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Human Type II GnRH Receptor Mediates Effects of GnRH on Cell Proliferation

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ABSTRACT—GnRH (gonadotropin-releasing hormone) is well-known as the central regulator of the reproductive system through its stimulation of gonadotropin release from the pituitary. Progress in studies on GnRH demonstrated that GnRH has both inhibitory and stimulatory effects on cell proliferation depending on the cell type, and the mechanisms of these effects have been intensively studied. However, even human GnRH receptors which mediate GnRH stimulation have not been completely identified. In the present study, we showed that the inhibitory and stimulatory effects of GnRH on colony-formation using four cell lines and have demonstrated that the inhibitory and stimulatory effects of GnRH exhibit distinctly different patterns of ligand sensitivity. This result strongly suggests that the two opposite effects of GnRH occur via different types of GnRH receptors, however expressional analyses of human GnRH receptors did not exhibit the significantly different pattern between negatively and positively responding cell lines. Then, in order to identify the GnRH receptors involved in the two opposite effects, effects of GnRH were analysed under the conditions that human GnRH receptors were knocked down by the technique of RNA interference. Consequently, it was found that human type II GnRH receptor mediates GnRH stimulation and its splice variant determines the direction of the response to GnRH. These results are the first clear evidence for the functionality of human type II GnRH receptor. Therefore our novel findings are quite noticeable and will greatly contribute to the studies on the mechanisms of the effects of GnRH on cell proliferation in the future.

Key words: gonadotropin-releasing hormone, human GnRH receptor, cell proliferation

INTRODUCTION

GnRH was originally identified as a hypothalamic decapeptide, which promotes gonadotropin secretion from pituitary gonadotropes and named gonadotropin-releasing hormone (Matsuo *et al*., 1971; Amoss *et al*., 1971). Thereafter, a number of studies demonstrated direct inhibitory effects of GnRH and its analogues on a number of tumour cell proliferation (Redding *et al*., 1983; Miller *et al*., 1985; Emons *et al*., 1993; Schally, 1999; Enomoto *et al*., 2001; Grundker *et al*., 2002) and these phenomena have been clinically applied to the treatment of hormone-related tumours (Schally, 1999; Emons *et al*., 2003). As for non-tumour cells, the direct inhibitory effect of GnRH agonist on cultured porcine granulosa cells was reported (Takekida *et al*., 2003). It was also demonstrated that GnRH has the direct stimulatory effects depending on the cell type (Batticane *et al*., 1991; Azad *et al*., 1997; Enomoto *et al*., 2001; Enomoto *et al*., 2004). However the mechanisms of the two opposite effects of GnRH on cell proliferation, especially, what determines the direction of the response to GnRH remain to be elucidated.

Whereas, wide range distribution of multiple GnRH isoforms and multiple types of GnRH receptors has been reported beyond vertebrates and thus GnRH is suggested to have diverse physiological functions (Iwakoshi *et al*., 2002; Millar, 2003; Adams *et al*., 2003; Kusakabe *et al*., 2003). In humans, type I GnRH receptor (hGnRHR-1) was firstly cloned and characterized (Kakar *et al*., 1992), and recently, the genomic loci encoding the human type II GnRH receptor (hGnRHR-2) has been cloned. However, previous studies revealed the puzzling fact on human type II GnRH receptor gene. The protein coding sequences contain a frameshift mutation and a TGA stop codon, when compared with the other primates type II GnRH receptor sequence which were

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demonstrated to be functional (Faurholm *et al.*, 2001; Neill, 2002; Morgan *et al.*, 2003). So it was suspected that hGnRHR-2 is translated into the functional receptor. Therefore, it is of great interest whether hGnRHR-2 is a functional receptor and related to the effects of GnRH on cell proliferation or not.

In the present study, we demonstrated that the colony-formation of HHUA (a human endometrial carcinoma cell line) or DU145 (a human prostatic carcinoma cell line) was decreased by GnRH, whereas that of TSU-Pr1 (a human prostatic carcinoma cell line) or Jurkat (a human mature leukemic cell line) was increased. Furthermore, in order to elucidate the human GnRH receptors involved in the effects of GnRH on cell proliferation, the effects of GnRH on the colony-formation in these four cell lines were analysed under the condition that each human GnRH receptor is knocked down by the technique of RNA interference.

MATERIALS AND METHODS

Cell culture

HHUA (a human endometrial carcinoma cell line) (Ishiwata *et al.*, 1984) and Jurkat (a human mature leukemic cell line) cells were purchased from Riken Cell Bank (Tsukuba, Japan). The DU145 cell line was a gift from the Cell Resource Centre for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). TSU-Pr1, a human prostatic carcinoma cell line, was established by Dr T. Iizumi *et al.* at the Department of Urology, Teikyo University School of Medicine (Iizumi *et al.*, 1987). Each cell line was maintained at 37°C in appropriate medium (DMEM-low glucose for TSU-Pr1, F12 for HHUA, and RPMI 1640 for Jurkat and DU145), containing 10% Nu-serum I, a semi-synthetic serum supplement (Collaborative Biomedical Products, Bedford, MA) (Enomoto *et al.*, 2002) in a humidified atmosphere of 5% CO_2/95% air.

Colony-forming efficiency assay

The colony-forming efficiency was assessed as described in our previous report (Enomoto *et al.*, 2001). A total of 48 wells in a flat-bottomed 96-well plate were inoculated with cells at each cell density indicated in the text. GnRH-I and II were purchased from Sigma (St. Louis, MO) and Cetrorelix was a gift from Prof. T. Minegishi (Department of Obstetrics and Gynaecology, School of Medicine, Gunma University). All GnRHs were dissolved at 1 mg/ml in 0.1N HCl and stored at –80°C. The final GnRH concentration added to the treatment group was 100 pM. The significance of the difference between the slopes of control and GnRH treatment groups was analyzed by the two-tailed P-value test using GraphPad Prism software 4 with default settings (GraphPad Software, San Diego, CA) (Zar, 1999).

Analysis of the ligand sensitivity

The ligand sensitivity of each cell line was examined by analyzing the number of colony-containing wells when cells were plated at the density of 8 cells per well (HHUA, DU145, and TSU-Pr1) or 20 cells per well (Jurkat). The effects of GnRH-I, II, and Cetrorelix were tested at 400 nM, 20 nM, 1.0 nM, 50 pM, 2.5 pM, 125 fM, 62.5 fM, 3.125 fM, 156.25 aM, and 7.8125 aM final concentration. The data obtained were analyzed using GraphPad Prism software 4 with default settings (GraphPad Software, San Diego, CA).

RT-PCR analysis

Total RNA was extracted from HHUA, DU145, TSU-Pr1, and Jurkat cells using ISOGEN (NIPPON GENE, Tokyo, Japan). mRNA was purified from 10 μg of total RNA, and cDNAs used as templates were synthesized using Dynabeads mRNA DIRECT^{TM} Kit (Dynal ASA, Oslo, Norway) according to the manufacturer's instructions. The primer sets used for RT-PCR analysis of hGnRHR-1 or 2 and 2v were as follows: SEhGnRHR-1 (5'-GACCTTGTCTGGAAAGATCC-3'), AShGnRHR-1 (5'-CAGGCTGATCACCACCATCA-3'), SEhGnRHR-2 (5'-TTCATCCTCCTCAGTTTCTCTCC-3'), and AShGnRHR-2 (5'-AGAAGTTTCCTTACACCTGAACG-3'). For confirming the efficacy of RNAi, the primer sets described above were also used.

RNA interference

RNAi was performed using a mammalian expression vector for siRNA-induced gene silencing, p*Silencer* 2.0-U6 (Ambion Inc., Austin, TX). The siRNA target region was selected referring to "The siRNA user guide" (Tuschl *et al.*, 2004), and designing hairpin siRNA inserts for p*Silencer* vector was done according to the manufacturer's instructions. The sequences of the siRNA inserts were as follows: hGnRHR-1SE (5'-GATCCCGATCCGAGTGACGGTTACTTTCAAGAGAAGTAACCGTCACTCGGATCTTTTTTGGAAA-3'), hGnRHR-1AS (5'-AGCTTTTCCAAAAAAGATCCGAGTGACGGTTACTTCTCTTGAAAGTAACCGTCACTCGGATCGG-3'), hGnRHR-2SE (5'-GATCCCGGTCCGAGTGGGAGTGACCTTCAAGAGAGGTCACTCCCACTCGGACCTTTTTTGGAAA-3'), hGnRHR-2AS (5'-AGCTTTTCCAAAAAAGGTCCGAGTGGGAGTGACCTCTCTTGAAGGTCACTCCCACTCGGACCGG-3'), hGnRHR-2vSE (5'-GATCCCCTAATGGCCACGTATTCTGTTCAAGAGACAGAATACGTGGCCATTAGTTTTTTGGAAA-3'), and hGnRHR-2vAS (5'-AGCTTTTCCAAAAAACTAATGGCCACGTATTCTGTCTCTTGAACAGAATACGTGGCCATTAGGG-3'). Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

In our previous study we used a synthetic GnRH agonist, Buserelin, to analyze the effects of GnRH on colony-forming efficiency (Enomoto *et al.*, 2001), but effects of native GnRH ligands, GnRH-I and II on colony-forming efficiency were examined in the present study. Fig. 1 shows the effect of GnRH-I on each cell line, and the results on GnRH-II were the same (data not shown). The colony-forming efficiencies of HHUA and DU145 were significantly decreased by GnRH, whereas those of TSU-Pr1 and Jurkat were significantly increased. It was demonstrated that a synthetic GnRH agonist, Buserelin, significantly decreases the rate of cell proliferation in HHUA cells, whereas in Jurkat cells GnRH significantly increases it in our previous study (Enomoto *et al.*, 2001). Thereafter, it was also confirmed that GnRH significantly decreases the rate of cell proliferation in DU145 cells and increases it in TSU-Pr1 cells (data not shown). The fact that the directions of the effect of GnRH on the colony-formation and on the rate of cell proliferation are identical suggests that the colony-forming efficiency assay is a useful method in order to analyze the effects of GnRH on cell proliferation.

Among the human GnRH receptors, type I (hGnRHR-1) and II (hGnRHR-2) GnRH receptors are known. Type I receptor has already cloned and well characterized. However the full-length sequence of type II receptor which is

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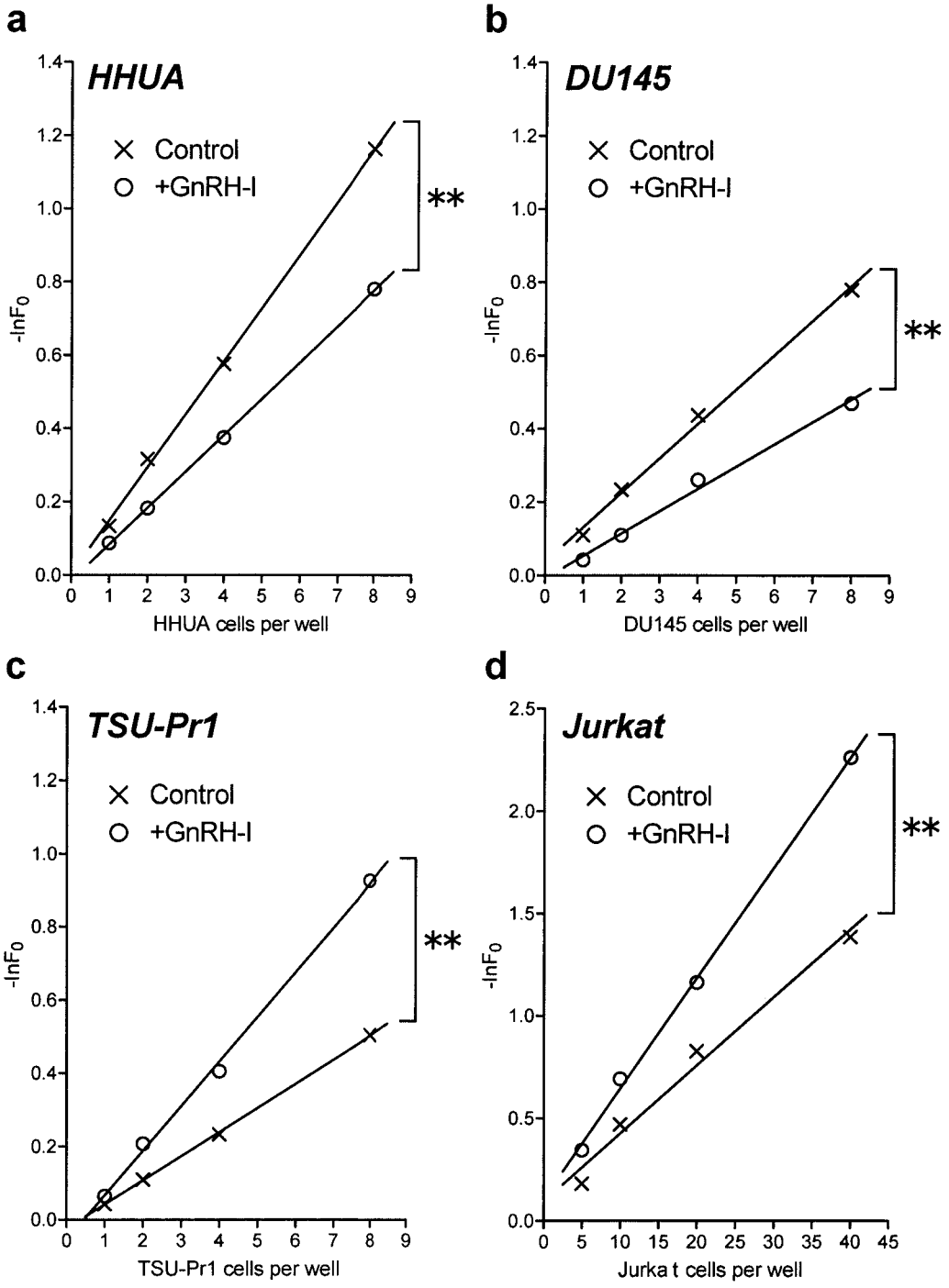
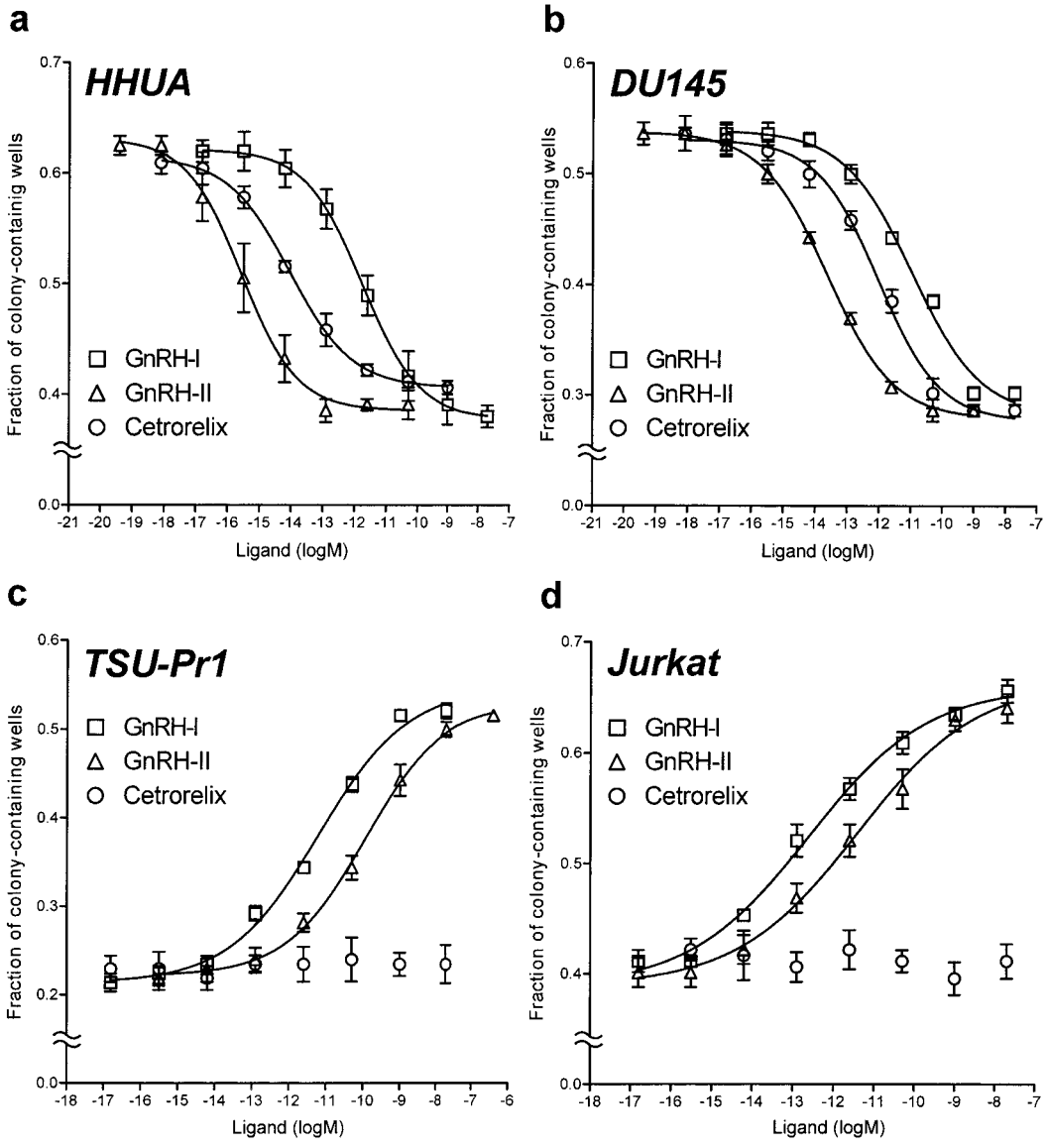


Fig. 1. Effects of GnRH-I (final concentration, 100 pM) on the colony-forming efficiency of HHUA (**a**), DU145 (**b**), TSU-Pr1 (**c**), and Jurkat (**d**) cells (**p<0.01). The number of cells plated per well was 1, 2, 4, and 8 cells in the cases of HHUA, DU145, and TSU-Pr1 cells and 5, 10, 20, and 40 cells in the case of Jurkat cells. The regression equation and coefficient of determination (r^2) of each experimental group were as follows: (**a**) ×: y = 0.145x+0.003, r^2=0.9987, ○: y=0.099x–0.016, r^2=0.9998; (**b**) ×: y = 0.094x+0.036, r^2=0.9954, ○: y=0.061x–0.079, r^2=0.9924; (**c**) ×: y = 0.066x–0.024, r^2=0.9997, ○: y=0.122x–0.055, r^2=0.9974; (**d**) ×: y = 0.033x+0.094, r^2=0.9826, ○: y=0.054x+0.106, r^2=0.9982.

translated into protein has not discovered yet. Thus, it is of great interest and importance to know whether hGnRHR-2 is a functional receptor or not and the two opposite effects of GnRH on cell proliferation occur via the same type of GnRH receptor or different types of GnRH receptors. At first, in order to obtain clues to answering these questions, the ligand sensitivities of the four cell lines used in the experiment in Fig.1 on colony-formation were analyzed, using GnRH-I, II and Cetrorelix. Humans GnRHs consist of GnRH-I (Matsuo *et al.*, 1971; Amoss *et al.*, 1971) and GnRH-II (Miyamoto *et al.*, 1984). Cetrorelix is a GnRH antagonist that has been shown to be safe and effective in inhibiting LH and

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	EC50 [logM]		
	GnRH-I	GnRH-II	Cetrorelix
HHUA	-11.77±0.248	-15.57±0.249	-14.05±0.183
DU145	-10.96±0.170	-13.62±0.115	-12.02±0.164
TSU-Pr1	-11.21±0.145	-9.95±0.191	no effect
Jurkat	-12.61±0.244	-11.42±0.369	no effect

Fig. 2. Ligand sensitivities of HHUA (**a**), DU145 (**b**), TSU-Pr1 (**c**), and Jurkat (**d**) cells with respect to colony-formation. The effects of GnRH-I, GnRH-II, and Cetrorelix on colony-forming activity were examined at 400 nM, 20 nM, 1.0 nM, 50 pM, 2.5 pM, 125 fM, 62.5 fM, 3.125 fM, 156.25 aM, and 7.8125 aM final concentration. The cell densities were 8 cells per well for HHUA, DU145, and TSU-Pr1 cells and 20 cells per well for Jurkat cells. The x and y-axis represents the concentration of GnRH ligand and the fraction of colony-containing wells (the number of colony-containing wells per the number of total wells), respectively. Results are expressed as means±SE (n=4). **e.** EC_{50} values of all nonlinearly fitted curves.

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sex-steroid secretion in a variety of animal species and to have direct inhibitory effects on tumour cells (Reissmann *et al*., 2000). The results showed distinct differences between the patterns of ligand sensitivity of negatively responding cell lines (HHUA and DU145) and those of positively responding cell lines (TSU-Pr1 and Jurkat) (Fig. 2). In HHUA and DU145 cells, GnRH-II was much more effective (1,000 to 10,000 fold) than GnRH-I, and Cetrorelix, the type I GnRH receptor antagonist, behaved as an agonist. (Fig. 2a, b). In contrast, in TSU-Pr1 and Jurkat cells, GnRH-I was more effective (about 10 fold), and Cetrorelix had no effect (Fig. 2c, d). This result strongly suggests that the inhibitory and stimulatory effects of GnRH occur via different GnRH receptors. Furthermore, the results that GnRH-II decreased colony-formation at 1,000 to 10,000 fold lower dose than GnRH-I in HHUA and DU145 cells (Fig. 2a, b) are also important information for clinical application of GnRH against hormone-related tumours.

From the results of Fig. 2, we simply presumed that the distinctly different patterns of ligand sensitivity between inhibitory and stimulatory effects of GnRH on colony-formation should be due to the different expression patterns of human GnRH receptors. In fact, Millar *et al.* (2001) demonstrated that human type I GnRH receptor and marmoset type II GnRH receptor, which has 90% amino acid identity with deduced human type II GnRH receptor amino acid sequence, exhibited distinctly different patterns of ligand sensitivity as shown in Fig. 2. Therefore, the expression patterns of human GnRH receptors were investigated (Fig. 3). For hGnRHR-1, a single PCR product was detected in all four cell lines (upper panel in Fig. 3). Whereas for hGnRHR-2, two PCR products were amplified with one primer set in all four cell lines (lower panel in Fig. 3). The results of direct sequencing demonstrated that the longer PCR product was identical with the human type II GnRH receptor (GenBank accession no. AY077708) (Van Biljon *et al*., 2002), and the lower one was identical with the human type II GnRH receptor splice variant (hGnRHR-2v) (GenBank accession no. AY081843) (Neill, 2002). As shown in Fig. 3, all four cell lines expressed hGnRHR-1, 2, and 2v, which suggests that expressional differences of human GnRH receptors could not explain the opposite response of cell proliferation to GnRH stimulation.

In results of expressional analyses of human GnRH receptors (Fig. 3), informative differences between negatively responding cell lines (HHUA and DU145) and positively responding cell lines (TSU-Pr1 and Jurkat) were not observed. Considering the possibility that hGnRHR-2 is not translated into protein, then, we performed experiments in which the expression of each of the human GnRH receptors was suppressed individually by the technique of RNA interference (RNAi) using p*Silencer* 2.0-U6, a hairpin short interfering RNA (siRNA) expression vector (Ambion Inc., Austin, TX), because it can efficiently induce sequence-specific messenger RNA degradation and thereby suppress the target gene expression (Brummelkamp *et al*., 2002; Hannon, 2002; Shi, 2003). About the specificity of the RNAi system, it was reported that a two-base mismatch between a hair pin siRNA and its target abolished inhibition (Yu *et al*., 2002). Human GnRHR-1, 2, or 2v was thus suppressed and then the effects of GnRH-I, II and Cetrorelix on the colony-forming efficiencies of the four cell lines were comprehensively analyzed. In designing experiments, the siRNA target region should be selected in a given cDNA sequence beginning 50 to 100 nucleotides downstream of the start codon (Tuschl *et al*., 2004). hGnRHR-2v is a splice variant in which 260 nucleotides are spliced out starting 26 nucleotides downstream of the start codon, and therefore different hairpin siRNA inserts were designed to silence hGnRHR-2 and 2v. Representative results are shown in Fig. 4a1-a3, and the complete results are summarized in Fig. 4c, in comparison with those of non-transfected cells. As negative controls, the same analyses were performed in cells transfected with the p*Silencer* negative control vector encoding a hairpin siRNA whose sequence is not found in the mouse, rat, or human genome databases (Ambion Inc., Austin, TX), and the resultant colony-forming efficiencies were the same as those of non-transfected cells (data not shown). The efficacy of the adopted RNAi system was confirmed by analyzing the expression of each receptor by RT-PCR in HHUA (Fig. 4b1) and Jurkat (Fig. 4b2) cells. As summarized in Fig. 4c, when hGnRHR-1 was silenced, all three GnRH ligands had no sig-

