

Fig. 1. Phenotypic characterization of AR knockout female mice. (a) Diagram of the wild-type *Ar* genomic locus (+), floxed *AR*L3 allele (L3), and *AR* allele (L-) obtained after Cre-mediated excision of exon 1. K, KpnI; E, EcoRI; H, HindIII; B, BamHI. LoXP sites are indicated by arrowheads. The targeting vector consisted of a 7.6-kb 5' homologous region containing exon 1, a 1.3-kb 3' homologous region, a single loXP site, and the neo cassette with two loXP sites. (b) Detection of the Y chromosome-specific *Sry* gene in *AR*^{-/-} mice by PCR. (c) Absence of AR protein in *AR*^{-/-} mice ovaries by Western blot analysis using a specific C-terminal antibody. (d) Normal weight gain in *AR*^{-/-} females. (e) Histology of pituitary, uterus, and bone tissues in *AR*^{+/+} and *AR*^{-/-} females at 8 weeks of age. (f) Female reproductive organs were macroscopically normal in *AR*^{-/-} mice. (g) Serum hormone levels at the proestrus stage in *AR*^{-/-} mice were not significantly altered. Serum 17 β -estradiol, progesterone, testosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels in *AR*^{+/+} ($n = 13$) and *AR*^{-/-} ($n = 10$) females at 8–10 weeks of age are shown. (h) Lobuloalveolar development is impaired in *AR*^{-/-} mammary glands. Whole mount of inguinal mammary glands (Left) and its higher magnification (Right) were prepared on day 3 of lactation. (i) Average number of pups per litter is markedly reduced in *AR*^{-/-} mice at 8 weeks of age. Data are shown as mean \pm SEM and analyzed by using Student's *t* test. (j) AR immunocytochemistry in *AR*^{+/+} and *AR*^{-/-} ovaries. Sections were counterstained with eosin.

more layers of granulosa cells with no antrum), or antral follicles (antrum within the granulosa cell layers enclosing the oocyte). Follicles were determined to be atretic if they displayed two or more of the following criteria within a single cross section: more than two pyknotic nuclei, granulosa cells within the antral cavity, granulosa cells pulling away from the basement membrane, or uneven granulosa cell layers (15).

Immunohistochemistry. Sections were subjected to a microwave antigen retrieval technique by boiling in 10 mM citrate buffer (pH 6.0) in a microwave oven for 30 min (16). The cooled sections were incubated in 1% H₂O₂ for 30 min to quench endogenous peroxidase and then incubated with 1% Triton X-100 in PBS for 10 min. To block nonspecific antibody binding, sections were incubated in normal goat serum for 1 h at 4°C. Sections were then incubated with anti-AR (1:100) or anti-cleaved caspase-3 (1:100) in 3% BSA overnight at 4°C. Negative controls were incubated in 3% BSA without primary antibody. The ABC method was used to visualize signals according to the manufacturer's instructions. Sections were incubated in biotinylated goat anti-rabbit IgG (1:200 dilution) for 2 h at room

temperature, washed with PBS, and incubated in avidin–biotin–horseradish peroxidase for 1 h. After thorough washing in PBS, sections were developed with 3,3'-diaminobenzidine tetrahydrochloride substrate, slightly counterstained with eosin, dehydrated through an ethanol series and xylene, and mounted.

Estrus Cycles and Fertility Test. To determine the stage of the estrus cycle (proestrus, estrus, and diestrus), vaginal smears were taken every morning and stained with Giemsa solution. For evaluation of female fertility for 15 weeks, an 8- or 24-week-old wild-type or *AR*^{-/-} female was mated with a wild-type fertile male, replaced every 2 weeks with the other fertile male. Cages were monitored daily and for an additional 23 days, and the presence of seminal plugs and number of litters were recorded.

RNA Extraction and Quantitative Competitive RT-PCR. Total ovarian RNA was extracted by using TRIzol (Invitrogen) (16). Oligo-dT-primed cDNA was synthesized from 1 μ g of ovarian RNA by using SuperScript reverse transcriptase (Gibco BRL, Gaithersburg, MD) in a 20- μ l reaction volume, 1 μ l of which was then diluted serially (2- to 128-fold) and used to PCR-amplify an internal control gene, *cycA*, to allow concentration estimation.

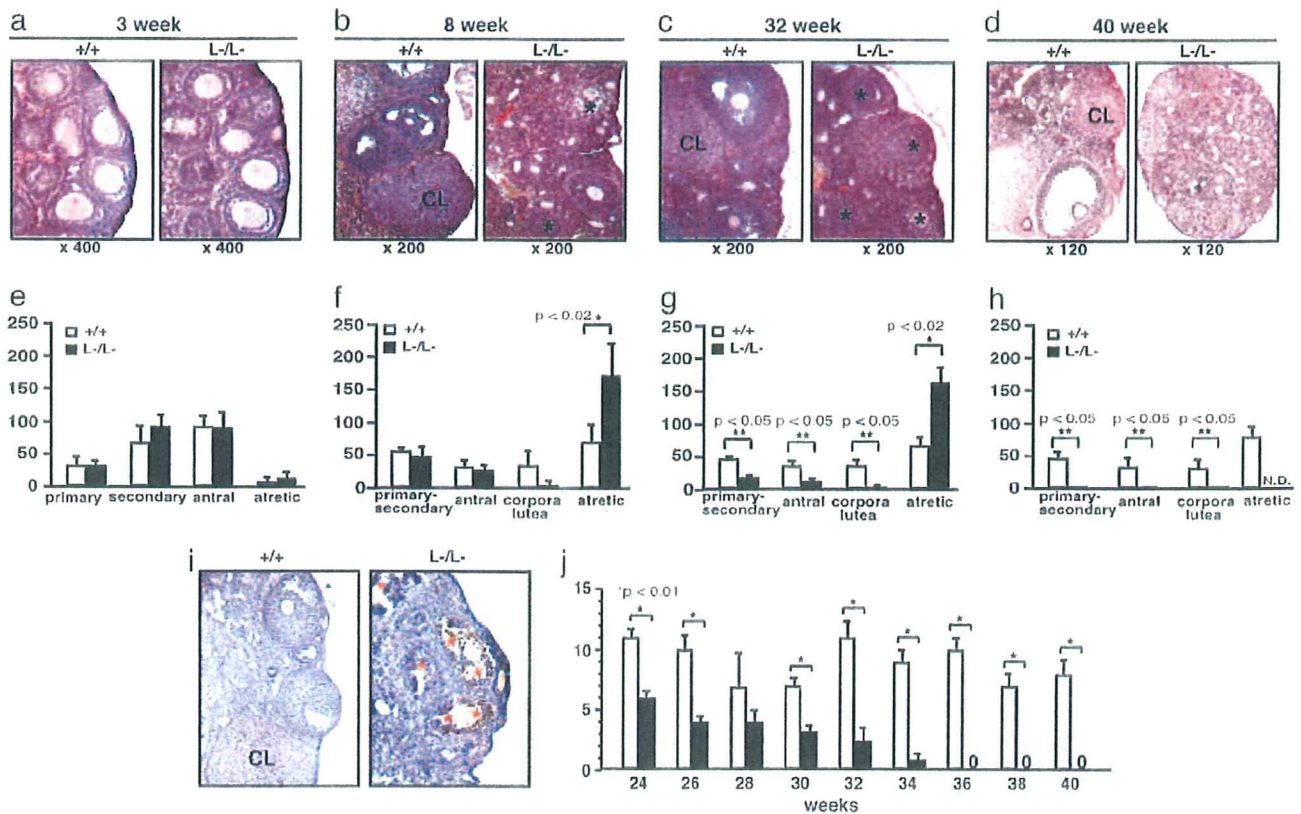


Fig. 2. POF in $AR^{-/-}$ female mice. (a–d) Histology of $AR^{+/+}$ and $AR^{-/-}$ ovaries at 3 weeks, 8 weeks, 32 weeks, and 40 weeks of age. All sections were stained with hematoxylin and eosin. An asterisk marks the atretic follicle. CL, corpus luteum. (e–h) Relative follicle counts at 3 weeks (e), 8 weeks (f), 32 weeks (g), and 40 weeks (h) of age. Numbers represent total counts of every fifth section from serially sectioned ovaries ($n = 4$ animals per genotype). (i) Immunohistochemical study for activated, cleaved caspase-3 revealed increased positive cells (apoptotic cells) in $AR^{-/-}$ ovaries. Sections were counterstained with hematoxylin. An asterisk marks the caspase-3-positive cell. CL, corpus luteum. (j) Age-dependent reduction in the number of pups per litter in $AR^{-/-}$ female mice. A continuous breeding assay was started at 24 weeks of age ($n = 6–10$ animals per genotype). For all panels, data are shown as mean \pm SEM and were analyzed by using Student's t test.

Primers were designed from cDNA sequences of *Kitl* (M57647; nucleotides 1099–1751), *Gdf9* (NM008110; nucleotides 720–1532), *Bmp15* (NM009757; nucleotides 146–973), *Ers2* (NM010157; nucleotides 1139–1921), *Pgr* (NM008829; nucleotides 1587–2425), *Cyp11a1* (NM019779; nucleotides 761–1697), *Cyp17a1* (M64863; nucleotides 522–932), *Cyp19* (D00659; nucleotides 699–1049), *Fshr* (AF095642; nucleotides 625–1427), *Lhr* (M81310; nucleotides 592–1331), *Prgs2* (AF338730; nucleotides 3–605), and *Cend2* (NM009829; nucleotides 150–1065) and chosen from different exons to avoid amplification from genomic DNA.

GeneChip Analysis. Ovaries were isolated and stabilized in RNA-later RNA Stabilization Reagent (Ambion, Austin, TX) before RNA purification (17). Total RNA was purified by using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. First-strand cDNA was synthesized from 5 μ g of RNA by using 200 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA), 100 pmol T7-(dT)₂₄ primer [5'-GGCCAGTGAATTGTAATACGACTCATATAGGGAGCGG-(dT)₂₄-3'], 1 \times first-strand buffer, and 0.5 mM dNTPs at 42°C for 1 h. Second-strand synthesis was performed by incubating first-strand cDNA with 10 units of *Escherichia coli* ligase (Invitrogen), 40 units of DNA polymerase I (Invitrogen), 2 units of RNase H (Invitrogen), 1 \times reaction buffer, and 0.2 mM dNTPs at 16°C for 2 h, followed by 10 units of T4 DNA polymerase (Invitrogen) and incubation for another

5 min at 16°C. Double-stranded cDNA was purified by using GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions and labeled by *in vitro* transcription by using a BioArray HighYield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Briefly, dsDNA was mixed with 1 \times HY reaction buffer, 1 \times biotin-labeled ribonucleotides (NTPs with Bio-UTP and Bio-CTP), 1 \times DTT, 1 \times RNase inhibitor mix, and 1 \times T7 RNA polymerase and incubated at 37°C for 4 h. Labeled cRNA was then purified by using GeneChip Sample Cleanup Module and fragmented in 1 \times fragmentation buffer at 94°C for 35 min. For hybridization to the GeneChip Mouse Expression Array 430A or 430B or Mouse Genome 430 2.0 Array (Affymetrix), 15 μ g of fragmented cRNA probe was incubated with 50 pM control oligonucleotide B2, 1 \times eukaryotic hybridization control, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated BSA, and 1 \times hybridization buffer in a 45°C rotisserie oven for 16 h. Washing and staining were performed by using a GeneChip Fluidic Station (Affymetrix) according to the manufacturer's protocol. Phycoerythrin-stained arrays were scanned as digital image files and analyzed with GENECHIP OPERATING SOFTWARE (Affymetrix) (17).

Luciferase Assay. The *Kitl* promoter region (–2866 to –1 bp) was inserted into the pGL3-basic vector (Promega) for assay using the Luciferase Assay System (Promega) (14, 16). Cells at 40–50% confluence were transfected with a reference pRL-CMV

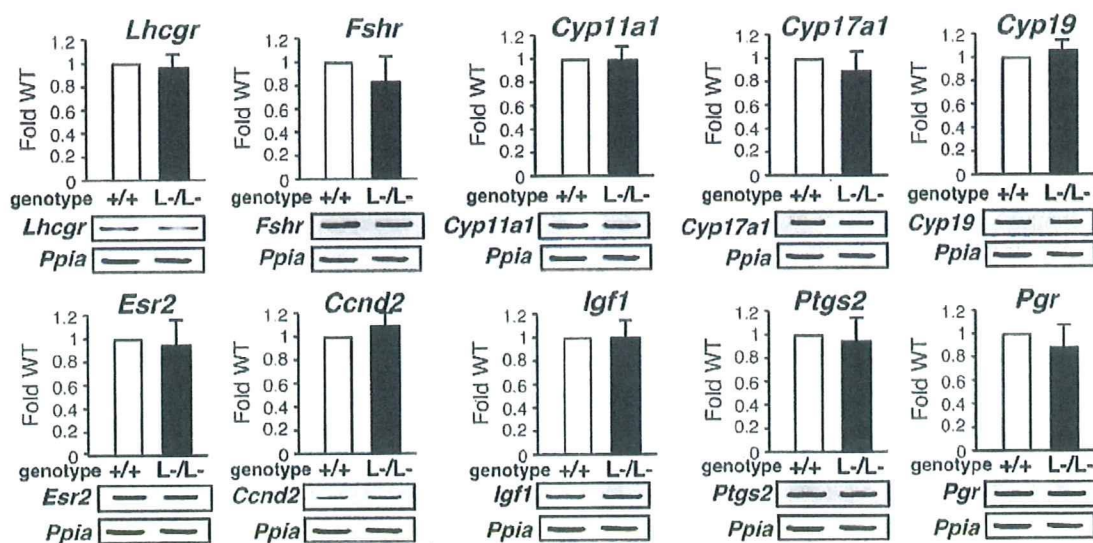


Fig. 3. No significant alterations in mRNA levels of several major regulators in folliculogenesis. Shown is semiquantitative RT-PCR of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17- α -hydroxylase (*Cyp17a1*), Aromatase (*Cyp19*), estrogen receptor- β (*Esr2*), cyclin D2 (*Ccnd2*), insulin-like growth factor 1 (*Igf1*), cyclooxygenase 2 (*Ptgs2*), or progesterone receptor (*Pgr*) gene expression in $AR^{+/+}$ and $AR^{-/-}$ ovaries. Results shown were representative (using one ovary per genotype in each experiment) of five independent experiments.

plasmid (Promega) using Lipofectamine reagent (GIBCO/BRL, Grand Island, NY) to normalize transfection. Results shown are representative of five independent experiments.

