Epigenetic regulation of *Kiss1* gene expression mediating estrogen-positive feedback action in the mouse brain

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This study aims to determine the epigenetic mechanism regulating *Kiss1* gene expression in the anteroventral periventricular nucleus (AVPV) to understand the mechanism underlying estrogen-positive feedback action on gonadotropin-releasing hormone/gonadotropin surge. We investigated estrogen regulation of the epigenetic status of the mouse AVPV *Kiss1* gene locus in comparison with the arcuate nucleus (ARC), in which *Kiss1* expression is down-regulated by estrogen. Histone of AVPV *Kiss1* promoter region was highly acetylated, and estrogen receptor α was highly recruited at the region by estrogen. In contrast, the histone of ARC *Kiss1* promoter region was deacetylated by estrogen. Inhibition of histone deacetylation up-regulated in vitro *Kiss1* expression in a hypothalamic non–*Kiss1*-expressing cell line. Gene conformation analysis indicated that estrogen induced formation of a chromatin loop between *Kiss1* promoter and the 3′ intergenic region, suggesting that the intergenic region serves to enhance estrogen-dependent *Kiss1* expression in the AVPV. This notion was proved, because transgenic reporter mice with a complete *Kiss1* locus sequence showed kisspeptin neuron-specific GFP expression in both the AVPV and ARC, but the deletion of the 3′ region resulted in greatly reduced GFP expression only in the AVPV. Taken together, these results demonstrate that estrogen induces recruitment of estrogen receptor α and histone acetylation in the *Kiss1* promoter region of the AVPV and consequently enhances chromatin loop formation of *Kiss1* promoter and *Kiss1* gene enhancer, resulting in an increase in AVPV-specific *Kiss1* gene expression. These results indicate that epigenetic regulation of the *Kiss1* gene is involved in estrogen-positive feedback to generate the gonadotropin-releasing hormone/gonadotropin surge.

metastin | GPR54 | DNA methylation

The gonadotropin-releasing hormone (GnRH) surge is well established as the cause of the luteinizing hormone (LH) surge (1). The continuous release of GnRH might be caused by the continuous excitation of GnRH neurons triggered by the estrogen-positive feedback action on the brain. Recently, investigations of kisspeptin, a neuropeptide encoded by the *Kiss1* gene, shed light on the mechanism mediating GnRH/LH surges (2–7). One of the major kisspeptin neuronal populations in rodents is found in the anteroventral periventricular nucleus (AVPV) (8, 9), which has been suggested as the brain region involved in the surge generation in rodents (10). GnRH neurons express GPR54, a kisspeptin receptor (11). Preovulatory and estrogen-induced LH surges are blocked by the infusion of anti-kisspeptin antibody in rats (8, 12). Therefore, the estrogen-induced increase in *Kiss1* gene expression in the AVPV might be closely associated with the induction of GnRH/LH surges in rodents. Primates may have a surge-generating mechanism different from that in rodents. The mediobasal hypothalamus might play a crucial role in surge induction (13, 14).

Estrogen signals responsible for the positive feedback are mediated by estrogen receptor (ER) α, because the LH surge is evoked by exogenous estrogen in ERβ-KO mice but not in ERα-KO mice (15–17). Kisspeptin neurons in the AVPV express ERα, and estrogen positively regulates kisspeptin expression (8, 9, 18), suggesting that the AVPV kisspeptin neurons would be a target of the estrogen action exerting a positive feedback effect on GnRH/LH release. On the other hand, estrogen negatively regulates kisspeptin expression in another population of kisspeptin neurons that are located in the arcuate nucleus (ARC) and coexpress ERα (8, 19). The molecular mechanism involved in these opposite effects of estrogen on AVPV and ARC kisspeptin neurons should be clarified to understand the estrogen-positive feedback mechanism more clearly.

Epigenetic modification of genomic DNA and histones has been tightly linked to chromatin organization and transcriptional regulation. Histone acetylation in gene promoter/enhancer regions generally is correlated with transcriptional activation (20–22). On the other hand, in mammals, genomic DNA is methylated at cytosine residues predominantly in CG dinucleotides (CpGs) (23). Methylation of DNA is essential for mammalian development and is associated with gene silencing in conjunction with histone core modifications, probably through chromatin remodeling (20, 22, 24, 25). These epigenetic mechanisms reportedly mediate estrogen actions in the brain. For example, estrogen decreased levels of histone deacetylase (HDAC) protein and increased DNA methyltransferase expression in the dorsal hippocampus, leading to impaired formation of hippocampal-dependent memory (26). Furthermore, the histone acetylation status during the early postnatal period plays a critical role in sexual differentiation of the brain, because masculinization of sexual behavior and the volume of the nucleus of the bed nucleus of the stria terminalis, a sexual

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Data deposition: The sequence reported in this paper has been deposited in the DNA Data Bank of Japan, EMBL, and Genbank nucleotide database [accession no. AB666166 (*Kiss1* mRNA)].

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dimorphic nucleus, were blocked by neonatal administration of HDAC inhibitors (27, 28).

The present study aims to determine the epigenetic regulatory mechanism underlying the effect of estrogen-positive feedback on *Kiss1* gene expression in the AVPV. We first identified the *Kiss1* gene promoter functioning in the mouse hypothalamus. To investigate the effect of estrogen on epigenetic status in the *Kiss1* promoter, the histone acetylation and DNA methylation status of the *Kiss1* gene locus in the mouse AVPV were analyzed in comparison with that in the ARC. A chromatin immunoprecipitation (ChIP) assay with ERα antibody was used to investigate if ERα is recruited at the AVPV *Kiss1* promoter region in the presence of estrogen. Further, we investigated the effect of estrogen on the interaction between the *Kiss1* promoter region and intergenic DNA regions of the *Kiss1* locus by a gene conformational analysis to obtain the candidate(s) for the AVPV-specific *Kiss1* enhancer region. Finally, we generated transgenic (Tg) mice carrying DNA containing the GFP-labeled *Kiss1* gene locus with or without a candidate 3′ intergenic region to determine whether the *Kiss1* gene 3′ intergenic region, an enhancer candidate locus, functions as an estrogen-dependent AVPV-specific *Kiss1* enhancer.

Results

Determination of the Core Promoter Region Driven In Hypothalamic Cells. The AVPV and ARC express two splice variants that were identified in the present study (DNA Data Bank of Japan, EMBL, and Genbank nucleotide database, accession nos. AY707858 and AY182231) (Fig. 1 *A* and *B* and Fig. S1). Representative splice variants including the kisspeptin amino acid sequence within the ORF derived from University of California, Santa Cruz database (http://genome.ucsc.edu/) are shown in Fig. S1*A*. The RT-PCR analysis for AVPV and ARC tissues showed the same positive bands in both brain regions (Fig. 1*B*). *Kiss1* mRNA were highly expressed in the AVPV of the ovariectomized (OVX) mice implanted with estradiol-17β (E2) (the OVX+E2 model) and in the ARC of OVX mice without E2 implantation (the OVX model). The variants were the products of alternative splicing from the same transcription start site (TSS), but their functional relevance is unknown.

To determine the region for epigenetic analyses, the *Kiss1* promoter was analyzed with luciferase reporter assays using the N7 cell line, a mouse hypothalamic immortalized neuronal cell line. All the constructs containing the *Kiss1* region upstream of −5198 (pGL4-A), −2630 (pGL4-B), −1089 (pGL4-C), and −180 (pGL4-D) showed a significant increase in luciferase activity compared with controls (pGL4-mock) (Fig. 1*C*). The D sequence region had the strongest promoter activity, and a sequence with repressive effects was located in the upstream region of D. E2 treatment did not affect promoter activity, although ERα was expressed in the cells.

Induction of in Vitro *Kiss1* Gene Expression in Hypothalamic Cell Lines by Treatment with Reagents Affecting Epigenetic Status. Fig. 1*D* shows the effects of trichostatin A (TSA), an inhibitor of histone deacetylation, and/or 5-aza-2′-deoxycytidine (5-aza-dC), an inhibitor of DNA methylation, on *Kiss1* expression in N6 cells, a mouse hypothalamic cell line that does not express *Kiss1* mRNA (Fig. S2). TSA induced *Kiss1* expression in the cell line, but 5-aza-dC alone had no effect on *Kiss1* gene repression.

Estrogen-Induced Brain Region-Specific Alteration of the Histone Acetylation Status of the *Kiss1* Gene Locus. To determine the brain region-specific alteration of estrogen-induced change in the histone acetylation status at the *Kiss1* locus, ChIP assays with anti-acetylated histone H3 antibody were performed using three sets of primers across the *Kiss1* gene locus (Fig. 2*A*): a further region upstream of the *Kiss1* gene (5′ region, −17588 to −17242), a region upstream of the *Kiss1* promoter (−707 to −270), and an intergenic

