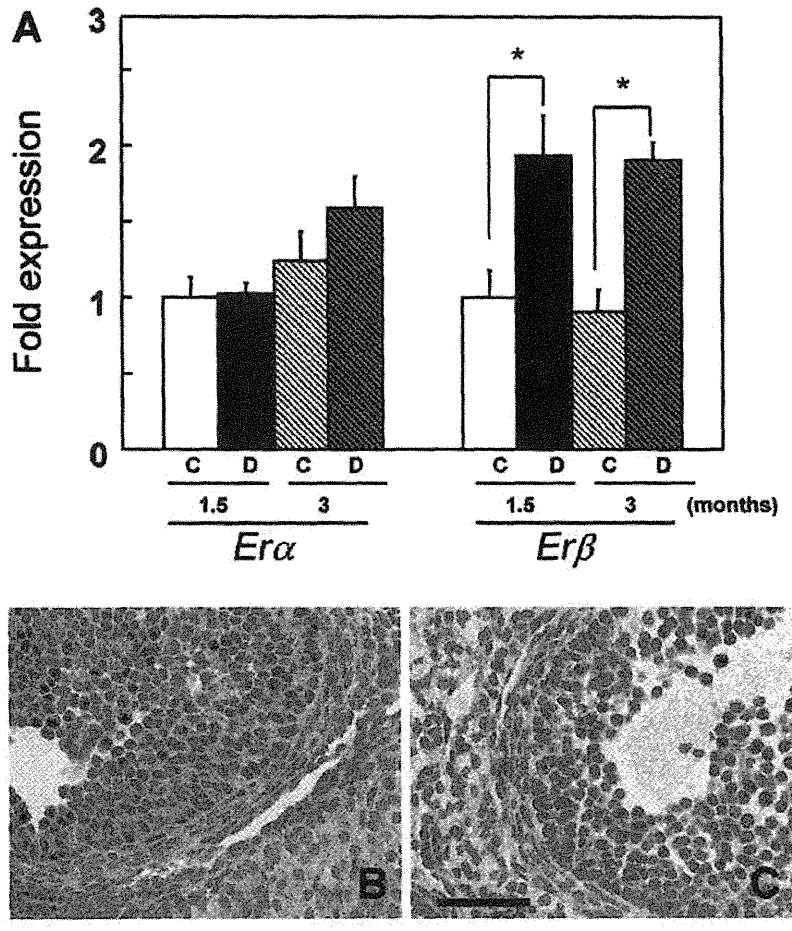

Fig. 5. mRNA expression in 1.5- and 3-month-old neonatally oil- or DES-treated C56BL/6J mice (A). C: neonatally oil-treated mice. D: neonatally DES-treated mice. Immunohistochemistry of ERβ in the 3-month-old neonatally oil- (B) and neonatally DES-treated mice (C). Bar = 50 μm. *; $p < 0.05$, compared with age-matched oil-treated mice.

significantly decreased compared with that in oil-treated mice, and *Cga* mRNA expression tended to be lower ($p = 0.068$, Fig. 6C). In contrast, *Fshb* mRNA expression was not changed (Fig. 6C). Ovariectomy stimulated the expression of *Cga*, *Fshb* and *Lhb* in the pituitary of 3-month-old neonatally oil-treated mice (data not shown).

3.7. Histology of grafted ovaries stained with Oil Red O

Various stages of follicles and CL (Fig. 7A and B) were found in both the grafted ovaries from neonatally oil- and DES-treated mice (group 1). Similarly, the grafted ovaries of neonatally oil- (group 2) and DES-treated mice (group 3) showed growing follicles and CL (Fig. 7C and D). Four out of 8 grafted ovaries in group 3 contained cystic follicles (CF) and 2 out of 8 grafted ovaries in group 3 contained hemorrhagic follicles (HF). Grafted ovaries of neonatally DES-treated 3-month-old mice (group 4) showed growing follicles, CL and CF (Fig. 7E). When ovaries of neonatally oil-treated 5-day-old (group 5) or 3-month-old mice were grafted into neonatally DES-treated mice (group 6), lipid droplets in interstitial and theca cells stained with Oil Red O were found and no CL was observed (Fig. 7F and G). The number of CL in ovaries of groups 2 and 3 were reduced compared to neonatally oil-treated 3-month-old mice (Table 1).

