy et al., 1981). Perinatal exposure of mice to DES generates a spectrum of reproductive tract lesions similar to those observed in humans (Forsberg, 1976; McLachlan et al., 1980; Plapinger and Bern, 1979). Using this animal model, many genes have been identified as a potential cause of DES-induced abnormalities in the female reproductive tract. For example, perinatal DES exposure disrupts expression of *Wnt7a* (Miller et al., 1998a), *Hoxa10* and *Hoxa11* (Ma et al., 1998) in the upper Müllerian duct. These genes play important roles in development and/or function of the uterus. However, the mechanism of DES-induced cervicovaginal adenosis is not understood. Estrogen receptor α (ERα) is essential for development of cervicovaginal adenosis induced by neonatal DES-exposure (Couse et al., 2001); however, the target of DES and ERα in the cervicovaginal adenosis is still unclear.

Columnar and squamous epithelia are dramatically different. The major functions of columnar epithelium are absorption and secretion, while stratified squamous epithelia form barriers. In addition, cytoskeletal and cell-adhesion molecules are different in columnar versus squamous epithelia. For example, cytokeratins 5 and 14 are expressed in squamous epithelial cells, and are essential to maintain the integrity of squamous epithelium (Chan et al., 1994; Ehrlich et al., 1995; Hutton et al., 1998; Rugg et al., 1994). It is not understood how squamous and columnar epithelia differentiate from their embryonic precursors. The female reproductive tract is an excellent model with which to study the program of epithelial differentiation because it is lined with two distinct types of epithelia that differentiate from a common precursor. The Müllerian vagina, cervix, uterus and oviduct develop from the embryonic Müllerian duct, which is composed of a uniform layer of pseudo-stratified columnar epithelial cells. In the mouse, the Müllerian duct epithelium undergoes organ-specific morphogenetic changes during postnatal development induced by uterine and vaginal mesenchyme (Cunha, 1976; Kurita et al., 2001a). In the uterus, the epithelium gives rise to columnar luminal and glandular epithelia. In the Müllerian vagina and cervix, the columnar epithelium transforms into a stratified squamous epithelium. In adulthood, columnar uterine and squamous cervicovaginal epithelia meet at the squamo-columnar junction (SCJ) in the cervix. In the mouse, epithelial cells of the Müllerian duct are fully capable of being induced by heterotypic mesenchyme to undergo uterine or vaginal differentiation prior to 7 days postnatal, after which this developmental plasticity is gradually lost. By adulthood, most

uterine and cervicovaginal epithelial cells do not change their phenotype in response to induction by heterotypic mesenchyme (Cunha, 1976; Kurita et al., 2001a).

Historically, uterine and vaginal epithelial phenotypes have been judged by histology. However, 17β-estradiol (E_2) and progesterone (P_4) modify epithelial morphology in the uterus and vagina, and thus the effects of ovarian steroids must be always considered. For example, uterine epithelium can stratify as a result of hyper-proliferation in response to E_2. In this case, the stratification is reversible, and does not involve expression of squamous-epithelial markers (Kurita et al., 2001a). The uterus of progesterone receptor (PR) knockout mice shows a stratified epithelial phenotype due to hyperplasia caused by unopposed estrogen action (Lydon et al., 1995), which is due to loss of PR in the stromal cells (Kurita et al., 1998). Thus, epithelial stratification (histology) per se is not the most reliable marker distinguishing uterine versus cervicovaginal epithelia. Unequivocal identification of cervicovaginal epithelial differentiation can be achieved by examination of squamous markers such as K14, which are not modified by steroid hormones (Kurita et al., 2001a). Likewise, uterine epithelial differentiation is best assessed by estrogen-independent expression of PR, which is a unique feature of rodent uterine epithelium (Kurita et al., 2000). In this study, we used multiple markers to assess differentiation of uterine and cervicovaginal epithelia.

We report the crucial role of p63 as an identity switch in differentiation of Müllerian duct epithelium. P63 (KET, p51A, p51B, p40 or p73L) is a homologue of the p53 tumor suppressor gene (Yang et al., 1998). *p63*$^{-/-}$ mice have skin defects and lack organs arising from epidermis such as mammary and salivary glands (Mills et al., 1999; Yang et al., 1999). As development of uterus, cervix and vagina occurs mostly during postnatal stages, the phenotype of *p63*$^{-/-}$ mice in the mature female reproductive tract is unknown because of newborn lethality. Through rescue of *p63*$^{-/-}$ cervicovaginal rudiments by grafting, we have shown that cervicovaginal epithelium of *p63*$^{-/-}$ mice expresses the full spectrum of uterine epithelial markers. In this study, we describe the ontogeny of p63 in the mouse female reproductive tract and demonstrate a key role for p63 in DES-induced cervicovaginal adenosis

Materials and methods

Animals and tissue recombination

Mice were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, and the UCSF LARC committee approved all procedures described here. BALB/c, CD-1, C57B6 and adult female nude mice were purchased from Charles River (Wilmington, MA).

p63$^{-/-}$ embryos were produced by heterozygous intercrosses of p63^{Brdm2} mice (Mills et al., 1999). Pregnant females were sacrificed at 16-18 days postcoitum, and embryos were harvested. Female reproductive organs from 18 *p63*$^{-/-}$ and 32 *p63*$^{+}$ (hetero *p63*$^{+/-}$ and wild-type *p63*$^{+/+}$) embryos were used. *p63*$^{-/-}$ mice were identified visually as limbless. The genotypes of embryos were confirmed by PCR. Uterine and vaginal rudiments were divided into four parts [uterus, lower cervix and upper Müllerian vagina (cervicovaginal), lower Müllerian vagina and sinus vagina] and were grafted under the kidney capsule of athymic mice. Hosts were ovariectomized 4 weeks after grafting. Two weeks after the ovariectomy, hosts were injected with 125 ng E_2 in 100 μl corn oil (tocopherol-stripped, ICN Biomedicals, Aurora, OH) or 100 μl corn oil alone daily for 3 days before termination. Silastic capsules filled with 25 mg DES (Wang et al., 2001) were implanted into some hosts bearing *p63*$^{-/-}$ and *p63*$^{+}$ uterine grafts at the time of grafting. Two weeks later, the grafts were harvested for analysis.

ERα$^{-/-}$ mice on a C57BL6J/129Svj mixed genetic background were produced and genotyped as described previously (Lubahn et al., 1993).

For the UtE/VgM recombination experiment with *ERα*$^{-/-}$ mice, hosts were ovariectomized at the time of grafting and 25 mg DES Silastic-capsules were implanted into half of the hosts. The capsules were removed 2 weeks after the grafting, and tissues were harvested 2 weeks after removal of the DES-capsule.

The detailed protocol for tissue recombination has been described previously (Kurita et al., 2001a). Results were based upon analysis of 8-11 tissue recombinants/group from at least three independent experiments.

Primary culture of uterine epithelial cells

The methods for epithelial separation and primary culture have been described previously (Kurita et al., 2000). Briefly, epithelial cells were embedded in a 1:1 mixture of growth factor reduced Matrigel (BD Bioscience, Franklin Lakes, NJ) and rat tail collagen, and cultured in a 1:1 mixture of DME and Ham's F-12 media (Gibco, Gaithersburg, NY) with transferrin (5 μg/ml) (Sigma) and insulin (10 μg/ml) (Gibco).

Immunohistochemistry (IHC)

Methods for IHC have been described (Kurita et al., 1998). Mouse monoclonal antibodies were used at the following concentrations: anti-ERα 1D5 (1:50), anti-cytokeratin 10 (1:25, DAKO, Carpenteria, CA), anti-p63 4A4 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p63 Ab2 (1:100, Lab Vision, Fremont, CA), anti-Ki67 (1:100, Novacastra Laboratories, Burlingame, CA), anti-K8 LE41 (1:5) and anti-K14 LE001 (1:5, gift from Dr E. B. Lane, University of Dundee, Dundee, UK). Rabbit and goat polyclonal antibodies were used at the following concentrations: anti-P-cadherin goat (1:100) and anti-p130 rabbit (1:200) (Santa Cruz Biotechnology), anti-PR rabbit (1:100, DAKO) and anti-involucrin rabbit (1:2000, Covance, Princeton, NJ). Anti-K19 rabbit monoclonal antibody (1:1) was obtained from Dr Robert Pytela, UCSF, San Francisco, CA. Positive signals were visualized as brown precipitates utilizing 3,3′-diaminobenzidine tetra-hydrochloride (Sigma).

