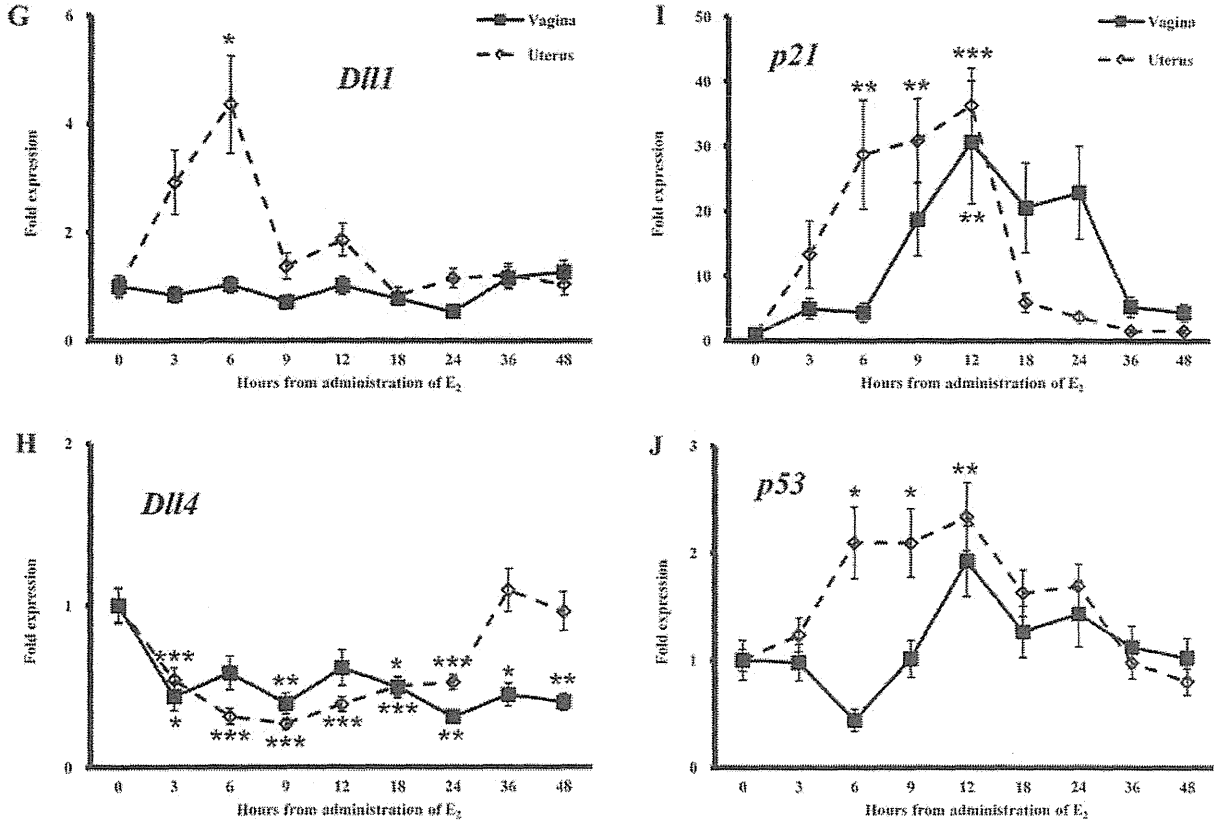

Figure 4. *Fold expression of wingless-related MMTV integration site-4 (Wnt4) (A), Wnt5a (B), Wnt 11 (C), hairy/enhancer-of-split related with YRPW motif-1 (Hey1) (D), Hey2 (E), Heyl (F), delta-like-1 (Dll1) (G), Dll4 (H), p21 (I) and p53 (J) in the vagina and uterus after a single injection of 50 ng 17β-estradiol (E_2)/g bw. Expression of these genes in control mice before E_2 injection (0 h) was regarded as the basal level (1.0). Data are the means±standard error (n=3). *p<0.05, **p<0.01 and ***p<0.001 vs. controls (one-way ANOVA).*

roles in maintaining undifferentiated quiescent cells, as in satellite muscle cells (22).

We reported that Notch ligand genes, *Dll1* and *Dll4*, are unaltered in the vagina exhibiting estrogen-independent epithelial cell proliferation in the neonatally DES-exposed mice (12). *Dll1* and *Dll4* genes had a differential expression pattern between the vagina and uterus after E_2 treatment in this study. Expression of *Dll1* was transiently increased 6 h after E_2 stimulation but it showed no change in the vagina. Dll4 is reported to be involved in tumor angiogenesis (23, 24), and is expressed in the arterial endothelium (25). *Dll4*, as well as *Wnt11*, exhibited down-regulation by E_2 in both organs, suggesting a negative correlation with cell proliferation.

Finally, we confirmed the expression of *p21* and *p53*. *p21* is a direct target of p53 tumor suppressor, and mediates p53-dependent cell cycle arrest in response to DNA damage (26). In this study, we found the expression patterns of p21 and p53 in the uterus to be similar.

In conclusion, we demonstrated the difference in the expression patterns of *Wnt4*, *Wnt5a*, *Wnt11*, *Hey1*, *Hey2*, *Heyl*, *Dll1*, *Dll4*, *p21* and *p53* between the vagina and uterus after E_2 stimulation. Moreover, we identified the localization of Wnt4 and p21 proteins in the ovariectmized adult mouse vagina exhibiting epithelial stratification, after a single injection of E_2. Additional elucidation of the molecular mechanism of cell proliferation in the vagina and uterus is essential.

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Wnt family genes and their modulation in the ovary-independent and persistent vaginal epithelial cell proliferation and keratinization induced by neonatal diethylstilbestrol exposure in mice

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ABSTRACT

Proliferation and differentiation of cells in female reproductive organs, the oviduct, uterus and vagina, are regulated by endogenous estrogen. In utero exposure to a synthetic estrogen, diethylstilbestrol (DES), induces vaginal clear-cell adenocarcinoma in humans. In mice, perinatal exposure to DES results in abnormalities such as polyovular follicles, uterine circular muscle disorganization and persistent vaginal epithelial cell proliferation. We reported the persistent gene expression change such as interleukin-1 (IL-1) related genes, insulin-like growth factor-I (IGF-I) and its downstream signaling in the mouse vagina exposed neonatally to DES. In this study, we found persistent up-regulation of *Wnt4* and persistent down-regulation of *Wnt11* in the vagina of mice exposed neonatally to DES and estrogen receptor α specific ligand. Also *Wnt4* expression in vagina is correlated to the stratification of epithelial cells with the superficial keratinization of vagina, but not epithelial cell stratification only.

1. Introduction

Estrogen-induced cell proliferation and differentiation in female reproductive organs such as oviduct, uterus and vagina are long being studied by several group of researchers (Takasugi et al., 1962; Dunn and Green, 1963; Takasugi and Bern, 1964; Forsberg, 1969; Herbst et al., 1971; McLachlan et al., 1980; Newbold and McLachlan, 1982; Newbold et al., 1985; Iguchi et al., 1986; Iguchi, 1992). Diverse biological effects of estrogens are primarily mediated via the activation of nuclear estrogen receptors, ERα and ERβ, which are ligand-inducible transcription factors (Tsai and O'Malley, 1994; Beato et al., 1995). Increase in specific gene expressions via ERα or ERβ after estrogen exposure in mice has been silenced by an ER antagonist, ICI 182,780 (Miyagawa et al., 2004a,b).

Vaginal epithelium is an intriguing model for analyzing the estrogen action in mice. It undergoes characteristic changes from a non-keratinized to a fully keratinized epithelium depending on the levels of the endogenous estrogen, estradiol (E_2), during the estrous cycle (Miller et al., 1998).

Estrogen exposure, during a critical period in the early development in mice, induces persistent, ovary-independent proliferation and keratinization in the vaginal epithelium at adulthood (Takasugi et al., 1962; Takasugi and Bern, 1964). In humans, trans-placental exposure to a synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for prevention of miscarriages from the 1940s to 1970s in the USA and European countries, resulted in vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). Although perinatal estrogenic chemical exposure induces various abnormalities, i.e., polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal adenosis, and cervico-vaginal carcinomas (Takasugi et al., 1962; Dunn and Green, 1963; Takasugi and Bern, 1964; Forsberg, 1969; Newbold and McLachlan, 1982; Newbold et al., 1985; Iguchi et al., 1986; Iguchi, 1992; Suzuki et al., 2002), the critical period of estrogen action during mouse development varies from organ to organ (Iguchi et al., 2002). DES exposure during critical developmental period results in alterations of the response to estrogens in mouse vagina, leading to a set of

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Table 1
Sequences of gene primer sets for real-time quantitative RT-PCR.

Gene	Primer (5′–3′)[a]	Product size (bp)	Gene accession no.
Wnt4	F: CATCGAGGAGTGCCAATACCA R: GACAGGGAGGGAGTCCAGTGT	70	NM_009523
Wnt11	F: ATGTGCGGACAACCTCAGCTA R: CGCATCAGTTTATTGGCTTGG	100	NM_009519

[a] F, forward; R, reverse.

subsequent abnormalities. Among them, vaginal epithelial proliferation persists even after ovariectomy in mice exposed to sufficient doses of DES during the early neonatal period (Takasugi et al., 1962; Takasugi and Bern, 1964).

Wnt genes are the vertebrate homologs of *wingless*, the Drosophila segment polarity gene is comprised of 16 members. They are a large group of highly conserved secreted glycoproteins, and play crucial roles in embryonic developmental processes (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Smalley and Dale, 1999), tumorigenesis (Tsukamoto et al., 1988; Smalley and Dale, 1999; Lustig and Behrens, 2003) and reproduction (Parr and McMahon, 1998; Vainio et al., 1999) mostly via Frizzled (Fz) receptor (Dale, 1998). Fzs constitute a large family of seven transmembrane G protein-coupled receptors and possess an extracellular cysteine-rich domain (CRD) for Wnt/binding (Wang et al., 1996; Liu et al., 1999). Among several Wnt-mediated intracellular signaling pathways (Willert and Nusse, 1998; Huelsken and Birchmeier, 2001; van Noort and Clevers, 2002), the canonical Wnt β-catenin pathway has been well studied.

The Wnt singling is highly responsive to variable hormone concentration and location (Weber-Hall et al., 1994). It is well known that Wnt singling plays roles in epithelial–mesenchymal interactions and cellular organization during embryonic and postembryonic development, involving in cell proliferation and differentiation, cell fate specification and cell-to-cell communication (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Smalley and Dale, 1999). Wnt signaling also plays a key role in murine female reproductive tract development (Miller et al., 1998; Daikoku et al., 2004), and has been suggested as a target for potential endocrine disruptors (Sassoon, 1999). Miller et al. (1998) reported that three Wnt family genes, *Wnt4*, *Wnt5a* and *Wnt7a*, were expressed in the uterus and cervix in specific epithelial–mesenchymal interactions during postnatal development and in the adult. However, the expression of Wnt genes in vagina has not yet been elucidated.

Previously, we examined the global expression of mRNA, focusing on factors involved in cell signaling in the vagina of mice exposed neonatally to DES showing persistent hyperplasia and the superficial keratinization (Miyagawa et al., 2004b). In the present study, we report that neonatal exposure of DES and ERα specific ligand induced persistent up-regulation of *Wnt4* and persistent down-regulation of *Wnt11* in mouse vagina. In addition, to clarify the role of Wnt4 in vaginal histological modulation by estrogen, we used Wnt4 hetero ($Wnt4^{+/-}$) mice, since $Wnt4^{-/-}$ mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). *Wnt4* expression was correlated to epithelial keratinization, in mouse vagina exposed neonatally to DES.

