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LH and hCG signalling in natural target cells and cell lines transfected with LHCGR

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Abstract

Chorionic gonadotropin (hCG) and luteinizing hormone (LH) are glycoprotein hormones mediating crucial events in human sexual development and fertility. Since they act by binding to the common lutropin/choriogonadotropin receptor (LHCGR), which belongs to the superfamily of the G-protein coupled receptors, LH and hCG were traditionally considered equivalent. However, some hints suggested that LHCGR is able to differentiate the LH/hCG binding, thus resulting in differential signaling. In the present study, we have compared the activity of recombinant LH and hCG, in terms of signaling pathways activation, by using different cell models. In HEK293 cells transiently transfected with the LHCGR, and in MLTC1 cells naturally expressing the mouse lhcgr, we have evaluated the cAMP accumulation, , β-arrestin 2 recruitment, IP1 production and steroid production. Also, in human granulosa-lutein cells (hGLC) naturally expressing the LHCGR, we analyzed the cAMP, phospo-CREB, -ERK1/2 and -AKT activation, gene expression and steroids production upon LH/hCG treatment, in the presence of a fixed FSH dose. In murine primary Leydig cells, we have compared the activity of LH/hCG, in terms of cAMP accumulation, phospo-CREB and -ERK1/2 activation, gene expression and testosterone synthesis. The results demonstrated that hCG and LH, exert quantitatively and qualitatively different intracellular events and biased signaling.

Introduction

1. The gonadotropins

Luteinizing hormone (LH), follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) are heterodimeric glycoprotein hormones playing crucial roles in development and reproduction. FSH and LH are produced by the anterior pituitary in a pulsatile fashion and stimulate follicular growth and maturation, ovulation and the development of the corpus luteum (Hillier et al 2001). In contrast, hCG is the pregnancy hormone produced by the trophoblast cells of the developing embryo in a constant fashion. It is present only in the primates and equids and is crucial for promoting progesterone production by ovarian corpus luteal cells (Richardson et al.1980; Schmitt et al. 1996).

Gonadotropins share a unique 92 amminoacids alpha subunit, *non* covalently linked to the receptor-specific beta subunit, which confers the unique biological properties (Lapthorn et al. 1994; Wu et al. 1994). The heterodimers, enriched by carbohydrate moieties, provides the effective hormonal activity exerted by each hormone (Vaitukaitis et al. 1976). These molecules are represented by a mixture of iso-hormones differing in the carbohydrate chains content due to post-translational modifications of the native proteins, leading to several truncated or nicked intermediates and natural sequence variants (Stanton et al. 1996; Cole et al. 1991; Kovalevskaya et al. 2002).

1.1 The luteinizing hormone

The common 92 amminoacids α-subunit of LH, FSH and hCG, is encoded by a single gene (*CGA*) located at the *locus* 6q21.1-23 (Fiddes et al. 1981) while the 121 aminoacids unique β-subunit of LH is encoded by the *LHB* gene, at 19q13.32 (Julier et al. 1984), belonging to a cluster with the multiple hCG beta subunits (*CGB)* genes (Boorstein et al. 1982). This gene cluster was cloned completely as a 58kb DNA fragment, arranged in single genes adjacent to each other, in tandem and inverted repeats (Policastro et al. 1986).

Due to their critical role in reproduction and fertility, *CGA*, *LHB* and *CGB* genes exhibit very rare mutations (Themmen and Huhtaniemi, 2000). The main polymorphisms and mutations are displayed within the encoding β- rather than the α-subunit genes. Four common genetic variants have been found in the LH β-subunit: one of them is known as the cV-LH, due to its worldwide occurrence in several human populations. It is characterized by two point mutations, $Trp^{8}Arg$ and $Ile^{15}Thr$ which result in a functional LH with a shorter half-life and possibly a decreased bioactivity compared to normal LH (Pettersson et al. 1994; Nilsson et al. 1998). A single base substitution, changing codon 54 of the LH β-chain from glutamine to arginine, is the only known mutation causing a deep functional effect, resulting in the uncorrect folding of the β-subunit structure and affecting hormone binding to the receptor (Weiss et al. 1992). Two LH β-subunit variants, characterized by aminoacid replacements ($Gln^{102}Ser$ and $Ala^{3}Thr$, respectively), are population-specific, according to screenings of various ethnic groups of Asian populations (Roy et al. 1996; Jiang et al. 2002).

LH exists in several isoforms, differing in glycosilation content. Due to the distinct size and conformation of the molecules and to their different features of molecular interaction with the receptor, these isoforms display peculiar half-life, bioactivity and signaling properties (Stanton PG et al. 1996; Arey and López, 2011). Variations in the composition of LH isoforms were measured over the menstrual cycle (Anobile et al. 1998) and, in general, for the duration of the reproductive life (Reader et al. 1983).

LH functions are exerted through its receptor, the lutropin/choriogonadotropin receptor (LHCGR), activating multiple signaling pathways. LHCGR is present in ovarian theca, granulosa and luteal cells in female, and in testicular Leydig cells in male (McFarland et al. 1989; Segaloff and Ascoli . 1993). In female, LH is fundamental for ovulation induction and maintenance of the corpus luteum.

During the first days of the follicular phase, LH promotes androgen production by theca cells and their conversion to estrogens by granulosa cells (Nahum et al. 1995). In the late follicular phase, the positive feedback generated by estrogen levels at the pituitary gland supports the LH surge which triggers ovulation. The subsequent arise in the enzyme 3β-hydroxysteroid dehydrogenase (3βHSD) expression results in progesterone production by corpus luteum. LH supports progesterone production and the maintenance of corpus luteum (Bomsel et al. 1979).

In male, LH, together with FSH, regulates spermatogenesis and steroidogenesis in the testes, by controlling testosterone synthesis and secretion from Leydig cells. (Saez. 1994).

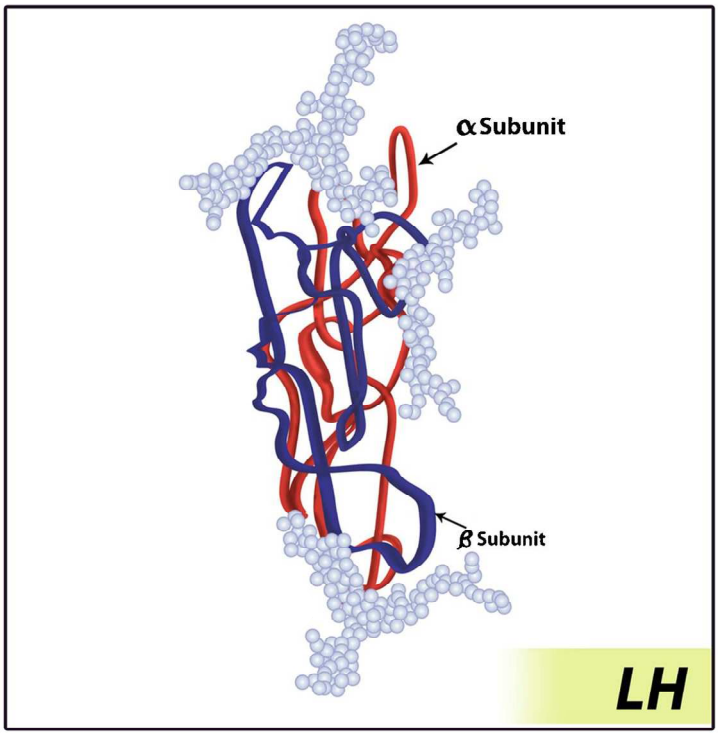

Figure 1 Luteinizing hormone. The α and β subunits are represented by red and blue strands, respectively. The carbohydrate chains are represented by light blue balls, two for the α subunit and one for the βsubunit.

1.2 The human chorionic gonadotropin

hCG is the first biochemical signal from the embryo to the maternal environment during pregnancy. It is required to rescue the corpus luteum from *atresia* and to maintain progesterone production, thereby ensuring embryo implantation and development (Pierce and Parsons, 1981). hCG is also crucial for male fetal sexual differentiation, due to its promoting activity on testosterone synthesis by Leydig cells (Huhtaniemi et al. 1977).

The 145 aminoacids hCG β-subunit is encoded by a cluster of eight genes located at *locus* 19q13.32 (Julier et al. 1984). Only four of them are coding genes (*CGB3,CGB5, CGB7, CGB8*) while the remaining two (*CGB1 and CGB2)* are pseudogenes. The aminoacid sequence of LHβ and hCGβ shares 85% identity in the first 114 aminoacids, suggesting that *CGB* evolved by repeated duplications and a single nucleotide deletion of an ancestral *LHB* gene (Hallast et al. 2005).

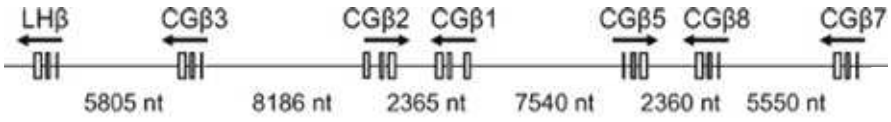

Figure 2. Structure of the *LH/CGB* gene cluster. Organisation of the human *LHB/CGB* gene cluster on chromosome 19.3. Directions of the gene transcription are indicated by arrows and the intergenic distances by numbers. nt = number of nucleotides.

This deletion, together with a two nucleotide insertion in codon 138, generate a termination codon from the AATAAA polyadenylation signal (Talmadge et al. 1984), resulting in a carboxyl terminal peptide (CTP) extension featured by multiple proline and serine/threonine residues with an extensive glycosylation pattern of four O-linked oligosaccharides (Birken and Canfield, 1977). The CTP domain and associated sugar chains confer to hCG higher stability and longer circulating half-life than LH (Bousfield and Ward, 2006). Even though hCG polymorphisms with functional significance are generally rare, β–subunit variants associated with increased risk of recurrent miscarriage have been identified within the genes encoding for the major fraction of transcripts, *CGB5* and *CGB8*. These mutations mainly leads to conformational changes in protein's structure, preventing the hCG heterodimer formation (Nagirnaja et al. 2012).

Since 70% of the structure of hCG is represented by the peptide while the 30% by carbohydrate residues, hCG is characterized by high glycosylation rate. The sugar branches bind covalently to the aminoacid sequence and consist of both four O-linked oligosaccharides containing an N-acetylgalactosamine residue linked to a serine residue, and two N-linked oligosaccharides containing an N-acetylglucosamine residue linked to an asparagine residue. The sialic acid content of hCG plays a role in receptor binding, biological activity and clearance from the maternal circulation (De Medeiros and Norman, 2009).

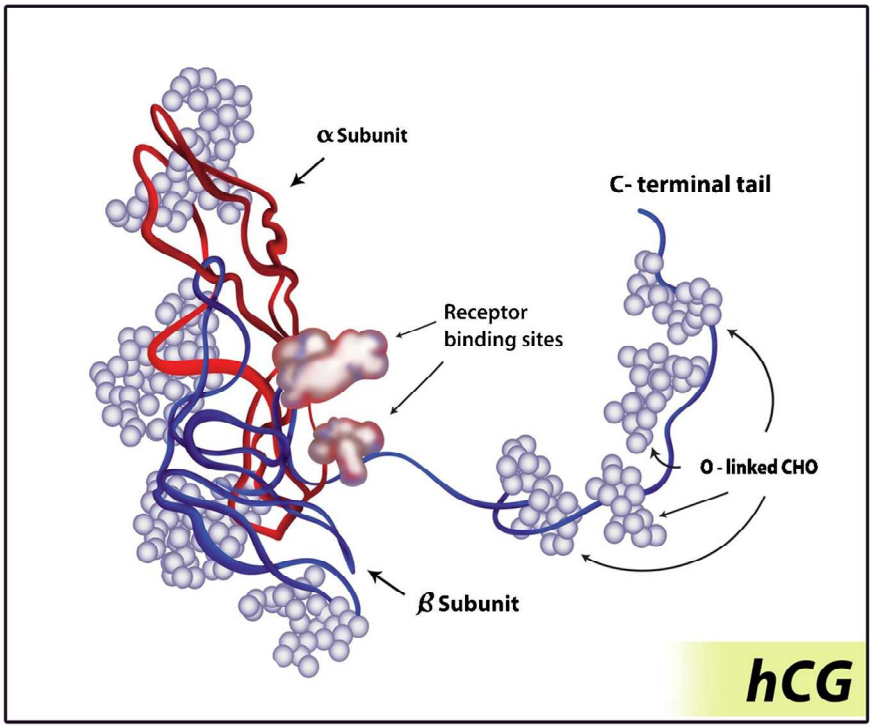

Figure 3. Human chorionic gonadotropin. The α and β subunits are represented by red and blue strand, respectively. A long carboxy-terminal segment (C-terminal tail), which is O-glycosylated (O-linked CHO), conferres a longer half-life to hCG. Receptor binding sites are represented by little grey clouds on the molecule.

At least four physiologically important hCG isoforms have been detected in serum samples (Butler et al. 2001), mainly differing in their oligosaccharide moieties and consequently in their size, half-lives and biological activities. These oligosaccharide variants originate from differences in the availability of sugars, variations in cellular metabolism and the differential expression of glycosyl transferases which are the cellular sugar-adding enzymes (Elliott et al. 1997). A hyperglycosylated hCG (hCG-H) is produced and secreted by the invasive

cytotrophoblast cells during the first trimester of pregnancy (Kardana et al. 1991). hCG-H has double size O-linked sugar structures and larger N-linked structures which may confer specific biological function (Kobata and Takeuchi, 1999). The high grade of glycosylation prevents the normal folding of the molecule, leading to the exposure of hidden sequences. It was proposed that hCG-H activity may occur independently to the LHCGR by antagonizing the transforming growth factor β receptor (TGFβR) (Berndt. et al. 2013), although this hypothesis has been recently challenged (Koistinen et al. 2015).

2. The LH and hCG common receptor

The lutropin/choriogonadotropin receptor (LHCGR) plays crucial roles in the regulation of LH and hCG activity. It is expressed in ovarian theca, granulosa, luteal and interstitial cells and in the Leydig cells of the testes (Ascoli et al. 2002).

LHCGR is a member of the G-protein coupled receptors (GPCRs) superfamily, belonging the subfamily of the rhodopsin/β2-adrenergic receptor. LHCGR is a trans-membrane receptor composed by three distinct domains: a large N-terminal extracellular region characterized by leucine-rich repeats domain (LRRD) and multiple sites for glycosylation, a serpentine region containing seven transmembrane segments (TM) connected by three extra- and three intra-cellular loops and a C-terminal tail (Ascoli et al. 2002).

