Estrogen-mediated Regulation of Igf1 Transcription and Uterine Growth Involves Direct Binding of Estrogen Receptor α to Estrogen-responsive Elements

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Estrogen enables uterine proliferation, which depends on synthesis of the IGF1 growth factor. This proliferation and IGF1 synthesis require the estrogen receptor (ER), which binds directly to target DNA sequences (estrogen-responsive elements or EREs), or interacts with other transcription factors, such as AP1, to impact transcription. We observe neither uterine growth nor an increase in Igf1 transcript in a mouse with a DNA-binding mutated ERα (KIKO), indicating that both Igf1 regulation and uterine proliferation require the DNA binding function of the ER. We identified several potential EREs in the Igf1 gene, and chromatin immunoprecipitation analysis revealed ERα binding to these EREs in wild type but not KIKO chromatin. STAT5 is also reported to regulate Igf1; uterine Stat5α transcript is increased by estradiol (E2), but not in KIKO or αERKO uteri, indicating ERα- and ERE-dependent regulation. ERα binds to a potential Stat5α ERE. We hypothesize that E2 increases Stat5α transcript through ERE binding; that ERα, either alone or together with STAT5, then acts to increase Igf1 transcription; and that the resulting lack of Igf1 impairs KIKO uterine growth. Treatment with exogenous IGF1, alone or in combination with E2, induces proliferation in wild type but not KIKO uteri, indicating that Igf1 replacement does not rescue the KIKO proliferative response. Together, these observations suggest in contrast to previous in vitro studies of IGF-1 regulation involving AP1 motifs that direct ERα-DNA interaction is required to increase Igf1 transcription. Additionally, full ERα function is needed to mediate other cellular signals of the growth factor for uterine growth.

Estrogen is a critical mediator of female reproductive system development and function. Additionally, estrogen is involved in nonreproductive tissues, such as the cardiovascular and skeletal systems, and is implicated in several diseases, including cancer and osteoporosis (1, 2). Estrogen interacts with the nuclear estrogen receptor (ER), which binds directly to palindromic target DNA sequences, estrogen-responsive elements (EREs). In vitro studies in model systems have shown that ERs can also interact with or be “tethered” to other transcription factors, such as AP1, to impact genes regulated by the corresponding motifs (3). A mutation that disrupts the direct DNA binding ability of the ERα (4, 5) has been “knocked in” at the ERα locus of a mouse (4, 6). Female mice that carried a single copy of this nonclassical ER knock-in “NERKI” mutation were infertile because of ovarian and uterine defects. To circumvent this issue, NERKI/ERαWT males were crossed to female mice heterozygous for the ERα null allele (αERKO/WT) to produce “KIKO” animals that express the NERKI mutant allele as their only functional ERα (7).

The ovariectomized rodent uterus exhibits a robust and rapid response to a single dose of E2, culminating in a synchronous wave of epithelial cell mitosis within 18–24 h (8). The uterine response to E2 is modulated by stromal factors, such as IGF1, that are induced by E2 and then impact epithelial responses (9). The Igf1 transcript is increased with a concomitant decrease of Igfbp3 (10) and activation of the Igf1 receptor and downstream effectors following E2 treatment (11). Igf1 transcript is increased in both stromal and epithelial compartments of the uterus by E2, with greater signal apparent in the stroma (12). Igf1 has been demonstrated to play an essential role in the uterine growth response, because Igf1−/− null mice lack a full uterine proliferative response and, more specifically, lack G2/M progression of the epithelial cells following E2 stimulation (13). Additionally, transgenic mice overexpressing Igfbp1, which sequesters and therefore decreases, the amount of available IGF1, have an attenuated uterine response to E2 (14). Uterine response is restored by transplanting Igf1KO uterine tissue into a WT host (15). Further, E2 treatment results in the activation of downstream mediators of Igf1 signaling, including the Igf1 receptor, IRS1 (11), AKT, and GSK3β (12). Additionally, inhibitors of AKT and GSK3β inhibit E2-stimulated uterine growth (12).

In vitro studies that employed a reporter gene to characterize the chicken Igf1 promoter indicated that Igf1 was an example of a transcript whose E2 regulation was mediated by indirect tethering (16), specifically involving association with AP1. However, we see no increase in vivo in Igf1 transcript in the tethered selective ERα containing KIKO uterus following E2 stimulation.
indicating that direct ERE binding was involved in E2 induction of uterine Igf1 transcripts.