Table 1
Number of mice with corpora lutea (CL), cystic follicles (CF) and hemorrhagic follicles (HF) observed in neonatally oil- or DES-treated mice and grafted neonatally oil- or DES-treated ovaries.

Group	No. of mice examined	No. of mice with			Number of CLs in the ovary
		CL	CF	HF	
Oil-treated 3-month-old mice	7	7	0	0	6.4 ± 0.65
DES-treated 3-month-old mice	10	0[a]	4	3	0[a]
Oil-treated ovaries, group 1	7	5[b]	1	0	2.0 ± 0.65[a,b]
DES-treated ovaries, group 1	7	6[b]	5[a]	0	1.7 ± 0.42[a,b]
Oil-treated ovaries, group 2	7	6[b]	1	0	2.9 ± 0.69[a,b]
DES-treated ovaries, group 3	12	8[b]	4	2	1.9 ± 0.60[a,b]
DES-treated ovaries, group 4	4	4[b]	2	0	3.0 ± 0.58[a,b]

Number of CLs; the mean ± SE.
[a] $p < 0.05$; the significant difference from the oil-treated 3-month-old mice.
[b] $p < 0.05$; the significant difference from the DES-treated 3-month-old mice.

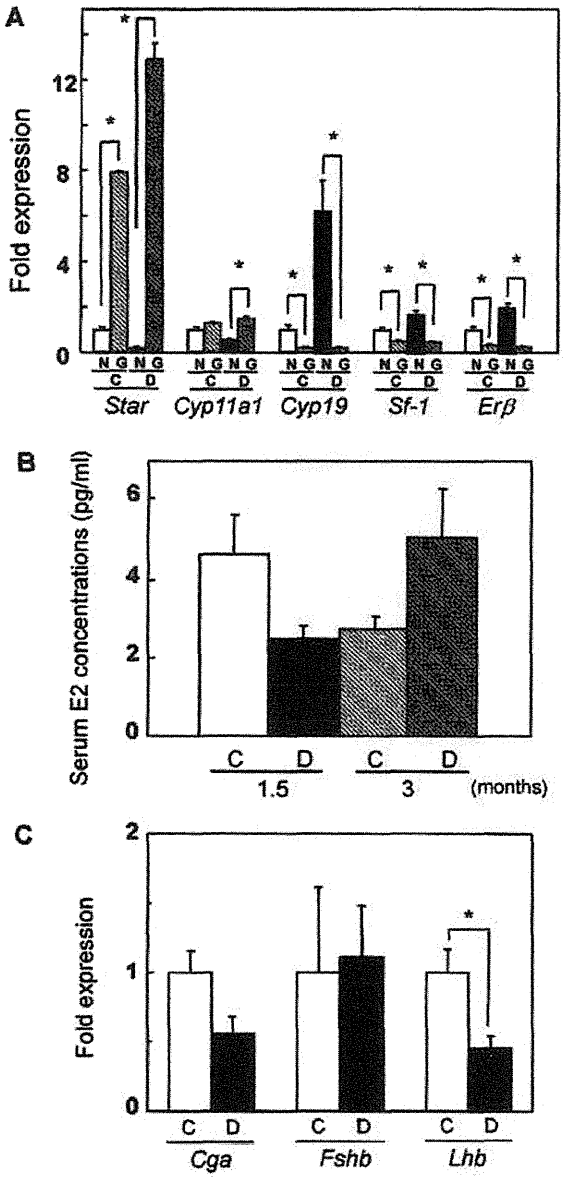

Fig. 6. Changes in the mRNA expression involved in ovarian steroidogenesis in the ovaries of 3-month-old neonatally oil- or DES-treated C56BL/6J mice after gonadotropin treatments (A) and serum levels of 17β-estradiol (E2) in 1.5- and 3-month-old neonatally oil- or DES-treated mice (B). Changes in the gonadotropin-related genes mRNA expression in the anterior pituitary of 3-month-old neonatally oil- or DES-treated C56BL/6J mice (C). N: non-treated with gonadotropins, G: treated with gonadotropins, C: neonatally oil-treated mice, D: neonatally DES-treated mice. *; $p < 0.05$ compared with age-matched oil control mice in the same treatment group.