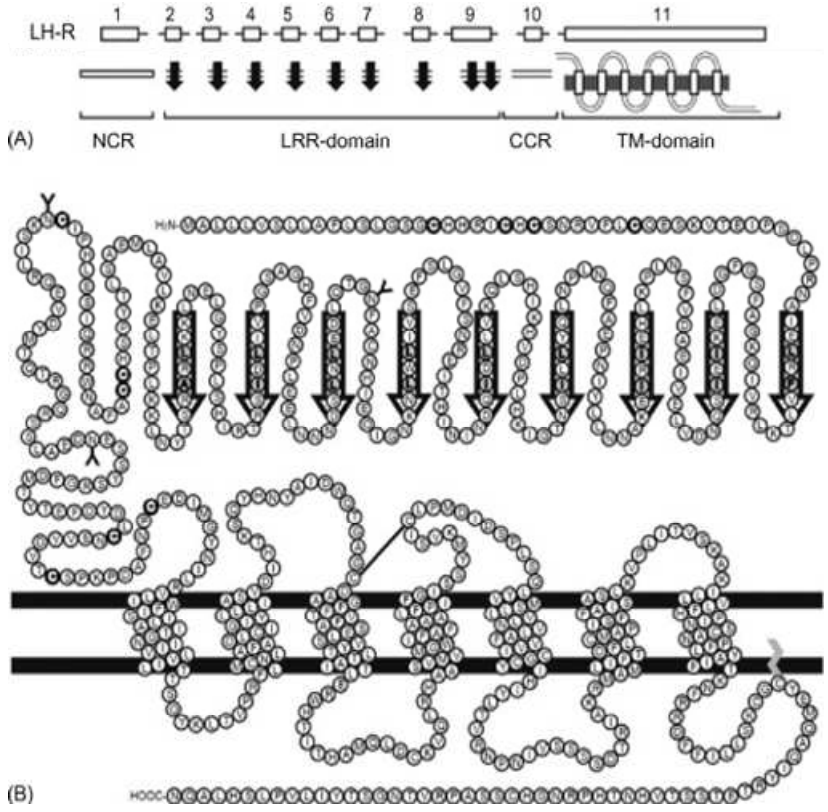

Figure 4. LHCGR gene and protein. A) *LHCGR* gene. The overall genomic organization is shown, with 11 coding exons . Exon 1 encodes the N-terminal cysteine-rich region (NCR) while exons 2-9 encode the leucine-rich repeats domain (LRR-domain), involved in direct hormone binding. Exon 10 codes for the C-terminal cysteine cluster (CCR) of the extracellular domain whereas exon 11 encodes the transmembrane domain (TM-domain). β-strand of each LRR unit is indicated by a black arrow. B) LHCGR protein. N-terminal extracellular domain, the seven transmembrane domain helices and the C-terminal intracellular tail are illustrated. Cell membrane is represented by two black bars. β-strands in the LRRD are indicated by black arrows.

LHCGR is encoded by a single gene spanning 80kbp on the short arm of chromosome 2 (2p21) and it is composed by 10 introns and 11 exons (Rousseau-Merck et al. 1990). Exon 1 encodes the signal peptide and the N-terminal cysteine-rich region while exons 2-9 encode the LRRD, involved in direct hormone binding. Exon 10 consists in 81 bp encoding 27 amino acids and contributes to the formation of a C-terminal cysteine cluster of the extracellular domain. Exon 11 encodes the entire serpentine region, the C-terminal intracellular part and the C-terminal segment of the hinge region, connecting the LRRD and the first transmembrane helix (Ascoli et al. 2002; Atger at al. 1995). The mature 699 aminoacids glycosylated protein has a molecular weight of about 85–95 kDa (Ascoli et al. 2002).

Human LHCGR, as well as *non* human lhcgr, is alternatively spliced during transcription by exon skipping or *via* alternative splice sites, resulting in several LHCGR isoforms. Most of them remain untranslated or not transported at the cell membrane (Aatsinki et al. 1992; Bacich et al. 1994; Reinholz et al. 2000). In the marmoset monkey *Calltithrix jacchus*, exon 10 of the lhr is naturally missing at the mRNA level. This mRNA lacking of exon 10 is the natural wild-type receptor in the Platyrrhini lineage (Gromoll et al. 2003). CGβ mRNA was detected at high level in the pituitary of the common marmoset and CTP was speculated to overcome exon 10 lacking, leading to normal receptor activation (Müller et al. 2004; Müller et al. 2004). A patient with Leydig cell hypoplasia type II caused by a genomic deletion of exon 10 of LHCGR was reported, representing the clinical counterpart of the normal male marmoset monkey. LH action was impaired and the patient was hypogonadic (Gromoll et al. 2000). Although both hCG and LH bind the LHCGR-exon 10, cAMP production by LH was impaired, but not by hCG, showing that exon 10 is necessary for ligand recognition (Müller et al. 2003).

Moreover, a DNA insertion of a 2.7 kb genomic region between exons 6 and 7 resulting in an additional exon, the exon 6A, displays composite characteristics of an internal/terminal exon and possesses stop codons, triggering nonsense-mediated mRNA decay (NMD) in LHCGR (Kossack et al. 2008). The resulting altered ratio of LHCGR transcripts lead to the generation of predominantly nonfunctional LHCGR isoforms, thereby preventing proper expression and functioning. (Kossack et al. 2008). This novel exon is confined to primates and humans, where is highly conserved; exon 6A-like sequences are missing in other species. The physiological importance of exon 6A is not yet fully understood, even if

naturally occurring mutations within this exon leads to severe disorders of sexual differentiation (Kossack et al. 2013).

A variety of mutations have been identified in *LHCGR* gene: activating mutations occur mainly within exon 11 (Latronico and Segaloff, 1999), while loss of function mutations at exon 10 result in developmental and reproductive abnormalities, including pseudohermaphrodism, micropenis, hypospadias and infertility (Segaloff. 2009).

At cell membrane, LHCGR forms functional homodimers or oligomers and constitutive LHCGR self-association was shown upon hCG stimulation (Tao et al. 2004). An inactive LHCGR mutant was seen to attenuate the signaling of the LHCGR wt or the constitutively active LHCGR due to receptor heterodimerization (Zhang et al. 2009). The main interface contact between the protomers is likely located between the transmembrane helices and the extracellular portion might modulate dimeric interrelations (Urizar, 2005). A study using transgenic mice coexpressing binding-deficient and signaling-deficient murine lhr demonstrated the cooperation between the two LHCGR protomers, in terms of ligand binding and signal transduction. Deficient LHCGR mutants are capable of ligand binding and convey the signal to activate the cell signaling by dimerization (Rivero-Muller et al. 2010).

3. hCG/LH binding to LHCGR

Although the crystallography of hCG was provided about two decades ago (Lapthorn et al. 1994), structures of the hCG or LH bound to the extracellular region of the LHCGR are not yet available. How hCG/LH interacts with the LHCGR at molecular level is controversial. Most of the binding and mutagenesis experiments were conducted using hCG or LH of mammalian origin, such as rat or bovine, against rat or human receptor, as they share high structural homology (Ascoli et al. 2002). Rat lhr binds hCG with 5–10 times higher affinity than human LHCGR, which is more discriminating, binding hCG with 1000 times higher affinity than mammalian LHs. All these issues demonstrate that hCG owns a higher affinity, known as the strength of the ligand-receptor interaction, for the receptor than LH (Huhtaniemi et al. 1981; Strickland et al. 1981; Jia et al. 1991).

LHCGR has two separate binding sites for ligands, located at the extracellular region: one at the LRRs domain and the other at the hinge region. The crystallized LRRs domain is featured by around 260 amino acids and 11 leucine-rich repeats, where hormone binding occurs (Smits et al. 2003; Fan and Hendrickson, 2007). Site-directed mutagenesis experiments demonstrated that repeats 2 to 8 of the LRRs are fundamental for high-affinity binding of LH and hCG (Bhowmick et al. 1996). The second hormone-binding site, constituted by Asp330 and Tyr331, is located at the C-terminal part of the LHCGR hinge region (Bruysters et al. 2008b), where the interaction between the sulphated tyrosine and the hormone occurs (Jiang et al. 2012). Hinge region is involved in ligand binding (Costagliola et al. 2002; Bonomi et al. 2006) and selectivity (Bernard et al. 1998), signaling pathway regulation (Nurwakagari et al. 2007) and features (Moyle et al. 2004).

LH and hCG efficacy is differently modulated by removing LHCGR residues encoded by exon 10 (Müller et al. 2003), resulting in structural changes at the hinge region. It is supposed that the missing aminoacid sequence leads to spatial displacement of the downstream part containing the sulphated Tyr331 at the hinge region. This impairs LH but not hCG signaling, suggesting different interactions exerted by the two hormones at the hinge region of the receptor. By studying the mechanism of LHCGR-ex 10 activation, hCG and LH differential interaction with the hinge region was demonstrated (Grzesik et al. 2015).

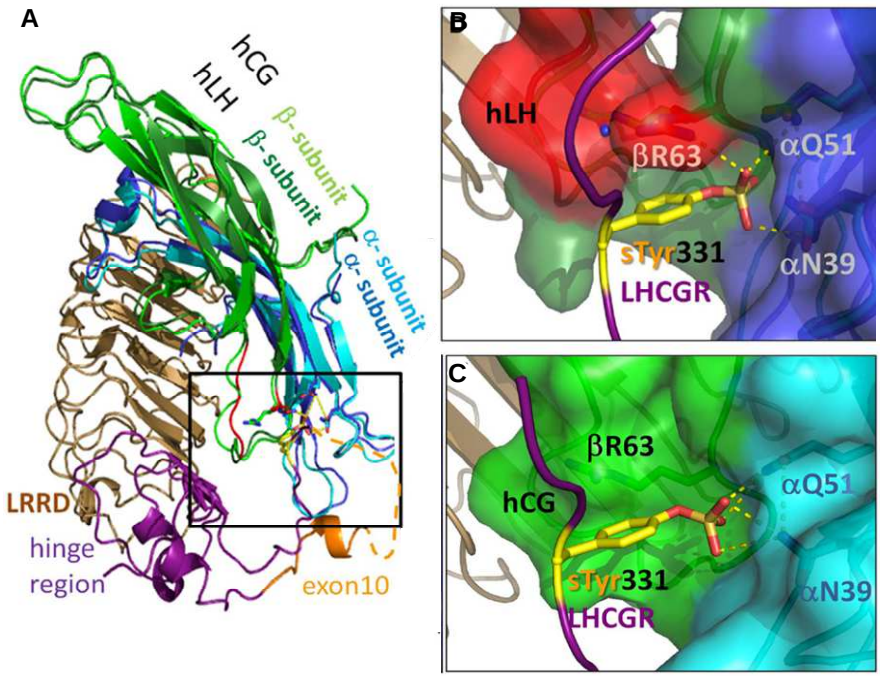

Figure 5. Differences between LH and hCG interaction with the LHCGR hinge region. **(**A) Homology model details of superimposed hormones LH and hCG bound to the leucine-rich repeats domain (LRRD) and hinge region of LHCGR, based on the crystal structure of FSH/FSHR. (B) Detailed view for hLH. The resulting binding cavity at sTyr331 is more surrounded in hLH comparing to (C) detailed view for hCG, where the binding pocket is much wider.

Cooperation between receptor protomers was described and two different mechanisms of activation were identified: one mediated by intramolecular communications (cis-activation) and the other through intermolecular cooperations with an adjacent receptor (trans-activation). Especially, human LH activates human LHCGR exclusively through a cis-mechanism while hCG is capable of inducing both cis- and trans-activation at of the receptor (Grzesik et al. 2014).

We can speculate that the differences between LH and hCG at the molecular level are depending on the structural variations related to the hormone structures (e.g. extended and glycosylated hCG β-subunit). Different contact points between the hormones and the receptors might be responsible for their different binding, affinity and the more and more divergent bioactivity exerted *in vitro* (Casarini et al. 2012).

4. LHCGR-mediated signaling

According to the classical receptor occupancy theory (Gether U, 2000; Urban et al. 2007), GPCRs exist in a dynamic equilibrium between an inactive (Ro) and an active (R*) state. The switch is given by ligand binding to the receptor, leading to structural re-arrangements between the extracellular loops and the hinge region.

The activation of GPCRs results in biochemical responses mediated by G proteins (Brzostowski and Kimmel, 2001), or alternative pathways such as the β-arrestin-mediated signaling occurring *via* Src and mitogen-activated protein kinase (MAPK) pathways (Lefkowitz and Shenoy, 2005).

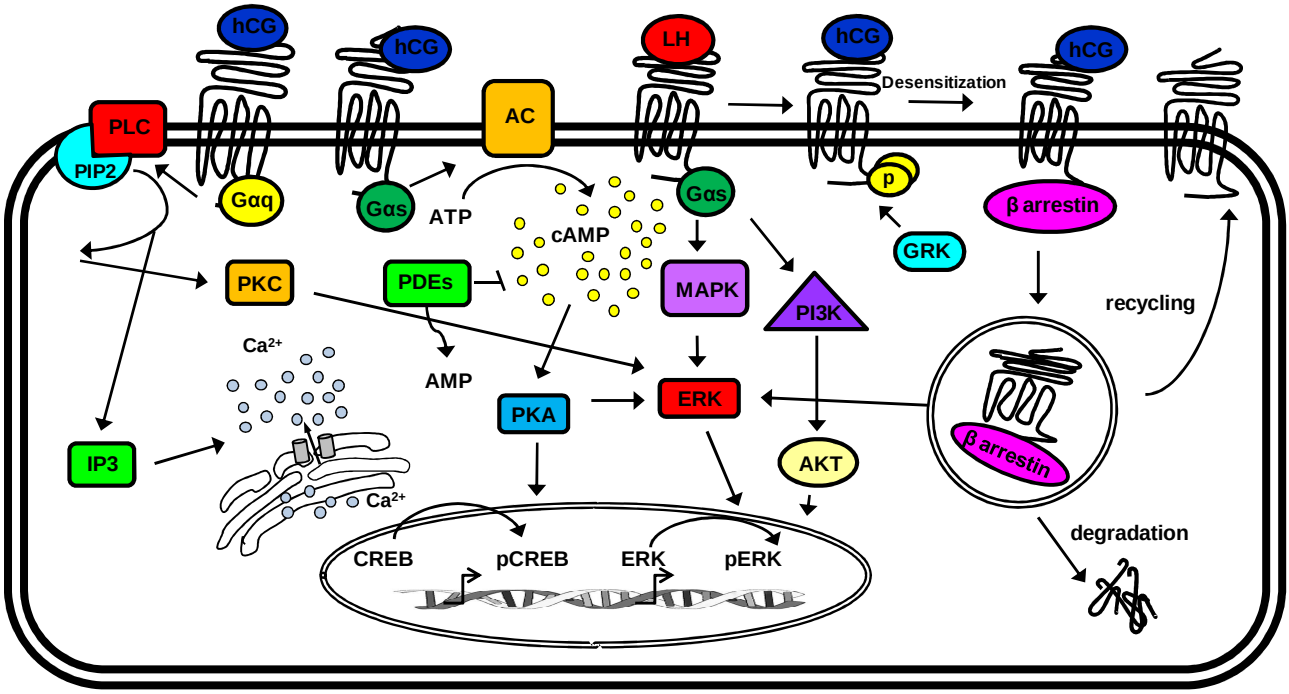

Figure 6. Signaling pathways activated upon hCG/LH binding of LHCGR in granulosa cells. Gs- and Gq-dependent pathways were illustrated together with β arrestins signaling. Arrows represents direct linking between intracellular effectors.