Results and Discussion

Subfertility of $AR^{-/-}$ Female Mice at 8 Weeks of Age. The *Ar* gene located on the X chromosome was disrupted in mice by using the Cre/Lox P system (6) (Fig. 1 *a-c*). Female $AR^{-/-}$ mice showed normal growth compared with the wild-type littermates (Fig. 1*d*), with no detectable bone loss (Fig. 1*e*) or obesity common for male $AR^{-/-}$ mice (8, 9). Young (8-week-old) $AR^{-/-}$ females appeared indistinguishable from the wild-type littermates, displayed normal sexual behavior (7), and produced the first offspring of normal body size at the expected age. Macroscopic appearance of their reproductive organs, including uteri, oviducts, and ovaries, also appeared normal (Fig. 1*f*). Histological analysis showed no significant abnormality in the uterus or pituitary (Fig. 1*e*), whereas mammary ductal branching and elongation were substantially reduced, as revealed by whole-mount analysis (Fig. 1*h*). Serum levels of 17 β -estradiol, progesterone, testosterone, luteinizing hormone, and follicle-stimulating hormone were also within normal range in 8-week-old mutant females at the proestrus stage (Fig. 1*g*), suggesting that the two-cell two-gonadotrophin system in female reproductive and endocrine organs (18) was intact in $AR^{-/-}$ mice at 8 weeks of age. The most obvious early sign of abnormal reproductive function in the $AR^{-/-}$ females was that their average numbers of pups per litter were only about half of those of the wild-type littermates, ($AR^{+/+}$, 8.3 ± 0.4 pups per litter; $AR^{-/-}$, 4.5 ± 0.5 pups per litter) (Fig. 1*i*).

$AR^{-/-}$ Female Mice Developed POF Phenotypes. Histological analysis of 8-week-old $AR^{-/-}$ ovaries clearly showed that numbers of atretic follicles were significantly increased, with decreased numbers of corpora lutea (Fig. 2 *b* and *f*). This finding suggests that the reduced pup numbers were due to impaired folliculogenesis in AR-deficient ovaries. Indeed, AR protein expression was readily detectable in the wild-type 8-week-old ovaries (Fig. 1*j*), with AR expressed at the highest levels in growing follicle granulosa cells at all developmental stages and at relatively low

levels in corpora lutea. Thus, AR appears to play a regulatory role in granulosa cells during their maturation to the luteal phase.

To investigate this possibility, we examined the ovarian phenotype of female $AR^{-/-}$ mice at different ages. At 3 weeks, ovaries contain various stages of follicles, including primary, secondary, and antral follicles in wild-type animals (Fig. 2*a*) (19). In $AR^{-/-}$ ovaries at 3 weeks of age, the folliculogenesis appeared to be unaltered, with normal numbers and localization of primary and secondary follicles (Fig. 2 *a* and *e*). However, degenerated folliculogenesis became evident with further aging. Although follicles and corpora lutea at all developmental stages were still present, corpora lutea numbers were clearly reduced in 8-week-old $AR^{-/-}$ mutants (Fig. 2 *b* and *f*), similar to that observed in another mouse line (20). Expected apoptosis was seen in atretic follicles by activated caspase-3 immunohistochemistry assays (Fig. 2*i*). But, by 32 weeks of age, defects in folliculogenesis in $AR^{-/-}$ ovaries became profound, with fewer follicles observed and increased atretic follicles (Fig. 2 *c* and *g*), and >40% (5 of 12 mice) of the $AR^{-/-}$ females were already infertile. By 40 weeks, all $AR^{-/-}$ females became infertile, with no follicles remaining (Fig. 2 *d* and *h*); at the same age, $AR^{+/+}$ females were fertile and had normal follicle numbers. Consistent with progressive deficiency in folliculogenesis, the pup number per litter steadily decreased in aging $AR^{-/-}$ females (Fig. 2*i*). These data indicate that AR plays an important physiological role at the preluteal phase of folliculogenesis.

Alteration in Gene Expressions of Several Major Regulators Involved in the Oocyte-Granulosa Cell Regulatory Loop. To explore the molecular basis underlying the impaired folliculogenesis in $AR^{-/-}$ ovaries, we analyzed expression of several major known regulators and markers of folliculogenesis (21-23). Surprisingly, no significant alterations in mRNA levels of LH receptor (*Lhr*), FSH receptor (*Fshr*), p450 side chain cleavage enzyme (*Cyp11a1*), 17- α -hydroxylase (*Cyp17a1*), aromatase (*Cyp19*), estrogen receptor- β (*Esr2*), cyclin D2 (*Ccnd2*), or insulin-like growth factor 1 (*Igf1*) of 8-week-old $AR^{-/-}$ ovaries at the proestrus stage, and further cyclooxygenase 2 (*Ptgs2*) or progesterone receptor (*Pgr*) at the estrus stage, were detected by

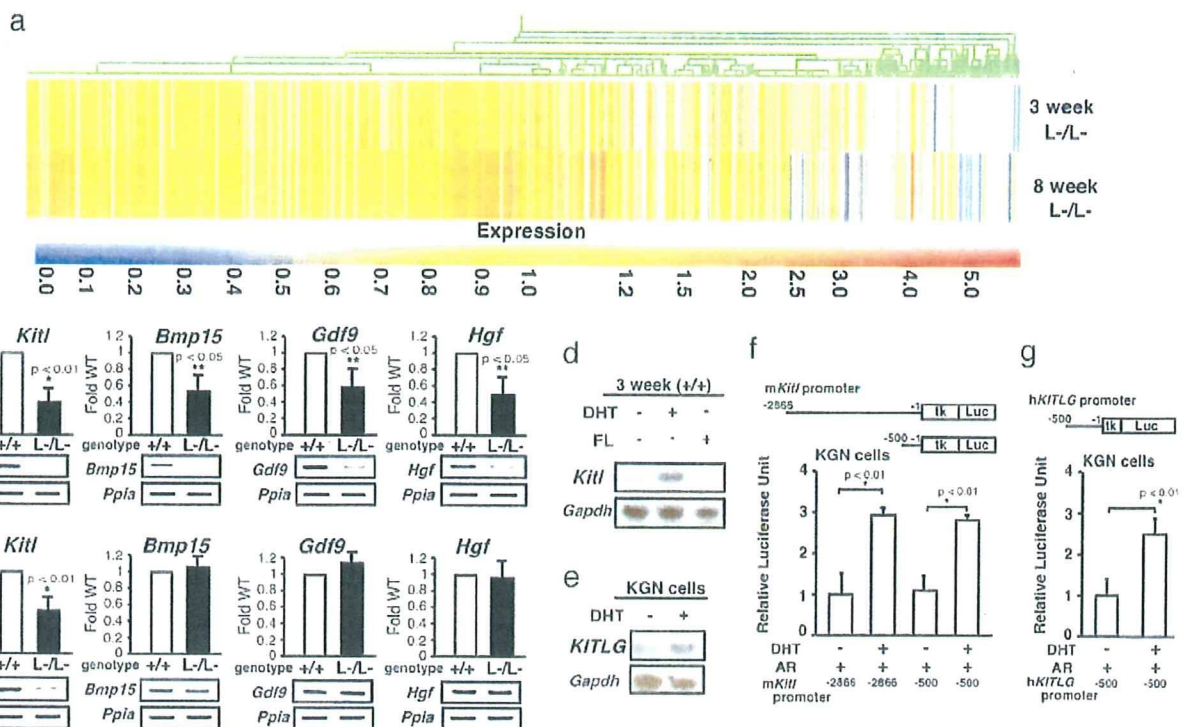


Fig. 4. Genome-wide microarray analysis and semiquantitative RT-PCR revealed that expression of the oocyte-granulosa cell regulator loop was down-regulated in $AR^{-/-}$ ovaries. (a) Microarray analysis of $AR^{-/-}$ compared with $AR^{+/+}$ ovaries at 3 and 8 weeks of age. Data obtained from microarray analysis as described in *Materials and Methods* were used to generate a cluster analysis. Each vertical line represents a single gene. The ratios of gene expression levels in $AR^{-/-}$ ovaries compared with wild type are presented. (b and c) Semiquantitative RT-PCR analysis of AR-regulated genes identified from the microarray study. Results shown are representative (using one ovary per genotype in each experiment) of five independent experiments. Data are shown as mean \pm SEM and were analyzed by using Student's *t* test. (d) Comparison of *Kitl* gene expression by Northern blot analysis among placebo-, DHT-, and flutamide (FL)-treated $AR^{+/+}$ mouse ovaries. (e) Induction of *KITLG* gene expression by DHT treatment in KGN cells. (f and g) Androgen responsiveness in the mouse and human *kit ligand* promoters by a luciferase assay performed by using KGN cells. Data are shown as mean \pm SEM and were analyzed by using Student's *t* test.

semiquantitative RT-PCR analysis (Fig. 3). Genome-wide microarray analysis (17) of RNA from 8-week-old $AR^{-/-}$ ovaries at the proestrus stage has been undertaken to identify AR-regulated genes. In comparison with $AR^{+/+}$ ovaries, expressions of 772 genes were down-regulated, whereas 351 genes were up-regulated in $AR^{-/-}$ ovaries (Fig. 4a; see also Tables 1 and 2, which are published as supporting information on the PNAS web site). Several genes known to be involved in the oocyte-granulosa cell regulatory loop (24) were identified as candidate AR target genes, including KIT ligand (*Kitl*) (25), morphogenetic protein 15 (*Bmp15*) (26), growth differentiation factor-9 (*Gdf9*) (27), and hepatocyte growth factor (*Hgf*) (28). Impaired folliculogenesis had been reported in mice deficient in each of these three regulators (26, 27, 29). To validate the microarray data, we performed semiquantitative RT-PCR analysis of 8-week-old $AR^{-/-}$ ovary RNA and confirmed that expression of these factors was down-regulated (Fig. 4b). To identify a regulator downstream of the AR signaling at an earlier stage of folliculogenesis, 3-week-old $AR^{-/-}$ ovaries that, as pointed out earlier, display no apparent phenotypic abnormality were examined. Fewer genes had altered expression levels (519 genes up-regulated; 326 genes down-regulated) (Fig. 4a; see also Tables 3 and 4, which are published as supporting information on the PNAS web site), and, of the four regulators tested by RT-PCR, only *Kitl* was found to be down-regulated at this age (Fig. 4c). Because *Kitl* is a granulosa cell-derived factor and stimulates oocyte growth and maturation (29–31), down-regulation of the *Kitl* expression in 3-week-old or even younger $AR^{-/-}$ ovaries may trigger impairment in folliculogenesis at a

later age. To test for possible *Kitl* gene regulation by AR, 3-week-old wild-type females were treated with 5 α -dihydrotestosterone (DHT). At 4 h after hormone injection, a clear induction of *Kitl* expression was observed in the ovaries, whereas a known antiandrogen flutamide attenuated the induction by DHT (Fig. 4d). The induction of endogenous human *kit ligand* (*KITLG*) gene by DHT was also observed in human granulosa-like tumor cells (KGN) in culture (Fig. 4e). Furthermore, androgen-induced transactivation of mouse and human *kit ligand* promoters (32) was observed by a luciferase reporter assay (33) in KGN (Fig. 4f and g), 293T, and HeLa (data not shown) cells. However, no response to DHT was detected in the similar assay using promoters of the *Bmp15*, *Gdf9*, and *Hgf* genes (data not shown). Thus, we have shown that, in a regulatory cascade controlling folliculogenesis, *Kitl* represents a direct downstream target of androgen signaling.

As an upstream regulator, AR may also be indirectly involved in control of expression of other genes critical for folliculogenesis, because an age-dependent down-regulation of *Bmp15*, *Gdf9*, and *Hgf* gene expression was also observed in $AR^{-/-}$ ovaries. *Bmp15* and *Gdf9* are oocyte-derived factors that promote the development of surrounding granulosa cells in growing follicles (34, 35), whereas *Hgf* is secreted by theca cells and acts as a granulosa cell growth factor (36). Down-regulation of these factors, presumably due to decreased *Kitl* expression, may lead to impaired bidirectional communication between oocyte and granulosa cells (24) and, eventually, to early termination of folliculogenesis, as in POF syndrome.

Thus, we have identified AR as a novel regulator of follicu-

logogenesis that apparently acts in the regulatory cascade upstream of the major factors controlling ovarian function, confirming the previous findings of the AR expression in granulosa cells of growing follicles (3). Although not immediately relevant to the ovarian physiology, abnormal development of the mammary glands observed in our AR-deficient mice adds further strong evidence of an essential role of the AR not only in male, but also in female, reproductive function.

With increasing age of the first childbirth by women in the modern society, POF syndrome has become an important social and medical problem. Our findings suggest that POF syndrome may be caused by an impairment in androgen signaling and that X chromosomal mutations affecting the AR gene function may

play a key role in hereditary POF. From clinical perspective, the present study provides evidence that AR can be a beneficial therapeutic target in treatment of POF syndrome patients.