The growth hormone signaling activated transcription factor, STAT5, is also a regulator of Igf1 transcript levels in the rodent liver via interaction with growth hormone-responsive element (GHRE) sites in the Igf1 gene (17, 18). The rodent liver contains STAT5 protein as well, and in this study we observed a WT ERα-dependent increase in Stat5a transcript. Although estrogen regulation of the Igf1 transcript has been extensively described in the rodent uterus, precise regulatory sequences have not been elucidated. The evaluation of potential mechanisms of estrogen regulation of the mouse Igf1 gene is important; therefore in this study we used KIKO and WT uterine models to identify ERE sequences in the Igf1 and Stat5a genes that could be involved in regulation by estrogen.

MATERIALS AND METHODS

Animals—All of the animal studies were done in accordance with National Institutes of Health Guidelines and a NIEHS Animal Care and Use Committee-approved animal studies proposal. The animals used were either an ERα null line (aERKO) (19), maintained at Taconic Farms (Germantown, NY) or were obtained by crossing NERKI+/− males (from Jameson lab) with ERα−/+ from our Taconic colony. These crosses were done at Charles River (Wilmington, MA). DNA was made from tail biopsies by using Direct PCR reagent (Viagen Biotech, Inc., Los Angeles, CA) according to manufacturer’s protocols. Offspring were screened for the presence of the NERKI allele as previously described (6) except that 2× RedTaq mix (Sigma) was used. The offspring were then screened for the presence of the ERα null allele using the following primers (purchased from Sigma): Esr1 Exon2 forward, CTGTGTTCAACTACCGAGG; Esr1 Intron2 reverse II, GGCGCGGGTACCTGTAGAA; Neo forward II, GATATCATATAATTTACAAGCAACACCAA in RedTaq reagent in a MBS Satellite PCR machine (Thermo Fisher, Milwaukee WI) with the following conditions: 95 °C for 2 min, 95 °C for 45 s, 54 °C for 45 s, 72 °C for 45 s (35 cycles) and then 72 °C for 7 min. The results showed either a band at ~350 bp (WT or NERKI allele) or bands at ~450 and 350 bp (ERα null and WT or NERKI alleles, respectively). Females that carried one copy each of the ERα null and the NERKI alleles (KIKO) and their ERα WT littermates were shipped to NIEHS and used in studies. The mice were ovariectomized after reaching at least 10 weeks of age, rested for 10–14 days, and then used in studies. The animals were then treated by injecting 100 μl of saline (vehicle controls) or E2 (100 μl of 2.5 μg/ml in saline) intraperitoneally. Long R3 Igf1, a synthetic IGF1 with low affinity for IGF-binding proteins (Cell Sciences, Canton, MA), was given by osmotic pumps (Durect Corporation, Cupertino, CA) as described previously (20) except the IGFI was dissolved at 0.5 mg/ml in 0.1 N acetic acid. Uterine tissue was collected at indicated times (1, 2, 6, 18, or 24 h after injection or pump insertion) and either snap frozen in liquid nitrogen for RNA, chromatin, or protein isolation or fixed in 10% formalin for immunohistochemical analysis.

Real Time PCR—RNA was isolated from frozen uteri, and cDNA was prepared as previously described (10). 10 ng of cDNA was used in a 25-μl reaction containing Power SYBER Master Mix (ABI, Foster City, CA) and 5 pmol each forward and reverse primers. The primers were designed using Primer Express software (ABI) and synthesized by Sigma and are listed in supplemental Table S2. An ABI 7900 instrument and SDS 2.1 software was used to carry out the PCRs. The values were calculated using the method of Pfaffl (21).

Identification of Potential ERE Sequences—The genomic sequence of Igf1 (NM_010512) plus its 2-kilobase pair upstream promoter sequence (81,992 bp in total) was downloaded from the UCSC Genome Browser (build mm9). We then scanned this sequence for putative ERE using the position weight matrix constructed from 48 experimentally identified ERE (15 bp in length) (22). We computed the log likelihood ratio score of each sliding window of the 15-bp segment for both the plus and reverse complementary strands. A segment was declared a putative ERE when the p value of its log likelihood ratio score is less than or equal to 0.0002 (23). Similarly, we scanned the ~5000-bp promoter sequence of Stat5a (NM_011488) for putative ERE using the same position weight matrix. A putative ERE was found at ~287 bp upstream from the Stat5a transcription start site.

Chromatin Immunoprecipitation (ChIP)—Pools of frozen uterine tissue (3–4 uteri/pool; 75–100 mg of tissue) were crushed using a metal pulverizer, and powdered frozen tissue was resuspended in 0.5% formaldehyde in phosphate-buffered saline at 1.5 ml/uterus and incubated on a rotating platform at room temperature for 5 min. Cross-linking was stopped by adding 225 µl/uterus of 1 M glycine and incubating 5 more min at room temperature. Tissue was collected by centrifugation at 3000 rpm for 5 min. Liquid was decanted, and the tissue was resuspended in 4 °C phosphate-buffered saline with added phosphatase inhibitors 1 and 2 (Sigma) and protease inhibitors (Sigma; 20 µg/ml each aprotinin and leupeptin, 4 µg/ml α-phenylmethylsulfonyl fluoride) and allowed to sit on ice for 2 min. The samples were then centrifuged at 3000 rpm for 5 min. The phosphate-buffered saline wash was repeated, and the tissue was resuspended in 0.5 ml/uterus of 50 mm Tris (Lanza, Rockland ME; pH 7.4), 1% Nonidet P-40 (Sigma), 0.25% deoxycholic acid (Sigma), 1 mm EDTA (Ambion/ABI) with added phosphatase and protease inhibitors. Tissue was homogenized with a PT1200C Polytron (Brinkman, Westbury, NY). Chromatin was pelleted by centrifuging for 15 min at 4 °C at 14,000 rpm (Eppendorf 5417R, Westbury NY) and resuspended in Lysis buffer (1% SDS, Ambion, 20 mm Tris, pH 8; Lanza) containing phosphatase and protease inhibitors. Resuspended chromatin was incubated on ice for 15 min, split into 250–300-μl aliquots, and sonicated in a 4 °C room in an ice water bath with a Bioruptor (Diogenode, Sparta, NJ) on high for two 3-min cycles of 30 s on and 30 s off with 1 min rest on ice between cycles. Sonicated chromatin was centrifuged for 1 min at 4 °C to remove insoluble material; supernatant was stored at −80 °C until use.

Chromatin was diluted 1:5 with HEP buffer (50 mm Tris, pH 7.5 (Lonza), 150 mm NaCl (Lonza) 1% Triton X-100 (Sigma)) containing protease and phosphatase inhibitors and was precleared with 60 µl/ml 10% protein A-Sepharose CL-4B (Suspended in HEP buffer); GE Healthcare) containing 200 µg/ml salmon sperm DNA (Stratagene, La Jolla, CA) for 1 h at 4 °C. Precleared supernatant was divided into 1-ml aliquots, and antibody was added to each. For ERα, sc7207 (Santa Cruz, Santa
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Cruz, CA) was added at a 1:25 dilution. For a control, rabbit IgG (Santa Cruz) was added at 1:25. For STAT5α, sc1081 (Santa Cruz) was added at 1:25 dilution. For SP3, sc644 (Santa Cruz) was added at 1:25. All were incubated for 4–18 h at 4 °C. 100 μl of protein A-Sepharose containing 200 μg/ml single-stranded DNA was added and incubated at 4 °C for 3 h. The tubes were centrifuged at 3000 rpm for 3 min to collect the pellets, which were then washed three times in 1 ml of HIP containing phosphatase and protease inhibitors for 10 min at 4 °C each wash. Cross-linked chromatin was then eluted twice using 100 μl of 1% SDS in 0.1 M NaHCO₃ at room temperature for 15 min. An aliquot was taken for Western blot analysis. The cross-link was reversed using a final concentration of 200 mM NaCl overnight at 65 °C, and then RNase (200 μg/ml final concentration) was added and incubated at 37 °C for 30 min, followed by inactivation with EDTA and proteinase K (Bioline Taunton MA) (at final concentrations of 5 mM and 1 μg/μl, respectively) at 45 °C for 1 h. The DNA was purified using the Qiaquick PCR purification kit (Qiagen). Purified DNA was analyzed by real time PCR using Power Syber master mix (ABI, Foster City, CA). Primer sequences for real time PCR of ChIP samples are shown in supplemental Table S3. The values were calculated relative to vehicle-treated samples.

**Gel Shift Assays**—Gel shift assays were done with the Gelshift ER kit (Active Motif, Carlsbad, CA) using the provided MCF7 extract according to the manufacturer protocol. The consensus ERE was end-labeled using [γ-32P]ATP (MP Biomedicals, Solon, OH) and T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). Other EREs were tested by synthesizing oligonucleotides (Sigma) substituting the Igf1 EREs (supplemental Table S4), which were annealed by heating to 95 °C for 5 min and then slowly cooling to room temperature in buffer containing 10 mM Tris, 1 mM EDTA, and 50 mM NaCl. These EREs or the WT ERE or mutated ERE provided in the kit were added to reaction mixtures to compete binding to 32P-labeled consensus ERE. The products were separated on DNA retardation gels (Invitrogen) in 0.5× TBE buffer, dried, and visualized with PhosphorImager screens (GE Storm 860; GE Biosciences).

**Western Blots**—Total protein homogenates were made from pulverized frozen uterine tissue by homogenizing with a Polytron in HIP buffer with containing 2.5 mg/ml sodium deoxycholate (Sigma), phosphatase, and protease inhibitors. Homogenates were centrifuged at 14,000 rpm for 10 min, the supernatant was collected, and protein levels were assayed using BCA assay (Pierce). Nuclear protein fractions were isolated from pulverized frozen uterine tissue using the NPER kit (Pierce). 10 μg of total uterine protein or 5 μg of nuclear protein was separated on a 10% NuPage gel using LDS loading buffer and reducing agent and MOPS SDS running buffer according to the manufacturer’s instructions (Invitrogen). The gels were then transferred to nitrocellulose filters using iBlot apparatus and gel transfer stacks (Invitrogen). The filters were stained with Ponceau Red (Sigma) to ensure even loading and transfer of proteins, then blocked with 5% milk (Santa Cruz) in TBST (20 mM Tris, pH 7.4, 180 mM NaCl, 0.1% Tween 20) for 30 min, and then incubated with primary antibody (details in figure legends) for 1 h. The filters were washed in TBST; incubated with anti-rabbit horseradish peroxidase IgG (Cell Signaling Technologies, Danvers, MA) for all but anti-β-actin, which was incubated with anti-goat IgG horseradish peroxidase (Santa Cruz); diluted 1:5000 in 5% milk for 1 h; and then washed in TBST. Signal was developed using ECL Plus reagent (GE Healthcare) and detected with Hyblot CL x-ray film (Denville Scientific, Metuchen, NJ).

**Immunohistochemistry**—Formalin-fixed uterine pieces were embedded on end in paraffin, and cross-sections were cut in 4-μm slices, mounted on Superfrost charged slides (Fisher), deparaffinized, and hydrated. Ki67 was detected as previously described (24). Phosphoserine 10 histone H3 was detected using a similar method, except blocking buffer contained 1.5% goat serum (Santa Cruz) and 1% bovine serum albumin and primary antibody (catalog number 06-570, Upstate Cell Signaling Solutions, Lake Placid, NY) diluted 1:500 in blocking buffer and incubated on slides for 1 h.

**RESULTS**

**Uterine Growth Is Impaired in the KIKO**—A previous report showed that although the KIKO uterus lacked any uterine weight increase in response to E₂, epithelial proliferation was retained (7). However, we were unable to detect this uterine response in our colony, as demonstrated by the lack of increase in the Ki67 proliferation marker or the perimitotic marker Ser(P)₁₀ histone H3 24 h after E₂ treatment (Fig. 1). Additionally, transcripts that are associated with cell cycle stages G₁-S
(supplemental Fig. S1A) or G2-M (supplemental Fig. S1B) were assessed by RT-PCR. All were induced in WT uteri, but responses were lost or dramatically diminished in the KIKO and absent in the αERKO (not shown), suggesting a lack of cell cycle progression.

Igf1 Is Not Increased by E2 in the KIKO—Igf1 is critical to the uterine growth responses (12, 13, 17), and the Igf1 transcript is reported to be an example of a gene response mediated by the tethered ERα mechanism, where ERs indirectly interacts with other transcription factors on promoter sequences rather than directly binding to DNA (16). Surprisingly, no increase in the Igf1 transcript was seen in the KIKO mouse uterus, which has a mutation in the ERα, rendering it unable to bind directly to ERE sequences, but selectively preserves its ability to interact with other transcription factors (5, 6). In WT mice, Igf1 transcript increases maximally at 6 h after E2 (Fig. 2A) (25, 26). The lack of Igf1 regulation in the KIKO and ERα null (αERKO) uterus indicates the sole dependence on ERα for the response (Fig. 2A) (26) and suggests the possible presence of ERE sequences in the Igf1 gene.

The mouse Igf1 gene has two promoters that result in production of transcripts with different first exons (Fig. 3) (27, 28). To determine whether the E2-mediated Igf1 increase was selectively regulated by one of these promoters, exon 1- or 2-selective probes were designed. RT-PCR indicated that both Igf1 promoters are E2-regulated because both exon 1- and 2-containing transcripts were increased by E2 (Fig. 2B), although exon 1 is more robustly increased. Neither transcript increased in the KIKO, indicating that both promoters require direct ERα DNA binding for estrogen-mediated response.

Potential ERE Sequences That Mediate E2 Regulation of Uterine Igf1—Sequence analysis of the Igf1 gene identified several putative ERE sequences upstream of and within intronic regions of the mouse Igf1 gene (supplemental Table S1). Using ChIP, we examined ERα binding to three of these putative EREs, selected because they varied from the consensus ERE sequence (GGTCAnnnTGACC) by no more than two bases and were located such that they might impact either promoter (Fig. 3).

ChIP Analysis of EREs in Igf1 Promoter—ERα binding to a previously described ERE sequence (29) upstream of both promoters 1 and 2 (−6215 and −8270 bp, respectively) was demonstrated by ChIP. E2 increased ERα association with this ERE by more than 5-fold after 1 h (Fig. 4A), and the level decreased to about 2-fold of the vehicle-treated level after 2–6 h. ERα binding to an ERE sequence adjacent to promoter 1 (−29 bp) but also upstream of promoter 2 (−2084 bp) was enhanced 10-fold above vehicle levels after 1 h of E2 treatment and decreased to 4–5-fold after 2–6 h (Fig. 4B). ERα binding to the ERE sequence in intron 3–4 was enriched 9–10-fold compared with the vehicle control 1–2 h after E2 injection and decreased after 6 h (Fig. 4C). Control experiments to demonstrate specificity of the ERα-chromatin interaction indicated there was no ERα interaction in a region 3000 bp distal from the ERE sequences assayed that lacked ERE and AP1 motifs (data not presented).

ERα is not detected on these three predicted ERE sequences in chromatin isolated from KIKO uterine tissues (Fig. 4, A–C).

Western blot of the immunoprecipitated chromatin indicates that the ERs in the KIKO samples is only 10% of the level found in WT samples (Fig. 4D). No ERα binding could be seen even when the amount of KIKO chromatin used in the real time PCR analysis was increased by 4-fold (data not presented).
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Previous reports have described an AP1 element in the chicken Igf1 promoter that mediates estrogen regulation (16, 30). Comparison of sequences in this vicinity of the chicken and mouse promoters indicated that no homologous region is present in mammals (supplemental Fig. S2 and Table S5). Evaluation of the promoter region of the mouse Igf1 gene for AP1-like motifs indicated the presence of an AP1-like sequence (number 15 in supplemental Table S5) adjacent to promoter 1. ChIP analysis of this sequence indicates E2-dependent ERα enrichment in this region in both WT (2.5 ± 0.4-fold increase after 1 h) and KIKO (2.3 ± 0.4-fold enrichment after 1 h).

ChIP Analysis of EREs in Stat5α Promoter—Igf1 transcript can be induced in the rat liver via binding of STAT5 to regulatory sequences in intron 2–3 (17, 31). Interestingly, Stat5α transcript is increased by E2 in our mouse samples after 2 h (Fig. 5A), which precedes the peak of Igf1 increase at 6 h (Fig. 2A). The increase in Stat5α transcript does not occur in the KIKO or αERKO, indicating that ERα DNA binding is required. A potential ERE sequence was found 287 nucleotides upstream of the Stat5α gene promoter. ChIP assays showed that ERα binding to this potential ERE sequence was enriched about 7-fold within 1 h of E2 treatment (Fig. 5B) and that the binding to this ERE was not detected in the KIKO.

ERE Sequences Bind ERα in Gel Shift Assay—ERα binding to Igf1 and Stat5α EREs was tested in a gel shift assay. ERα that was complexed with 32P-labeled WT consensus ERE was coincubated with 100-fold excess unlabeled WT ERE, mutant ERE, or 100 or 200-fold excess unlabeled ERE from Stat5α or Igf1 genes (Fig. 6). The consensus WT ERE sequence effectively competed the complexes, whereas the mutant ERE sequence did not. The Stat5α ERE and the Igf1 ERE from intron 3–4 were equally effective at competing as the WT ERE at 100-fold excess. The Igf1 ERE from −6215 bp was less effective, requiring 200-fold excess to achieve competition similar to the WT ERE at 100-fold excess. The Igf1 ERE from −29 bp was the weakest, with only partial competition at 200-fold excess. The relative strength of these EREs in the gel shift assay agrees with the differences in sequence between each ERE and the consensus ERE sequences, because the Stat5α and Igf1 intron 3–4 oligonucleotides contain EREs that differ by one base from the consensus GGTCAnnnTGACC and differ from the sequence in the 32P labeled WT ERE probe by three bases (supplemental Table S3). The Igf1 −6215-bp ERE also varies from consensus
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**FIGURE 6.** Gel shift demonstrates Stat5a and Igf1 ERE sequences bind to ERα. MCF-7 nuclear proteins were bound with 32P-labeled WT consensus ERE sequence and competed with 100- or 200-fold excess of unlabeled WT ERE (W) ERE mutated so it does not bind ERα (M) or the ERE sequences from Stat5a, or from Igf1 −6215. −29, or intron 3–4. The doublet of ERα-bound probe (B) or free probe (F) are indicated by arrows.

levels but leads to nuclear accumulation of STAT5a selectively in the WT.

We hypothesized that E2 increases nuclear STAT5a protein, which may contribute to the observed increase of Igf1 transcription. ChIP was used to determine whether STAT5a is recruited to a previously described STAT5-binding site in intron 2–3 of the Igf1 gene GHRE-1; (31)). STAT5a recruitment to this region was enriched nearly 5-fold within 1 h of E2 treatment (Fig. 7B) and was retained throughout the 6 h. There is less recruitment of STAT5a to the intron 2–3-binding site in the KIKO (2.5-fold enrichment after 1 h of E2; Fig. 7B). Western blot of proteins in the WT and KIKO immunoprecipitated chromatin indicates STAT5a is present in the KIKO at ~25% of the WT level (Fig. 7C), which correlates with the decreased amount of STAT5a binding observed in the ChIP assay. In the KIKO, STAT5a binding to the GHRE1 is not seen in the 2- or 6-h samples, unlike the WT (Fig. 7B).

To ensure in vivo function of the KIKO ERα on a uterine gene, recruitment of the ERα to a previously described Sp1 site in the Cdkn1a (p21) promoter (32) was evaluated. SP3 and ERα are both enriched in E2-treated chromatin from WT or KIKO mice (supplemental Fig. S3).

**Exogenous Igf1 Does Not Rescue Proliferation in KIKO**—Because of the lack of IGF1 expression, KIKO mice were treated with exogenous IGF1, alone or in combination with E2, to determine whether epithelial proliferation could be stimulated. The combined treatments were either administered simultaneously (E2 for 24 h and IGF for 24 h), or the IGF1 was given 6 h after E2 (E2 for 24 h and IGF1 for 18 h) to mimic the time at which IGF1 increases following E2 dosing. The treatment was successful in WT mice, as demonstrated by the increase in the Ki67 proliferation marker but was not effective in KIKO uterine samples (Fig. 8), indicating that IGF1 replacement does not suffice to rescue the inactivity of the mutant KIKO ERα in uterine proliferative response and
ERα binds EREs in mouse uterine Igf1 gene

A

WT

KIKO

0h 2h 6h

0h 2h 6h

B

Stat5a ChIP of Intron 2-3 Stat5 Site
(GHRE1)

Relative Enrichment

*** ***

0 1 2 3 6

Hours After E2

WT-Stat5a KIKO-Stat5a WT-IgG KIKO-IgG

C

WT

KIKO

IP: Stat5a

IB: Stat5a

% WT Input

100 0.2 38 44 21 0.1 6.4 12

WT Input

Stat5a

V, E1h

Stat5a

V, E1h

FIGURE 7. E2 does not increase STAT5a protein but causes accumulation in the nuclear fraction. A, Western blots from total uterine proteins (left panels) or nuclear extracts (right panels) from WT or KIKO mice treated with vehicle (0 h) or with E2 for 2 or 6 h detected with anti-STAT5a (top panels, both left and right; Santa Cruz, sc1081 diluted 1:1000 in TBST with 5% Milk), β-Tubulin (bottom left panel; Santa Cruz sc-9104 diluted 1:2000 in TBS and milk) or β-actin (bottom right panel; Santa Cruz sc1614 diluted 1:5000 in TBS and milk) is the loading control. B, ChIP analysis of STAT5-binding site in intron 2–3 of the Igf1 gene. Chromatin isolated from mice treated with vehicle or E2 for 1, 2, or 6 h was immunoprecipitated with anti-STAT5a or normal IgG. Precipitated DNA was quantified by real time PCR using primers that flank the GHRE1 site. The levels are expressed relative to the values for WT vehicle anti-STAT5a or sample, which are plotted as time 0. The time 0 Stat5a value was compared with each time point Stat5a value by two-way ANOVA tests with Bonferroni correction. For each time point p < 0.001 (**). C, Western blot of input (In) and chromatin associated proteins in ChIP sample aliquots after IP with normal IgG control (IgG) or anti-STAT5a and blotted as in A. The samples were from WT or KIKO treated with saline vehicle (V) or for 1 h with E2 (E1h). The gel images were quantified using ImageQuant software (GE Healthcare), and the values were calculated as percentages of WT input, shown below the gel. IP, immunoprecipitation; IB, immunoblot.

Emphasizes the additional requirement for direct ERα DNA binding (ERE) activity.

Igf1 receptors were present in the KIKO uterus at levels modestly lower than those of WT samples (supplemental Fig. S4). AKT and GSK3β are both downstream mediators of IGFL1 signaling. Examination of the phosphorylated forms of these proteins in WT and KIKO uterine extracts indicates that E2 leads to phosphorylation of both proteins more effectively in the WT samples (supplemental Fig. S5), indicating that this aspect of the signaling is impaired in the KIKO.

Discussion

Although in vitro Igf1 has been reported to be E2-regulated via indirect tethering (16), we saw no increase of Igf1 transcript in the KIKO uterus. Similarly, O2ES cells stably expressing the NERKI ERα lacked E2-dependent Igf1 regulation (33), and a different DNA ERO binding mutant also failed to increase uterine Igf1 transcript in response to E2 (34). Comparison of the previously described AP1 element in the chicken Igf1 promoter (16, 30) to mammalian sequences indicated that there was no homologous region in the mammalian Igf1 genes (supplemental Table S5 and Fig. S2). An AP1-like motif near promoter 1 of mouse Igf1 had E2-dependent ERO binding but apparently did not contribute to increased Igf1 transcription, because the KIKO EROS were also enriched in this motif, but no increase in transcript occurred. Because the reproductive cycle of avian egg-laying species differ greatly from mammals, mechanisms of Igf1 regulation may have evolved to accommodate the different modes of reproduction, where a more involved temporal regulation, including other factors such as STAT5, has developed.

The mouse Igf1 gene is encoded by six exons, with as many as six to eight reported splice variant transcripts. The five RefSeq transcripts utilize either exon 1 or 2 as their first exon; thus two alternate promoters adjacent to these exons have been described (27). Our RT-PCR analysis indicated that both of these promoters are targets for ERα-dependent regulation, because exon 1- and 2-containing transcripts were both increased by E2 (Fig. 2B), as are transcripts containing exons 4 (Fig. 2A), 5, and 6 (data not presented); therefore, estrogen regulatory elements should be common to both of the promoters. A potential ERE sequence upstream of both promoters was described as a result of genome-wide sequence analysis for high affinity estrogen-responsive elements (29). Our analysis identified 32 more putative ERE sequences; of these nine are half-sites (supplemental Table S1). Among the remaining 23 palindromic EREs, one is upstream of exon 1, two are in intron 2–3, 15 are in the very large 48 kb intron 3–4, and five are in intron 5–6 (data not presented); therefore, estrogen regulatory elements should be common to both of the promoters. A potential ERE sequence upstream of both promoters
samples when the amount of KIKO IP chromatin was increased 4-fold (data not presented). The complexes detected in the WT samples are consistent with a mechanism of Igf1 transcript regulation by E2 that utilizes direct ERα-ERE interaction. Additionally, all of the tested ERE sequences competed away the interaction between ERα and a consensus ERE in a gel shift assay with effectiveness that mirrored their degree of variation from the consensus sequence (Fig. 6).

Binding to the ERE sequences was temporally correlated to the E2-stimulated increase in the Igf1 transcript, with binding occurring within 1 h after E2 injection, which precedes the maximal increase of Igf1 transcript after 6 h. Together, our observations support a mechanism by which ERα interacts in an E2-dependent manner with the ERE sequences located within the vicinity of the mouse Igf1 gene promoter to increase mouse uterine Igf1 transcription. Our future studies will develop experimental reporter gene systems in vitro to examine the remaining potential ERE sequences and will evaluate whether these interactions are regulating Igf1 transcription.

Although interactions between estrogen and Stat5 signaling have been investigated, the Stat5a gene as a target for ER has only recently been suggested in a study involving mammary tissue (36), where levels of STAT5a protein were low following ovariectomy but increased after E2 and progesterone treatments. We noted that uterine Stat5a transcript was increased by E2 after 2 h in the WT and that no increase was seen in either KIKO or ERα null uteri. We also identified an ERE sequence in the vicinity of the mouse Stat5a promoter that recruits ERα binding after E2 stimulation; thus we propose Stat5a is a target for ERα-mediated regulation and involves direct DNA binding.

Two highly homologous isoforms of Stat5, Stat5a and Stat5b, are produced by neighboring genes (37). Stat5b is the predominant isoform found in the liver, whereas Stat5a is more abundant in the mammary gland (37, 38). In liver and breast cancer cell models, E2-dependent effects on STAT5 phosphorylation, interaction with ERα or ERβ, nuclear accumulation, and transcriptional activity have been reported (39–43). Studies in the rat liver (31) show Igf1 regulation via interaction between STAT5b and two STAT5-binding sites (GHRE1 and GHRE2) in the intron between exons 2 and 3. These STAT5-binding sites are conserved in the mouse (31).

In our studies, we detected abundant STAT5a protein in the WT uterus prior to E2 injection, and no increase in total STAT5a protein was observed after the E2 treatment. Although the total uterine level of WT STAT5a protein was unchanged, it is interesting that nuclear accumulation increased after 6 h, which coincides with the peak of increasing Igf1 transcript. More STAT5a protein was in the nuclear fraction of the KIKO uterus than the WT prior to E2 treatment, and the nuclear accumulation of STAT5a was unaffected by E2 in the KIKO, which correlates with the lack of Igf1 regulation in the KIKO uterine samples. Experimentally, we were able to see E2-dependent STAT5a binding to the GHRE1-binding site in intron 2–3 in the WT, preceding the peak of Igf1 increase.

Estrogen-dependent interaction between ERα and STAT5a proteins has been described in human kidney (HEK293) cells transfected with ERα, STAT5a, and prolactin receptor (44). Similarly, interaction between GST-ERα and STAT5b has been reported in HC11 mammary epithelial cells and shown to involve the DNA-binding domain of the ERα (40). These and other studies have lead to a proposed mechanism of integration of growth factor and estrogen signals via interaction between ER and STAT proteins on promoters (39). A similar DNA-binding mutant form of the ERα (point mutations in the first zinc finger at amino acids C201A/C204A) is unable to interact with STAT5 (40). Thus, this tethered mechanism of STAT5a interaction with ERα signaling could be disrupted in the KIKO (which also has point mutations in the first zinc finger but at amino acids E207A/G208A). Because STAT5a nuclear accumulation was inhibited in the KIKO, it is difficult to address whether STAT5/ERα interaction is altered in KIKO tissues. Our studies did show that STAT5a protein was present in levels comparable with those of WT (Fig. 7A); however, KIKO ERα did not interact with the Igf1 EREs (Fig. 4), and there was little STAT5a binding to GHRE1 detected (Fig. 7B), suggesting a potential effect of ERα-ERE binding on the STAT5a recruitment to GHREs. Future analysis in model systems will facilitate more thorough evaluation of relationships or interactions between STAT5a and ERα recruitment and uterine Igf1 regulation.

Previously, it was reported that E2 was not able to increase the uterine weight of the KIKO but was effective in increasing
epithelial cell proliferation (7) as evidenced by increases in epithelial cell height and DNA synthesis as assessed by bromodeoxyuridine incorporation. Since that report, we established a separate colony for further analysis using NERKI/WT males on a mixed background (129/SvJ and C57B1/6J) (6, 7) from the Jameson lab and ERαko/WT females from our aERKO colony, which are on a pure BL6 background. Female mice expressing only the NERKI ERα allele no longer show any indication of epithelial proliferation as evidenced by the proliferative marker Ki67 or the perimittotic marker Ser(P)10 histone H3 (Figs. 1 and 8) or epithelial cell height increase (data not presented). The reason for the difference between our current observations and those reported previously are unclear at the present time. They may be explained by the drift of the mixed genetic background of the current breeders. Whatever the reason, it is clear that in our colony, we are unable to detect any E2-dependent uterine growth.

Studies described elsewhere have used microarray to demonstrate that E2 regulates numerous ERα-dependent uterine transcripts in the KIKO, indicating that the NERKI ERα allele is functional (35). Cdkn1a (p21) is one example of an ERα-dependent transcript response that is preserved in the KIKO (supplemental Fig. S1A) (7). This gene has been demonstrated to recruit ERs to SP1 motifs in its promoter (32). We observe E2-dependent recruitment of both ERα and SP3 to one of the SP1 sites in the p21 promoter in the KIKO (supplemental Fig. S3).

Because Igf1 plays such an essential role in uterine response to E2, the lack of increase in Igf1 in the KIKO might have explained the lack of growth in this model. However, the supply of systemic exogenous IGFl either alone or together with E2 did not restore uterine response (Fig. 8). Our previous studies have shown that the ERα is required for the uterine proliferative response to EGF or IGF1, because aERKO mice administered either growth factor lacked uterine epithelial proliferation (45, 46). We conclude that Igf1 signaling together with other ERO-mediated ERE-dependent gene regulations are necessary for a full uterine response to E2.

Together, our observations suggest that, in contrast to previous reports indicating a tethered interaction between AP1 and ERs on the Igf1 promoter (16), E2-mediated regulation of Igf1 in the mouse uterus requires the DNA binding ability of ERα, and we propose this mechanism of mouse uterine Igf1 regulation: E2 induces ERα and Stat5 recruitment to ERαEs and GHERs in the Igf1 gene as early as 1 h after E2 treatment, leading to increased transcription. Igf1 transcript does not increase in the KIKO uterus because of a lack of nuclear accumulation of STAT5a and impaired recruitment of ERα to the Igf1 gene. In addition, the KIKO uterus lacks other responses to E2 that would be necessary for uterine proliferation, because no increase in growth can be induced by E2 even if supplemented with exogenous IGFl, indicating that both Igf1 signaling and ERα-ERE-mediated responses are necessary for a full uterine response to estrogen and growth.

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**ERα Binds EREs in Mouse Uterine Igf1 Gene**