Some grafted ovaries from groups 1, 3 and 4 had CF, and some grafted ovaries from group 3 also had HF (Table 1).

4. Discussion

Ovulation and steroidogenesis have begun by 1.5 months of age in neonatally oil-treated (Neo Oil) mice, since CL were observed in their ovaries. In contrast, CL were not found but lipid droplets were observed in the ovary of 1.5- and 3-month-old neonatally DES-treated (Neo-DES) mice, suggesting a lack of LH surge caused by neonatal DES treatment as previously reported [8].

The expression of *Star* and *Cyp11a1* was decreased in the ovary of 1.5- and 3-month-old Neo DES mice due to the alterations of gonadotropin levels [16,21,22]. Ronen-Fuhrmann et al. [36] showed that *Star* is expressed in interstitial and theca cells in response to PMSG, and then expressed in interstitial, theca and granulosa cells in response to hCG. *Cyp11a1* is also expressed in the interstitial, theca and granulosa cells in response to PMSG and reaches a peak after hCG administration. Thus, lack of the LH surge may result in a decrease of *Star* and *Cyp11a1* expression in Neo-DES mice. In addition, the expression of *Cyp19* increases in response to PMSG but decreases after hCG administration [36]. The expression of *Erβ* in the ovary is also reduced by hCG whereas PMSG has no effect [37]. In this study, the expression of *Star*, *Cyp11a1*, *Cyp19* and *Erβ* was restored after PMSG and hCG treatments in Neo-DES mice suggesting that insufficiency of gonadotropin levels. In addition, exogenous gonadotropins can affect steroidogenesis in the ovary of Neo-DES mice as well as oil-treated mice.

Hypertrophy of interstitial tissues is found in the ovaries of FSH receptor KO (FORKO) mice showing elevated levels of plasma FSH, LH and T, but decreased E2 levels with normal *Cyp19* expression compared with those in WT mice [38,39], and FSHβ subunit KO (FSHKO) mice showing decreased *Cyp11a1* and *Cyp19* despite high circulating levels of LH [19,40]. However, hypertrophy of interstitial tissues is not found in the ovary of αERKO mice showing high LH levels, normal FSH levels and *Star* and *Cyp11a1* expression [41]. Hasegawa et al. [12] showed that ovaries of *Star* KO mice appear normal, however, prominent lipid deposits accumulated in the interstitial region after the time of normal puberty. Targeted disruption of *Cyp11a1* results in the accumulation of lipid droplets in the interstitial cells [14]. Thus, hypertrophy and accumulation of lipid droplets in interstitial cells of Neo-DES mice is caused by failure of steroidogenesis due to the alterations of gonadotropin levels. Indeed, grafted ovaries of Neo-DES mice showed various stages of follicles and CL, but no lipid accumulation in the interstitial tissue, whereas grafted ovaries of Neo Oil mice into 3-month-old Neo-DES hosts showed lipid accumulation in the interstitial tissue. In addition, when ovaries from 1-month-old Neo-DES mice were grafted into control female hosts, the pattern of ^{3}H-steroids in *in vitro* steroid synthesis changes the pattern characteristic to controls and vice versa [4]. These results support the idea that hypertrophy of interstitial tissue in Neo-DES mice is caused by the alteration of gonadotropin levels and lack of the LH surge.