4.1 G proteins signaling

The guanine nucleotide-binding proteins (G proteins) are heterotrimeric proteins located at the cell membrane functioning as signal transducers. They belong to the larger GTPase superfamily, consisting of an α subunit, which contains the guanine nucleotide binding site and an intrinsic GTPase activity, and the βγ subunit complex. Four main classes of G proteins can be distinguished: Gs which activates adenylyl cyclase, Gi which inhibits adenylyl cyclase, Gq which activates phospholipase C and G12 and G13 (Rens-Domiano et al. 1995).

When LHCGR is in the Ro state, G protein is bound to GDP through the α-subunit in a heterotrimeric inactive state. Upon stabilization of the receptor in the R* state, several conformational changes occur both at the receptor and G protein structural level, inducing GDP release and its exchange with GTP. GTP binding leads to the dissociation of the heterotrimer in the α subunit alone and the βγ dimer, leading to the activation of different downstream effectors (Lambright et al. 1994). βγ complex increases the affinity of the Gα subunit for the receptor and is implicated in the recruitment of G-protein-coupled receptor kinases (GRK) at the cell membrane, which is crucial for receptor downregulation (Clapham and Neer, 1993).

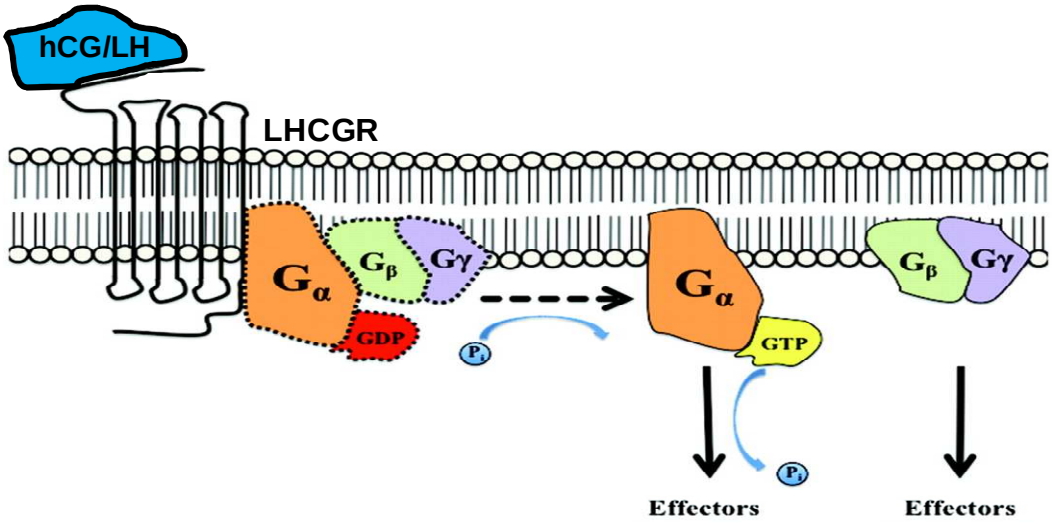

Figure 7. LHCGR activation. Ligand binding induces LHCGR conformational change whereby the G protein α subunit is activated by exchanging bound GDP for GTP.The α and βγ subunits dissociate to activate downstream signaling cascades.

The specific recruitment of G-proteins, as well as the production of related second messengers is cell-type specific and differs depending on LH or hCG as ligand (Ascoli et al. 2002; Casarini et al. 2012). LHCGR was one of the first GPCRs shown to independently activate at least two G protein-dependent signaling pathways, adenylyl cyclase and phospholipase C (PLC) (Gudermann et al. 1992; Herrlich et al. 1996).

4.1.1 Gαs signaling

Upon ligand binding to LHCGR, Gαs activates adenylyl cyclase (AC) which catalyzes the conversion of ATP into cAMP. cAMP production is controlled by phosphodiesterase enzymes (PDEs), which catalyze the degradation of cAMP into AMP (Houslay et al. 2003). cAMP mediates its biological effects through several intracellular effectors such as the serine/threonine protein kinase A (PKA) (Pierce et al. 2002). cAMP binding to PKA activates its catalytic activity, leading to phosphorylation of both cytosolic and nuclear substrates. PKA-anchoring proteins (AKAPs) provide specificity in cAMP signal transduction by maintaining PKA close to specific effectors and substrates (Wong et al. 2004). PKA phosphorylates different transcription factors such as cAMP-response element-binding protein (CREB), cAMP-responsive modulator (CREM), and the activating transcription factor 1 (ATF1) (Mayr et al. 2002), regulating the expression of several target genes related to steroidogenesis and circadian clock (Manna et al. 2002; Dibner et al. 2010)

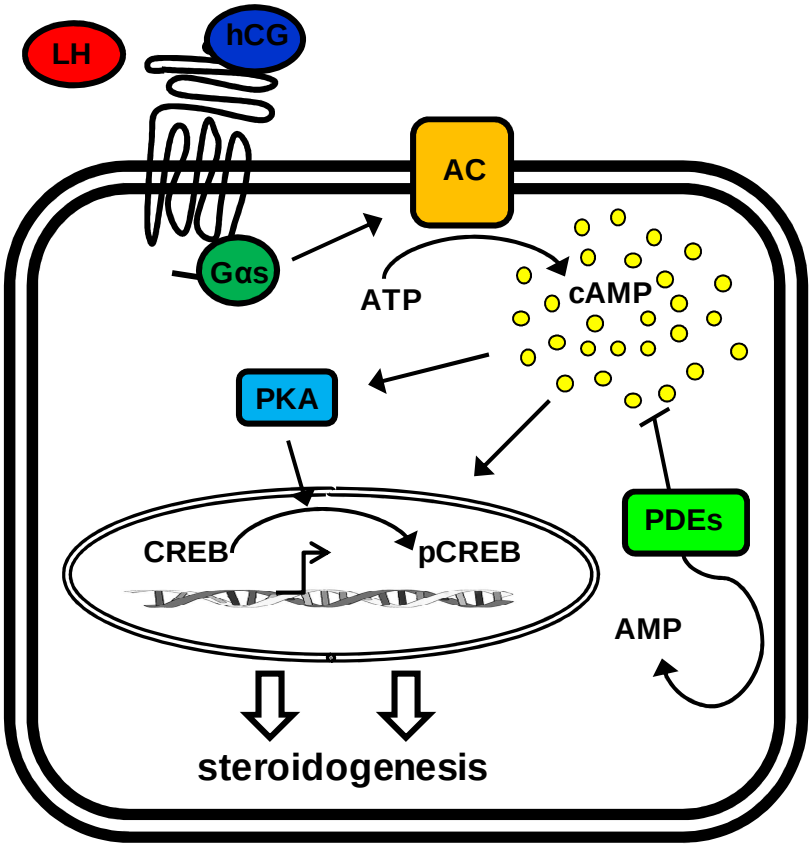

Figure 8. cAMP/PKA pathway. LHCGR activated by ligand (hCG/LH), once bound to Gαs, activates adenylyl cyclase (AC) which in turn catalyzes ATP conversion in cAMP. PKA activation occurs, resulting in CREB phosphorylation into the nucleus. Modulation of target genes related to steroidogenesis occurs. PDEs catalyze the degradation of cAMP into AMP.

Besides cAMP/PKA signaling, pathways including extracellular signal-related protein kinases (ERKs) and protein kinase B (AKT) signaling have been identified as major downstream players in LHCGR-mediated cascades (Casarini et al. 2012). AKT phosphorylation occurs through phosphatidylinositol-3-kinase (PI3K) recruitment and it is related to anti-apoptotic effects in granulosa cells (Cecconi. 2012). ERKs include the p42/44 mitogen activated protein kinase (MAPK) (ERK1/2) and, upon phosphorylation, it regulates proliferation, differentiation and cell survival (Palaniappan et al. 2010; Shiraishi, 2007). It was demonstrated that the cAMP/PKA pathway regulates ERK1/2 signaling through several distinct molecular mechanisms, including the involvement of either Ras (*Rat Sarcoma*) and Ras-related protein 1 (Rap1) or cAMP- independent recruitment of PKA (Stork et al. 2002).

4.1.2 Gαq signaling

Gαq protein activates phospholipase C (PLC), which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol trisphosphate (IP3), resulting in the activation of protein kinase C (PKC) and Ca2+ release from intracellular stores (Berridge, 1993). DAG remains bound to the membrane due to its lypophilic nature and promotes ERK1/2 phosphorylation by PKC activation (Woods et al. 2007). IP3 is released into the cytosol and binds to calcium channels in the endoplasmic reticulum (ER), leading to the increase of cytosolic calcium levels which regulate steroidogenesis (Sullivan et al. 1986).

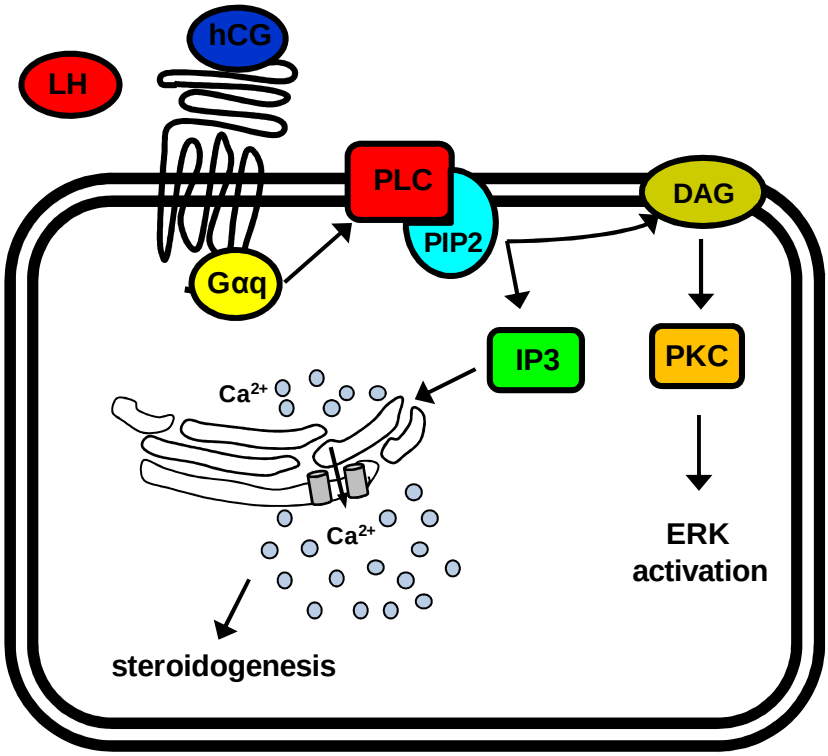

Figure 9. PLC/IP3 pathway. Gαq activate phospholipase C (PLC) which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) to diacyl glycerol (DAG) and inositol trisphosphate (IP3), resulting in activation of protein kinase C (PKC) and extracellular signal-related protein kinase (ERK) and Ca2+ release from the endoplasmic reticulum into the cytosol, respectively. Modulation of steroidogenesis occurs.

4.1.3 G proteins-independent signaling: β-arrestins and G proteins receptor kinases (GRKs)

β arrestins and GRKs are mainly linked to G protein-signaling silencing and receptor trafficking. Upon ligand binding, GRKs phosphorylate the receptors in the active state (Lefkowitz. 1998). β-arrestins are subsequently recruited to the GRK-phosphorylated receptor, increasing G proteins uncoupling (receptor desensitization). β-arrestins interact also with phosphodiesterase 4D (PDE4D), further desensitizing Gαs-coupled receptors by dual inhibition of cAMP production and degradation (Perry et al. 2002). Moreover, GRK-mediated phophorylation and β-arrestin recruitment are crucial for clathrin-dependent internalization (Shenoy et al. 2003), resulting in dephosphorylation, resensitization and recycling of the receptor to the cell membrane or its degradation into the lysosomes.

Recent studies reveal the activation of additional intracellular pathways β arrestins- and GRKs-mediated (Reiter et al. 2006), which are independent from G proteins activation. So far, the best-characterized β-arrestins-dependent signaling mechanism is ERK1/2 signaling pathway. β-Arrestins have been shown to scaffold components of the MAPK cascade in complex with the receptor, resulting in ERK activation. Spatial distribution and kinetics of G protein- or β-arrestin-dependent ERK activation are distinct: G protein-dependent pathway triggers a rapid and transient nuclear translocation of pERK, while β-arrestin-activated ERK is confined to the cytoplasm and it is slower and persistent (Luttrell et al. 2001; Ahn et al. 2003). Moreover, GRKs might modulate cellular function through their interaction with a variety of proteins involved in signaling and trafficking, such as Gαq, Gβγ, PI3K and proteins involved in endocitosis (Penela et al. 2003; Kara et al. 2006).

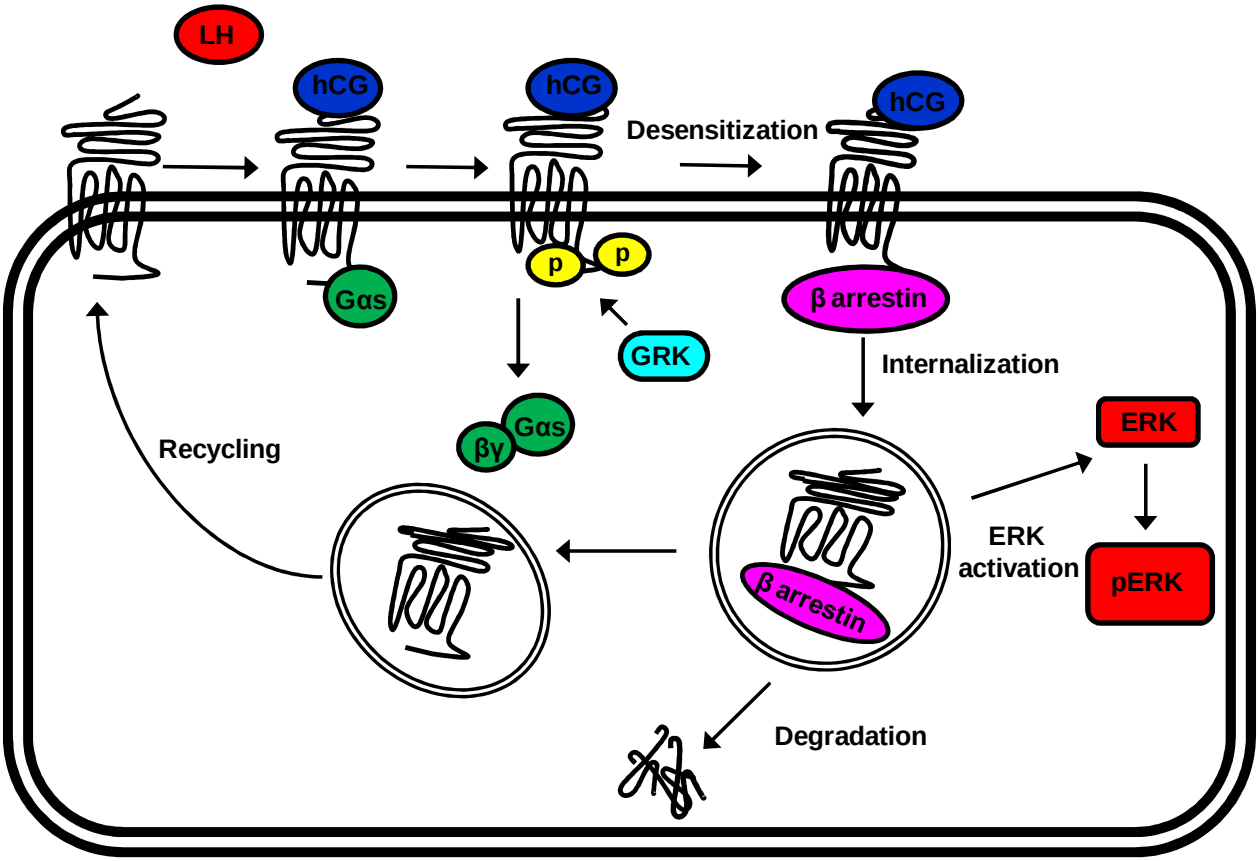

Figure 9. G proteins-independent signaling. Upon hCG/LH binding to LHCGR, GRK phosphorylates the activated receptor, resulting in receptor desensitization. β-arrestins is recruited to the phosphorylated receptor leading to receptor internalization. Receptor degradation or recycling to the cell membrane can occur, as well as the activation of additional signaling, such as ERK activation.