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- Laml, T., Preyer, O., Umek, W., Hengstschlager, M. & Hanzal, H. (2002) *Hum. Reprod. Update* **8**, 483–491.
- Davison, R. M., Davis, C. J. & Conway, G. S. (1999) *Clin. Endocrinol. (Oxford)* **51**, 673–679.
- Tetsuka, M., Whitelaw, P. F., Bremner, W. J., Millar, M. R., Smyth, C. D. & Hillier, S. G. (1995) *J. Endocrinol.* **145**, 535–543.
- Ehrmann, D. A., Barnes, R. B. & Rosenfield, R. L. (1995) *Endocr. Rev.* **16**, 322–353.
- Norman, R. J. (2002) *Mol. Cell. Endocrinol.* **191**, 113–119.
- Kato, S. (2002) *Clin. Pediatr. Endocrinol.* **11**, 1–7.
- Sato, T., Matsumoto, T., Kawano, H., Watanabe, T., Uematsu, Y., Sekine, K., Fukuda, T., Aihara, K., Krust, A., Yamada, T., *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101**, 1673–1678.
- Sato, T., Matsumoto, T., Yamada, T., Watanabe, T., Kawano, H. & Kato, S. (2003) *Biochem. Biophys. Res. Commun.* **300**, 167–171.
- Kawano, H., Sato, T., Yamada, T., Matsumoto, T., Sekine, K., Watanabe, T., Nakamura, T., Fukuda, T., Yoshimura, K., Yoshizawa, T., *et al.* (2003) *Proc. Natl. Acad. Sci. USA* **100**, 9416–9421.
- Li, M., Indra, A. K., Warot, X., Brocard, J., Messaddeq, N., Kato, S., Metzger, D. & Chambon, P. (2000) *Nature* **407**, 633–636.
- Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N. & Kato, S. (1999) *Nat. Genet.* **21**, 138–141.
- Yoshizawa, T., Handa, Y., Uematsu, Y., Takeda, S., Sekine, K., Yoshihara, Y., Kawakami, T., Arioka, K., Sato, H., Uehiyama, Y., *et al.* (1997) *Nat. Genet.* **16**, 391–396.
- Gubbay, J., Collignon, J., Koopman, P., Capel, B., Economou, A., Munsterberg, A., Vivian, N., Goodfellow, P. & Lovell-Badge, R. (1990) *Nature* **346**, 245–250.
- Yanagisawa, J., Yanagi, Y., Masubiro, Y., Suzawa, M., Watanabe, M., Kashiwagi, K., Toriyabe, T., Kawabata, M., Miyazono, K. & Kato, S. (1999) *Science* **283**, 1317–1321.
- Britt, K. L., Drummond, A. E., Cox, V. A., Dyson, M., Wreford, N. G., Jones, M. E., Simpson, E. R. & Findlay, J. K. (2000) *Endocrinology* **141**, 2614–2623.
- Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., *et al.* (2003) *Nature* **423**, 545–550.
- Fujimoto, N., Igarashi, K., Kanno, J., Honda, H. & Inoue, T. (2004) *J. Steroid Biochem. Mol. Biol.* **91**, 121–129.
- Couse, J. F. & Korach, K. S. (1999) *Endocr. Rev.* **20**, 358–417.
- Elvin, J. A. & Matzuk, M. M. (1998) *Rev. Reprod.* **3**, 183–195.
- Hu, Y. C., Wang, P. H., Yeh, S., Wang, R. S., Xie, C., Xu, Q., Zhou, X., Chao, H. T., Tsai, M. Y. & Chang, C. (2004) *Proc. Natl. Acad. Sci. USA* **101**, 11209–11214.
- Elvin, J. A., Yan, C., Wang, P., Nishimori, K. & Matzuk, M. M. (1999) *Mol. Endocrinol.* **13**, 1018–1034.
- Zhou, J., Kumar, T. R., Matzuk, M. M. & Bondy, C. (1997) *Mol. Endocrinol.* **11**, 1924–1933.
- Burns, K. H., Yan, C., Kumar, T. R. & Matzuk, M. M. (2001) *Endocrinology* **142**, 2742–2751.
- Matzuk, M. M., Burns, K. H., Viveiros, M. M. & Eppig, J. J. (2002) *Science* **296**, 2178–2180.
- Joyce, I. M., Pendola, F. L., Wigglesworth, K. & Eppig, J. J. (1999) *Dev. Biol.* **214**, 342–353.
- Yan, C., Wang, P., DeMayo, J., DeMayo, F. J., Elvin, J. A., Carino, C., Prasad, S. V., Skinner, S. S., Dunbar, B. S., Dube, J. L., *et al.* (2001) *Mol. Endocrinol.* **15**, 854–866.
- Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N. & Matzuk, M. M. (1996) *Nature* **383**, 531–535.
- Parrott, J. A., Vigne, J. L., Clu, B. Z. & Skinner, M. K. (1994) *Endocrinology* **135**, 569–575.
- Driancourt, M. A., Reynaud, K., Cortvrindt, R. & Smits, J. (2000) *Rev. Reprod.* **5**, 143–152.
- Huang, E. J., Manova, K., Packer, A. I., Sanchez, S., Bachvarova, R. F. & Besmer, P. (1993) *Dev. Biol.* **157**, 100–109.
- Packer, A. I., Hsu, Y. C., Besmer, P. & Bachvarova, R. F. (1994) *Dev. Biol.* **161**, 194–205.
- Grimaldi, P., Capolunghi, F., Geremia, R. & Rossi, P. (2003) *Biol. Reprod.* **69**, 1979–1988.
- Kitagawa, H., Fujiki, R., Yoshimura, K., Mezaki, Y., Uematsu, Y., Matsui, D., Ogawa, S., Unno, K., Okubo, M., Tokita, A., *et al.* (2003) *Cell* **113**, 905–917.
- Otsuka, F. & Shimasaki, S. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 8060–8065.
- Joyce, I. M., Clark, A. T., Pendola, F. L. & Eppig, J. J. (2000) *Biol. Reprod.* **63**, 1669–1675.
- Parrott, J. A. & Skinner, M. K. (1998) *Endocrinology* **139**, 2240–2245.

Vascular Biology, Atherosclerosis and Endothelium Biology

E4F1, a Novel Estrogen-Responsive Gene in Possible Atheroprotection, Revealed by Microarray Analysis

Yasuhiro Nakamura,^{*,†} Katsuhide Igarashi,[‡]
Takashi Suzuki,^{*} Jun Kanno,[‡] Tohru Inoue,[§]
Chika Tazawa,^{*} Masayuki Saruta,^{*}
Tomoko Ando,[‡] Noriko Moriyama,[‡]
Toru Furukawa,[¶] Masao Ono,^{*} Takuya Moriya,^{*}
Kiyoshi Ito,^{||} Haruo Saito,[†] Tadashi Ishibashi,[†]
Shoki Takahashi,[†] Shogo Yamada,[†] and
Hironobu Sasano^{*}

From the Departments of Pathology,^{*} Radiology,[†] Molecular Pathology,[¶] and Gynecology,^{||} Tohoku University School of Medicine, Sendai; and the Division of Toxicology[‡] and the Biological Safety Research Center,[§] National Institute of Health Sciences, Tokyo, Japan

Estrogen has been postulated to be involved in inhibition of vascular smooth muscle cell (VSMC) proliferation mainly via estrogen receptor (ER), but the detailed mechanism has remained primarily unknown. Therefore, in this study, microarray analysis was used in two types of cultured human VSMCs: one positive for ER α , and the other for ER β , which were treated by estrogens to detect the estrogen-responsive genes. We also used quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to evaluate mRNA levels of selective target gene (TG) in these cells. We further studied whether the TG product was involved in inhibition of proliferation using small interfering RNA (siRNA) of the TG transfection. We subsequently used quantitative RT-PCR and *in situ* hybridization analysis to evaluate the expression of these gene products in human aorta. E4F1, a possible inducer of cell growth arrest, was markedly increased only in ER α -positive VSMCs by estrogens in both microarray and RT-PCR analyses. Blocking of E4F1 using siRNA suppressed estrogenic inhibition of ER α -positive VSMC proliferation. E4F1 mRNA was abundant in premenopausal female aorta with mild atherosclerotic changes. E4F1 is therefore considered one of the estrogen-responsive genes involving ER α -mediated inhibition of VSMC proliferation and may play an

important role in estrogen-related atheroprotection of human aorta. (*Am J Pathol* 2004, 165:2019–2031)

Estrogen has been proposed as a cardioprotective agent.¹ However it is also true that the significance of hormone replacement therapy has remained controversial because recent randomized controlled trials failed to show protective effects of hormone replacement therapy in reducing the risk of coronary artery disease and instead revealed undesirable side effects such as an increment of breast cancer incidence.^{2–4} Results of experimental, clinical, and epidemiological studies have, however, also demonstrated that estrogen is predominantly involved in the suppression of development of atherosclerosis.^{2,5,6} Therefore, it is still very important to examine the detailed mechanisms of estrogenic actions especially in relation to its atheroprotective effects in the human cardiovascular system.

Estrogens have been considered to exert direct anti-atherogenic effects through an initial interaction with estrogen receptor (ER) in vascular smooth muscle cells (VSMCs) in addition to various systemic estrogenic effects. Results of recent studies demonstrated that there are two subtypes of ERs, ER α and ER β .^{7,8} The presence of both ER α and ER β has been also reported in the vascular wall of cardiovascular systems in human and various experimental animals.⁹ Between both ERs, ER α has been considered to be important for anti-atherogenic effects of estrogen in the great majority of the cases.^{9–13} However, the detailed genomic mechanisms of estrogen-ER α actions in inhibition of VSMC proliferation remain virtually unknown. Especially the target gene (TG) induced by estrogen, ie, estrogen-responsive gene, has not been examined in the human cardiovascular system.

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Address reprint requests to Yasuhiro Nakamura, M.D., Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, 980-8575 Japan. E-mail: nakamura@patholo2.med.tohoku.ac.jp.

Table 1. Primer Sequences Used in RT-PCR Analysis for VSMC Characterization

cDNA	Sequence	Size (bp)	Reference no.
ER α	Forward 5'-AAGAGCTGCCAGGCCTGCC-3'	167	56
	Reverse 5'-TTGGCAGCTCTCATGTCTCC-3'		
ER β	Forward 5'-GCTCAATCCAGTATGTA-3'	241	57
	Reverse 5'-CCTGGTGTA AAAACGTGA-3'		
GAPDH	Forward 5'-TGAACGGGAAGCTCACTGG-3'	307	58
	Reverse 5'-TCCACCACCTGTTGCTGTA-3'		

Recently, expression profiling analysis using cDNA microarray technology has been demonstrated to provide very important information as to the elucidation of the scheme of estrogen-signaling and improvement of clinical decisions in ER α -positive breast cancer.¹⁴ A number of investigators also identified several novel estrogen-responsive genes using a cDNA microarray method in other tissues and cancer cells.^{14,15} However, there has been little information regarding estrogen-responsive genes involving anti-atherogenic effects induced by estrogens in the human cardiovascular system. Therefore, in this study, we first screened the estrogen-responsive gene involving inhibition of VSMC proliferation using a microarray in a cell line derived from ER α -positive human VSMCs. We then used quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) to evaluate the expression level of this TG mRNA in both dose- and time-dependent manners to further confirm the results of microarray analysis. We also examined whether the TG detected in ER α -positive VSMCs can be also induced by estrogens in ER β -positive VSMCs. Double-stranded RNAs (dsRNAs) have been recently reported to be remarkably effective at suppressing specific gene expression in various kinds of cells by a pathway involving RNA interference (RNAi) through small interfering RNAs (siRNAs).¹⁶⁻¹⁸ Therefore, we used this procedure to confirm whether the gene products derived from the TG detected in microarray analysis were associated with estrogenic inhibition of cell proliferation in ER α -positive cells.

It then becomes very important to study the expression of the genes detected by microarray analysis in human VSMCs to obtain their clinical relevance. We therefore examined the expression levels of the above TG in VSMCs of human abdominal aorta obtained by autopsy using both quantitative RT-PCR and the *in situ* hybridization method. We then correlated these findings with the degree of atherosclerosis, sex, ER α expression levels, and other features of the patients for further characterization of the findings.

Materials and Methods

Cell Culture and Characterization

Two types of human VSMCs, ie, HUVS-112D (derived from human umbilical cord; CRL-2481), and T/G HA-VSMC (derived from human aorta; CRL-1999) were commercially obtained from American Type Culture Collection (Manassas, VA). They were cultured in a 75-cm² flask with F12-K

medium (American Type Culture Collection) containing 5% fetal bovine serum (FBS) at 37°C in a 5% CO₂ atmosphere. We first characterized these cell lines using several methods, such as morphological, immunohistochemical, and microarray analyses described below. In addition, we examined whether these cells expressed both ERs, especially ER α , using quantitative RT-PCR and Western blot analysis in combination with semiquantification, as described below.

Quantitative RT-PCR

Total RNA was extracted from both VSMCs in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA) followed by a phenol-chloroform phase extraction and isopropanol precipitation. The Superscript Preamplification System RT kit (Life Technologies, Inc., Grand Island, NY) was used in the synthesis and amplification of complementary DNA (cDNA). cDNA was synthesized from total RNA (2 μ g) using 25 ng/ μ L of Oligo (dT)₁₂₋₁₈ Primer (Life Technologies Inc., Gaithersburg, MD) on a PTC-200 Peltier thermal cycler DNA engine (MJ Research Inc., Watertown, MA). To test for the presence of genomic DNA contamination, we performed the RT step in the absence of Superscript II RNase H⁻ reverse transcriptase (Life Technologies, Inc.) followed by PCR. RT-PCR products lacking reverse transcriptase in the initial RT step were run on an ethidium bromide-stained 2% agarose gel. No band was observed in these samples (data not shown). The resulting cDNA was used as a template for real-time PCR. Real-time PCR was performed with the Light Cycler System (Roche Diagnostics GmbH, Mannheim, Germany) using the DNA binding dye SYBR Green I (Roche Diagnostics GmbH) for the detection of PCR products. Primers are summarized in Table 1. As a positive control, T-47D human breast cancer cells were used for ER α and ER β .¹⁹ Negative control experiments lacked cDNA substrate to check for the presence of exogenous contaminant DNA. No amplified products were detected under these conditions. The mRNA levels for both ERs in each VSMC are summarized as a ratio of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and evaluated as a ratio (%) compared with that of each control cDNA, which were synthesized from each PCR products and purified by using the pGEM-T Easy vector. The detailed procedures above were previously described in detail.²⁰ The analyses with real-time PCR were triplicated.

Western Blot Analysis

The procedures were based on these reported previously.²¹ The above VSMCs were washed with ice-cold phosphate-buffered saline (PBS) and then lysed in a triple detergent buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate, 100 μ g/ml phenylmethyl sulfonyl fluoride, 1 μ g/ml aprotin, 1% Nonidet P-40, and 0.5% sodium deoxycholate. A Protein Assay Rapid Kit (Wako, Osaka, Japan) and a SpectraMax 190 microplate reader (Molecular Devices Corp., Sunnyvale, CA) were used to determine the concentration of protein according to the manufacturers' instructions. A Western blot analysis was performed using 60 μ g of each protein. After optimizing the conditions of experiments, 60 μ g of protein samples were denatured at 95°C in Tris/glycine/sodium dodecyl sulfate buffer (25 mmol/L Tris, 192 mmol/L glycine, 0.1% sodium dodecyl sulfate, pH 8.3) and electrophoresed at 25-mA constant current through a 10 to 20% polyacrylamide gradient gel (Bio-Rad, Hercules, CA). The proteins in the gel were electrophoretically transferred to a polyvinylidene difluoride membrane (Clear Blot Membrane-P; ATTO Co. Ltd., Tokyo, Japan). Nonspecific binding sites were blocked by immersing the membrane in 5% skim milk (Becton Dickinson and Company, NJ) for 1 hour at room temperature, washed twice in 0.05% Tween 20 and PBS (PBS-T), and then incubated with ER α polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or control IgG overnight at 4°C. After washing in PBS-T, membranes were incubated for 1 hour with horseradish peroxidase-conjugated anti-mouse IgG (Amersham), and the target protein was detected using the ECL Western blotting detection reagent (Amersham). Equal loading of protein in each lane was confirmed by probing the membrane with anti-human β -actin monoclonal antibody (Sigma, St. Louis, MO). The relative amounts of ER α and β -actin protein levels for each band were standardized to the relative OD units obtained by an Image Gauge system (Fuji Photo Film Co. Ltd., Minamishinagawa, Kanagawa, Japan). In addition, the relative amount of ER α protein was adjusted by the β -actin protein level, and then evaluated as a ratio (%) to untreated MCF-7 cell lines.²² The analyses were also triplicated.