In Neo-DES mice, serum FSH concentrations are increased but LH levels are not altered on day 19 [35], however, the levels of FSH and LH are similar in Neo-Oil and Neo-DES mice on day 56 [42]. In males, the reduced concentrations of gonadotropins in plasma are observed in neonatally estrogenized rats [43]. In this study, the expression of *Lhb* was reduced but *Cga* and *Fshb* mRNA levels were not altered in the anterior pituitary of 3-month-old Neo-DES mice. In addition, the percentage of LH-producing cells in the anterior pituitary of Neo-DES mice was partially decreased at 3-month-old (unpublished data), suggesting that serum levels of gonadotropins may be altered in Neo-DES mice. Although the levels of FSH and LH in 3-month-old Neo-DES mice have not been investigated yet, it is speculated that reduced serum levels of gonadotropins in Neo-DES mice may result in hypertrophy of interstitial tissues in the ovary. Furthermore, the expression of gonadotropin-related genes is increased after ovariectomy [44] and our data were coincident with the previous reports, however, it is not clear whether ovariectomy can affect the pituitary gonadotropins in 3-month-old Neo-DES mice. Thus, it is necessary to examine the effects of neonatal DES treatment on the pituitary in the near future.

In this study, the serum levels of E2 were not different between Neo-Oil and Neo-DES mice although the expression of *Cyp19* was significantly increased in the ovary of Neo-DES mice. This fact suggests 2 possibilities: (1) transcription of *Cyp19* may be inhibited and/or (2) CYP19 may not have the ability to stimulate E2 synthesis in the ovary of Neo-DES mice. In addition, CYP19 is localized in