5. Clinical use of LH and hCG

Animal pituitary extracts of FSH provided the first commercially available gonadotropins for the management of infertility in the 1930s, while the first use of cadaveric human gonadotropins for induction of ovulation was reported in 1958 (Gemzell et al. 1958). The increasing demand for gonadotropins led to the extraction and isolation of human menopausal gonadotropin (hMG) from urine of women in 1950 (Lunenfeld. 2002). Highly purified urinary preparations were recently isolated through advanced immunopurification and fractionation techniques using specific monoclonal antibodies. Genetic engineering technology allow to obtain recombinant highly pure of FSH, LH and hCG preparation (Howles. 1996). Commercially available gonadotropins are used for controlled ovarian stimulation in assisted reproduction, ovulation induction for WHO group I and II anovulatory infertility and in men with hypogonadotrophic hypogonadism (HH) or idiopathic oligo-asthenospermia.

Some authors believe that hCG is ideal for follicular development because its long half-life provides a more sustained LH stimulation. Recombinant LH, in contrast, has a shorter half-life and requires multiple daily injections to sustain follicle development. On the other side, accumulation of hCG bioactivity may have detrimental effects on follicular development and oocyte quality (Baer et al. 2003). Some clinical studies in hypogonadotropic hypogonadal women have demonstrated the efficacy of recombinant hLH, together with recombinant hFSH, in promoting optimal follicular development, oestrogen secretion and endometrial thickness (Baer et al. 2003).

Due to their structural similarities and their binding to the common LHCGR, for several decades hCG and LH were considered two gonadotropins biologically equivalent, inducing similar intracellular cascades. Basing on these assumptions, hCG is still added to hMG preparations used in reproductive techniques, in order to mimic LH activity (Wolfenson et al. 2005; Van de Weijer et al. 2003), although several clinical outcomes depending on LH/hCG differential use in treatment protocols occurs (Hanson et al. 1971; Razi et al. 2014; Requena et al. 2014). Recently, some *in vivo* studies supported the higher ability of LH and FSH to improve oocyte quality and fertilization rate, rather than hCG (Ruvolo et al. 2007; Carone et al. 2012; Fábregues et al. 2013). These data could be supported by the non

biological equivalence exerted by hCG and LH reported in different *in vitro* models, (Casarini et al. 2012; Gupta et al. 2012), documented also in the presence of FSH (Casarini et al. 2015).

6. hCG *vs* LH: common receptor but biased signaling

The major differences between LH and hCG are resumed in Table 1.

	LH	hCG
Major site of production	Anterior pituitary gland	Placental trophoblast cells
Secretion pattern	Pulsatile release	*non* pulsatile secretion
Period of physiological production	Reproductive age and menopause	Pregnancy
Species	All vertebrates	Equids and primates
Sex	Men and women	Pregnant women and men
Major function	Ovulation and synthesis of sex steroids	Establishment of pregnancy in women; male fetal sexual differentiation
Peptide length	121 aminoacids	145 aminoacids
Glycosylation pattern of the β-subunit	2× N-glycans no O-glycans	2× N-glycans 4× O-glycans
Molecular weight (Da)	28.000-32.000	36.000-45.000

Table 1. Main differences between hCG and LH

In the last years, the arising concept of hCG/LH biased signaling. In vitro experiments performed using human primary granulosa cells revealed that hCG is more potent in activating the steroidogenic cAMP/PKA pathway, while LH treatment results in higher levels of the anti-apoptotic ERK1/2 and AKT pathways (Casarini et al. 2012). Prolonged, constant exposure of granulose cells to gonadotropins results in cyclic cAMP production is cyclic in granulosa cells, with a period of approximately 4-5 hours. Higher cAMP peaks were induced by hCG comparing to those induced by LH, in spite of similar progesterone production. In goat ovarian granulosa cells, sustained exposure to hCG leads to higher levels of cAMP, decreasing growth and increasing cell doubling time. Prolonged LH treatment

promotes growth and proliferation characterized by higher ERK1/2 levels to those mediated by hCG (Gupta et al. 2012).

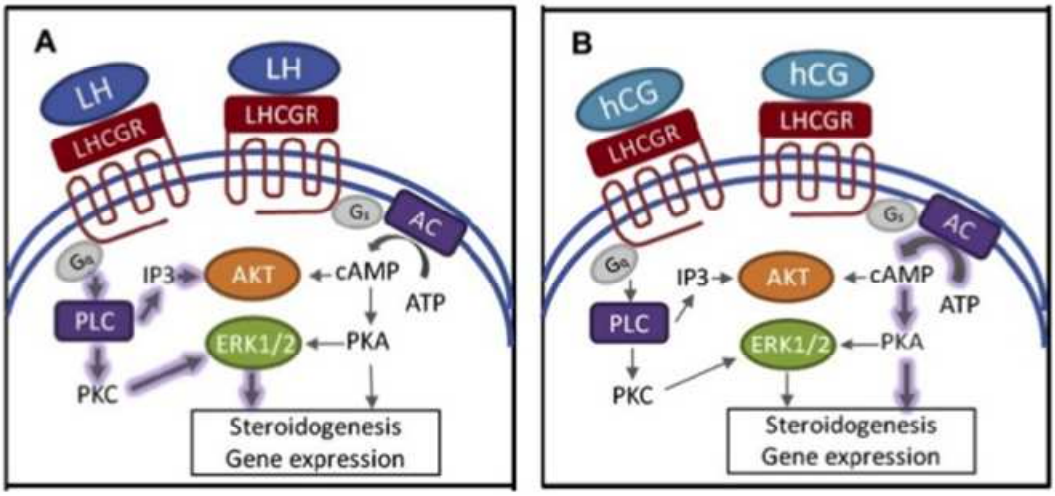

Figure 10. Theoretical pathways representing the divergence in LH- and hCG-mediated signaling. Although acting on the same receptor, accumulating evidence suggests that LH binding (A) has a greater impact (thicker arrow) on AKT and extracellular signal-regulated protein kinase (ERK1/2) phosphorylation than hCG does, whereas binding of hCG (B) generates a greater intracellular accumulation (thicker arrow) of cAMP than does LH.

hCG/LH-mediated intracellular signaling was evaluated in human granulosa cells, in the presence of a fixed dose of FSH, demonstrating that FSH increases the anti-apoptotic effects of LH and the steroidogenic potential of hCG (Casarini et al. 2015).

Taken together, these results support the *in vitro* non equivalence of hCG and LH and prompting the need of in vitro experiments to dissect their activated signaling pathways in different conditions.

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Aim of the study

The action of LH and hCG is mediated by LHCGR, which belongs to GPCRs family, by inducing multiple signaling pathways. The arising concept of hCG and LH biased signaling is shown by their ability to direct specific and distinct biological responses *via* activation of select signaling pathways in a ligand-specific manner, probably by multiple ligand-receptor conformations.

In this *in vitro* study, I have compared the activity of recombinant LH and hCG, in terms of cell response in both primary cells and transfected LHCGR-expressing cell lines. Gs/cAMP/PKA pathway, Gq/PLC pathway, ERK1/2 and AKT signaling, β-arrestin 2 recruitment, gene expression and steroidogenesis were analyzed. Biased agonism exerted by hCG and LH was found, revealing interesting differences in term of hormone potency and efficacy. These results highlight the proliferative and anti-apoptotic activity of LH and the high steroidogenic activity of hCG, revealing ligand-dependent preference for both G-protein signaling and β-arrestin signaling.

Chapter 1

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Follicle-stimulating hormone potentiates the steroidogenic activity of chorionic gonadotropin and the anti-apoptotic activity of luteinizing hormone in human granulosa-lutein cells *in vitro*

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ABSTRACT

Luteinizing hormone (LH) and choriogonadotropin (hCG) are glycoprotein hormones regulating ovarian function and pregnancy, respectively. Since these molecules act on the same receptor (LHCGR), they were traditionally assumed as equivalent in assisted reproduction techniques (ART), although differences between LH and hCG were demonstrated at molecular and physiological level. In this study, we demonstrated for the first time that co-treatment with a follicle-stimulating hormone (FSH) dose in the ART therapeutic range potentiates different LH- and hCG-dependent responses *in vitro*, measured in terms of cAMP, phospho-CREB, -ERK1/2 and -AKT activation, gene expression, progesterone and estradiol production in human granulosa-lutein cells (hGLC). We show that in the presence of FSH, hCG bio-potency is about 5-fold increased, in the presence of FSH, in terms of cAMP activation. Accordingly, CREB phosphorylation and steroid production is increased under hCG and FSH co-treatment. LH effects, evaluated as steroidogenic cAMP/PKA pathway activation, do not change in the presence of FSH, which, however, increases LH-dependent ERK1/2 and AKT, but not CREB phosphorylation, resulting in anti-apoptotic effects. The different modulatory activity of FSH on LH and hCG action *in vitro* corresponds to their different physiological functions, reflecting proliferative effects exerted by LH during the follicular phase and before trophoblast development, and the high steroidogenic potential of hCG requested to sustain pregnancy from the luteal phase onwards.

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; hCG, human chorionic gonadotropin; TSH, thyroid-stimulating hormone; FSHR, FSH receptor; LHCGR, LH and hCG receptor; GPCRs, G protein-coupled receptors; PKA, protein kinase A; CREB, cAMP-responsive element binding protein; ERK1/2, extracellular-regulated kinase; PI3K, phosphatidylinositol-3-kinases; AKT, protein kinase B; ART, assisted reproduction techniques; hGLC, human granulosa-lutein cells; HRM, High Resolution Melting; IBMX, 3-isobutyl-1-methylxanthine; androstenedione, 4-androstene-3, 17-dione; RPS7, ribosomal subunit protein 7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD, standard deviation; EC_{20}, 20% maximal effective dose; EC_{50}, half maximal effective dose; H-slope, hill slope value; EGF, epidermal growth factor; *AREG*, amphiregulin gene; *EREG*, epiregulin gene; *CASP3*, procaspase 3 gene; *TP53*, p53 gene; *XIAP*, X-linked inhibitor of apoptosis factor gene; *STARD1*, steroidogenesis-related StAR gene; *CYP19A1*, aromatase gene; Tβ-RII, transforming growth factor β receptor.

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1. Introduction

1.1. The gonadotropins: structure and differences

The gonadotropins human follicle-stimulating hormone (FSH), luteinizing hormone (LH) and choriogonadotropin (hCG) belong to the family of glycoprotein hormones. In women of reproductive age, FSH and LH regulate ovarian cycle, follicle growth and ovulation, while hCG induces progesterone production to maintain pregnancy. They act *via* the specific FSH receptor (FSHR) and the common LH/hCG receptor (LHCGR) located in the gonads, belonging to the superfamily of the G protein-coupled receptors (GPCRs).

Several differences characterize gonadotropins; at the molecular level, they share a common α and a unique hormone-specific β subunit. FSH and LH are produced by pituitary in a pulsatile fashion, following a cyclic rhythm during the reproductive age in women. hCG is present only in primates and equids and is produced by the trophoblast cells in a constant manner. These molecules exist in several glycosylation isoforms with different half-lives and ligand binding affinity (Arey and López, 2011; Bousfield et al., 2014; Crochet et al., 2012; Fournier et al., 2015). Moreover, LH and hCG share the particular aminoacid residues to bind the same receptor (Ascoli et al., 2002) despite previous studies evaluating the displacements of hCG radioligands suggested that they may differ in the binding affinity to LHCGR (Galet and Ascoli, 2005; Müller et al., 2003). However, a clear comparison between LH and hCG binding is still lacking and further binding experiments evaluating the displacement of both LH and hCG radioligands are required to exactly define which gonadotropin has the highest binding affinity to LHCGR.

1.2. Cell signaling

As a consequence of such structural sharing (Schaarschmidt et al., 2014), FSHR- and LHCGR-mediated pathways overlapping in large part (Casarini et al., 2012, 2014; Zhang et al., 2009). Upon ligand binding, GPCRs undergo conformational changes resulting in sequential activation of $G_{\alpha}s$ protein and adenylyl cyclase, which catalyzes the conversion of ATP to the second messenger cAMP, leading to a complex cross-talk between steroidogenic and death-related pathways (Amsterdam et al., 1999), which are simultaneously counterbalanced by proliferative and anti-apoptotic signals (Craig et al., 2007). The intracellular cAMP increase results in the canonical cAMP/protein kinase A (PKA) pathway activation, leading to phosphorylation of the cAMP-responsive element binding protein (CREB), and the extracellular-regulated kinase (ERK1/2). While pCREB activation results in progesterone production and androgen conversion to estradiol (Manna et al., 2002), pERK1/2 exerts a modulatory role of steroidogenesis (Casarini et al., 2014; Ryan et al., 2008). pERK1/2 recruitment may occur *via* alternative molecules coupled to FSHR and LHCGR, such as β-arrestins, activated upon hormone binding simultaneously to G proteins (Ayoub et al., 2015; Cervantes et al., 2010; Musnier et al., 2010). Also, the phosphorylation of ERK1/2 is linked to intracellular signaling pathways mediating the internalization of the receptor (Amsterdam et al., 2002) and cell survival (Ben-Ami et al., 2009). Moreover, phosphatidylinositol-3-kinases (PI3K) recruitment leads to protein kinase B (AKT) phosphorylation, which is traditionally related to anti-apoptotic effects in granulosa cells (Cecconi e al. 2012). Although the FSHR- and LHCGR-mediated signaling cascades are highly similar (Casarini et al., 2012, 2014), the activation of specific receptor-dependent signaling involving second messengers, such as calcium ions and phospholipids (Conti, 2002; Lee et al., 2002), was associated with LHCGR, rather than FSHR. Also, different gonadotropin-dependent gene expression and steroid synthesis were observed (Donadeu and Ascoli, 2005; Freimann et al., 2004), likely as a result of structural diversities of the glycoprotein hormone receptors (Bonomi et al., 2006; Vassart et al., 2004) and fine-tune regulations of the early cAMP/PKA-, ERK1/2- and AKT-mediated signaling (Bebia et al., 2001). At the cell membrane level, the GPCRs exist as a functional unit of homo- or heterodimers (Dean et al., 2001), which increase the complexity of regulation of the signal transduction through several *cis*- or *trans*-interactions (Ji et al., 2002). LHCGR-FSHR heterodimerization was recently observed by fluorescence resonance energy transfer (FRET) techniques (Feng et al., 2013; Mazurkiewicz et al., 2015), suggesting that it may play a relevant role in the regulation of granulosa cell differentiation (Mazurkiewicz et al., 2015).