2. Materials and methods

2.1. Reagents

Diethylstilbestrol (DES) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4′,4″-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT), ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN) and estrogen receptor antagonist, ICI 182,780, were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

2.2. Animals and treatments

C57BL/6J mice and 129^{+Ter}/Sv mice were purchased from CLEA Japan (Tokyo, Japan). Wnt4 mutant mice (129^{+Ter}/Sv strain) were from Jackson Laboratory (Bar Harber, ME, USA) through Prof. K.-I. Morohashi. They were maintained under 12 h light/12 h dark at 23–25 °C and fed laboratory chow (CE-2, CLEA) and tap water ad libitum. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

C57BL female newborn mice were given 5 daily subcutaneous (s.c.) injections of 0.025 ($n=6$), 0.25 ($n=6$) or 2.5 μg ($n=6$) DES/g body weight (bw) dissolved in sesame oil or the oil vehicle alone ($n=6$) beginning from day 0 (the day of birth). Ovariectomy was performed in all mice exposed neonatally to DES, since the aim of the present study was to understand the underlying molecular mechanisms of ovary (estrogen)-independent persistent vaginal changes. These mice ovariectomized at 8 weeks and sacrificed at 10 weeks of age were used for DNA microarray analysis, reverse transcriptase polymerase chain reaction (RT-PCR), histology and immunohistochemistry. In addition, mice exposed to 2.5 μg DES/g bw neonatally and ovariectomized as adults ($n=8$) were given 5 daily intraperitoneal injections of 5 μg ICI 182,780/g bw or oil vehicle alone beginning from day 65 and killed 24 h after the last injection. Tissues were used for real-time quantitative RT-PCR and histological examination for counting number of vaginal epithelial cell layers.

Newborn female C57BL mice were given 5 daily s.c. injections of 2.5 μg DES/g bw ($n=4$), 25 μg/g bw PPT ($n=4$) or DPN ($n=4$) dissolved in 5.6% DMSO or the vehicle alone ($n=4$) beginning from day 0. These mice ovariectomized at 13 weeks were sacrificed at 15 weeks of age, and used for real-time quantitative RT-PCR and histology.

$Wnt4^{+/+}$ and $Wnt4^{+/-}$ newborn mice were given 5 daily s.c. injections of 2.5 μg DES/g bw dissolved in oil ($n=10$ or 4, respectively) or the oil vehicle alone ($n=5$ each). These mice ovariectomized at 8 weeks were sacrificed at 10 weeks of age, and analyzed *Wnt4* mRNA expression and histology.

2.3. DNA microarray analysis

Total RNA from vaginae exposed neonatally to 0.025, 0.25 or 2.5 μg DES/g bw or oil vehicle alone were extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy mini kit (QIAGEN, Chatsworth, CA, USA). Quality and quantity of total RNA were confirmed by the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Santa Clara, CA USA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for use on their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix) containing approximately 12,500 genes, and the scanned data were analyzed with GeneChip software (Affymetrix) and processed as described previously (Watanabe et al., 2004). To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment twice. The expression data were analyzed with GeneSpring software (Agilent) as described previously (Watanabe et al., 2004).

For the clustering analysis, genes expressed more than 2-fold or less than a half by neonatal DES treatment to controls were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described previously (Watanabe et al., 2002, 2003, 2004).

2.4. RT-PCR and real-time quantitative RT-PCR

Total RNA, isolated with RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each group of vaginae, was used in RT-PCR or real-time quantitative RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen). RT-PCR was carried out using AmpliTaq Gold (TAKARA, Ohtsu, Japan). Sequences of gene primer sets are given in Table 1. PCR conditions were as follows: 94 °C for 10 min, and 32 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, and 72 °C for 10 min in 25 μl volumes.

Changes in gene expression were confirmed and quantified using ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 36 cycles of 95 °C for 15 s and 60 °C for 1 min in 15 μl volumes. Relative RNA equivalents

Table 2
Microarray date of Wnt genes in vaginas of adult mice (10-week old) exposed neonatally to DES.

Gene accession no.	Name	Fold change			Prove set ID
		0.025	0.25	2.5	
NM_021279	Wingless-related MMTV integration site 1	NC	NC	NC	1425377_at
NM_023653	Wingless-related MMTV integration site 2	NC	NC	NC	1449425_at
NM_009520	Wingless-related MMTV integration site 2b	NC	NC	NC	1421465_at
NM_009521	Wingless-related MMTV integration site 3	NC	NC	NC	1450763_x_at
NM_009522	Wingless-related MMTV integration site 3A	NC	NC	NC	1422093_at
NM_009523	Wingless-related MMTV integration site 4	**4.15**	**5.95**	**3.23**	1450782_at
NM_009524	Wingless-related MMTV integration site 5A	0.82	0.91	1.33	1436791_at
NM_009524	Wingless-related MMTV integration site 5A	1.29	1.13	1.03	1448818_at
NM_009525	Wingless-related MMTV integration site 5B	NC	NC	NC	1422602_a_at
NM_009525	Wingless-related MMTV integration site 5B	NC	NC	0.80	1439373_x_at
NM_009526	Wingless-related MMTV integration site 6	NC	NC	NC	1419708_at
NM_009527	Wingless-related MMTV integration site 7A	NC	NC	NC	1423367_at
NM_001163634	Wingless-related MMTV integration site 7B	NC	**2.42**	1.97	1420891_at
NM_001163634	Wingless-related MMTV integration site 7B	NC	NC	NC	1420892_at
NM_009290	Wingless-related MMTV integration site 8A	NC	NC	NC	1422228_at
NM_011720	Wingless-related MMTV integration site 8b	NC	NC	NC	1421439_at
NM_011720	Wingless-related MMTV integration site 8b	NC	NC	NC	1421440_at
NM_139298	Wingless-type MMTV integration site 9A	NC	NC	NC	1425889_at
NM_011719	Wingless-type MMTV integration site 9B	NC	NC	NC	1451711_at
NM_009518	Wingless-related MMTV integration site 10a	NC	NC	NC	1460657_at
NM_011718	Wingless-related MMTV integration site 10b	NC	NC	NC	1426091_a_at
NM_009519	Wingless-related MMTV integration site 11	**0.21**	**0.21**	**0.29**	1450772_at
NM_053116	Wingless-related MMTV integration site 16	NC	NC	NC	1422941_at

The values shown as bold indicate statistically significant change compared to controls.

for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 1. More than three pools of samples per group were run in 3–7 groups to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control group normalized to an average of 1.0.

2.5. HE staining and immunohistochemistry

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 8 μm. Some sections were stained with standard hematoxylin and eosin. Other sections deparafinized were incubated with 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidase. After washing with PBS, the sections were incubated anti-Wnt4 antibody (R&D Systems, Inc., Minneapolis, MN, USA) at 1:200 dilution in PBS containing 1% BSA (Sigma) overnight at 4 °C. The sections were visualized with LSAB™ 2 kit, Universal (Dako, Carpinteria, CA, USA) according to the manufacturer-supplied protocol. For negative controls, normal goat immunoglobulin fraction (Dako) was used at the same dilution.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA), Student's *t*-test or Welch's *t*-test followed by *F*-test as appropriate. Differences with $P < 0.05$ were considered significant.

3. Results

3.1. DNA microarray analysis

Microarray analyses were performed to get an idea about the expression profiles of different Wnt genes, especially, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt7b* and *Wnt11* mRNA in the mouse vagina (Table 2). Surprisingly, only *Wnt4* and *Wnt7b* showed higher (3 to 6-fold) to moderate (1.97 to 2.42-fold) spikes in the neonatally DES-exposed mouse vagina than controls. On the other hand, *Wnt11* showed a decrease (0.21 to 0.29-fold) after DES treatment. However, other Wnt genes remained unaffected in vaginal epithelia after neonatal DES treatment. To verify the results of microarray analysis, we examined the expression of *Wnt4* and *Wnt11* mRNA using RT-PCR. Similar to microarray analysis, *Wnt4* or *Wnt11* expression was up- or down-regulated, respectively, in the vaginal epithelium of DES-exposed mice than controls (data not shown). Interestingly, mRNAs of all *Frizzled* family (*Fz 1–10*) were detected in the mouse vagina regardless of the neonatal DES exposure (data not shown). Henceforth, further studies were conducted with *Wnt4* and *Wnt11* only.

3.2. Estrogen responsive changes of Wnt genes in mouse vagina

Neonatal DES exposure induced vaginal epithelial stratification with superficial keratinization which was not abolished by ovariectomy (Fig. 1A and D). By contrast, neonatally oil-treated control mice had atrophied vaginal epithelium after ovariectomy (Fig. 1C and D). Expressions of *Wnt4* mRNA was high and *Wnt11* mRNA was low in the vagina of ovariectomized mice exposed neonatally to DES, however, the expression patterns in these genes in the vagina of ovariectomized mice exposed neonatally to oil vehicle alone were reversed (Fig. 1E and F). To investigate the transcriptional regulation of *Wnt4* and *Wnt11* mRNA by exogenous estrogen, we administrated ER antagonist, ICI 182,780, to neonatally 2.5 μg DES-exposed, ovariectomized mice, showing vaginal epithelial stratification and superficial keratinization. *Wnt4* expression in the neonatally DES-exposed mouse vagina, which treated with ICI 182,780, was significantly decreased, but *Wnt11* expression was not changed by anti-estrogen exposure. Surprisingly, the number of vaginal epithelial cell layers in ICI 182,780-treated mice exposed neonatally to DES, were significantly decreased (Fig. 1B, E, and F). This suggested that the DES-responsive changes in Wnt expressions and estrogen responsive epithelial cell proliferation are actually correlated.

To ascertain the role of Wnt genes in vaginal epithelial cell proliferation, we performed immunohistochemistry (IHC) of Wnt4. Ten-week-old ovariectomized mice exposed neonatally to 2.5 μg DES or oil vehicle alone, were used for IHC with anti-Wnt4 antibody (Fig. 2). Wnt4 staining was observed in the basal and middle layers of epithelial cells in vagina of mice exposed neonatally to DES (Fig. 2A), but no Wnt4 staining was observed in oil-treated control mouse vagina (Fig. 2C). This suggests that Wnt4 might be associated with epithelial cell proliferation and further keratinization. We also found that *Wnt4* was expressed in the vagina showing epithelial cell proliferation, while *Wnt11* was restricted to the atrophic vagina having 2–3 epithelial cell layers.

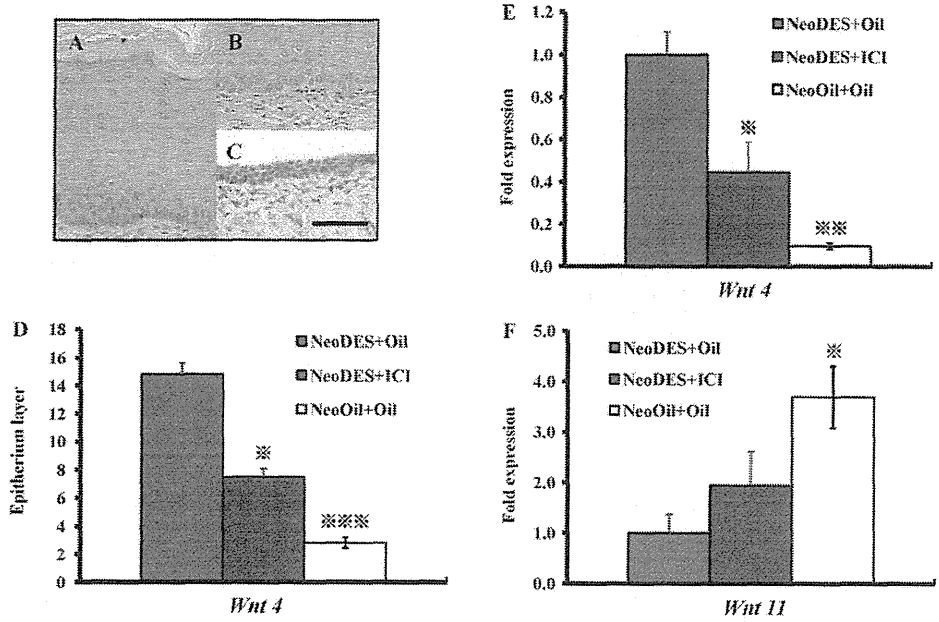

Fig. 1. Administration of anti-estrogen, ICI 182,780, reduced proliferation of vaginal epithelial cells in 10-week-old, ovariectomized mice exposed neonatally to 2.5 μg DES. Vaginal histology of ovariectomized mice exposed neonatally to DES (NeoDES) treated with oil vehicle [NeoDES + oil (A)] or 5 μg ICI 182,780/g bw [NeoDES + 5 μg/g bw ICI 182,780 (B)] and ovariectomized mice exposed neonatally to oil vehicle alone and oil before sacrifice [NeoOil + oil (C)]. Sections were stained with hematoxylin and eosin. Number of vaginal epithelial cell layers was significantly decreased in mice treated with ICI 182,780 (D). Expression profiles of Wnt gene mRNA in vagina of NeoDES mice treated with oil or ICI 182,780. *Wnt4* mRNA expression was significantly decreased in mice treated with ICI 182,780 (E), but *Wnt11* mRNA was not changed (F). Bar: 50 μm. $^{*}P < 0.05$ vs. NeoDES + oil, $^{**}P < 0.01$ vs. NeoDES + oil, $^{***}P < 0.001$ vs. NeoDES + oil (Student's *t*-test or Welch's *t*-test followed by *F*-test).