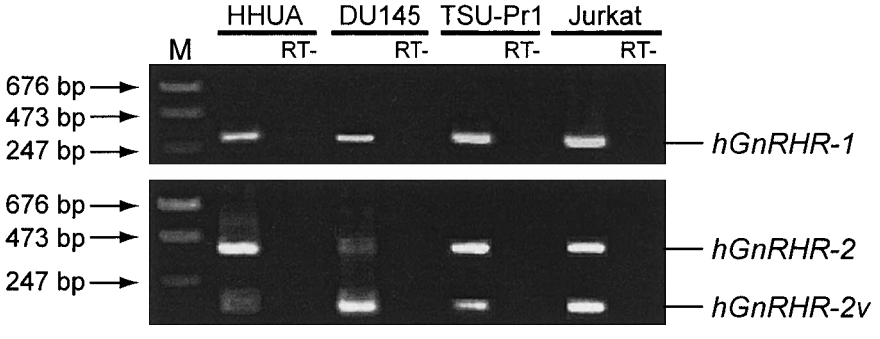
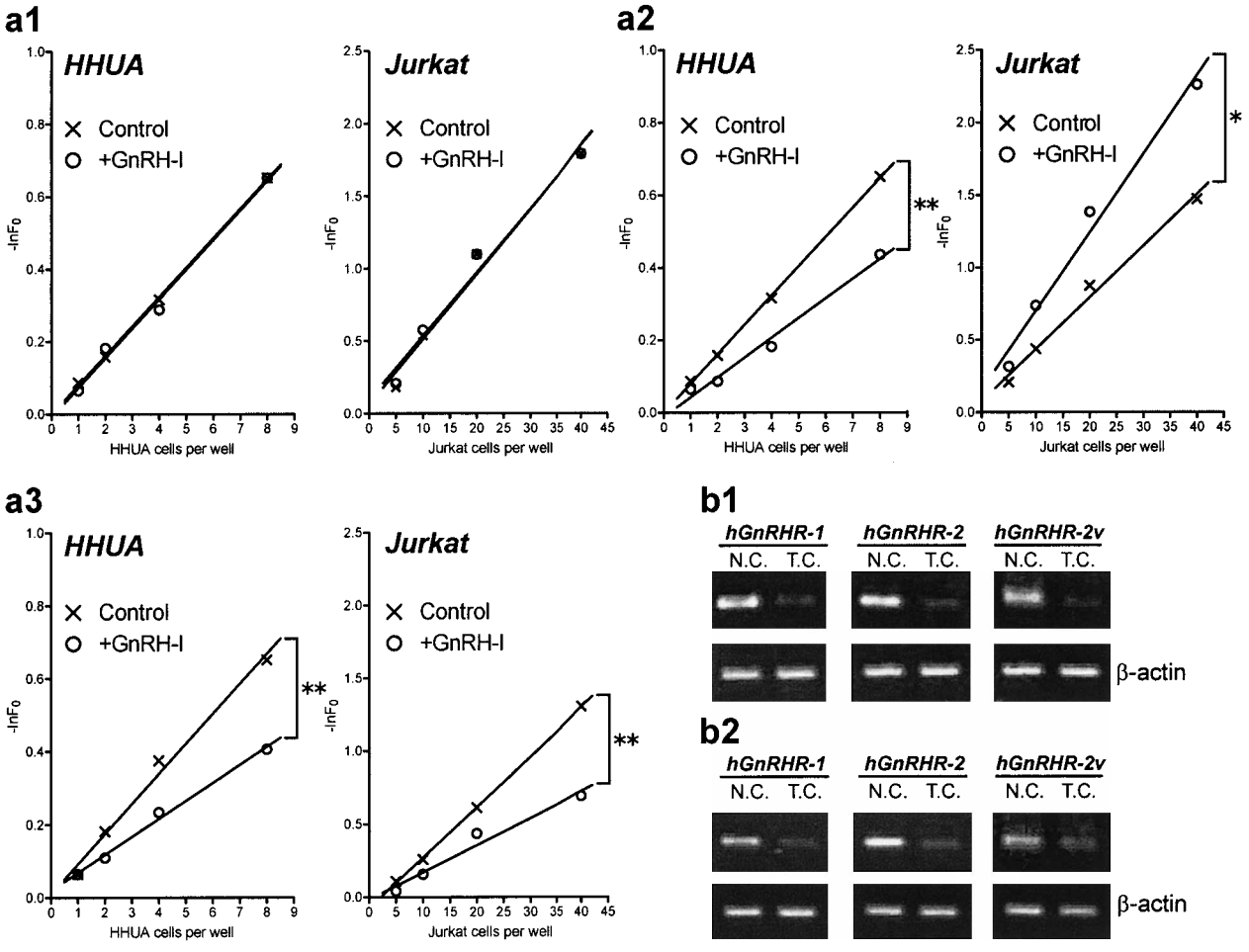


Fig. 3. Expressions of human GnRH receptor 1 (the upper panel), 2 and 2 variant (the lower panel) in HHUA, DU145, TSU-Pr1, and Jurkat cells. RT- are negative controls in which non-reverse transcribed mRNA of each cell line was used as the template. The left-hand lane contains the molecular marker.

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c

		HHUA	DU145	TSU-Pr1	Jurkat
Normal cells	GnRH-I	−	−	+	+
	GnRH-II	−	−	+	+
	Cetrorelix	−	−	n.e.	n.e.
hGnRHR-1 silenced	GnRH-I	n.e.	n.e.	n.e.	n.e.
	GnRH-II	n.e.	n.e.	n.e.	n.e.
	Cetrorelix	n.e.	n.e.	n.e.	n.e.
hGnRHR-2 silenced	GnRH-I	−	−	+	+
	GnRH-II	n.e.	n.e.	n.e.	n.e.
	Cetrorelix	n.e.	n.e.	n.e.	n.e.
hGnRHR-2v silenced	GnRH-I	−	−	−	−
	GnRH-II	−	−	−	−
	Cetrorelix	−	−	−	−