1.3. Non-equivalence of gonadotropins

In clinical practice, a number of naturally occurring and recombinant gonadotropins are traditionally used to induce follicle maturation in women undergoing assisted reproduction techniques (ART). Concerning LH and hCG, they are assumed to be equivalent, although molecular (Galet and Ascoli, 2005), biochemical (Ludwig et al., 2003), physiologic (Khan-Sabir et al., 2008) and evolutionary (Henke and Gromoll, 2008) features differentiate these molecules. The clinical use of gonadotropins classically aims at obtaining a large number of follicles, as the main endpoint of a successful treatment, and at maximizing embryo quality and take-home baby rate without multiple gestations (Society of Obstetricians and Gynaecologists of Canada et al., 2014). However, several studies demonstrated that important clinical implications may result from different treatment protocols, due to risk of ovarian hyperstimulation (Soave and Marci, 2014), phenotypic features of the patients (Casarini and Brigante, 2014) and tumorigenic potential of these molecules (Cole and Butler, 2008; Reigstad et al., 2015). In ART, FSH may be used alone or in combination with LH or hCG to optimize follicular maturation, but the choice of the therapeutic regimen is empirical. Our idea is that LH and hCG are not equivalent at molecular level, and that their effects may be affected by the concomitant activation of the FSH-FSHR complex to further modulate cell signaling and gene expression, steroid synthesis, and proliferative and anti-apoptotic signals in granulosa cells.

1.4. Aim of the study

In this work we confirm that LH and hCG are not equivalent at molecular level *in vitro*, and demonstrate that FSH modulates signaling and downstream survival/proliferative and steroidogenic signals of LH and hCG. The biopotency of the gonadotropins, and the modulatory activity of FSH was evaluated using short-term stimulation of human granulosa-lutein cells (hGLC) by dose–response experiments, in the presence of a fixed, minimal, effective FSH dose previously identified (Casarini et al., 2014). The response was measured in terms of signal transduction, gene expression and steroidogenesis activation.

2. Materials and methods

2.1. Recombinant gonadotropins

Human recombinant FSH (Gonal-F), LH (Luveris) and hCG (Ovitrelle) were kindly provided by Merck-Serono S.p.A. (Rome, Italy), a division of Merck KGaA (Darmstadt, Germany).

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HealthCare, Little Chalfont, UK), then acquired and semi-quantitatively evaluated by an image analysis system (VersaDoc Imaging System and QuantityOne software, Bio-Rad Laboratories Inc.). Our previous experiments demonstrated that 15 min is the optimal time-point to detect and compare pERK1/2, pAKT and pCREB signals in hGLC (Casarini et al., 2012, 2014). Indeed, the analysis of the pCREB, pERK1/2 and pAKT activation kinetics demonstrated that the phosphorylation of these proteins occurs within 5 min, achieves the maximal level in about 15 min, decreases after 20 min and it is barely detectable at 1 h time-point, upon LH, hCG and FSH treatment. Also, blockade of the pCREB, pERK1/2 and pAKT by selective inhibitors affected cAMP production, gene expression and steroid synthesis, revealing the importance and effectiveness of their early phosphorylation for a proper regulation of the cell signaling (Casarini et al., 2012, 2014).

2.9. Gene expression analysis

To avoid the achievement of a steady metabolic *plateau* by continuously maintaining the cells in the presence of gonadotropins, a short-term stimulation protocol was applied before the gene expression evaluation (Casarini et al., 2014). To this purpose, cells stimulated for 2 h by 1×10^1 nM FSH, in the presence or in the absence of 1x10 ^{1} nM LH or hCG, were washed twice with 37 °C PBS to stop the reaction and left in the incubator in serum-free medium for additional 12 h. Then they were lysed and subjected to RNA extraction. Total RNA was extracted and retro-transcribed and quantitative real-time RT-PCR was performed using primer probes previously validated (Casarini et al., 2012, 2014): gene encoding for *AREG* (NCBI gene ref. seq.: NM_001657.2): forward primer sequence 5′-GACACCTACTCTGGGAAGCG-3′ and reverse primer sequence 5′-AAGGCATTTCACTCACAGGG-3′; *EREG* (NM_001432.2): 5′- TACTGCAGGTGTGAAGTGGG-3′ and 5′-TGGAACCGACGACTGTGATA-3′; *STARD1* (NM_000349.2): 5′-AAGAGGGCTGGAAGAAGGAG-3′ and 5′-TCTCCTTGACATTGGGGTTC-3′; *CYP19A1* (NM_000103.3): 5′-CCCTTCTGCGTCGTGTCAT-3′ and 5′-GATTTTAACCACGATAGCACTTTCG-3′; *XIAP* (NM_001167.3): 5′-TTGAAAATAGTGCCACGCAG-3′ and 5′-TGTGTCTCAGATGGCCTGTC-3′; *TP53* (NG_017013.2): 5′-GGAGACACCGCTTGGAACTA-3′ and 5′-AGCCCACTTACAGCCTTTCA-3′; *CASP3* (AY219866.1): 5′-TGTTTGTGTGCTTCTGAGCC-3′ and TCTACAACGATCCCCTCTGAA-3′. The expression of *ribosomal subunit protein 7* gene (*RPS7*; NM_001011.3: 5′-AATCTTTGTTCCCGTTCCTCA-3′ and 5′-TTCTGCCTAAGCCAACTCG-3′) was used as loading control. Normalized gene expression was evaluated using the 2-ΔCt method (Livak and Schmittgen, 2001) and expressed as fold increase over its unstimulated sample (basal level).

2.10. Cell viability assay

Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Mosmann, 1983) after a short-term stimulation protocol (Casarini et al., 2014). 2 h-stimulated cell were washed twice with 37 °C PBS and left in an incubator in serum-free medium. After 2 days they were lysed in 100 μL of isopropanol (Sigma–Aldrich) and the absorbance was evaluated in a plate reader (Victor3, Perkin Elmer Inc., Waltham, MA, USA) at the wave length of 570 nm, then graphically represented as means ± standard deviation (SD).

2.11. Statistical analysis

Each value obtained from stimulated cells was normalized for the corresponding control value, as follows: in cAMP, progesterone and estradiol dose–response experiments, data were normalized as percentage of the highest value. In the experiments for ERK1/2 and CREB, the semi-quantitative evaluations were graphically expressed in relative units. Data are expressed as means ± SD and Mann–Whitney's U-tests, two-way Anova coupled with Bonferroni post-test, *non*-linear or linear regression, and F test were performed as appropriate. Values were considered statistically significant for $p < 0.05$, but lower p values were also indicated. Statistical analysis were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

3. Results

In a previous study, we demonstrated that LH and hCG stimulation results in different biopotency on cAMP and progesterone production, cell signaling activation and gene expression, revealing that LH treatment leads to higher activation of the proliferative and anti-apoptotic ERK1/2- and AKT-pathways than hCG, which, instead, results in the uppermost activation of the steroidogenic and cell death-related cAMP/PKA-pathway (Casarini et al., 2012). Here we evaluated for the first time the modulation of LH and hCG action by 1×10^1 nM FSH, as fixed, minimally effective dose, as previously assessed (Casarini et al., 2014). hGLC exposure to 1×10^1 nM FSH alone resulted in slight, not statistically significant changes in terms of signaling pathway activation and steroid synthesis, compared to unstimulated cells *in vitro*; thus, 1×10^1 nM FSH-treatment was assumed as basal experimental condition, as previously demonstrated (Casarini et al., 2014). This FSH concentration corresponds to about the 20% maximal effective dose (EC_{20}) in hGLC and is in the therapeutic range used in ART (Weisman et al., 1989). Cells maintained without FSH served as control. In these individual LH and hCG-stimulated controls (Supplementary Fig. 1), the phosphorylation of ERK1/2 and AKT was evaluated by Western blotting, after treatment with increasing doses of LH or hCG, in the presence and in the absence of FSH. By replicating results previously observed and discussed (Casarini et al., 2012), we validated the experimental protocol and the comparison of the present results with those of Casarini et al. (2012).

3.1. cAMP dose–response curves in the presence of FSH

To evaluate the modulation of FSH on LH- or hCG-stimulated cAMP production, dose–response experiments were performed in hGLC. cAMP accumulation was evaluated by ELISA after stimulation by increasing doses of LH or hCG for 2 h, in the presence of 1×10^1 nM FSH and 500 μM IBMX (Fig. 1). Under these conditions, increasing LH doses result in significantly different half maximal effective concentration (EC_{50}) values compared to those of hCG (LH + FSH: $EC_{50} = 440.9 \pm 271.4$ pM; hCG + FSH: $EC_{50} = 20.3 \pm 1.2$ pM; means ± SD; Mann–Whitney's U-test, $p = 0.0006$, $n = 6$) revealing a 15.6-fold higher biopotency of hCG *versus* LH, in the presence of a fixed, minimally effective FSH dose (Table 1). However, the hill slope value (H-slope) of LH is significantly different from that of hCG, suggesting that the mechanisms of interaction between ligand-receptor complex and/or downstream molecules are differently modulated by the two gonadotropins, in the presence of FSH (LH + FSH H-slope = 2.9 ± 1.0; hCG + FSH H-slope = 1.0 ± 0.2; Mann–Whitney's U-test, $p = 0.0023$, $n = 6$; means ± SD). This indicates that a small number of binding sites trigger maximal cAMP response to LH while hCG interaction with the LHCGR is stoichiometric, since their H-slopes are higher than and about similar to 1, respectively (Prinz, 2010). Further ligand-receptor binding experiments should be performed to confirm this statement. Finally, the maximal levels of cAMP activation significantly differs between LH and hCG treatment in the presence of FSH (LH + FSH max cAMP = 650.3 ± 97.8 pmol/ml; hCG + FSH

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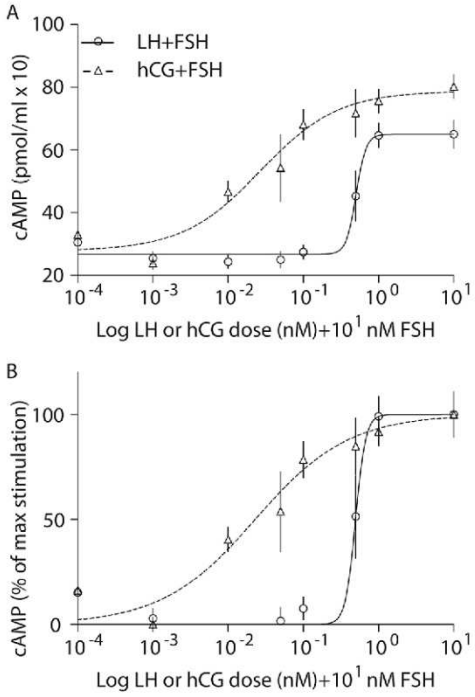

Fig. 1. cAMP dose–response to LH or hCG in the presence of 1×10^1 nM FSH treatment. hGLC were stimulated 2 h by 0–1x10¹ nM LH or hCG together with the fixed FSH dose, in the presence of IBMX, then cAMP was measured by ELISA. A) cAMP levels expressed as pmol/ml. B) cAMP response normalized in percentage of the maximal response. All the results are represented as means ± SD (n = 6) in a logarithmic X-axis, then *non*-linear regressions were plotted. The EC50, H-slope and maximal cAMP values were compared by Mann–Whitney's *U*-test as indicated in Table 1.

max cAMP = 795.4 ± 46.10 pmol/ml; Mann–Whitney's U-test, p = 0.0175, n = 6; means ± SD), suggesting that the gonadotropins have different agonism, where LH may act as a partial agonist or hCG as a superagonist (Fig. 1A; Table 1).

Moreover, by comparing the EC_{50} and H-slope values with those extracted from our previous study performed under identical experimental conditions (Casarini et al., 2012), it is evident that hCG, but not LH treatment, results in about 5.0- and 2.3-fold lower EC_{50} and H-slope, respectively, in the presence of FSH (Table 2). Thus, the presence of FSH increases hCG biopotency. On the contrary, FSH does not affect LH EC_{50} and H-slope.

3.2. cAMP/PKA, ERK1/2 and PI3K/AKT signaling pathways activation

The effects of LH or hCG and FSH co-treatment for 15 min was evaluated in terms of signaling pathways activation in dose–response experiments. hGLC were stimulated for 15 min with increasing LH or hCG doses, together with the fixed, minimally activating 1×10^1 nM FSH dose, which alone results in slight activation of the signaling pathways (Casarini et al., 2014). Then phosphorylation of CREB, ERK1/2 and AKT were evaluated by Western blotting (Fig. 2a) and semi-quantitatively measured (Fig. 2b–d).

Reflecting cAMP activation, the downstream phosphorylation of CREB occurs differently in LH- or hCG-treated cells in the presence of FSH (Fig. 2b). pCREB activation occurs upon stimulation by the highest (1×10^1 nM) LH dose used, while hCG induces CREB phosphorylation in a range of concentrations spanning two orders of magnitude (1×10 ¹ nM-1x10¹ nM) (Mann–Whitney's *U*-test, $p < 0.05$; n = 4), demonstrating a more potent stimulation of the cAMP/PKA pathway by hCG than by LH (two-way Anova and Bonferroni post-test, $p < 0.05$; n = 4). Interestingly, a discrepancy between cAMP production and pCREB activation exists at the 1×10^0 nM LH dose-point (Fig. 1), likely resulting from the inhibitory, negative feedback exerted *via* pERK1/2 on the GPCR-dependent cAMP/PKA-pathway activation (Pitcher et al., 1999).

The activation of pERK1/2 (Mann–Whitney's *U*-test, $p < 0.05$; n = 4) was induced by LH in a wider range of concentrations compared to hCG (1x10 ¹ nM-1x10¹ nM LH, 1×10^1 nM hCG; two-way Anova and Bonferroni post-test, $p < 0.05$; n = 4), in the presence of FSH (Fig. 2c). Moreover, pERK1/2 achieved higher levels of activation upon LH compared to hCG stimulation at the 1×10^1 nM dose (two-way Anova and Bonferroni post-test, $p < 0.05$; n = 4), confirming that luteotropin predominantly activates the proliferation-related ERK1/2-pathway (Casarini et al., 2012; Gupta et al., 2012) also in the presence of FSH. However, a modulatory effect of FSH occurs in both LH- and hCG-dependent activation of pERK1/2, which increases in a progressive, dose-dependent manner (LH $R^2 = 0.83$; hCG $R^2 = 0.57$; linear regression, $p < 0.0001$; n = 4). It differs from what we previously observed in the absence of FSH (Casarini et al., 2012) when the maximal activation was obtained at 1x10 ¹ nM LH or hCG and decreased at higher doses.