To pinpoint the role of specific estrogen receptor on such transcriptional modulation of Wnt genes and related cell proliferation, we analyzed both *Wnt4* and *Wnt11* mRNA expression and epithelial cell proliferation and keratinization in vagina of 15-week-old ovariectomized mice treated neonatally with 25 μg DPN, 25 μg PPT or 2.5 μg DES. The vaginal epithelium of these ovariectomized mice exhibited epithelial cell proliferation, stratification and superficial keratinization (Fig. 3A–D). *Wnt4* expression was found to increase after neonatal DES or PPT treatment (Fig. 3E). A simultaneous decrease in *Wnt11* expression was also observed in DES- or PPT-treated vagina (Fig. 3F). However, DPN treatment neither changed the *Wnt4* and *Wnt11* expression nor epithelial cell proliferation. Vaginal epithelia of ovariectomized mice treated neonatally with oil (Fig. 3A) or 25 μg DPN (Fig. 3D) were composed of 2–3 layers of cuboidal cells only. This highlights only *Wnt4*, but not *Wnt11*, is responsible for the persistent vaginal epithelial cell proliferation and persistent activation of ERα (Miyagawa et al., 2004a).

To clarify the role of Wnt4 in vaginal histological modulation by estrogen, we used Wnt4 hetero ($Wnt4^{+/-}$) mice, since

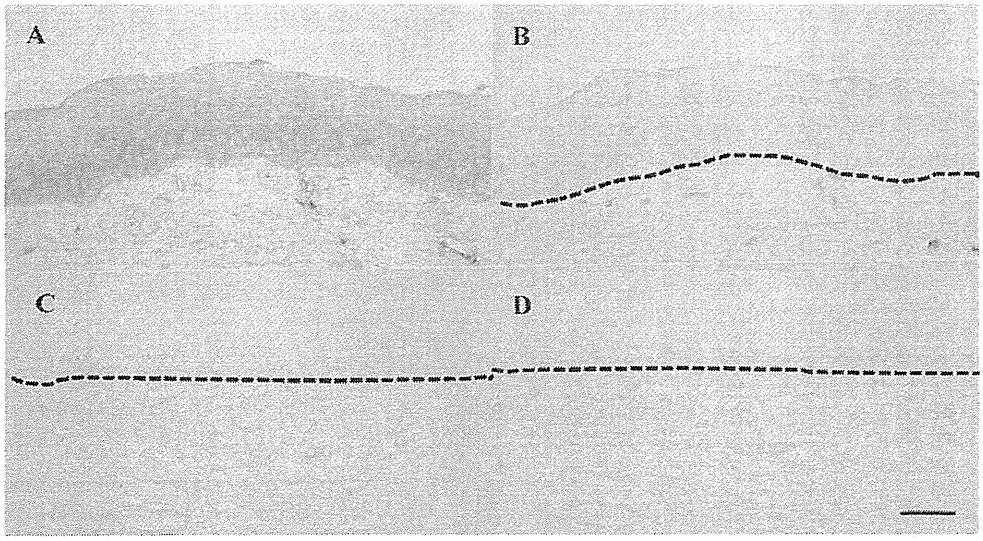

Fig. 2. Immunohistochemistry by Wnt4 antibody. Vaginae of 10-week-old, ovariectomized mice exposed neonatally to 2.5 μg DES (A, B) or oil vehicle alone (C, D). Wnt4 localized in epithelium cells of DES-exposed vagina, especially, in basal layer and middle layers (A). Control mouse vagina was not expressed Wnt4 protein (C). No immunostaining was noted when sections were incubated with preimmune serum instead of primary antibody (B, D). Bar: 50 μm. The boundary between epithelium and stroma is indicated by a dotted line.

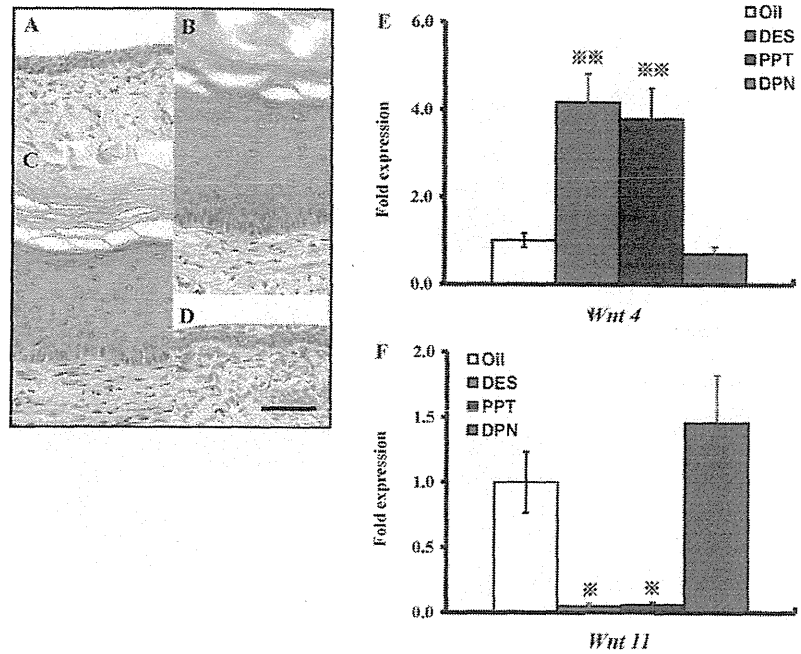

Fig. 3. Histology of vaginae of 15-week-old, ovariectomized mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note ovary-independent persistent proliferation of vaginal epithelium in DES- and PPT-treated mice. Neonatal 2.5 μg DES or 25 μg PPT treatment for the first 5 days induced persistent up-regulation of *Wnt4* mRNA (E), and persistent down-regulation of *Wnt11* mRNA (F) in mouse vagina. The expression of each mRNA in vagina of the oil-treated controls was regarded as the basal level (1.0). Bar: 50 μm. $^{*}P < 0.05$ vs. controls, $^{**}P < 0.01$ vs. controls (one-way ANOVA).

$Wnt4^{-/-}$ mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). We thought that Wnt4 expression levels in the vagina of wild type ($Wnt4^{+/+}$) mice were higher than $Wnt4^{+/-}$ mice. All $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice treated neonatally with oil, vaginal epithelia were composed of 2–3 layers of cuboidal cells (Table 3). While, all neonatally DES-exposed $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice exhibited vaginal epithelial stratification or stratification with superficial keratinization (Table 3). *Wnt4* expression levels and histology in vaginae between $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice were not different. *Wnt4* was highly expressed in neonatally DES-exposed mice both in $Wnt4^{+/+}$ and in $Wnt4^{+/-}$ mice (Fig. 4A), showing epithelial stratification with superficial keratinization (Fig. 4B and C). The vagina of $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice exposed neonatally to DES having only epithelial stratification show no up-regulation of Wnt4 expression (Fig. 4B and C) suggesting that Wnt4 plays a role in epithelial keratinization in the vagina.

4. Discussion

In the present study, we intended to clarify the mechanism of ovary-independent proliferation of vaginal epithelial cells. First, we analyzed global gene expression patterns in the DES-exposed mouse vagina. Both microarray analysis and RT-PCR showed differential interplay of Wnt family genes after DES-exposure. Especially, neonatal DES and ERα specific ligand exposure induced persistent up-regulation of *Wnt4* or persistent down-regulation of *Wnt11* in mouse vagina. In addition, we found that DES induces ER-mediated epithelial stratification and keratinization regulated by *Wnt4*.

During embryonic development, members of the Wnt gene family express in a diverse fashion. Pavlova et al. (1994) have previously noted that murine Wnt gene family, *Wnt5a*, were abundant in the adult female reproductive tract, but become relatively scarce during gestation. In addition to *Wnt4*, *Wnt5a* and *Wnt7a* are also detected at high levels in the murine female reproductive tract and had a specific mesenchymal–epithelial expression pattern (Miller et al., 1998). However, these expressions fluctuate along with estrus cycle progression (Miller et al., 1998). In present study, we confirmed the expressions of several Wnt family genes, i.e., *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt7b* and *Wnt11* mRNA in the neonatally DES-exposed or oil control mouse vagina using DNA microarray analysis. Although we recorded an elevated expression for *Wnt4* and *Wnt7b*, and reduced expression of *Wnt11*, but *Wnt5a* and *Wnt5b* remain unchanged. Therefore, we decided to focus on *Wnt4* and *Wnt11* genes in the vagina exposed neonatally to DES.

Wnt4 is known to be involved in multiple development processes, such as the formation of kidney, adrenal gland, female reproductive tracts and various cancers (Connoly and Schnitt, 1993; Stark et al., 1994; Kispert et al., 1998; Brisken et al., 2000; Smalley and Dale, 2001; Jeays-Ward et al., 2004; Yu et al., 2006). *Wnt11* is a non-canonical Wnt family, regulates ureteric branching (Majumdar

Table 3
Effect of neonatal treatment of DES on vaginae of $Wnt4^{+/-}$ and $Wnt4^{+/+}$ mice ovariectomized 2 weeks before sacrifice.

