

Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) action on the same receptor results in different in vitro intracellular signaling in mouse primary Leydig cells.

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Introduction

Human luteinizing hormone (LH) and chorionic gonadotropin (hCG) are two glycoprotein hormones involved in reproduction. Despite binding the same receptor (LHCGR), are both used in clinical practices for assisted reproduction. LHCGR mediates specific LH/hCG-specific signaling, likely through the hinge region of the receptor, which is responsible for LH/hCG differentiation [3]. Quantitative and qualitative *non*-equivalence of hCG and LH was previously demonstrated in human primary granulosa cells *in vitro* [1,2]. However, experiments in steroidogenic cells from males are missing. Since Mice Leydig cells are naturally expressing the murine LH receptor (Lhr), they are a suitable model to evaluate the action of the gonadotropins at molecular level. Although Lhr and LHCGR doesn't share a complete sequence identity (80%), the murine receptor retains the ability of human LH and hCG binding.

Aim

The aim of this study is to compare the LH- and hCG-mediated signaling and downstream events in mouse primary Leydig cells *in vitro*.

Study design

Leydig cells were collected from testis of 3/5-months-old C57BL6 mice by density gradient, then they were cultured and treated by increasing doses of recombinant LH and hCG (1 pM-100 nM range). cAMP accumulation experiments were performed in the presence of 500 μM the phosphodiesterase inhibitor IBMX, then measured by ELISA as well as testosterone synthesis. ERK1/2 and CREB phosphorylation was evaluated by Western blotting, gene expression by real-time PCR.

Results

Dose-response curves revealed that hCG is about 10-fold more potent than LH in inducing cAMP recruitment (hCG EC₅₀=18.64±10.14 pM; LH EC₅₀=192.00±53.96 pM; Mann-Whitney's U-test; p<0.05; n=4), despite achieving the same plateau level at the 100 nM dose. LH and hCG stimulated the activation of ERK1/2 in the 10 pM-100 nM range over basal, moreover achieving higher levels upon hCG *versus* LH stimulation in the 0.1 pM-100 nM range (two-way Anova and Bonferroni post-test; p<0.05; n=5), reflecting its prevalent cAMP-dependence. LH and hCG treatments induced about equal levels of CREB phosphorylation, both resulting in a significant pCREB increase over basal between the 10-100 pM doses (two-way Anova and Bonferroni post-test; p<0.05; n=5). Downstream cell signaling-mediated events, i.e. gene expression and testosterone production, were not significantly different upon LH and hCG treatments. Indeed, the two gonadotropins induced similar *Stard1* gene expression and EC₅₀ for steroid synthesis (Mann-Whitney's U-test; p<0.05; n=4).

Discussion

Murine Lhr mediates a different cAMP and PKA-dependent pERK1/2 activation upon human LH and hCG treatment. However, no downstream CREB phosphorylation, gene expression and testosterone production occur, likely due to an opposite, balancing effect between cAMP and pERK1/2 on steroidogenesis. The *non*-human receptor mediates quantitatively but not qualitatively different LH/hCG-specific signaling, oppositely to what previously described in human primary granulosa cells. In fact, the comparison between LHCGR and Lhr amino acid sequences revealed no identity within the hinge regions. This finding is relevant to improve the method for gonadotropin characterization based on the rat bioassay [4], which rely on the evaluation of *in vivo* effects mediated by *non*-human receptor.

References

1. Casarini et al. PLoS One. 2012. 7(10); 2. Casarini et al. Mol Cell Endocrinol. 2016. 422:103-14.; 3. Grzesik et al. Front Endocrinol (Lausanne). 2015. 6:140; 4. Christin-Maitre et al. Methods 2000; 21(1): 51-7.