Table 1
Comparison between LH and hCG EC50, H-slopes and maximal levels from cAMP dose–response curves, in the presence of 1×10^1 nM FSH. $P < 0.05$ were indicated in bold (Mann–Whitney's *U*-test).

	EC50 (pM; means ± SD; n = 6)	p^a	H-slopes (pM; means ± SD; n = 6)	p^a	Max cAMP value (pmol/ml; means ± SD; n = 6)	p^a
LH + FSH	440.9 ± 271.4	**0.0006**	2.9 ± 1.0	**0.0023**	650.3 ± 97.8	**0.0175**
hCG + FSH	20.3 ± 1.2		1.0 ± 0.2		795.4 ± 46.10	
Fold difference	15.6		9.3		0.8	

[a] Mann-Whitney's U-test.

Table 2
Comparison between LH and hCG EC50 and H-slopes from cAMP dose–response curves, in the presence (present data) or absence (Casarini et al., 2012) of 1×10^1 nM FSH.

EC50 (means±SD)		H-slopes (means±SD)			
LH	hCG	LH	hCG	+FSH (nM)	Reference
475.8 ± 137.4	101.8 ± 44.6	2.8 ± 0.6	2.3 ± 0.5	0.0	Casarini et al., 2012 (n = 4)
440.9 ± 271.4	20.3 ± 1.2	2.9 ± 1.0	1.0 ± 0.2	10^1	Present article (n = 6)
1.1	5.0	1.0	2.3		Fold difference

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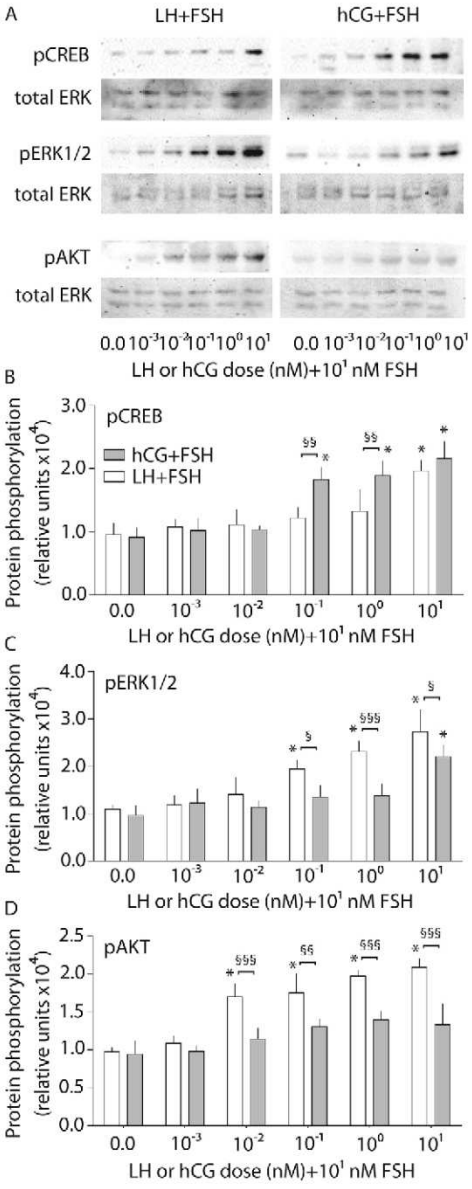

Fig. 2. Phosphorylation of CREB, ERK1/2 and AKT induced by increasing LH or hCG concentrations, in the presence of a fixed FSH dose. hGLC were stimulated 15 min by 0–1x10¹ nM LH or hCG together with 1 × 10¹ nM FSH, then the signaling pathways activation was evaluated. A) Qualitative analysis of pCREB, pERK1/2 and pAKT by Western blot using specific antibodies (image representative of 4 experiments). Total ERK served as normalizer. B-D) Semi-quantitative analysis of pCREB, pERK1/2 and pAKT activation, respectively. The signals detected by Western blotting were semi-quantitatively evaluated using an image analysis software and graphically represented as means ± SD after background subtraction. * = significantly different *versus* LH- or hCG-unstimulated (basals) (Mann–Whitney's *U*-test; $p < 0.05$; n = 4). The results obtained by LH and hCG treatments were compared by two-way Anova and Bonferroni post-test (LH *versus* hCG; §$p < 0.05$, §§$p < 0.01$, §§§$p < 0.001$; n = 4).

LH stimulation results in higher pAKT activation compared to hCG treatment, in the presence of FSH (two-way Anova and Bonferroni post-test, $p < 0.05$; n = 4), with activation in the 1x10 ^{2} nM-1x10¹ nM LH range. No statistically detectable activation of pAKT was obtained by hCG (Mann–Whitney's *U*-test, $p < 0.05$; n = 4) (Fig. 2d). Moreover, the pAKT lowest stimulatory LH concentration was reduced (1x10 ^{2} nM) in the presence of FSH, compared to the results obtained previously (1x10 ^{1} nM) in the absence of FSH (Casarini et al., 2012). This suggests an amplification of the LH-dependent PI3K/AKT signaling by FSH.

3.3. Gene expression

A previous study failed to find any difference depending on the stimulations (Segers et al., 2012). Thus, we avoided long-term, continuous exposure of the cells to gonadotropins, which may results in long-term masking of the different cell signaling activation due to the achievement of a steady-state of the cell system. The expression of cell cycle-related epidermal growth factor (EGF)-like factors amphiregulin (*AREG*) and epiregulin (*EREG*), of the pro- and anti-apoptotic caspase 3 (*CASP3*), p53 (*TP53*) and X-linked inhibitor of apoptosis factor (*XIAP*), and of the steroidogenesis-related StAR (*STARD1*) and aromatase (*CYP19A1*) genes was evaluated by real time PCR analysis (Fig. 3).

Consistently with the action in the proliferative ERK1/2-pathway, co-stimulation by LH or hCG together with FSH results in *AREG* and *EREG* gene expression increase over basal (Mann–Whitney's U-test, $p < 0.05$; n = 4) (Fig. 3a).

CASP3 gene expression was significantly more increased under hCG and FSH co-treatment compared to LH and FSH, or FSH treatment alone ($p < 0.05$; Mann–Whitney's U-test; n = 4). Moreover, both LH and hCG treatments, in the presence of FSH, result in increase of the pro-apoptotic *TP53* gene expression over basal (Mann–Whitney's U-test, $p < 0.05$; n = 4). The positive modulation of p53 is accompanied by a significant increase of the anti-apoptotic *XIAP* gene expression mediated by LH, but not hCG together with FSH (Mann–Whitney's U-test, $p < 0.05$; n = 4) (Fig. 3b).

Both LH and hCG together with FSH increase the expression of the steroidogenic *STARD1* and *CYP19A1* genes over basals (Mann–Whitney's U-test, $p < 0.05$; n = 4); however, the *CYP19A1* gene expression achieves higher levels by hCG, than by LH stimulation (Mann–Whitney's U-test, $p < 0.05$; n = 4) (Fig. 3c), corroborating the important role played by the placental gonadotropin in progesterone and estrogen synthesis (Cole, 2010; Daveseelan et al., 2010).

3.4. Cell viability

Following intracellular cAMP increase, pERK1/2 and pAKT activation were related with opposite, pro- and anti-apoptotic signals in steroidogenic cells *in vitro* (Amsterdam et al., 2003; Craig et al., 2007; Matsuda-Minehata et al., 2006). Cell viability was evaluated by MTT assay in hGLC stimulated by gonadotropins. The cells were stimulated 2 h by increasing LH or hCG doses, in the presence of 1 × 10¹ nM FSH, then washed twice and maintained 48 h in an incubator, in the absence of serum. The MTT solution was administred to the cells 2 h before lysis by isopropanol and evaluation of absorbance. The absorbance values represent cell viability, which was graphically showed as mean ± SD (Fig. 4).

Reflecting the intracellular hCG-mediated cAMP increase (Fig. 1), x10 ^{1}–1x10¹ nM hCG together with 1 × 10¹ nM FSH treatment significantly decreases cell viability compared to LH (Mann–Whitney's U-test, $p < 0.05$; n = 4). On the contrary, LH stimulation results in no changes compared to unstimulated cells (Mann–Whitney's U-test, $p \geq 0.05$; n = 4). Surprisingly, cell viability is preserved in spite of the similar, maximal cAMP increase elicited by LH and hCG (*non*-normalized cAMP concentrations at the maximal dose-point: LH = 649.4 ± 112.9 pmol/ml;

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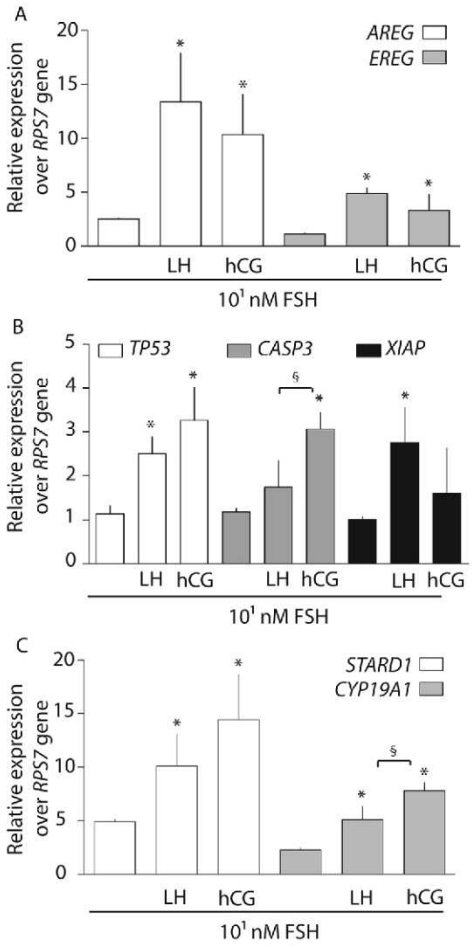

Fig. 3. Gene expression analysis. The expression of target genes was evaluated in 2-h 1x10 ^{1} nM LH- or hCG-stimulated hGLC, in the presence of 1×10^{1} nM FSH, after 12 h, by real time PCR. Controls were prepared by incubating the cells in media without LH or hCG. Each value was normalized by the *RPS7* control gene expression and graphically represented as fold increase over unstimulated controls in relative units scale (n = 4; means ± SD). A) Evaluation of the expression of EGF-like factor genes involved in cell cycle regulation. B) Analysis of pro- and anti-apoptotic genes. C) Steroidogenesis-related genes. * = statistically significant difference *versus* FSH alone (basal); § = statistically significant difference between LH- and hCG-stimulated cells (Mann–Whitney's *U*-test; $p < 0.05$; n = 4).

hCG = 806.7 ± 147.0 pmol/ml; means ± SD; Mann–Whitney's *U*-test, $p = 0.34$; n = 4; Fig. 1), suggesting a preponderance of the proliferative and anti-apoptotic ERK1/2- and PI3K/AKT-pathways activation in the presence of LH.

3.5. Evaluation of the steroid synthesis

The effect of increasing LH or hCG doses in the presence of 1×10^{1} nM FSH was evaluated in terms of progesterone and estradiol production. Since hGLC does not produce estradiol by itself (Patel et al., 2009), androstenedione was added as a substrate for aromatase (Nordhoff et al., 2011). A previous 36-h time-course experiment evaluating progesterone synthesis upon continuous stimulation by the EC_{50} dose of LH and hCG in hGLC resulted in no differences depending on the type of gonadotropin (Casarini et al., 2012). Thus, steroid production was evaluated using a short-term 2-h stimulation of hGLC by LH or hCG, thereafter the cells were washed twice and maintained 24 h in an incubator in the absence of serum before steroid measurement in the supernatants by ELISA.

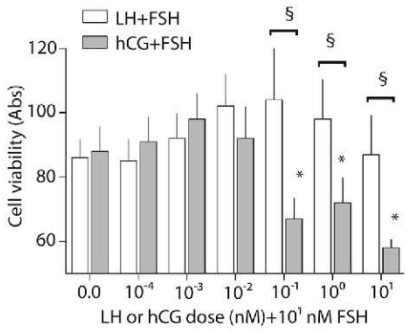

Fig. 4. Cell viability assay in hGLC stimulated by different LH or hCG doses, in the presence of 1×10^{1} nM FSH. The cells were treated using the short-term stimulation protocol, then they were washed twice. The cell viability was assessed after 2 days by MTT assay and measured as absorbance using a spectrophotometer. The results were graphically represented as means ± SD after background subtraction. * = statistically significant difference *versus* FSH (basal); § = statistically significant difference between LH + hCG- and LH + FSH-stimulated cells (Mann–Whitney's *U*-test; $p < 0.05$; n = 4).

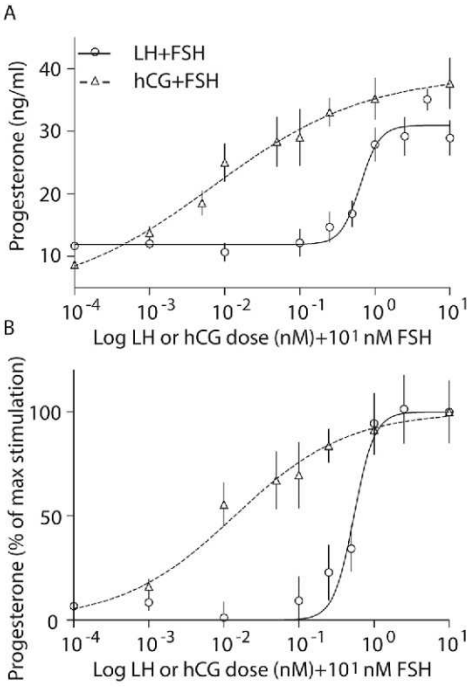

Fig. 5. Progesterone dose–response in hGLC upon 2-h stimulation by increasing LH or hCG doses, together with 1×10^{1} nM FSH. Progesterone was measured by ELISA after 24 h. A) Progesterone levels expressed as ng/ml. B) Progesterone values normalized as percentage of the maximal response. All the results are graphically represented as means ± SD (n = 6) in a logarithmic X-axis. *Non*-linear regressions were calculate and the EC50, H-slopes and maximal progesterone levels were extracted from the curves and compared as indicated in the Table 3.

Upon stimulation by LH or hCG (in the presence of 1×10^{3} nM FSH) highly statistically significant different EC_{50} values were measured in terms of progesterone synthesis (LH EC_{50} = 671.2 ± 240.6 pM; hCG EC_{50} = 82.7 ± 46.8 pM; Mann–Whitney's *U*-test, p = 0.0095; n = 6) (Fig. 5; Table 3), suggesting

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Table 3
Comparison between LH and hCG EC50, H-slopes and maximal levels from progesterone dose–response curves, in the presence of 1×10^1 nM FSH. $P < 0.05$ were indicated in bold (Mann–Whitney's *U*-test).