Treatments	Genotypes	No. of mice used	No. of mice showing vaginal epithelial		
			Atrophy	Stratification	Stratification with keratinization
Oil	$Wnt4^{+/+}$	5	5	0	0
	$Wnt4^{+/-}$	5	5	0	0
2.5 μg/g bw DES	$Wnt4^{+/+}$	10	0	3	7
	$Wnt4^{+/-}$	4	0	1	3

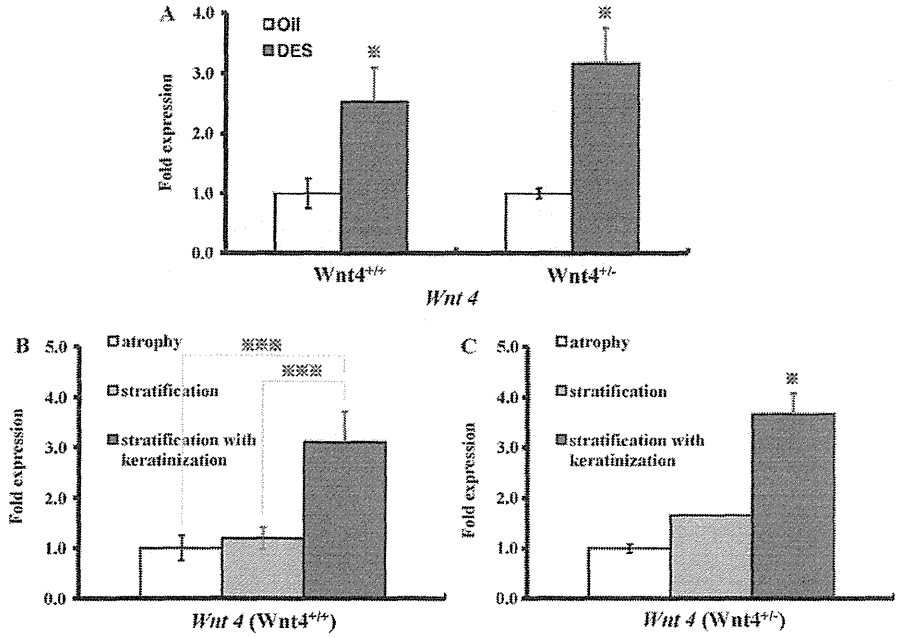

Fig. 4. Vaginae of 10-week-old, ovariectomized $Wnt4^{+/+}$ or $Wnt4^{+/-}$ mice exposed neonatally to 2.5 μg DES or oil vehicle alone. Neonatal exposure to 2.5 μg DES induced persistent up-regulation of *Wnt4* mRNA in vaginae of both $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice (A). *Wnt4* mRNA expression was significantly correlated to the vaginal epithelial cell proliferation with the superficial keratinization but not for the proliferation only in $Wnt4^{+/+}$ (B) and $Wnt4^{+/-}$ mice (C). Since only one $Wnt4^{+/-}$ mouse showed epithelial stratification only, therefore, statistical analysis could not be done. The number of mice showing vaginal epithelial atrophy, stratification only and stratification with keratinization are correlated to Table 3. $^{*}P < 0.05$ vs. controls, $^{***}P < 0.001$ vs. controls (Student's *t*-test or Welch's *t*-test followed by *F*-test).

et al., 2003), and cardiogenesis (Pandur et al., 2002). In the line of microarray results, our tissue distribution data also suggested similar respective up- and down-regulation of *Wnt4* and *Wnt11* expression after neonatal DES exposure. The reduction in *Wnt11* after DES exposure suggests their repressive role in Wnt pathway (Maye et al., 2004). However, the expression of Fz genes, receptors of *Wnt4* (Lyons et al., 2004), did not change in DES-treated vagina, suggested that *Wnt4* might have other function unrelated to Fzs.

Cellular localization of protein gives an idea about the potential target. Miller et al. (1998) reported the localization of *Wnt4* mRNA in mouse reproductive tract using in situ hybridization, however, no information of the localization of Wnt4 protein in the vagina. In the present study, Wnt4 protein was localized in the vaginal epithelium of mice exposed neonatally to DES, especially in the basal epithelial cell layer. Saitoh et al. (1998) reported that Wnt4 protein plays a role in epidermal–dermal (presumably keratinocyte–fibroblast) interactions in the skin. Wnt4 is possibly participating in cell proliferation or keratinization in the mouse vaginal epithelium.

In this regard, our earlier reports suggest that DES-induced persistent proliferation in vagina is actually mediated through ERα (Nakamura et al., 2008). Moreover, in wild-type mice, uterine expression of *Hoxa10*, *Hoxa11* and *Wnt7a* genes exhibited significant decrease shortly after DES treatment (Ma et al., 1998; Kitajewski and Sassoon, 2000; Couse et al., 2001), whereas this effect was not observed in the αERKO mice (Couse et al., 2001). This supports the idea about the obligatory role for ERα in DES-induced alteration of mouse reproductive tract. Interestingly, in the present study, only PPT, but not DPN, induced a similar magnitude of *Wnt4* and *Wnt11* expression as in DES-exposed vagina. This suggests that the changes in *Wnt4* and *Wnt11* profile are ERα responsive. But anti-estrogen mediated reduction of *Wnt4*, but not *Wnt11*, confirms that *Wnt4* action is regulated by ERα, and *Wnt11* might be regulated by androgen receptor as in prostate cancer (Zhu et al., 2004).

Finally, we used $Wnt4^{+/-}$ mutant mice to study the function of Wnt4 in the estrogen-induced vaginal epithelial stratification and keratinization, since $Wnt4^{-/-}$ mouse show fetal lethality (Vainio et al., 1999; Majumdar et al., 2003). $Wnt4^{+/-}$ mice exposed neonatally to DES showed vaginal epithelial stratification with the superficial keratinization similar to wild-type mouse exposed neonatally to DES. However, *Wnt4* was highly expressed in vagina showing epithelial stratification with the superficial keratinization. Keratins have long and extensively been used as immunohistochemical markers in diagnostic tumor pathology (Moll et al., 2008; Karantza, 2011). Interestingly, *Wnt11* was significantly down-regulated in the vagina of mice showing ovary-independent persistent epithelial proliferation. This confirms that *Wnt4* and *Wnt11* might show the opposite behavior in the mouse vagina. *Wnt4* expression was correlated to the keratinization of vaginal epithelium.

In conclusion, we suggested that *Wnt4* mRNA is permanently up-regulated, and *Wnt11* mRNA is permanently down-regulated in the vagina exposed neonatally to DES or ERα specific ligand, PPT. *Wnt4* might be participated in the irreversible superficial keratinization in the mouse vagina. However, the ER-independent repressive role of *Wnt11* in vaginal keratinization, need to address more critically in the near future.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2012.02.010.

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Dose-Related Estrogen Effects on Gene Expression in Fetal Mouse Prostate Mesenchymal Cells

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Abstract

Developmental exposure of mouse fetuses to estrogens results in dose-dependent permanent effects on prostate morphology and function. Fetal prostatic mesenchyme cells express estrogen receptor alpha (ERα) and androgen receptors and convert stimuli from circulating estrogens and androgens into paracrine signaling to regulate epithelial cell proliferation and differentiation. To obtain mechanistic insight into the role of different doses of estradiol (E2) in regulating mesenchymal cells, we examined E2-induced transcriptomal changes in primary cultures of fetal mouse prostate mesenchymal cells. Urogenital sinus mesenchyme cells were obtained from male mouse fetuses at gestation day 17 and exposed to 10 pM, 100 pM or 100 nM E2 in the presence of a physiological concentration of dihydrotestosterone (0.69 nM) for four days. Gene ontology studies suggested that low doses of E2 (10 pM and 100 pM) induce genes involved in morphological tissue development and sterol biosynthesis but suppress genes involved in growth factor signaling. Genes involved in cell adhesion were enriched among both up-regulated and down-regulated genes. Genes showing inverted-U-shape dose responses (enhanced by E2 at 10 pM E2 but suppressed at 100 pM) were enriched in the glycolytic pathway. At the highest dose (100 nM), E2 induced genes enriched for cell adhesion, steroid hormone signaling and metabolism, cytokines and their receptors, cell-to-cell communication, Wnt signaling, and TGF- β signaling. These results suggest that prostate mesenchymal cells may regulate epithelial cells through direct cell contacts when estrogen level is low whereas secreted growth factors and cytokines might play significant roles when estrogen level is high.

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Introduction

The mouse prostate begins to differentiate from the urogenital sinus (UGS) at gestation day 17, soon after the onset of testosterone secretion by the fetal testes [1–2]. Prostate duct development is initiated by mesenchymal influences and results in the formation of epithelial cell outgrowths, or epithelial buds. This event is dependent on mesenchymal conversion of testosterone to 5α-dihydrotestosterone (DHT), which is a higher-affinity androgen receptor (AR) ligand [1,3]. Androgen receptor gene (Ar) expression in prostatic mesenchyme is required for the continued normal growth and branching morphogenesis of epithelial ducts [4,5]. Although differentiation of the prostate is androgen-dependent, there is now considerable evidence that estrogens act to modulate the activity of androgen in regulating prostate development. During development, the mouse and rat UGS mesenchyme expresses both Ar and estrogen receptor- α (Esr1). In contrast, epithelial cells exhibit little androgen binding at this time, and Ar expression in UGS epithelium is not required for differentiation [6,7,8]. Since the growth of epithelial cells requires signals from the UGS mesenchyme [9], and since fetal UGS epithelial cells do not express estrogen receptors (ER) [8,10], proliferative responses of the epithelial compartment to estrogens have been presumed to be driven by stimuli from mesenchymal cells.

We and others have shown that prenatal exposure of male mouse fetuses to estradiol-17β (E2), estrogenic drugs such as diethylstilbestrol (DES) and ethinylestradiol, or industrial estrogenic chemicals such as bisphenol A (BPA), induce an increase in the number of developing prostatic glands and an increase in prostate gland size during fetal life due to basal epithelial cell hyperplasia [11,12,13]; there is also a permanent increase in prostatic AR [11]. However, effects of prenatal estrogen exposure do not follow a monotonic dose response [14], and effects on the developing prostate at high and low concentrations may be very different [11,12,13]. We recently reported that the exposure of primary culture fetal mouse prostate mesenchyme cells to E2 enhanced expression of both Ar and Esr1 [14] in a non-monotonic manner. In the present study, we sought to identify other estrogen-regulated genes in mesenchymal cells and to compare effects of low (physiological) and high (pharmacological) concentrations of

E2 on gene expression while the concentration of DHT was held constant. We show here that gene expression is dose-dependent but that expression profiles differ at low and high doses.

Materials And Methods

Ethics Statement

All animal procedures were approved by the University of Missouri Animal Care and Use Committee (protocol number: 6489) and conformed to the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The program is fully accredited by the Association for Assessment & Accreditation of Laboratory Animal Care, International (AAALAC).

Animals

CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained as an outbred stock at the University of Missouri. Animals were housed on corncob bedding in standard (11.5×7.5×5″) polypropylene cages. Water was purified by reverse osmosis and carbon filtration and provided in glass bottles *ad libitum*. Pregnant and lactating females were fed Purina 5008 chow, and otherwise were maintained on Purina 5001 chow. Rooms were maintained at 25±2°C under a 12:12 L:D cycle.

Tissue collection, primary cell culture, and dosing

Timed-pregnant females were killed on gestation day (GD) 17 (mating = GD 0) by CO_2 asphyxiation, and fetuses were removed from the uterine horns. The bladder and UGS were removed from male fetuses as previously described [8,11], and the prostatic region of the UGS was separated from the bladder at the bladder neck and the lower UGS just below the ejaculatory ducts. UGS tissue was disrupted by collagenase treatment as described [14]. Epithelial and mesenchymal cells in the suspension were separated by gravity, since the epithelial cells settle and the mesenchymal cells remain suspended. The composition of the two cell type fractions was confirmed by immunofluorescence staining of cytokeratins with mouse anti-pan-cytokeratin clone PCK-26 fluorescein isothiocyanate conjugate (Sigma), and co-staining with the mesenchymal cell marker vimentin with goat anti-vimentin (Sigma) and rabbit anti-goat Cy3 conjugate (Sigma) ([15], data not shown). For these studies, epithelial cells were discarded and the collected mesenchymal cells were cultured at 37°C under 5% CO_2 in RPMI-1640 medium without phenol red (Gibco, Grand Island, NY), supplemented with 2 mM L-glutamine, 100 units penicillin G sodium/ml, 100 mg streptomycin sulfate/ml, and 0.25 mg fungizone/ml. 10% fetal bovine serum (FBS) (U.S. Bio-Technologies, Parkerford, PA) was added to this initial growth medium and was not stripped of endogenous steroid hormones. Cells were grown to 95% confluence (approximately 3–5 days), and then passaged by digestion with 0.05% trypsin in 0.53 mM EDTA (Gibco) for 5 min at room temperature.

First passage cells were used in these experiments and were seeded at 3.2×10^5 cells/well in 35 mm dishes. Cells were seeded in complete RPMI medium with endogenous hormones removed by substituting 5% (v/v) charcoal-stripped FBS and 5% (v/v) charcoal-stripped horse serum (Sigma, St. Louis, MO) for the 10% whole FBS, and further supplementing with ITS supplement (Cambrex, Walkersville, MD), for final concentrations of 10 μg insulin/ml, 10 μg transferrin/ml, and 10 ng selenium/ml. This medium was further supplemented with 690 pM DHT (200 pg/ml). Cells were treated with DHT rather than testosterone for two reasons. First, we wanted to control E2 exposure, since the developing prostate expresses aromatase [16] and unlike testosterone, DHT is not aromatized to E2 [5]. Second, we aimed to control the androgen concentration due to the potential for these compounds to alter 5α-reductase activity [17]. Cells were maintained in estrogen-free medium for three days, with one medium change, before the start of estrogen treatment, and then treated with either low doses (10 pM and 100 pM) or a high dose (100 nM) of E2 [14], selected based on our previous work (14). Negative controls were treated with the treatment vehicle, 0.05% ethanol. Cells were treated for four days with daily medium changes, with three replicate samples per treatment. At the end of the treatment period the cells were washed once with PBS, and immediately lysed on ice in Trizol (Invitrogen, Carlsbad, CA).

Microarray Analysis

Total RNA was isolated from the Trizol lysate and purified with the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturers' instructions, and RNA quality was checked on an Agilent Bioanalyzer (Agilent, Palo Alto, CA). The transcriptomal profiles were determined using Affymetrix mouse ST 1.0 or 430A microarrays. Scanned image data were converted into numerical tables using Affymetrix GeneChip Operating Software and Gene Expression Console. Data analysis and mining, including gene ontology enrichment analysis, were performed using GeneSifter server (Giospiza Inc., Seattle, WA) and Partek Genomics Suite (Partek Inc., St. Louis, MO). Microarray data were deposited in NCBI Gene Expression Omnibus (accession numbers GSE16854 and GSE36630).

Quantitative PCR (qPCR) measurement of gene expression

To confirm the relative changes in gene expression induced by estradiol, we used a real-time quantitative reverse transcriptase-polymerase chain reaction (qPCR) approach for selected transcripts [18]. These data were previously reported elsewhere, compared with results for BPA treatment [19]; that article is attached here in Supporting Information. Fetal mouse UGS mesenchyme cells were treated *in vitro* for four days as described with either 100 nM 17β-estradiol or the ethanol vehicle (0.05%) alone; treatments were performed in triplicate wells within each experiment, and analyses were conducted on RNA preparations from three independent experiments. Total RNA was isolated with the RNAqueous kit (Ambion, Austin, TX) according to the manufacturer's instructions, and quantified by absorbance at 260 nm. Expression of specific mRNAs were measured by one-step real time Rt-PCR as described [20] using the TaqMan EZ RT-PCR kit (PE Applied Biosystems, Foster City, CA) on the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). Assays for each mRNA were carried out in duplicate. The primer/probe set for Ar was designed using Primer Express software (PE Applied Biosystems), as described [14]. Ar primers were synthesized by Invitrogen, and the Ar probe was synthesized by Applied Biosystems. The concentrations of Mn^{2+}, probe and primers were optimized for the primer/probe set. Other analyses were performed using validated ABI Taqman Gene Expression assays (Applied Biosystems). Assays for each mRNA were carried out in duplicate. ABI Taqman Gene Expression assays used for specific transcripts were: Mm00433149_m1 (Esr1), Mm00432087_m1 (Bmp4), Mm00500361_m1 (Capn6), Mm00484157_m1 (Cyp7b1), Mm00840104_m1 (Sfrp4) and Mm00449036_m1 (Thbs2). These primers spanned *Esr1* exons 3–4, Bmp4 exons 2–3, Capn6 exons 2–3, Cyp7b1 exons 4–5, Sfrp4 exons 4–5 and Thbs2 exons 1–2.

The relative concentrations of specific mRNAs in each sample were normalized to total RNA per well, as described [20,21].

Normalization to total RNA allowed for comparisons between independent experiments and provided a conservative estimate of relative amounts of mRNA. Differences between control and estradiol -treated cells were evaluated using the ANOVA GLM procedure in SAS. Comparisons of mean reciprocals for each dose relative to controls were made using the LS Means Test in SAS. The criterion for statistical significance was $P \leq 0.05$ (two-tailed).

Results

Microarray analysis of effects of low (10 pM and 100 pM) concentrations of E2 on gene expression in fetal mouse prostate cells

Exposure of the primary culture mouse prostate mesenchymal cells significantly affected expression of 628 genes (ANOVA, $p<0.01$, unadjusted). Benjamini-Hochberg correction of multiple testing eliminated these effects, reflecting the relatively low statistical power of the present analysis due to the limited numbers of samples in each group.

These 628 genes were subjected to hierarchical clustering, which classified them into seven groups, based on induction or suppression of gene activity and on relative sensitivity to E2 (Figure 1). The seven groups were categorized as: 1) E2-inducible, high sensitivity genes; 2) E2-inducible, moderate sensitivity genes; 3) E2-inducible, low sensitivity genes; 4) U-shaped dose-response genes; 5) E2-suppressible, high sensitivity genes; 6) E2-suppressible, low sensitivity genes; and 7) Inverted U-shaped dose-response genes. Gene Ontology (GO) analysis was performed on these groups of genes using g:Profiler [22,23] and DAVID [24,25].

Analysis of GO enrichment of E2 inducible genes (Figure 2) indicated effects on pathways for Cell Adhesion, EGF-like Calcium Binding, Sterol Biosynthesis, and Actin Filament & Cytoskeleton. Results for all E2-inducible genes, from groups 1, 2 and 3, were similar, and together suggested changes in cell adhesion, morphology, and sterol biosynthesis. Analysis of genes with a U-shaped dose response (Group 4, data not shown) did not yield specific GO/pathway effects. Analysis of E2- suppressible genes (Figure 3) indicated no specific pathway effects within the highly sensitive gene set (Group 5), but nor indicated significant effects within Group 6, the E2-suppressible, low sensitivity genes, on pathways related to extracellular matrix, Cell adhesion, EGF-like growth factor binding, IGF binding, Thyroglobulin, Thrombospondin, Ossification, and Somatomedin B. Overall, these pathways suggested changes in cell adhesion and reduced growth factor signaling.

For the 34 genes showing an inverted U-shape dose response (Figure 4A), pathway analysis strongly indicated effects on sugar metabolism. Synchronized changes in mRNA expression of key genes suggest enhancement of glycolysis by 10 pM E2 but significant suppression by 100 pM E2. (Figure 4B). Specific effects within the Glycolysis pathway are illustrated in Figure 5.

Microarray analysis of effects of a high (100 nM) concentration of estradiol on gene expression in fetal mouse prostate cells

After filtering the data in GeneSifter, using a 1.5-fold expression ratio criterion between control and estradiol treatment and a statistical cutoff at $P \leq 0.05$, and discarding genes with expression levels less than 10 fluorescence units in both treated and control samples, it was determined that 181 genes were activated by 100 nM E2 exposure and 86 genes were repressed.

The results of Gene Ontology functional enrichment analysis, within the categories of Biological Process and Molecular Function, are shown in Table 1, and categories significantly affected by the 100 nM E2 treatment were identified by z-scores. These included effects on growth, reproductive processes, and

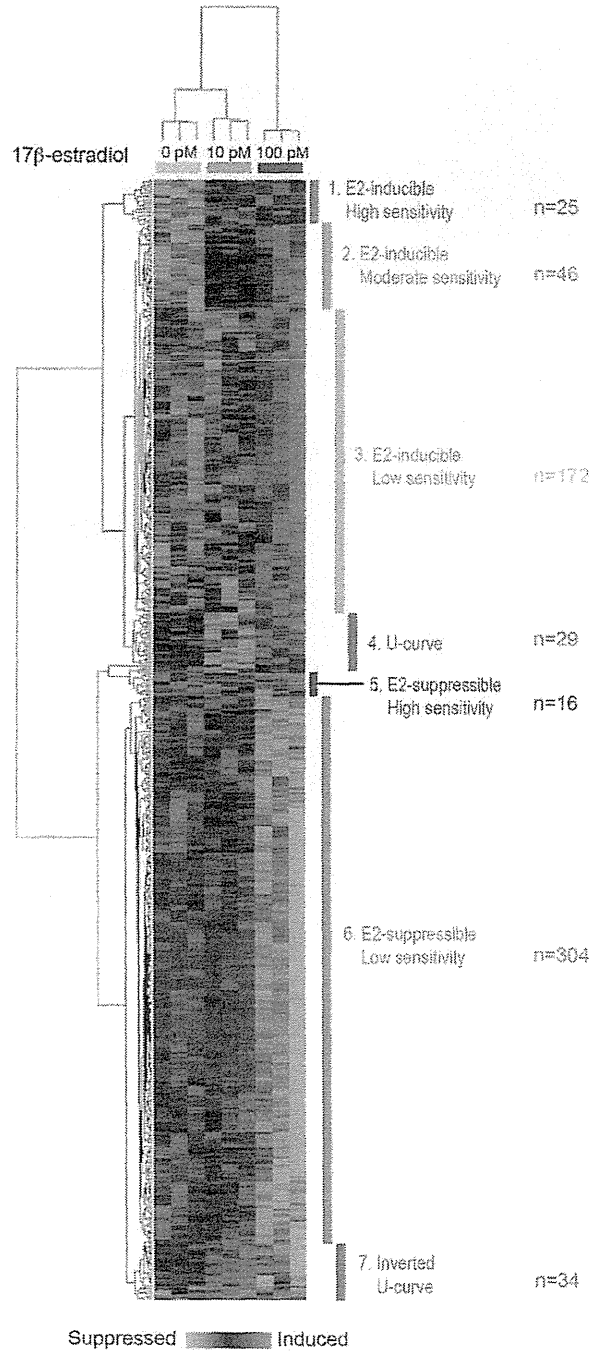

Figure 1. Cluster analysis of estrogen-responsive genes in fetal UGS mesenchyme cells after estrogen treatment with the two lower doses, showing strong separation of responses to control, 10 pM E2, and 100 pM E2 treatments. Based on clustering, genes were identified as falling into one of 7 groups that differed in their responses to low-dose E2.
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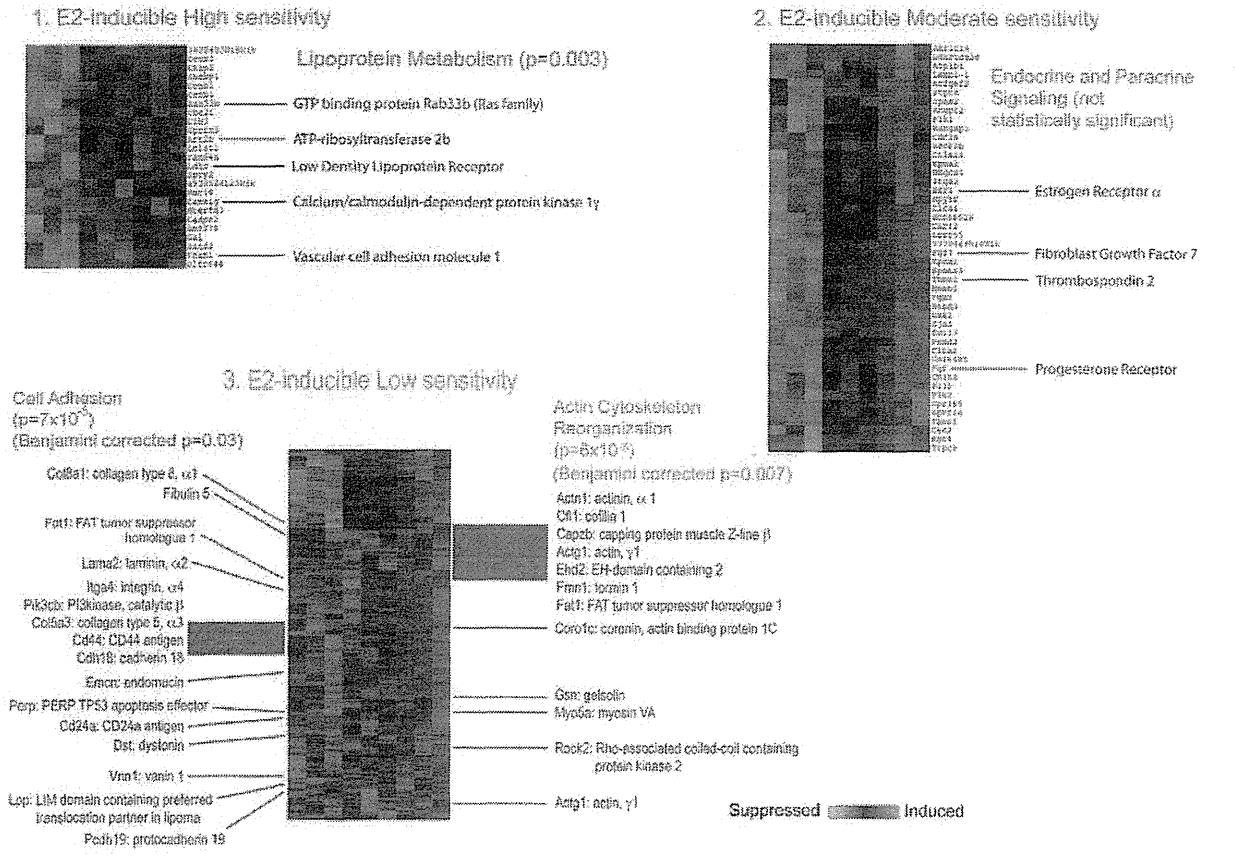

Figure 2. Detail of E2-inducible genes in groups identified by clustering analysis. Set 1: E2-inducible high sensitivity. Set 2: E2- inducible moderate sensitivity. Set 3: E2-inducible low sensitivity. Select genes of interest are highlighted. The figures show raw p values as well as, where indicated, Benjamini-Hochberg corrected p values.
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metabolic processes, and generally indicated effects of E2 on promotion of growth and inhibition of apoptosis. Table 2 lists genes in selected signaling pathways influenced by E2 treatment, again identified using z-scores. Additional genes affected by E2 treatment, selected as being "of interest" in these and other pertinent pathways, were identified manually. These data indicated significant effects of 100 nM E2 treatment on three key pathways: cell communication, androgen and estrogen metabolism, and the TGF-β signaling pathway. E2-regulated genes were also identified in other pathways of interest, namely the Wnt signaling pathway, cytokine-cytokine receptor interaction, sonic hedgehog signaling and apoptosis.

Confirmation of estrogen-regulated genes

The expression patterns of several genes demonstrated to be up- or down-regulated by estradiol treatment using microarray analysis were validated in independent samples using quantitative PCR. The genes selected were: Ar, Bmp4, Capn6, Cyp7b1, Esr1, Sfrp4, and Thbs2. Ar and Esr1 were chosen because we have shown by qPCR [14] that estradiol stimulates Ar and Esr1 mRNA expression. The other genes were selected based on strength of response and relevance to cell growth. In this particular study, the effects of estradiol on Ar expression, while in the same direction as predicted by earlier studies, did not quite reach significance by microarray analysis. The results of the follow-up qPCR analysis are shown in Fig. 6. The data obtained for cells treated with estradiol are consistent with the microarray expression profiles.

Discussion

The effects of fetal E2 exposure on prostate development do not follow a monotonic dose-response [11,12]. Previous studies have shown that exposure of male mouse fetuses to a very small increase in serum E2 [11], or to very low maternal doses of the estrogenic drugs DES and ethinylestradiol or the xenoestrogen BPA, lead to basal epithelial cell hyperplasia and to a permanent increase in prostate AR binding activity, resulting in an increase in prostate size in adulthood [11,12,13,26]. Those findings showed that at low doses, estrogen has a stimulatory effect on the action of androgen in regulating prostate differentiation and subsequent prostate function, including development of early stage prostate cancer in adulthood [27,28]. In contrast, opposite effects have been found at much higher doses of E2 and xenoestrogens. Prenatal or neonatal exposure of rats or mice to high doses of estrogens led to a decrease in prostate growth during the time of exposure in development, which led to reduced prostate size and androgen responsiveness in adulthood [3,11,12,13,29,30].

Non-monotonic dose responses were seen in our initial examination of the effects of estradiol and BPA on Ar and Esr1 expression in fetal mouse UGS mesenchyme [14], and dose-related variation in the pattern of gene expression was also

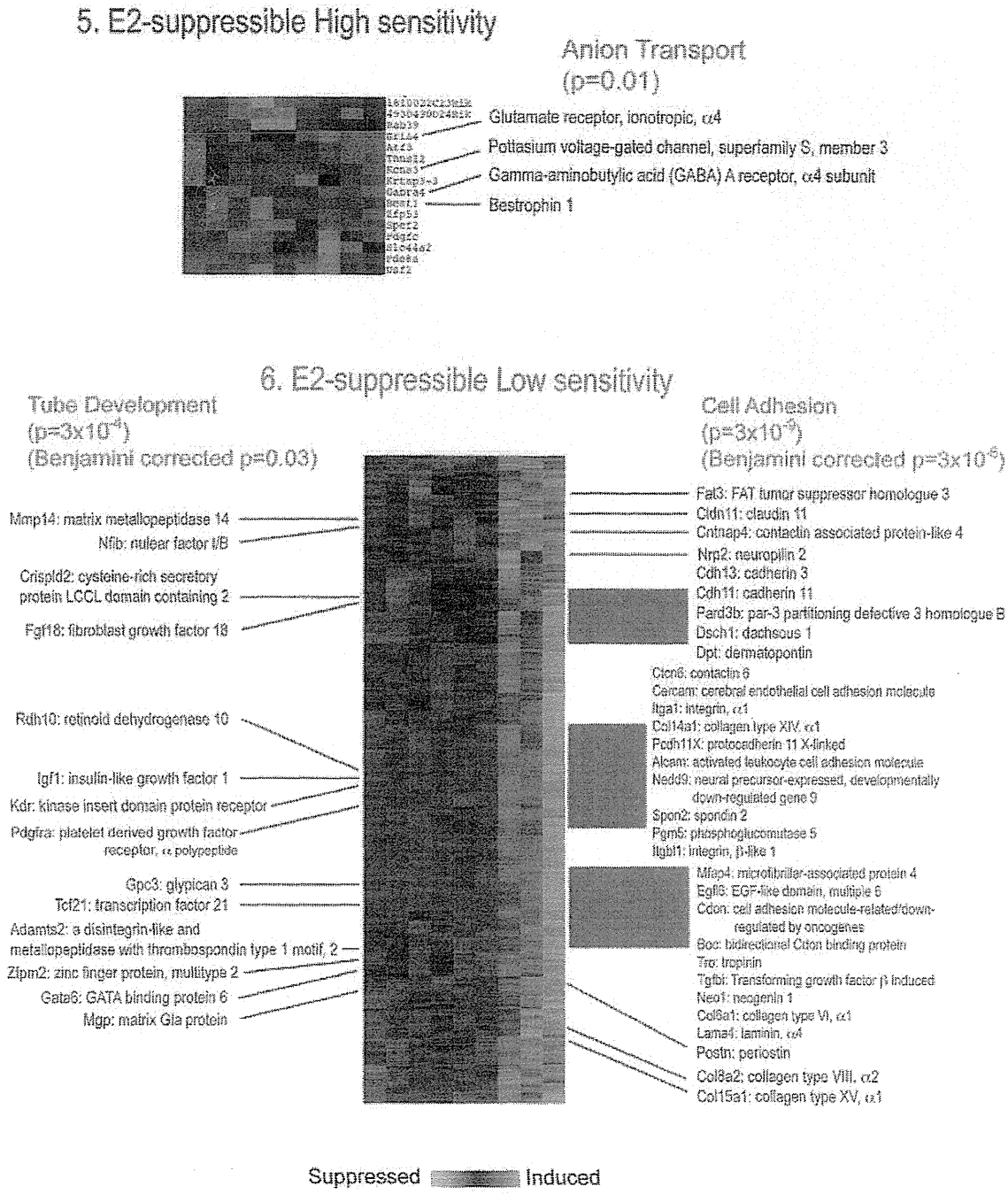

Figure 3. Detail of E2-suppressible genes in groups separated by clustering analysis. Set 5: E2-suppressible high sensitivity. Set 6: E2-suppressible low sensitivity. The figures show raw p values as well as, where indicated, Benjamini-Hochberg corrected p values.
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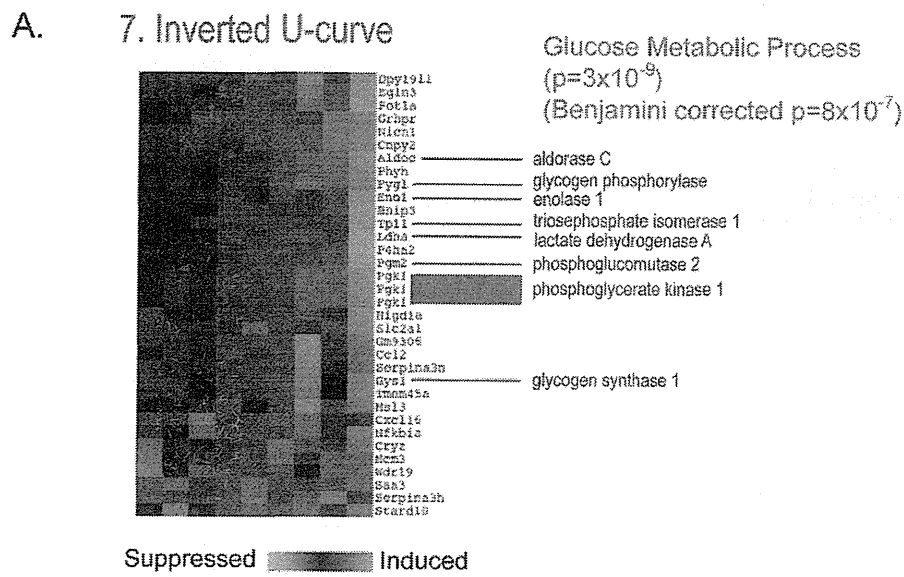
observed for a large number of genes in human MCF-7 breast cancer cells, in response to doses of E2 between 10–100 pM [31]. Because of these prior findings as well as different *in vivo* effects of high and low doses of estrogen, we chose to examine the effects of E2 on gene expression in fetal prostate mesenchyme cells by microarray analysis, using two low doses (10 pM and 100 pM) as well as a high dose (100 nM) that had resulted in maximal Ar expression in our prior study with the same fetal mesenchyme cells [14]. In laboratory rats and mice, the free serum concentration of E2 (unbound to plasma proteins and unconjugated) is about 2 pM during the initial period of prostate development [11] although calculation of the actual biologically active fraction of E2 during sexual differentiation is complicated by uncertainty regarding the bioavailability of albumin-bound E2 and the capacity for the



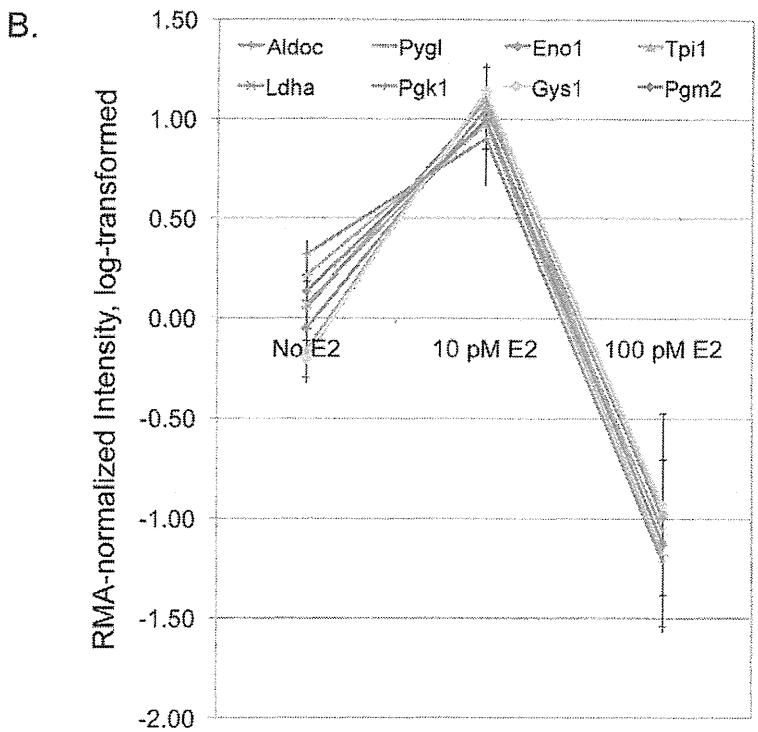


Figure 4. Detail of E2-suppressible genes in groups separated by clustering analysis. A) Set 7: Inverted U-curve. Both raw p values and Benjamini-Hochberg corrected p values are given. B) Genes identified as part of the glucose metabolic pathway in panel A depicted as relative values to illustrate the high association between dose and gene expression.
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maternal-placental-fetal tissues to deconjugate sulfated estrogens [32]. Total serum E2 during this period is in the range of 300 pM [33], and thus the low doses of E2 used in this study are physiologically relevant.

