GONADOTROPIN-RELEASING HORMONE TYPE-I (GNRH-I) EXPRESSION IN PERIPHERAL LYMPHOCYTES AND POSSIBLE IMMUNE ACTION*

Periferik lenfositlerde gonadotropin-releasing hormon tip-I (GnRH-I) ekspresyonu ve muhtemel immün etki

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Abstract

Purpose: *Expression of GnRH receptor type-I (GnRHR) and GnRH-I are not restricted to the hypothalamic area, and have been demonstrated in the immune system. GnRH expression in human B lymphocytes has not yet been explored. We therefore investigated GnRH-I expression in human peripheral mononuclear blood cells (PMBC) and B lymphoblastoid cells (B-LCLs). We also investigated the regulation of B-LCL proliferation by GnRH in the presence and absence of interleukin-2 (IL-2). In the present study, we report the preliminary results from our ongoing collaborative study.*

Materials and Method: *RT-PCR was used for expression of locally produced GnRH-I. The effects of GnRH-I on B-LCL proliferation were investigated using a non-radioactive cell proliferation assay.*

Results: *GnRH-I was present in PMBC and in B lymphocyte groups (normal and GnRHR defective B-LCLs). Treatment of normal B-LCLs with GnRH and with Interleukin-2 (IL-2) resulted in a significant increase in proliferation compared with the untreated control ($p<0.05$). Normal B-LCLs co-treated with IL-2 and GnRH demonstrated higher proliferative responses than IL-2 treatment alone. In GnRHR defective B-LCLs, there were no significant proliferative responses to either GnRH or IL-2 treatment, nor to IL-2 and GnRH co-treatment when compared to controls.*

Conclusion: *We therefore confirm the presence of GnRH transcript in PMBC and B-LCLs. GnRH induced a proliferative response in B-LCLs, an effect mediated by a functional GnRHR. Furthermore, the IL-2 mediated proliferative response in B lymphocytes is modulated by GnRH. Further experimental data are necessary to understand the physiological effects of local GnRH in the immune system.*

Key Words: *B lymphocytes, GnRH-I, Immune modulation, Proliferation*

Özet

Amaç: *GnRH-I ve GnRH reseptor tip-I (GnRHR) ekspresyonu santral sinir sistemiyle sınırlı değildir ve immün sistemde de gösterilmiştir. İnsan B lenfositlerinde GnRH ekspresyonu daha önce çalışılmamıştır, bu nedenle biz periferal mononükleer hücreler (PMBC) ve B lenfoblastoid hücreler (B-LCLs)'de GnRH ekspresyonunu araştırdık. Ayrıca GnRH'nın (IL-2 ile birlikte veya olmadan) B-LCL proliferasyonu üzerine etkisi de araştırıldı. Bu çalışmada halen sürmekte olan ortak çalışmamızın preliminer sonuçlarını sunduk.*

Gereç ve Yöntem: *Lokal olarak üretilen GnRH ekspresyonunu saptamak için RT- PCR kullanıldı. GnRH'nın B-LCL proliferasyonuna etkisi non-radioaktif hücre proliferasyon tetkiki ile araştırıldı.*

Bulgular: *GnRH PMBC ve B lenfosit guruplarında (normal and GnRHR defektif B-LCLs) gösterildi. Normal B-LCLs'in GnRH ve Interleukin-2 (IL-2) ile muamelesi, kontrolle karşılaştırıldığında proliferasyonda anlamlı artışa neden oldu ($p<0.05$). Normal B-LCLs'de IL-2 ve GnRH'nın birlikte uygulanması, yalnız IL-2 uygulanmasına göre artmış proliferatif cevap gösterdi. GnRHR defektif B-LCLs'de, ne GnRH ne de IL-2'ye anlamlı proliferatif cevap saptanmadı.*

Sonuç: *PMBC ve B-LCLs'de GnRH transkriptinin olduğunu konfirme etmiş olduk. GnRH B-LCLs'de proliferatif cevabı fonksiyonel GnRHR aracılığıyla indüklemektedir. Ayrıca IL-2'ye bağlı B lenfosit cevabı GnRH ile modüle edilmektedir. Lokal GnRH'nın immün cevaptaki fizyolojik rolünün aydınlatılması için ileri deneysel veriye ihtiyaç vardır.*

Anahtar Kelimeler: *B lenfosit, GnRH-I, İmmün modülasyuon, Proliferasyon*

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Gonadotropin-releasing hormone type-I (GnRH-I) is a decapeptide generally known for its role in reproductive function, through regulation of GnRH receptor (GnRHR) mediated FSH and LH secretion from pituitary gonadotropes (1). GnRH-I and

GnRHR-I expression are not however, limited to the central nervous system, and have been demonstrated in different tissues such as the ovary, breast and prostate (2, 3).

