Supporting Information

Zheng and Cohn 10.1073/pnas.1108312108

SI Methods

Animals and Treatments. Flutamide (120 mg/kg, 40 mg/mL, dissolved in corn oil with 1% ethanol), fulvestrant (1 mg/kg, 50 mg/mL, dissolved in ethanol and diluted 100 times in corn oil), DHT (2 mg/kg, 100 mg/mL, dissolved in ethanol diluted 100 times in corn oil), estradiol (300 μg/kg, 15 mg/mL dissolved in ethanol and diluted 100 times in corn oil), or corn oil control vehicle (corn oil with 1% ethanol) was administered by oral gavage (flutamide) or IP injection (DHT, estradiol, fulvestrant) of pregnant females from E12.5 to E15.5 once daily, except for fulvestrant which was administered at E12.5 and E14.5 to prevent induction of labor. The effect of treatment on embryos was validated by measurements of anogenital distance (Fig. S7*A*). Anogenital distance is a confirmed sexually dimorphic marker in rodents, is permanently affected by prenatal androgen exposure (1, 2), and is obvious as early as E15.5 (Fig. S7*B*). Sample sizes for pharmacological treatment groups (Fig. 3 and Figs. S3, S4, and S7) range from a minimum of $n = 15$ to a maximum of $n = 23$. Wild-type sample sizes are provided in the figure legends. All animal experiments were performed in accordance with institutional guidelines.

Quantitative RT-PCR. Total RNA was extracted from 2D and 4D by using RNeasy plus micro kit (Qiagen), and RNA quantity (>100 ng/μL) and purity (260/280 > 2.0, 260/230 > 1.65) were determined by using a Nanodrop. RNA integrity (RIN > 8.5) and 28S/18S ratio (>1.5) were assessed by using a Bioanalyzer 2100 (Agilent Technologies). A quantity of 500 ng of high-quality RNA for each pooled sample ($n = 3$) was converted into cDNA by using the RT^2 First Strand cDNA Kit (SABiosciences). Skeletogenic gene expression was determined by using the Osteogenesis PCR Array (PAMM-026; SABiosciences) and the 7900 HT Fast Real-Time PCR system (Applied Biosystems) according to the manufacturer's protocol. The complete list of genes assayed on the array can be found at the manufacturer's Web site. For other genes, expression was detected by using the CFX96 Real Time system (Bio-Rad) and QPCR system (Bio-Rad) with Actb (3) and Gapdh (4) as controls. Primers not previously published were designed by using Beacon Designer Software, except for Hoxa13, which was purchased from SABiosciences. The qRT-PCR primers designed for this study are listed in Table S2. The Web-Based PCR Array Data Analysis system (SABiosciences) was used to analyze PCR array results, and results of qRT-PCR assays not represented on the array were determined using ΔΔCt method (5).

1. Hotchkiss AK, et al. (2007) Prenatal testosterone exposure permanently masculinizes anogenital distance, nipple development, and reproductive tract morphology in female Sprague-Dawley rats. *Toxicol Sci* 96:335–345.
2. Manno FA, 3rd (2008) Measurement of the digit lengths and the anogenital distance in mice. *Physiol Behav* 93:364–368.
3. Seifert AW, Zheng Z, Ormerod BK, Cohn MJ (2010) Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics. *Nat Commun* 1:23.
4. Crusselle-Davis VJ, Vieira KF, Zhou Z, Anantharaman A, Bungert J (2006) Antagonistic regulation of beta-globin gene expression by helix-loop-helix proteins USF and TFII-I. *Mol Cell Biol* 26:6832–6843.
5. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45.