These microarray experiments were performed as a hypothesis generation step for a study of effects of estrogens on prostate development and differentiation, and the sample size is small. Because of this, the data must be seen as preliminary, but the results do indicate activation of different patterns of gene expression and dominance of different pathways at low, physiologically relevant, compared to high, pharmacological, doses of E2. Results from the lowest (10 pM and 100 pM) doses of E2

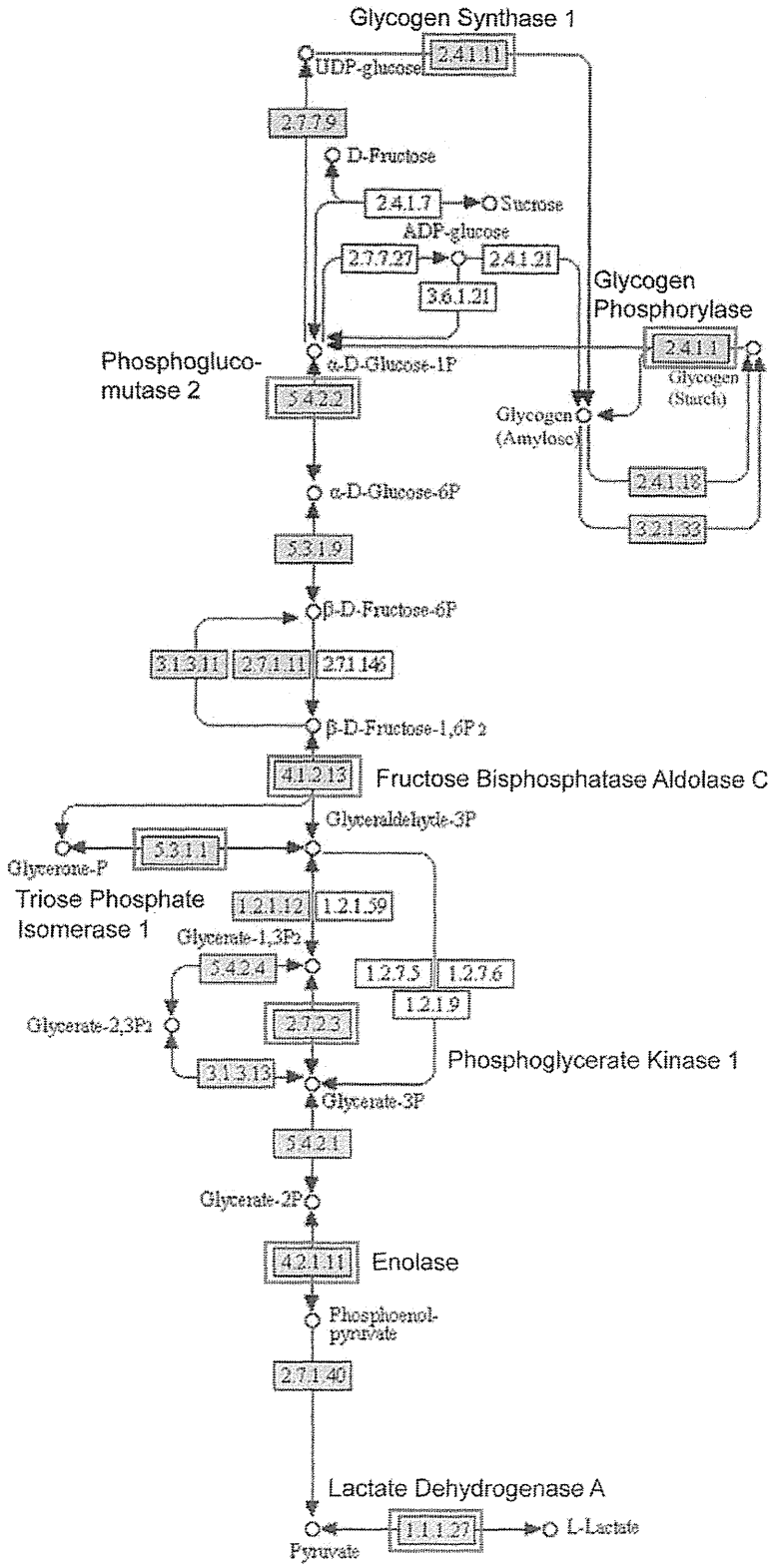

Figure 5. Glucose metabolism pathway. Highlighted genes (outlined in red) were influenced by lower dose estradiol treatment (10 pM and 100 pM) in an inverted U manner, suggesting enhancement of glycolysis by 10 pM E2 but suppression by 100 pM E2.
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Table 1. Functional characterization by gene ontology (GO) terms of gene expression profiles in cells treated with 100 nM E2.

		Number of genes		z-scores	
Category	**Gene Ontology Term**	**up-regulated**	**down- regulated**	**up-regulated**	**down- regulated**
Biological process	metabolic process	121	69	−2.08*	−1.43
	biological regulation	100	40	2.70**	−0.99
	growth	17	3	5.11**	−0.03
	reproduction	11	9	0.94.	2.02*
	reproductive process	5	6	0.42.	2.42*
	rhythmic process	6	1	3.92**	0.23
Molecular function	catalytic activity	135	76	−2.3*	0.82
	transporter activity	53	35	2.76**	1.42

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treatments indicate E2-inducible genes within pathways related to cell adhesion, actin cytoskeleton reorganization, EGF-like calcium binding, sterol biosynthesis and lipoprotein metabolism, and E2-suppressible genes within pathways related to growth factor signaling, tube development and additional effects on cell adhesion. At the high (100 nM) concentration, E2 induced genes enriched for steroid hormone signaling and metabolism, cytokines and their receptors, cell-to-cell communication, and TGF-β signaling (Table 2). Results from the 100 nM E2 treatment thus indicated effects on cell adhesion pathways, but also emphasized a stimulation of a positive feedback loop involving steroid hormone receptors and genes related to growth and metabolism that promote rather than inhibit cell growth. Taken together, these results suggest that fetal prostate mesenchymal cells may regulate epithelial cells through direct cell contacts when estrogen levels in mesenchyme are in the pM range, whereas growth factors might play significant roles when estrogen levels are higher in the nM range.

Importantly, an inverted U (non-monotonic) response was seen within the low-dose results, with enhancement of glycolysis observed at 10 pM E2 but significant suppression at 100 pM E2 (Figure 5). The expression of these specific genes was not influenced by 100 nM E2, indicating that the stimulation of glycolysis is highly dependent on dose and only seen at low pM E2 concentrations. This is of particular interest given the Warburg effect, the observation that most cancer cells rely on glycolysis to generate the energy needed for cellular processes, in contrast to normal differentiated cells that use mitochondrial oxidative phosphorylation [34–35]. The enhancement of glycolysis seen in our culture was only at the lowest dose tested here, 10 pM (2.72 pg/ml), and as such is intriguing because mice exposed prenatally to a very similar concentration of estradiol have enlarged prostates in adulthood [11] relative to mice exposed to higher doses. It is interesting to speculate on whether there is a relationship between the enhancement of cell proliferation rate and glycolysis seen in cancer cells, and the enhancement of glycolysis in fetal prostate mesenchymal cells and increased prostate size due to hyperplasia seen in mice.

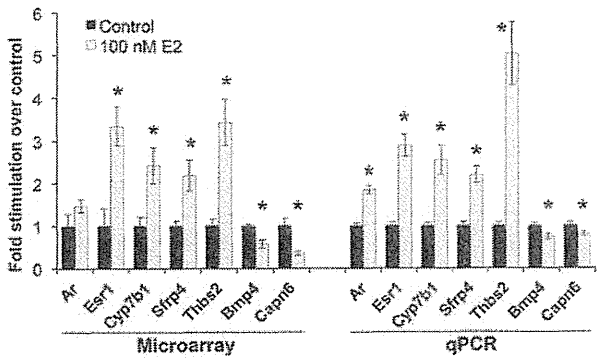

Figure 6. Comparison of expression of selected genes measured by microarray and by Q-PCR. Gene expression in cells treated with 100 nM 17β-estradiol (grey bars) is compared to that in untreated control cells (black bars). * Control vs. treated cells statistically different, $p<0.05$. The qPCR data were previously published elsewhere [19].
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Only 29 genes out of those screened were influenced by all doses of estradiol examined (Table 3). For approximately half of these genes the dose-response relationship was monotonic, although some of these were maximally up- or down-regulated at the 100 pM dose. For the rest, the direction of the effect (stimulation or suppression of gene expression) was either strongly reversed at the highest (100 nM) E2 concentration (a non-monotonic response), or simply showed a suggestion of reversal at the highest dose. Of the monotonic profiles, two genes showed particularly strong linearity with dose: Angpt2 (angiopoetin 2) and Sprr1a (small proline-rich protein 1a). Angpt2 expression is strongly correlated with prostate cancer progression [36] and is stimulated by growth factors, especially VEGF [37–38]; Vegf expression is stimulated by androgen treatment in fetal prostate fibroblasts [39], but we did not observe an effect of estrogen on Vegf expression here. Expression of Sprr genes is typically restricted to cells committed to terminal differentiation [40]. Although strong up-regulation of Sprr1a has been associated with abnormal cell differentiation in uterine tissue from neonatal CD-1 mice treated with diethylstilbestrol [41], effects in the developing prostate have not previously been reported.

Also of interest in this 29-gene subset are the clear inverse U effects on Perp and Gja1 expression. Perp is typically upregulated during apoptosis [42] but is also important for promoting desmosomal cell-cell adhesion [43], and loss of Perp is associated with dysregulation of cell adhesion and promotion of tumor development and progression [44]. Decreased expression of Gja1 (Cx43) is similarly consistent with loss or reduction of cell-cell communication. Only one gene in this 29-gene subset, Enpp2, showed a U-shaped response to increasing E2 concentrations; Enpp2 codes for autoaxin, an ecto-enzyme responsible for

Table 2. Effects of 100 nM estradiol treatment on gene expression within specific regulatory pathways identified as being of interest.