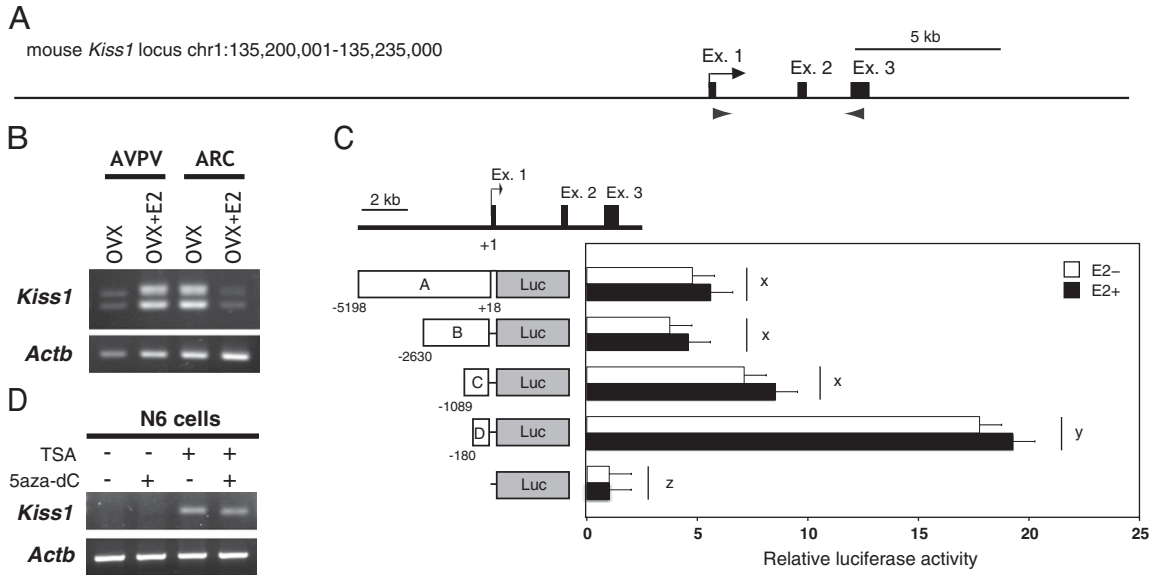

Fig. 1. Characterization of the *Kiss1* gene expressed in the mouse AVPV and ARC. (*A*) Schematic illustration of the genomic structure around the mouse *Kiss1* gene. Filled boxes and arrowheads show exons and primer regions, respectively, used in RT-PCR analyses. (*B*) Expression pattern of *Kiss1* mRNA. RT-PCR analysis of *Kiss1* expression in the AVPV and ARC is shown. RT-PCR analysis of AVPV and ARC tissues showed the same positive bands in both brain regions. (*C*) Identification of the core promoter region of *Kiss1* expression in the hypothalamus. (*Upper*) A map of the *Kiss1* locus. (*Lower*) Empty plasmid (pGL4-mock) or constructs carrying different lengths of the *Kiss1* 5′-flanking regions (labeled *A–D*) were transfected into immortalized hypothalamic N7 cells, together with pcDNA-ERα plasmids. Luciferase activities were determined relative to that of pGL4-mock. Open and filled bars indicate the absence and presence of E2 treatment, respectively. Each bar represents the mean of triplicate experiments ± SEM. x, y, z, significantly different from each other ($P < 0.01$). (*D*) Effects of inhibitors of histone deacetylation and/or DNA methylation on *Kiss1* expression in the immortalized hypothalamic cell line. RT-PCR analysis of *Kiss1* gene expression in N6 cells treated with 200 nM of TSA (an inhibitor of histone deacetylation) and/or 1 μM of 5-aza-dC (an inhibitor of DNA methylation). Total RNA (1 μg) extracted from cells was used for RT-PCR analyses.

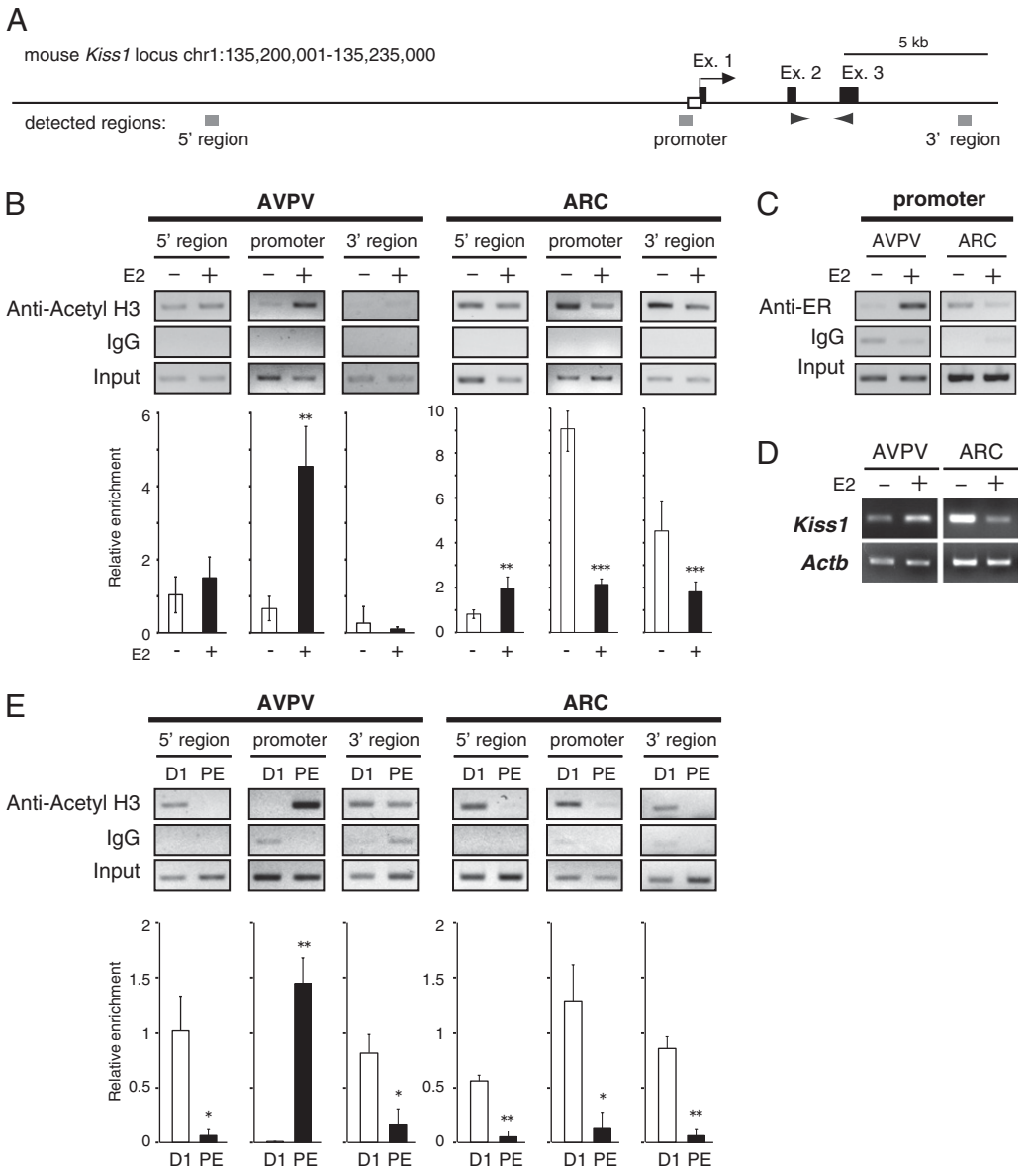

Fig. 2. Chromatin modification status of the *Kiss1* locus. (*A*) Schematic diagram of the *Kiss1* gene locus (exons 1–3 indicated by black squares; promoter region indicated by open square) and location of three regions for ChIP assay (5′, promoter, and 3′ regions indicated by gray squares). Arrowheads show primer regions used in RT-PCR analysis in Fig. 2*D*. (*B*) Histone acetylation status of the *Kiss1* locus in the AVPV and ARC nuclei before and after E2 replacement. (*Upper*) Punched-out brain tissues from OVX ($E2^-$) and estrogen-treated OVX ($E2^+$) mice were subjected to ChIP assays with antibody against acetylated histone H3 (Acetyl H3). Normal mouse IgG was used as a negative control for the specificity of immunoprecipitation. An amount of chromatin fragments equal to that used for each immunoprecipitation also was subjected to PCR without immunoprecipitation as a positive control (input). (*Lower*) Semiquantitative analysis of the relative levels of histone acetylation was performed. The intensity of each band in the gel images was measured using the ImageJ program and normalized by input. Each bar represents the mean ± SEM of two independent experiments performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.005$. (*C*) ChIP analysis of ERα binding with the *Kiss1* gene locus. Brain tissues punched out from OVX and OVX+E2 mice were subjected to ChIP assays with antibody against ERα. PCR products were analyzed by agarose gel electrophoresis. (*D*) Expression pattern of the *Kiss1* gene in the AVPV and ARC following E2 replacement. RT-PCR analysis using AVPV and ARC tissues taken from OVX and OVX+E2 mice. (*E*) Histone acetylation status of the *Kiss1* locus in the AVPV and ARC nuclei at diestrous and proestrous stages. (*Upper*) Punched-out brain tissues from diestrous and proestrous mice were subjected to ChIP assays with antibody of acetyl H3. (*Lower*) Semiquantitative analysis of relative levels of histone acetylation was performed. The intensity of each band in gel images was measured using the ImageJ program and normalized by input. Each bar represents the mean ± SEM performed in triplicate. D1, diestrus-1 phase; PE, proestrus. $^{*}P < 0.05$, $^{**}P < 0.01$.

region downstream of the *Kiss1* gene (3′ region, +9238 to +9670). Estrogen treatment significantly increased H3 acetylation at the *Kiss1* promoter region in the AVPV (Fig. 2*B*), but significantly decreased H3 acetylation of the same promoter region in the ARC. Estrogen treatment also significantly increased and decreased ARC histone acetylation at 5′ and 3′ regions, respectively. ChIP assays using anti-ERα antibody further showed that estrogen treatment increased ERα binding within the *Kiss1* promoter in the AVPV but not in the ARC (Fig. 2*C*). *Kiss1* expression was up-regulated by estrogen in the AVPV and was down-regulated by estrogen in the ARC (Fig. 2*D*). Furthermore, the histone acetylation status within the AVPV *Kiss1* promoter was significantly higher during proestrus than during diestrus (Fig. 2*E*), whereas histones of the ARC *Kiss1* promoter were significantly higher

during diestrus than during proestrus. Histones of the 5′ and 3′ regions of both the AVPV and ARC were more highly acetylated during diestrus than during proestrus (Fig. 2*E*).

DNA Methylation Status of *Kiss1* Core Promoter in Vivo and in Vitro. *Kiss1* neurons visualized by in situ hybridization were isolated from the AVPV and ARC sections by microdissection (Fig. 3*A*). Bisulfite sequencing analysis of the *Kiss1* upstream region harboring strong promoter activity (−180 to +29) revealed that the CpGs upstream of the TSS (Fig. 3*B*) appeared hypermethylated in all samples examined (Fig. 3*C*). No apparent difference was found between *Kiss1*-expressing cells (AVPV, ARC, N7, and differentiated trophoblast stem cells) and non–*Kiss*-expressing cells (cortex and N6 cells) (Fig. S2).

Effects of Estrogen on Chromatin Loop Formation in AVPV and ARC *Kiss1* Loci. We analyzed the effects of estrogen on chromatin conformation of the *Kiss1* locus in the AVPV and ARC by a chromatin conformation capture (3C) assay using 3C primers as indicated in Fig. 4*A*. PCR for loading control showed no difference in the amount of samples among groups (Fig. 4*B*). The 3C assay with AVPV tissue showed that estrogen treatment increased 3′ PCR products with H2F–H4F primers, but no band was detected without estrogen treatment (Fig. 4*C*). These results indicate that estrogen enhances the formation of the chromatin loop between the promoter and the 3′ intergenic region. The assay also showed an estrogen-dependent decrease in 5′ PCR products (H2F–H1F) in the AVPV, indicating the presence of another association between the promoter region and the 5′ region at the *Kiss1* locus in the AVPV.

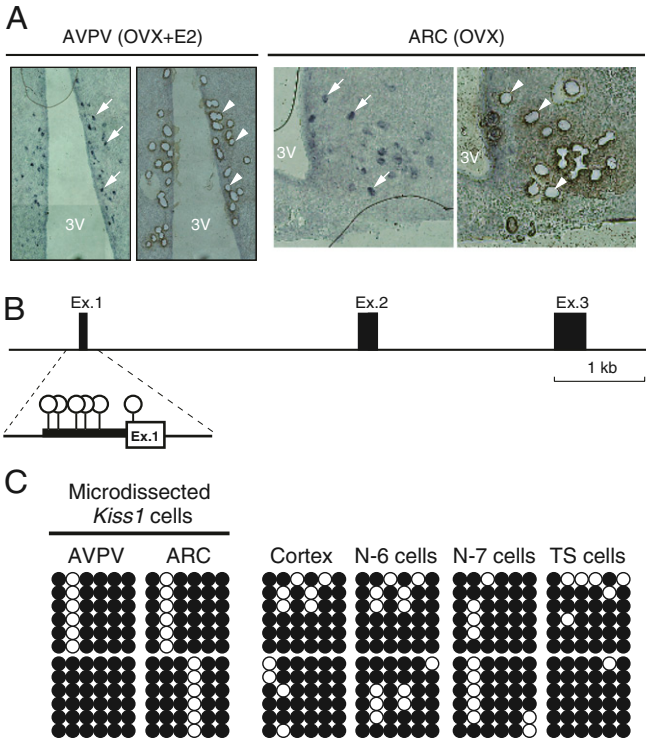

Fig. 3. DNA methylation status of the *Kiss1* promoter region in *Kiss1*-expressing or -nonexpressing cells. (*A*) Images of sections including AVPV or ARC nuclei before (*Left* in AVPV and ARC) and after (*Right* in AVPV and ARC) laser microdissection. *Kiss1* neurons were visualized by in situ hybridization and were isolated based on in situ hybridization signals (indicated by arrows in left panels and by arrowheads in right panels). *Kiss1*-positive cells were collected from the AVPV in OVX+E2 mice and from the ARC in OVX mice. 3V, third ventricle. (*B*) Six CpG sites in the *Kiss1* core promoter region analyzed by bisulfite sequencing. Open circles indicate CpG sites. (*C*) DNA methylation status of individual CpGs at the *Kiss1* core promoter region in each sample. Bisulfite sequencing analysis was performed with DNA extracted from each sample. Each row of circles represents a single clone randomly picked from each of two independent PCRs. Open and filled circles represent unmethylated and methylated cytosines, respectively. TS cells, trophoblast stem cells.

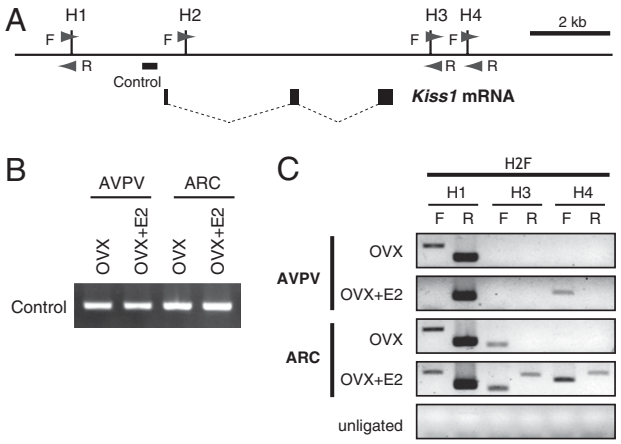

Fig. 4. Chromatin conformational change at the *Kiss1* gene locus in the AVPV and ARC. (*A*) Diagram of the *Kiss1* locus; filled boxes indicate exons, and thin dotted lines indicate introns. HindIII restriction endonuclease sites are indicated by vertical lines (labeled by H1–H4). Arrowheads show positions of primers used in 3C assays. The thick horizontal bar indicates the region used in loading control PCR. (*B*) PCR for loading control. OVX mice with (OVX+E2) or without (OVX) E2 treatment were subjected to AVPV and ARC tissue sampling. (*C*) 3C analysis of the *Kiss1* locus obtained from AVPV and ARC tissues in the absence and presence of E2 stimulation. 3C assays were performed, and PCR products were analyzed by agarose gel electrophoresis. PCR products were generated using primer H2 forward (H2F) in combination with one of the other HindIII primers (H1–H4) as indicated.

The 3C assay of the ARC showed a pattern of loop formation within *Kiss1* locus different from that seen in the AVPV. In the ARC, the 5′ region (H2F–H1F) and 3′ proximal region (H2F–H3F) were associated with a promoter region regardless of E2 treatment. Furthermore, an extensive range of the 3′ region (H2F–H3R, H2F–H4F, and H2F–H4R) was associated with the promoter region after E2 treatment (Fig. 4*C*).

In Vivo Reporter Assay to Confirm the Candidate Estrogen-Dependent AVPV-Specific Enhancer Region in Tg Mice. Two lines of Tg mice were generated using DNA constructs, the 22-kb upstream and 10- kb downstream sequence from the *Kiss1* gene TSS (Tg-1) and the same sequence without the 3′ region of the third exon (Tg-2) (Fig. 5*A*). Dual-labeling immunohistochemistry revealed that GFP-positive neurons are located in two predicted nuclei, the AVPV and ARC. Photomicrographs show kisspeptin and GFP immunoreactivity in the AVPV (Fig. 5*B*) and ARC (Fig. 5*C*) of representative Tg-1 and Tg-2 mice. Fig. 6 shows the integrated density of fluorescence values for kisspeptin and GFP determined by Image J software in each nucleus. Tg-1 mice showed E2-enhanced GFP immunoreactivity in the AVPV (Fig. 6), most of which overlapped with kisspeptin immunoreactivity (Fig. 5*B* and Fig. S3). GFP immunoreactivity was greatly reduced, even with E2 treatment, in Tg-2 mice (which lacked the 3′ region) as compared with Tg-1 mice (Figs. 5*B* and 6 and Fig. S3). In the ARC, E2 reduced kisspeptin expression but did not cause an obvious reduction in GFP expression (Figs. 5*B* and 6 and Fig. S3).

Discussion

This study demonstrates that epigenetic regulation of *Kiss1* is involved in the up-regulation of *Kiss1* expression in the AVPV in response to estrogen. Histone H3 acetylation in the *Kiss1* pro-

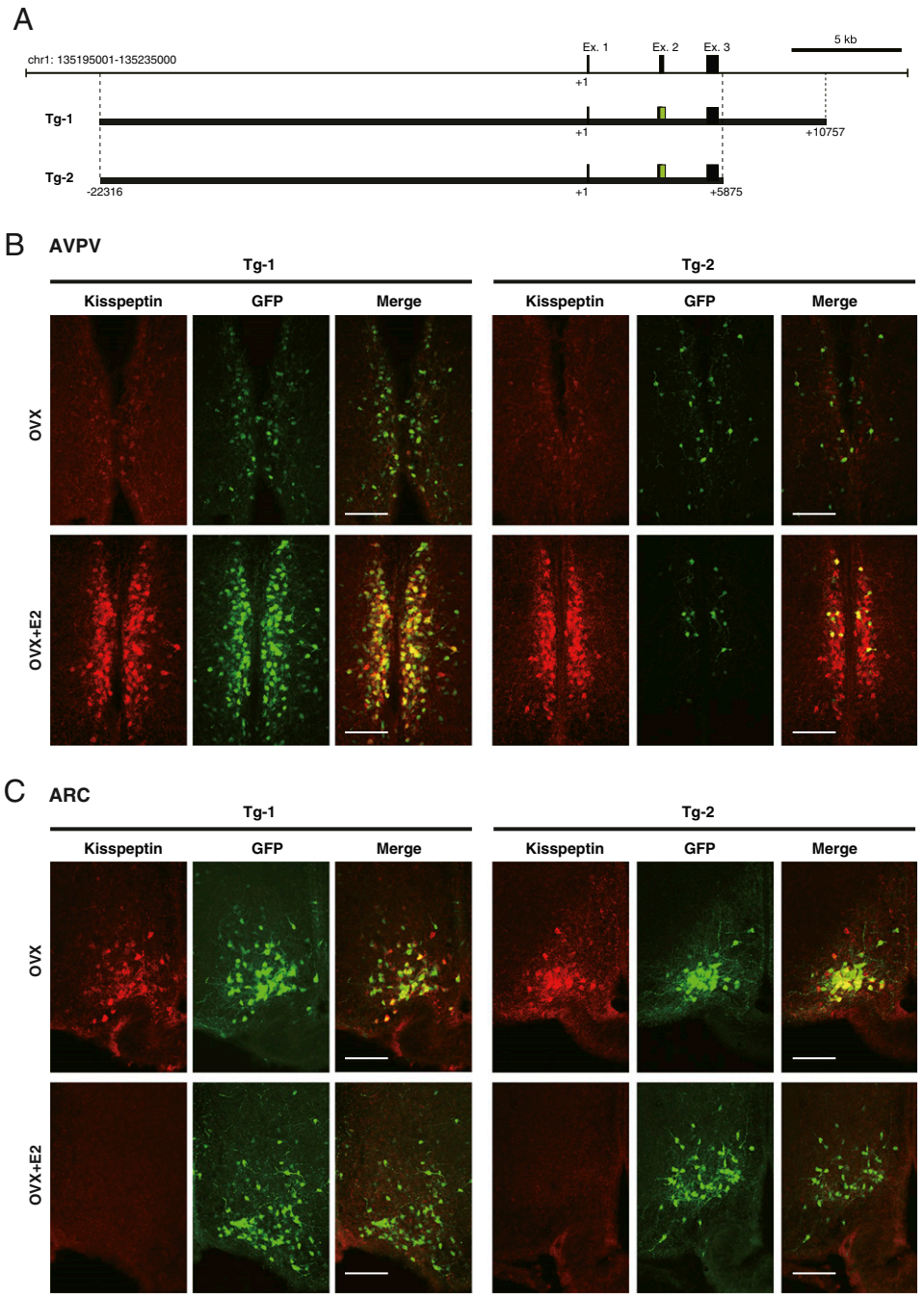

Fig. 5. In vivo reporter assay using constructs including the *Kiss1* gene locus. (*A*) Schematic illustration of constructs for the generation of Tg mice. Green boxes indicate the inserted AcGFP sequence. (*B* and *C*) Kisspeptin and GFP expression in the AVPV and ARC of OVX and OVX+E2 Tg mice. Photomicrographs show sections of the AVPV and ARC stained by immunohistochemistry for kisspeptin (*Left*) and GFP (*Center*). Computer-aided merged images of immunoreactive signals for kisspeptin and GFP are shown on the right. (Scale bars, 100 μm.)

moter region in the AVPV, which is increased by estrogen, is closely associated with an increase in *Kiss1* expression in the AVPV. Indeed, histone H3 acetylation in the *Kiss1* promoter region of the AVPV was much higher in proestrous animals than in diestrous animals. These results suggest that estrogen in the AVPV causes the *Kiss1* promoter region to switch from an inactive to an active, open chromatin structure. In support of this idea, in vitro *Kiss1* expression in hypothalamic cell lines was induced by TSA, an inhibitor of histone deacetylation, indicating that histone acetylation is involved in *Kiss1* expression. Importantly, the present study demonstrates that the 3′ intergenic region of *Kiss1* is essential for activation of the estrogen-induced *Kiss1* promoter in the AVPV. This requirement is demonstrated clearly by the present in vivo reporter assay. Tg-1 mice, with the complete sequence of *Kiss1* locus, showed GFP expression in kisspeptin neurons in both the AVPV and ARC, but the deletion of the 3′ region in Tg-2 mice greatly reduced estrogen-induced GFP expression in the AVPV, indicating that the 3′ region of the *Kiss1* gene functions as an estrogen-responsive enhancer. The 3C assay showed interaction between the promoter and 3′ region of *Kiss1* via a chromatin loop in the AVPV in the presence of estrogen, indicating that the 3′ region enhances the induction of

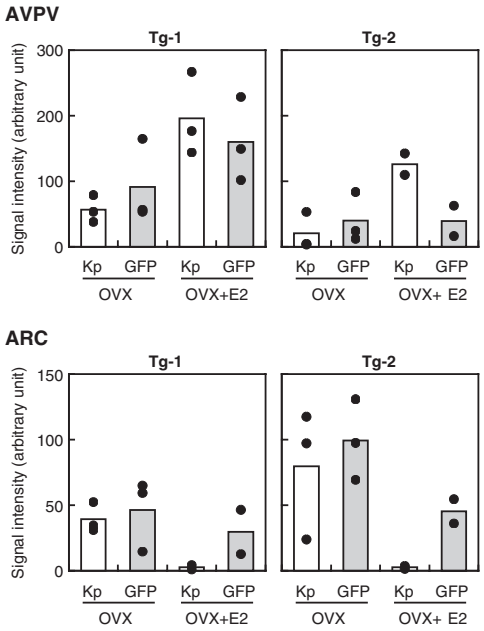

Fig. 6. Expressions of kisspeptin (Kp) and GFP in the AVPV and ARC of Tg-1 and Tg-2 mice with or without E2 replacement. Integrated density of fluorescence in each microscopic field was determined by Image J software. Each bar represents the mean value, and solid circles indicate individual values.

estrogen-dependent AVPV *Kiss1* promoter activity. Taken together, these results demonstrate that estrogen induces histone acetylation in the region of the *Kiss1* promoter in the AVPV and consequently enhances the formation of a chromatin loop in the *Kiss1* promoter, resulting in an increase in the estrogen-dependent, AVPV-specific expression of *Kiss1*. Functional in vivo data would be ultimately needed to completely demonstrate the role of histone acetylation in generating GnRH/LH surges.

The present study shows that, in the presence of estrogen, ERα is highly recruited at the region of the *Kiss1* promoter in the AVPV (Fig. 2*C*), suggesting that the estrogen–ERα complex recruited to the region may be responsible for the histone acetylation at the *Kiss1* promoter region and the subsequent expression of *Kiss1* in the AVPV. Indeed, a potential estrogen-responsive element half-site was located within the predicted promoter region. The present finding is consistent with previous studies showing that ERα has a critical role in transcriptional activation of *Kiss1* in the AVPV (8, 17, 18, 29). Furthermore, previous studies using knockin mice expressing a mutant form of ERα lacking a functional estrogen-responsive element-binding domain also suggested that estrogen-positive feedback is mediated via the classical estrogen receptor pathway, whereas estrogen-negative feedback is mediated via the nonclassical pathway (30, 31). An increase in estrogen-induced ERα binding in the *Kiss1* promoter region in the AVPV might recruit some histone acetyl-transferases (HATs) and other transcription regulators within the *Kiss1* promoter in the AVPV. Indeed, the previous study using the human *pS2* promoter as a model indicates that liganded ERα induced transcriptional activation of *pS2* involving an orchestrated recruitment of components of basal transcriptional machinery and intermediate factors, such as HATs and the ATP-dependent chromatin remodeling complex, concomitant with an alteration in local chromatin structure (32). In vitro reporter analysis showed that E2 treatment failed to enhance *Kiss1* promoter activity even in the presence of ERα (Fig. 1*C*). This result is consistent with the 3C and Tg analyses indicating that the 3′ region is a possible enhancer region.

An interesting issue in the mechanism of *Kiss1* regulation in the hypothalamus is the opposite responses to circulating estrogen in kisspeptin neurons in the ARC and AVPV (8, 33). Here, we show that, in the AVPV, estrogen increased the histone H3 acetylation level in the *Kiss1* promoter as correlated with the estrogen-dependent transcriptional activation of *Kiss1*. In contrast, estrogen decreased the histone acetylation level in the ARC, where estrogen negatively regulates *Kiss1* expression. Indeed, the acetylation level of the *Kiss1* promoter in the AVPV was higher during proestrus than during diestrous, whereas the level in the ARC was higher during diestrus than during proestrus (Fig. 2*E*). Unlike the AVPV, the histone H3 acetylation level in the ARC also was affected by estrogen in the 5′ and 3′ regions of *Kiss1* (Fig. 2 *B* and *E*). It is possible that estrogen induces a broad range of changes in the *Kiss1* locus in the ARC. We detected formation of an ARC-specific chromatin loop within the 3′ region of the *Kiss1* locus after E2 treatment, suggesting that the 3′ region is involved in the suppression of *Kiss1* expression in the ARC. Therefore, we speculate that the transcriptional cofactors that control *Kiss1* gene expression could be different in the AVPV and ARC. Interestingly, we detected ERα binding to the *Kiss1* promoter region taken from the ARC of OVX mice without E2 treatment (Fig. 2*C*), suggesting that unliganded ERα may have a role in *Kiss1* regulation, as previously reported (34–36). The ligand-independent ERα activity was increased by phosphorylation at specific serine residues in the N-terminal domain as activated by the growth factor pathway (37, 38). Furthermore, ERα is known to interact with other transcription factors, such as Sp1 and AP-1, and it may use the constitutive transcriptional function of these factors (39, 40). Thus, unliganded ERα may play a role in regulating basal *Kiss1* transcription in the ARC. Estradiol reduced kisspeptin expression in the ARC but did not cause an obvious reduction of GFP expression in the ARC (Figs. 5C and 6 and Fig. S3). The long half-life of conventional GFP may prevent the repression of reporter expression. Another possibility is that the *cis* element regulating the action of estrogen-negative feedback in the *Kiss1* expression in the ARC might not be included within the transgene. The precise mechanism involved in the down-regulation of *Kiss1* in the ARC by estrogen should be clarified in the future.

In the present study, we put more emphasis on the regulation of the *Kiss1* gene by histone acetylation than by DNA methylation. In vitro *Kiss1* expression was not affected by 5-aza-dC, an inhibitor of DNA methylation, in N6 hypothalamic cell lines. The *Kiss1* promoter region was hypermethylated even in the *Kiss1*-expressing cells microdissected from both AVPV and ARC regions. However, we could not exclude the possibility that DNA methylation is involved in *Kiss1* gene regulation. Our DNA methylation analysis did not cover the entire region of the *Kiss1* locus, and transient cyclical change of DNA methylation status in the *pS2* promoter in MCF-7 cells has been reported (41, 42). Further studies will be needed to elucidate the involvement of DNA methylation in regulating the *Kiss1* gene.

In conclusion, the present study demonstrates an epigenetic mechanism underlying the estrogen regulation of AVPV *Kiss1* expression to mediate estrogen positive feedback action to induce GnRH/LH surges. Our results suggest that estrogen stimulates ERα recruitment on the *Kiss1* promoter region to induce histone H3 acetylation and the formation of a chromatin loop between the *Kiss1* promoter and the 3′ enhancer region, leading to *Kiss1* up-regulation in the AVPV. This notion suggests that epigenetic regulation of *Kiss1* is involved in the estrogen-positive feedback generating the GnRH/gonadotropin surge.

Materials and Methods

Animals and tissue preparation, cell culture, RNA analyses, transient transfection, luciferase assays, and immunohistochemistry, including validity of

the current E2 treatment (Fig. S4) and specificity of anti-kisspeptin antibody (Fig. S5), are described in detail in *SI Materials and Methods*.

Microdissection of *Kiss1*-Expressing Cells from Hypothalamus. To dissect out *Kiss1*-positive cells, *Kiss1* mRNA was visualized by in situ hybridization in coronal sections (20-μm thickness) of the hypothalamus taken from OVX or OVX+E2 mice as previously described (8, 43). Briefly, the hypothalamic tissues were sectioned on a cryostat. Digoxigenin (DIG)-labeled antisense and sense cRNA probes for mouse *Kiss1* were synthesized by in vitro transcription from the cDNA clones. Hybridization with DIG-labeled cRNA probes was carried out at 60 °C overnight, and hybridized probes were detected using an alkaline phosphatase-conjugated anti-DIG Fab fragment (Roche Diagnostics) and 5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium chloride (Roche Diagnostics). Signal-positive cells were taken from the sections with the PALM MicroBeam System (Carl Zeiss Microimaging) according to the manufacturer's instructions. About 100 cells for each nucleus were pooled and stored at −80 °C until used for DNA extraction.

Sodium Bisulfite Sequencing. The conditions for using the bisulfite reaction to determine DNA methylation status have been described previously (22). The DNA fragment covering the 5′-flanking sequence of the *Kiss1* gene was amplified by PCR using the forward primer GGGTATTGAGGAGTTTTTGGGTTAGATTGT and the reverse primer ACCTACTTCTCCAAACCCTCCCTAAATCAA. The PCR products were cloned into pCR2.1 TOPO (Invitrogen), and six clones randomly picked from each of two independent PCRs were sequenced using the BigDye Terminator v.3 System (Applied Biosystems) and an ABI3100 sequencer (Applied Biosystems).

ChIP Assay. The ChIP assay was performed with 10 mg of punched-out tissue per assay using the ChIP Assay Kit (Millipore) according to the manufacturer's instructions. Fixed tissues were homogenized, lysed, and sonicated until chromatin fragments became 200–1,000 bp in size. Antibody against acetylated histone H3 (Millipore) or ERα (Millipore) was used for immunoprecipitation. Mouse nonimmunized IgG (Millipore) was used as a negative control to check the specificity of immunoprecipitation. After immunoprecipitation, recovered chromatin samples were subjected to PCR with the primers TACAGCACCAGGAAAGTTGAGA and GAAGAATCCGAGACTGCAGAAC for 5′ region, promoter CAACCACCCAGGAGGTAGAA and GAAGAGAAAGTGGCTGAGCAG for the promoter region, and GAGCTAGTGTACCCGCTTCTGT and GAAGTGACTCAAAGGTCCTGCT for the 3′ region. The PCR products were run on agarose gel, and the intensity of each band was measured using ImageJ software v. 1.40G (National Institutes of Health). Results from two independent experiments performed in triplicate were quantified and averaged.

3C Assay. The 3C assay was conducted as described by Dekker et al. (44) with some modification. Crosslinked chromatin was digested with 500 U of HindIII (Roche) overnight at 37 °C, and was ligated in 6 mL of 1× ligation buffer. The 3C products were extracted by phenol/chloroform, precipitated by ethanol, and dissolved in Tris-EDTA buffer. The *Kiss1* locus contains four HindIII sites; the primers flanking HindIII sites were designated as H1F (GCAGCTGGTGACATCAAGAA), H1R (CACCGACAGTCCAAGTTCAA), H2F (CAGGGCTTATCTGAGCCTTTC), H3F (GAGACTTCCCTTCTTTCCTGGT), H3R (GTTCGGGATGATTACAAAGAGC), H4F (AACATGTTTGGGCAGTAGTGTG), and H4R (AACTAGGGATGCACTTGGTTG). The *Kiss1* chromatin loop was detected using primer H2F in combination with one of the other primers. A region serving as a loading control was amplified with GTTGTTTGGGGTGGAATGAGTC and TGGCTCCTGGGCTTACTCTA. Each PCR was performed under the following conditions: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min; final extension 72 °C for 10 min. PCR products were analyzed by agarose gel electrophoresis.

Generation of Transgenic Mice. To generate transgenic mice, the two types of DNA construction shown in Fig. 5*A* were subcloned from BAC clone RP24-299J2 (BACPAC Resources). All final constructs were verified by sequencing. The linearized DNA fragments were purified by precipitation with ethanol and injected into fertilized eggs of the B6D2F1 mouse strain. Manipulated eggs were transplanted into foster mothers. We generated two types of Tg mice containing the *Kiss1* locus sequence, Tg-1 and Tg-2. Tg-1 includes the sequences 22-kb upstream and 10-kb downstream from the *Kiss1* TSS. Tg-2 also has a 22-kb upstream sequence but lacks the downstream sequence of the third exon. Both Tg mice contained the AcGFP sequence within the second exon of the *Kiss1* gene. Mouse ear DNA was screened by PCR to check the presence of the transgenes using primers for the DNA sequence of AcGFP, AAGTTCATCTGCACCACCG and CCTGGGTATCTCTCAAGTGCAGAAA. F1 and F2 mice were identified by PCR analysis.

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