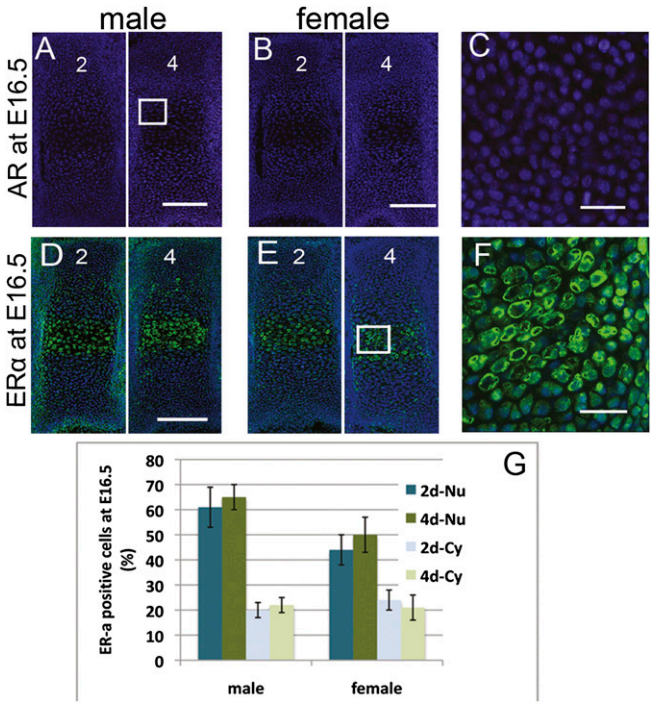

Fig. S1. AR and ER-α localization in 2D and D4 in E16.5 CD-1 mice. *(A, B, D,* and *E)* Longitudinal sections through proximal phalanges stained with DAPI (blue) and antibodies against AR (red, which is undetectable in *A* and *B*) and ER-α (green in *D* and *E*). D2 and 4D are numbered. White boxes in *A* and *E* are enlarged in *C* and *F*. (Scale bars: 50 μm in *A*, *B*, *D*, and *E*; 10 μm in *C* and *F*.) (*G*) Quantitative analysis of ER-α–positive cells in E16.5 proximal phalanges with nuclear (Nu) and cytoplasmic (Cy) staining. AR staining at this stage was below detectable levels. Error bars show ± SEM.

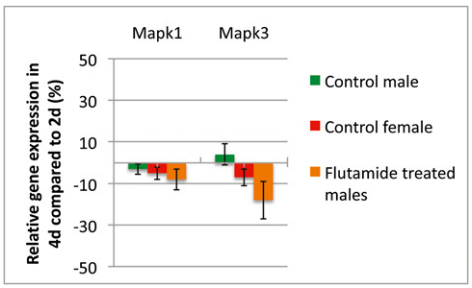

Fig. S2. Quantitative RT-PCR expression analysis of *Mapk1* and *Mapk3* in 2D and 4D. Graphs show relative transcript levels in 4D compared with 2D. Note that there is no significant difference between 2D and 4D. Error bars show ± SEM.

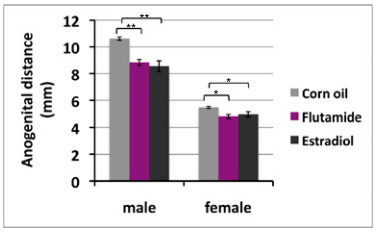

Fig. S3. Postnatal antiandrogen or estradiol treatment reduces anogenital distance in mice. Anogenital distance was measured at P21 following daily treatments from P0 to P3 with flutamide (120 mg/kg) and estradiol (300 μg/kg). The results show that either flutamide ($P = 0.001$) or estradiol ($P = 0.005$) can significantly reduce anogenital distance during postnatal growth and development. Digit ratios are not affected by treatments at these stages.