P63-positive epithelial index

To determine the p63-positive epithelial index, images of p63-IHC were captured with a DC330 camera (Dage-MTI, Michigan City, IN), interfaced with a computer. Lengths of basement membrane were measured on the images with Scion Image 1.62a (Scion, Frederick, MD), and the length of basement membrane associating with p63-positive cells against the length of total basement membrane was calculated. Approximately 2000-14,000 μm of basement membrane was analyzed for each tissue recombinant.

Neonatal DES-treatment

Neonatal CD-1, BALB/c and C57B6 mice and neonatal mice from *ERα*$^{+/-}$ breeding cages were treated with DES by the following protocol previously described (McLachlan et al., 1980). Briefly, newborn mice were injected subcutaneously with 2 μg DES in 20 μl corn oil or 20 μl corn oil from day 1. The neonatal mice received DES or oil every 24 hours for 5 or 7 days. mice were sacrificed at P1, P2, P3, P4, P5, P7, P10, P14, P21, P35 and P60. mice to be harvested at P60 were ovariectomized at P35 and treated with 125 ng E_2 in 100 μl corn oil or 100 μl corn oil alone everyday for 3 days before termination.

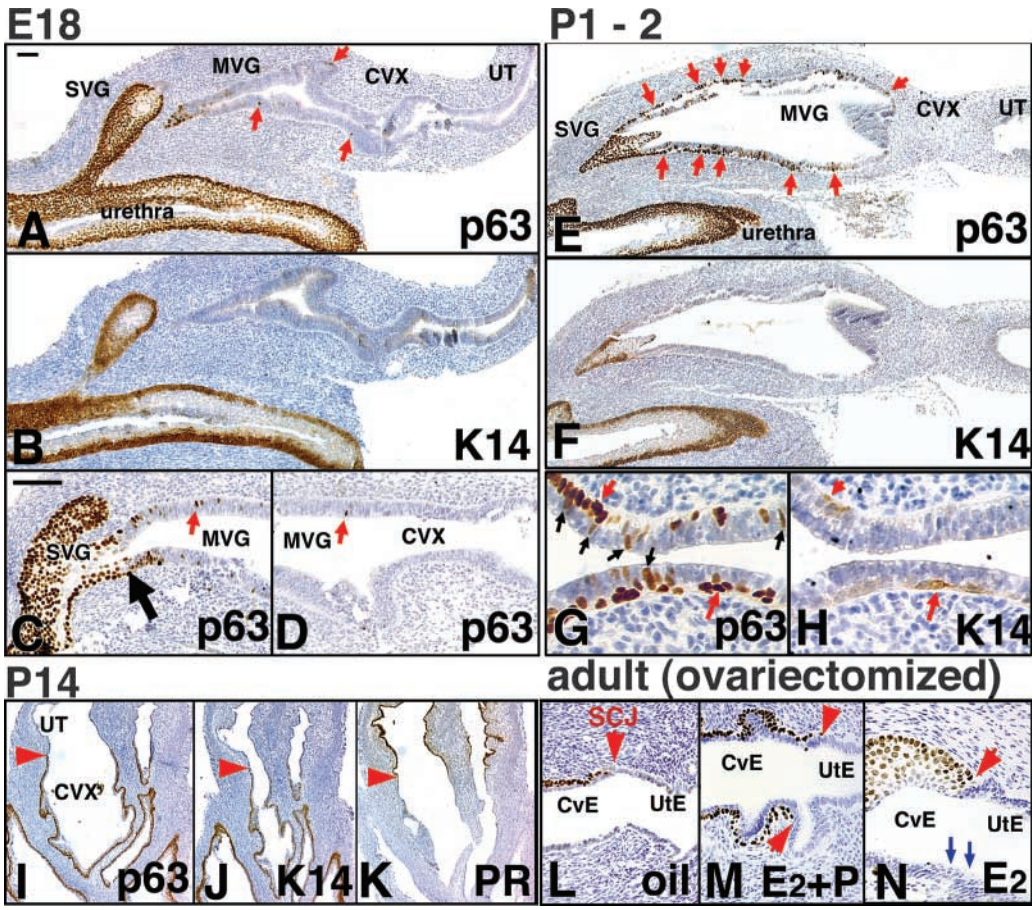

Fig. 1. Ontogeny of p63 in mouse female reproductive tract. P63, K14 and PR proteins were detected by IHC in mouse female reproductive tract. At E18, the sinus vagina (SVG) was uniformly positive for p63 (A) and K14 (B). By contrast, p63 was detected only in a small subset of columnar epithelial cells in the Müllerian vagina (MVG) (A,C,D) and the cervix (CVX) (A). Uterus (UT) was negative for p63 and K14. Red arrows indicate p63-positive epithelial cells. The black arrow (C) indicates boundary between sinus and Müllerian vaginal epithelia. At postnatal day 1 (P1), although p63-positive epithelial cells increased (E, red arrows), K14 was still undetectable in CVX and MVG (F). In the P2 CVX, p63-positive squamous basal epithelial cells were detected (G, red arrows) and some basal epithelial cells were weakly positive for K14 (H, red arrow). Some luminal columnar epithelial cells were also positive for p63 (G, black arrows). Note change in nuclear polarity in the emerging basal cells (G). By P14, the SCJ (red arrowheads) was formed, and p63 (I), K14 (J) and PR (K) expression abruptly changed at the SCJ. To test the effect of steroid hormones, ovariectomized adult female BALB/c mice were treated with oil (L), E_2+P_4 (M) or E_2 alone (N). Hormone treatments did not change the boundary of p63 expression at the SCJ. E_2 induced stratification but not p63 expression in UtE (N, blue arrows).

Results

Ontogeny of p63 in the female reproductive tract

P63 was undetectable in the Müllerian duct on embryonic day 16 (E16) (not shown). At E18, the sinus vagina (SVG) consisted of a solid epithelial cord that was uniformly positive for p63 and K14 (Fig. 1A-C). By contrast, p63 was only detected in a small subset of columnar epithelial cells in the Müllerian vagina (MVG) (Fig. 1A,C,D) and the cervix (Fig. 1A). Other vaginal epithelial differentiation markers including K14 (Fig. 1B) were not detectable in MVG at this stage (Kurita et al., 2001a). In the MVG, p63-positive cells were consistently detected at the junction between SVG and MVG (Fig. 1C).

During postnatal development, the number of p63-positive cells gradually increased in cervicovaginal epithelium (CVE) as the p63-positive epithelial layer extended cranially from the MVG into cervix. On postnatal day 1 (P1), p63 was strongly detected in a substantial portion of Müllerian-vaginal and cervical epithelial cells (Fig. 1E), while K14 (Fig. 1F) and other markers of CVE (K5, K19 and p-cadherin) were still undetectable (data not shown). By P2, some p63-positive epithelial cells became squamous (with a ~90° change in nuclear polarity) and formed a basal layer in the cervix and MVG (Fig. 1G). Some of these p63-positive basal cells were weakly positive for K14 (Fig. 1H). However, many p63-positive epithelial cells in the cervix and MVG were columnar and remained in the luminal position (Fig. 1G, black arrows). These observations suggest that in Müllerian duct epithelium, expression of p63 proceeds squamous differentiation. As p63 was already detected in cervicovaginal epithelium at E18, there was at least a three-day gap between expression of p63 and K14. By P5, a p63-positive basal epithelial layer was fully formed and continuous throughout MVG and cervix (not illustrated). By P14, the SCJ was formed, and differences in expression of p63 (Fig. 1I), K14 (Fig. 1J) and PR (Fig. 1K) were clearly visible in CVE versus uterine epithelium (UtE).

Although E_2 and/or P_4 regulate histodifferentiation and functional cytodifferentiation of UtE and CVE in adulthood, E_2 and/or P_4 did not modify the adult expression pattern of p63 in the female reproductive tract. Therefore, p63 was always expressed in CVE but not in UtE (Fig. 1L-N). E_2-treatment stimulated proliferation and caused stratification of UtE (Fig. 1N, blue arrows), but the uterine epithelial cells remained negative for p63 and K14 (not shown), which were always expressed in adult CVE.