	EC50 (pM; means ± SD; n = 6)	p^a	H-slopes (pM; means ± SD; n = 6)	p^a	Max progesterone value (ng/ml; means ± SD; n = 6)	p^a
LH + FSH	671.2 ± 240.6	**0.0095**	3.1 ± 1.2	**0.0087**	35.0 ± 2.11	0.3939
hCG + FSH	82.7 ± 46.8		1.0 ± 0.2		39.0 ± 3.4	
Fold difference	8.1		3.1		0.9	

[a] Mann-Whitney's U-test.

that hCG is about 8-fold more biopotent than LH. Moreover, the simultaneous stimulation by FSH together with LH or hCG results in different H-Slope values (LH H-slope = 3.1 ± 1.2; hCG H-slope = 1.0 ± 0.2; Mann–Whitney's *U*-test, p = 0.0087; n = 6), reflecting the fact that progesterone synthesis is immediately dependent on cAMP increase, as suggested by the similar response in terms of cAMP and progesterone.

hCG together with FSH is about 275-fold more biopotent than LH, in terms of androstenedione conversion to estradiol by aromatase (LH + FSH: EC_{50} = 225.1 ± 80.2 pM; hCG + FSH: EC_{50} = 1.3 ± 0.8 pM; Mann–Whitney's U-test, p = 0.0022; n = 6). This occurred in spite of no significant differences between the LH- and hCG-dependent H-Slopes and similar, maximal levels of estradiol synthesis (Fig. 6; Table 4). These results corroborate the preferential activation of the steroidogenic cAMP/PKA pathway by hCG compared to LH.

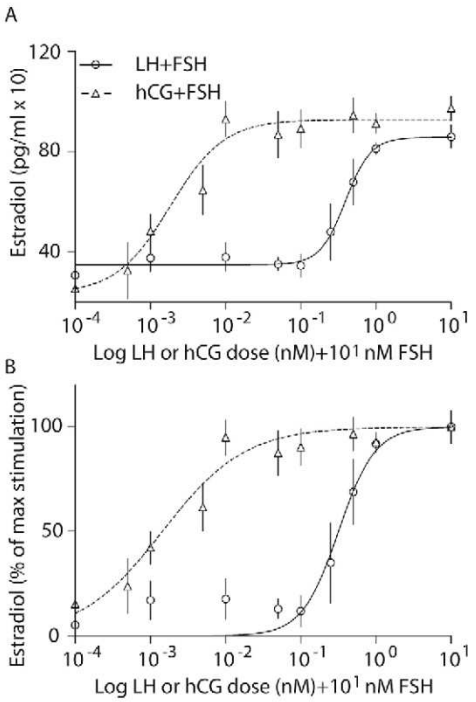

Fig. 6. Evaluation of estradiol production. hGLC were stimulated 2 h by increasing LH or hCG doses, in the presence of 1×10^1 nM FSH, then they were washed twice with PBS and the estradiol was measured by ELISA after 24 h 1 μM androstenedione was added to the cells media as substrate for the enzymatic conversion to estradiol. A) Estradiol levels expressed as pg/ml. B) Estradiol values represented by *non*-linear regression (n = 6). The EC50, H-slopes and maximal estradiol levels were compared as indicated in Table 4.

4. Discussion

Our study demonstrates for the first time that, in hGLC, the activities of LH and hCG are different in the presence of FSH, resulting in different biopotency and H-slopes, in terms of cell signaling activation, gene expression, cell viability and steroid synthesis *in vitro*. In particular, in the presence of FSH, hCG treatment results in significantly lower cAMP EC_{50} values and more potent cAMP production compared to LH, and the initially lower cAMP production by LH leads to higher ERK1/2 and AKT phosphorylation compared to hCG. The different activation of the signaling pathways leads to the prevalence of proliferative or steroidogenic downstream events mediated by LH and hCG, respectively. Especially, the concomitant presence of FSH increases hCG biopotency in terms of cAMP/PKA pathway activation and steroid synthesis, and the LH-dependent activation of ERK1/2 and PI3K/AKT signaling, which is linked to proliferative and anti-apoptotic events (Ben-Ami et al., 2009; Cecconi et al., 2012). Considering previous studies demonstrating different cell responses *in vitro* depending on the ligand (Casarini et al., 2012; Gupta et al., 2012), these results highlight the *non*-equivalence of LH and hCG at the molecular level although they act on the same receptor, and the modulatory role on events downstream the ligand-LHCGR complex elicited by FSH.

4.1. Molecular mechanisms of gonadotropins' action

A previous *in vitro* study found that LH and hCG were not equivalent at molecular level, revealing that hCG treatment of hGLC resulted in a higher activation of the steroidogenic cAMP/PKA pathway compared to LH, which rather led to higher activation of the proliferation- and anti-apoptotic-related ERK1/2 and AKT pathways (Casarini et al., 2012). Especially, upon hCG treatment the cAMP EC50 is about 5-fold lower than that of LH, but corresponds to a weaker activation of the proliferation-related and anti-apoptotic ERK1/2 and AKT pathways by hCG than LH (Casarini et al., 2012). Since intracellular cAMP increase was related to granulosa cell death (Aharoni et al., 1995), we could speculate that hCG have a higher pro-apoptotic potential than LH *in vitro*. In goat ovarian granulosa cells, LH promotes growth and proliferation while hCG lead to opposite results, suggesting in addition a different tumorigenic potential of the two hormones (Gupta et al., 2012). *In vivo* studies further suggest that different molecular mechanisms underlie the LH and hCG action. In genetically manipulated female mice, hCG overexpression result in excessive progesterone production leading to ovarian pathologies such as cysts or hemorrhagies and infertility (Matzuk et al., 2003; Peltoketo et al., 2011), while the lack of LH signaling prevents the preovulatory follicular maturation and ovulation (Pakarainen et al., 2005). The different activation of the signaling pathways likely relies on peculiar LH- or hCG-dependent molecular features of the ligand-receptor complex and dimerization of LHCGRs. In fact, predictive homology models have suggested that LH and hCG interact with distinct sites in the L2-beta loop and in the hinge region of the LHCGR (Grzesik et al.,

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Table 4
Comparison between LH and hCG EC50, H-slopes and maximal levels from estradiol dose–response curves, in the presence of 1×10^1 nM FSH. $P < 0.05$ were indicated in bold (Mann–Whitney's *U*-test).

	EC50 (pM; means ± SD; n = 6)	p^a	H-slopes (pM; means ± SD; n = 6)	p^a	Max estradiol value (pg/ml; means ± SD; n = 6)	p^a
LH + FSH	225.1 ± 80.2	**0.0022**	2.0 ± 1.1	0.4848	921.1 ± 86.6	0.5887
hCG + FSH	1.3 ± 0.8		1.2 ± 0.8		954.5 ± 39.5	
Fold difference	173.1		1.7		1.0	

[a] Mann-Whitney's U-test.

2015; Jiang et al., 2012). In the Human Embryonic Kidney cell line HEK 293 transfected with mutants LHCGRs, it was recently demonstrated that LH is able to stimulate only the targeted receptor *via cis*-activation, while hCG treatment results in *trans*-activation of multiple receptors (Grzesik et al., 2014) potentially leading to amplification of signal transduction. We could hypothesize that the increased biopotency of hCG in the presence of FSH may be explained by LHCGR-FSHR heterodimerisation at the cell membrane, which may occur in different proportion depending on whether LH or hCG is the ligand, modulating the intracellular signaling. The LHCGR-FSHR heterodimer is a complex system in which the activity of one receptor may allosterically regulate the other (Feng et al., 2013; Mazurkiewicz et al., 2015; Segaloff, 2012) and this feature may be more pronounced upon hCG *versus* LH binding, resulting in different receptor coupling by the two hormones. Indeed, a previous *in vitro* study failed to demonstrate differential progesterone accumulation between LH and hCG after a 24 h-treatment of hGLC (Casarini et al., 2012); conversely, the current results suggest that the two hormones differentially modulate the steroids synthesis in the presence of FSH, corroborating *in vivo* data (Ruvolo et al., 2007), which may result from different interactions with the FSH-FSHR complex. However, gonadotropins bioactivity may be affected by the amount of O-linked oligosaccharides, which are higher in hCG than LH, thus resulting in different half-lives and affecting *in vivo*, rather than *in vitro* bioactivity (Fares, 2006). In fact transgenic mice expressing longer half-life chimeric LH beta subunit develop ovarian pathologies such as granulosa cell tumors (Risma et al., 1995). Interestingly, our cAMP and progesterone dose–response experiments demonstrate that LH or hCG together with FSH co-treatment results in different hill slopes and maximal cAMP levels. It suggests that positive cooperativity (Prinz, 2010) may occur upon hCG and FSH co-treatment, rather than LH (which may act as a partial agonist), increasing the range of effective hCG concentrations. On the other hand, hCG may display LHCGR superagonism leading higher receptor signaling output (Schrage et al., 2015, in press) than LH, which may be assumed as the classical, endogenous ligand (except during pregnancy). Positive cooperativity among gonadotropin receptors were previously described in the amphibian *Rana catesbeiana*, which lacks the choriogonadotropin, likely to avoid that one hormone competitively inhibits the action of the other when they are secreted together (Yamanouchi and Ishii, 1990). We could speculate that, in humans, the same, intrinsic characteristics of these receptors are unmasked upon LH or hCG binding, further differentiating their physiological roles, at least *in vitro*. By this point of view, the proliferative and anti-apoptotic role of LH are enhanced, optimizing the hormone's functions exerted during folliculogenesis, while the pro-apoptotic potential of hCG remains hidden since this hormone is physiologically lacking in the presence of FSH. However, opposite results describing negative cooperativity between FSHR and LHCGR were previously found (Feng et al., 2013), revealing decreased $G_{\alpha}s$ protein-dependent signaling in co-transfected HEK293 cells under FSH or hCG treatment. It suggests that cell-dependent, peculiar FSHR:LHCGR ratio and intracellular *milieu* may result in partial receptor homo-oligomers (Thomas et al., 2007) and different coupling to receptor-specific intracellular interactors (Kanamarlapudi et al., 2012; Nechamen et al., 2007), thus modulating the downstream signaling. Therefore, further studies are needed to fully elucidate the mechanisms underlying gonadotropin receptor heterodimers.

Interestingly, the range of LH and hCG concentration resulting in pERK1/2 activation was extended in the presence of FSH, compared to that described in our previous LH and hCG dose–response experiments in the absence of FSH (Casarini et al., 2012) and confirmed here (Supplementary Fig. 1). As reasonably suggested by a study using prepubertal gilts (Breen and Knox, 2012), although speculative, the slight, tonic activity of FSH enhances the LHCGR-mediated pathway activation to optimize its activity when high efficiency of LH signaling is required, e.g. during an LH surge to achieve the ovulation. Previous studies demonstrated that LH and FSH co-treatment improved the ovarian response by potentiating the efficacy of gonadotropins in a concentration-unrelated manner (Lehert et al., 2014; Papaleo et al., 2014; Yazıcı Yılmaz et al., 2015), corroborating our results.

4.2. Steroidogenic/apoptotic versus proliferative signals

The higher activity of hCG *versus* LH in cAMP/PKA pathway activation *via* their common receptor may reflect the different physiological role of these molecules (Casarini et al., 2011; Choi and Smitz, 2014). Together with FSH or alone, LH regulates several proliferative and anti-apoptotic events such as folliculogenesis and granulosa cells growth, ovulation induction and maintenance of the corpus luteum during the luteal phase of the menstrual cycle. Also, some hints suggested that LH may play a role in endometrial receptivity, thus increasing the implantation rate (Altmäe et al., 2013; Revelli et al., 2015). Physiologically, hCG maintains pregnancy by inducing progesterone production as revealed by the importance of its steroidogenic potential exerted *via* the cAMP/PKA pathway (Weedon-Fekjær and Taskén, 2012). Our data corroborate this view; both LH and hCG treatments result in the increase of steroidogenic-related gene expression, in the presence of FSH, but *CYP19A1* achieves higher expression levels upon hCG *versus* LH treatment. Interestingly, the EGF-like factor *AREG* and *EREG* gene expression is accompanied by the positive regulation of the pro-apoptotic *TP53* gene; however, the *CASP3* gene expression is increased upon hCG, more than LH stimulation, suggesting opposite actions on cell death exerted by the two gonadotropins, likely counterbalanced by mechanisms involving the expression of the anti-apoptotic *XIAP* gene. The gene expression pattern reflects the protective effect of LH compared to that of hCG (together with FSH), measured in terms of cell viability. Consistently, the action of hCG and FSH was linked to death signals (Breckwoldt et al., 1996; Jolly et al., 1994) *via* a cross-talk between the predominantly activated steroidogenic cAMP/PKA and apoptotic pathways (Aharoni et al., 1995; Amsterdam et al., 1999; Keren-Tal et al., 1995; Liao et al., 2014; Zwain and Amato, 2001), decreasing cell viability. A previous study using caprine granulosa cells demonstrated that LH increased

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cell proliferation oppositely to hCG treatment, which results in cell death (Gupta et al., 2012), supporting our results and two distinct physiological roles of LH and hCG.

hCG rescues the corpus luteum from atresia and regulates the maternal invasion of embryonic tissues, suggesting a key role in cell growth and proliferative events, likely exerted by different hCG glycosylation isoforms rather than the "classical" hCG (Cole, 2010; Cole and Butler, 2008; Crochet et al., 2012). Indeed the *CGB* gene cluster, which is present only in primates, has an increasing complexity (in humans and in the taxonomically closest *non*-human primates) together with the placental invasiveness of the maternal tissues, suggesting a complex regulatory system of proliferative events, rather than steroidogenic (Crochet et al., 2012) at the early stages of pregnancy (Gabay et al., 2014), possibly supported by a receptor different than LHCGR (Berndt et al., 2013).

4.3. Clinical use of LH and hCG

Given the limitations of the *in vitro* model as a too simplistic system to reproduce more complex *in vivo* dynamics, these results provide the basis for further investigations *in vivo*. Clinical evidences demonstrating different effects depending on LH and hCG are not unanimous (Hanson et al., 1971; Moro et al., 2015; Razi et al., 2014; Requena et al., 2014; Ziebe et al., 2007), although some recent *in vivo* data support that LH, together with FSH, improves oocyte quality and fertilization rate compared to hCG (Carone et al., 2012; Fábregues et al., 2013; Revelli et al., 2015; Ruvolo et al., 2007). This could result from the synergistic activity of LH and FSH in the activation of anti-apoptotic- and proliferative-related pathways regulating follicular growth (Kawamura et al. 2013; Shoham et al., 1993; Wang and Greenwald, 1993; Yuan and Greenwald, 1994).