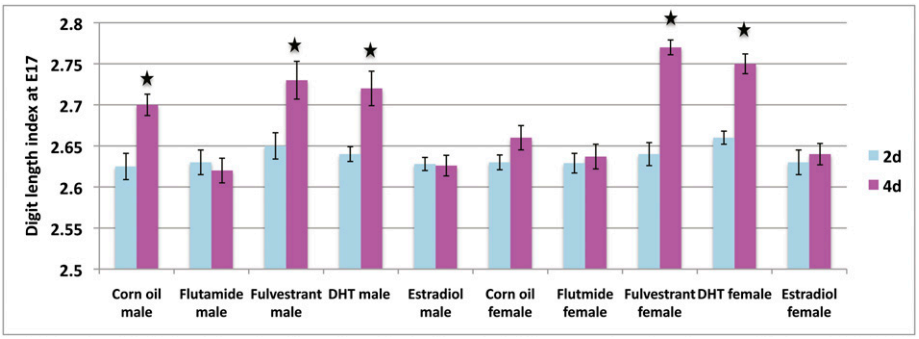

Fig. S4. Modulation of prenatal androgen and estrogen signaling alters 4D length in both males and females. All treatments were done prenatally, from E12.5 to 15.5, except for fulvestrant, which was administered at E12.5 and 14.5 to avoid labor induction. Digit length index was calculated by dividing digit length by tibia length to control for systemic changes in skeletal growth. Error bars show ± SEM. *$P < 0.05$.

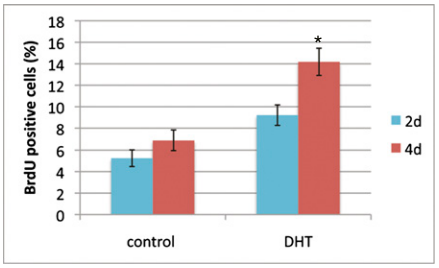

Fig. S5. Effects of DHT treatment on cell proliferation in 2D and 4D. Mitotic indices, calculated from longitudinal sections through 2D and 4D, show that DHT significantly increases cell proliferation in 4D relative to 2D. Error bars show ± SEM. *$P < 0.05$.

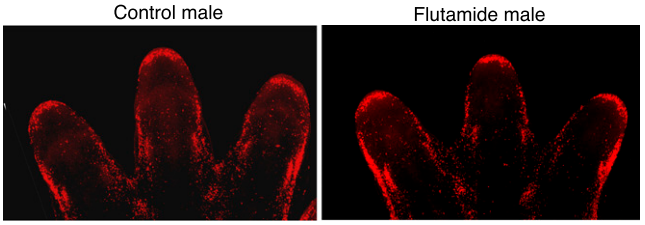

Fig. S6. Comparison of cell death in the digits of control (*Left*) and flutamide-treated (*Right*) male mice at E14.5. Lysotracker red staining showed no obvious differences in apoptosis between control and flutamide-treated digits 24 h after treatment.

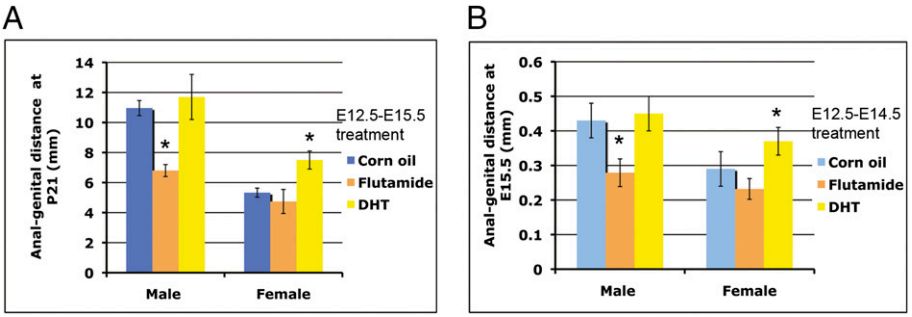

Fig. S7. Effects of prenatal flutamide and DHT treatment on anogenital distance. (*A*) Prenatal administration of flutamide (120 mg/kg from E12.5 to 15.5, once daily) significantly reduces ($P < 0.001$) anogenital distance in male mice at P21. DHT treatment (2 mg/kg from E12.5 to 15.5, once daily) significantly increases ($P = 0.007$) the anogenital distance in female mice at P21. (*B*) Anogenital distance is affected as early as E15.5 following treatment with flutamide ($P = 0.012$) or DHT ($P = 0.027$) between stages E12.5 and 14.5.

Table S1. Relative gene expression in 4D compared with 2D after flutamide and fulvestrant treatment

	Flutamide treatment		Fulvestrant treatment	
Gene	*P**	Fold change†	*P**	Fold change†
Ahsg	NA	NA	NA	NA
Alpl	0.399206	1.48	0.266898	1.37
Ambn	0.963548	1.02	0.817367	1.01
Anxa5	*0.026445*	1.36	0.363989	1.16
Bgn	0.393466	1.12	0.57909	−1.05
Bmp1	*0.005414*	1.15	0.899569	1.02
Bmp2	0.059722	1.33	0.078897	1.11
Bmp3	0.444235	1.14	0.670644	−1.04
Bmp4	0.306083	−1.07	0.913614	−1.01
Bmp5	0.749093	−1.04	0.882607	−1.04
Bmp6	*0.034389*	**2.62**	0.171142	1.16
Bmpr1a	0.067409	−1.09	0.62312	−1.06
Bmpr1b	0.948885	−1	0.544714	1.09
Cd36	0.295599	1.77	0.130393	−1.5
Cdh11	0.201374	1.17	0.635321	−1.11
Col10a1	*0.018254*	**−1.66**	0.393528	1.28
Col11a1	0.264219	1.29	0.608184	−1.43
Col12a1	*0.026702*	**−1.68**	0.799201	1.33
Col14a1	0.186024	1.19	*0.0278857*	**2.29**
Col1a1	0.157679	1.5	0.304379	1.18
Col1a2	0.142395	1.26	0.454542	−1.04
Col2a1	0.456362	1.18	0.411452	1.17
Col3a1	0.123003	1.51	0.280583	1.17
Col4a1	0.072995	1.59	0.615775	1.07
Col4a2	*0.040047*	**−1.44**	*0.003599*	**1.47**
Col5a1	0.060589	1.28	*0.036235*	**1.46**
Col6a1	0.053092	1.51	0.19521	1.48
Col6a2	0.052914	1.26	*0.017826*	**1.51**
Col7a1	0.450404	1.17	0.572001	1.17
Comp	0.8629	−1.01	0.810341	−1.06
Csf2	NA	NA	NA	NA
Csf3	NA	NA	NA	NA
Ctsk	0.054292	2.54	0.389205	−1.75
Dmp1	0.85548	NA	0.501604	NA
Egf	0.566286	−1.46	*0.018661*	1.29
Enam	NA	NA	NA	NA
Fgf1	0.615475	1.27	*0.0302511*	1.22
Fgf2	0.625374	1.79	0.715929	−1.03
Fgf3	NA	NA	*0.0397*	**−1.74**
Fgfr1	0.83189	1.02	0.5264	−1.06
Fgfr2	0.031945	**−1.48**	0.935737	−1.01
Fgfr3	0.10594	1.33	0.327585	−1.26
Flt1	0.305575	1.27	0.934638	−1
Fn1	0.675327	−1.1	0.908728	1.01
Gdf10	0.318849	1.17	0.599319	1.05
Hoxa13	*0.028932*	1.37	*0.041667*	−1.27
Icam1	0.40052	1.16	0.460418	−1.19
Igf1	0.652132	1.05	*0.0135072*	−1.16
Igf1r	0.100419	1.32	0.620577	1.06
Igfbp2	*0.034528*	**1.46**	*0.020395*	**−2.39**
Igfbp5	*0.041755*	**1.49**	*0.033681*	**−1.85**
Ihh	*0.027431*	**−1.68**	*0.015481*	**1.92**
Itga2	0.503951	1.22	0.610009	1.05
Itga2b	0.649112	1.11	0.441788	1.11
Itga3	0.932126	−1.08	0.351632	−1.09
Itgam	*0.011399*	**2**	0.090589	−1.24
Itgav	0.585689	−1.07	0.960074	−1
Itgb1	*0.002062*	1.35	0.218512	1.19
Mmp10	0.921681	−1.16	0.901799	1.07
Mmp2	*0.026878*	1.37	0.178692	1.17

Table S1. Cont.

Gene	Flutamide treatment		Fulvestrant treatment	
	*P**	Fold change†	*P**	Fold change†
Mmp8	NA	NA	NA	NA
Mmp9	*0.023637*	**3.9**	0.20607	1.23
Msx1	0.06553	−1.25	*0.007759*	**−1.49**
Nfkb1	*0.027757*	1.17	0.607161	1.03
Pdgfa	0.360583	1.15	*0.040907*	−1.17
Phex	0.060886	1.79	0.059236	1.81
Runx2	*0.034153*	**1.77**	0.114711	1.02
Scarb1	0.77894	1.03	0.939359	1.01
Serpinh1	0.682536	1.14	0.862305	−1.02
Smad1	0.540028	1.09	0.54183	−1.08
Smad2	0.073445	2.9	*0.0327889*	1.13
Smad3	*0.048333*	**1.45**	0.513535	1.09
Smad4	0.17833	1.21	0.056494	−1.45
Sost	0.633648	−1.12	0.465883	−1.38
Sox9	*0.02586*	**−1.76**	0.0818246	1.15
Tfip11	0.15807	1.27	0.669522	−1.04
Tgfb1	*0.00128*	1.19	*0.0445893*	1.08
Tgfb2	0.644308	1.13	0.129208	1.19
Tgfb3	0.151979	1.33	0.198769	1.15
Tgfbr1	*0.026563*	1.2	0.764842	1.03
Tgfbr2	0.871591	1.02	0.471603	−1.03
Tgfbr3	0.076085	2.67	0.094265	1.04
Tnf	NA	NA	NA	NA
Tuft1	0.426009	1.21	0.496134	−1.05
Twist1	0.375008	−1.5	0.076953	−1.55
Vcam1	0.057933	1.19	0.366577	−1.09
Vdr	0.116554	1.55	0.899057	−1.08
Vegfa	0.152263	1.9	0.790228	−1.17
Vegfb	0.097637	1.38	0.788172	−1.14
Wnt5a	*0.03783*	**1.48**	0.17962	−1.18

*Italic type indicates significant ($P < 0.05$) changes in expression levels.
†Positive fold change indicates up-regulation in 4D vs. 2D; negative fold change indicates down-regulation in 4D vs. 2D. Fold changes for genes shown in Fig. 4 are in boldface type.

Table S2. Primers designed in this study

Name	Sequences	GenBank accession no.
Igfbp2		NM_008342
For	ATCCCGAACACCAGCAGAAATG	
Rev	GCCCTCCATACCACCCTTCC	
Igfbp5		NM_010518
For	TGCTCACTCCTCTTCTCCTTCC	
Rev	TCTCTTCTCCTTGGCTCACTCC	
Ihh		NM_010544
For	GAGACACCATTGAGACTTGACCAG	
Rev	GTGAAGAATCGCAGCCAGAGC	
Wnt5a		NM_009524
For	AAAGTAGCCTTTCTGCTTCCTGCC	
Rev	TATGTGGTGAGCTGGTTTGCTTCG	
Fgfr3		NM_001163216
For	GCCAGGAGCACCAAACAAGAATG	
Rev	AAGGTAGCAGTGGGAAATGAGAGG	
Mapk1		NM_011949
For	TTGTGGCTTTGGGACTGTGTG	
Rev	GCATATTCATCCGTTACCTTCTTACC	
Mapk3		NM_011952
For	AGGAGCGGCTGAAGGAGTTG	
Rev	GCAGAGAAGGAGCAGGTAGGAG	