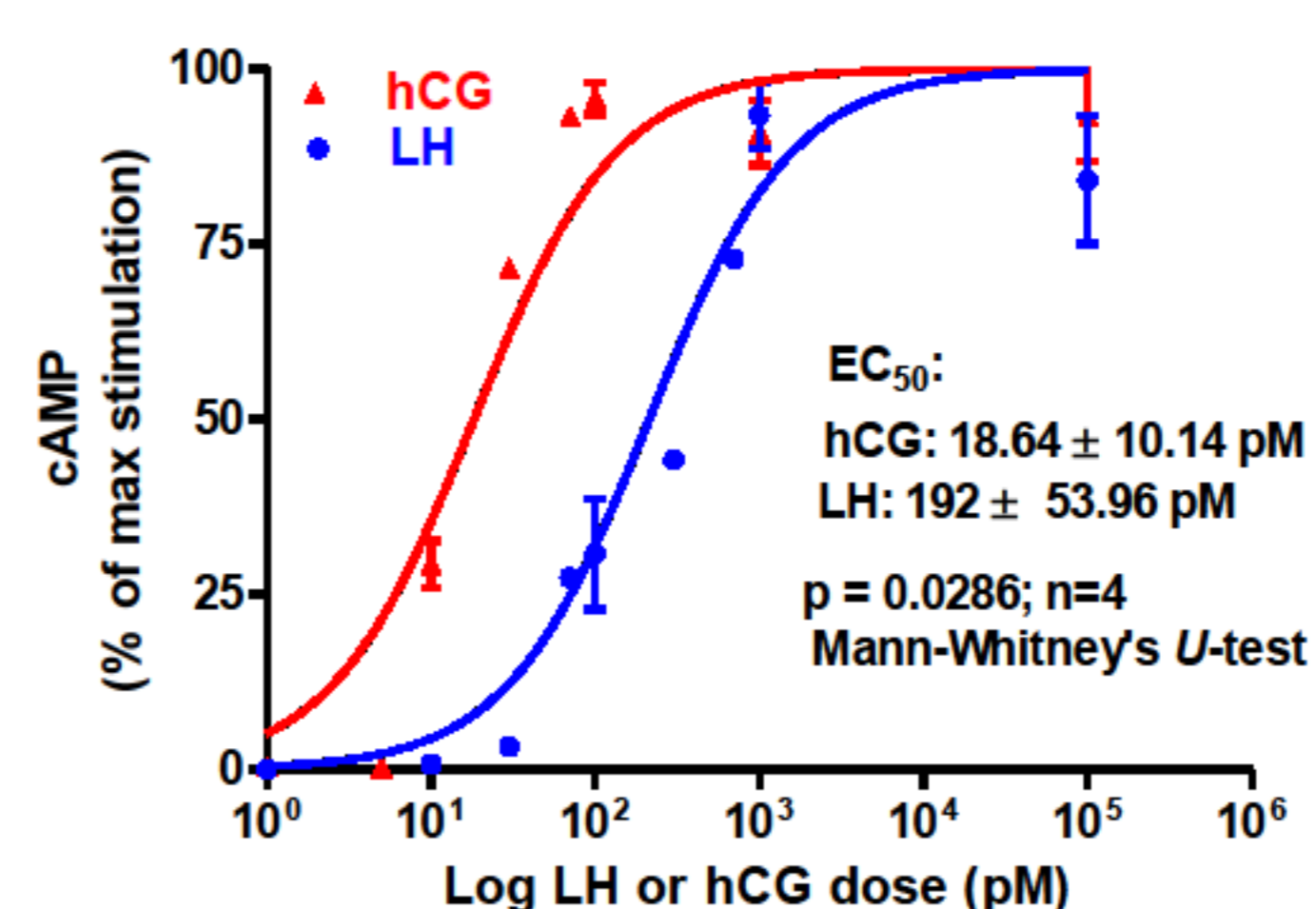


Figure 1. Evaluation of cAMP production. Murine primary Leydig cells were stimulated with increasing doses of hCG and LH in the presence of IBMX. Total cAMP was measured after 3 hours of incubation. Total cAMP levels normalized as percentage of the maximal response. All the results are represented as means SEM in a logarithmic X-axis, then non-linear regressions were plotted. The EC₅₀ values were compared by Mann-Whitney's U-test (p<0.05; n=4).