Previous clinical comparisons between commercially available gonadotropins focused on different endpoints, such as live birth or pregnancy rate, which may be not fully indicative of the gonadotropins action at molecular level. Especially, the follicular phase is a complex physiological process characterized by cell proliferation, apoptosis and other biological events, orchestrated mainly by LH, FSH and estrogens. Assisted reproduction aims at multiple follicular maturation. It is not known whether using gonadotropin combinations with the best steroidogenic potential (FSH plus hCG rather than LH) results in better follicular maturation and oocyte quality. Indeed, plausibly due to the pro-apoptotic effects, hCG is physiologically absent during the follicular phase and at the very early stage of the oocyte fecundation, before the trophoblast development, although its fundamental role in the prevention of the corpus luteum atresia and placentation suggests that choriogonadotropin exerts anti-apoptotic and proliferative functions in case of pregnancy. Some hints suggested that the gonadotropin-mediated cell fate rely on the capability to recruit cAMP, which may have a dual, proliferative and anti-apoptotic role depending on the its intracellular concentration (Yong et al., 1992). In this regard, it is plausible that the lack of FSHR expression together with LHCGR, as in the corpus luteum (Yamoto et al., 1992), is necessary for hCG to be anti-apoptotic. Finally, recent studies proposed that several hCG isoforms and glycosylation variants, instead of the "regular" hCG, support the cell proliferation, angiogenesis and maternal tissue invasion at the early stage of pregnancy (Cole, 2012), likely acting *via non*-canonical receptors, such as the transforming growth factor β receptor (Tβ-RII) (Berndt et al., 2013). *In vivo* experiments using transgenic mice models producing hCG, instead of endogenous LH, revealed that choriogonadotropin was not optimal for optimal control of folliculogenesis (Ahtiainen et al., 2007; Huhtaniemi et al., 2006). However, further studies should be performed to better address this issue given that the action of hCG trough Tβ-RII was recently challenged (Koistinen et al., 2015). Therefore, different clinical protocols and experimental settings may have arisen not indicative or conflicting results. Proper clinical trials enrolling a wide number of patients should be performed to evaluate clearly whether LH or hCG is more properly suitable for *in vitro* fertilization (IVF) protocols.

5. Conclusions

Although two decades have passed since the advent of recombinant gonadotropins, clinical comparisons of recombinant molecules, which provide a set of reliable and clear-cut results is still lacking. However, increasing evidences pinpoint the complexity and the diversity of the interaction between FSH, LH and hCG at molecular level. Time is ripe to review the approach to fertility treatments, featuring hCG as prevalent on the activation of steroidogenesis while LH is a proliferative and anti-apoptotic factor. In this scenario, FSH acts as modulator of the cell response elicited by the other two gonadotropins, especially hCG.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2015.12.008.

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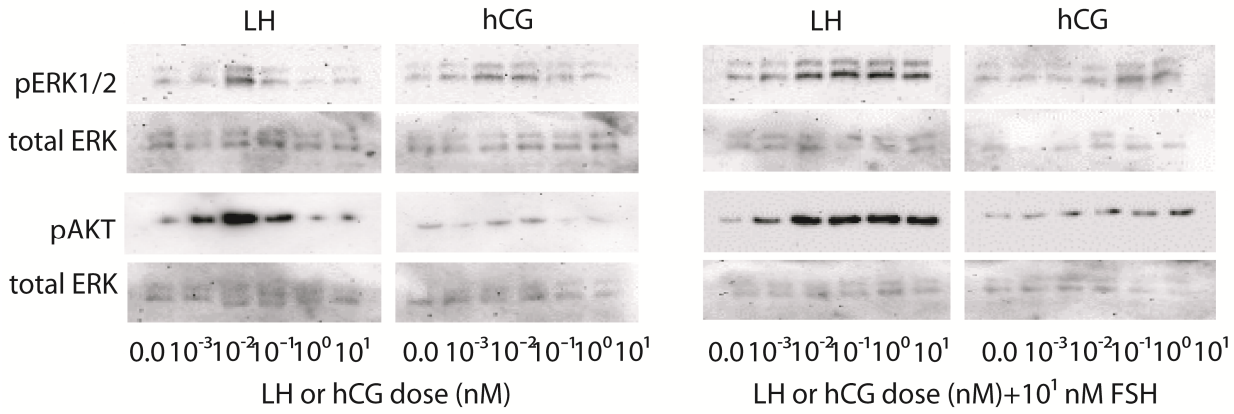
Supplementary Figures



Supplementary Figure 1. Phosphorylation of ERK1/2 and AKT induced by increasing LH or hCG concentrations, in the presence or in the absence of $1x10^1$ nM FSH. A representative experiment using hGLC from one patient is shown. Cells were stimulated 15 min by 0-$1x10^1$ nM LH or hCG without (left panels) or with $1x10^1$ nM FSH (right panels), then pERK1/2 and pAKT activation was evaluated by Western blotting. Total ERK served as normalizer. In the absence of FSH, the LH/hCG dose-dependent pattern of phospho-proteins activation is identical to that previously observed (Casarini et al. 2012), with decreasing pERK1/2 and pAKT signals at concentrations higher than $1x10^{-2}$ nM. The pattern of pERK1/2 and pAKT activation upon LH/hCG treatment in the presence of FSH changes and the activation persists at the highest LH/hCG concentrations used. The original results presented in this paper derive from identical experimental conditions allowing direct comparison with the results described previously (Casarini et al. 2012). This experiment proves that both the results are reproducible and that they can be compared as shown in the table 2.

Chapter 2

Human luteinizing hormone and chorionic gonadotropin treatment results in different intracellular signaling in mouse primary Leydig cells *in vitro*

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1. Abstract

Human luteinizing hormone (LH) and chorionic gonadotropin (hCG) regulate development and reproductive functions by acting on the same receptor (LHCGR), which conserves high sequence homology among mammals. The aim of this study is to compare LH and hCG activity, in primary mouse Leydig cells *in vitro*, naturally expressing the murine receptor (lhr). Leydig cells were treated by increasing doses of LH and hCG, and cell signaling, gene expression and steroid synthesis were evaluated. hCG treatment results in higher total cAMP production. hCG is about 10-fold more potent than LH (hCG EC_{50}=18.64±10.14 pM; LH EC_{50}=192.00±53.96 pM; Mann-Whitney's *U*-test; $p<0.05$; n=4). Moreover, hCG stimulation induces higher ERK1/2 and CREB phosphorylation than LH (Mann-Whitney's *U*-test; $p<0.05$; n=4). Gene expression and testosterone production were not significantly different between LH and hCG treatments. We demonstrated that hCG treatment results in higher cAMP/PKA-dependent, lhr-mediated cell signaling activation than LH. This reveals that the murine receptor mediates quantitatively but not qualitatively different response to human gonadotropins, oppositely to what previously described in human primary granulosa cells.

Keywords:

hCG, LH, lhr, Leydig, signaling

1. Introduction

Human chorionic gonadotropin (hCG) and luteinzing hormone (LH) are glycoproteins which bind the same receptor (LHCGR). LH is produced by the pituitary in a pulsatile fashion, inducing ovulation and maintenance of the corpus luteum in females, and regulating spermatogenesis in males. hCG is the pregnancy hormone produced by trophoblast cells only in primates and equids (Pierce and Parsons, 1981). During development, maternal hCG stimulates the proliferation of the male foetus' Leydig cells. After birth, LH replaces hCG, and induces Leydig cells activation, regulating spermatogenesis in the testes, by controlling testosterone synthesis and secretion (Saez J. 1994; Huhtaniemi. 1996). Previous studies demonstrated that the two gonadotropins induce different intracellular signaling in goat and human granulosa lutein cells *in vitro* (Casarini et al. 2012; Gupta et al. 2012; Casarini et al. 2015), however, an in-depth comparison between the hCG- and LH-mediated signaling in Leydig cells *in vitro* is still lacking.

LH and hCG binding to LHCGR triggers the activation of adenylyl cyclase, resulting in cyclic adenosine-monophosphate (cAMP) production increase and downstream activation of the protein kinase A (PKA) (Dufau et al. 1977; Smith and Walker, 2015), and, in turn, of the extracellular signal-regulated kinases 1 and 2 (ERK1/2) pathway (Matzkin et al. 2013). PKA regulates the activation of the cAMP-response element-binding protein (CREB), modulating the expression of genes related to steroidogenesis, including the steroidogenic acute regulatory protein (StAR) gene (*STARD1*) (King et al. 2012), and testosterone production.

Since hCG is acting on the LHCGR, it is used in clinical practice for the treatment of hypogonadotrophic hypogonadism (HH) or idiopathic oligo-asthenospermia in males. Previous experiments conducted in human primary granulosa cells demonstrated higher steroidogenic potential of hCG than LH, leading to cAMP/PKA pathway activation and progesterone production, while LH treatment induced the activation of the proliferative and anti-apoptotic ERK1/2 and AKT pathways at higher levels than hCG (Casarini et al. 2012). Since Leydig cells are naturally expressing the LHCGR, it should be an optimal system, alternative to granulosa cells, for *in vitro* studies focused on gonadotropin-mediated signaling. However, human Leydig cells expressing the human LHCGR are not available in proper amounts, therefore we compared the cell responses to hCG or LH using mouse primary Leydig cells isolated from testis of C57BL6 adult mice. Mouse Leydig cells are

expressing the murine lhr, which share a high sequence homology with the human receptor and human LH and hCG binding capability (Matzkin et al. 2013; Yamashita et al. 2011). The activity of LH and hCG was analyzed by dose-response experiments, evaluating cAMP production, phosphorylation levels of ERK1/2 (pERK1/2) and CREB (pCREB), gene expression and testosterone synthesis.

3. Materials and Methods

3.1 Recombinant Gonadotropins

Human recombinant LH (Luveris) and CG (Ovitrelle) were provided by Merck KGaA (Darmstadt, Germany).

3.2 Leydig cells isolation and culture

Leydig cells were collected from 3-5 months-old C57BL6 mice following a validated protocol (Schumacher et al. 1978). Briefly, testes were mechanically dissociated after decapsulation and subjected to 20mg/ml collagenase (Sigma Aldrich, St. Louis, MO, USA) treatment by gentle shacking at 37°C. The cell suspension was filtered by a 100µm Nylon mesh and Leydig cells were obtained after centrifugation in a 0-100% Percoll (GE Healthcare, Little Chalfont, UK) gradient (800*g* for 45 minutes). Cell isolation was confirmed by 3β-hydroxysteroid dehydrogenase (3βHSD) assay (Ge et al. 2006). Leydig cells were seeded depending on the endpoint measured, in multi-well plates, in minimal essential medium (MEM) (Gibco, Life Technologies, Carlsbad, California, USA), supplemented with 0,07% serum albumin (Sigma Aldrich), 100 U/ml penicillin, 50 µg/ml streptomycin and 25 mM Hepes, pH 7.4 (Gibco, Life Technologies). Cells were maintained two days in an incubator at 37°C and 5% CO_2 before stimulation.

3.3 cAMP stimulation protocol and measurement

For cAMP analysis, a validated protocol was followed (Nordhoff et al. 2011). Briefly, Leydig cells were seeded in 24-well plates ($5x10^4$ cells/well) and cultured 2 days before stimulation. Cells were treated with increasing doses of hCG or LH (1 pM–100 nM range), in the presence of 500µM of phosphodiesterases inhibitor 3-isobutyl-1-methylxanthine (IBMX) (#I5879, Sigma-Aldrich) (Lindsey and Channing, 1978). Cells stimulated by 1 µg/ml Cholera Toxin (CTX) (Sigma-Aldrich) (May et al. 1984) were used as positive control while unstimulated cells served as control (basal). After 3 hours of incubation, samples were frozen. Total cAMP was evaluated using the Cyclic AMP Direct EIA kit (Arbor Assays, Ann Arbor, MI, USA), following the supplier's instructions and signals were measured by a Victor3 multilabel plate reader (Perkin Elmer Inc., Waltham, MA, USA). Data were entered into a curve fitting software and represented using a log regression analysis.

3.4 Western blot analysis

Stimulations and Western blotting procedures were performed as previously described (Casarini et al. 2012; Casarini et al. 2014). Briefly, Leydig cells were seeded in 96-well plates ($1x10^5$ cells/well) and treated 15 minutes by increasing doses of LH or hCG (1 pM-100 nM range). Cells were immediately lysed for protein extraction in 4 °C-cold RIPA buffer added with protease and phosphatase inhibitors. pERK1/2 and pCREB activation was evaluated by Western blotting after 12% SDS-PAGE, using specific antibodies (#9101 and #9198; Cell Signaling Technology Inc., Danvers, MA, USA). Total ERK1/2 served as loading control (#4695; Cell Signaling Technology Inc.). Signals were revealed by ECL chemiluminescent compound (GE HealthCare, Little Chalfont, UK), after incubation of the membranes with a secondary horseradish peroxidase-conjugated antibody (#NA9340V; GE HealthCare), then acquired and semi-quantitatively evaluated by an image analysis system (VersaDoc Imaging System and QuantityOne software, Bio-Rad Laboratories Inc.).

3.5 Stimulation for gene expression analysis and Real-time PCR

The lowest doses of hCG and LH maximally activating cAMP (100 pM hCG; 1 nM LH) were calculated from the dose-response experiments described above. These doses are corresponding to the LH and hCG EC_{80} and were used as fixed-stimulating doses for gene expression analysis. Leydig cells were seeded in 12-well plates ($1x10^5$ cells/well) and stimulated 12 hours by LH and hCG. Samples were lysed and subjected to RNA extraction using the automated extractor EZ1 Advanced XL (QUIAGEN, Hilden, Germany). Total RNA was quantified, retrotranscribed with iScript reverse transcriptase according to the following protocol: 25°C for 5 m followed by 42°C for 30 m and 85°C for 5 m. Quantitative real-time PCR was performed in triplicates with the primers shown in Table 1. The settings for the real-time PCR reactions were as follows: 95.0 °C for 30 s followed by 45 cycles of 3 s at 95.0 °C and 30 s at 57.0 °C. Normalized gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method (Livak et al. 2001) and expressed as fold-increase over unstimulated control.

Gene	Oligonucleotide sequences	Product leght (bp)	Protein name
m-STAR	F: ACAGACTCTATGAAGAACTT	20	Steroidogenic acute regulatory protein
	R: GACCTTGATCTCCTTGAC	18	
m-AREG	F: AGAGTTGAACAGGTGATTA	19	Amphiregulin
	R: TCTTATTCCTTCTGCCTTT	19	
m-EREG	F: AGATGTGAAGTGGGCTAC	18	Epiregulin
	R: GTATTCTTTGCTCAAGGGTT	20	
m-HPRT	F: TTGCTCGAGATGTCATGAAGGA	22	Hypoxanthine-guanine phosphoribosyltransferase
	F: AGCAGGTCAGCAAAGAACTTATAG	24	

Table 1. Primer sequences used in real-time PCR experiments.

3.6 Testosterone measurement

Leydig cells were seeded in 12-well plates ($1x10^5$ cells/well) and stimulated by increasing doses of hCG and LH (1 pM-100 nM range) in the presence of 500µM IBMX. Testosterone production was measured in the supernatants after 24 hours by immunoassay, using the reader ARCHITECT 2nd Generation Testosterone system (Abbot, Chicago, Illinois, USA).

3.7 Statistical analysis

For cAMP and testosterone dose-response experiments, data were normalized as percentage of the maximal response. For pERK1/2 and pCREB analysis, results were normalized over total ERK, then semi-quantitative evaluations were graphically expressed in relative units. For gene expression analysis, the results were represented as relative expression over the basal condition. Data are expressed as means±SEM. