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Proliferative *versus* apoptotic signals in granulosa cells: β-arrestins as switch between life and death signals *in vitro*

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INTRODUCTION

Molecular mechanisms underlying folliculogenesis result from a delicate balance between proliferative, steroidogenic and apoptotic stimuli mediated by gonadotropins. In this study we used the human granulosa cell line hGL5 [1], which are traditionally assumed as not responsive to gonadotropins, to demonstrate how follicle-stimulating hormone (FSH) and luteinizing hormone (LH) oppositely propend towards proliferative or apoptotic signals, revealing novel, unexpected mechanisms regulating ovarian folliculogenesis.

METHODS

We evaluated the FSHR and LHCGR expression in hGL5 cells maintained under different serum (FBS-cs) concentrations (between 0 and 15%) by Western blotting. The response to 50 nM FSH or 100 pM LH was evaluated by measuring cAMP and progesterone production by ELISA, as well as ERK1/2 and CREB phosphorylation by Western blotting. Cell viability was assessed by proliferation assay and confocal imaging. These endpoints were evaluated in the presence and in the absence of selective inhibitors or agonists (i.e. the PKA inhibitor H-89, the phorbol ester PMA as a PKC-ERK1/2 activator, and siRNA against β-arrestin1/2). The effects mediated by FSHR and LHCGR were confirmed using long-term culture of transfected hGL5 cells permanently overexpressing these receptors.

RESULTS

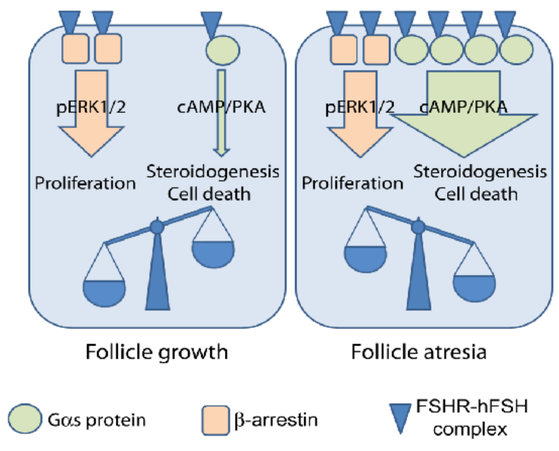
The expression of FSHR and LHCGR was serum-dependent (fig.1A), being absent under starvation and increasing progressively with serum concentrations, as well as oxytocin receptor [2]. However, in 15% serum-cultured hGL5 cells, FSH/LH stimulation was ineffective both on cAMP and progesterone production and CREB phosphorylation (fig.1B-D), suggesting uncoupling of the receptors to the Gs alpha protein.

Stimulation by FSH/LH, as well as by the PKC activator PMA, results in ERK1/2 phosphorylation in the presence of serum (fig.1E), and in a significant increase of cell proliferation over 4 days (fig.1F). hGL5 cell proliferation was damped by MEK inhibitor U0126 (fig.1G).

β-arrestin1/2 siRNA transfection unlocked the cAMP/PKA pathway, leading to cAMP and progesterone accumulation and CREB phosphorylation (fig.2A-C), at high basal levels. Moreover, siRNA-treated cells underwent cell rounding, pro-caspase 3 cleavage and apoptosis (fig.2D-I). The pro-apoptotic effects of cAMP/PKA pathway activation were augmented by FSH- but not LH treatment, and inhibited by selective PKA blockade by H-89 (fig.2J,K).

The FSHR-mediated cell death was reproduced by permanently overexpressing FSHR, but not LHCGR, in hGL5 cells. hGL5/FSHR cells died within 4-8 weeks (fig.3A), showing high basal cAMP levels and procaspase 3 cleavage (fig.3B,C), cell rounding and apoptosis (fig.3D,E), revealing the dual role of the FSHR in the activation of proliferative and apoptotic signals.

CONCLUSIONS



FSH should be probably revisited as a pro-apoptotic rather than a proliferative factor, suggesting the existence of cell-protective mechanisms exerted by β-arrestins, which block GPCRs-mediated apoptosis. LH may act as a switch from the FSH-dependent death signals to proliferative signals to rescue the dominant follicle, which indeed becomes LH-responsive.

REFERENCES. [1] Rainey et al., 1994. J Clin Endocrinol Metab. 78(3):705-10. [2] Copland et al., 2002. Biol Reprod. 66(5):1230-6.