The presence of specific GnRH binding sites and expression of GnRH mRNA in the immune system has also been extensively reported, including in cultured porcine lymphocytes (4, 5), as well as in the murine thymus and spleen (6-9).

Immunoactive and bioactive production of GnRH-I has been demonstrated in human peripheral T-cells (CD4+, CD8+) and in the leukaemic (Jurkat) cell line similar to T lymphocytes (10,11). Although the lymphocyte subtypes were not specified, GnRHR-I and GnRH-I mRNA expression have been identified in human peripheral lymphocytes suggesting a potential autocrine and/or paracrine effect of GnRH in immune system regulation (12,13). In an animal model of immunodeficiency, total IgG levels and CD4+ lymphocytes increased after native GnRH administration (14). IL-2 plays an important role in lymphocyte activation and proliferation through the IL-2R complex. In several human and rat studies, regulation of IL-2R expression by GnRH-I has been demonstrated in peripheral lymphocytes although the physiological consequences of these effects remain unknown. These data do however suggest that endogenous and/or exogenous GnRH-I might have a regulatory effect on IL-2 induced cell proliferation in lymphocytes (8, 12).

Understanding the roles of GnRH in immune function might have clinical importance for several reasons. First, in recent years GnRH agonists have become widely used in a variety of disorders, even though little is known about their potential immunological effects. Second, the potential immune modulatory and programming effects of currently used drugs (especially GnRH analogues) might have potential clinical applications in severe immune deficiency conditions.

B lymphocytes are important activators in the humoral immune response and to our knowledge, the local production of GnRH-I in human B lymphocytes and its possible modulatory role in this lymphocyte subset have not previously been investigated. The present study was therefore designed to investigate the expression of GnRH-I m-RNA level in human peripheral mononuclear blood cells (PMBC) and in EBV transformed B-lymphoblastoid cells (B-LCLs). In addition, the regulation of B-LCL proliferation by GnRH-I with and without interleukin-2 (IL-2) was examined. Finally, using a naturally occurring human GnRHR-I knockout model, we analysed the functional capability of lymphocyte GnRHR-I.

MATERIAL AND METHODS

Isolation of peripheral mononuclear cells and EBV transformation

The use of human mononuclear cells for research was approved by the Royal Free and University College Medical School Local Ethics Committee. Peripheral venous blood samples were obtained from both a 43y hypogonadotrophic hypogonadism patient with an inactivating GnRHR-I gene mutation, [whose lymphocytes were previously shown in our laboratory to express a defective GnRH (13)] and an age/sex matched normal control.

The Ficoll-Hypaque method was used to isolate PMBC as previously described (12). B lymphocytes from both samples were immortalized by Epstein-Barr virus (EBV) transformation, a standard method for obtaining stable B-lymphoblastoid cell lines (15). Cells were resuspended in RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) containing 10% FBS, 100 µg/mL streptomycin, 100µg/mL ampicillin, 25 mmol/L HEPES, 2 mmol L-glutamine and incubated at 37°C, with 5% CO_2. The viability of lymphocytes was confirmed by a trypan blue exclusion test (>95%). After harvesting, the cells were prepared at a concentration of 2 x 10^6 cells/ml for RNA extraction.

Total RNA and RT-PCR amplification for GnRH-I

Total RNA was extracted from control PMBC (normal total lymphocytes), and from control and GnRHR-defective patient B-lymphoblastoid cells (normal B-LCLs and GnRHR defective B-LCLs) using a commercially available kit (RNeasy midi kit, Qiagen, West Sussex, UK). RNA concentration was quantified by absorbance at 260 nm. Total RNA (2µg) was reverse-transcribed using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK), according to the manufacturer's instructions. The integrity of the RNA samples was verified by examining the RT-PCR product of GAPDH mRNA.