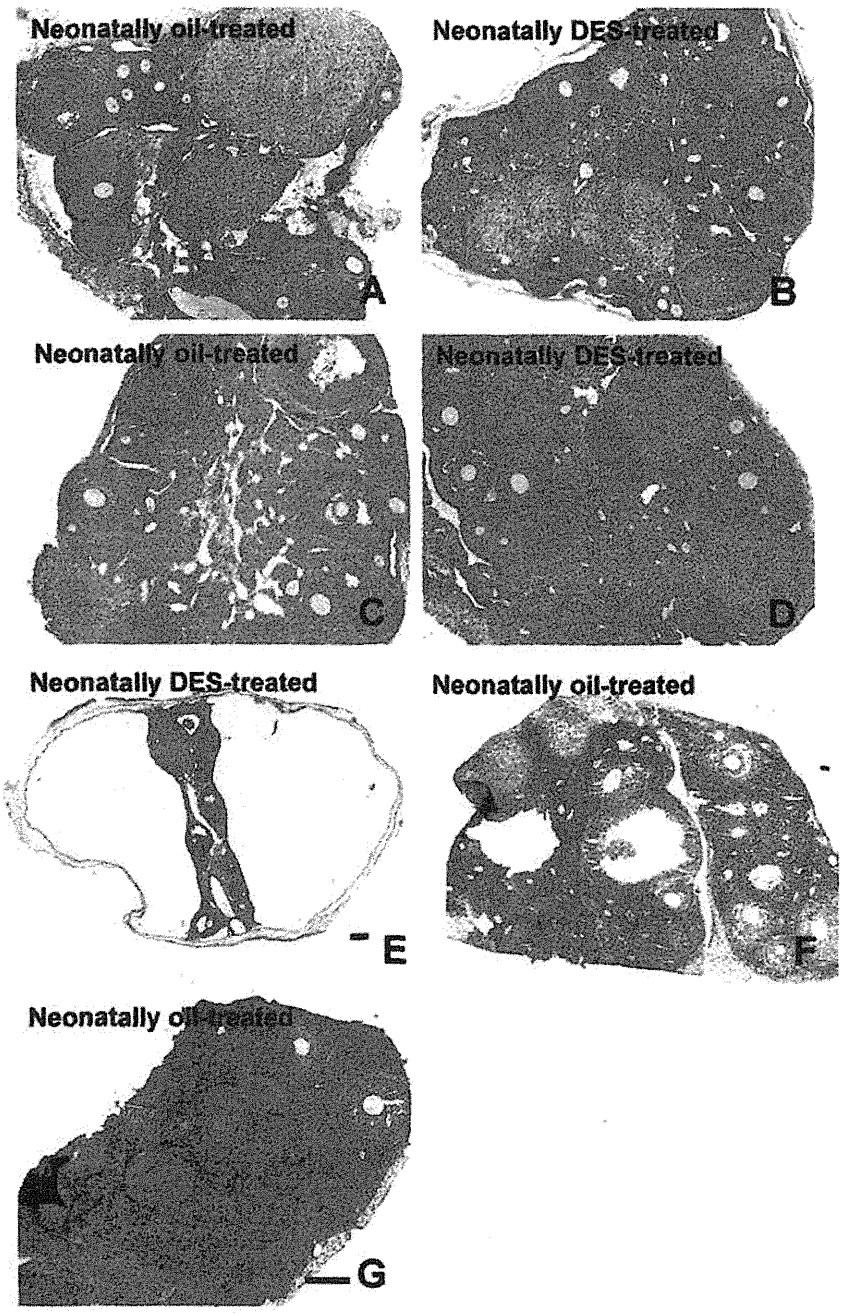

Fig. 7. HE and Oil Red O staining in grafted ovaries after the 3-month growth period. HE staining of the grafted ovaries from neonatally oil- (A) or DES-treated mice in 3-month-old neonatally oil-treated hosts (B, group 1). Oil Red O staining of the grafted ovaries from neonatally oil-treated mice (C, group 2), the grafted ovaries from neonatally DES-treated mice (D, group 3), the grafted ovaries from 3-month-old neonatally DES-treated mice (E, group 4) in 3-month-old neonatally oil-treated hosts, the grafted ovaries from neonatally oil-treated mice in 3-month-old neonatally DES-treated hosts (F, group 5) and the grafted ovaries from 3-month-old neonatally oil-treated mice in 3-month-old neonatally DES-treated hosts (G, group 6), respectively. Bar = 200 μm.

granulosa cells of the preovulatory follicle [15], therefore, it is possible that the function of granulosa cells can be altered in the ovary of Neo-DES mice. Indeed, inhibin secreted by granulosa cells is consisted of α and β subunits and the expression of inhibin α mRNA is high in the ovary of 2-month-old Neo-DES mice [45]. The number of oocytes is reduced in the Neo-DES mouse ovary [46,3]. Although the direct effects of neonatal DES treatments on oocytes are unclear, the oocyte–somatic cell interactions may also be impaired.

The expression of *Sf-1* is most abundant in the interstitial and theca cells in immature and cycling adult rats and also in granulosa cells of healthy growing follicles [33]. In this study, the expression of *Sf-1* both in the ovary of Neo-Oil or Neo-DES mice is significantly decreased with age, but *Sf-1* expression in the ovary of 3-month-old Neo-DES mice was higher than that in the age-matched Neo-Oil mice. Reduced expression of *Sf-1* after gonadotropin treatment is consistent with the previous report [33]. Thus, up-regulation of *Sf-1* in the ovary of 3-month-old Neo-DES mice may be due to lack of the LH surge. In contrast, the expression of *Lrh-1* is selective in granulosa and CL but not in interstitial and theca cells in cycling and immature rats [33]. Similar to *Sf-1*, the expression of *Lrh-1* in

the ovary is also decreased in response to the combination of E2, FSH and hCG treatments [33]. Interestingly, the expression of *Lrh-1* in the ovary of 1.5-month-old Neo-DES mice was significantly increased compared with that in the age-matched Neo-Oil mice, but not at 3 months of age. Both granulosa and luteal cells in the ovary of Neo-Oil mice may be responsible for the expression of *Lrh-1*, however, only granulosa cells may be involved in *Lrh-1* expression in the ovary of Neo-DES mice. In addition, *Sf-1* and *Lrh-1* have qualitatively similar actions on FSH-stimulated steroidogenesis in the ovary [24]. The localization of those genes raises the possibility that *Sf-1* may contribute to the interstitial and theca cells and *Lrh-1* may be specific for luteal cells, however, the function of these genes in granulosa cells where both genes are expressed is still unclear. Indeed, the expression of *Hsd3b1*, a target of both *Sf-1* and *Lrh-1* [15,47], was not altered in the ovary of Neo-DES mice. In the future study, therefore, it is needed to isolate different cell types from the ovaries of Neo-DES mice and quantify the expression of *Sf-1* and *Lrh-1*, respectively.

HSD17B1 is expressed in granulosa cells and converts estrone to E2, while HSD17B3 is expressed in Leydig cells and coverts androstenedione to T [15]. The ovary of αERKO mice exhibits testis-like levels of *Hsd17b3* expression [48]. The expression of *Hsd17b1* and *Hsd17b3* in the ovary of Neo-DES mice was not altered, suggesting that the interstitial and theca cells of Neo-DES mice maintain a female phenotype.

