Insulin-like growth factor 1 (IGF1) is primarily synthesized in and secreted from the liver; however, estrogen (E2), through E2 receptor α (ERα), increases uterine Igf1 mRNA levels. Previous ChIP-seq analyses of the murine uterus have revealed a potential enhancer region distal from the Igf1 transcription start site (TSS) with multiple E2-dependent ERα-binding regions. Here, we show E2-dependent super enhancer-associated characteristics and suggest contact between the distal enhancer and the Igf1 TSS. We hypothesized that this distal super-enhancer region controls E2-responsive induction of uterine Igf1 transcripts. We deleted 430 bp, encompassing one of the ERα-binding sites, thereby disrupting interactions of the enhancer with gene-regulatory factors. As a result, E2-mediated induction of mouse uterine Igf1 mRNA is completely eliminated, whereas hepatic Igf1 expression remains unaffected. This highlights the central role of a distal enhancer in the assembly of the factors necessary for E2-dependent interaction with the Igf1 TSS and induction of uterus-specific Igf1 transcription. Of note, loss of the enhancer did not affect fertility or uterine growth responses. Deletion of uterine Igf1 in a PgrCre;Igf1f/f model decreased female fertility but did not impact the E2-induced uterine growth response. Moreover, E2-dependent activation of uterine Igf1 signaling was not impaired by disrupting the distal enhancer or by deleting the coding transcript. This indicated a role for systemic IGF1, suggested that other growth mediators drive uterine response to E2, and suggested that uterine-derived IGF1 is essential for reproductive success. Our findings elucidate the role of a super enhancer in Igf1 regulation and uterine growth.

Insulin-like growth factor 1 (IGF1) is primarily secreted from the liver (1) and circulates in serum bound to IGF1-binding proteins, which serve to regulate availability of IGF1 to its transmembrane receptor, IGF1R (2). IGF1 binding to the extracellular IGF receptor activates intracellular mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways (3). Rodent uterine Igf1 transcript is increased by E2, with a peak of induction 4–6 h following acute E2 injection, unlike the earliest responding uterine transcripts, such as Fos, which exhibit peak induction 1–2 h after E2 injection (4–7). E2 initiates a growth response in the epithelial cells of the rodent uterus. Numerous studies support a mechanism in which growth factors, including IGF1, are induced by E2 through the estrogen receptor α (ERα) and secreted from uterine stromal cells, subsequently activating growth factor receptor signaling of adjacent epithelial cells in a paracrine manner, leading to their proliferation. A precise approach that supports this mechanism is the selective deletion of ERαs from uterine epithelial cells using Wnt7aCre;Esr1f/f mice or from anti-mesometrial stromal cells using Amhr2Cre;Esr1f/f mice. Administering E2 to Wnt7aCre;Esr1f/f mice results in uterine epithelial cell proliferation (8), whereas E2 treatment of Amhr2Cre;Esr1f/f mice causes proliferation only in those uterine epithelial cells that are adjacent to mesometrial stromal cells that still express ERα but not in anti-mesometrial epithelial cells adjacent to stromal cells where ERα has been deleted (9). Evaluation of the mouse uterine ERα cistrome using ChIP-seq revealed ERα binding proximal to the two transcription start sites (TSSs) at the two Igf1 alternate first exons (exons 1 and 2); however, an upstream enhancer region 40–70 kb 5′ of the Igf1 TSS exhibited very strong ERα binding (5, 10). Based on ranked acetylation of histone H3 lysine 27 (H3K27Ac) ChIP-seq enrichment, this distal Igf1 can be classified as a super enhancer (11). We hypothesize that this distal super-enhancer region controls the E2-respon-
sive induction of uterine Igf1 transcript via a looping mechanism by which the distal regions are brought into close contact with each other to facilitate induction of the Igf1 transcript. We tested whether such deletion would subsequently result in loss of ERα-stimulated growth. Here, we report studies that evaluate the distal Igf1 super enhancer and its roles in Igf1 transcriptional regulation and uterine growth response.

Results
Super enhancer–associated characteristics of region distal from Igf1 TSS

Our previous findings described a potential enhancer region 40–70 kb 5′ of the Igf1 TSS that exhibited E2-dependent ERα and RNA polymerase II (PolII) binding to five discrete regions (10); we designated these ERα-binding peaks IGFI enhancers 1–5 (Fig. 1A and Fig. S1A). To further assess potential enhancer activity of this region, we evaluated several histone H3 modifications, including monomethylation of histone H3 lysine 4 (H3K4Me1) and trimethylation of histone H3 lysine 4 (H3K4Me3). Increased ratios of H3K4Me1/H3K4Me3 are often associated with enhancers, whereas decreased ratios are often observed at promoters (12–14). We also examined acetylation of histone H3 lysine 27 (H3K27Ac) found in both enhancer and promoter regions (12, 15). The two enhancer-associated modifications (high H3K4Me1/H3K4Me3 ratio and presence of H3K27Ac) were apparent in the Igf1 enhancer region (Fig. 1, A and B, and Fig. S1). Furthermore, E2 treatment increased enhancer-associated H3K27Ac. We ranked the H3K27Ac ChIP-seq signal at 4600 ERα-bound regions to define ERα-binding super enhancers and classified this region as a...
**Estrogen-dependent Igf1 super enhancer**