Fig.1. FSHR and LHCGR serum-dependent expression in hGL5 cells, and uncoupling to the cAMP/PKA pathway. **A**) Analysis of the serum-dependent increase of FSHR and LHCGR by Western blotting (n=3). **B-D**) Evaluation of cAMP and progesterone production by ELISA, and CREB phosphorylation by Western blotting in serum-starved and *non*-starved hGL5 cells stimulated by FSH/LH. Total ERK was the normalizer (means±SDMann-Whitney's U-test; $p<0.05$; n=4). **E,F**) Analysis of ERK1/2 phosphorylation and cell viability increase in 2-days FSH/LH treated and 15% serum-cultured hGL5 cells, by Western blotting and MTT assay, respectively. **G**) Inhibition of the cell growth by 20 μM of the U0126 MEK blockade (means±SD; Mann-Whitney's U-test; $p<0.05$; n=4). *=significant vs basal.

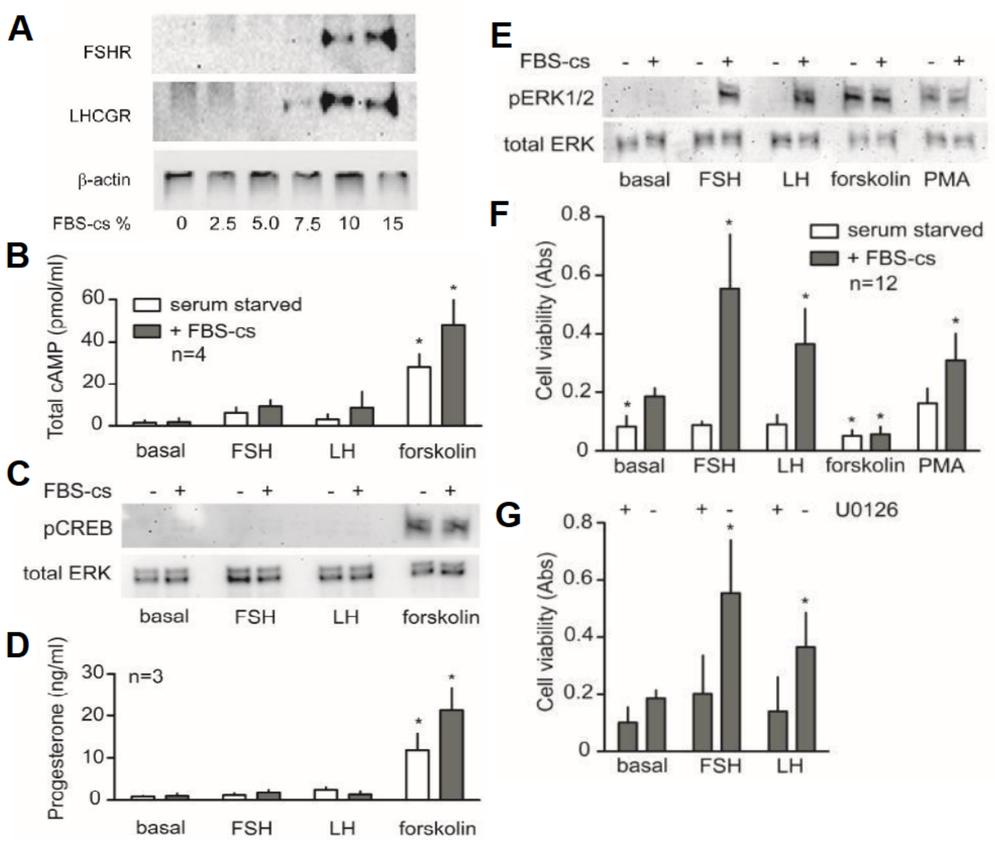
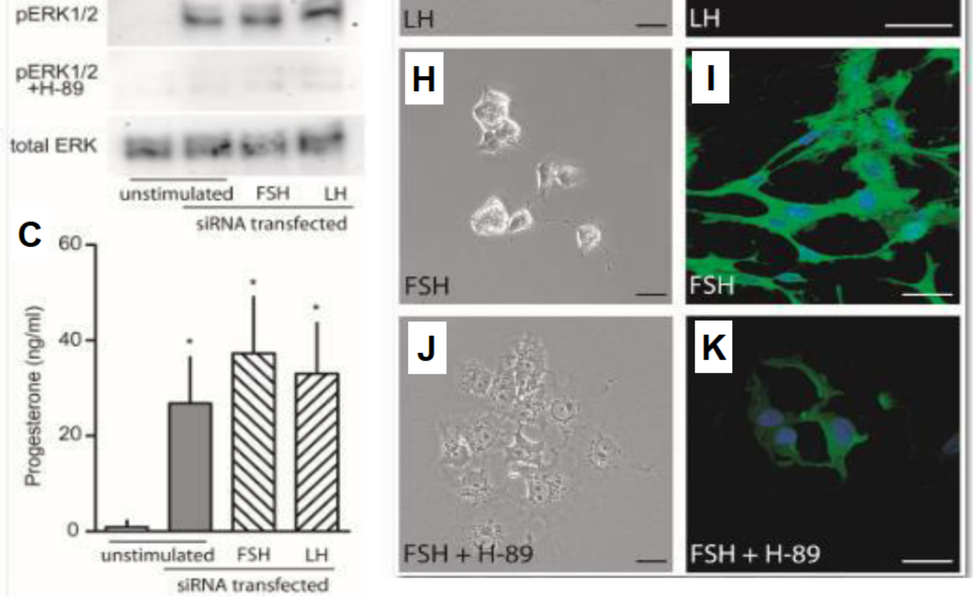


Fig.2. Effects of β-arrestin1/2 silencing by siRNA in hGL5 cells. **A-C**) cAMP and progesterone production and CREB phosphorylation in 2-days siRNA transfected hGL5 cells stimulated by FSH/LH, evaluated by ELISA and Western blotting, respectively. *=significant vs untransfected, unstimulated (means±SD; Mann-Whitney's U-test; $p<0.05$; n=4). **D-K**) 1-day siRNA transfected hGL5 cells were cultured in the presence of FSH (panels H,I) or LH (F,G) in a medium containing 15% serum, then cell rounding (D,F,H,J) and caspase 3 activation (E,G,I,K) were evaluated by optical and fluorescence microscopy, respectively. 10 μM of the PKA inhibitor H-89 were added where appropriate (G,H). FSH- and LH-untreated cells served as control (A,B). Green=cleaved caspase 3 staining; blue=nucleus (DAPI); bars=10 μm. The FSH-dependent cell rounding (H) and caspase 3 cleavage (I) were dampened by PKA inhibition (J,K) (bars= 10 μm; images representative of three independent experiments).



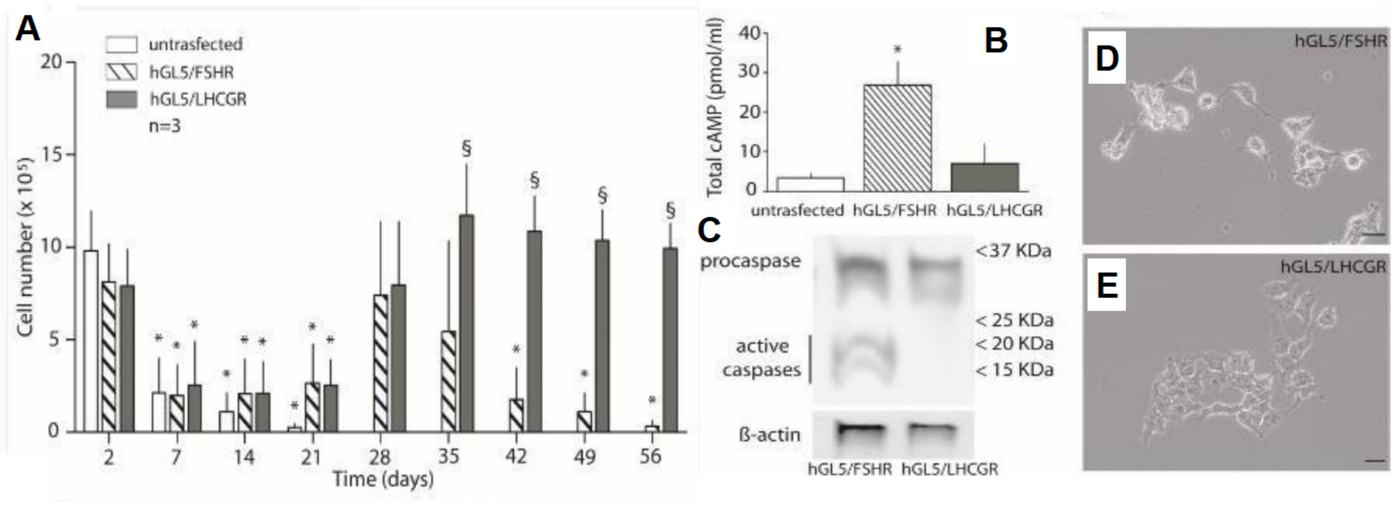


Fig.3. Long-term effects of stable FSHR and LHCGR overexpression. **A**) hGL5 cells were transfected with FSHR or LHCGR by electroporation, then maintained in the presence of 80 μg/ml zeocin for the selection of the stable transfectants. Cell viability was evaluated by Trypan blue count. §=weekly 1:10 dilution to avoid cell confluency. *=significant vs untransfected cells at day 2 after the electroporation (means±SD; Mann-Whitney's U-test; $p<0.05$; n=4). **B**) Total cAMP was measured in 28-days FSHR- and LHCGR-transfected hGL5 cells by ELISA. *=significant vs untransfected (means±SD; Mann-Whitney's U-test; $p<0.05$; n=4). **C**) Evaluation of procaspase 3 cleavage By Western blotting. β-actin served as normalizer (images representative of three independent experiments). **D,E**) Evaluation of cell rounding by optical microscopy (bars= 10 μm; images representative of three independent experiments).

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