In this study, HF were occasionally found in the ovaries of Neo-DES mice, even after the grafting ovaries from Neo-DES mice into the normal host mice. HF are also seen in the ovaries of αERKO mice due to the high circulating levels of LH [49] but not in βERKO mice [50]. The transgenic LHβ-C-terminal peptide (LHβCTP) mice exhibiting LH hypersecretion also show HF, however, LHβCTP mice lacking ERβ show no HF [51]. In addition, in αERKO mice, plasma levels of FSH, and expression of *Star* and *Cyp11a1* expression are normal but the expression of *Cyp17* and *Cyp19* is elevated in the ovary [41]. Therefore, HF in the ovary of Neo-DES mice is not due to high circulating levels of LH and elevated steroidogenesis as in αERKO. Previously, we reported that neonatal DES treatment inhibited follicle formation and development through ERβ in the neonatal mouse ovaries [52], indicating that DES may directly affect follicular growth and development.

In summary, our findings indicate that hypertrophy and accumulation of lipid droplets in ovarian interstitial cells of Neo-DES mice is caused by impaired steroidogenesis due to low levels of gonadotropins, especially lack of the LH surge. Neonatal DES treatment can affect the HPG axis via ERα and indirectly disrupt ovarian steroidogenesis.

Conflict of interest statement

There is no conflict 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.reprotox.2011.10.013.

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PLOS ONE

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

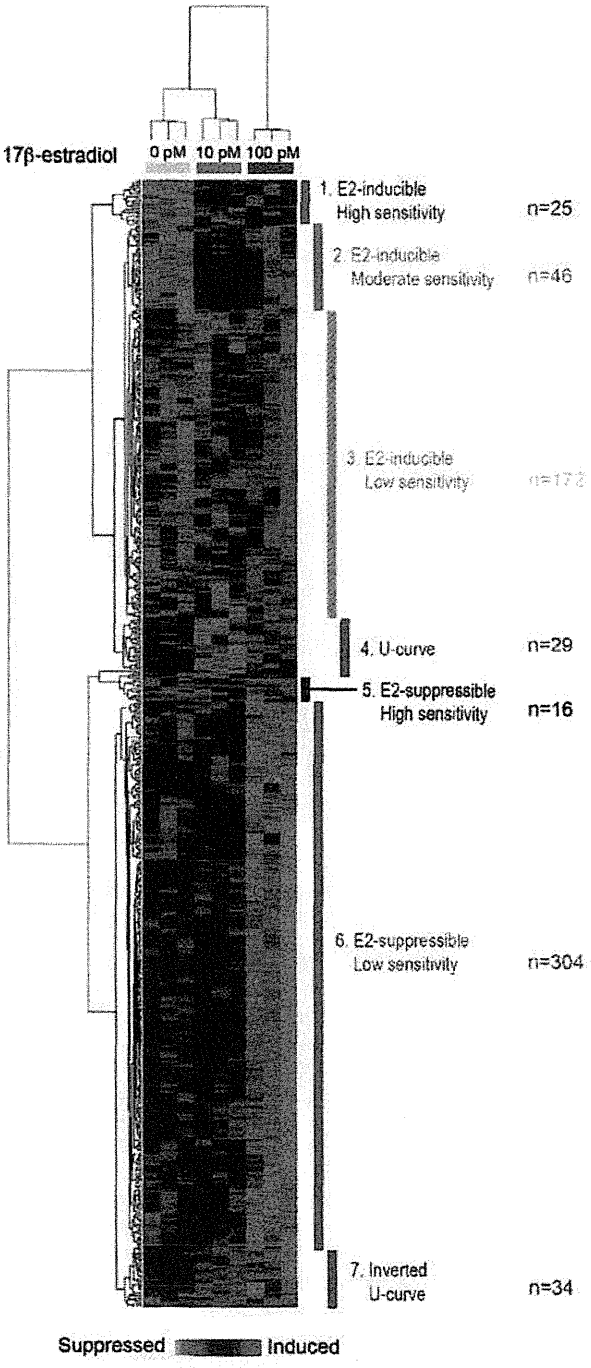

Figure 1. Cluster analysis of estrogen-responsive genes in fetal UGS mesenchyme cells after estrogen treatment with the two lower doses, showing strong separation of responses to control, 10 pM E2, and 100 pM E2 treatments. Based on clustering, genes were identified as falling into one of 7 groups that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