GeneChip Microarray Assay

Estrogen Treatment

The two types of VSMC cells described above were seeded in a 75-cm² flask at an initial concentration of 100,000 cells/flask with F12-K medium (American Type Culture Collection) containing 5% FBS and cultured until a subconfluent state was obtained. The medium was then replaced with FBS-free and phenol red-free medium (modified Eagle's medium) (Sigma) to arrest the cell growth. After 24 hours, the medium was replaced again with phenol red-free and FBS-free medium in the presence of estrogen (10 nmol/L) or vehicle (0.1% ethanol). After incubation for 8 hours, the cells were subsequently subjected to total RNA extraction for microarray analysis.

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was further purified using RNeasy columns (Qiagen, Valencia, CA) and treatment with ribonuclease-free deoxyribonuclease I (Qiagen).

Labeling

Isolated total RNA was labeled as described in the Affymetrix (Santa Clara, CA) GeneChip Expression Analysis Technical Manual (revision 3). The labeling is performed in two steps. In the first step, double-stranded cDNAs (ds-cDNAs) were synthesized from total RNA by reverse transcriptase reaction with SuperScript Reverse Transcriptase II (Life Technologies) and an oligo (dT) primer linked to a T7 RNA polymerase-binding site sequence and second strand synthesis reaction with *Escherichia coli* DNA polymerase, DNA ligase, and RNaseH. In the second step, the resulted ds-cDNAs were used as templates to produce biotin-labeled cRNA (the target) using T7 RNA polymerase in the presence of biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY). The biotin-labeled cRNAs were fragmented in fragmentation buffer and used for the preparation of target solution.

GeneChip Genome Array Hybridization

Fragmented targets are combined with control oligomer (Control Oligo B2, Affymetrix) and control cRNAs (Eukaryotic Hybridization Control kit, Affymetrix) in a hybridization buffer (100 mmol/L MES, 1 mol/L Na⁺, 20 mmol/L ethylenediaminetetraacetic acid, 0.01% Tween 20). Each target was hybridized to HGU133A using protocols described in the Affymetrix Expression Analysis Technical Manual.

Data Analysis

The procedure was basically based on the previously reported study.²³ The fluorescent signals on the slides were scanned by a GeneArray scanner (Affymetrix) and further data processing was performed using Microsoft Excel software (Microsoft, Seattle, WA). Image analysis was performed using Microarray Suite 5.0 software (Affymetrix). In this study, the ratios represented the values changed by 10 nmol/L E₂ treatment compared to control values. The values obtained from each experiment were log transformed and normalized so that the median log-transformed ratio equaled zero. To confirm the estrogen-related changes in gene expression obtained from microarray analysis, we independently repeated the same experiment twice and the differences were calculated. To further maintain reproducibility, we also selected for further analysis only those genes in which the difference of expression levels at the same condition in two separate experiments did not exceed more than 2.5-fold in further analysis. The average ratios yielded by these independent experiments were then used to denote the gene expression levels. As the average ratios increased more than twofold by both of duplicated 10 nmol/L E₂ treatment

Table 2. The Sequence Information of Primers, siRNA, and Probes for *in Situ* hybridization for Target Gene (E4F1)

Primers for RT-PCR	Forward	5'-ACACCACACAGGCGAGAA-3'
	Reverse	5'-TCCTCAGACACCAGCAACT-3'
Gene-specific sequences for siRNA	Sense	5'-AAGCTCTACAAGACCATTGCC-3'
	Anti-sense	5'-AAGCTCTACAAGACCATTGCC-3'
Gene-specific probes for <i>in situ</i> hybridization	Anti-sense	5'-CCACGGAGATCATCGAGGGCACCCAGACAGAGGTGGACAGCCACATCATG-3'
	Sense	5'-CATGATGTGGCTGTCCACCTCTGTCTGGGTGCCCTCGATGATCTCCGTGG-3'

It was determined that the target gene in this study was E4F1 by microarray analysis. The reason for this determination was described in detail in the Results section.

were considered to have been up-regulated when compared to control values. In this study, among the genes detected in microarray analysis, we regarded a gene that was up-regulated and was significantly associated with inhibition of cell growth in ER α -positive cells as the TG in this study. In an analysis of the function of these detected genes, we used the homepage of HUGO Human Gene Nomenclature Committee (<http://www.gene.ucl.ac.uk/nomenclature/>) for further clarification. We then examined whether there was an estrogen-responsive element (ERE) in the promoter region of TG by analyzing 10,000 bp upstream of the transcription start sites for TG with sequence information of chromosome mapping provided by the above homepage.

Real-Time PCR for TG mRNA

Estrogen Treatment

The ER α -positive cells were seeded in a 75-cm² flask at an initial concentration of 100,000 cells/flask with F-12K medium containing 5% FBS and cultured until a subconfluent state was obtained. The medium was then replaced with phenol red-free and FBS-free medium to arrest the cell growth. After 24 hours, the medium was replaced again with FBS-free F12-K medium with E₂ (100 pmol/L, 10 nmol/L), E₂ (10 nmol/L) with ICI 182780 (1 μ mol/L; Tocris, Ballwin, MO), tamoxifen (TAM, Sigma) alone (10 nmol/L), raloxifene (RAL, Sigma) alone (10 nmol/L), or vehicle. In addition, after pretreatment of the cells with inhibitors of RNA transcription, actinomycin D (ACD) (1 μ mol/L, Sigma), or protein translation (Sigma), cycloheximide (CHX) (1 μ mol/L, ICN Biomedicals Inc.), other two flasks were replaced again with FBS-free medium with E₂ (10 nmol/L). After incubation for 8 hours, the cells were subsequently subjected to total RNA extraction for real-time RT-PCR analysis described above for target product mRNA expression. In addition, the cells were also incubated for 24 hours and 48 hours, respectively, with E₂ alone (10 nmol/L) or vehicle and also subsequently subjected to total RNA extraction for real-time RT-PCR analysis described above for expression levels of TG product mRNA. The mRNA levels for target product in each VSMC are summarized as a ratio of GAPDH, and evaluated as a ratio (%) compared with that of each control cDNA, which were synthesized from each PCR product and purified by using the pGEM-T Easy vector. The procedures were also previously described in detail.²⁰ As a contrast, we also examined the relative ex-

pression levels of TG products mRNA treated with E₂ (100 pmol/L, 10 nmol/L) or vehicle for 8 hours, and treated with E₂ (10 nmol/L) or vehicle for 24 hours and 48 hours in ER β -positive cells. The analyses with real-time PCR were triplicated by using three flasks per treatment or nontreatment. The sequence information of primers for TG is shown in Table 2.

siRNA Preparation, Transfection, and Cell Count Assay

siRNAs corresponding to the TG mRNAs designed with 5' phosphate, 3' hydroxyl, and two base overhangs on each strand were synthesized and transfected to the ER α -positive VSMCs. The ER α -positive cells were seeded in a 25-cm² flask at an initial concentration of 50,000 cells/flask with F-12K medium containing 5% FBS and cultured until a subconfluent status was achieved. The medium was then replaced with phenol red-free and FBS-free medium to arrest the growth. After 24 hours, transfections of siRNA for endogenous gene targeting were performed with TransMessenger transfection reagent (Qiagen). The TG siRNA (2 μ g per flask) was condensed with 4 μ l of Enhancer R and formulated with 8 μ l of TransMessenger reagent, according to the manufacturer's instructions. The transfection complex was added directly to the cells; it was replaced with phenol red-free medium containing 5% dextran-coated charcoal-stripped FBS (DCC-FBS) with E₂ (10 nmol/L), TAM (10 nmol/L), RAL (10 nmol/L), or vehicle (0.1% ethanol) after 3 hours. After incubation for 48 hours, we then measured the number of cells in each sample as described above. We also examined the number of cells treated with E₂ (10 nmol/L) or vehicle (0.1% ethanol) with transfection of negative control siRNA and treated by E₂ (10 nmol/L), TAM (10 nmol/L), RAL (10 nmol/L), or vehicle into both ER α -positive cells. After incubation for 48 hours, the cells were trypsinized and suspended. We then used Cell Counting Kit-8 system (Wako) for measurement of the number of cells in each sample. As a contrast, we also examined the number of cells treated with E₂ (10 nmol/L) or vehicle with transfection of TG or negative control siRNA in ER β -positive cells treated by E₂. The effective ratio of transfection into the cells was more than 80% by using fluorescein-labeled negative control siRNA before transfection of the TG siRNA (data not shown). The sequence information of siRNA for TG is shown in Table 2.

Quantitative RT-PCR Analysis for mRNA of the TG Expression in Human Aorta

Human abdominal aortae without hormone therapy were collected at the time of autopsy performed in Tohoku University Hospital (Sendai, Japan) within 2 hours post-mortem from 22 patients (6 male, 6 premenopausal female, 10 postmenopausal female; mean, 52.2 ± 5.9 years of age). The Ethics Committee at Tohoku University School of Medicine approved the research protocol for this study. Aortic specimens were tentatively classified into the following five groups as previously described,²⁰ A, B, C, D, and E based on the sex of the deceased patient, degree of atherosclerosis, and status of menstruation (group A: male, normal or mild atherosclerosis, corresponding to group I to III in the AHA classification; group B: male, advanced atherosclerosis, corresponding to group IV to VI in the AHA classification; group C: premenopausal female, normal or mild atherosclerosis; group D: postmenopausal female, advanced atherosclerosis; and group E: postmenopausal female, mild atherosclerosis). The distribution of the cases among these groups is summarized as follows: A, three cases; B, three cases; C, six cases; D, six cases; and E, four cases. The adventitia and fat tissues around the aorta were immediately and carefully removed using clean surgical scissors and forceps at the time of autopsy. After this procedure, these specimens were immediately frozen in liquid nitrogen and stored at -80°C until use and also subsequently subjected to total RNA extraction for real-time RT-PCR analysis described above for expression levels of TG product mRNA and the correlation of ER α mRNA abundance in those samples. The mRNA levels for target product and ER α in each sample are summarized as a ratio of GAPDH, and evaluated as a ratio (%) compared with that of each control cDNA, which were synthesized from each PCR product and purified by using the pGEM-T Easy vector, as described above.

In Situ Hybridization Study for the TG mRNA Expression in Human Aorta

Unstained and duplicated, 5- μm -thick, formalin-fixed, paraffin-embedded human aorta sections were mounted onto clear glass slides (Matsunami, Tokyo, Japan) and processed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ) on the Ventana Discovery (Ventana Medical Systems) automated *in situ* hybridization instrument. *In situ* hybridization step protocols after the deparaffinization step were designed based on the standard protocol described in the manufacturer's RiboMap application note. The first fixation step was performed using formalin-based RiboPrep reagent (Ventana Medical Systems) for 30 minutes at 37°C . The reacted sections were then acid-treated using hydrochloride-based RiboClear reagent (Ventana Medical Systems) for 10 minutes at 37°C . The slides were subsequently processed for the protease digestion using ready-to-use a protease 2 reagent. The sections were incubated for hybridization with the anti-sense riboprobe

(2 ng/slide) using RiboHybe hybridization buffer (Ventana Medical Systems) for 6 hours at 37°C after a denaturing step for 6 minutes at 70°C . After stringency wash step using 2 \times RiboWash (equivalent to 0.2 \times standard saline citrate, Ventana Medical Systems) for 6 minutes at 42°C , the second fixation step was performed using RiboFix reagent for 20 minutes at 37°C followed by incubation of biotin-labeled anti-digoxigenin antibody (Sigma) for 30 minutes at 37°C . After streptavidin-alkaline phosphatase conjugate incubation for 16 minutes at 37°C , the signal was detected automatically using the BlueMap NBT/BCIP substrate kit for 4 hours at 37°C . Finally, the sections were counterstained with Kernechtrot as a marker stain and covered with a glass coverslip. We also used double immunostaining with diaminobenzidine (DAB) for ER α using a monoclonal antibody (Novocastra Laboratories, Newcastle, UK) and Vector-blue for α -smooth muscle actin using a monoclonal antibody (DAKO Corp., Carpinteria, CA), respectively, in adjacent tissue sections to further characterize these positive cells in the human aorta of the cases positive for the target mRNAs. We also used a monoclonal antibody against CD31 antigen (DAKO) for endothelial cells and a monoclonal anti-human macrophage antibody (PG-M1, DAKO) for macrophages in adjacent tissue sections for further characterization. In addition, we performed labeling index (LI), a quantitative value that evaluates the number of TG mRNA-positive cells present in VSMCs of the aortic neointima and media.²⁴ After determining the areas of evaluation by simultaneous observation using a multiheaded light microscopy, three authors (T.S., M.S., and H.S.) independently evaluated 100 VSMCs. When interobserver differences were less than 5%, the mean value was determined as the LI. When interobserver differences were greater than 5%, the three aforementioned authors above re-evaluated the discrepant immunostained slides simultaneously using a multiheaded light microscope, after which the mean value was obtained. The sequence information of probes for *in situ* hybridization for TG is shown in Table 2.

Statistical Analysis

Values for all results are shown as mean \pm SE of means (SEM). For comparisons between two groups, we used one-way analysis of variance followed by unpaired *t*-test. To examine the correlation of the two factors, we used a correlation coefficient (*r*) and a regression equation. A *P* value less than 0.05 was considered significant in this study.

