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Title: Mutual interaction of kisspeptin, estrogen and bone morphogenetic protein-4 activity in GnRH regulation by GT1-7 cells.

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Abstract: Reproduction is integrated by interaction of neural and hormonal signals converging on hypothalamic neurons for controlling gonadotropin-releasing hormone (GnRH). Kisspeptin, the peptide product of the *kiss1* gene and the endogenous agonist for the GPR54 receptor, plays a key role in the regulation of GnRH secretion. In the present study, we investigated the interaction between kisspeptin, estrogen and BMPs in the regulation of GnRH production by using mouse hypothalamic GT1-7 cells. Treatment with kisspeptin increased GnRH mRNA expression and GnRH protein production in a concentration-dependent manner. The expression levels of *kiss1* and GPR54 were not changed by kisspeptin stimulation. Kisspeptin induction of GnRH was suppressed by co-treatment with BMPs, with BMP-4 action being the most potent for suppressing the kisspeptin effect. The expression of kisspeptin receptor, GPR54, was suppressed by BMPs, and this effect was reversed in the presence of kisspeptin. It was also revealed that BMP-induced Smad1/5/8 phosphorylation and Id-1 expression were suppressed and inhibitory Smad6/7 was induced by kisspeptin. In addition, estrogen induced GPR54 expression, while kisspeptin increased the expression levels of ER $\alpha$  and ER $\beta$ , suggesting that the actions of estrogen and kisspeptin are mutually enhanced in GT1-7 cells. Moreover, kisspeptin stimulated MAPKs and AKT signaling, and ERK signaling was functionally involved in the kisspeptin-induced GnRH expression. BMP-4 was found to suppress kisspeptin-induced GnRH expression by reducing ERK signaling activity. Collectively, the results indicate that the axis of kisspeptin-induced GnRH production is bi-directionally controlled, being augmented by an interaction between ER $\alpha/\beta$  and GPR54 signaling and suppressed by BMP-4 action in GT1-7 neuron cells.

**Dear Editor-in-Chief:**  
**Raymond J Rogers**  
*Mol Cell Endocrinol*

**Ms. Ref. No.: MCE-D-13-00008-R1**

**Title: “Mutual interaction of kisspeptin, estrogen and bone morphogenetic protein-4 activity in GnRH regulation by GT1-7 cells.” by Terasaka *et al.***

We would like to thank the referees for their careful review of the manuscript and constructive comments. In accordance with the reviewers' recommendations, we have made significant modifications to the manuscript including addition of new data. Our manuscript was thoroughly revised by modifying each section on the basis of the reviewers' useful comments and critiques (changes indicated by underlines).

We would also like to thank you for allowing us to resubmit our revised manuscript. We hope that the revised manuscript is now acceptable for publication in MCE. Our responses to the referees' specific comments are provided in the attachment file.

Sincerely yours,

Fumio Otsuka, M.D., Ph.D.

## Response to Reviewer #1:

### Reviewers' comments:

Reviewer #1: The paper by Terasaka et al. examines the mutual interaction of kisspeptin, estrogen and BMP-4 in the regulation of GnRH production in GT1-7 cells.

The paper is well written and the results are sound. My major area of concern is with regards to the interpretation of results. These concerns are outlined below.

### ... Answer:

We appreciate the reviewer's comments on our manuscript. The comments provided by the reviewer were very important to further discuss and reconsider our present data. According to the reviewer's comments and suggestions, we added new data and thoroughly revised the manuscript. Please refer to our revised manuscript.

1) What is the physiological relevance of GT1-7 cells? The authors state that these cells express Kiss1, GPR54, ER $\alpha$ , ER $\beta$  and GnRH mRNA making them unlike any neuronal population in an *in vivo* setting. GnRH neurons do not express Kiss1 and do not express ER $\alpha$ . Thus, one must be extremely cautious when making conclusions using this cellular model. The authors state "The expression of kiss1, GPR54 and ERs, including ER $\alpha$  and ER $\beta$ , in GT1-7 cells implied that the functional kisspeptin-ER system exists as an autocrine mechanism for GnRH secretion" Such a statement (while true in regards to the data) can be wildly misleading as it could imply to GnRH secretion in animals. The authors should carefully revise their document to account for this.

### ... Answer 1)

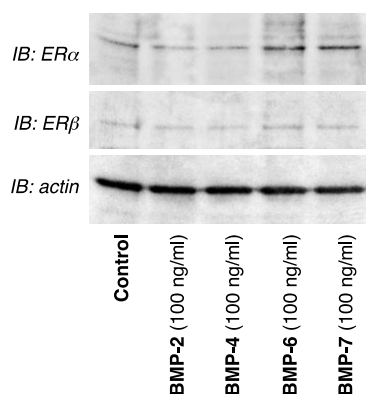
We agree. The physiological relevance of utilizing GT1-7 cells is critical for this study. At present, this cell model is the closest physiologically to GnRH neurons *in vivo*, although, as with any cell line, its use and conclusions drawn from it must be considered with caution. Estrogens influence the GnRH neuronal network to modulate the final output of GnRH into the median eminence in females. To know the detailed mechanism by which estrogen exerts positive and negative feedback effects, we utilized the GT1-7 cell line. Immortalized GnRH-producing GT1-7 cells are valuable for studying the molecular biology of GnRH neurons. Both GnRH neurons and GT1-7 cells express GPR54, ER $\beta$  and the estrogen membrane receptor GPR30. The cells differ from confirmed observations *in vivo* in the expression of ER $\alpha$  and kisspeptin. We focused on the action of GPR54 and report the interaction with BMPs, which are also expressed in the hypothalamus. Although we also examined the action of estrogen, it is not clear if its action is mediated by GPR30, ER $\alpha$  or ER $\beta$ . As the referee commented, our findings may not fully translate to an *in vivo* model, but they do formulate hypotheses that can eventually be tested. The inability to detect ER $\alpha$  in native GnRH neurons has been well documented (Herbison and Pape, 2001; Herbison, 2008), whereas kiss1 neurons predominantly express ER $\alpha$  with a modest fraction expressing ER $\beta$  (Smith et al., 2005a; Smith et al., 2005b), suggesting

that kiss1 neurons mediate estrogen actions for GnRH secretion (Pinilla et al., 2012). To avoid confusion regarding the data for GT1-7 cells in terms of the physiology of GnRH regulation, we amended the discussion including the sentence stating "The expression of kiss1, GPR54 and ERs, including ER $\alpha$  and ER $\beta$ , in GT1-7 cells implied that the functional kisspeptin-ER system exists as an autocrine mechanism for GnRH secretion." In the present study, we also focused on interaction of the BMP system and ER action that is linked to hypothalamic GnRH production. Regarding this interaction between BMP and ER, we carefully interpreted the data obtained from the cell line of GT1-7 in the discussion and focus on GPR54 and BMP receptor action on GnRH gene expression and secretion, not on kisspeptin expression. The point the referee suggested here is very important for readers. We thoroughly amended the discussion in the revised manuscript. We appreciate the referee's comments.

2) The authors show an effect of estradiol on GT1-7 cells. In the paper no attempt is made to explain which of these receptors (or both) is mediating the estrogen effect. This is again important, as GnRH neurons do not possess ER $\alpha$ . In the discussion (and Figure 5) the authors simply state regulation by ER, without discriminating which receptor. This needs to be reconciled.

... Answer 2)

We agree. GT1-7 neuron cells express both ER $\alpha$  and ER $\beta$  (as shown in the lower panel (Otani et al., 2009)), unlike native GnRH neurons, which express only ER $\beta$ . Based on our results including results of our previous study using GT1-7 cells, we consider that both ER $\alpha$  and ER $\beta$  are involved in the process of GnRH regulation via a combination of kisspeptin, BMP-4 and estrogen. It is actually difficult to compare the absolute expression levels of both ERs, however, based on Western blots, we speculate that ER $\alpha$  expression is more abundant than ER $\beta$  in GT1-7 cells. We revised the discussion and Fig. 5 accordingly. Thank you for your comment.



GT1-7 cells ( $1 \times 10^5$  cells/ml) were treated with BMP-2, -4, -6 and -7 (100 ng/ml) in serum-free condition. After 24-h culture, cells were lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-ER $\alpha$ , anti-ER $\beta$  and anti-actin antibodies. The results shown are representative of those obtained from three independent experiments. (Otani and Otsuka et al. J Endocrinol 203: 87, 2009)

3) Is BMP expressed in neurons in vivo? Is it expressed in GnRH neurons? Kisspeptin neurons? Such information would strengthen the relevance of the data.

... Answer 3)

We agree. *In vivo* information regarding BMP expression is very important. The interaction of neural and hormonal signals including BMPs and kisspeptin may integrate the neuronal activity for GnRH secretion. However, information regarding BMPs in the hypothalamus is limited. The neuro-regulatory factors TGF- $\beta$ 1 and TGF- $\alpha$  secreted by astrocytes may be candidates for modulation of GnRH secretion. Given the findings that several BMPs elicit a neurotropic capacity for dopaminergic neurons in cooperation with other members of the TGF- $\beta$  superfamily through glial cells, hypothalamic GnRH production and/or secretion may be functionally associated with BMP activity in the neural network. However, in order to clarify expression of the BMP system including BMP ligands and receptors in the hypothalamus, we additionally examined the mRNA expression of BMP system components by RT-PCR. The key BMP ligands (BMP-2, -4, -6 and -7) and receptor components (ALK-2, -3, -4, -6, BMPRII, ActRIIA and IIB) were clearly expressed in hypothalamic tissues. We added these data in new Fig. 1B in the revised manuscript. Thank you for your important comments.