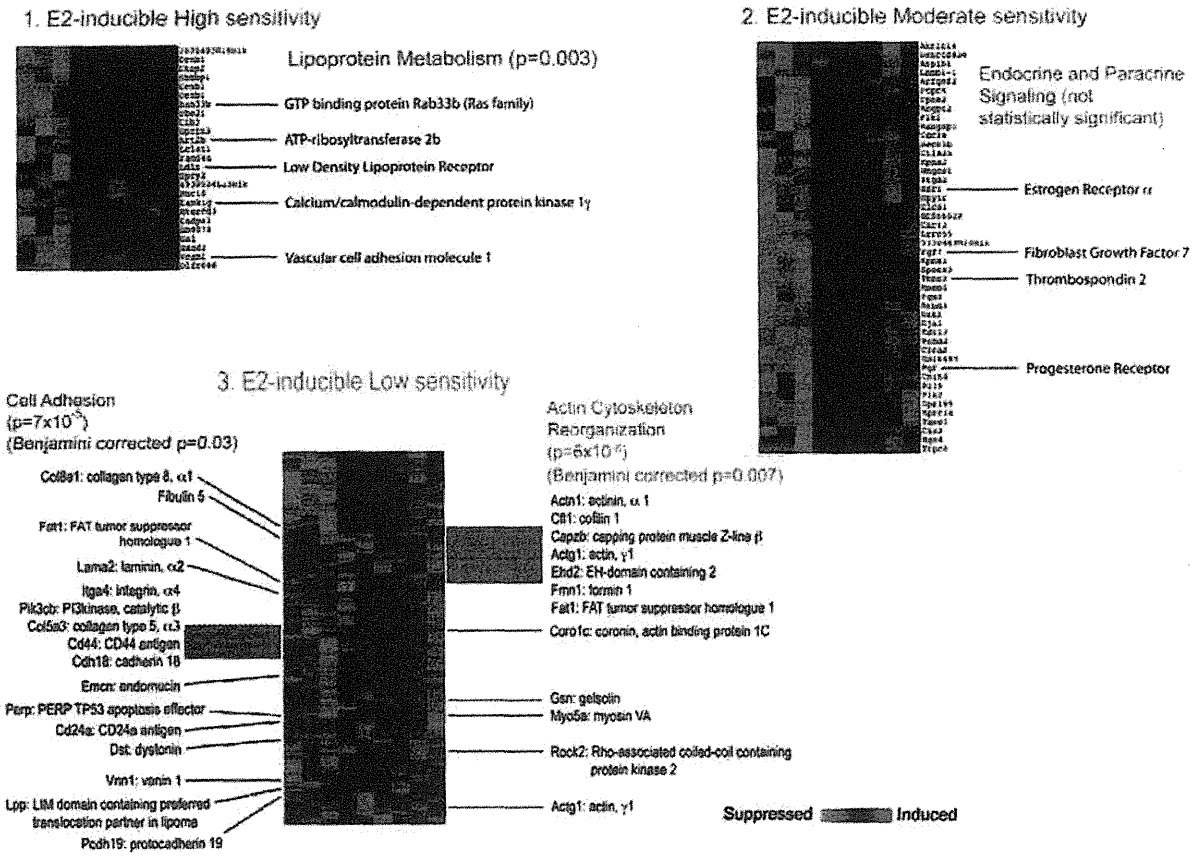

Figure 2. Detail of E2-inducible genes in groups identified by clustering analysis. Set 1: E2-inducible high sensitivity. Set 2: E2- inducible moderate sensitivity. Set 3: E2-inducible low sensitivity. Select genes of interest are highlighted. The figures show raw p values as well as, where indicated, 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