**A.**

| Treatment | 1 | 2 | 3 | 4 | 5 |
|-----------|---|---|---|---|---|
| Uterus, E2, 1 h | Liver PolI | Uterus, H3K27Ac, 1 h | Liver H3K27Ac, 1 h | Liver H3K4Me3, 1 h | Liver H3K4Me1, 1 h |
| Liver PolI | Uterus, Adult E2, 1 h | Liver E2, 1 h | Liver E2, 1 h | Liver E2, 1 h | Liver E2, 1 h |
| Uterus, 21d | Uterus, Adult | Liver | Liver | Liver | Liver |
| H3K27Ac, 1 h | H3K27Ac, 1 h | H3K27Ac, 1 h | H3K27Ac, 1 h | H3K27Ac, 1 h | H3K27Ac, 1 h |
| Uterus, 21d | Uterus, Adult | Liver | Liver | Liver | Liver |
| H3K4Me3 | H3K4Me3 | H3K4Me3 | H3K4Me3 | H3K4Me3 | H3K4Me3 |
| Uterus, 21d | Uterus, Adult | Liver | Liver | Liver | Liver |
| H3K4Me1 | H3K4Me1 | H3K4Me1 | H3K4Me1 | H3K4Me1 | H3K4Me1 |
| Uterus, 21d | Uterus, Adult | Liver | Liver | Liver | Liver |
| E2, 1 h | E2, 1 h | E2, 1 h | E2, 1 h | E2, 1 h | E2, 1 h |
| Liver | Liver | Liver | Liver | Liver | Liver |
| RNA E2, 1 h, + strand | RNA E2, 1 h, + strand | RNA E2, 1 h, + strand | RNA E2, 1 h, + strand | RNA E2, 1 h, + strand | RNA E2, 1 h, + strand |
| Liver | Liver | Liver | Liver | Liver | Liver |
| RNA E2, 1 h, - strand | RNA E2, 1 h, - strand | RNA E2, 1 h, - strand | RNA E2, 1 h, - strand | RNA E2, 1 h, - strand | RNA E2, 1 h, - strand |
| Liver | Liver | Liver | Liver | Liver | Liver |
| RNA GroSeq, RNA, + strand | RNA GroSeq, RNA, + strand | RNA GroSeq, RNA, + strand | RNA GroSeq, RNA, + strand | RNA GroSeq, RNA, + strand | RNA GroSeq, RNA, + strand |
| Liver | Liver | Liver | Liver | Liver | Liver |
| RNA groove, RNA, - strand | RNA groove, RNA, - strand | RNA groove, RNA, - strand | RNA groove, RNA, - strand | RNA groove, RNA, - strand | RNA groove, RNA, - strand |
| Liver | Liver | Liver | Liver | Liver | Liver |

**B.**

**Figure 2. Igf1 distal region develops as a super enhancer in uterine but not in liver tissue.** A, UCSC Genome Browser screen shot of the Igf1 distal enhancer region and TSSs with comparison of liver and uterus ChIP-seq data. Shown are PolII ChIP-seq (green) from ovariectomized female uterus injected with E2 (GEO GSE36455 (10)) and from adult male liver (GEO GSE44571 (18)); H3K27Ac ChIP-seq (red) from 21-day-old or ovariectomized adult female uterus (this study) or liver (GEO GSE44571 (17)) treated with E2 or female liver (GEO GSE44571 (16)); H3K4Me3, ChIP-seq (purple) and H3K4Me1, ChIP-seq (blue) from 21-day-old or ovariectomized adult female uterus treated with E2 (this study) or female liver (GEO GSE44571 (16)); E1a ChIP-seq (dark blue) from 21-day-old or ovariectomized adult female uterus (GEO GSE36455 (10)) or liver (GEO GSE70346 (17)) treated with E2; and stranded RNA-seq positive strand (pink) and negative strand (blue) from ovariectomized female uterus treated with E2 (this study) or GroSeq from adult male liver (GEO GSE59486 (19)) sampled ZT16 collected 4 h after lights off. Enhancer 4 is highlighted in blue. B, focus on E1a and H3K27Ac ChIP-seq at each enhancer peak, 1–5.

super enhancer, ranked 97th of 281 super enhancers (Fig. 1C). Promoter-associated modifications (H3K27Ac and H3K4Me3) are detected near the Igf1 TSSs (Fig. 1A and Fig. S1A). Transcription of enhancer RNA (eRNA) is characteristic of transcriptional enhancers. Therefore, we used stranded RNA-seq of total uterine RNA to calculate signal strength fragments per kilobase of transcript per million mapped reads (FPKM) within 1500 bp 5′ (− strand) or 3′ (+ strand) of PolII of each of the two Igf1 TSSs, the five enhancers, and a control region that lacked PolII (Fig. 1D and Fig. S1, A and B). E2 treatment of the mice resulted in robustly increased signal from the positive strand of the TSS. E2 also significantly increased signal from enhancers 3, 4, and 5 that returned to basal level by 24 h (Fig. 1D and Fig. S1, A and B).

To impact transcription of Igf1, the distal super enhancer must physically interact with the TSSs. We therefore analyzed samples from vehicle (V)- or E2-treated uterine tissue using chromatin capture sequencing (HiC), which indicated an interaction between the Igf1 super-enhancer region and the Igf1 TSS (Fig. 1A and Fig. S1A). We also examined binding of the cohesin subunit, SMC1a, using ChIP-seq and observed an E2-dependent increase of cohesin binding to enhancer 4 (Fig. 1A and Fig. S1A), consistent with the interaction detected using HiC. Observation of the super enhancer–associated modifications and contact suggests the distal region is bound by E1a and forms a loop enabling transcriptional regulation of Igf1.

Evaluation of PolII, H3K27Ac, H3K4me3, and H3K4me1 ChIP-seq from published liver data sets indicates this region lacks the super enhancer characteristics seen in the uterus (Fig. 2, A and B; GEO GSE44571 (16), GSE70346 (17), and GSE49847 (18)). In mouse liver samples, H3K27Ac is associated with peak 1, and moderate H3K4Me3/H3K4Me1 is seen (Fig. 2, A and B). GroSeq data from mouse liver reveals eRNA synthesis from an enhancer 1 and 2–positive strand and enhancer 1–negative strand (Fig. 2A; GEO GSE59486 (19)), indicating that this region of the enhancer may have some activity in the liver. E1a ChIP-seq from mouse liver revealed a small amount of E1a binding to enhancer 2 (Fig. 2, A and B; GEO GSE70346), indicating that enhancer 2 was less likely to modulate uterine-specific regulation. We also note that the E1a binding and histone modifications seen in adult ovariectomized uterine samples is apparent in prepubertal (21-day-old) uterine samples (Fig. 2, A and B), indicating that this region is developmentally programmed to function as an E1a-binding super enhancer in uterine cells, but not in liver tissue.