4) The dose of kisspeptin used to elicit a GnRH response seem excessively high compared to other published manuscripts. Here the authors only saw effects at 300 nM, 1  $\mu$ M and higher. In the referenced paper by Novaira et al (2009) greater stimulation was seen with only 1 nM. This leads to concerns over the viability of cells or the kisspeptin used. Can the authors comment?

... Answer 4)

Certainly, the GnRH responsiveness (detected by RIA and qPCR) by kisspeptin was weaker than that reported by Novaira et al. (Novaira et al., 2009). Based on Navaira's report, the response of GnRH production by GT1-7 cells was quickly but only transiently shown after 1-h to 4-h incubation with 1 nM kisspeptin. In their study using GT1-7 cells, GnRH protein levels were increased approximately 2-fold compared with the vehicle control. In a study by Tonsfelt et al., 10 nM of kisspeptin was used for GT1-7 cells, resulting in a similar increase in the level of GnRH secretion by 2-3 fold for 30-h to 48-h of treatment (Tonsfeldt et al., 2011). In the present study, GnRH response was modest and increased up to 1.5 to 2 fold after 24-h culture with 300-1000 nM of kisspeptin. This effect was certainly weak but not too weak compared with those observed in the previous studies. This could be due to time-course (24 h) and serum-starvation (serum-free) conditions in our study. Much earlier time points and serum inclusion in the medium might be suitable to see higher responsiveness of GnRH in response to exogenous kisspeptin. However, in the present study, we focused on interaction of the BMP system and ER action that is linked to hypothalamic GnRH production. To clarify the interaction between BMP and a genomic effect of ER, it usually takes 24 to 48 h and serum contents in the medium often disturb the effect of BMPs as well as steroids. Considering these points, we decided to use a 24-hour culture condition with higher concentrations of kisspeptin and the data obtained were stable and reproducible. We added discussion regarding the

differences in our treatment regime with that of others to assist the reader in interpretation of our data. We appreciate your important comments.

#### References:

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- Tonsfeldt, K.J., et al., 2011. Oestrogen induces rhythmic expression of the Kisspeptin-1 receptor GPR54 in hypothalamic gonadotrophin-releasing hormone-secreting GT1-7 cells. *J Neuroendocrinol.* 23, 823-30.

Thank you for your review.

## **Response to Reviewer #2:**

### Reviewers' comments:

Reviewer #2: This paper uses the immortalized GT1-7 cell line well characterized to investigate the interaction between kisspeptin, estrogen and BMPs in the regulation of GnRH production.

The authors provide several lines of evidence that the axis of kisspeptin-induced GnRH production is controlled by opposite signaling pathways; one increased by an interaction between estrogen receptor and kisspeptin receptor, another suppressed by BMP-4 action.

Molecular mechanisms are needed to support the physiological relevance of these experiments.

The data are interesting but enthusiasm is reduced by the interpretation and robustness of the results. Specific issues are addressed below:

### ... Answer:

We appreciate the reviewer's favorable comments on our manuscript. The comments provided by the reviewer were very important to further discuss and reconsider our present data. According to the reviewer's comments and suggestions, we added new data and thoroughly revised the manuscript. Please refer to our revised manuscript.

1. Fig. 1B. The crude values of GnRH release must be presented since the differences appear very low according to the representation (fold increase).

### ... Answer 1:

We agree. We added the raw data of GnRH in the revised manuscript (Fig. 1C).

2. Fig. 2 A and B. The effect of BMP-6 alone is not valid.

### ... Answer 2:

We agree. There were mistakes in Fig. 2A and 2B. We amended the figures in the revised manuscript. Thank you for your comment.

3. Fig. 3B. The data obtained using BMP-6 and -7 are not shown. Why??

### ... Answer 3:

Based on the data in Fig. 2A, among BMP ligands we focused on the effects of BMP-4, which suppressed kisspeptin-induced GnRH expression by GT1-7 cells. As shown in Fig. 3B, Id-1 transcription induced by BMP-4, but not by BMP-2, was significantly suppressed by kisspeptin. The Id-1 mRNA levels induced by BMP-6 and -7 were also reduced by co-treatment with kisspeptin. We added these data in the revised manuscript. Please refer to our revised Fig. 3B.

4. Fig. 4. The western blots are very difficult to interpret. Quantitative values should be added.

... Answer 4:

We agree. We added the data regarding quantitative values of Western blots in the revised Fig. 4D. The relative integrated density of each protein band was digitized, in which the phosphorylated levels were normalized by the total levels, and then the results were statistically analyzed and expressed as fold changes. Thank you for your comments.

5. The estradiol effect in Fig 4B is only evaluated on the transcript. It is possible to measure the GnRH levels as in the Fig1.

... Answer 5:

We agree. We have measured the protein levels of GnRH in the medium as well. As shown in the new Fig. 4B, treatment with estradiol (100 nM) suppressed GnRH mRNA levels after 24-h culture of GT1-7 cells. In terms of the protein level, the same concentration of estradiol tended to reduce GnRH secretion for 24-h culture, but significantly suppressed GnRH production after 48-h culture (new Fig. 4C). It is informative for the readers to show the protein levels of GnRH in this study. Thank you for your comments.

6. A number of typographical errors should be corrected (Fig 1.).

... Answer 6:

We feel sorry for this point. Again, we carefully checked up throughout the manuscript including figures. Please refer to our revised manuscript.

Thank you for your review.



1 **Mutual interaction of kisspeptin, estrogen and bone morphogenetic**  
2 **protein-4 activity in GnRH regulation by GT1-7 cells.**

3  
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5 Kenichi Inagaki<sup>1</sup>, Kishio Toma<sup>1</sup>, Kanako Ogura-Ochi<sup>1</sup>, Christine Glidewell-  
6 Kenney<sup>3</sup>, Mark A. Lawson<sup>3</sup> and Hirofumi Makino<sup>1</sup>

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12  
13 *Short title:* Kisspeptin and BMP regulate GnRH production.

14 *Key words:* BMP, estradiol, GnRH, GPR54, kisspeptin, and MAPK

15 *Disclosure statement:* All authors have nothing to disclose.

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21  
22 *Abbreviations:* ActRII, activin type II receptor; ALK, activin receptor-like kinase;  
23 ARC, arcuate nucleus, AVPV, anteroventral periventricular nucleus; BMP, bone  
24 morphogenetic protein; BMPRII, BMP type II receptor; ER, estrogen receptor;  
25 ERK, extracellular signal-regulated kinase; FSH, follicle-stimulating hormone;  
26 GPR54, G-protein-coupled receptor 54; GnIH, gonadotropin-inhibitory hormone;  
27 GnRH, gonadotropin-releasing hormone; JNK, c-Jun NH<sub>2</sub>-terminal kinase; LH,  
28 luteinizing hormone; MAPK, mitogen-activated protein kinase.

29  
30 Word count: 4252 words

Figure number: 5 figures

**ABSTRACT**

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3       Reproduction is integrated by interaction of neural and hormonal signals  
4 converging on hypothalamic neurons for controlling gonadotropin-releasing  
5 hormone (GnRH). Kisspeptin, the peptide product of the kiss1 gene and the  
6 endogenous agonist for the GRP54 receptor, plays a key role in the regulation  
7 of GnRH secretion. In the present study, we investigated the interaction  
8 between kisspeptin, estrogen and BMPs in the regulation of GnRH production  
9 by using mouse hypothalamic GT1-7 cells. Treatment with kisspeptin increased  
10 GnRH mRNA expression and GnRH protein production in a concentration-  
11 dependent manner. The expression levels of kiss1 and GPR54 were not  
12 changed by kisspeptin stimulation. Kisspeptin induction of GnRH was  
13 suppressed by co-treatment with BMPs, with BMP-4 action being the most  
14 potent for suppressing the kisspeptin effect. The expression of kisspeptin  
15 receptor, GPR54, was suppressed by BMPs, and this effect was reversed in the  
16 presence of kisspeptin. It was also revealed that BMP-induced Smad1/5/8  
17 phosphorylation and Id-1 expression were suppressed and inhibitory Smad6/7  
18 was induced by kisspeptin. In addition, estrogen induced GPR54 expression,  
19 while kisspeptin increased the expression levels of ER $\alpha$  and ER $\beta$ , suggesting  
20 that the actions of estrogen and kisspeptin are mutually enhanced in GT1-7  
21 cells. Moreover, kisspeptin stimulated MAPKs and AKT signaling, and ERK  
22 signaling was functionally involved in the kisspeptin-induced GnRH expression.  
23 BMP-4 was found to suppress kisspeptin-induced GnRH expression by  
24 reducing ERK signaling activity. Collectively, the results indicate that the axis of

1 kisspeptin-induced GnRH production is bi-directionally controlled, being  
2 augmented by an interaction between ER $\alpha$ / $\beta$  and GPR54 signaling and  
3 suppressed by BMP-4 action in GT1-7 neuron cells. (254 words)

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## INTRODUCTION

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Reproduction is regulated by a complex interaction of neural and hormonal signals that converge on hypothalamic neurons responsible for the pulsatile secretion of gonadotropin-releasing hormone (GnRH) (Pinilla et al., 2009). Coordination of the GnRH impulse from the hypothalamus and the subsequent secretion of pituitary gonadotropins are critical for the central control of reproductive function (Pinilla et al., 2009). The pattern of GnRH secretion is regulated by the intrinsic oscillatory activity of GnRH neurons and the integration of presynaptic inputs of various neurotransmitters such as kisspeptin and gonadotropin-inhibitory hormone (GnIH) (Tsutsui et al., 2010) and the feedback loop from gonadal steroids (Petersen et al., 2003). Immortalized GnRH-producing GT1-7 cells have proven to be a valuable tool to study the biology of GnRH neurons (Liposits et al., 1991; Wetsel et al., 1991). In females, estrogens act directly or indirectly on the GnRH neuronal network to modulate the final output of GnRH into the median eminence (Garcia-Galiano et al., 2012; Hameed et al., 2011). However, the detailed mechanism by which estrogen exerts positive and negative feedback effects is still controversial.

Progress has been made in elucidation of the indirect mechanism of estrogen-GnRH regulation following the discovery of kisspeptin. Recently, an understanding of the physiological control of the reproductive axis has been advanced by identification of the essential role of kisspeptin. Kisspeptins are products of the kiss1 gene and these peptides are derived from the plasma proteolytic cleavage of the 145-amino-acid gene product. The kisspeptin

1 fragments commonly have a C-terminal decapeptide including Arg-Phe residues  
2 that are critical for the biological activity (Clements et al., 2001; Kotani et al.,  
3 2001; Ohtaki et al., 2001). Kisspeptin and its receptor, G-protein-coupled  
4 receptor 54 (GPR54), are key components in the regulation of GnRH secretion  
5 in humans and other mammals (Goodman and Lehman, 2012). Kisspeptin is  
6 found in both the peripheral and central nervous systems. In the periphery,  
7 kisspeptin has been identified in the testis, ovary, pituitary gonadotrope,  
8 pancreas, small intestine and placenta (Gaytan et al., 2009; Horikoshi et al.,  
9 2003; Muir et al., 2001; Ohtaki et al., 2001; Richard et al., 2008). In the central  
10 nervous system, both kiss1 mRNA and kisspeptin protein are abundantly  
11 expressed within the hypothalamus in the arcuate nucleus (ARC), anteroventral  
12 periventricular nucleus (AVPV) and periventricular nucleus (Gottsch et al.,  
13 2004) in rodents. Hypothalamic kiss1 mRNA is predominantly found in the  
14 infundibular nucleus, the equivalent of the ARC in primates (Rometo et al.,  
15 2007). Accumulated data have shown that central or systemic administration of  
16 kisspeptin increases GnRH and gonadotropin secretion in both prepubertal and  
17 adult animals, which is crucial for the neuroendocrine regulation of reproduction  
18 (Castellano et al., 2005; Funes et al., 2003; Pinilla et al., 2012; Seminara et al.,  
19 2003; Tena-Sempere, 2006). GPR54 is also critical for the integrity of  
20 reproductive activity, as shown in patients with idiopathic hypogonadotropic  
21 hypogonadism and in GPR54-knockout mice (de Roux et al., 2003; Funes et al.,  
22 2003; Seminara et al., 2003).

23 Bone morphogenetic proteins (BMPs), which belong to the transforming  
24 growth factor (TGF)- $\beta$  superfamily, were originally identified as active

1 components in bone extracts that are capable of inducing bone formation at  
2 ectopic sites. Recent studies have shown that BMPs are crucial molecules in  
3 normal folliculogenesis by regulating gonadotropin-induced steroidogenesis and  
4 mitosis in ovarian granulosa cells (Otsuka, 2010; Otsuka et al., 2011; Shimasaki  
5 et al., 2004). The major regulatory process by BMPs in ovarian steroidogenesis  
6 is control of follicle-stimulating hormone (FSH)-receptor activity, leading to  
7 normal follicular development in the ovary. It has also been reported that BMPs  
8 are involved in the regulation of various functions of the anterior pituitary  
9 including gonadotropin secretion (Otsuka and Shimasaki, 2002; Otsuka et al.,  
10 2012; Otsuka, 2013; Takeda et al., 2007; Tsukamoto et al., 2010). Bi-  
11 directional communication between the ovary and central nervous system  
12 ensures that the neural signal for ovulation occurs at the time point when follicle  
13 maturation is completed. The BMP system is also required for the early  
14 developmental stage of the hypothalamus for regulating cell identities in the  
15 neural tube (Liu and Niswander, 2005; Ulloa and Briscoe, 2007).

16 We have reported expression of the BMP system including BMP type I  
17 and type II receptors in hypothalamic neuron GT1-7 cells (Otani et al., 2009). In  
18 that study, it was found that BMPs modulate the suppressive effects of estrogen  
19 on GnRH production by regulating estrogen receptor (ER) expression and  
20 estrogen-induced MAPK signaling. Interaction of the BMP system and ER  
21 action may be linked to hypothalamic GnRH production in an  
22 autocrine/paracrine fashion. Hence, the BMP system plays a regulatory role in  
23 female reproduction not only by altering gonadotropin sensitivity in ovarian  
24 folliculogenesis but also by modulating the hypothalamo-pituitary-ovarian (HPO)

1 axis. However, the direct effects of BMPs on hypothalamic GnRH production  
2 and secretion have yet to be elucidated. Reproduction is regulated by mutual  
3 interaction of neural and hormonal factors that direct the production and  
4 secretion of GnRH. In the present study, we investigated the effects of BMPs  
5 on GnRH production controlled by kisspeptin. We uncovered a novel  
6 interaction between BMP-4, kisspeptin and estrogen, which is involved in the  
7 control of hypothalamic GnRH production and secretion by GT1-7 neuron cells.

8

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## MATERIALS AND METHODS

### *Reagents and supplies*

Dulbecco's Modified Eagle's Medium (DMEM), dimethylsulfoxide (DMSO), penicillin-streptomycin solution, human metastin (45-54) amide (kisspeptin-10), and 17 $\beta$ -estradiol were purchased from Sigma-Aldrich Co. Ltd. (St. Louis, MO).

Mouse hypothalamus total RNA was purchased from Clontech Laboratories, Inc.

(Mountain View, CA). Recombinant human BMP-2, -4, -6 and -7 were purchased from R&D Systems Inc. (Minneapolis, MN), the extracellular signal-regulated kinase (ERK) inhibitor U0126 and p38-MAPK inhibitor SB203580 were from Promega Corp. (Madison, WI), the stress-activated protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) inhibitor SP600125 was from Biomol Lab. Inc. (Plymouth Meeting, PA), and the AKT inhibitor SH-5 was from Calbiochem (San Diego, CA).

### *GT1-7 cell culture*

GT1-7 cells were kindly provided by Dr. Pamela L. Mellon, University of California, San Diego, CA. The GT1-7 cells were maintained in DMEM supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin (Sigma-Aldrich Corp.) at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. The culture medium was changed twice a week, and cultures were passaged at ~80% confluence. Changes in cell morphology and growing conditions were carefully monitored under an inverted microscope.



1 *Measurement of GnRH production*

2 To assess the effects of BMPs on GnRH protein synthesis, GT1-7 cells ( $2 \times 10^5$   
3 viable cells/ml) were cultured in 6-well plates with DMEM containing 10% FCS  
4 for 24 h. The medium was then changed to serum-free DMEM and was  
5 subsequently treated with the indicated concentrations of kisspeptin. After 24-h  
6 culture, the supernatant of the culture media was collected and stored at  $-80^\circ\text{C}$   
7 until assay. GnRH concentration (pg/ml) in the conditioned medium was  
8 determined by a radioimmunoassay as reported previously (Chappell et al.,  
9 2003; Otani et al., 2009). Briefly, 0.1-ml aliquots of each sample were  
10 incubated for 48 h with the GnRH antibody EL-14 at  $4^\circ\text{C}$ , after which time  
11 10,000 cpm sample of radio-iodinated GnRH (Amersham Pharmacia,  
12 Piscataway, NJ) was added. Following ethanol precipitation of the bound  
13 fractions after 48-h incubation, radioactivity was detected by a gamma  
14 scintillation counter (Micromedic, Huntsville, AL), and standard curve analysis  
15 was performed at each assay. The inter-assay variability and intra-assay  
16 variability in the radioimmunoassay are 8.7% and 7.7%, respectively.

17

18 *RNA extraction, RT-PCR and quantitative real-time PCR analysis*

19 GT1-7 cells ( $2 \times 10^5$  viable cells/ml) were precultured in serum-free DMEM, and  
20 the cells were treated with indicated concentrations of kisspeptin in combination  
21 with BMPs, estradiol and various chemical inhibitors including U0126,  
22 SB203580, SP600125 and SH-5. After 24-h culture, the medium was removed,  
23 and total cellular RNAs were extracted using TRIzol® (Invitrogen Corp.,  
24 Carlsbad, CA) and subsequently quantified by measuring absorbance at 260

1 nm and then stored at -80°C until assay. The expression of GnRH, kiss1,  
2 GPR54, ERs (ER $\alpha$  and ER $\beta$ ), BMP ligands (BMP-2, -4, -6 and -7), BMP type I  
3 (ALK-2, -3, -4 and -6) and type II (ActRIIA, ActRIIB and BMPRII) receptors, and  
4 Smads (Smad6 and 7) was detected by RT-PCR. The extracted RNA (1  $\mu$ g)  
5 was subjected to a RT reaction using First-Strand cDNA Synthesis System®  
6 (Invitrogen Corp.) with random hexamer (2 ng/ $\mu$ l), reverse transcriptase (200 U)  
7 and deoxynucleotide triphosphate (dNTP; 0.5 mM) at 42°C for 50 min and at  
8 70°C for 10 min. Subsequently, hot-start PCR was performed using MgCl<sub>2</sub> (1.5  
9 mM), dNTP (0.2 mM) and Taq DNA polymerase (2.5 U) (Invitrogen Corp.).  
10 Oligonucleotides used for PCR were custom-ordered from Invitrogen Corp.  
11 PCR primer pairs were selected from different exons of the corresponding  
12 genes to discriminate PCR products that might arise from possible chromosome  
13 DNA contaminants. The primer pairs for mouse BMP ligands, receptors, Id-1,  
14 Smads, and the house-keeping gene ribosomal protein-L19 (RPL19) were  
15 selected as we previously reported (Otani et al., 2009; Takeda et al., 2007;  
16 Tsukamoto et al., 2010). For ERs, GnRH, kiss1 and GPR54 genes, the  
17 following sequences were used: ER $\alpha$ , 1523-1543 and 1737-1757 from  
18 GenBank accession number NM\_007956; ER $\beta$ , 701-721 and 1004-1024 from  
19 NM\_010157; GnRH, 90-110 and 316-336 from BC116897; kiss1, 61-81 and  
20 328-352 from NM\_178260; and GPR54, 1959-1980 and 2122-2144 from  
21 NM\_053244. Aliquots of the PCR products were electrophoresed on 1.5%  
22 agarose gels and visualized after ethidium bromide staining. For the  
23 quantification of the receptors for BMP-4 (ALK-3 and BMPRII), ERs (ER $\alpha$  and

1 ER $\beta$ ), GnRH, GPR54, Id-1, kiss1, Smads (Smad6 and Smad7) and RPL19  
2 mRNA levels, real-time PCR was performed using LightCycler-FastStart DNA  
3 master SYBR Green I system<sup>®</sup> (Roche Diagnostic Co., Tokyo, Japan) under  
4 each optimized condition of annealing at 59 to 63°C with 4 mM MgCl<sub>2</sub>, following  
5 the manufacturer's protocol. Accumulated levels of fluorescence for each  
6 product were analyzed by the second derivative method after melting-curve  
7 analysis (Roche Diagnostic Co.), and then, following assay validation by  
8 calculating amplification efficiency of each amplicon, the expression levels of  
9 target genes were quantified on the basis of standard curve analysis for each  
10 product. For each transcript, all treatment groups were quantified  
11 simultaneously in a single LightCycler run. To correct for differences in RNA  
12 quality and quantity between samples, the expression levels of target gene  
13 mRNA were normalized by dividing the quantity of the target gene by the  
14 quantity of RPL19 in each sample. The raw data of each target mRNA level  
15 (/RPL19) were statistically analyzed as indicated and are shown as fold  
16 changes in the figures.

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#### 18 *Western immunoblot analysis*

19 GT1-7 cells ( $1 \times 10^5$  viable cells/ml) were precultured in 12-well plates in serum-  
20 free DMEM. The cells were treated with BMPs (100 ng/ml) and kisspeptin (300  
21 nM) either alone or in combination with estradiol (100 nM). After 15- and 60-  
22 min stimulation indicated, cells were solubilized in 100  $\mu$ l RIPA lysis buffer  
23 (Upstate Biotechnology, Lake Placid, NY) containing 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF,

1 2% SDS and 4%  $\beta$ -mercaptoethanol. The cell lysates were then subjected to  
2 SDS-PAGE/immunoblotting analysis as we previously reported (Otani et al.,  
3 2009; Tsukamoto et al., 2010), using anti-phospho- and anti-total-extracellular  
4 signal-regulated kinase (ERK) 1/2 MAPK antibody (Cell Signaling Technology,  
5 Inc., Beverly, MA), anti-phospho- and anti-total-p38 MAPK antibody (Cell  
6 Signaling Technology, Inc.), anti-phospho- and anti-total-stress-activated  
7 protein kinase/c-Jun NH2-terminal kinase (SAPK/JNK) MAPK antibody (Cell  
8 Signaling Technology, Inc.), anti-phospho- and anti-total-AKT antibody, anti-  
9 phospho-Smad1/5/8 (pSmad1/5/8) antibody (Cell Signaling Technology, Inc.),  
10 and anti-actin antibody (Sigma-Aldrich Co. Ltd.). The relative integrated density  
11 of each protein band was digitized by NIH image J 1.34s.

12

### 13 *Statistical analysis*

14 All results are shown as means  $\pm$  SEM of data from at least three separate  
15 experiments, each performed with triplicate samples. The data were subjected  
16 to ANOVA or the unpaired *t*-test (StatView 5.0 software, Abacus Concepts, Inc.,  
17 Berkeley, CA). If differences were detected by ANOVA, Tukey-Kramer's post  
18 hoc test was used to determine which means differed (StatView 5.0 software).  
19 *P* values < 0.05 were accepted as statistically significant.

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## RESULTS

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3 First, expression of the kisspeptin system in GT1-7 cells was confirmed  
4 by RT-PCR. As shown in **Fig. 1A**, in addition to GnRH expression, kiss1,  
5 GPR54, ER $\alpha$  and ER $\beta$  were clearly expressed in mouse hypothalamic GT1-7  
6 neuron cells. To clarify the existence of kisspeptin and BMP system *in vivo*, we  
7 examined the expression of these molecules in the mouse hypothalamus. As  
8 shown in Fig. 1B, the GnRH-kisspeptin system, including GnRH, kiss1,  
9 GPR54, ER $\alpha$  and ER $\beta$ , BMP ligands (BMP-2, -4, -6 and -7), BMP type I (ALK-2,  
10 -3, -4 and -6) and BMP type II (BMPRII, ActRIIA and ActRIIB), and inhibitory  
11 Smads (Smad6 and 7) were detected in the mouse hypothalamus. Although  
12 kisspeptin expression is not demonstrated in GnRH neurons *in vivo*, GPR54  
13 expression is present and influences GnRH secretion and expression. To  
14 examine the functional role of kisspeptin in GnRH production by GT1-7 cells,  
15 cells were treated with kisspeptin (0.1 to 1  $\mu$ M) and GnRH concentration in the  
16 serum-free medium was determined by RIA. As shown in **Fig. 1C**, kisspeptin  
17 treatment significantly increased GnRH release in a concentration-dependent  
18 manner. The mRNA level of GnRH was also increased by kisspeptin (0.01 to 3  
19  $\mu$ M) treatment (**Fig. 1D**), whereas the expression levels of kiss1 and GPR54  
20 were not altered by kisspeptin (0.03 to 3  $\mu$ M) treatment (**Fig. 1E**).

21 Of note, the stimulating effects of kisspeptin on GnRH mRNA levels  
22 were suppressed by co-treatment with BMPs (100 ng/ml) including BMP-2, -4, -  
23 6 and BMP-7 (**Fig. 2A**). BMP-4 action was the most potent inhibitor of GnRH

1 induction by kisspeptin. It was also revealed that the expression of the  
2 kisspeptin receptor, GPR54, was potently suppressed by treatment with BMP-2,  
3 -4, -6 and -7 (**Fig. 2B**). Interestingly, the BMP actions that suppress GPR54  
4 expression were significantly reversed by co-treatment with kisspeptin (300 nM).

5 To describe the mechanism by which kisspeptin interferes with the  
6 effects of BMPs, the downstream signals of BMP receptors, Smad1/5/8  
7 phosphorylation and Id-1 transcription, were examined (**Fig. 3**). As shown in  
8 **Fig. 3A**, treatments with BMP-2 and -4 (100 ng/ml) readily stimulated  
9 Smad1/5/8 phosphorylation in GT1-7 cells; however, BMP-induced Smad1/5/8  
10 phosphorylation, in particular that induced by BMP-4, was suppressed by co-  
11 treatment with kisspeptin (300 nM). To confirm the effects of kisspeptin on BMP  
12 receptor signaling, Id-1 mRNA levels were examined by real-time PCR analysis.  
13 In accordance with the results of immunoblots for Smad1/5/8, Id-1 mRNA  
14 activation induced by BMP-2, -4, -6 and -7 (100 ng/ml) was suppressed by  
15 kisspeptin (300 nM) (**Fig. 3B**). Of note, the suppressive effect of kisspeptin on  
16 BMP-2-induced Id-1 expression was less potent than that on Id-1 expression  
17 induced by BMP-4, -6 and -7.

18 To investigate the molecular mechanism by which kisspeptin (300 nM)  
19 regulates BMP activities in GT1-7 cells, the expression changes in ALK-3 and  
20 BMPRII, which are major type I and type II receptors for BMP-2 and -4,  
21 respectively, were examined in the presence or absence of estradiol (100 nM).  
22 As shown in **Fig. 3C**, kisspeptin treatment had no effect on ALK-3 or BMPRII  
23 mRNA levels regardless of the presence of estradiol. The effects on inhibitory  
24 Smads, including Smad6 and Smad7, were also examined. As shown in **Fig.**

1 **3D**, mRNA levels of Smad6 and Smad7 were increased in the presence of  
2 BMP-2 and -4. Kisspeptin treatment had no significant effect on Smad6 and  
3 Smad7 mRNA expression in the presence of BMP-2; however, it was notable  
4 that kisspeptin significantly increased Smad6 and Smad7 levels in the presence  
5 of BMP-4. Thus, kisspeptin regulates BMP activity by increasing inhibitory  
6 Smads expression in GT1-7 cells.

7 Next, the interaction between kisspeptin and estrogen action was studied.  
8 Treatment with kisspeptin (100 nM) significantly increased the expression levels  
9 of ER $\alpha$  and ER $\beta$  mRNAs (Fig. 4A). Treatment with estradiol (100 nM)  
10 suppressed GnRH mRNA expression (Fig. 4B, left) and GnRH protein levels in  
11 the media (Fig. 4C) by GT1-7 cells as a negative feedback effect, while  
12 estradiol significantly increased kisspeptin receptor GPR54 mRNA expression  
13 levels in GT1-7 cells (Fig. 4B, right). These findings suggest that the actions of  
14 estrogen and kisspeptin are mutually enhanced in GT1-7 cells.

15 We further investigated the underlying mechanism by which BMP-4  
16 suppressed kisspeptin-induced GnRH expression. As shown in Fig. 4D,  
17 kisspeptin (300 nM) stimulated ERK and AKT phosphorylation and the actions  
18 of kisspeptin were enhanced in the presence of estradiol (100 nM), although it  
19 failed to increase P38 and JNK phosphorylation in GT1-7 cells. As shown in  
20 Fig. 4E, treatment with chemical inhibitors for MAPKs and AKT, including  
21 U0126, SB203580, SP600125 and SH5 (1 to 10  $\mu$ M), revealed that kisspeptin-  
22 induced GnRH mRNA expression was suppressed by the ERK inhibitor (U0126),  
23 suggesting that ERK signaling is functionally involved in the kisspeptin-induced  
24 GnRH expression. As shown in Fig. 4F, BMP-4 treatment (100 ng/ml) was

1 found to suppress kisspeptin-induced ERK phosphorylation in GT1-7 cells. It  
2 was thus thought that BMP-4 suppresses kisspeptin-induced GnRH production  
3 by inhibiting ERK activation.  
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## DISCUSSION

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3 The mechanism by which estrogen acts on the GnRH regulatory system  
4 has yet to be clarified. Direct actions on GnRH neurons as well as indirect  
5 actions through other linked neurons via trans-synaptic interaction seem to be  
6 involved in this machinery. It was revealed that kisspeptin stimulated GnRH  
7 synthesis and the GnRH induction was suppressed by BMP-4 in mouse  
8 hypothalamic GT1-7 cells, suggesting the presence of a mutual balance  
9 between the actions of kisspeptin and BMP-4 for regulating GnRH (Fig. 5). In  
10 this interaction, estrogen upregulated GPR54 expression, and kisspeptin, in turn,  
11 increased the expression levels of ER $\alpha$  and ER $\beta$  in addition to the role of  
12 estrogen as a GnRH repressor of negative feedback. On the other hand,  
13 GPR54 expression was suppressed by BMPs. The suppressive effect of BMPs  
14 on GPR54 expression was reversed by kisspeptin via upregulation of inhibitory  
15 Smad6/7 expression. Hence, in GT1-7 neuron cells, the axis of kisspeptin-  
16 induced GnRH production is bi-directionally controlled, being augmented by the  
17 estrogen-to-GPR54 pathway and being suppressed by BMP-4-to-Smad1/5/8  
18 signaling (Fig. 5).

19 Our results showing that estrogen acts directly on GT1-7 neurons to  
20 suppress GnRH expression as well as to enhance the GPR54 pathway have  
21 added more complexity to the system for gonadotropin regulation. Estrogen  
22 also has been shown to regulate kiss1 mRNA differentially in various nuclei that  
23 exert major physiological activity in the forebrain (Jacobi et al., 2007). Estrogen  
24 stimulation promotes kiss1 expression in the ventromedial hypothalamus AVPV,

1 which is implicated in positive feedback regulation of the luteinizing hormone  
2 (LH) surge, whereas it reduces kiss1 expression in the ARC, which is implicated  
3 in negative feedback regulation of GnRH in female reproduction (Smith et al.,  
4 2005). In the present study, a mutual interaction of the expression control of  
5 ERs and GPR54 was shown by GT1-7 cells, in which both ER $\alpha$  and ER $\beta$   
6 mRNAs were increased by kisspeptin, while GPR54 expression and the  
7 GPR54-induced ERK1/2 signaling were enhanced by estradiol. Expression of  
8 ER $\alpha$  in GnRH neurons is not clearly demonstrated, but GnRH neurons do  
9 express ER $\beta$  and the estrogen membrane receptor GPR30 (Kenealy et al.,  
10 2011; Terasawa and Kenealy, 2012). It was also reported that estrogen elicited  
11 circadian profiles of GPR54 expression *in vitro* possibly through ER $\beta$  action  
12 (Tonsfeldt et al., 2011). The upregulation of GRP54 was likely involved in the  
13 stimulation of estradiol-induced GnRH secretion as a positive feedback induced  
14 by estrogen (Tonsfeldt et al., 2011). These results suggest that sensitivity of  
15 GnRH secretion to kisspeptin is partly dependent on estrogen activity. Elevated  
16 gonadal estrogen level may increase kisspeptinergic tone at the same time as  
17 increasing GnRH neuronal sensitivity.

18 Interestingly, effects of kisspeptin on GnRH induction were suppressed  
19 by co-treatment with BMPs, with BMP-4 action effectively suppressing induction  
20 of GnRH expression by kisspeptin (**Fig. 5**). A complex interaction of neural and  
21 hormonal signals including BMPs and kisspeptin may integrate the neuronal  
22 activity for GnRH secretion. The neuroregulatory factor TGF- $\beta$ 1 secreted by  
23 astrocytes is a putative candidate for modulation of GnRH secretion (Buchanan

1 et al., 2000; Galbiati et al., 1996; Melcangi et al., 1995; Zwain et al., 2002). A  
2 regulatory role of TGF- $\alpha$  originating from astrocytes in GnRH release has also  
3 been demonstrated (Ma et al., 1992; Ma et al., 1997; Ojeda et al., 2000). In the  
4 present study, expression of the BMP system including BMP ligands and key  
5 receptors was shown in the hypothalamus. Given the findings that several  
6 BMPs elicit a neurotropic capacity for dopaminergic neurons in cooperation with  
7 other members of the TGF- $\beta$  superfamily through glial cells (Jordan et al., 1997;  
8 Reiriz et al., 1999; Samanta et al., 2007), hypothalamic GnRH synthesis may be  
9 functionally associated with BMP activity in a neural network in the  
10 hypothalamus.

11 The BMP system has been shown to play critical roles in initial  
12 development of the anterior pituitary (Scully and Rosenfeld, 2002), and BMPs  
13 modulate GnRH responses in gonadotropes (Takeda et al., 2012). BMP-4 is  
14 necessary for the first step of pituitary organogenesis including the proliferation  
15 of Rathke's pouch, which gives rise to Pit-1 lineage cells. BMP-4 not only  
16 governs pituitary organogenesis but also plays a role in the pathogenesis of  
17 differentiated pituitary lineages (Otsuka et al., 2012; Tsukamoto et al., 2010).  
18 The BMP system also acts as a key molecule in the early developmental stages  
19 of the hypothalamus in the neural tube in conjunction with hedgehog activity  
20 (Liu and Niswander, 2005; Ulloa and Briscoe, 2007). Hypothalamic neuronal  
21 differentiation is regulated by a series of activities induced by sonic hedgehog  
22 (Shh) and BMP-7 via specification of hypothalamic progenitors (Ohyama et al.,  
23 2008). The elaborate neural patterning seems to be achieved by integrated  
24 antagonism between Shh and BMP actions. In addition, it was recently

1 reported that BMP type IB receptor (ALK-3) signaling is critical for postnatal  
2 establishment of the hypothalamic circuit from ARC to multiple nuclei, which  
3 regulate energy homeostasis and feeding behavior (Peng et al., 2012).

4         The receptors for TGF- $\beta$  superfamily members consist of type I and type  
5 II receptors, each of which exhibits Ser/Thr kinase activity. Among the several  
6 combinations of BMP ligands and receptors, BMP-4 readily binds to ALK-3  
7 and/or ALK-6 and preferentially binds to BMPRII (Shimasaki et al., 2004).  
8 Since ALK-6 is not expressed in GT1-7 cells (Otani et al., 2009), the receptor  
9 pairs of ALK-3/BMPRII are likely to be the major functional complex for BMP-4.  
10 Our data demonstrated that kisspeptin suppressed BMP-Smad1/5/8 signaling  
11 by augmenting the expression of inhibitory Smad6 and/or Smad7 in GT1-7 cells.  
12 The expression of GPR54 mRNA was potently suppressed by BMPs, and the  
13 suppressive effects of BMPs on GnRH expression were reversed in the  
14 presence of kisspeptin. It was also revealed that BMP-4-activated Smad1/5/8  
15 phosphorylation and Id-1 expression were suppressed by kisspeptin-induced  
16 increase of inhibitory Smad6/7. Smad6/7 expression was increased by  
17 kisspeptin, particularly in the presence of BMP-4. This new interaction between  
18 the BMP-Smad pathway and kisspeptin-GPR54 signaling may be involved in  
19 the control of GnRH-neuronal activity.

20         The neuronal mechanism through which estrogen regulates GnRH  
21 neurons is a key for elucidating reproductive control of the HPO axis. In our  
22 study, kisspeptin stimulated MAPKs and AKT signaling, particularly in the  
23 presence of estrogen, in which ERK1/2 signaling was functionally involved in  
24 the kisspeptin-induced GnRH expression. It was also revealed that BMP-4

1 suppressed kisspeptin-induced GnRH expression by reducing ERK signaling  
2 activity. There have been several reports on signaling of kisspeptin for GnRH  
3 release. The pulsatile pattern of GnRH release, which results in the intermittent  
4 release of gonadotropins from the pituitary, is critical for maintenance of  
5 reproductive function. Ozcan *et al.* revealed the pattern of kisspeptin-induced  
6 intracellular signaling and the role of protein kinase C (PKC) in the intracellular  
7 signaling cascade by fluorescence calcium imaging using GT1-7 cells (Ozcan et  
8 al., 2011), and their results suggested the importance of intracellular calcium  
9 flux downstream from GPR54 through the PKC signaling pathway. Novaira *et*  
10 *al.* reported an interesting finding that the negative regulation of GnRH secretion  
11 by estrogen was antagonized by kisspeptin (Novaira et al., 2009). In our study,  
12 GnRH response to kisspeptin was weaker than the previous reports (Novaira et  
13 al., 2009; Tonsfeldt et al., 2011). This could be due to the culture condition with  
14 serum-free medium for 24 h in our experiments, although this condition was  
15 chosen to simplify the interaction between BMP and a genomic effect of ER.  
16 Much earlier time points and serum inclusion might be suitable to see higher  
17 responsiveness of GnRH. Further approaches to the intracellular signaling  
18 pathway revealed the involvement of ERK1/2 and phosphatidylinositol-3-kinase  
19 (PI3K)-AKT pathways in the effects of kisspeptin on GnRH regulation (Novaira  
20 et al., 2009). This work demonstrated that the kisspeptin-GPR54 system plays  
21 a role not only in stimulating GnRH secretion but also in positive regulation of  
22 GnRH mRNA levels via MAPK and AKT signaling in hypothalamic neurons.  
23 Taken together, both MAPK and AKT pathways seem to be substantially linked  
24 to the activation of GnRH regulation by kisspeptin and estrogen.

1        Collectively, the results indicate that the axis of kisspeptin-induced GnRH  
2 production was positively controlled by an interaction between ER $\alpha$ / $\beta$  and  
3 GPR54 and, on the other hand, negatively regulated by BMP-4 signaling in  
4 GT1-7 cells (Fig. 5). Considering that GT1-7 cells exhibit daily changes in  
5 cellular peptide expression and GnRH secretion in response to kisspeptin and  
6 other hormones (Zhao and Kriegsfeld, 2009), the regulatory mechanism of  
7 GnRH modulated by intrinsic circadian cycles may also be taken account for  
8 approaching the role in the reproductive axis. However, we cannot fully  
9 translate this interaction shown in a GT1-7 cell model into the physiological  
10 situation *in vivo* because of the lack of ER $\alpha$  in native GnRH neurons (Herbison  
11 and Pape, 2001; Herbison, 2008). Given that kisspeptin neurons predominantly  
12 express ER $\alpha$  with a modest fraction expressing ER $\beta$  (Pinilla et al., 2012), it can  
13 be postulated that kisspeptin neurons are also involved in the regulatory  
14 mechanism of estrogen for GnRH secretion in the presence of BMP-4. Further  
15 studies on the molecular mechanism are needed to elucidate the detailed  
16 mechanism of hypothalamic release of GnRH and the *in vivo* role of the BMP  
17 system in the physiologic gonadotropin surge and onset of puberty. A mutual  
18 interaction of kisspeptin, estrogen and BMP-4 may be a key for fine-tuning of  
19 GnRH regulation under the dynamic influence of estrogen level.

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1 **FIGURE LEGENDS**

2  
3 **Fig. 1. Expression of kisspeptin system and kisspeptin actions on GnRH**

4 **production by GT1-7 cells.** A) Total cellular RNAs were extracted from GT1-7

5 cells. The expression of GnRH (247 bp), kiss1 (392 bp), GPR54 (186 bp), ER $\alpha$

6 (235 bp), ER $\beta$  (324 bp) and the housekeeping gene RPL19 (195 bp) in GT1-7

7 cells was examined by RT-PCR. Aliquots of PCR products were

8 electrophoresed on 1.5% agarose gel and visualized by ethidium bromide

9 staining, and the results shown are representative of those obtained from three

10 independent experiments. MM indicates molecular weight marker. B) The

11 expression of GnRH, kiss1, GPR54, ER $\alpha$ , ER $\beta$ , BMP ligands including BMP-2

12 (212 bp), -4 (241 bp), -6 (224 bp) and -7 (287 bp), BMP receptors including

13 ALK-2 (483 bp), -3 (510 bp), -4 (528 bp), -6 (456 bp), BMPRII (522 bp), ActRIIA

14 (492 bp) and ActRIIB (271 bp), and Smad6 (165 bp) and Smad 7 (169 bp) in the

15 mouse hypothalamus was also examined by RT-PCR. C) GT1-7 cells ( $2 \times 10^5$

16 cells/ml) were treated with indicated concentrations of kisspeptin in serum-free

17 conditions. After 24-h culture, the culture media were collected and GnRH

18 concentrations (pg/ml) were determined by a radioimmunoassay. D, E) Cells ( $2$

19  $\times 10^5$  cells/ml) were treated with indicated concentrations of kisspeptin in

20 serum-free conditions for 24 h. Total cellular RNA was extracted and mRNA

21 levels of GnRH, kiss1 and GPR54 were examined by real-time RT-PCR. The

22 expression levels of target genes were standardized by RPL19 level in each

23 sample. Results in all panels are shown as means  $\pm$  SEM of data from at least

24 three separate experiments, each performed with triplicate samples. The

1 results were analyzed by ANOVA (C-E) and, when a significant effect due to  
2 treatment was observed, subsequent comparisons of group means were  
3 conducted. For each result within a panel, the values with different superscript  
4 letters are significantly different at  $P < 0.05$ . \*,  $P < 0.05$  vs. control group.

5  
6 **Fig. 2. Kisspeptin and BMP actions on GnRH and GPR54 expression by**  
7 **GT1-7 cells.** GT1-7 cells ( $2 \times 10^5$  cells/ml) were treated with indicated  
8 concentrations of kisspeptin (300 nM) in combination with BMP-2, -4, -6 and -7  
9 (100 ng/ml) in serum-free conditions for 24 h. Total cellular RNA was extracted  
10 and (A) GnRH and (B) GPR54 mRNA levels were examined by real-time RT-  
11 PCR. The expression levels of target genes were standardized by RPL19 level  
12 in each sample. Results in all panels are shown as means  $\pm$  SEM of data from  
13 at least three separate experiments, each performed with triplicate samples.  
14 The results were analyzed by ANOVA and unpaired *t*-test and, when a  
15 significant effect due to treatment was observed ( $P < 0.05$ ), subsequent  
16 comparisons of group means were conducted. \*,  $P < 0.05$  and \*\*,  $P < 0.01$   
17 between the indicated groups; #,  $P < 0.05$  vs. control group. n.s., not  
18 significant.

19  
20 **Fig. 3. Effects of kisspeptin on BMP-Smad signaling in GT1-7 cells.** A)  
21 GT1-7 cells ( $1 \times 10^5$  cells/ml) were precultured in serum-free conditions in the  
22 absence or presence of kisspeptin (300 nM) for 24 h, and then stimulated with  
23 BMP-2 and -4 (100 ng/ml). After 60-min culture with BMP treatment, cells were  
24 lysed and subjected to SDS-PAGE/immunoblotting (IB) analysis using anti-

1 phospho-Smad1/5/8 (pSmad1/5/8) and anti-actin antibodies. The results shown  
2 are representative of those obtained from three independent experiments. B)  
3 Cells ( $2 \times 10^5$  cells/ml) were treated with BMP-2, -4, -6 and -7 (100 ng/ml) in  
4 combination with kisspeptin (300 nM) in serum-free conditions for 24 h. Total  
5 cellular RNA was extracted and Id-1 mRNA level was examined by real-time  
6 RT-PCR. The expression levels of target genes were standardized by RPL19  
7 level in each sample. C) Cells ( $2 \times 10^5$  cells/ml) were treated with kisspeptin  
8 (300 nM) in combination with estradiol (100 nM) in serum-free conditions for 24  
9 h. Total cellular RNA was extracted and ALK-3 and BMPRII mRNA levels were  
10 examined by real-time RT-PCR. D) Cells were treated with kisspeptin (300 nM)  
11 in the presence of BMP-2 and -4 (100 ng/ml) in serum-free conditions for 24 h.  
12 Total cellular RNA was extracted and Smad6 and Smad7 mRNA levels were  
13 examined by real-time RT-PCR. Results in all panels are shown as means  $\pm$   
14 SEM of data from at least three separate experiments, each performed with  
15 triplicate samples. The results were analyzed by ANOVA and, when a  
16 significant effect due to treatment was observed ( $P < 0.05$ ), subsequent  
17 comparisons of group means were conducted. #,  $P < 0.05$  vs. control group;  
18 and \*,  $P < 0.05$  between the indicated groups.