Expression of p63 in the Müllerian duct epithelium is induced by vaginal mesenchyme

Tissue recombinants were constructed with newborn uterine (UtM) or Müllerian-vaginal mesenchyme (VgM) plus UtE or

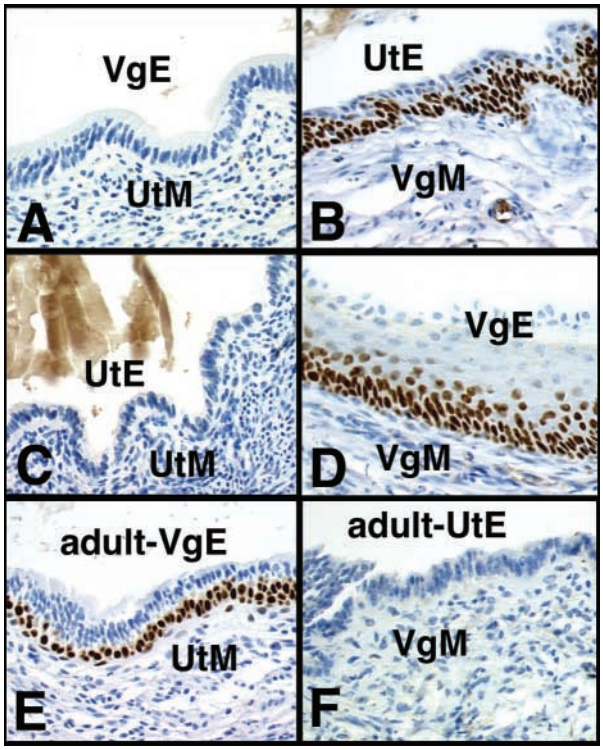

Fig. 2. Induction of p63 expression by vaginal mesenchyme. UtE and VgE isolated from newborn (A-D) or adult (2-months-old, E and F) BALB/c mice were recombined with newborn UtM or VgM. The tissue recombinants were grown as renal grafts in nude mice for 4 weeks. When newborn VgE (A) or UtE (C) was recombined with UtM, the epithelium was p63-negative and columnar. By contrast, when newborn UtE (B) or VgE (D) was recombined with VgM, epithelium was p63-positive and squamous. Thus, mesenchyme determines expression of p63 in the newborn UtE and VgE. However, the original expression of p63 in adult-VgE (positive) (E) and UtE (negative) (F) was not altered by heterotypic mesenchyme.

Müllerian-vaginal epithelium (VgE) derived from either P1 neonatal or adult BALB/c mice. When VgE from neonatal mice was recombined with UtM (VgE+UtM), the epithelial tissue developed a columnar uterine phenotype as previously reported (Cunha, 1976; Kurita et al., 2001a) (Fig. 2A). Even though a substantial portion of Müllerian-vaginal epithelial cells expressed p63 at the time of tissue recombination (Fig. 1E), the entire epithelium became negative for p63 in VgE+UtM tissue recombinants after 1 month of growth (Fig. 2A). Thus, UtM turned-off p63 expression in VgE. When neonatal-UtE was recombined with VgM (UtE+VgM), a squamous vaginal epithelium developed that was positive for p63 (Fig. 2B). Homotypic recombinants (UtE+UtM and VgE+VgM) behaved as expected (Fig. 2C,D). Thus, based upon p63 ontogeny and tissue recombination studies, p63 expression is induced in Müllerian duct epithelial cells by VgM during embryonic-neonatal development.

In contrast to newborn UtE and VgE, p63 expression was not changed when adult UtE and VgE were grown in association with heterotypic mesenchyme. When adult-VgE was recombined with UtM, the adult-VgE maintained its squamous phenotype, and p63 was still highly expressed in the basal layer after 1 month of in vivo growth (Fig. 2E). Likewise, adult-UtE recombined with VgM retained the original p63-negative columnar uterine phenotype (Fig. 2F). Therefore, most epithelial cells of the uterus and vagina lose their developmental plasticity by adulthood as reported previously (Cunha, 1976; Kurita et al., 2001a).

Phenotype of p63$^{-/-}$ mice

The rudiments of uterus, cervix and Müllerian vagina were rescued from *p63*$^{-/-}$, wild-type (*p63*$^{+/+}$) and heterozygous (*p63*$^{+/-}$) embryos (E16-18) by grafting. Uterine and cervicovaginal phenotypes of *p63*$^{+/+}$ and *p63*$^{+/-}$ mice were indistinguishable by all criteria examined. Thus, both *p63*$^{+/+}$ and *p63*$^{+/-}$ progeny were referred as *p63*$^{+}$. In uterine grafts of *p63*$^{-/-}$ mice, the epithelium appeared completely normal in morphology and expression of differentiation markers (Table 1). In cervicovaginal and Müllerian-vaginal grafts from *p63*$^{-/-}$

Table 1. Phenotypes in normal and abnormal uterine and cervicovaginal epithelia

		Müllerian vagina/cervix			Uterus			
	Müllerian duct	*p63*$^{+}$			*p63*$^{+}$			UtE/VgS-TR
	(E16)	Normal†	Adenosis*	*p63*$^{-/-}$*	Normal	SQM†	*p63*$^{-/-}$	*p63*$^{+}$*
Epithelial type	Columnar	Squamous	Columnar	Columnar	Columnar	Squamous	Columnar	Columnar
Inclusion/gland	–	–	+	+	+	-	+	+
ERα	–	+	+	+	+	+	+	+
p130	–	+	+	+	+	+	+	+
p63	–	+	–	–	–	+	–	–
K14	–	+	–	–	–	+	–	–
K19	–	+	–	–	–	+	–	–
P-cadherin	–	+	–	–	–	+	–	–
K10	–	+	–	–	–	+	–	–
Involucrin	–	+	–	–	–	+	–	–
K8	+	–‡	+	+	+	–	+	+
PR (–E2)	–	–	+	+	+	–	+	+
PR (+E2)	–	+	+	+	–	+	–	+

*Epithelial phenotypes of p63^{+}cervicovaginal adenosis, *p63*$^{-/-}$ cervix/vagina and adult-UtE+VgS tissue recombinants were identical.
†Epithelial phenotypes of p63^{+} cervix/vagina and uterine SQM were identical.
‡Vagina only.