Results

Characterization of Two VSMC Cell Lines

Results are summarized in Figures 1 and 2. We confirmed that these cells were positive for α -smooth muscle actin immunoreactivity but both of these cell lines represented relatively dedifferentiated VSMCs on the basis of their morphological features and expression of specific markers such as caldesmon, α -tropomyosin, and others (data not shown). In RT-PCR analysis, HUVS-112D cells

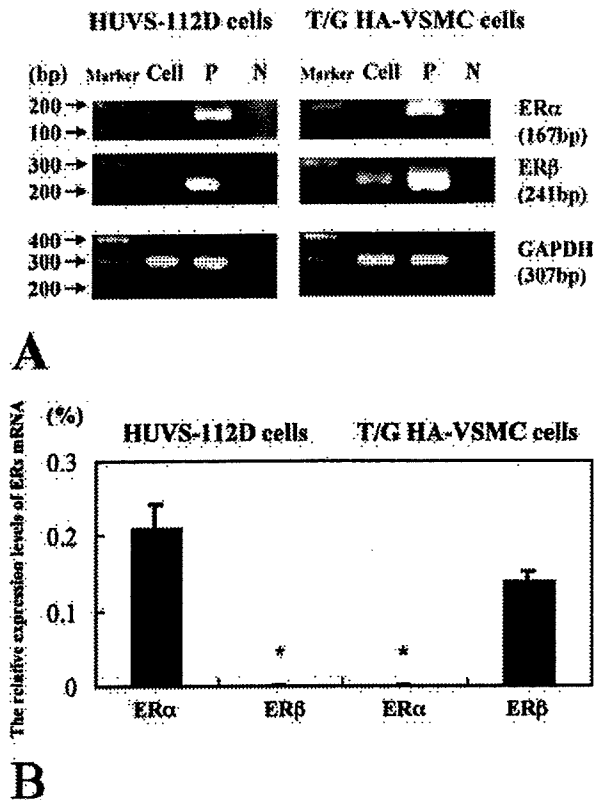


Figure 1. A: Results of real-time RT-PCR analysis for ER α and ER β in two cultured human VSMC lines (HUVS-112D and T/G HA-VSMC), positive controls, and negative controls. Cell, each type of cultured VSMC; P, positive controls (T-47D breast cancer cell lines); N, negative controls (no cDNAs). T-47D breast cancer cell lines were both positive for ER α and ER β . Negative controls yielded no bands for both ER α and ER β . In HUVS-112D cells, only the bands of ER α were detected, whereas only the bands of ER β were detected in T/G HA-VSMC cells. **B:** The relative mRNA expression levels for both ERs in two types of VSMC lines. The data for RT-PCR were adjusted by ratios (%) of GAPDH mRNA compared with that of each control cDNA, which were synthesized from each PCR product and purified by using the pGEM-T Easy vector. *, PCR products were not detected and the relative expression levels resulted in zero.

were positive only for ER α (0.21 \pm 0.03%, adjusted by ratios of GAPDH mRNA), and T/G HA-VSMCs were positive only for ER β (0.14 \pm 0.01%, adjusted by ratios of GAPDH mRNA) (Figure 1).

In Western blot analysis, only HUVS-112D cells were positive for the full-length ER α protein (66 kd) (10.4 \pm 0.8%, adjusted by ratios of β -actin protein compared to the ratio of MCF-7 cells) (Figure 2). In addition, HUVS-112D cells were also positive for the exon 7 deletion variant of ER α protein (52 kd), which is known to be expressed in both human VSMCs and MCF-7 cells, and known to inhibit transcriptional activation of wild-type ERs with little transcriptional activity by itself (Figure 2).^{25,26} On the other hand, MCF-7 cells were also positive for another splicing variant of ER α protein (46 kd) (Figure 2).²⁷

Gene Chip Microarray Assay

Table 3 shows the 12 genes that demonstrated expression ratios of above 2.0 after 8 hours of duplicated 10 nmol/L E₂ treatment of ER α - and/or ER β -positive cells.

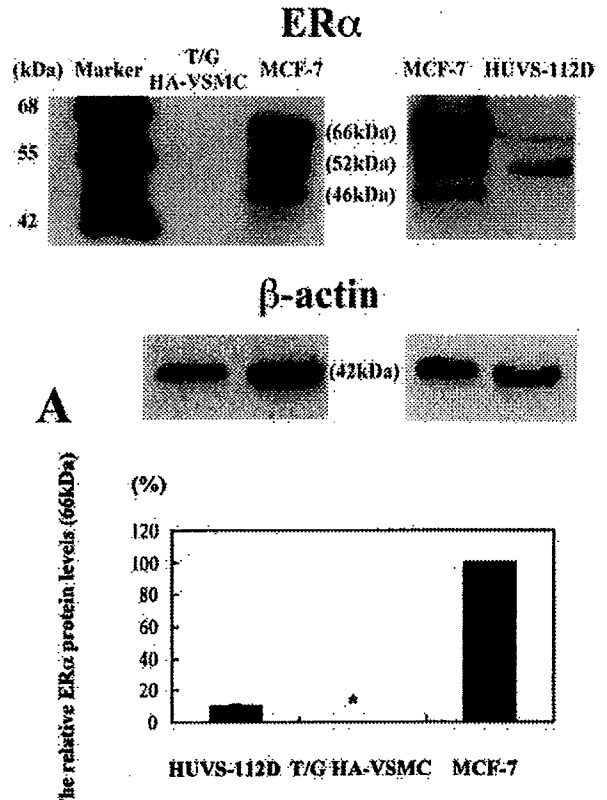


Figure 2. A: Western blot analysis of ER α and β -actin in HUVS-112D cells, T/G HA-VSMCs, and MCF-7 cells. Total protein was extracted and 60 μ g of protein from each cell was loaded. Western blot analysis demonstrated both the full-length ER α protein (66 kd) in HUVS-112D and MCF-7 cells, but not in T/G HA-VSMCs. In addition, both HUVS-112D and MCF-7 cells were also positive for the exon 7 deletion variant of ER α protein (52 kd). On the other hand, MCF-7 cells were positive for another splicing variant of ER α protein (46 kd). **B:** The relative protein expression levels for the full-length ER α protein (66 kd) in two types of VSMC lines. The results were evaluated as a ratio (%) compared with that of untreated MCF-7 cells. *, Bands of protein were not detected and the relative expression levels resulted in zero.

Among these genes, E4F transcription factor 1, ie, E4F1 was detected in HUVS-112D cells (ER α -positive cells), and its expression was associated with the second highest ratios by duplicated 10 nmol/L E₂ treatment (2.4-fold) (Table 3). In addition, among the detected genes, only E4F1 has been reported to inhibit cell growth and cell-cycle progression.²⁸⁻³³ In addition, more than 20 half-EREs in the promoter region of E4F1 were detected in this search (data not shown). Therefore we further examined the features of E4F1 as an estrogen-responsive gene in human VSMCs and whether E4F1 was associated with estrogenic inhibition of ER α -positive cell proliferation using quantitative RT-PCR, and siRNA transfection assay described above. In addition, we examined the expression levels of E4F1 mRNA in human abdominal aorta using both quantitative RT-PCR and *in situ* hybridization. In this study, E4F1 was not detected in T/G HA-VSMC cells (ER β -positive cells). In addition, among the genes detected by microarray analysis, the gene associated with inhibition of VSMC proliferation was not demon-

Table 3. Genes Up-Regulated by Estrogen Treatment for 8 Hours in Cultured VSMCs

Cell lines	Approved gene symbols	RefSeq accession numbers	Ratios
HUVS-112D (ER α -positive)	INPP4B	NM_003866	2.8
	E4F1	NM_004424	2.4
	CKLF	NM_016326	2.2
	RYR3	NM_001036	2.2
	PLAG1	NM_002655	2.1
T/G HA-VSMC (ER β -positive)	RFP2	NM_005798	2.5
	CPM	NM_001874	2.4
	EGR3	NM_004430	2.3
	POFUT1	NM_015352	2.3
	ADPRTL2	NM_005484	2.1
	HAP1	NM_177977	2.1
	RBM12	NM_152838	2.1

Ratios represents the mean ratios of expression levels of each gene mRNA between biologically duplicated experiments with estrogen and without estrogen.

strated in ER β -positive VSMCs according to the results of our research.

E4F1 mRNA Expression in VSMCs by Estrogen Treatment

E₂ significantly increased E4F1 mRNA levels of ER α -positive VSMCs compared to controls (131.9 \pm 5.9% by E₂ 100 pmol/L, 267.0 \pm 19.7% by E₂ 10 nmol/L, respectively; $P < 0.05$) (Figure 3A). In addition, E₂ with CHX also significantly increased those of ER α -positive VSMCs compared to controls (172.4 \pm 20.3%, $P < 0.05$) (Figure 3A). However, E₂ with ICI 182780, TAM, RAL, and E₂ with ACD suppressed expression of these mRNAs (33.4 \pm 0.1% by E₂ 10 nmol/L with ICI 182780 1 μ mol/L, 63.5 \pm 16.9% by TAM 10 nmol/L, 32.3 \pm 1.8% by RAL 10 nmol/L, 48.5 \pm 10.6% by E₂ 10 nmol/L with ACD 1 μ mol/L, respectively; $P < 0.05$) (Figure 3A). In addition, E4F1 mRNA levels of ER α -positive VSMCs were the highest at 8 hours after E₂ 10 nmol/L treatment compared to control (267.0 \pm 19.7%) (Figure 3C). However, none of E₂ significantly increased or inhibited E4F1 mRNA levels of ER β -positive VSMCs compared to controls (Figure 3, B and D). In summary, results of quantitative RT-PCR analyses also demonstrated that E4F1 is one of the genes induced by estrogen via ER α , but not via ER β , in our cultured human VSMCs.

E4F1 siRNA Transfections and Cell Proliferation Assay

We confirmed the down-regulation of E4F1 mRNA levels in the cells by transfection of E4F1 siRNAs using RT-PCR (data not shown). After transfection of negative control siRNA, all E₂ and estrogen receptor modulators (SERMs) used in this study significantly inhibited proliferation of ER α -positive VSMCs compared to controls (20.0 \pm 2.2% by E₂ 10 nmol/L, 28.2 \pm 1.0% by TAM 10 nmol/L, and 12.0 \pm 0.2% by RAL 10 nmol/L, respectively; $P < 0.05$) (Figure 4A). In addition, TAM and RAL significantly inhibited proliferation of ER α -positive VSMCs compared to controls (30.7 \pm 1.6% by TAM 10 nmol/L, and 13.7 \pm 1.0% by RAL 10 nmol/L, respectively; $P < 0.05$) after

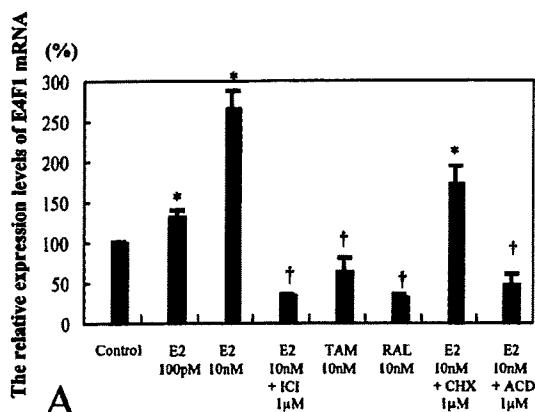
transfection of E4F1 siRNA (Figure 4A). However, E₂ with transfection of E4F1 siRNA did not suppress the proliferation of ER α -positive VSMCs (Figure 4A). In addition, none of the agents examined in this study significantly inhibited the cell proliferation of ER β -positive VSMCs compared to controls by transfection of both E4F1 siRNA and negative control siRNA (Figure 4B). In summary, siRNA analysis indicated that E4F1 may be associated with inhibition of VSMC proliferation through ER α not ER β in our cultured VSMCs.

E4F1 mRNA Expression in Human Aorta

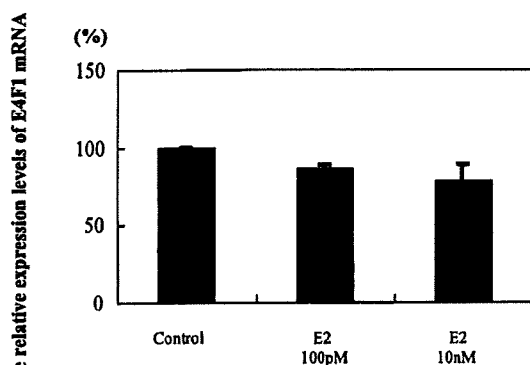
The relative abundance of E4F1 mRNA determined by real-time PCR analysis was significantly higher in the premenopausal female aorta with a mild degree of atherosclerotic changes (group C, 2.22 \pm 0.37%) than in the male aorta with a mild degree of atherosclerosis (group A, 0.09 \pm 0.05%), with a severe degree of atherosclerosis (group B, 0.31 \pm 0.27%), and in the postmenopausal female aorta with a severe degree of atherosclerosis (group D, 0.24 \pm 0.12%) ($P < 0.05$) (Figure 5B). In addition, there was a significant positive correlation between expression levels of ER α mRNA and relative abundance of E4F1 mRNA expression in female aorta ($y = 0.571 + 1.641 * x$, $r = 0.65$; $P < 0.05$) (Figure 5C). In addition, there was a significant inversed correlation between ages of the patients and the abundance of E4F1 mRNA expression in female aorta ($y = 2.482 - 0.026 * x$, $r = -0.575$; $P < 0.05$) (data not shown). However, no significant correlations were detected between relative abundance of E4F1 mRNA and ages and degree of atherosclerosis in male aorta (data not shown). In summary, results of quantitative RT-PCR in human aorta demonstrated that E4F1 was markedly expressed in premenopausal female aorta in an early stage of atherosclerosis with abundant ER α .