Pathway/Category	Direction	Ratio	Gene Identifier	Gene Name
Cell Communication	Up	2.50	AV239646	Gjb2
z-score (up) = 3.32	Up	1.96	BE197934	Krt1-14
	Up	2.62	AV330726	Gja1
	Up	*3.16*	*BC006894*	*Gja1*
	Up	*3.81*	*M63801*	*Gja1*
	Up	4.48	L06421	Thbs2
	Up	*3.43*	*NM_011581*	*Thbs2*
	Up	1.83	BI455189	Col6a2
Androgen and estrogen metabolism	Down	7.25	NM_023135	Sult1e1
z-score (up) = 2.4	Up	2.42	NM_007825	Cyp7b1
	Up	8.12	NM_01378	Hsd17b9
TGF-beta signaling pathway	Down	1.93	NM_010496	Idb2
z-score (down) = 2.19	Down	1.83	NM_008046	Fst
	Down	1.75	NM_007554	Bmp4
	Down	3.27	BM230984	Tgfb14i
	Up	3.67	BB353211	Inhbb
	Up	4.48	L06421	Thbs2
	Up	*3.43*	*NM_011581*	*Thbs2*
Steroid hormone receptors	Up	3.34	NM_007956	Esr1
	Up	5.29	NM_008829	Pgr
Wnt signaling	Down	2.22	NM_009519	Wnt11
	Up	3.72	NM_009526	Wnt6
	Up	1.89	W29605	Wnt7b
	Up	5.85	NM_020265	Dkk2
	Up	2.18	BB221995	Sfrp4
Cytokine-cytokine Receptor interaction	Up	2.77	NM_019583	Il17rb
	Up	2.21	NM_011330	Ccl11
	Up	6.53	NM_021443	Ccl8
	Up	3.67	BB353211	Inhbb
	Up	3.85	AF000304	Il4ra
	Up	*2.91*	*NM_010557*	*Il4ra*
Hedgehog signaling	Down	2.22	NM_009519	Wnt11
	Down	1.75	NM_007554	Bmp4
	Up	3.72	NM_009526	Wnt6
	Up	1.89	W29605	Wnt7b
Apoptosis	Up	2.07	BF137345	Birc4
	Down	2.85	NM_007603	Capn6
	Down	*2.23*	*AI747133*	*Capn6*
Prostate cancer	Up	3.40	BC010786	Creb3l3
	Up	2.21	AJ252157	Foxo1
	Up	2.60	NM_019739	Foxo1
Basal cell carcinoma	Down	2.22	NM_009519	Wnt11
	Down	1.75	NM_007554	Bmp4
	Up	3.72	NM_009526	Wnt6
	Up	1.89	W29605	Wnt7b

All genes listed are significantly altered at $P<0.05$. Where the z-score for the entire pathway was significant, the score is given below the pathway name. Where multiple probes for the same gene are represented in these lists (indicated by italics), agreement was good between the probes.

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Table 3. Genes whose expression was significantly (P≤0.05) influenced by estradiol (E2) treatment at all doses tested.

		Log2 fold expression relative to control				
Low-dose cluster group	**Gene ID**	**No E2**	**10 pM E2**	**100 pM E2**	**100 nM E2**	**Monotonic trend?**
Inducible_moderate	Sprr1a	0.00	0.98	2.23	5.57	Y
Inducible_moderate	Angpt2	0.00	1.20	2.25	3.77	Y
Inducible_moderate	Dkk2	0.00	0.67	2.21	2.55	Y
Inducible_moderate	Pgr	0.00	0.98	2.22	2.40	Y
Inducible_low	Fabp7	0.00	0.75	2.18	2.26	Y
Inducible_low	Fbxo32	0.00	−0.33	1.52	1.57	Y
Inducible_moderate	Esr1	0.00	1.00	2.19	1.74	I
Inducible_moderate	Rgs4	0.00	0.64	2.09	1.81	I
Inducible_moderate	Thbs2	0.00	1.49	2.20	1.78	I
Inducible_moderate	Btbd3	0.00	0.95	2.25	1.05	N
Inducible_moderate	Gja1	0.00	0.66	2.21	1.51*	N
Inducible_moderate	Npy1r	0.00	1.14	2.16	1.49	N
Inducible_low	Perp	0.00	0.21	2.06	0.71	N
Suppressible_low	Sult1e1	0.00	0.20	−1.81	−2.86	Y
Suppressible_low	Lcn2	0.00	0.49	−1.63	−2.44	Y
Suppressible_low	Egfl6	0.00	−0.20	−2.04	−1.93	Y
Suppressible_low	Pdlim3	0.00	−0.14	−1.96	−1.95	Y
Suppressible_low	Cdkn1c	0.00	−0.19	−1.99	−1.16	Y
Suppressible_low	Capn6	0.00	−0.43	−1.93	−1.51	I
Suppressible_low	Igfbp2	0.00	0.15	−1.87	−0.96	I
Suppressible_low	Wnt11	0.00	−0.55	−2.11	−1.15	I
Suppressible_low	Cyb561	0.00	0.57	−1.52	−0.93	I
Suppressible_low	Gda	0.00	−0.49	−2.06	−0.77	I
Suppressible_low	Dpep1	0.00	0.97	−1.19	−0.67	I
Suppressible_low	Zfp161	0.00	0.10	−1.70	0.74	N
Suppressible_low	Sfrp4	0.00	0.16	−1.77	1.12	N
Suppressible_low	Penk1	0.00	−0.53	−1.95	1.06	N
Suppressible_low	Enpp2	0.00	0.90	−1.17	1.85	N
U-curve	Cd80	0.00	−1.04	1.12	0.65	I

For each gene the log2 value of the fold change is given, and thus up-regulated and down-regulated genes are reflected in positive and negative numbers respectively. Genes are sorted first according to the cluster groups identified for the low-dose treatments (see Methods), and then by whether the trend at the high dose (100 nM) is consistent with the results seen at lower doses. Y = monotonic trend; gene expression at 100 nM E2 continues the trend at lower doses or has reached a plateau at that point. N = trend is clearly not monotonic; gene expression at 100 nM E2 is in the reverse direction of the trend at lower doses. I = suggestion of non-monotonic trend; gene expression at 100 nM shows slight reversal of trend at lower doses. *Value is average value for all probes for this gene (n = 5).

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producing lysophophatidic acid (LPA), known to be a mitogen for both ovarian and prostate cancer cells, which stimulates cell proliferation, survival and migration (reviewed in [45]). These non-monotonically expressed genes reinforce the general conclusion that pathways related to cell adhesion are influenced by estrogen treatment, but also suggest a different effect of the highest dose relative to the lower doses, with a progression toward increased cell proliferation and migration at increasing dose.

The Wnt signaling pathway was influenced at all E2 doses examined, but with an emphasis toward up-regulation of canonical Wnt/β-catenin stabilization signaling at the high dose, and non-canonical (PCP) signaling at lower doses. The high-dose effect may be mediated through the known association of β-catenin with AR and ER. Truica *et al.* have shown that β-catenin significantly enhances androgen-stimulated transcriptional activation by the AR, and that β-catenin also increases AR transcriptional activation by E2 [46]. Although many Wnt genes are differentially expressed in the prostate according to age [47], their role in prostate development, and particularly their interactive and temporal roles, is only starting to be described.

At the high dose of E2 we observed changes in genes related to steroid hormone metabolism, and alterations in steroid hormone signaling that would lead in turn to disruption of the normal expression of other developmentally important genes. Of particular interest was the observed up-regulation of Cyp7b1, which catalyzes the metabolism of the DHT metabolites 3α-Adiol and 3β-Adiol, and is thought to control cellular levels of both androgens and estrogens [48]. We verified by quantitative PCR (qPCR) that the up-regulation of Esr1 observed in these estrogen-treated cells was dose-dependent and consistent with our prior data ([14], data not shown); up-regulation of Ar was seen by qPCR but did not reach statistical significance by microarray. Esr1 was stimulated across the entire E2 dose range in this study, and thus is a potential common mechanism for the initiation of consequent

signaling events. Stimulation of Esr1 and Ar serve to amplify estrogen and androgen signaling respectively, and in the intact gland there would be further potential for signal amplification, with local conversion of testosterone not only via Srd5a1 to the more potent androgen DHT, but also via aromatization to E2.

It is important to note that the intracellular concentration of E2 within the urogenital sinus during development is still unknown. The dose of E2 that reaches ER in male mouse UGS mesenchyme cells would depend not only on E2 uptake from the blood but also on local aromatization of testosterone to E2. Because of this issue, we administered E2 over a wide dose range, but also ensured that the opportunity for aromatization was controlled by the use of DHT rather than testosterone in the culture medium. Total testosterone circulates in the range of 5–8 nM in the male rat and mouse fetus during prostate differentiation [11,32]. Because there is no high-affinity testosterone binding protein in the blood at this time, and testosterone is only weakly bound to albumin, the result is that the percentage of total testosterone in blood that is bioactive is high, particularly compared to E2, which binds to the high-affinity plasma protein alphafetoprotein. Serum testosterone thus provides a substantial pool from which intracellular E2 can be formed by aromatization in fetal prostate mesenchyme cells [32,49]. Arase and colleagues [50] have measured E2 concentrations in fetal male mouse UGS tissue at GD17 and postnatal day (PD) 1, which approximated 10 and 25 pg/g, respectively. These concentrations are consistent with the low doses of E2 that we administered in this study, although again we do not know how much of this E2 reaches ER (the actual dose at target). Future work should address the dynamics of estrogen concentration and receptor activation both *in vitro* and *in vivo*.

The up-regulation of Pgr by all doses of E2 administered here to UGS mesenchyme cells is in general agreement with Risbridger et al., who reported up-regulation of progesterone receptors (PR) in the adult mouse prostate after estrogen treatment [51], and with data from Nishino et al. that showed enhancement of progesterone's proliferative effects on the adult rat prostate after co-treatment with E2 or DHT [52]. The presence of PR may be more relevant during fetal life, when progesterone levels are higher, than in adulthood when progesterone levels are low. The issue of fetal responsiveness to progestins is complex in that there is evidence that progestins can have anti-androgenic influences on sexual differentiation, through inhibition of 5α-reductase [53,54]. Up-regulation of Pgr is thus a potential mechanism for disruptive effects of estrogens on male accessory reproductive organ development, but its impact will require further study.

Neonatal estrogen treatment is known to affect the expression of several genes critical to prostate development. Notable examples are Hoxb13, Nkx3.1, Shh, Fgf10 and Bmp4 [55]. Some of the genes that responded to E2 treatment in our cells agree with the findings of others (Hoxb13, Bmp4), but several of the "candidate" genes were not affected at the doses we examined. There may be several reasons for this, but two are critical. First, we deliberately cultured only the mesenchyme cells, to specifically examine effects of E2 on gene expression in the cells that initiate early prostate differentiation. Without the two-way communication that occurs between epithelial and mesenchymal cells in the developing prostate the full range of gene expression will not be seen [1,56,57]. For example, Nkx3.1 is expressed only in epithelial cells in regions of ductal growth, although its expression is dependent on the presence of UGS mesenchyme [58]. Similarly, Ptc and Gli, components of the Shh signaling pathway that are important for directing ductal growth, are expressed in the mesenchyme but are regulated by Shh signaling from the epithelium [59]. Additionally, in studies performed *in vivo*, other factors provided via blood circulation (known or unknown), as well as shifts in hormone levels that occur during late fetal life, parturition and early postnatal life [32,59], will influence gene expression. Consequently, studies performed in whole tissues of intact animals are bound to yield different and more complex results.

Developmental estrogen exposure has the potential to acutely stimulate abnormal growth and induction of hyperplasia in the developing prostate [12], and this clearly establishes the potential for abnormal function in later life and a predisposition toward adult prostate disease [28]. The growth of fetal prostate epithelial cells and duct formation are driven by signals from the UGS mesenchyme [9], and our results suggest that the developmental effects of estrogens or xenoestrogens on UGS differentiation may be mediated initially by enhanced mesenchymal cell responsiveness to sex steroid hormones through up-regulation of steroid hormone receptor concentrations, with subsequent effects on other genes that differed based on the dose of E2. The differing patterns of gene expression at low and high E2 concentrations and the presence of non-monotonic responses of some genes to the wide (10,000-fold) range of E2 concentrations studied are consistent with non-monotonic dose effects on prostate development *in vivo* [11,12,13].

Supporting Information

Article S1 Previously published article [19] that included qPCR data from validation tests for microarray experiments. The data presented (Figure 5, page 89) show effects of 100 nM estradiol on expression of seven genes, and effects of 1000 nM bisphenol A on the same seven genes. Microarray data are not included.
(PDF)

Author Contributions

Conceived and designed the experiments: FVS CAR. Performed the experiments: JAT CAR AS KC HW. Analyzed the data: JAT KC TS CAR. Contributed reagents/materials/analysis tools: FVS TS TI . Wrote the paper: JAT FVS TS.

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