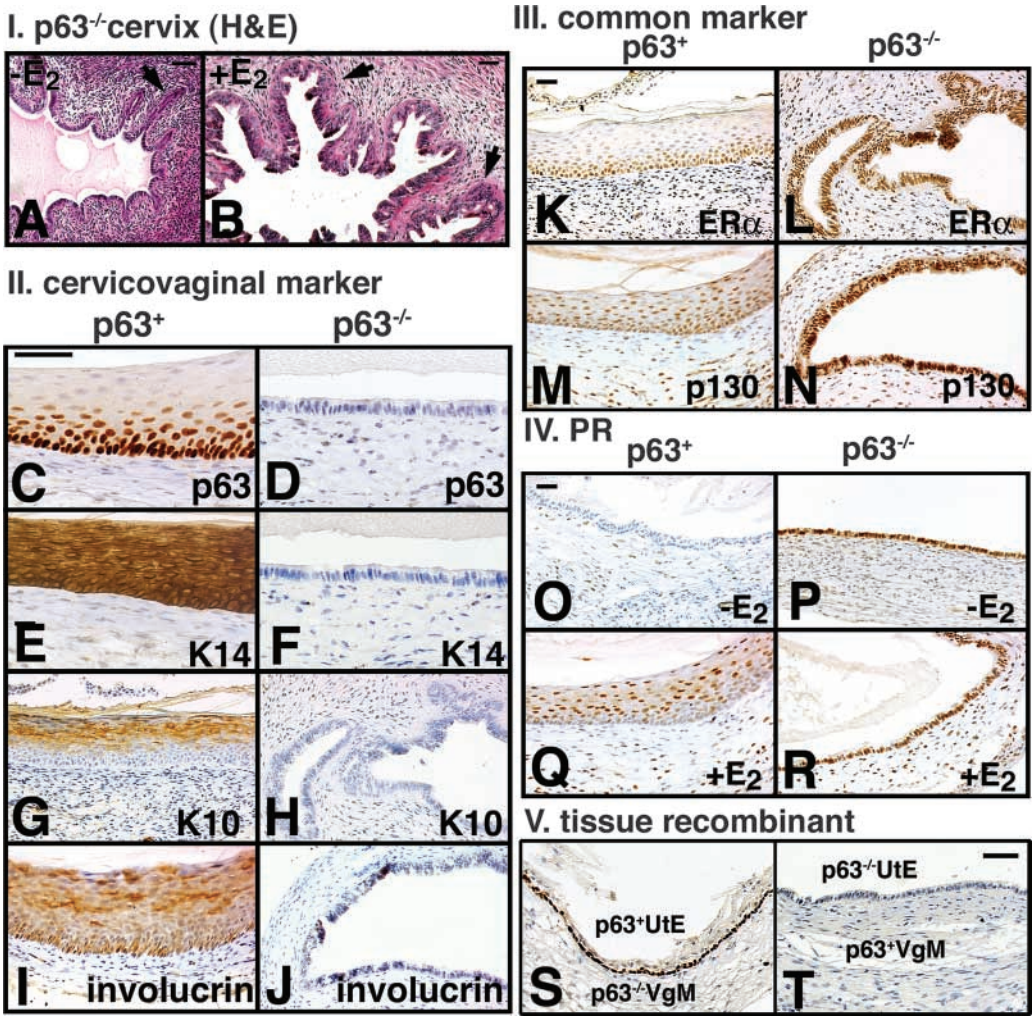

Fig. 3. Phenotype of *p63*$^{-/-}$cervicovaginal epithelium (CVE). I. Epithelial morphology. The *p63*$^{-/-}$ CVE was columnar and developed deep inclusions or glands (black arrows). The *p63*$^{-/-}$cervicovaginal grafts in the ovariectomized hosts showed uterine-like epithelial morphology (A). When the hosts were treated with E_2, the glandular p63$^{-/-}$ CVE remained columnar but became hyperplastic (B). II. Cervicovaginal epithelial markers. Markers for squamous differentiation [p63 (C,D) and K14 (E,F)] and keratinization [K10 (G,H) and involucrin (I,J)] were assessed in *p63*$^{+}$ (C,E,G,I) and *p63*$^{-/-}$ (D,F,H,J) cervicovaginal grafts by IHC. The *p63*$^{-/-}$ CVE failed to express squamous and keratinization markers. III. Common markers for uterine and cervicovaginal epithelia. Markers common for UtE and CVE [ERα (K,L) and p130 (M,N)] were examined in *p63*$^{+}$ (K,M) and *p63*$^{-/-}$ (L,N) cervicovaginal grafts by IHC. Both *p63*$^{+}$ and *p63*$^{-/-}$ CVE strongly expressed ERα and p130. IV. Regulation of PR by E_2. Expression of PR was assessed by IHC in p63^{+} (O,Q) and p63$^{-/-}$ (P,R) cervicovaginal grafts in the ovariectomized (–E_2, O and P) or E_2-treated ovariectomized (+E_2, Q and R) hosts. In the *p63*$^{+}$ CVE, PR was detectable only when the host was treated with E_2 (compare O with Q). By contrast, *p63*$^{-/-}$ CVE expressed a high level of PR in the absence of E_2 (P), which is a unique phenotype of uterine epithelium. V. VgM/UtE tissue recombination. Tissue recombinants were made with UtE and VgM from E17 *p63*$^{-/-}$ and *p63*$^{+}$ embryos. Expression of p63 was examined in the tissue recombinants. The *p63*$^{+}$ UtE developed into normal vaginal epithelium and expressed p63 when it was recombined with *p63*$^{-/-}$ VgM (S). By contrast, *p63*$^{-/-}$ UtE recombined p63^{+} VgM failed to undergo squamous differentiation (T). Scale bar: 50 μm.

mice, the epithelium failed to differentiate into squamous and instead formed a uterine-like columnar epithelium (Fig. 3). Moreover, in the *p63*$^{-/-}$cervicovaginal grafts deep epithelial invaginations or glands were always observed (Fig. 3A,B, black arrows). The glandular epithelium in the *p63*$^{-/-}$cervicovaginal grafts became hyperplastic and multilayered in response to E_2-treatment (Fig. 3B). However, markers for squamous differentiation (Fig. 3C-F) or keratinization (Fig. 3G-J) were never detected in *p63*$^{-/-}$ CVE (Table I). Although negative for cervicovaginal differentiation markers, *p63*$^{-/-}$ CVE expressed markers common to both uterine and cervicovaginal epithelia such as ERα (Esr1 – Mouse Genome Informatics) and p130 (Fig. 3K-N, Table 1). Most importantly, p63$^{-/-}$ CVE expressed a high level of PR in the absence of E_2 [Fig. 3, compare *p63*$^{+}$ (O) and *p63*$^{-/-}$ (P)], which is a feature unique to rodent UtE. Unlike normal UtE, which downregulates PR in response to E_2, the epithelium of *p63*$^{-/-}$ cervicovaginal grafts remained strongly positive for PR when treated with E_2 (Fig. 3R). However, this pattern of PR expression is actually identical to that of adult-UtE + VgM tissue recombinants (Table 1). It is known that PR in UtE is downregulated by an action of uterine stroma induced by E_2 (Kurita et al., 2000), and the stromal activity to downregulate PR in UtE is specific for uterine stroma. Therefore, when adult-UtE is recombined with VgM, the adult-UtE retained the original uterine phenotype and expressed high levels of PR in the absence of E_2, but the UtE also strongly expressed PR in the presence of E_2 (Kurita et al., 2001a). In conclusion, the profile of differentiation markers for *p63*$^{-/-}$ CVE was identical to that of normal UtE for the all criteria examined (Table 1). Hence, in the absence of p63, Müllerian CVE differentiated into UtE.

To determine if the *p63*$^{-/-}$ cervicovaginal mesenchyme (CVM) was able to induce cervicovaginal epithelial differentiation in Müllerian duct epithelial cells, E17.5 UtE from *p63*$^{-/-}$ and *p63*$^{+}$ embryos were recombined with *p63*$^{-/-}$ and *p63*$^{+}$ VgM. When p63^{+} UtE was recombined with *p63*$^{-/-}$ VgM (Fig. 3S), p63 expression and cervicovaginal differentiation was induced by the *p63*$^{-/-}$ VgM in the p63^{+} UtE.

By contrast, *p63*$^{-/-}$ UtE could not be induced to become CVE by *p63*$^{+}$ VgM (Fig. 3T). The epithelial marker expression profile in the *p63*$^{-/-}$ UtE + *p63*$^{+}$ VgM tissue recombinants was identical to *p63*$^{-/-}$ cervicovaginal grafts (not shown). Thus, the uterine phenotype of p63$^{-/-}$ CVE was due to the absence of p63 in the epithelial cells even though *p63*$^{-/-}$ CVM has ability to induce normal cervicovaginal epithelial differentiation.

Uterine mesenchyme is required to induce uterine epithelial differentiation

As CVE differentiated into UtE in the absence of p63, it appeared that the uterine phenotype might be the default differentiation endpoint for embryonic Müllerian duct epithelium, suggesting that induction by UtM may not be required for differentiation of UtE. Whether embryonic UtE requires instructive induction from UtM to establish the proper uterine marker expression pattern was tested in vitro and in vivo.

At E18, UtE did not express PR (Fig. 4A) or ERα (Fig. 4B). When undifferentiated UtE from E18 embryos (E18-UtE) was cultured in an extracellular matrix for 7 days, only a small subset of the cultured epithelial cells expressed PR (Fig. 4E), which was expressed in the entire UtE in situ by P5 (Fig. 4C). In addition, whereas ERα was undetectable in UtE in situ at P5 (Fig. 4D), ERα was expressed in a substantial proportion of cultured E18-UtE cultured to the age equivalent of P5 (Fig. 4F). The comparison of PR and ERα expression in vivo versus in vitro demonstrates that the gene expression pattern of developing UtE is strongly affected by presence or absence of UtM (compare Fig. 4C,D with E,F). By contrast, when adult (2-month-old) UtE were cultured under the same conditions as the E18-UtE, the adult-UtE retained its original expression pattern of PR and ERα (Fig. 5G,H). Thus, the absence of PR expression in the cultured E18-UtE was not due to the culture conditions.

The role of UtM in the differentiation of UtE was also examined in vivo by tissue recombination experiments. E18-UtE was recombined with bladder mesenchyme (BLM) or UtM, and the UtE + UtM and UtE + BLM tissue recombinants were grafted onto contralateral kidneys of the same hosts. The grafts were grown for a month, and then marker expression was analyzed. The UtE recombined with UtM expressed both PR and ERα as expected (Fig. 4I,J). By contrast, UtE recombined with BLM in the same host failed to express PR (Fig. 4K). Thus, UtM appears to play an essential role in inducing proper uterine epithelial differentiation in Müllerian duct epithelium. As grafts of *p63*$^{-/-}$ CVE expressed the full and correct set of uterine differentiation markers, mesenchymal cells within the Müllerian-vaginal, cervical and uterine domains appear to have uterine inductive activity.