19

20 **Fig. 4. Interaction of kisspeptin and estrogen receptor signaling in GnRH**  
21 **induction by GT1-7 cells. A, B) GT1-7 cells ( $2 \times 10^5$  cells/ml) were treated**  
22 **with kisspeptin (10 to 100 nM) and estradiol (100 nM) in serum-free conditions**  
23 **for 24 h. Total cellular RNA was extracted and ER $\alpha$ , ER $\beta$ , GnRH and GPR54**  
24 **mRNA levels were examined by real-time RT-PCR. The expression levels of**

1 target genes were standardized by RPL19 level in each sample. C) Cells ( $2 \times$   
2  $10^5$  cells/ml) were treated with indicated concentrations of estradiol in serum-  
3 free conditions. After 24-h and 48-h culture, the culture media were collected  
4 and GnRH concentrations (pg/ml) were determined by a radioimmunoassay. D,  
5 F) Cells ( $1 \times 10^5$  cells/ml) were precultured in serum-free conditions in the  
6 absence or presence of estradiol (E2; 100 nM) and BMP-4 (100 ng/ml) for 24 h,  
7 and then stimulated with kisspeptin (Kp; 300 nM). After 15-min culture with  
8 kisspeptin treatment, cells were lysed and subjected to SDS-  
9 PAGE/immunoblotting (IB) analysis using anti-phospho-ERK1/2 (pERK) and  
10 anti-total-ERK1/2 (tERK), and anti-phospho-AKT (pAKT) and anti-total-AKT  
11 (tAKT), anti-phospho-JNK (pJNK) and anti-total JNK (tJNK), and anti-phospho-  
12 p38 (pP38) and anti-total-p38 (tP38) antibodies. The results shown are  
13 representative of those obtained from three independent experiments. The  
14 relative integrated density of each protein band was digitized, in which the  
15 phosphorylated levels were normalized by the total levels, and then the results  
16 were expressed as fold changes. E) Cells ( $2 \times 10^5$  cells/ml) were treated with  
17 MAPK and AKT inhibitors (1 to 10  $\mu$ M), including U0126 (U01), SB203580  
18 (SB20), SP600125 (SP600) and SH-5 (SH5), in combination with kisspeptin  
19 (300 nM) in serum-free conditions for 24 h. Total cellular RNA was extracted  
20 and GnRH mRNA levels were examined by real-time RT-PCR. Results in all  
21 panels are shown as means  $\pm$  SEM of data from at least three separate  
22 experiments, each performed with triplicate samples. The results were  
23 analyzed by ANOVA (A, D-F) and unpaired *t*-test (B, C) and when a significant  
24 effect due to treatment was observed ( $P < 0.05$ ), subsequent comparisons of

1 group means were conducted. \*,  $P < 0.05$  and \*\*,  $P < 0.01$  vs. control group  
2 and between the indicated groups.

3

4 **Fig. 5. Interaction of kisspeptin, estrogen and BMP-4 in regulation of**

5 **GnRH production by GT1-7 cells.** Kisspeptin stimulates GnRH production via

6 GPR54 and its downstream signaling of the ERK pathway in GT1-7 cells.

7 Estrogen upregulates GPR54 expression, while kisspeptin increases the

8 expression levels of  $ER\alpha/\beta$ , suggesting that the actions of estrogen and

9 kisspeptin are mutually enhanced. Kisspeptin effects on GnRH induction were

10 suppressed by BMP-4, by inhibiting ERK phosphorylation and GPR54

11 expression. On the other hand, by upregulating inhibitory Smads6/7, kisspeptin

12 suppressed Smad1/5/8 phosphorylation and Id-1 expression induced by BMP-4

13 through the ALK-3/BMPRII receptor. In GT1-7 neuron cells, the axis of

14 kisspeptin-induced GnRH production is augmented by an interaction between

15  $ER\alpha/\beta$  and GPR54 and it is also negatively controlled by BMP-4.

16

17



Title:

**Mutual interaction of kisspeptin, estrogen and bone morphogenetic protein-4 activity in GnRH regulation by GT1-7 cells.**

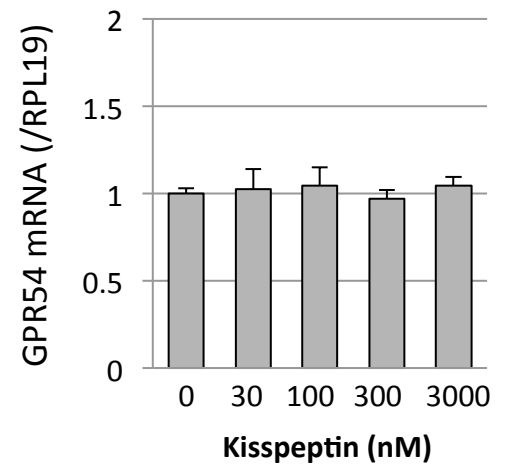
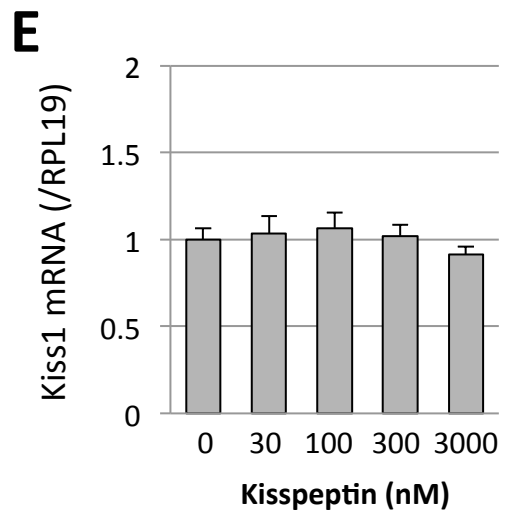
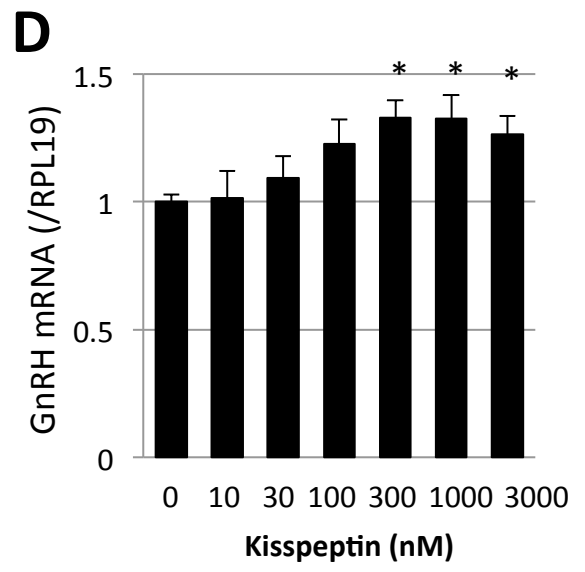
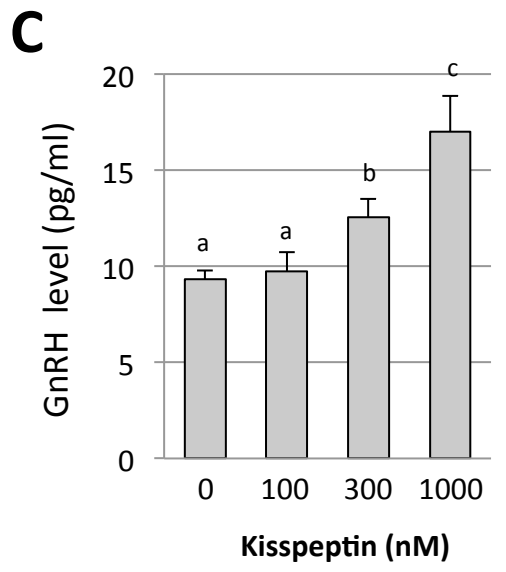
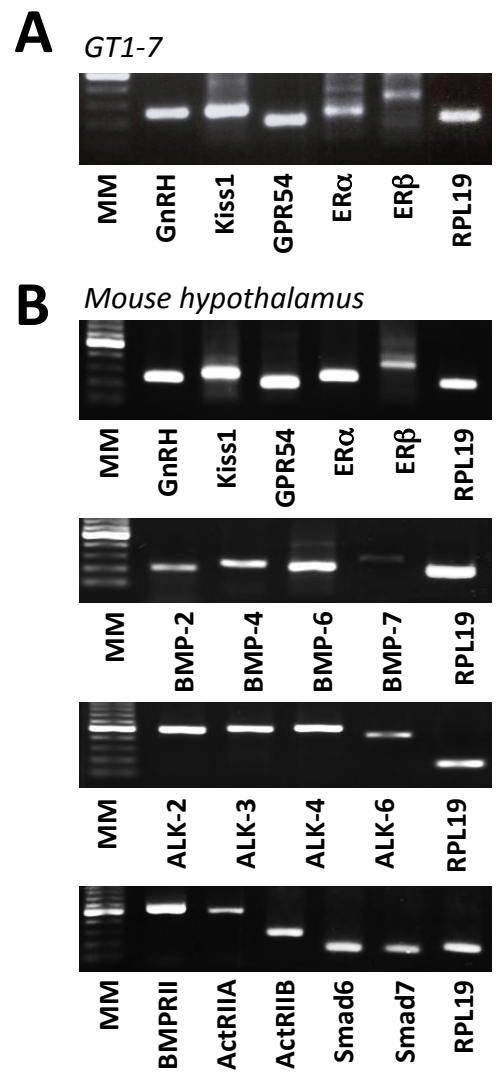
Authors:

Tomohiro Terasaka, Fumio Otsuka, *et al.*

Highlight:

- Kisspeptin and estrogen mutually enhanced the expression of ERs and GPR54 in GT1-7 cells.
- Kisspeptin increased GnRH production by ERK activation, which was suppressed by BMP-4.
- GPR54 expression was reduced by BMP-4, while BMP-Smad signaling was inhibited via kisspeptin-induced Smad6/7.
- Under the influence of estrogen, the interaction of kisspeptin and BMP-4 is a key for fine-tuning of GnRH level.

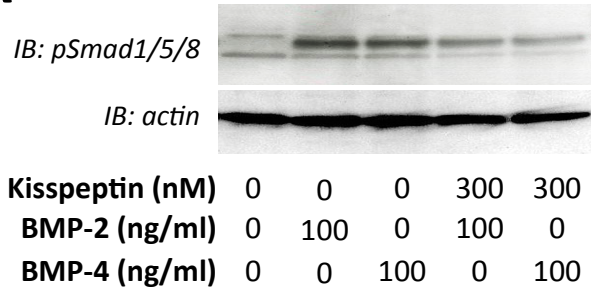
# Revised Fig. 1



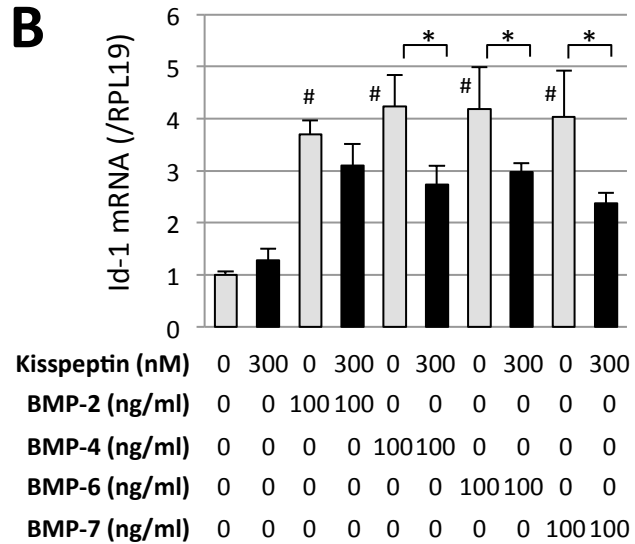


# Revised Fig. 3

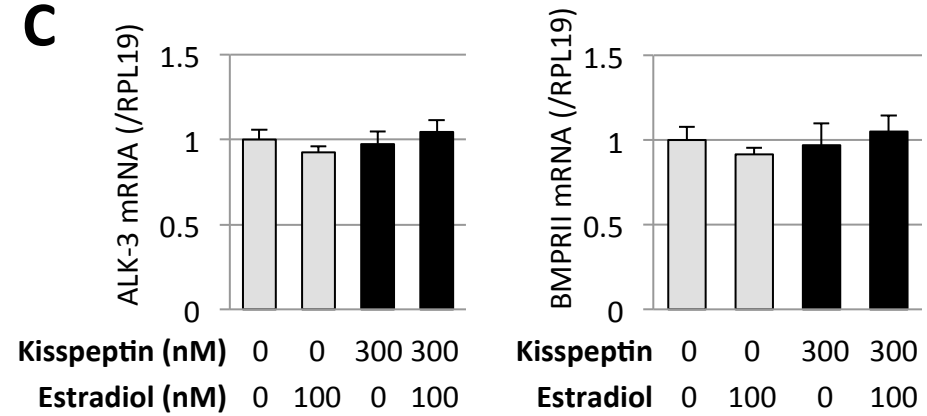
**A**



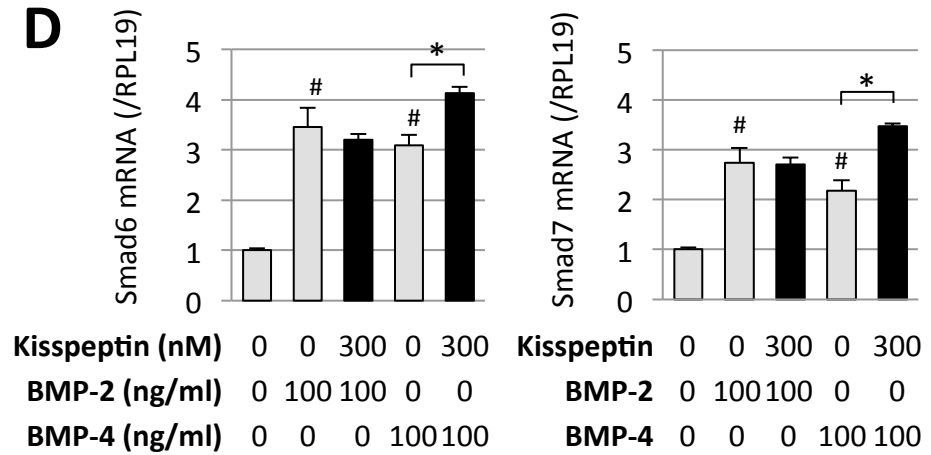
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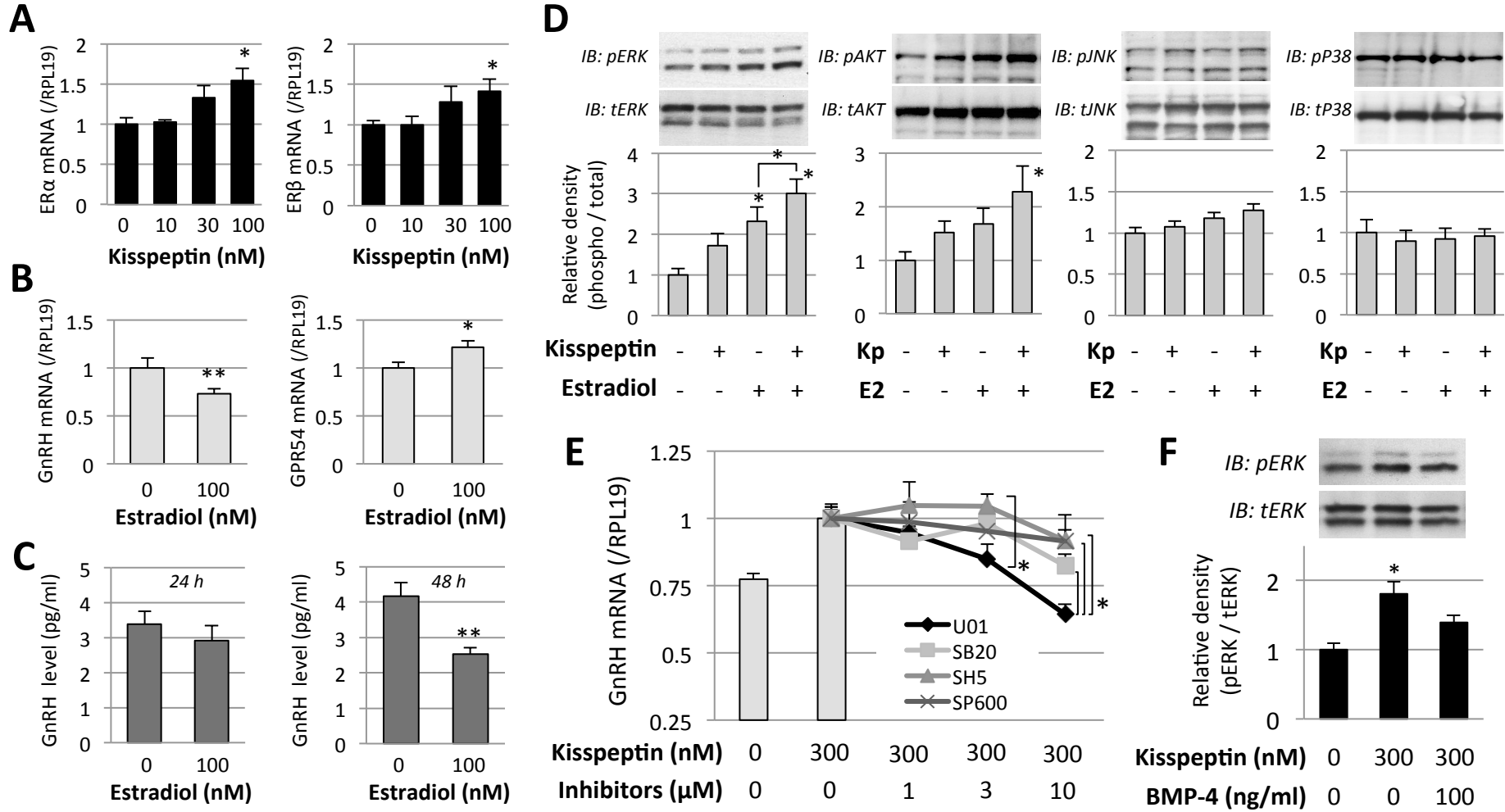
**C**



**D**



# Revised Fig. 4



# Revised Fig. 5