The resultant cDNAs were submitted to PCR amplification using specific primers for GnRH-I (sense 5'-ATTCTACTGACTTGGTGCGTG-3' and anti sense 5'-GGAATATGTGCAACTT GGTGT-3') (12). These primers were used to amplify the major portion of cDNA encoding the precursor protein and GAP (GnRH associated peptide). The template cDNAs (2 ml) were amplified in a 50 ml PCR reaction containing, 50 pmol of each primer, 200 mmol of dNTPs, 2.5 U HotstarTaq polymerase (Qiagen, West Sussex, UK), 50 mmol/L KCl, 10 mmol/L Tris-HCl(pH 8.3) and 2 mmol/L $MgCl_2$. PCR reaction for GnRH -I was carried out for 40 in the following sequence; denaturation at 94°C for 1 min, annealing at 55°C for 40 s, extension at 72°C for 1 min, final extension at 72°C for 15 min.

*The quality and predicted size of PCR products in 3 groups (***380 bp for GnRH-I***) were assessed by 1.5 % agarose gel electrophoresis. PCR products from normal total lymphocytes, normal B-LCLs and GnRHR-defective B-LCLs were sequenced by automatic DNA sequencing using dye terminator chemistry in an ABI 377 sequencer (MWG, Ebersberg, Germany). The sequencing results were matched with the available sequences using the BLAST (basic length alignment search tool) program provided by the National Centre for Biotechnology Information.*

Proliferation Assay

Normal B-LCLs and GnRHR defective B-LCLs were plated in 96-well flat bottom tissue culture plates (Nunclon, NY, USA) at a concentration of $1x10^4$ cells/100 ml in the culture medium described above. Just after the onset of culture, cells were treated in triplicate with either IL-2 50 IU/ml (Sigma-Aldrich, Dorset, UK), human GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2; Sigma-Aldrich, Dorset, UK; 10^{-5} M and 10^{-9} M), IL-2 (50 IU/ml) + GnRH-I (10^{-10} M to 10^{-5} M), or GnRH antagonist (Cetrorelix; Ac-d-Nal-d-Cpa-D-Pal-Ser-Tyr-d-Cit-Leu-Arg-Pro-d-Ala-NH_2; ASTA medica, Frankfurt, Germany; 10^{-6} M) + IL-2 (50 IU/ml)+ hGnRH-I (10^{-5} M). In addition, normal B-LCLs were treated with the GnRH antagonist (10^{-6} M) + IL-2 (50 IU/ml). Plates were incubated for 72 hours at 37 °C with 5% CO_2. Cell proliferation was estimated according to the manufacturer's instructions with the Cell Titer 96 AQ_{ueous} non-radioactive cell proliferation assay using tetrazolium compounds (Promega, Southampton, UK). At the end of a 72 hours incubation period, 20 ml of the MTS/PMS solution was added directly to each well and incubated for 4 h. The absorbance (Mean Optic Density: mean OD) was measured at 492 nm using an ELISA plate reader (Anthos ht III). The blank comprised 100 ml of culture medium, and absorbances of samples were corrected by subtracting blank absorbance. To confirm the reproducibility of the assay, each experiment design was performed 3 times and means of each experiment were pooled for subsequent analysis.

Statistical Analysis

Data in the proliferation assay were expressed as mean and standard error of the mean (mean ± SEM) OD. Pooled data were compared by one-way ANOVA followed by post-hoc comparison of individual groups by unpaired t-test (adjusted for multiple hypothesis testing) after Levene's test had shown that variances were homogenous.

RESULTS

Expression of GnRH-I in peripheral lymphocytes

*The predicted size of RT-PCR products (***380-bp for GnRH-I***) from normal total lymphocytes, normal B-LCLs and GnRHR defective B-LCLs were confirmed using agarose gel electrophoresis (Fig.1). These cDNAs encoding GnRH-I precursors were sequenced and found to be identical to their hypothalamic counterparts in the 3 lymphocyte groups. These data established the expression of GnRH-I mRNA in PMBC and in B-LCLs with and without the GnRHR-I mutation.*

Effects of GnRH-I and IL-2 on B-Lymphoblastoid cell (B-LCL) proliferation

To evaluate the modulatory role of GnRH-I, IL-2 and their combined effect on B lymphocyte proliferation, the MTS/PMS assay was performed in both B-lymphoblastoid cells (normal and GnRHR defective B-LCLs).

Treatment of normal B-LCLs with GnRH-I (10^{-5} M and 10^{-9} M) and with IL-2 (50 IU/ml) resulted in a significant increase in proliferation compared with untreated control ($p<0.05$; Table 1). Co-treatment with IL-2 and GnRH (10^{-10} to 10^{-5} M) increased proliferation significantly in normal B cells at all concentrations of GnRH when compared with control ($p< 0.05$); the highest response was seen with IL-2 + GnRH 10^{-5} M ($p<0.001$; Table 1). In all groups, normal B-LCLs co-treated with IL-2 and GnRH demonstrated higher proliferative responses than IL-2 treatment alone, although the enhancement of GnRH on IL-2 response was significant only at 10^{-7} M, 10^{-6} M, 10^{-5} M GnRH concentrations ($p<0.05$). The GnRH antagonist (Cetrorelix; 10^{-6} M) co-incubated with IL-2+ GnRH 10^{-5} M treated normal B-LCLs, resulted in a significant decrease in proliferation when compared to IL-2+GnRH 10^{-5} M alone ($p< 0.05$). To demonstrate the autocrine/paracrine effect of locally produced GnRH-I on IL-2 induced lymphocyte proliferation, we treated normal B-LCLs with IL-2+GnRH antagonist and a significant decrease in proliferation was observed when compared with IL-2 treatment alone ($p< 0.05$). Treatment effects and mean OD values in normal B-LCLs are shown in Table I.

In GnRHR defective B-LCLs, there were no significant proliferative responses to either GnRH (10^{-9} and 10^{-5} M) or IL-2 treatment, nor to co-treatment with IL-2 and GnRH (10^{-10} to 10^{-5} M) when compared with control level ($p>0.05$) (Table II). Although there was a trend for a mean OD increase in IL-2 treated group, this was not statistically significant; the GnRHR antagonist (Cetrorelix; 10^{-6} M) did not affect the proliferative profile of GnRHR defective B-LCLs either (Table II).

When we compared the proliferation responses of GnRHR defective B-LCLs and normal B-LCLs for each particular group treated with GnRH-I and/or IL2, normal transformed B-LCLs demonstrated a significant difference in proliferation over GnRHR mutant B-LCLs ($p <0.05$), although there was no baseline difference ($p>0.05$) (Table III).

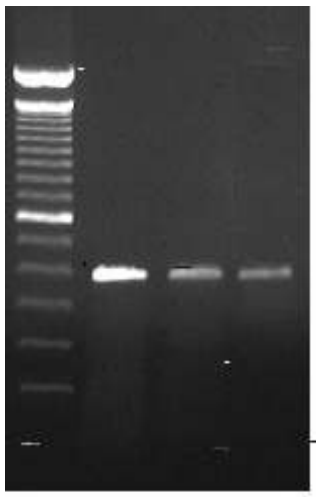

Figure 1. *Agarose gel-electrophoresis (1.5 %) showing RT-PCR products for GnRH-I mRNA using specific primers. Lane 1, Normal total lymphocyte cDNA; Lane 2, GnRHR defective B-LCL cDNA; Lane 3, normal B-LCL cDNA*

Table I. *Effects of GnRH -I (10^{-10} M to 10^{-5} M) and /or IL-2 (50 IU/ml) on normal B-LCL proliferation. The mean OD's (mean ± SEM) represent three separate experiments in triplicates. Levels of mean OD's are also expressed as a percentage (mean ± SEM) of the level in control (without GnRH-I treatment). The treatment groups are; control (TG 1) , GnRH 10^{-9} M (TG 2), GnRH 10^{-5} M (TG 3), IL-2 (TG 4), IL-2+ GnRH 10^{-10} M (TG 5), IL-2+ GnRH 10^{-9} M (TG 6), IL-2+ GnRH 10^{-8} M (TG 7), IL-2+ GnRH 10^{-7}M (TG 8), IL-2+ GnRH 10^{-6} M (TG 9), IL-2+ GnRH 10^{-5} M (TG 10), IL-2+ GnRH Antagonist 10^{-6} M+ GnRH 10^{-5} M (TG 11), IL-2+ GnRH Antagonist 10^{-6} M (TG 12) , respectively.*

	TREATMENT GROUPS (TG)											
	TG 1	*TG 2*	*TG 3*	*TG 4*	*TG 5*	*TG 6*	*TG 7*	*TG 8*	*TG 9*	*TG 10*	*TG 11*	*TG 12*
Mean OD	*0.374± 0.004*	*0.447± 0.01* [a]	*0.494± 0.02* [a]	*0.496± 0.01* [a]	*0.535± 0.03* [a]	*0.582± 0.03* [a]	*0.547± 0.01* [a]	*0.624± 0.01* [a,b]	*0.615± 0.03* [a,b]	*0.646± 0.05* [a,b]	*0.411± 0.02* [c]	*0.432± 0.01* [b]
% of Control	*100 %*	*119.5 ± 4.7%* [a]	*131.9 ± 6.9%* [a]	*132.6± 3.3%* [a]	*143.0 ± 8.6%* [a]	*155.6 ± 9.5%* [a]	*146.1 ± 4.7%* [a]	*166.7 ± 5.2%* [a,b]	*164.4 ± 8.1%* [a,b]	*172.7 ± 13 %* [a,b]	*109.9 ± 5.6%* [c]	*115.4 ± 4.0%* [b]

Table II. *Effects of GnRH –I (10^{-10} M to 10^{-5} M) and /or IL-2 (50 IU/ml) on GnRHR defective B- LCL proliferation. The mean OD's (mean ± SEM) represent three separate experiments in triplicates. Levels of mean OD's are also expressed as a percentage (mean ± SEM) of the level in control (without GnRH-I treatment). The treatment groups are; control (TG 1) , GnRH 10^{-9} M (TG 2), GnRH 10^{-5} M (TG 3), IL-2 (TG 4), IL-2+ GnRH 10^{-10} M (TG 5), IL-2+ GnRH 10^{-9} M (TG 6), IL-2+ GnRH 10^{-8} M (TG 7), IL-2+ GnRH 10^{-7}M (TG 8), IL-2+ GnRH 10^{-6} M (TG 9), IL-2+ GnRH 10^{-5} M (TG 10), IL-2+ GnRH Antagonist 10^{-6} M+ GnRH 10^{-5} M (TG 11) , respectively.*

	TREATMENT GROUPS (TG)										
	TG 1	*TG 2*	*TG 3*	*TG 4*	*TG 5*	*TG 6*	*TG 7*	*TG 8*	*TG 9*	*TG 10*	*TG 11*
Mean OD	*0.335± 0.01*	*0.288± 0.01*	*0.328± 0.01*	*0.372± 0.003*	*0.336± 0.009*	*0.362± 0.01*	*0.354± 0.02*	*0.326± 0.01*	*0.328± 0.01*	*0.326± 0.01*	*0.336± 0.02*
% of Control	*100 %*	*86.1 ± 5.9%*	*98.1 ± 4.6%*	*111.0± 1.1%*	*100.3 ± 2.8%*	*100.8 ± 3.0%*	*105.7 ± 7.1%*	*97.4 ± 3.6%*	*97.9 ± 5.0%*	*97.3 ± 3.7%*	*100.2 ± 3.0%*

Table III. *Comparison of proliferation responses to GnRH-I and/or IL-2 treatments of normal B- LCLs and GnRHR defective B-LCLs*

Treatment Groups	*Mean Optic Densities* *normal B-LCLs*	*GnRHR defective B-LCLs*	*P*
Control	*0.374±0.004*	*0.335±0.01*	*>0.05*
GnRH 10^{-9} M	*0.447±0.01*	*0.288±0.01*	*<0.05*
GnRH 10^{-5}M	*0.494±0.02*	*0.328±0.01*	*<0.05*
IL-2 (50 IU/ml)	*0.496±0.01*	*0.372±0.003*	*<0.05*
GnRH 10^{-10} M+IL-2	*0.535±0.03*	*0.336±0.009*	*<0.05*
GnRH 10^{-9} M+ IL-2	*0.582±0.03*	*0.362±0.01*	*<0.05*
GnRH 10^{-8}M+ IL-2	*0.547±0.01*	*0.354±0.02*	*<0.05*
GnRH 10^{-7} M+ IL-2	*0.624±0.01*	*0.326±0.01*	*<0.05*
GnRH 10^{-6} M+ IL-2	*0.615±0.03*	*0.328±0.01*	*<0.05*
GnRH 10^{-5}M+ IL-2	*0.646±0.05*	*0.326±0.01*	*<0.05*