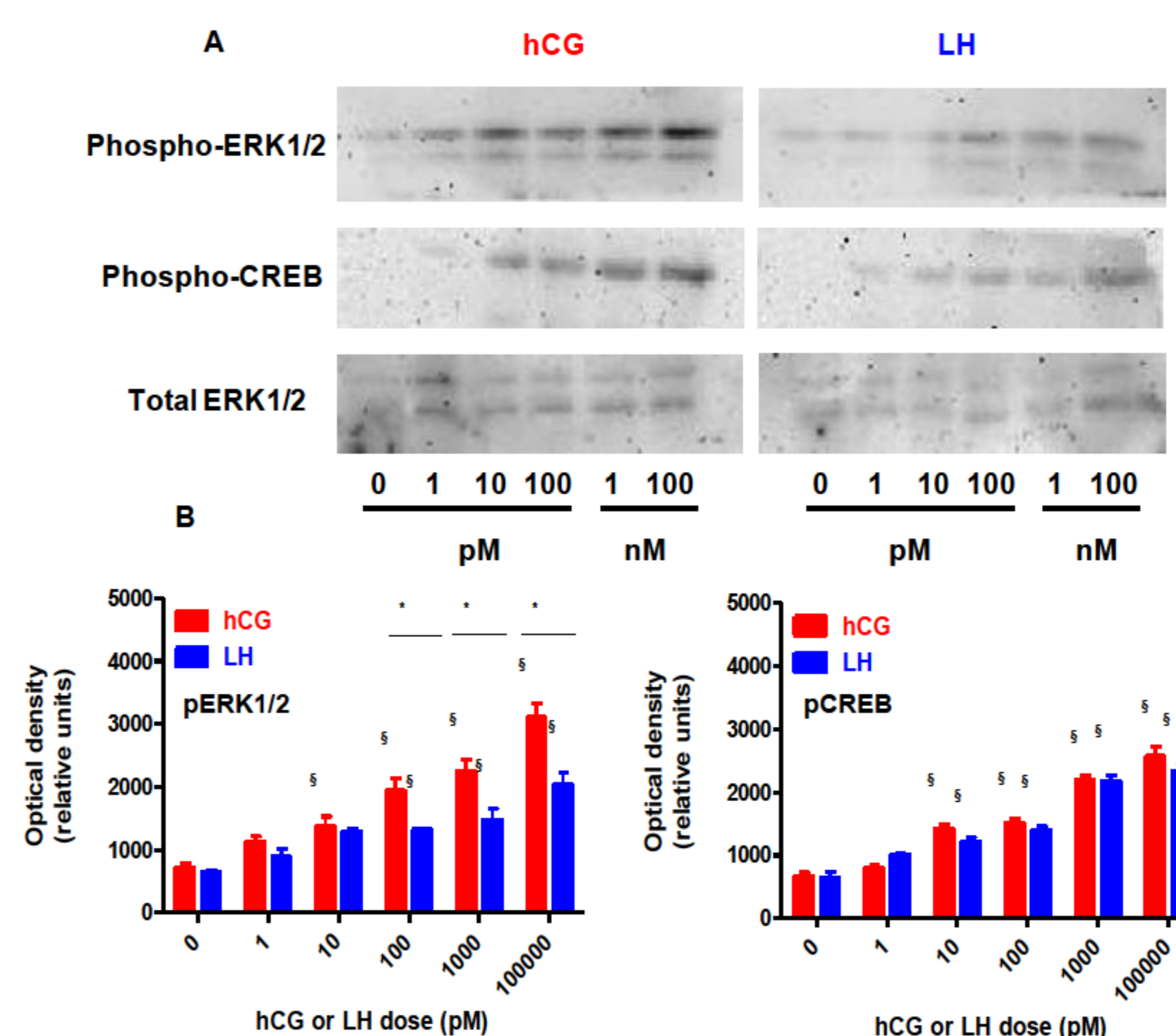


Figure 2. Evaluation of phospho-ERK1/2 and phospho-CREB activation in murine Leydig cells. A) The cells were stimulated for 15 minutes by increasing doses of hCG and LH, then pERK1/2 and pCREB signals were evaluated by Western blotting (image representative of five independent experiments). B) Semiquantitative analysis of pERK1/2 and pCREB signals. The values were normalized over the loading control total ERK (means SEM; n=5). =significant vs control; *=significant hCG vs LH (2-way ANOVA and Bonferroni post-test; p<0.05).



Figure 4. Amino acid sequence alignment of human LHCGR and murine Lhr hinge region. *Homo sapiens* LHCGR and *Mus musculus* Lhr sequences (NP_000224 and NP_038610.1, respectively) were obtained from the National Center for Biotechnology Information (NCBI) database (<http://www.ncbi.nlm.nih.gov/>). No color indicates aminoacidic residues fully conserved; green indicates conservation of strong groups; purple indicates conservation of weak groups and gray indicates no consensus.

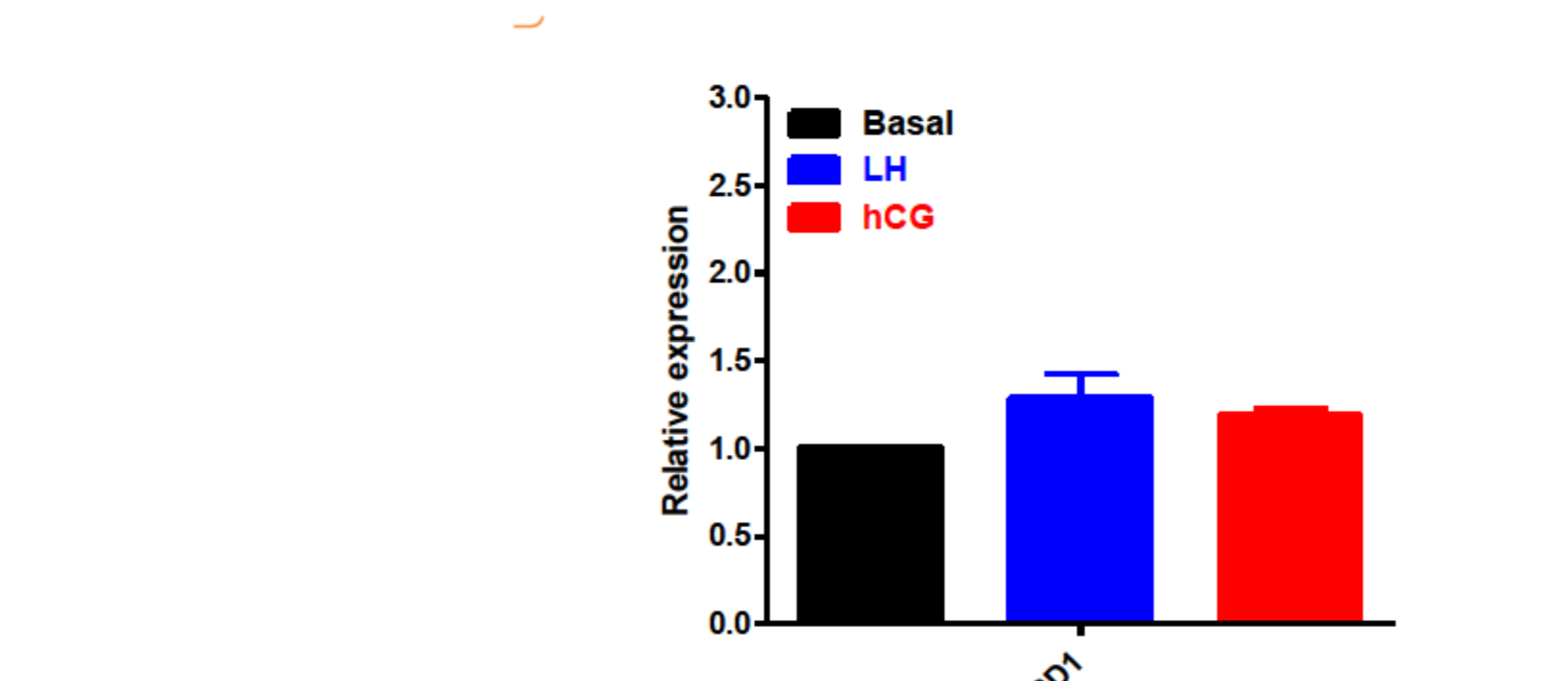


Figure 5. *Stard1* gene expression analysis. LH- or hCG-induced *Stard1* gene expression in 12 h-stimulated mouse Leydig cells. The cells were treated using the LH and hCG EC₈₀ doses. *Hprt* gene expression served as normalizer (mean SEM; =significant versus basal; Mann-Whitney's U-test; p<0.05; n=4).

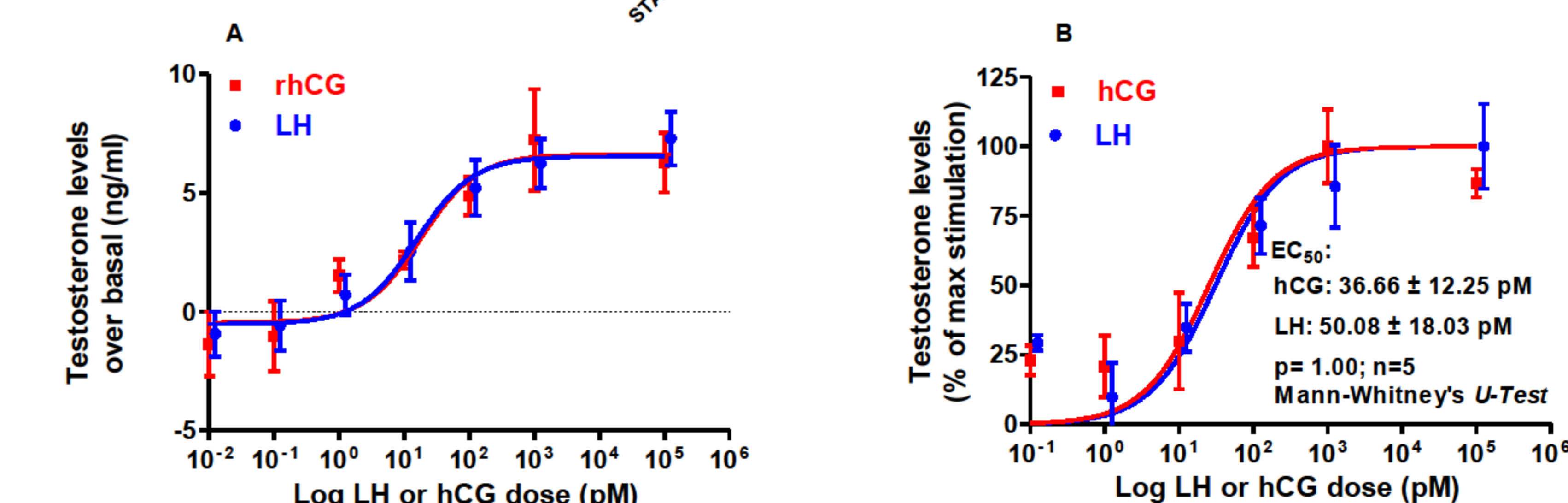


Figure 6. Evaluation of Testosterone production upon hCG or LH stimulation. Murine primary Leydig cells were stimulated by increasing LH or hCG doses, in the presence of 500μM IBMX, and total testosterone was measured by immuno-assay after 24 h. A) Testosterone levels expressed as ng/ml. B) Testosterone production normalized in percentage of maximal response. All the results are represented as means SEM in a logarithmic X-axis, then non-linear regressions were plotted. The EC₅₀ values were compared by Mann-Whitney's U-test (p<0.05; n=5).

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