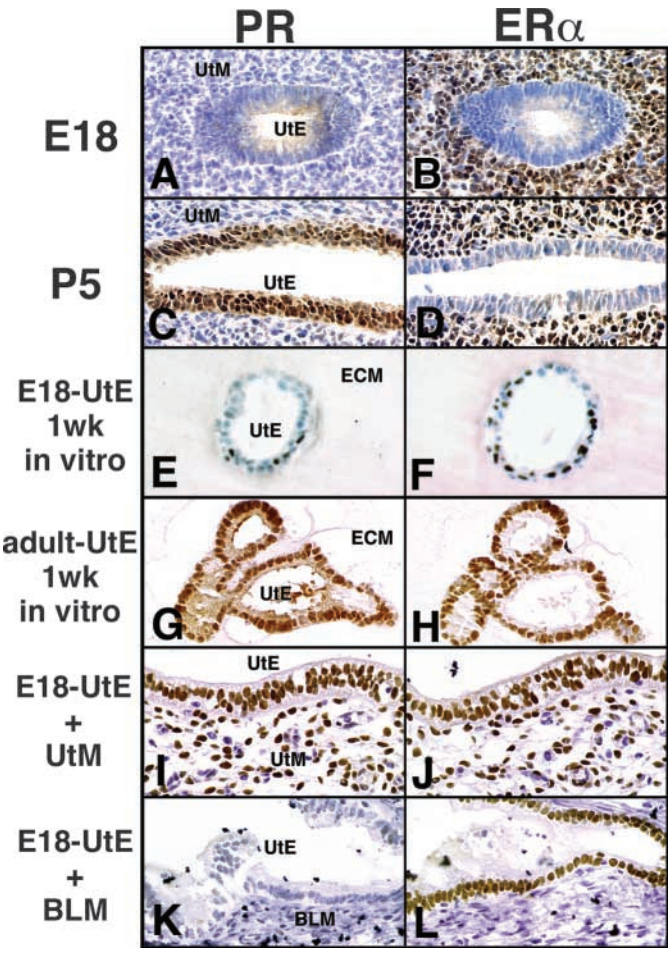

Fig. 4. Role of mesenchyme in the induction of uterine epithelial phenotype. ECM, extracellular matrix. Expression of PR (left column) and ERα (right column) in UtE was examined in vivo (A-D, I-L) and in vitro (E-H). In the E18 uterine anlage, both PR (A) and ERα (B) were undetectable in the epithelium. By P5, UtE in situ became strongly positive for PR (C) but was negative for ERα (D). By contrast, when isolated E18-UtE was cultured in vitro for 7 days, PR was detected in only a subset (<20%) of epithelial cells (E), while ERα was detected in more than a half of epithelial cells. By contrast, adult-UtE maintained a high level of PR (G) and ERα (H) under the same culture conditions. When E18-UtE was recombined with UtM, the epithelium expressed PR (I) and ERα (J) after 1 month of in vivo growth. In the same host, E18-UtE recombined with BLM did not express PR (K), while ERα (L) was strongly expressed.

P63 expression in DES-induced epithelial lesions in uterus, cervix and vagina

Cervicovaginal adenosis consists of focal areas of columnar surface epithelium or simple columnar submucosal glands in the cervix or vagina, which are normally lined with squamous epithelium (Robboy et al., 1986). In the cervix and Müllerian vagina of neonatally DES-treated mice cervicovaginal adenosis appeared as deep-inclusions or glands with openings into the lumen of cervicovaginal canal (Fig. 5A-D), or simple columnar surface epithelium within the vaginal or cervical canal (Fig. 5E-G). In DES-induced cervicovaginal adenosis, squamous differentiation markers were not expressed (Table 1). Instead, the columnar adenosis lesions expressed high levels of PR in the absence of E_2 (Fig. 5C,G) and other markers indicative of UtE. Indeed, cervicovaginal adenosis expressed the full spectrum of uterine markers (Table I).

In DES-induced uterine SQM of the mouse, the metaplastic foci (Fig. 5H-J, red arrows) expressed p63, K14 (Fig. 5H,I), K19, P-cadherin and involucrin (Table 1). In the metaplastic squamous epithelium, PR was expressed in the presence of E_2 (Table 1), but was undetectable in the absence of E_2 (Fig. 4J, red arrows). Taken together, all of these markers of uterine SQM are identical to that of CVE. In both cervicovaginal adenosis and uterine squamous lesions, the epithelium expressed both ERα (Fig. 5D) and p130 (Table 1). Thus, based upon the

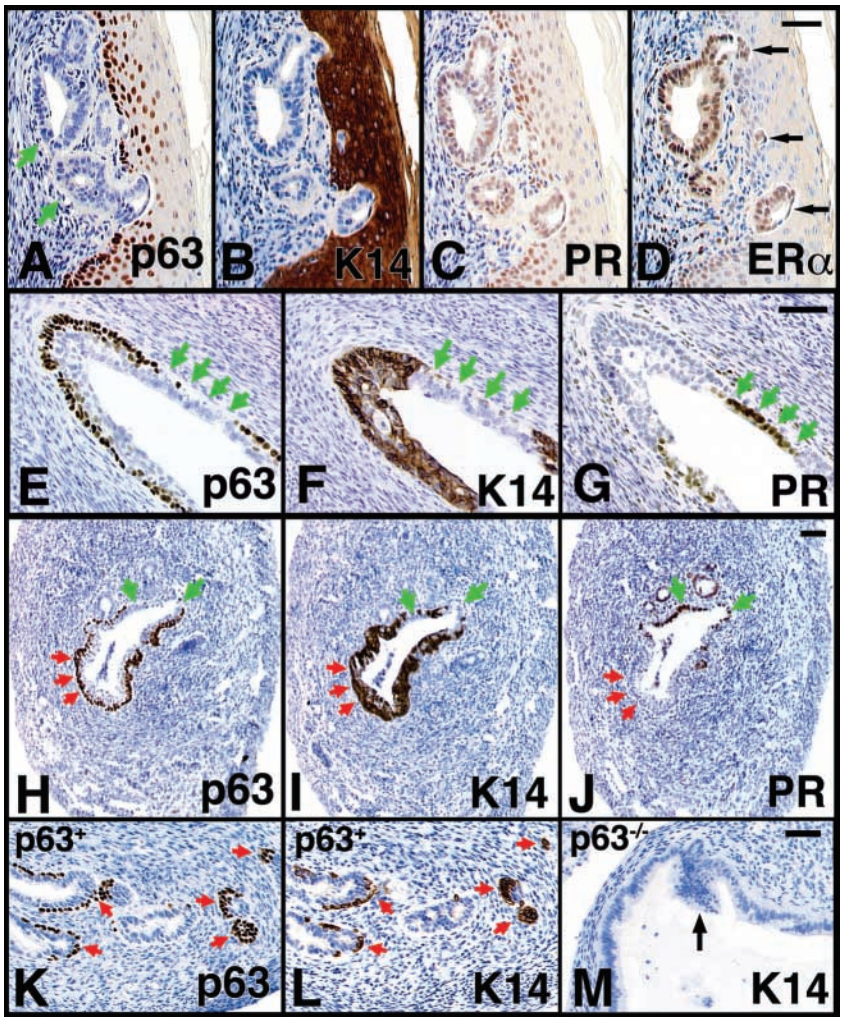

Fig. 5. Expression of differentiation markers in the DES-induced uterine and cervicovaginal epithelial lesions. Differentiation marker expression was examined in the serial sections of vaginal adenosis (A-D), cervical adenosis (E-G) and uterine SQM (H-J) in the OVX adult (P60) mice neonatally treated with DES. The openings of adenotic glands in the vagina are indicated by black arrows (D). Green and red arrows indicate columnar and squamous cells, respectively. Epithelial cells in cervicovaginal adenosis were negative for p63 (A,E) and K14 (B,F) and expressed PR in the uterine pattern (C,G). Thus, adenotic epithelium was strongly positive for PR in the absence of E_2 (in ovariectomized host, C and G). In the cervix, co-existing squamous cervical epithelium retained the normal cervicovaginal phenotype and was negative for PR in the absence of E_2 (G). In the vaginal epithelium of neonatally DES-exposed mice, PR (C), C/EBP-β, involucrin and K10 (not shown) were constitutively activated. Accordingly, in the vagina PR was detected not only in the adenotic but in the entire vaginal epithelium (C) in the absence of E_2. In the adenotic epithelium, ERα was also strongly expressed (D). Metaplastic squamous cells in the uterus were positive for ERα (not shown), p63 and K14 (H and I). In the absence of E_2, PR (J) was undetectable in the SQM (red arrows), while co-existing columnar UtE was strongly positive for PR (green arrows). In $p63^+$ uterine grafts, DES induced squamous cells, which were positive for p63 (K) and K14 (L). By contrast, K14 positive squamous epithelial cells were never detected in the $p63^{-/-}$ uterine grafts (M). Scale bar: 50 μm.