Values are (mean ± SEM) of triplicates for three separate experiment;
P, normal B-LCLs versus GnRHR defective B-LCLs

DISCUSSION

The present study clearly indicates the expression of GnRH-I mRNA (identical hypothalamic GnRH-I) in human PMBC and B-LCLs.

EBV transformed B lymphocytes (LCLs), widely used to study B cell physiology, were used to evaluate the modulatory role of GnRH-I in lymphocyte proliferation (15). Over the past several years, there has been increasing interest in the role of B cells in autoimmune diseases and the factors regulating B cells (16-18). Findings in rats have demonstrated the stimulation of splenocyte and thymocyte proliferative activity (with and without mitogen) after incubation with native GnRH-I and following a GnRH analogue (8;19). In a human study, both local production of GnRH in a T cell line and a stimulatory effect of exogenous and endogenous GnRH have been demonstrated (11). Although observations in the human are not extensive, such studies support a regulatory effect of GnRH in cell-mediated immunity.

In the present study, treatment of normal B-LCLs by native GnRH-I produced a significant proliferative response when compared with control, suggesting a potential regulatory role of GnRH-I in B lymphocyte proliferation. The mechanism of GnRH-I induced proliferation is unknown, but may be mediated by activation of PKC and a decrease in cAMP level (8, 11). A further aim of this study was to confirm the functional capacity of GnRHR-I on lymphocytes. In our study, the absence of a significant proliferative response to GnRH stimulation in GnRHR defective B-LCLs confirms that GnRH-I action on lymphocytes is via a functional GnRHR-I.

IL-2 induces proliferation and/or activation of T and B cells, and its receptor expression (IL-2R) is stimulated in rat thymocyte and splenocyte cultures incubated with native GnRH and its analogue (8). Stimulation of IL-2Rγ mRNA expression by GnRH and GnRH analogue has also been observed in human PMBC (12). Regulation of IL-2R expression may therefore be an important target of GnRH action in immune cells. In normal B-LCLs, co-treatment of GnRH and IL-2 demonstrated significant dose-dependent enhancement of proliferation when compared with IL-2 treatment alone. However, in the GnRH receptor mutant cells, we could not observe a significant proliferative response to IL-2 stimulation (alone and/or co-treatment) confirming that absence of GnRH action has a significant influence on IL-2 mediated cell proliferation. Moreover, when normal B-LCLs were treated with IL-2+GnRH antagonist, an apparent decrease in proliferation was observed compared to IL-2 treatment, implying that local tonic expression of GnRH contributes to the IL-2 response. Based on these experimental findings, an interaction between IL-2 induced cell proliferation and both exogenous and local GnRH-I via GnRHR can be envisaged in B-LCLs. Further studies are necessary to identify whether regulation of the IL-2 response might be at the IL-2R mRNA level and /or at the post-receptor level.

The clinical significance of local lymphocyte GnRH-I expression, the prominent proliferative effect of exogenous GnRH-I on lymphocytes and the interaction with IL-2 response are unclear. Jacobson et al. have suggested that exogenous or local GnRH may play a role in the exacerbation of autoimmune disorders (20). Although clinical data are at present rudimentary, exacerbation of lupus nephritis and thrombocytopenia has been reported in 2 separate case reports after GnRH analogue administration (21,22). The immune stimulatory effects of GnRH and the relation between IL-2 response and GnRH at immune system level may have potential clinical implications for GnRH and GnRH analogues, in immune deficiency conditions such as HIV infection or after bone marrow transplantation when immune reconstitution is crucial.

In conclusion, the preliminary data in our ongoing study shows the expression of GnRH-I mRNA in PMBC and B-LCLs. To our knowledge, our observations provide the first evidence for a functional effect of GnRH-I via a functional GnRHR-I in B-LCLs. Further experimental data in human are warranted to explore the clinical implications of these data.

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