Constitutive E1a binding prior to E2 treatment was seen in the uterus at enhancers 2 and 5 (Fig. 1, A and B), indicating that these two enhancers were less likely to mediate E2 induction. We selected enhancer 4 for deletion via CRISPR Cas9-mediated genetic disruption, because it exhibited E1a and PolII binding, eRNA synthesis as well as the enhancer-associated histone modification H3K27Ac, and interaction with the TSS, and all these characteristics were E2-dependent.
Figure 3. Deletion of 430 bp of IGF1enh4 using CRISPR-Cas9. A, targeting strategy showing sgRNA4G and sgRNA4H, the guide RNAs used to target the region for deletion (sequences in Table S1). Underlined letters are the matching part of a full ERE palindrome. Red text indicates boundary of targeted region. B, deleted DNA sequence, with ERE motif sites highlighted in yellow (perfect match) or gray (1–2 nt not matched to consensus). Underlined letters are the matching part of a full ERE palindrome. Red text indicates boundary of targeted region. C, transcription factor–binding motifs within the deleted sequence.
Deletion of Igf1 enhancer 4 selectively disrupts uterine Igf1 induction

To test the effect of the ERα-binding site, we deleted 430 bp of DNA comprising the ERα-binding peak of IGF1 enhancer 4 (Fig. 3), generating IGF1enh4KO mice. To assess any impact deletion of the IGF1 enhancer 4 has on Igf1 transcription, RNA was prepared from uteri and livers of ovarioctomized mice that were treated with saline V or with E2 for 6 h for RT-PCR analysis. In WT mice, uterine Igf1 RNA increased after E2 treatment (Fig. 4A), whereas the response was lost from IGF1enh4 KO uteri. Igf1 is detected in WT and IGF1enh4KO liver RNA at comparable levels and is not significantly changed after E2 treatment of the mice (Fig. 4A). These findings indicate that deletion of Igf1 enhancer 4 inhibits E2 induction of uterine Igf1 mRNA without impacting expression of the Igf1 gene in the liver. To ensure the disruption of IGF1enh4 did not affect uterine E2 transcriptional responses in general, we confirmed that two well-characterized E2-induced uterine transcripts (Fst (Follistatin) and Lif (leukemia-inhibiting factor)), which are both expressed from epithelial cells, as well as the stromal gene Ramp3 (receptor activity–modifying protein 3), were induced 6 h after E2 treatment of IGF1enh4KO mice (Fig. S2).

We then used RT-PCR to confirm the presence and E2 induction of positive-strand eRNA transcripts from IGF1 enhancers 3, 4, and 5. All three eRNAs could be detected in uterine RNA (Fig. 4B) and were increased after E2 treatment. When IGF1enh4 was disrupted, the uterine eRNAs from enhancers 3, 4, and 5 were no longer increased by E2 treatment (Fig. 4B). The correlation of Igf1 coding and eRNA induction in the uterus is consistent with a role for eRNA in facilitating transcription of Igf1 mRNA.

IGF1 enhancer 4 orchestrates assembly of mediators of E2-dependent transcription

Next, we evaluated the impact of the enhancer deletion on the E2-dependent assembly of transcriptional mediators. Using
ERα ChIP-PCR, we evaluated the impact of deletion of IGF1enh4 on ERα recruitment at IGF1enh1–5 and near the Igf1 TSS in the uterus. Treatment of mice with E2 for 1 h increased ERα binding to chromatin at a site proximal to the Igf1 TSS, as well as at IGF1enh1–5 (Fig. 5A). When IGF1enh4 is deleted, E2 still induces ERα binding to enhancers 1, 2, 3, and 5, yet Igf1 transcription is not increased. This indicates that ERα binding to IGF1enh4 is critical for E2 induction of Igf1 transcript in the uterus and that binding to the other sites is unable to compensate for the loss of the enhancer 4 site. Recruitment of p300 and its associated histone acetyltransferase activity to enhancers, and the TSS is indicative of enhanced transcription (20). Therefore, we evaluated p300 association with IGF1enh1–5 and near the Igf1 TSS in uterus samples. p300 binds the IGF1 TSS and IGF1enh1 (Fig. 5B), with a slight E2-dependent increase in WT mice. Treatment of WT mice with E2 for 1 h induced a pronounced increase in p300 associated with IGF1enh2–5. Administering E2 produced significantly less p300 binding at IGF1enh2–5 when IGF1enh4 was deleted and lead to loss of the modest p300 increase at TSS and IGF1enh1. Therefore, IGF1enh4 appears to be critical for modulation of this key component of enhancer function and consequent eRNA synthesis needed to transduce the E2 response to the TSS. Interaction with cohesin is indicative of loop structure formation (21).
Therefore we compared cohesin subunit, SMC1a, interaction using ChIP-PCR. Prior to E2 treatment, SMC1a is prominently bound to a region ~4500 bp 5’ of Igf1 TSS1 (Fig. 6A). E2 treatment increases SMC1a interaction with all five IGFI enhancers but most dramatically with IGFI enhancer 4 (Fig. 6A). Deletion of this enhancer does not impact SMC1a binding to any sites prior to E2 treatment, except for the interaction with IGFI enhancer 4 itself (Fig. 6A). Deletion of IGFI enhancer 4 attenuates the E2-induced increase in SMC1a binding to all the enhancers and the TSSs (Fig. 6A), consistent with an impact on E2-dependent interaction between the super-enhancer region and the TSS. E2-dependent interaction between enh4 and the TSS in WT mice was confirmed using 3C-PCR (Fig. 6B). After E2 treatment, increased interaction could be detected. Interaction could also be detected in some of the enh4 KO samples (Fig. 6B), irrespective of E2 treatment. Thus, the interaction between the regions is not dependent on the enhancer 4 site. Sequencing of the PCR amplicor revealed a ligation of IGFIenh4 and TSS fragments (Fig. 6C).

Disrupting E2 induction of uterine Igf1 does not impact fertility or uterine epithelial growth

Fertility of IGFIenh4KO females was assessed during a 6-month-long continuous breeding trial. IGFIenh4KO females were fertile, in terms of both the number of litters produced and the number of pups per litter (Table 1). We had expected that disrupting E2 regulation of uterine IGFI would impact uterine function and fertility. To directly examine the impact of loss of uterine IGFI, we deleted the coding transcript. We used the PgrCre, which has Cre recombinase activity in all cells that express progesterone receptor, including all uterine cells (22). We bred PgrCre mice to mice with loxP sites flanking exon 4 of Igf1 (23), which encodes the IGFI peptide, to produce mice that lack IGFI in uterine cells; we refer to these mice as “PIGFcKO.” Deletion of the Igf1 coding transcript decreased female fertility after 6 months of continuous breeding (Table 1), with fewer litters and fewer pups per litter, indicating a requirement for uterine IGFI for optimal fertility.

To further examine response within the uterine tissue, uterine epithelial cell growth was evaluated using the proliferation-associated marker, Ki67, 24 h after E2 treatment of ovariec- tomized mice. IGFIenh4KO and PIGFcKO females exhibited robust growth response that was similar to WT littermates (Fig. 7A). Previous work indicated a role for IGFI in facilitating cell cycle progression, as uterine epithelial cells of IGFI knockout mice that were treated with E2 exhibited G2/M arrest (24). We assessed the ability of IGFIenh4KO and PIGFcKO uterine epithelial cells to undergo mitosis in response to E2 by using colchicine to trap and quantify mitotic cells. E2 increased mitotic epithelial cells, shown by the mitotic marker phosphoserine 10 of histone H3 (Fig. 7, A and B).

RT-PCR revealed that E2 did not increase Igf1 in PIGFcKO uterine sample (Fig. 7C), indicating successful deletion of the coding transcript. Uterine response to E2 after three daily injections results in dramatic increases in tissue weight and epithelial cell layer height. The response observed in IGFIenh4KO and PIGFcKO mice was a blunted response relative to WT, with significantly lower uterine weight (Fig. 7D). The height of the epithelial cell layer increased comparably with WT littermates (Fig. 7D). Overall, we observed similar responses when E2 induction of uterine Igf1 was disrupted either by deleting the enhancer or the coding transcript.

Uterine Igf1 disruption does not impair E2-induced uterine IGFI1 receptor signaling

There is a long-standing debate regarding the relative contributions of systemic IGFI (endocrine) and IGFI synthesized within a tissue (paracrine/autocrine). Studies that support roles for systemic IGFI in uterine response include the restoration of E2-mediated growth response following transplantation of IGFI null uterine tissue into an IGFI WT host (25). Such findings suggest that E2 increases VEGF in uterine tissue, leading to increased vascular permeability and influx of systemic IGFI into the tissue (26, 27), and were further supported by a study using sFLT1-1, a VEGFα inhibito, to prevent E2-induced uterine growth response (27). We next examined E2-initiated IGFI receptor activation within uterine tissues of the two models with uterine IGFI disruption. As has been previously noted by others (28), 6 h following E2 treatment, phosphorylated IGFI receptor is detected in uterine tissue extracts (Fig. 8A). E2 increases uterine IGFI1 phosphorylation in both the IGFIenh4KO and PIGFcKO (Fig. 8A) and also increases phosphorylation of AKT, which is a downstream target of IGFI1 signaling (Fig. 8A). These findings support the concept that E2 facilitates the import of circulating IGFI into the tissue. To directly assess whether E2 response included uptake of IGFI1 into uterine tissue, an ELISA was used to measure IGFI content in uterine homogenates. E2 increased the amount of IGFI detected in uterine tissue (Fig. 8B), in WT mice but also in the PIGFcKO and IGFIenh4KO mice, which lack the E2-mediated increase of Igf1 transcript. This finding is consistent with a role for E2 in facilitating entry of systemic IGFI1 into uterine tissue.

Discussion

Our previous studies using deletion of uterine ERα from epithelial or anti-mesometrial stromal cells showed that epithelial cell ERα is dispensable but that stromal ERα is necessary for adjacent epithelial cell proliferative response induced by E2 (8, 9), supporting our hypothesis that ERα-mediated induction of growth factor synthesis in and secretion from stromal cells leads to growth of neighboring epithelial cells. IGFI1 is an E2-induced stromal factor and thus is a candidate for a paracrine growth mediator. We were surprised, then, that E2 growth response is unimpaired when we prevent E2 induction of uterine IGFI1; this indicates that paracrine factors other than IGFI1 are involved in the response. FGF10 and BMP8a (29) have also

Table 1

| Cross: male × female | Litters/pair/6 months | Pups/pair/6 months | Pups/litter |
|----------------------|----------------------|-------------------|-------------|
| WT × WT (n = 5)      | 4.2                  | 22.8              | 5.45        |
| WT × IGFIenh4KO (n = 5) | 5.0                 | 28.8              | 5.0         |
| WT × IGFI/Δf (n = 5) | 4.8                  | 24.4              | 4.76        |
| WT × PIGFcKO (n = 5) | 2.4*                 | 8.6*              | 3.33*       |

*p < 0.05 versus WT by one-way ANOVA.
been proposed to mediate uterine growth response and are expressed at comparable levels in WT and IGF1enh4KO uterine samples (data not shown) and may therefore be mediating uterine growth responses. Uterine-derived IGF1 does play a role in optimal response when E2 is administered for 3 days, because both IGF1enh4KO and PIGFcKO showed an attenuated tissue weight increase with less fluid accumulation.

We were also somewhat surprised by the observation of normal fertility when we deleted the Igf1 distal enhancer. Fertility of PIGFcKO of females was significantly lower than Cre−/H11002 littermates (Table 1), as evidenced by fewer and smaller litters. The PIGFcKO ovary functions appeared to be normal, with normal estrous cycles and ovulation responses comparable with IGFf/f (data not shown), suggesting impairment of their uterine function led to the decreased fertility. In general, the fertility of PIGFcKO females decreased over the course of the 6-month trial (data not shown), indicating that loss of uterine IGFl may not prevent pregnancy per se but rather that uterine IGFl may be needed for post-partum uterine recovery. The normal fertility of the IGF1enh4KO females indicates that E2-mediated uterine Igf1 regulation is not necessary for fertility. Igf1 expression is highest during diestrous (when progesterone is rising (30)) and proestrous (when estrogen and progesterone are elevated and ovulation is triggered by luteinizing hormone (30, 31)) and quite low during the estrous phase (when progesterone and estrogen are low and mating occurs (30, 31)) of the mouse estrous cycle (32). Together, these findings suggest other regulators of Igf1, including progesterone,

Figure 7. Disrupting uterine Igf1 enhancer or coding transcript does not impair uterine growth. A, uterine cross-sections after immunohistochemical analysis of ovariectomized WT, IGF1enh4KO, or PIGFcKO females that were treated with E2 for 24 h (K67) or treated with E2 for 22 h and colcemid for 2 h (phosphoserine 10 of histone H3; Ph-ser10Hist H3). B, mitotic index, calculated by counting proportion of epithelial cells that were positive for the mitotic marker of phosphoserine 10 of histone H3. *, p < 0.001 versus V; +, p < 0.05 versus WT. C, expression levels of Igf1 mRNA using primers targeting exon 4 analyzed by RT-PCR of total RNA isolated from uterus samples from ovariectomized female mice. Tissue samples were collected 6 h after injection of either saline (V) or E2. Samples were taken from mice with deletion of the Igf1 coding transcript (PIGFcKO) or their f/f littermates (PIGFWT). The data were plotted relative to WT V uterus = 1. The data are represented as means ± S.D. *, p < 0.05 versus V, n = 3 samples/group; +, p < 0.05 versus WT; tested using two-way ANOVA with Fisher’s LSD post test. D, uterine weights of ovariectomized WT, IGF1enh4KO, or PIGFcKO females that were treated with E2 daily for 3 days and then collected on the 4th day (E2, 72 h), n = 5–10/group. *, p < 0.0001 versus V; +, p < 0.0001 versus WT. Height of luminal epithelial cell layer (LEH), measured in μm.
**Estrogen-dependent Igf1 super enhancer**

![Image](image.png)

**Figure 8. Uterine Igf1 disruption does not impair E2-induced uterine Igf1 receptor signaling.** A, representative Western blotting of uterine proteins demonstrates that E2 (6 h) induces activation of the Igf1R, as indicated by the phosphorylation of its B subunit (pIGF1RB;Tyr-1135/1136). The same blot was then reprobed for total IGF1R. The same samples were analyzed for phosphorylation of AKT (pAKT; Ser-473) and AKT. Molecular masses of marker bands are shown in kilodaltons. PhIGF1RB was normalized to total IGF1R using a LI-COR Fc imager, and ratios of normalized phIGF1RB after E2 treatment relative to V treatment were calculated. Three pools of three uteri per sample were analyzed per group, and the average values ± S.E. are shown in the table. B, ELISA for mouse IGF1 using uterine proteins. E2 induces increase in IGF1 protein within the uterine tissue, despite the disruption of Igf1 transcript in the IGF1enh4KO and PgrCrexIgf1f/f models. Fold E2/V was calculated for each sample group and is shown above each E2 set (n = 3 pools of 3 uteri/sample). *, p < 0.05 versus V; +, p < 0.001 versus WT.

interaction between distal enhancer regions and coding gene TSSs is proposed to be mediated by cohesin driven loop extrusion, which brings converging CCCTC-binding factor CTCF sites into contact (21). The resulting loop structure facilitates transcriptional regulation via mediator complexes, together with tissue specific transcription factors, including ERα (39). Estrogen-responsive element (ERE) motifs present in the Igf1enh4 sequence (Fig. 3B), as well as in the other binding peaks (10), serve to facilitate ERα binding. Other transcription factor-binding motifs are within the deleted DNA (Fig. 3C); some, such as HOX, FOX, and KLF, are known mediators of uterine transcriptional responses (40–42). We propose that ERα binding to enhancer 4 orchestrates assembly of transcriptional activators, as illustrated by the E2-dependent interaction with p300 (Fig. 5B). Although ERα binding to other sites was not impacted by deletion of enhancer 4 (Fig. 5A), p300 recruitment and consequent eRNA transcription from enhancers 3, 4, and 5 was disrupted. eRNA may serve to stabilize interaction between the distal super-enhancer region and the regulated gene’s TSS via interaction between eRNA and mediator/cohesin subunits (43). Consistent with this view, we observe E2-dependent interaction of enhancer 4 with cohesin subunit SMC1a (Fig. 6A). The significance of eRNA transcription and its role in transcriptional regulation of coding transcripts is an emerging area of investigation. There is evidence that eRNA transcription may simply reflect the presence of PolII at enhancer regions (44). On the other hand, transcription of eRNA is involved in mechanisms of coding transcript regulation, because disrupting eRNA levels prevents regulation of its target transcripts (44, 45). Our findings are not sufficient to distinguish whether the eRNA per se or the process of its transcription is what is required for gene regulation. eRNA is widely reported to appear transiently, and consistent with this characteristic, the uterine eRNA transcripts detected return to basal levels within 24 h of E2 injection (Fig. S1, A and B). It is possible that deletion of any of the three eRNA-producing enhancers would impact Igf1 transcription and that these three enhancers function as a unit. Our continuing studies will further elucidate mechanistic details that underlie the critical role of this distal super enhancer in uterine induction of Igf1 transcription by E2. It is also interesting to note that this region develops as an ERα-binding super enhancer specifically in uterine tissue, as evidenced by enhancer-associated histone modifications and ERα binding in prepubertal uterine tissue and the lack of either in liver samples (Fig. 2).

Our observations have led us to conclude that uterine induction of Igf1 mRNA after E2 treatment of mice is conferred by a distal super enhancer containing five ERα-binding sites. We have demonstrated the critical role of Igf1enh4 within this enhancer region in the E2 regulatory mechanism by showing that deletion of Igf1enh4 completely eliminates any increase in uterine Igf1 transcript following E2 treatment. Overall our work indicates that a distal super enhancer is responsible for driving E2-dependent uterine induction of Igf1 and that uterine-derived IGF1 is not required for pregnancy but is important for maintaining fertility.

growth hormone, and STAT activators (33–35), may drive the expression of IGF1 needed for optimal uterine function.

Deleting E2-induced uterine IGF1, by disrupting the distal super enhancer (IGF1enh4KO), or by deleting the coding transcript (PIGFeKO) did not dramatically affect uterine response and function. Experimentally, our findings support the view that systemic endocrine IGF1, rather than IGF1 produced directly by uterine cells, is needed for E2 growth responses and fertility. However, findings that female mice with liver-specific disruption of IGF1 are fertile (36) but that global deletion of Igf1 results in female reproductive problems (24) point to a more complex interplay of these systems. Observations indicating increased serum IGF1 levels are associated with better reproductive performance of cows (37) and that higher levels of serum IGF1 are associated with better IVF outcome (38) support a role for levels of systemic IGF1 in optimal reproductive function.
Experimental procedures

Animals

All mice were used in accordance with an NIEHS-approved animal study protocol and using the 2015 edition of the Public Health Service Policy on Humane Care and Use of Laboratory Animals. IGF1enh4KO mice were made as described below. PgrCre;Igf11/4 (PIGFcKO) mice were made by breeding PgrCre mice (22) (provided by Dr. Francesco J. DeMayo) with Igf11/4 mice (23) (purchased from the Jackson Laboratory). For E2 response experiments, adult (10 + week old) female mice were ovarioectomized and then housed for 10–14 days before the experiments to allow endogenous ovarian hormones to diminish. There was no blinding, and mice were randomly assigned to treatment groups. Homozygous PgrCre;Igf11/4 or IGF1enh4KO mice were used in all studies, together with control (Igf11/4 or WT, respectively) littersmates. The mice were given a single intraperitoneal injection of 250 ng of E2 (Research Plus Inc., Barnegat, NJ) dissolved in 0.1 ml of normal saline. Control V animals were injected with 0.1 ml of normal saline. Tissue samples (uterus or liver) were collected 1, 2, 6, 22, or 24 h after the injections. For 3-day bioassays, the mice were subcutaneously injected daily with 250 ng of E2 dissolved in 0.1 ml of sesame oil (Sigma) for 3 days, and uterine tissue was collected on the 4th day. For the colchicine block experiment, demecolcine (Sigma; 20 g/ml leupeptin (Sigma), and 4 g/ml a-phenylmethylsulfonyl fluoride (Calbiochem)). Other frozen uterine samples were shipped to Active Motif for H3K4Me1, H3K27Ac per 15 g of mouse uterine tissue chromatin.

Chromatin immunoprecipitation

Frozen mouse tissue was submersed in PBS + 1% formaldehyde, cut into small pieces, and incubated at room temperature for 15 min. Fixation was stopped by the addition of 0.125 M glycine (final). The tissue pieces were then treated with a TissueTearer and finally spun down and washed twice in PBS. Chromatin was isolated by the addition of lysis buffer, followed by disruption with a Dounce homogenizer. Lysates were sonicated, and the DNA was sheared to an average length of 300–500 bp. Genomic DNA (Input) was prepared by treating aliquots of chromatin with RNase, proteinase K, and heat for de-cross-linking, followed by ethanol precipitation. Pellets were resuspended, and the resulting DNA was quantified on a NanoDrop spectrophotometer. Extrapolation to the original chromatin volume allowed quantitation of the total chromatin yield.

An aliquot of chromatin (15 g for histone marks; 25 g for ERα) was precleared with protein A–agarose beads (Invitrogen). Genomic DNA regions of interest were isolated using 4 g of antibody. Complexes were washed, eluted from the beads with SDS buffer, and subjected to RNase and proteinase K treatment. Cross-links were reversed by incubation overnight at 65 °C, and ChIP DNA was purified by phenol-chloroform extraction and ethanol precipitation.

Quantitative PCRs (qPCRs) were carried out in triplicate on specific genomic regions using SYBR Green Supermix (Bio-Rad). The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using Input DNA.

Illumina sequencing libraries were prepared from the ChIP and Input DNAs by the standard consecutive enzymatic steps of end polishing, dA addition, and adaptor ligation. After a final PCR amplification step, the resulting DNA libraries were quantified and sequenced on Illumina’s NextSeq 500 (75-nt reads, single end). All of the new data sets are deposited under GEO accession number GSE125972.

ChIP-PCR

The above steps for processing the tissue and performing the ChIP reactions were followed. Each ChIP reaction contained 30 μg of tissue chromatin and 4 μg of ERα antibody (Santa Cruz Biotechnologies, catalog no. sc-542) p300 antibody (Santa Cruz, catalog no. sc-585) or 10 μl of SMC1a antibody (Active Motif, catalog no. 61067).

qPCRs were carried out in triplicate using SYBR Green Supermix (Bio-Rad, catalog no. 170-8882) on a CFX ConnectTM real-time PCR system. One positive control site was tested (Untr6), plus the test sites of interest. The resulting signals were normalized for primer efficiency by carrying out qPCR for each primer pair using input DNA (pooled unprecipitated genomic DNA from each sample). Active Motif’s ChIP-qPCR normalization strategy, which considers the starting amount of chromatin, the final ChIP volume, and the primer efficiency, was used to calculate binding events per 1000 cells.
**Estrogen-dependent Igf1 super enhancer**

**HiC**

HiC experiments were performed by Arima Genomics (San Diego, CA) according to the Arima-HiC protocol described in the Arima-HiC kit. After the Arima-HiC protocol, Illumina-compatible sequencing libraries were prepared by first shearing purified Arima-HiC proximally ligated DNA and then size-selecting DNA fragments from ~200–600 bp using solid-phase reversible immobilization magnetic beads. The size-selected fragments were then enriched for biotin and converted into Illumina-compatible sequencing libraries using the KAPA Bio-systems Hyper prep kit (Wilmington, MA). After adapter ligation, DNA was PCR-amplified and purified using solid-phase reversible immobilization beads. The purified DNA underwent standard quality checks (qPCR and Bioanalyzer) and was shipped to the NIEHS sequencing core. Libraries were loaded on an Illumina flow cell (Illumina, San Diego, CA) for paired-end 50-nucleotide read length sequencing on an Illumina NovaSeq instrument.

**3C-PCR**

3C-PCR was done using a modification of the method described in Ref. 46. One-fourth of a pulverized frozen uterus was cross-linked and homogenized, and nuclei were resuspended in 500 μl of 1.2× New England Biolabs restriction buffer 2.1 (New England Biolabs) denatured with SDS and Triton X-100 and digested overnight with 400 units of BsrGI HF (New England Biolabs). Digestion was stopped with SDS, samples were diluted 10-fold with 1.1× New England Biolabs T4 ligation buffer, and Triton X-100 was added to neutralize the SDS. 1000 units of T4 ligase (New England Biolabs) was added, and samples were incubated for 4 h at 16 °C followed by 30 min at room temperature. DNA was isolated and analyzed by PCR using RedTaq mix (Sigma) and primers to detect the ligation product (see Table S1) and analyzed on a 3% GenePure agarose gel (GenePure, Kaysville, UT). The ligation product was purified using QiaQuick PCR kit (Qiagen) and sequenced by Genewiz (Morrisville, NC).

**CRISPR CAS**

Two CAS9 sgRNA (TAGCCCTTCATGCATCAGATNGG and AGAGGTCTACGTCACTACACNGG) were designed to excise the enhancer element. Complementary oligonucleotides were ordered from IDTDNA (Coralville, IA), cloned into a T7 transcription AmpliScribe T7 high-yield transcription kit (Madison, WI). C57BL/6J one-cell embryos were microinjected with both guides (10 ng/μl each) and 5′ capped and poly(A) tailed Cas9 RNA (100 ng/μl) derived from pCAG-T3-hCAS-pA, a gift from Wataru Fujii and Kunihiko Naito (47). Microinjected embryos were surgically transferred to SWISS pseudo-pregnant females. At weaning, potential founders were genotyped by PCR and amplicon sequencing (IGFI_E4 forward, 5′-TGTTCAACAG-ACCTTCCAGCC-3′; reverse, 5′-CAGGCAGTGTAGTTTTT-CAACTTGCT-3′). Founders of interest were bred to WT C57BL/6 mice, and F1 offspring were re-screened to confirm germ-line transmission. Phenotyping was done with founder line 3-7, which carries a 432-bp deletion between the two guides used, corresponding to chr10:87,812,231–87,812,662 (GRCm38/mm10 assembly). Correct deletion was confirmed by sequencing; the deleted sequence is shown in Fig. 3. In experiments, the mice with homozygous deletion (IGF1enhKO) and their WT littermates were used. Mouse genotyping was subsequently done with either the screening primers above or primer/probe assay by Transnetyx (WT forward, 5′-CACACACACAGACCTTAATGGT-3′; WT reverse, 5′-CCCTGACATCTCTGTGATCCT-3′; WT probe, 5′-CATGCGCCCTTCCTCCTGC-3′; KO forward, 5′-GTTTCCTCCCTCAGTTGTAATGGA-3′; KO reverse, 5′-GGCCTCAGGGATAGCAAGAAG-3′; KO probe, 5′-CTGCAGTGGACCTCTCA-3′).

**RNA-seq**

Uterine RNA from mice treated with V or with E2 for 2, 6, or 24 h was isolated using miRNeasy mini kit (Qiagen) according to the manufacturer’s protocol and treated with DNase (Qiagen). RNA was sent to the National Institutes of Health Intramural Sequencing Center for stranded sequencing library preparation and sequencing. Stranded RNA-seq libraries were constructed from 1 μg of total RNA after rRNA depletion using Ribo-Zero GOLD (Illumina, San Diego, CA). The Illumina TruSeq stranded total RNA sample prep kit was used according to the manufacturer’s instructions except where noted. Amplification was performed using 10 cycles optimized for the input amount and to minimize the chance of overamplification. Unique barcode adapters were applied to each library. Libraries were pooled together for sequencing. The pooled libraries were sequenced on multiple lanes of a HiSeq2500 using version 4 chemistry to achieve a minimum of 55 million 125-base read pairs. The data were processed using RTA version 1.18.64 and CASAVA 1.8.2.

**RNA-seq data processing**

Libraries were sequenced as paired-end 126-mers on an Illumina HiSeq2500. Read pairs were filtered based on a mean base quality score >20. Filtered read pairs were mapped to the mm10 reference genome with STAR version 2.5 (48) (parameters −outMultimapperOrder Random −outSAMattrHstart 0 −outFilter-Type BySJout -alignSoverhangMin 8 −limitBAMsortRAM 55000000000 −outSAMstrandField intronMotif −outFilterIntronMotifs RemoveNoncanonical). Stranded depth tracks were generated by STAR version 2.5 (parameters −outWigType bedGraph −outWigStrand Stranded −outWigNorm None) and subsequently normalized by size factors reported from DESeq2 (49).

**eRNA analysis**

Under each ERα peak of interest, the local maxima of PolII ChIP-seq signal (WT, E2 treatment condition) was identified. Additionally, the midpoint between each pair of consecutive local maxima were selected as control sites. Evaluated regions were defined as 1500-nt windows 5′ (− strand) or 3′ (+ strand) of the selected PolII max (or control) sites, and the FPKM of each region was calculated per sample from strand-specific RNA-seq data.

**ChIP-seq data processing**

PolII ChIP-seq libraries were sequenced as single-end 36-mers by Active Motif. ERα ChIP-seq libraries were
Assessment of histone modifications for super enhancers

Super enhancers were identified based on the method described by Bojcuk et al. (11). First, ERα peak calls with high stringency were called using Homer (parameters —fdr 0.00001 —F 12 —style factor) for the ovariectomized adult and 21-day-old samples and then combined via BEDtools mergeBed (version 2.24.0). BEDtools mergeBed (version 2.24.0) was then rerun, this time to merge all peak calls within 12.5 kb. This set was subsequently filtered to retain only those regions with more than one contributing called peak. Each region was scored by counting the number of overlapping uniquely mapped nonduplicate reads with BEDtools multiBamCov (version 2.24.0); reads in this calculation were H3K27ac, H3K4me1, and H3K4me3 histone modifications, converted to bedGraph format via BEDTools genomeCoverageBed (51), and then normalized to either 15 million (for ERα) or 20 million (all others) uniquely mapped nonduplicate reads per sample.

Identification of super enhancers

Super enhancers were identified based on the method described by Bojcuk et al. (11). First, ERα peak calls with high stringency were called using Homer (parameters —fdr 0.00001 —F 12 —style factor) for the ovariectomized adult and 21-day-old samples and then combined via BEDtools mergeBed (version 2.24.0). BEDtools mergeBed (version 2.24.0) was then rerun, this time to merge all peak calls within 12.5 kb. This set was subsequently filtered to retain only those regions with more than one contributing called peak. Each region was scored by counting the number of overlapping uniquely mapped nonduplicate reads with BEDtools multiBamCov (version 2.24.0); reads in this calculation were H3K27ac, H3K4me1, and H3K4me3 histone modifications, converted to bedGraph format via BEDTools genomeCoverageBed (51), and then normalized to either 15 million (for ERα) or 20 million (all others) uniquely mapped nonduplicate reads per sample.

HiC data processing

The Juicer (version 1.5.6) platform was used for processing the HiC samples (52). For each sample, a .hic file was generated based on mapped to the mm10 reference genome with Bowtie version 0.12.8 (50), allowing uniquely mapped alignments only. Duplicates were removed with MarkDuplicates.jar from the Picard tool suite (http://broadinstitute.github.io/picard). For visualization purposes, mapped reads were extended to their estimated fragment length (150 nt for PolII and ERα; 200 nt for SMC1α and H3K27ac, H3K4me1, and H3K4me3 histone modifications), converted to bedGraph format via BEDTools genomeCoverageBed (51), and then normalized to either 15 million (for ERα) or 20 million (all others) uniquely mapped nonduplicate reads per sample.

RT-PCR

RNA was isolated from livers and uteri using TRIzol reagent, and then 1–2 μg was treated with DNase (Invitrogen) and used to synthesize cDNA using random hexamers (Applied Biosystems, Grand Island, NY) and Super Script II (Invitrogen) per the manufacturer’s protocols. Resulting cDNA was diluted 1:10 (for eRNA real time PCR) or 1:100 (for mRNA real-time PCR) and analyzed using Fast SYBR Green Master Mix (Applied Biosystems). Primer sequences are listed in Table S1. The data were analyzed using the method described by Pfaffl (53).

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anti-rabbit IgG (LI-COR) diluted 1:20,000 in Odyssey buffer for 1 h at room temperature, and data were collected using the LI-COR Fc imager. The blot was then stripped with LI-COR 1× stripping buffer and reprobed with IGF-1 receptor β (Cell Signaling Technologies catalog no. 3018) diluted 1:1000 in 5% Blotto (Santa Cruz Biotechnologies, Dallas, TX) and detected with IRDye 680 anti-rabbit IgG (LI-COR). Separate blots were run with the same samples and probed with antibodies for phospho-Akt (Ser-473) antibody diluted 1:1000 in Odyssey blocking buffer (Cell Signaling Technologies, catalog no. 9721), stripped, and then reprobed with Akt antibody diluted 1:1000 in 5% Blotto (Cell Signaling Technologies, catalog no. 9272).

IGF1 ELISA

Serum or uterine homogenate IGF1 levels were determined using the ALPCO IGF-1 mouse/rat ELISA (ALPCO, Salem NH; catalog no. 22-IG1MS-E01). Serum was diluted 100-fold, and uterine homogenates were diluted 1:10 and assayed according to the protocol provided with the ELISA kit. Uterine homogenate results were normalized to total protein levels.

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