morphology and the expression of all markers tested (Table 1), cervicovaginal adenosis is not persistent undifferentiated Müllerian duct epithelium, but the development of UtE in the cervix and vagina. Likewise, uterine SQM appears to be the development of CVE in the uterus. Although uterine SQM was always associated with expression of p63, whether p63 was essential for the development of uterine SQM was not clear. To answer the question, uterus from E17.5 $p63^{-/-}$ and $p63^+$ embryos were grafted into untreated or DES-treated nude mice. Expression of squamous differentiation markers was examined 2 weeks after the grafting. In the absence of DES, no epithelial cells expressed squamous markers in the *p63*$^{-/-}$ and *p63*$^+$ uterine grafts. With DES-treatment, p63- (Fig. 5K) and K14- (Fig. 5L)-positive basal epithelial cells were detected in four out of 12 $p63^+$ uterine grafts, but these markers were not detected in 16 *p63*$^{-/-}$ uterine grafts (Fig. 5M). In the presence of DES, *p63*$^{-/-}$ uterine grafts showed stratification of epithelium (Fig. 5M, black arrow), but the stratified epithelial cells were negative for K14 and involucrin (not shown). Therefore, p63 is not just a marker but is essential for development of uterine SQM.

P63 expression in the development of cervicovaginal adenosis

The ontogeny of p63 expression was examined during treatment of neonatal mice with DES. In 5-day-old oil-treated mice (control), a continuous layer of p63-positive basal epithelial cells extended from the Müllerian vagina into the cervical canal (Fig. 6A). However, when neonatal wild-type mice were treated with DES from P1 through P5, induction of p63 was greatly inhibited in the upper part of Müllerian vagina and the cervix. At P5, this was manifest as large gaps in the p63-positive basal epithelial layer in the fornix and common cervical canal (Fig. 6B, green arrows), which are the primary site for development of cervicovaginal adenosis (Forsberg and Kalland, 1981). Two days after the last DES-injection (P7) (Fig. 6D), most of the gaps in the p63-positive layer in the CVE were filled-in with p63-positive cells. However, small patches of p63-negative epithelial cells in the fornix and common cervical canal (Fig. 6D,F, green arrows) persisted into adulthood as adenosis (Fig. 6G).

When DES-treatment was extended from birth to P7, p63 expression in CVE did not recover (compare Fig. 6D,E), and many large patches of p63-negative columnar cells were observed in CVE (Fig. 6E, green arrows). This result suggests that a continuous high level of DES is required to fully block induction of p63 expression in CVE. Presumably, discontinuation of DES-treatment at P5 allows mesenchymal and epithelial tissues to interact and induce p63 in Müllerian duct epithelium.

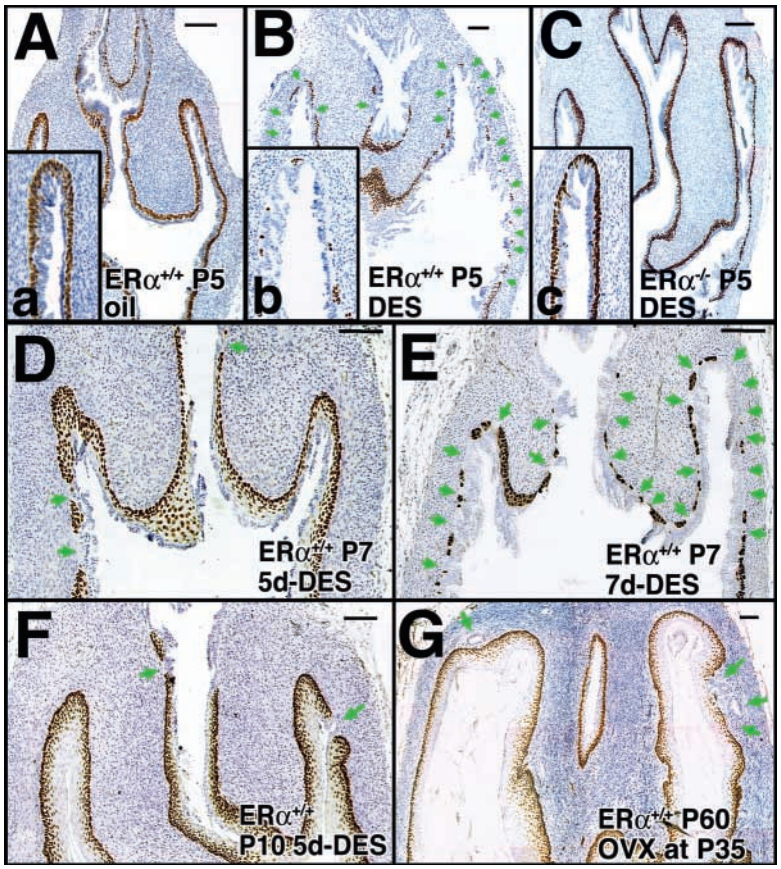

Fig. 6. Ontogeny of p63 in female reproductive tract of DES-exposed mice. P63 was detected by IHC in developing [postnatal days 5 (A-C), 7 (D,E) and 10 (F)] and adult (P60, G) mouse female reproductive tract treated with oil (A) or DES (B-G). Green arrows indicate p63-negative Müllerian-vaginal and cervical epithelial cells. DES disrupted p63 expression in Müllerian vagina and cervix via ERα [compare oil-treated $ER\alpha^{+/+}$ (A), DES-treated $ER\alpha^{+/+}$ (B) and DES-treated $ER\alpha^{-/-}$groups (C)]. Although most cervicovaginal epithelial cells expressed p63 by 2 days after the last DES-treatment (D), a small number of p63-negative columnar epithelium persisted at P7 (D) and P10 (F) (green arrows) in the Müllerian vagina and cervix, and developed into adenosis in adulthood (G). When DES-treatment was extended from birth to P7 (E), formation of p63-positive basal layer was largely inhibited at P7 (compare D with E). Scale bar: 50 μm.

DES disrupts p63 expression in CVE via ERα in the epithelial cells

$ER\alpha^{+/+}$, $ER\alpha^{+/-}$ and $ER\alpha^{-/-}$ mice were treated with DES from P1 to P5. Confirming the results described above, DES disrupted p63 expression in CVE in $ER\alpha^{+/+}$ and $ER\alpha^{+/-}$ mice. By contrast, the p63-positive basal layer of cervicovaginal epithelial cells developed normally in DES-treated $ER\alpha^{-/-}$ mice (Fig. 6C), which was identical to that of the oil-treated $ER\alpha^{+/+}$ control (Fig. 6A). Therefore, DES action on p63 in developing CVE requires signaling through ERα.

In the cervix and Müllerian vagina, ERα is highly expressed in both epithelial and mesenchymal cells by P3 (Kurita et al., 2001a). Therefore, DES action may be mediated by ERα in either epithelial and/or mesenchymal cells. To test whether DES disrupts induction of p63 via ERα in epithelial or mesenchymal cells, the four types of tissue recombinants were constructed with UtE and VgM from $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice ($ER\alpha^{+/+}$ *UtE+*$ER\alpha^{+/+}$ VgM, $ER\alpha^{+/+}$ UtE+$ER\alpha^{-/-}$ VgM, $ER\alpha^{-/-}$ UtE+$ER\alpha^{+/+}$ VgM and $ER\alpha^{-/-}$ UtE+$ER\alpha^{-/-}$ VgM) and grafted into ovariectomized female nude mice. In untreated hosts, a p63-positive squamous basal epithelial layer formed in all four types of UtE+VgM tissue recombinants (Fig. 7A, parts a and b, Fig. 7B) indicative of normal cervicovaginal epithelial differentiation. The basal cells were also strongly positive for K14 (not shown). When hosts were treated with DES, squamous basal cells were not detected in the UtE+VgM tissue recombinants prepared with $ER\alpha^{+/+}$ UtE ($ER\alpha^{+/+}$ UtE+$ER\alpha^{+/+}$ VgM and $ER\alpha^{+/+}$ UtE+$ER\alpha^{-/-}$ VgM) as assessed by histology and K14 expression (not shown), indicating that DES had inhibited cervicovaginal differentiation (Fig. 7A, parts c and e). By contrast, when $ER\alpha^{-/-}$ UtE were used to construct the tissue recombinants ($ER\alpha^{-/-}$ UtE+$ER\alpha^{+/+}$ VgM and $ER\alpha^{-/-}$ UtE+$ER\alpha^{-/-}$ VgM tissue recombinants), a p63-positive squamous basal epithelial layer was induced even when the hosts were treated with DES (Fig. 7A, parts d and f, red arrows). These results clearly demonstrate that DES acts via ERα in the epithelial cells to inhibit induction of p63 in CVE. DES action via mesenchymal ERα does not inhibit p63 expression and squamous differentiation of CVE.