In Situ Hybridization Study for E4F1 mRNA Expression in Human Aorta

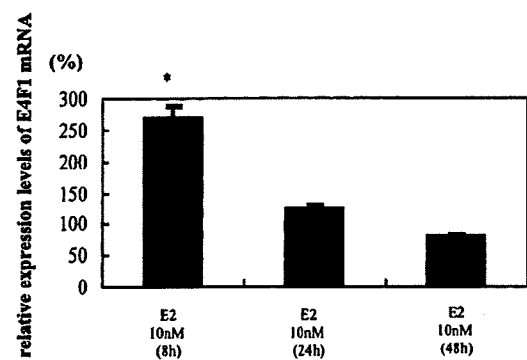
Expression of E4F1 mRNA products were markedly present in ER α -positive VSMCs at neointima of aorta in



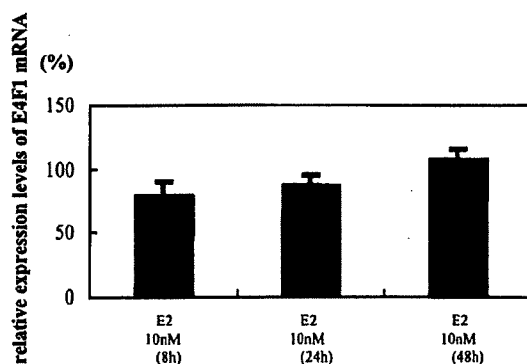
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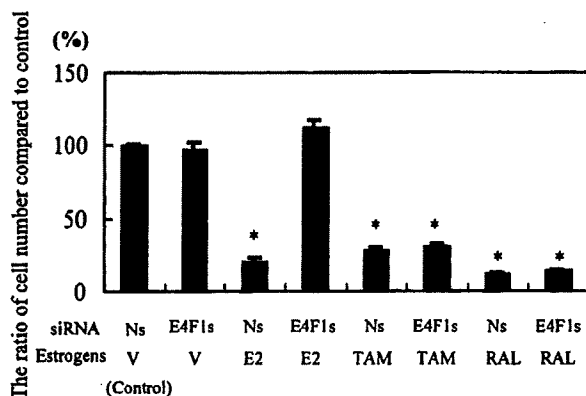
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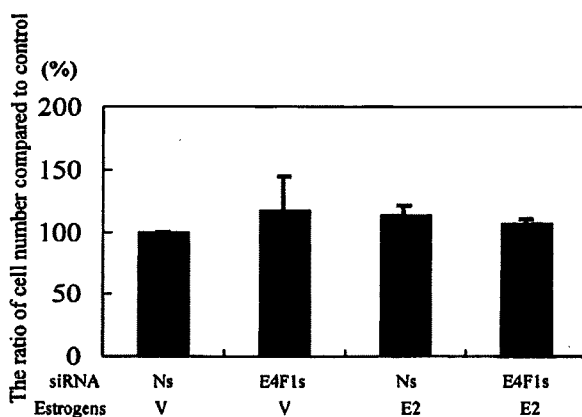
C



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Figure 4. A: The relative levels of cell numbers in HUVS-112D cells (ER α -positive cells) among cells treated with vehicle (control), estrogen (E₂) alone (10 nmol/L), tamoxifen (TAM; 10 nmol/L), raloxifene (RAL; 10 nmol/L), or vehicle (V; 0.1% ethanol) after transfection of E4F1 siRNA (E4F1s) or negative control siRNA (Ns). *, Significantly decreased compared to control; $P < 0.05$. **B:** The relative levels of cell numbers in 1/G HA-VSMCs (ER β -positive cells) among cells treated with vehicle (control), E₂ alone (10 nmol/L), or vehicle (V; 0.1% ethanol) after transfection of E4F1 siRNA (E4F1s) or negative control siRNA (Ns). *, Significantly decreased compared to control; $P < 0.05$.

group C. However, a very low level of expression of both E4F1 mRNA and ER α immunoreactivity was demonstrated in the nuclei of VSMCs of aorta in other groups. In addition, none of CD31-positive cells or PG-M1-positive cells demonstrated any hybridization signals of E4F1 mRNAs (data not shown). Figure 6 shows representative illustrations of an abdominal aorta specimen obtained from a 38-year-old woman with a mild degree of athero-

Figure 3. A: Results of real-time RT-PCR analysis for E4F1 in HUVS-112D cells (ER α -positive cells) among cells treated with vehicle (control), E₂ alone (100 pmol/L, 10 nmol/L), E₂ (10 nmol/L) with ICI 182780 (1 μ mol/L), E₂ (10 nmol/L) with actinomycin D (ACD; 1 μ mol/L), E₂ (10 nmol/L) with cycloheximide (CHX; 1 μ mol/L), tamoxifen (TAM) alone (10 nmol/L), and raloxifene (RAL) alone (10 nmol/L), respectively, after 8 hours. **B:** Results of real-time RT-PCR analysis for E4F1 in 1/G HA-VSMCs (ER β -positive cells) among cells treated with vehicle (control), E₂ (100 pmol/L, 10 nmol/L) alone, respectively, after 8 hours. **C and D:** The relative levels of E4F1 mRNA expression in ER α -positive cells (C) and ER β -positive cells (D) treated by E₂ alone (10 nmol/L) after 8, 24, and 48 hours compared to control (treated by vehicle). *, Significantly increased compared to control; †, significantly decreased compared to control; $P < 0.05$.

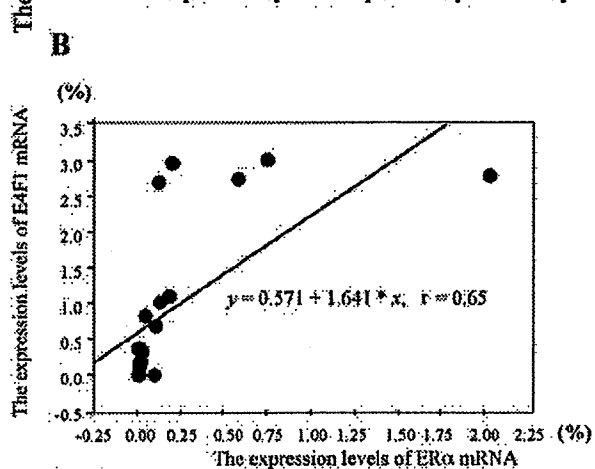
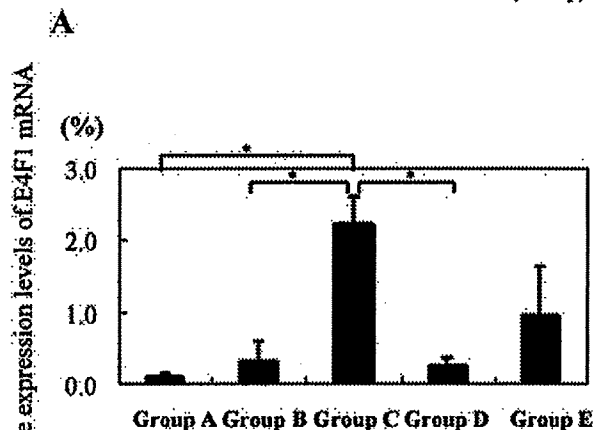
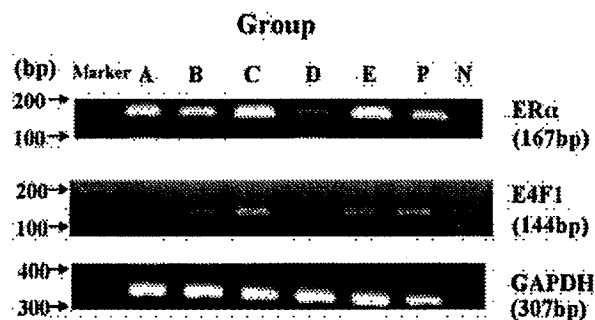


Figure 5. A: Results of real-time RT-PCR analysis for E4F1 in human aortas. Bands for PCR products were detected as specific single bands (167 bp for ER α , 144 bp for E4F1, and 307 bp for GAPDH). The amplified products were run on a 2% agarose gel stained with ethidium bromide. Representative photographs for these real-time RT-PCR gene products are illustrated. A, Aorta of a 32-year-old man with mild atherosclerotic change; B, Aorta of a 65-year-old man with severe atherosclerotic change; C, aorta of a 38-year-old premenopausal woman with mild atherosclerotic change; D, aorta of a 76-year-old postmenopausal woman with severe atherosclerotic change; E, aorta of a 71-year-old postmenopausal woman with mild atherosclerotic change; P, positive controls; N, negative controls. B: The results for mRNA expression levels for E4F1 (*, $P < 0.05$). C: Correlation between the levels of mRNA expression for ER α and E4F1 in 22 samples of human aorta. A significant correlation was detected ($y = 0.571 + 1.641x$; $r = 0.65$; $P < 0.05$).

sclerosis (group C). The number of E4F1 mRNA-positive cells in the neointima was significantly higher in the premenopausal female aorta with a mild degree of atherosclerotic changes (group C, 24.0 ± 2.0 LI) than in the male aorta with a mild degree of atherosclerosis (group

A, 5.6 ± 0.5 LI), in the male aorta with a severe degree of atherosclerosis (group B, 3.2 ± 0.4 LI), in the postmenopausal aorta with a severe degree of atherosclerosis (group D, 2.4 ± 0.5 LI), and in the postmenopausal aorta with a mild degree of atherosclerosis (group E, 6.2 ± 1.5 LI) ($P < 0.05$) (Table 4). However, LI for E4F1 mRNAs in the media was not significantly different among these groups (Table 4). In summary, results of *in situ* hybridization study in human aorta demonstrated that E4F1 was markedly expressed in ER α -positive VSMCs present at neointima of premenopausal female aorta in an early stage of atherosclerosis.

Discussion

In this study, results of microarray and quantitative RT-PCR analyses all suggest that E4F1 is one of the genes induced by estrogen via ER α , but not via ER β , in cultured human VSMCs. In addition, siRNA analysis demonstrated that E4F1 might be associated with inhibition of VSMC proliferation through ER α . Results of quantitative RT-PCR and *in situ* hybridization studies in human aorta further showed that E4F1 was markedly expressed in ER α -positive VSMCs present at neointima of premenopausal female aorta in an early stage of atherosclerosis.

In this study, we used duplicated microarray analyses as an initial screening. We then confirmed whether the gene was induced by estrogens or not by triplicated (using three flasks per treatment or nontreatment) quantitative RT-PCR. We then used siRNA assay for further examination of the findings. These findings all demonstrated that results of microarray analysis were consistent with those of RT-PCR, and the gene revealed by microarray analysis was actually involved in estrogenic inhibition of ER α -positive VSMC proliferation as demonstrated by the cell count assay with siRNA transfection.

E4F1 is well-known as a Krüppel-like family member.²⁸ The growth inhibitory activity of E4F1 has also been demonstrated to be associated with the posttranscriptional elevation of several cell-cycle regulatory proteins, including the CDK inhibitors p21^{Waf1} and p27^{Kip1} with reduced cdk2 and cdk4/6 activities, and with the down-regulation of cyclin A and cyclin E gene expression.²⁸⁻³⁰ E4F1-induced cell-cycle arrest has been also reported to be enhanced by its interaction with the p53 transcription factor and hypophosphorylated pRb.^{28,32,33} All of these findings above are consistent with the results of previous studies reported, and with inhibitory effects of estrogens on VSMC proliferation.³⁴

Stimulation of responsive genes in response to estrogen is postulated to be mediated by the direct binding in which E₂-liganded ER binds directly to a specific sequence called an ERE and interacts directly with co-activator proteins and components of the RNA polymerase II transcription initiation complex which result in enhanced transcription.³⁵ This is also consistent with results of our computer-based search of ERE in the promoter region of E4F1. In addition, quantitative RT-PCR analysis in our present study also demonstrated that ACD suppressed estrogen-induced E4F1 mRNA expression,

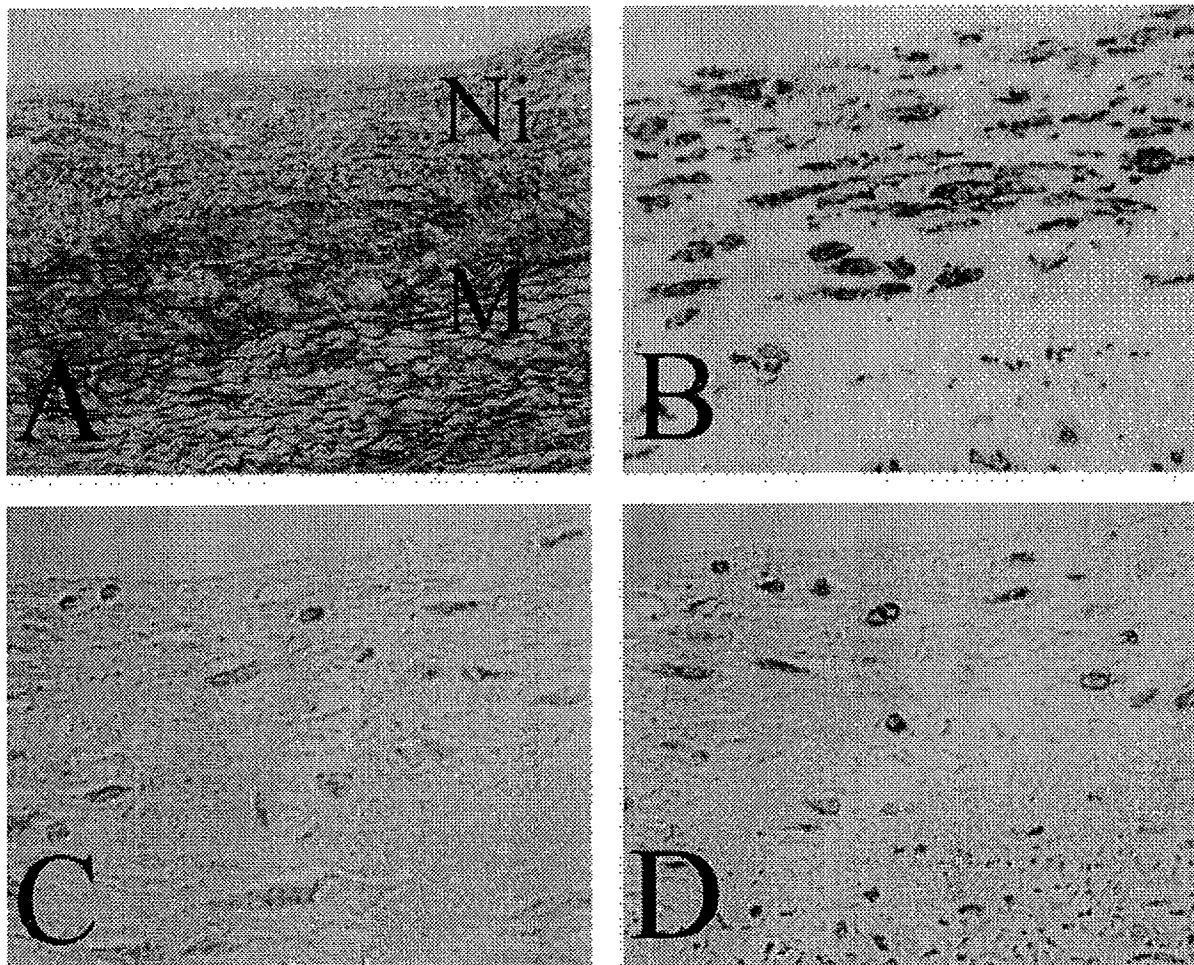


Figure 6. Modified Masson-Goldner's stains (A), double-immunohistochemical staining photos for α -smooth muscle actin and ER α in the neointima (B), and *in situ* hybridization of E4F1 mRNA in the neointima (C and D) of an abdominal aorta specimen obtained from a 38-year-old premenopausal woman with a mild degree of atherosclerosis (group C). B: Immunopositive cells for ER α appear brown as a result of diaminobenzidine colorimetric reaction. Immunopositive cells for α -smooth muscle actin appear blue as a result of Vector-blue colorimetric reaction. Double-immunopositive cells are confirmed. C: Negative control hybridized with sense oligonucleotide probes showed no detectable specific mRNA hybridization signals. Nuclear staining, appearing red, was performed by Kernechtrot. D: E4F1 mRNA hybridization signals, appearing blue, were detected in the marginal region of VSMCs in the neointima. Nuclear staining, appearing red, was performed by Kernechtrot. Ni, neointima; M, media. Original magnifications: $\times 100$ (A); $\times 400$ (B-D).

but CHX did not inhibit its expression. Therefore, these findings all indicated that E4F1 is considered one of the first established responsive genes in ER α -positive VSMCs. However, it is not known whether these half-EREs are associated with estrogenic actions or not.