Fig. 4. Effects of RNAi of human GnRH receptors on the colony-forming efficiency of HHUA, DU145, TSU-Pr1, and Jurkat cells. **a.** Effects of GnRH-I (100 pM) on the colony-forming efficiency of HHUA and Jurkat cells when hGnRHR-1 (**a1**), hGnRHR-2 (**a2**), or hGnRHR-2v (**a3**) was silenced (*p<0.05, **p<0.01). **b.** Expression levels of human GnRH receptors in normal cells (N.C.) and cells transfected with siRNA expression vector (T.C.) in HHUA (**b1**) and Jurkat (**b2**) cells. **c.** Summary of the effects of GnRH-I,II, and Cetrorelix (100 pM, respectively) on the colony-forming efficiency of the four cell lines when hGnRHR-1, hGnRHR-2, or hGnRHR-2v was silenced. Normal cells means non-transfected cells. +, significant stimulatory effect of GnRH (p<0.05); −, significant inhibitory effect of GnRH (p<0.05); n.e., no significant effect of GnRH.

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nificant effects and the colony-forming efficiencies are similar with those of the controls. When hGnRHR-2 was silenced, GnRH-II and Cetrorelix had no significant effects and only GnRH-I was significantly effective. For hGnRHR-2v, quite interesting and remarkable results were obtained. When hGnRHR-2v was silenced, the direction of response to the stimulation of GnRH-I and II in TSU-Pr1 and Jurkat cells was reversed from a positive effect to a negative one, and Cetrorelix behaved as an agonist. To the best of our knowledge, this result is the first example that a receptor can regulate the direction of the response to the ligand stimulation. As for HHUA and DU145 cells, no such alteration was observed upon the suppression of hGnRHR-2v. Although hGnRHR-2v mRNA is expressed in HHUA and DU145 cells, it appears to have no function in these cells. This fact may be due to the balance of the expression levels of hGnRHR-2 and 2v. In fact, as shown in Fig. 3, in HHUA and DU145 cells these levels are quite different, whereas in Jurkat and TSU-Pr1 cells they are much less different; however further studies will be necessary to clarify this point. The results shown in Fig. 4 reveal that hGnRHR-1 was indispensable for the effectiveness of all three GnRH ligands, and hGnRHR-2 was necessary for the effectiveness of GnRH-II and Cetrorelix. Furthermore, hGnRHR-2v plays a role in mediating the stimulatory effects of GnRH-I and II and the effectiveness of Cetrorelix. Judging from the results of this study, Cetrorelix has only inhibitory effects, and this fact has important implications for the clinical application of GnRH analogues.

GnRH-I, which acts as the central regulator of the reproductive system through its stimulation of gonadotropin release from the pituitary via type I GnRH receptor, has a variable structure in vertebrates, whereas GnRH-II is ubiquitous and is conserved in structure from jawed fish to humans. Recently, the cognate receptor for GnRH-II has been cloned from amphibians and mammals (Millar, 2003). Especially, in primates, full-length cDNAs encoding the mammalian type II GnRH receptor have been cloned from rhesus, African green, and marmoset monkeys have been well characterized (Neill *et al.*, 2001; Millar *et al.*, 2001). The amino acid sequences of these monkey type II GnRH receptors are more than 90% identical with the putative human type II GnRH receptor. Our results indicate that hGnRHR-2 is necessary to mediate both the inhibitory and stimulatory effects induced by GnRH-II, suggesting the possibility that the cognate and functional type II GnRH receptor also exists in humans. Furthermore, silencing of hGnRHR-2 diminished the effects of GnRH-II and Cetrorelix and silencing of hGnRHR-2v only changed the stimulatory effects to the inhibitory effects and did not diminish the effects of any of the three GnRHs. These complicated results suggest that hGnRHR-2 and 2v directly interact with hGnRHR-1 and form heterodimers or that there is cross talks among signalings of hGnRHR-1, 2 and 2v. Moreover, it is also possible that hGnRHR-2 and 2v mRNAs function as non-coding RNAs (Storz, 2002) to modulate the signaling mediated by hGnRHR-1. However, in any case, further investigations of the human type II GnRH receptor and splice variants will be required to elucidate the detailed mechanisms that human type II GnRH receptor and its splice variant are involved in the effects of GnRH on cell proliferation. To the best of our knowledge, this is the first report distinctly demonstrating that the human type II GnRH receptor is functional, and, interestingly, that its splice variant determines the direction of the response to GnRH stimulation. These findings will provide quite important clues for elucidating the mechanisms of the effects of GnRH on cell proliferation.

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