Discussion

The endocrine disruptor, DES, perturbs morphogenesis and epithelial differentiation in the mouse and human female reproductive tract. Accordingly, DES elicits cervicovaginal adenosis, which involves permanent changes in epithelial cell fate. This paper integrates development of DES-induced epithelial abnormalities into the context of normal differentiation of the Müllerian duct. P63 is proposed to be an essential epithelial identity switch, which allows Müllerian duct epithelial cells to undergo cervicovaginal squamous differentiation. Based upon the data presented here, we propose the following models of epithelial differentiation in the Müllerian duct (Fig. 8).

p63 is the identity switch for the Müllerian duct epithelial fate determination

Epithelial cells in the Müllerian duct are initially p63-negative and undifferentiated (Fig. 8A, stage 1). When induced by CVM, p63 is expressed in the Müllerian duct epithelial cells. This step is essential for subsequent squamous differentiation. If p63 is not expressed, the Müllerian duct epithelial cells differentiate into UtE. When p63 is initially expressed in Müllerian duct epithelial cells, these cells remain columnar and undifferentiated for about 3 days (Fig. 8A, stage 2). At this stage, epithelial cell fate can be altered by heterotypic mesenchymal induction. The expression pattern of p63 is subsequently stabilized by mesenchymal induction, and the squamous cervicovaginal and columnar uterine cell fate gradually becomes manifest and irreversible by adulthood (Fig. 8A, stage).

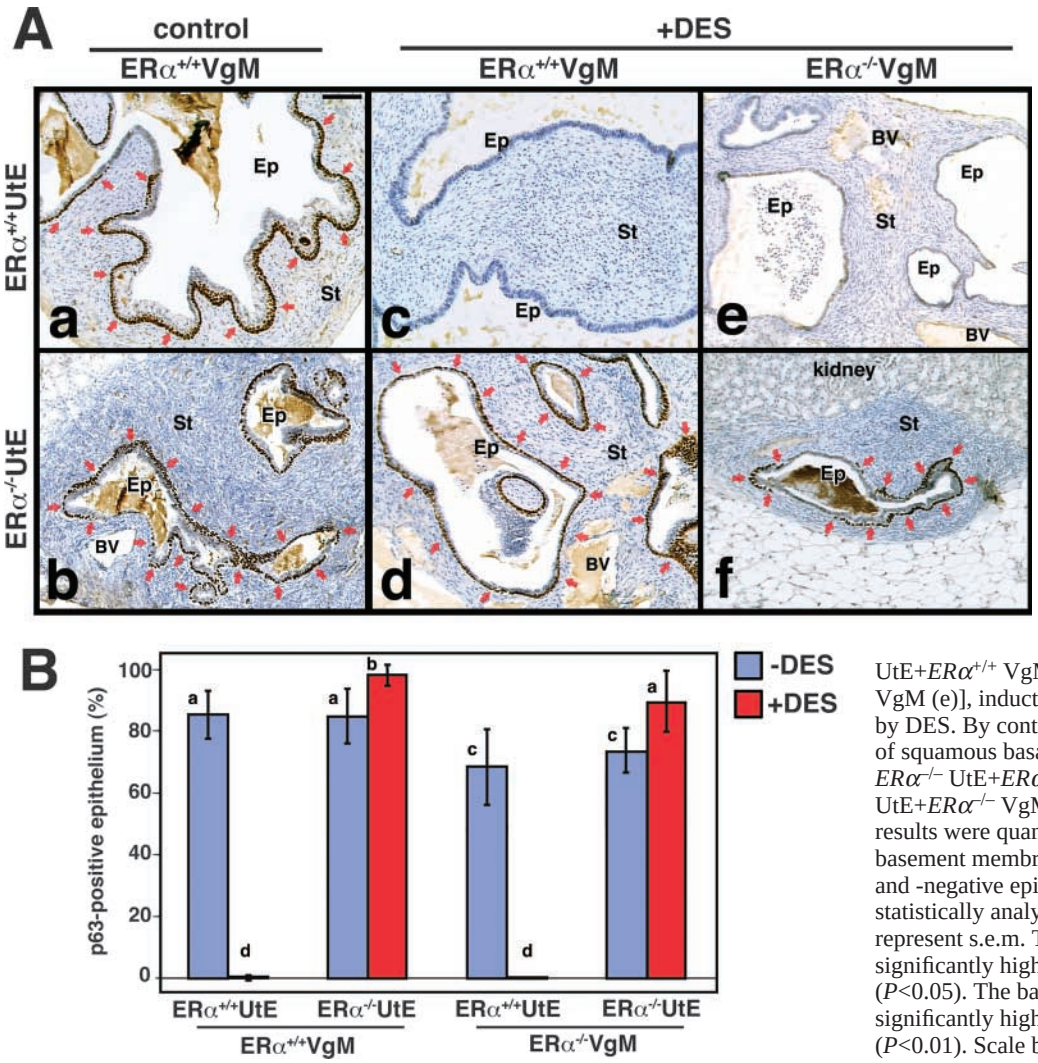

Fig. 7. Role of epithelial and mesenchymal ERα in the disruption of p63. Ep, epithelium. St, stroma. BV, blood vessel. (A) Four types of tissue recombinants were constructed with UtE and VgM from P2 $ER\alpha^{+/+}$ and $ER\alpha^{-/-}$ mice ($ER\alpha^{+/+}$ UtE+$ER\alpha^{+/+}$ VgM, $ER\alpha^{+/+}$ UtE+$ER\alpha^{-/-}$ VgM, $ER\alpha^{-/-}$ UtE+$ER\alpha^{+/+}$ VgM and $ER\alpha^{-/-}$ UtE+$ER\alpha^{-/-}$ VgM) and grafted into ovariectomized female nude mice hosts without (a,b; control) or with (c-f; +DES) DES-treatment. Without the DES-treatment, p63-positive basal layer was induced in all 4 types of tissue recombinants (a,b). When ERα was expressed in the epithelial cells [$ER\alpha^{+/+}$ UtE+$ER\alpha^{+/+}$ VgM (c) and $ER\alpha^{+/+}$ UtE+$ER\alpha^{-/-}$ VgM (e)], induction of p63 in the UtE was blocked by DES. By contrast, induction of p63 and formation of squamous basal cells were not affected by DES in $ER\alpha^{-/-}$ UtE+$ER\alpha^{+/+}$ VgM (d) and $ER\alpha^{-/-}$ UtE+$ER\alpha^{-/-}$ VgM (f) tissue recombinants. The results were quantitated by measuring the length of basement membrane associating with p63-positive and -negative epithelial cells (B). The result was statistically analyzed with ANOVA test. Error bars represent s.e.m. The bar marked with b is significantly higher than bars marked with c and d (P<0.05). The bars marked with a, b and c are significantly higher than bars marked with d (P<0.01). Scale bar: 100 μm.

This process seems to be a general principle among many organs. Besides female reproductive tract, we have studied the ontogeny of p63 in other developing mouse organs. For all organs examined, expression of p63 precedes the expression of squamous markers and morphological changes (90° nuclear polarity change) by several days. Squamous differentiation appears to consist of at least three steps: induction of p63, stabilization of p63 and subsequent squamous differentiation. As proposed above, expression of p63 itself does not transform columnar epithelium into squamous immediately, but p63 expression appears to be an essential step for squamous differentiation in general, because epithelial cells in the forestomach and seminal vesicle of $p63^{-/-}$ mice also failed to undergo squamous differentiation (T.K., unpublished).

Role of p63and epithelial-mesenchymal tissue interaction in normal and abnormal Müllerian duct development

Normal development (Fig. 8B, part a)

In embryo, the Müllerian duct is composed of uniform, undifferentiated columnar epithelial cells (i.e. stage 1 in Fig. 8A). UtM and CVM already have acquired their organ-specific inductive identities. Therefore, squamous epithelial differentiation inducing signals (red arrows) are expressed only in the CVM, whereas common uterine epithelial differentiation inducing signals (blue arrows) are expressed throughout the cervicovaginal and uterine regions of the Müllerian duct. In response to induction by CVM (red arrows), epithelial cells in the cervicovaginal area express p63 (P1-5). Subsequently, the p63-positive epithelial cells differentiate into squamous CVE as instructed by CVM. Because the signals inducing squamous differentiation are expressed only in CVM, epithelium in the uterus never expresses p63 and differentiates into columnar UtE, which requires mesenchymal inductive activity (blue arrows). By P5, the adult expression pattern of p63 is established in CVE and UtE, but the status of p63 and thus the developmental fate of the epithelial cells can still be altered by heterotypic mesenchyme (stage 2 in Fig. 8A). By P7, a substantial percentage of uterine and vaginal epithelial cells are insensitive to heterotypic uterine or cervicovaginal mesenchymal induction (Cunha, 1976), and the uterine and cervicovaginal epithelial phenotypes become irreversibly determined in the first few weeks of postnatal development. Accordingly, in adulthood almost all uterine and cervicovaginal epithelial cells cannot be re-programmed by

uterine or vaginal mesenchyme to express an alternative epithelial phenotype (stage 3 in Fig. 8A).

P63$^{-/-}$ mice (Fig. 8B, part b)

In the *p63*$^{-/-}$ mice, mesenchymal activity to induce uterine (blue arrows) and cervicovaginal (red) epithelial differentiation is normal. However, as the Müllerian duct epithelial cells cannot express p63, the entire Müllerian duct epithelium differentiates into UtE both in the uterine and cervicovaginal anlagen.

Neonatal DES treatment (Fig. 8B, part c)

DES binds to ERα in the Müllerian duct epithelial cells and blocks expression of p63, which is a prerequisite for subsequent squamous differentiation. As a result, p63 is induced in only a subpopulation of cervicovaginal epithelial cells, even though the mesenchymal signals to induce squamous epithelial differentiation (red arrow) are not affected by DES. Like most developmental processes, the timing of mesenchymal specification of uterine and cervicovaginal epithelial differentiation is tightly regulated. When DES treatment is stopped at P5, most epithelial cells in cervix and vagina respond to the mesenchymal signals (red arrows) and express p63 by P7. However, at least some Müllerian duct epithelial cells have lost sensitivity to the mesenchymal signals, as epithelial cell fate is already irreversibly fixed in some epithelial cells by this stage. These p63-negative epithelial cells in cervix and vagina have missed the time window to express p63, and thus, remain simple columnar and differentiate into UtE, which persists into adulthood as cervicovaginal adenosis. The tissue recombinant studies using *ERα*$^{+/+}$ and *ERα*$^{-/-}$ UtE and VgM demonstrate that DES inhibits expression of p63 in Müllerian duct epithelial cells through epithelial ERα. Because DES elicits abnormal epithelial differentiation via epithelial ERα, epithelial (and not mesenchymal/stromal) regulatory molecules are clearly the immediate targets of DES action in inducing adenosis. However, our model does not exclude a subordinate role of mesenchymal genes such as homeobox genes in the development of adenosis.

The correct and full expression of uterine epithelial markers in the *p63*$^{-/-}$ CVE implies the expression of uterine inductivity in the CVM as well as in the UtM. However, the molecular signals comprising uterine inductive activity may not be identical in CVM versus UtM. Many developmental regulatory genes have been detected only in the uterine regions of Müllerian duct, e.g. Wnt and Hox genes (Ma et al., 1998; Miller et al., 1998b; Pavlova et al., 1994). Our data do not exclude involvement of these molecules in normal uterine epithelial development. However, it is unlikely that a single molecule regulates all aspects of uterine epithelial differentiation, and instead a balance of several genes may be important for induction of normal UtE. CVM appears to express a reasonably complete combination of signals for inducing uterine epithelial differentiation based upon analysis of *p63*$^{-/-}$ cervix and Müllerian vagina. The squamous differentiation inducing activity in CVM is also likely to be a combination of multiple factors.

Wnt7a has been suggested as an immediate target of DES in development of vaginal adenosis because Wnt7a was downregulated by DES in the Müllerian duct, and the *Wnt7a*$^{-/-}$ mouse developed 'vaginal adenosis' (Miller et al., 1998a). However, in the paper by Miller et al., the term 'adenosis' was used to describe 'epithelial inclusions'. The glands in the *Wnt7a*$^{-/-}$ mouse appeared to be lined by keratinized squamous epithelium, which is not strictly adenosis. Such glands seem to be absent in younger *Wnt7a*$^{-/-}$ mice (more than 4 months old), which is also not in agreement with the phenotype of neonatally DES-treated mice. Therefore, it is not clear if downregulation of Wnt7a plays a role in development of columnar epithelium in cervix/vagina. Wnt7a may be upstream of p63, or the glands observed in *Wnt7a*$^{-/-}$ mice may have possibly developed via a different mechanism from DES-induced adenosis.

In DES-daughters, cervical/vaginal clear cell adenocarcinoma is rare, even though cervical/vaginal adenosis is commonly found. Although adenosis is thought to be the substrate from which clear cell adenocarcinoma develops, the mechanism by which adenosis develops into adenocarcinoma

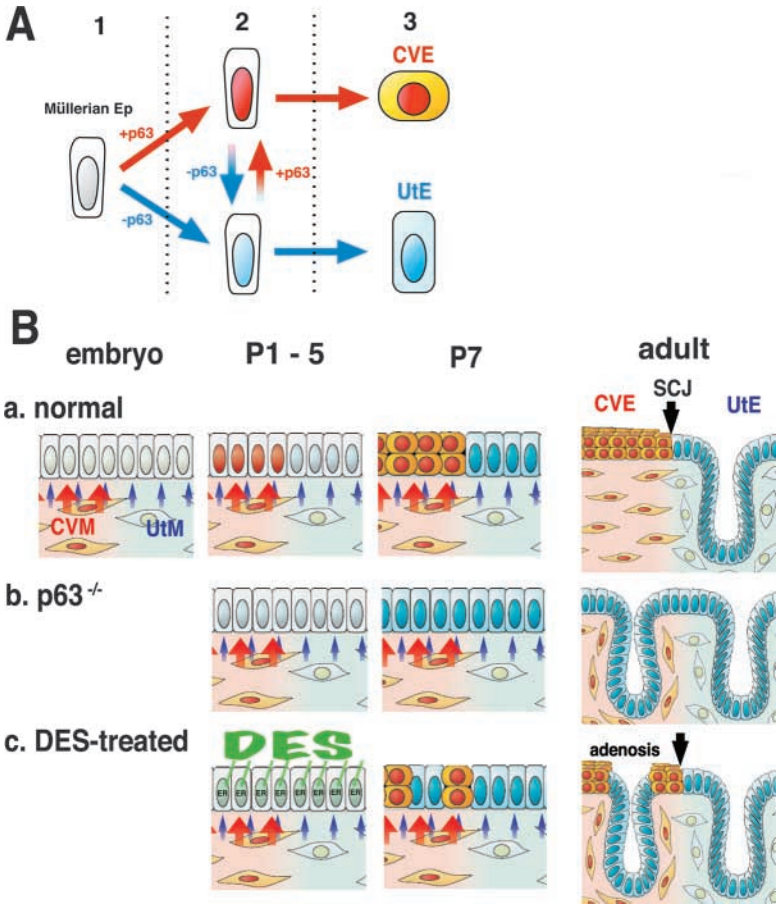

Fig. 8. Models for Müllerian duct epithelial differentiation. See the detail in the Discussion. (A) Model 1: p63 is the identity switch for the cervicovaginal epithelial fate determination. (B) Model 2: role of p63 and epithelial-mesenchymal tissue interaction in normal and abnormal Müllerian duct development.

is unclear. We have shown that DES actions on CVM do not play a role in formation of adenosis. However, growth, cell death and differentiation of epithelial tissue in the female reproductive tract are regulated by stromal cells during embryogenesis as well as adult period (Buchanan et al., 1998; Cooke et al., 1997; Kurita et al., 2001b; Kurita et al., 1998). Thus, it is likely that the DES-caused changes in the stromal cells also play important roles in the subsequent development of cervicovaginal adenocarcinoma.

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