Table 4. Results of Labeling Index (LI) for Target Gene (E4F1) mRNAs in VSMCs of Human Aorta

Group	n	Neointima	Media
Group A	3	5.6 \pm 0.5	2.0 \pm 0.5
Group B	3	3.2 \pm 0.4	2.0 \pm 0.4
Group C	6	24.0 \pm 2.0	4.8 \pm 1.0
Group D	6	2.4 \pm 0.5	3.4 \pm 0.7
Group E	4	6.2 \pm 1.5	4.8 \pm 0.7
Total	22	7.3 \pm 1.8	3.4 \pm 0.4

Data are the mean \pm SEM. A statistical significance was evaluated among the groups using one-way analysis of variance followed by unpaired *t*-test.

* *P* < 0.05.

Therefore, it awaits further investigation for clarification to detect a strict E4F1 promoter region and then to demonstrate whether ER α is actually binding to each ERE present at upstream region of E4F1 promoter using a chromatin immunoprecipitation assay.^{36,37}

Results of our study in human aorta also demonstrated that levels of E4F1 mRNA expression were significantly higher in VSMCs of neointima of premenopausal female aorta with mild atherosclerotic changes than in those of other groups. This finding therefore suggests that E4F1 is mainly expressed in dedifferentiated/proliferating VSMCs positive for ER α in neointima of premenopausal female aorta. This finding also demonstrated that the expression of E4F1 may be strongly induced by estrogens via ER α in VSMCs present at neointima of those aorta, possibly involved in inhibitory actions of VSMC proliferation by estrogens to prevent neointimal formation.³⁸

SERMs are postulated to be an attractive alternative with respect to cardiovascular risk reduction, providing

that they exert cardioprotective effects like estrogens. SERMs act as ER antagonists in the breast and uterus, avoiding the harmful effects of estrogens but presumably preserving the beneficial effects of estrogens on bone, lipids, and the cardiovascular system.³⁹⁻⁴¹ In addition, it is known that TAM binds with similar affinity to both ERs, whereas RAL has a higher affinity for ER α .^{42,43} Moreover, a previous report demonstrated that TAM has partial agonist activities on anti-atherogenic effects and vascular reactivity through interaction with ER α but not with ER β in human VSMCs.⁹ These all may be consistent with results of our present study. It is also postulated that estrogenic effects by SERMs are induced through AP-1 pathway.³⁵ However, several previous reports also documented that pretreatment of SERMs blocked the anti-atherogenic effects exerted by E₂ and that they have mixed estrogenic and anti-estrogenic effects on anti-atherogenesis in VSMCs and endothelial cells.⁴⁴⁻⁴⁶ Both TAM and RAL are reported to inhibit the classical ERE pathway described above activated by E₂ when these cells are treated together with E₂ and SERMs,³⁵ which is also consistent with these above reports.⁴⁴⁻⁴⁶ Results of quantitative RT-PCR and cell proliferation studies also demonstrated that E4F1 was induced by estrogens and suppressed the cell proliferation in ER α -positive VSMCs, but not by TAM or RAL, which may be partially consistent with results of these above reports. Therefore, SERMs may also be involved in inhibition of ER α -positive VSMC proliferation not through the above classical pathway induced by E4F1 based on results of our present investigations. These results all suggest the possible involvement of other pathways of estrogenic inhibition of VSMC proliferation. This may be also consistent with a previous report demonstrating that SERMs exert an anti-proliferative effect in VSMCs, at least in part through a p38 cascade whose activation is mediated by ER α via a nongenomic mechanism.⁴⁷ However, further investigations are required to determine how these above pathways interact with each other in estrogenic and/or anti-estrogenic effects on VSMCs, and to examine whether there are other possible pathways involved in estrogenic anti-atherogenic processes in human VSMCs *in vivo*, including an analysis of the difference between E₂ and SERMs.

In our study, estrogens demonstrated no inhibitory effects in proliferation of ER β -positive VSMCs. In addition, results of our present study also demonstrated that the inhibitory actions of VSMC proliferation via E4F1 were not detected in these VSMCs, whereas ER β was reported to be predominantly expressed in VSMCs.⁹ Furthermore, ER β has recently been demonstrated not only to play an essential role in the regulation of vascular function and blood pressure, but also to be associated with several mechanisms of anti-atherogenic effects.⁴⁸⁻⁵⁰ These previous reports of *in vitro* studies all suggest that E₂ was associated with growth inhibitory actions in porcine and/or rat VSMCs predominantly through ER β stimulation.^{50,51} The signaling pathway mediating growth inhibition via ER β has been also reported to be through reduction of p42/44 and p38 MAPK activity, and/or the cyclic AMP-adenosine pathway.⁴⁹⁻⁵² These signaling pathways may be attributable to the nongenomic action of E₂.^{53,54} However, ER β is well-

known as a relatively less potent transactivator than ER α at low receptor concentrations, such as in our T/G HA-VSMC cell lines, in response to E₂.⁹ This may be one of the reasons to explain the absence of an inhibitory role of estrogenic signals via ER β on VSMC proliferation demonstrated in our present study. ER β is also reported to predominantly increase after injury to the carotid artery of rat, and may be also consistent with promoting inhibitory actions through increment of ER β concentrations.⁵⁵ On the other hand, previous studies on vascular injury using fully null ER α knockouts indicated that ER α is basically and predominantly important for anti-atherogenic effects of estrogen in mice, which is consistent with results of our present study.¹³ However, according to these several previously published reports, it is difficult to exclude the possibility that which ER is more important for anti-atherogenesis depends on difference of species. Therefore, it still remains unclear which subtype of ER is more important for estrogenic anti-atherogenic effects in human, although our present study demonstrated the above pathway through ER α and E4F1. In addition, all previous reports have demonstrated several pathways of estrogen-induced anti-atherogenic effects *in vitro* but not *in vivo*, and therefore it remains unclear which pathway is most important for ER α -mediated anti-atherogenic effects *in vivo*. Further investigations in atherosclerotic models or in human cardiovascular system are required for clarification.

In conclusion, E4F1 is considered one of the estrogen-responsive genes involving ER α -mediated inhibition of VSMC proliferation and may play important roles in estrogen-related atheroprotection of human aorta.

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References

1. Stampfer MJ, Colditz GA, Willett WC, Manson JE, Rosner B, Speizer FE, Hennekens CH: Postmenopausal estrogen therapy and cardiovascular disease. *N Engl J Med* 1991, 325:756-762
2. Viscoli C, Brass L, Kernan W, Sarrel P, Suissa S, Horowitz R: A clinical trial of estrogen-replacement therapy after ischemic stroke. *N Engl J Med* 2001, 345:1243-1249
3. Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, Jackson BD, Beresford SA, Howard BV, Johnson KC, Kotchen JM, Ockere J: Writing Group for the Women's Health Initiative Investigators: Risk and benefits of estrogen plus progestin in healthy postmenopausal women. Principal results from the Women's Health Initiative randomized controlled trial. *JAMA* 2002, 288:321-333
4. Hulley S, Furberg C, Barrett-Connor E, Cauley J, Grady D, Haskell W, Knopp R, Lowery M, Satterfield S, Schrott H, Vittinghoff E, Hunninghake D: HERS Research Group. Noncardiovascular disease outcomes during 6.8 years of hormone therapy. HERS II. *JAMA* 2002, 288:58-66
5. Sullivan TR, Karas RH, Aronovitz M, Faller GT, Ziar JP, Smith JJ, O'Donnell TF, Mendelson ME: Estrogen inhibits the response-to-injury in a mouse carotid artery model. *J Clin Invest* 1995, 96:2482-2488
6. Hodgins JB, Kregel JH, Reddick RL, Korach KS, Smithies O, Maeda N: Estrogen receptor α is a major mediator of 17 β -estradiol's anti-atherogenic effects on lesion size in Apoe^{-/-} mice. *J Clin Invest* 2001, 107:333-340
7. Kulper GG, Enmark E, Pelto-Hulkko M, Nilsson S, Gustafsson JÅ:

- Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* 1996, 93:5925-5930
8. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JÅ: Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptor α and β . *Endocrinology* 1997, 138:863-870
 9. Hodges YK, Tung L, Yan XD, Graham JD, Horwitz KB, Horwitz LD: Estrogen receptors α and β ; prevalence of estrogen receptor β mRNA in human vascular smooth muscle and transcriptional effects. *Circulation* 2000, 101:1792-1798
 10. Brouchet L, Krust A, Dupont S, Chambon P, Bayard F, Arnal JF: Estradiol accelerates reendothelialization in mouse carotid artery through estrogen receptor- α but not estrogen receptor- β . *Circulation* 2001, 103:423-428
 11. Losordo DW, Kearney M, Kim EA, Jekanowski J, Isner JM: Variable expression of the estrogen receptor in normal and atherosclerotic coronary arteries of premenopausal women. *Circulation* 1994, 89:1501-1510
 12. Sudhir K, Chou TM, Chatterje K, Smith EP, Williams TC, Kane JP, Malloy MJ, Korach KS, Rubanyi GM: Premature coronary artery disease associated with a disruptive mutation in the estrogen receptor gene in a man. *Circulation* 1997, 96:3774-3777
 13. Pare G, Krust A, Karas RH, Dupont S, Aronovitz M, Chambon P, Mendelsohn ME: Estrogen receptor- α mediates the protective effects of estrogen against vascular injury. *Circ Res* 2002, 90:1087-1092
 14. Grunberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meitzer PS: Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. *Cancer Res* 2001, 61:5979-5984
 15. Soulez M, Parker MG: Identification of novel oestrogen receptor target genes in human ZR75-1 breast cancer cells by expression profiling. *J Mol Endocrinol* 2001, 27:259-274
 16. Krichevsky AM, Kosik KS: RNAi functions in cultured mammalian neurons. *Proc Natl Acad Sci USA* 2002, 99:11926-11929
 17. Park WS, Kurosaki MN, Hayafune M, Nakajima E, Matsuzaki T, Shimada F, Takaku H: Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. *Nucleic Acids Res* 2002, 30:4830-4835
 18. Wilda M, Fuchs U, Wossmann W, Borkhardt A: Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). *Oncogene* 2002, 21:5716-5724
 19. Vladusic EA, Hornby AE, Guerra-Vladusic FK, Lakins J, Lupu R: Expression and regulation of estrogen receptor beta in human breast tumors and cell lines. *Oncol Rep* 2000, 7:157-167
 20. Nakamura Y, Miki Y, Suzuki T, Nakata T, Darnel AD, Moriya T, Tazawa C, Saito H, Ishibashi T, Takahashi S, Yamada S, Sasano H: Steroid sulfatase and estrogen sulfotransferase in the atherosclerotic human aorta. *Am J Pathol* 2003, 163:1329-1339
 21. Kondo E, Horii A, Fukushima S: The human PMS2L proteins do not interact with hMLH1, a major DNA mismatch repair protein. *J Biochem (Tokyo)* 1999, 125:818-825
 22. Guzeloglu-Kayisli O, Kayisli UA, Al-Rejjal R, Zheng W, Luleci G, Arici A: Regulation of PTEN (phosphatase and tensin homolog deleted on chromosome 10) expression by estradiol and progesterone in human endometrium. *J Clin Endocrinol Metab* 2003, 88:5017-5026
 23. Watanabe H, Suzuki A, Kobayashi M, Lubahn DB, Handa H, Iguchi T: Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens. *Mol Endocrinol* 2003, 31:487-497
 24. Konishi H, Steinbach G, Terry NH, Lee JJ, Dubin JA, Glover GA, Fujita K, Spaulding D, Cass L, Hittelman WN: Histone H3 messenger RNA in situ hybridization correlates with in vivo bromodeoxyuridine labeling of S-phase cells in rat colonic epithelium. *Cancer Res* 1996, 56:434-437
 25. Mompeo B, Tschuschiluren G, Aust G, Metz S, Spaniel-Borowski K: Estrogen receptor expression and synthesis in the human internal thoracic artery. *Ann Anat* 2003, 185:57-65
 26. Garcia Pedrefo JM, Zuazua P, Martinez-Campa C, Lazo PS, Ramos S: The naturally occurring variant of estrogen receptor (ER) ERDeltaE7 suppresses estrogen-dependent transcriptional activation by both wild-type ERalpha and ERbeta. *Endocrinology* 2003, 144:2967-2976
 27. Flouriot G, Brand H, Denger S, Metivier R, Kos M, Reid G, Sonntag Buck V, Gannon F: Identification of a new isoform of the human estrogen receptor- α (hER- α) that is encoded by distinct transcripts and that is able to repress hER- α activation function 1. *EMBO J* 2000, 19:4688-4700
 28. Rizos H, Diefenbach E, Badhwar P, Woodruff S, Becker TM, Rooney RJ, Kefford RF: Association of p14ARF with the p120E4F transcriptional repressor enhances cell cycle inhibition. *J Biol Chem* 2003, 278:4981-4989
 29. Fajas L, Paul C, Vie A, Estrach S, Medema R, Blanchard JM, Sardet C, Vignais ML: Cyclin A is a mediator of p120E4F-dependent cell cycle arrest in G1. *Mol Cell Biol* 2001, 21:2956-2966
 30. Fernandes ER, Zhang JY, Rooney RJ: Adenovirus E1A-regulated transcription factor p120E4F inhibits cell growth and induces the stabilization of the cdk inhibitor p21WAF1. *Mol Cell Biol* 1998, 18:459-467
 31. Rooney RJ: Cell cycle attenuation by p120E4F is accompanied by increased mitotic dysfunction. *Cell Growth Differ* 2001, 12:505-516
 32. Sandy P, Gostissa M, Fogal V, Cecco LD, Szalay K, Rooney RJ, Schneider C, Del Sal G: p53 is involved in the p120E4F-mediated growth arrest. *Oncogene* 2000, 19:188-199
 33. Fajas L, Paul C, Zugasti O, Le Cam L, Polanowska J, Fabbriozzi E, Medema R, Vignais ML, Sardet C: pRB binds to and modulates the transrepressing activity of the E1A-regulated transcription factor p120E4F. *Proc Natl Acad Sci USA* 2000, 97:7738-7743
 34. Dzau VJ, Braun-Dullaeus RC, Sedding DG: Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. *Nat Med* 2002, 8:1249-1256
 35. Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson JÅ, Kushner PJ, Scanlan TS: Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* 1997, 277:1508-1510
 36. Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F: Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* 2003, 115:751-763
 37. Oesterreich S, Deng W, Jiang S, Cui X, Ivanova M, Schiff R, Kang K, Hadsell DL, Behrens J, Lee AV: Estrogen-mediated down-regulation of E-cadherin in breast cancer cells. *Cancer Res* 2003, 63:5203-5208
 38. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993, 362:801-809
 39. Mittak BH, Cohen FJ: Selective estrogen receptor modulators: a look ahead. *Drugs* 1999, 57:653-663
 40. Weryha G, Pascal-Vigneron V, Klein M, Leclere J: Selective estrogen receptor modulators. *Curr Opin Rheumatol* 1999, 11:301-306
 41. Goldfrank D, Haytuglu T, Frishman WH, Mohammad Z: Raloxifene, a new selective estrogen receptor modulator. *J Clin Pharmacol* 1999, 39:767-774
 42. Mendelsohn ME, Karas RH: The protective effects of estrogen on the cardiovascular system. *N Engl J Med* 1999, 340:1801-1811
 43. Gruber CJ, Tschugguel W, Schneeberger C, Huber JC: Production and actions of estrogens. *N Engl J Med* 2002, 346:343-352
 44. Mikkola TS, Clarkson TB: Estrogen replacement therapy, atherosclerosis, and vascular function. *Cardiovasc Res* 2002, 53:605-619
 45. Esplnosa E, Oemar BS, Luscher TF: 17 Beta-estradiol and smooth muscle cell proliferation in aortic cells of male and female rats. *Biochem Biophys Res Commun* 1996, 221:8-14
 46. Somjen D, Knoll E, Kohen F, Stern N: Effects of phytoestrogens on DNA synthesis and creatine kinase activity in vascular cells. *Am J Hypertens* 2001, 14:1256-1262
 47. Mori-Abe A, Tsutsumi S, Takahashi K, Toya M, Yoshida M, Du B, Kawagoe J, Nakahara K, Takahashi T, Ohnishi M, Kurachi H: Estrogen and raloxifene induce apoptosis by activating p38 mitogen-activated protein kinase cascade in synthetic vascular smooth muscle cells. *J Endocrinol* 2003, 178:417-426
 48. Zhu Y, Bian Z, Lu P, Karas RH, Bao L, Cox D, Hodgins J, Shaul PW, Thorén P, Smithies O, Gustafsson JÅ, Mendelsohn ME: Abnormal vascular function and hypertension in mice deficient in estrogen receptor β . *Science* 2002, 295:505-508
 49. Watanabe T, Akishita M, Nakaoka T, Kozaki K, Miyahara Y, He H, Ohike Y, Ogita T, Inoue S, Muramatsu M, Yamashita N, Ouchi Y: Estrogen receptor beta mediates the inhibitory effect of estradiol on vascular smooth muscle cell proliferation. *Cardiovasc Res* 2003, 59:734-744
 50. Geraldine P, Sirois MG, Tanguay JF: Specific contribution of estrogen

- receptors on mitogen-activated protein kinase pathways and vascular cell activation. *Circ Res* 2003, 93:399-405
51. Geraldes P, Sirolis MG, Bernatchez PN, Tanguay JF: Estrogen regulation of endothelial and smooth muscle cell migration and proliferation: role of p38 and p42/44 mitogen-activated protein kinase. *Arterioscler Thromb Vasc Biol* 2002, 22:1585-1590
52. Dubey RK, Gillespie DG, Mi Z, Rosselli M, Keller PJ, Jackson EK: Estradiol inhibits smooth muscle cell growth in part by activating the cAMP-adenosine pathway. *Hypertension* 2000, 35:262-266
53. Morey AK, Pedram A, Razandi M, Prins BA, Hu RM, Biesiada E, Levin ER: Estrogen and progesterone inhibit vascular smooth muscle proliferation. *Endocrinology* 1997, 138:3330-3339
54. Takeda-Matsubara Y, Nakagami H, Iwai M, Cui TX, Shiuchi T, Akishita M, Nahmias C, Ito M, Horiuchi M: Estrogen activates phosphatases and antagonizes growth-promoting effect of angiotensin II. *Hypertension* 2002, 39:41-45
55. Lindner V, Kim SK, Karas RH, Kuiper GG, Gustafsson JA, Mendelsohn ME: Increased expression of estrogen receptor-beta mRNA in male blood vessels after vascular injury. *Circ Res* 1998, 83:224-229
56. Pujol P, Rey JM, Nirde P, Roger P, Gastaldi M, Laffargue F, Rochefort H, Maudelonde T: Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis. *Cancer Res* 1998, 58:5367-5373
57. Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, Horne EL, Su JL, Kliewer SA, Lehmann JM, Willson TM: Cloning and characterization of human estrogen receptor beta isoforms. *Biochem Biophys Res Commun* 1998, 247:75-78
58. Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, Sakiyama S: Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. *Cancer Res* 1987, 47:5616-5619



Intrauterine position and postnatal growth in Sprague–Dawley rats and ICR mice

Tetsuji Nagao^{a,*}, Kazuyoshi Wada^b, Makiko Kuwagata^b, Madoka Nakagomi^a,
Chiaki Watanabe^b, Shinsuke Yoshimura^b, Yoshiaki Saito^b, Kenji Usumi^b, Jun Kanno^c

^a Department of Life Science, Faculty of Science and Technology, Kinki University, Kowakae 3-4-1, Higashiosaka, Osaka 577-8502, Japan

^b Safety Testing Laboratory, Hatano Research Institute, Food and Drug Safety Center, Hadano, Kanagawa, Japan

^c Division of Cellular and Molecular Toxicology, National Institute of Health Science, Tokyo, Japan

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Abstract

In rodents, steroid hormones are thought to be transported between adjacent fetuses, and male or female fetuses that develop in utero between female fetuses may have higher serum levels of estradiol, and lower serum levels of testosterone, relative to siblings of the same sex that develop between two male fetuses. The consequence in the variation of postnatal growth, development, and function in the intrauterine position, using various parameters such as anogenital distance, preputial separation and vaginal opening, estrous cycle, locomotor activity, and growth of reproductive organs, were examined in Sprague–Dawley rats. ICR mice were treated with 17 β -estradiol before copulation and during pregnancy to address the interaction with endogenous estradiol during pregnancy. In rats, no evidence of effects of prior intrauterine position was observed for any of the parameters examined. Mouse fetal exposure via the mother to low-dose 17 β -estradiol revealed no changes in the rate of postnatal growth in males and females that developed in any intrauterine position in utero. The results of this study suggested that the intrauterine position of the embryos/fetuses did not affect the postnatal growth of the reproductive organs, sexual maturation, or behavior in rats and mice.

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1. Introduction

The development of sexually differentiated phenotypes depends upon the hormonal environment during a critical period of growth [1]. Testosterone secretion by the fetal testis causes a longer anogenital distance (AGD), seen in neonatal males, relative to females. The AGD of newborn rats, mice, and gerbils is longer in males than in females and varies as a function of the intrauterine position of the animals [1–4]. A longer AGD is associated with the presence of males on either side of the developing fetus in utero, and a shorter AGD is associated with the absence of males on either side of the developing female fetus. Females with a male fetus on only one side are immediate [4].

In all litter-bearing species that have been examined to date, the intrauterine position that a fetus occupies relative to fetuses of the same or opposite sex has profound effects on its reproductive, behavioral, and morphological traits measured during adult life [4–7]. Gerbil males and females that

developed in utero between two female fetuses or two male fetuses, respectively, did not differ in relative hippocampal size [8].

The effects of intrauterine position are apparently not the result of the position itself, but rather of the movement of steroid hormones between the fetuses, and variations in the hormonal environment relative to the proximity of an individual fetus to other fetuses of the same or opposite sex [9]. Male rats located between two females had elevated serum estradiol and larger prostates than males located between two males, which had elevated serum testosterone and larger seminal vesicles [10]. The effect of intrauterine position in mice has been correlated with concentrations of steroid hormones in amniotic fluid and subsequent sexual activity [11,12].

Recently, intrauterine position has been the focus of discussions in the toxicology community because of its potential to alter the susceptibility of fetuses to endogenous hormones and endocrine disrupting chemicals [13,14]. In this regard, failure to account for intrauterine position in endocrine disrupting chemical toxicology studies could lead to false negative results, especially when adverse alterations

* Corresponding author. Tel.: +81-6-6721-2332; fax: +81-6-6723-2721.
E-mail address: tnagao@msa.kindai.ac.jp (T. Nagao).

are produced by low doses in fetuses from only one intrauterine position [14,15]. This possibility has been raised because of investigations into estrogenic compounds in mice. In rats, consistent effects due to intrauterine position on testosterone concentrations, and therefore potential interactions with endocrine disrupting chemicals, have not been found. Howdeshell and vom Saal [16] demonstrated that the greatest response to the estrogenic chemical, bisphenol A, occurred in males and females with the highest background levels of endogenous estradiol during fetal life, due to their intrauterine position, while fetuses with the lowest endogenous levels of estradiol showed no response to maternal bisphenol A within the range of human exposure, suggesting that estrogen-mimicking chemicals interact with endogenous estrogen in altering the course of development. It has been demonstrated that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin interacted with endogenous estradiol to disrupt prostate gland morphogenesis in male rat fetuses [17].

The objectives of this study were to determine the effects of intrauterine position, under normal physiological conditions, on the development of rat offspring, as well as sexual maturation, estrous cycle, behavior, and reproductive organ development. Another objective of this study was to determine whether the intrauterine position of mouse fetuses, which is related to background levels of estradiol and testosterone, would influence the response of the postnatal growth of gonads, including sexual maturation, to low dose 17 β -estradiol.

2. Materials and methods

2.1. Animals

Sprague–Dawley rats (Crj:CD, IGS), and ICR mice (Crj:CD-1) were purchased from Charles River, Laboratories, Inc. (Atsugi, Japan). Twenty-seven male rats (9 weeks of age), 84 female rats (8 weeks of age), 130 male mice (9 weeks of age), and 130 female mice (8 weeks of age), were used. The rats and mice arrived with mean weights of 301.1 ± 7.9 g for males and 216.2 ± 8.1 g for females, and 37.2 ± 1.2 g for males and 29.1 ± 0.9 g for females (mean \pm S.D.), respectively. The animals were acclimated to the laboratory for 7–14 days prior to the start of the experiments to evaluate weight gain and any gross signs of disease or injury. The animals were housed individually in stainless steel, wire-mesh cages in a room with controlled temperature (22–25 °C) and humidity (50–65%), with lights on from 07:00 to 19:00 h daily. The animals were given access to food (NIH-07-PLD: phytoestrogen low diet, Oriental Yeast Co., Japan) and tap water through metal pipes (distilled water, Wako Pure Chem., Japan) ad libitum. In a few instances, the temperature and humidity were outside the standard ranges, but the magnitude and duration of these incidents were minimal and judged to be of no consequence. The contents of genistein and daidzein in the diet

and wood bedding (ALPHA-dri, Shepherd Specialty Paper, USA) used in the present study were determined. Neither genistein nor daidzein were not detected in the diet or wood bedding (detection limit: 0.5 mg/100 g in each individual phytoestrogen, by HPLC).

Animal care and use conformed to published guidelines [18].

2.2. Experiment I (examination of intrauterine position effect on postnatal growth in rats)

2.2.1. Cesarean delivery and fostering

Estrous female rats at 10–11 weeks of age were cohabited overnight with a single male to obtain 66 pregnant females within 4 days. The next morning, females with sperm in their vaginal smears were regarded as pregnant, and this day was designated as day 0 of gestation. Thirty-three pregnant females were killed by CO₂ asphyxiation and cervical dislocation, and subjected to cesarean sectioning on day 21 of gestation. The fetuses were rapidly collected, and their intrauterine position was recorded, identified by tattoo, weighed, and sexed. Anogenital distance (AGD) was measured with a digital micrometer (reproductive precision of 0.01 mm, Digimatic caliper CD-15C, Mitutoyo Co., Kanagawa, Japan) under an Olympus dissecting microscope for each fetus, and the average was taken. The subject was held steady and in the same position during measurement. Measurements were made without knowledge of intrauterine position by one person. The AGD was measured from the center of the phallus to the center of the anus. The fetuses obtained by cesarean delivery were fostered to 33 dams that had just given birth naturally (one litter to each female). The original littermates remained together when cross-fostered. The litter sizes were similar for each cross-fostered dam. The day of cesarean section was considered as postnatal day (PND) 0. Pup body weights were recorded on PND 21 (day of weaning). Following weaning, and until 10 weeks of age, offspring were weighed once a week.

Neonates from 33 pregnant females were categorized as occupying six different intrauterine positions: 2M (male fetus located between two male fetuses; number of pups and litters on PND 0 = 36 and 19); 1M (male fetus that located between a male fetus and a female fetus; $n = 73$ and 27); 0M (male fetus located between two female fetuses; $n = 45$ and 24); 2F (female fetus located between two female fetuses; $n = 38$ and 18); 1F (female fetus located between a female fetus and a male fetus; $n = 83$ and 29); 0F (female fetus located between two male fetuses; $n = 41$ and 27). Fetuses adjacent to dead embryos (resorptions or macerated fetuses), and fetuses that were closest to each ovary or the cervix, were discarded from further analyses.

2.2.2. Observations of postnatal growth

2.2.2.1. Measurement of AGD and reproductive organ weights, and evaluation of sexual maturation. On PND