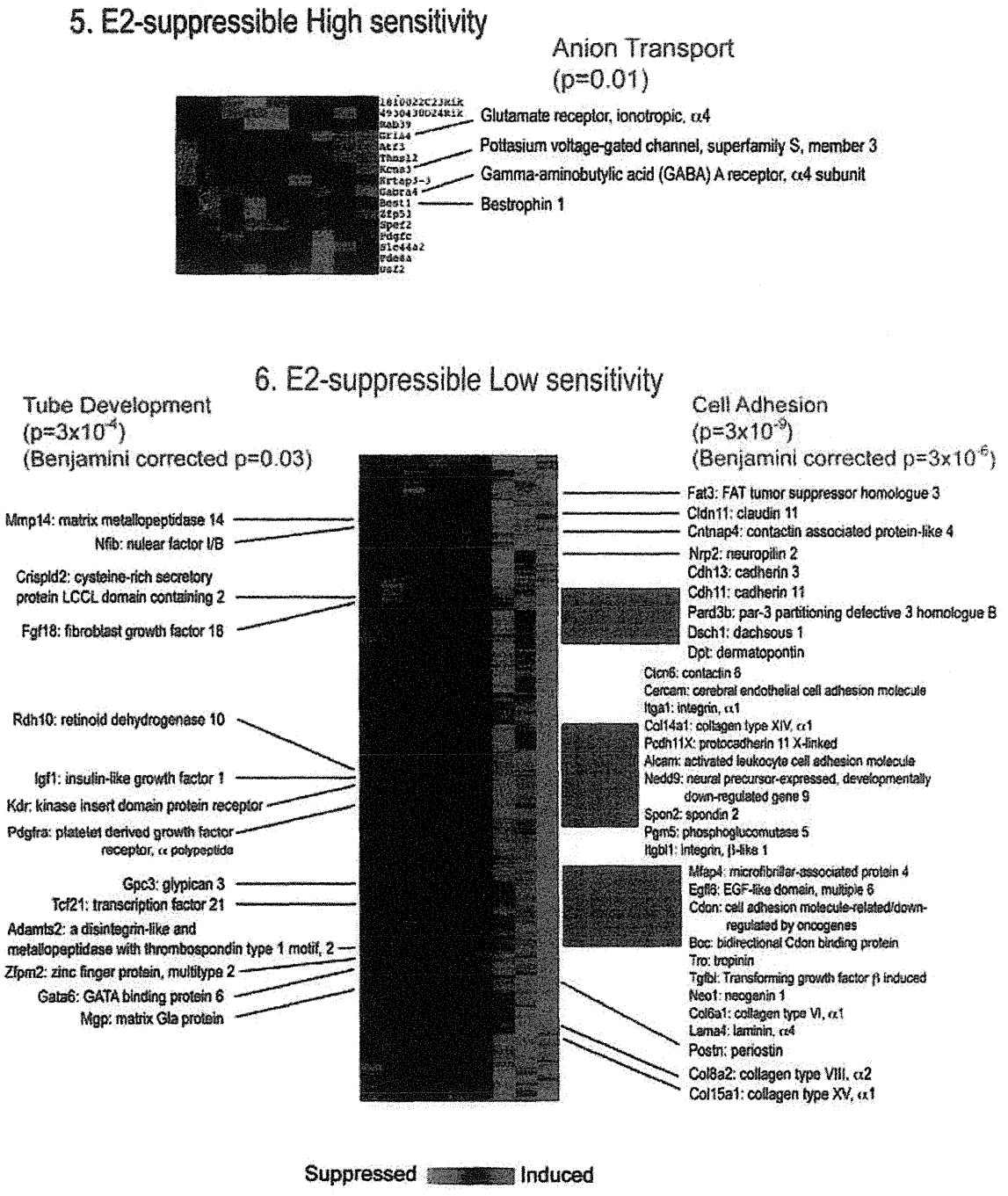

Figure 3. Detail of E2-suppressible genes in groups separated by clustering analysis. Set 5: E2-suppressible high sensitivity. Set 6: E2-suppressible low sensitivity. The figures show raw p values as well as, where indicated, Benjamini-Hochberg corrected p values.
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observed 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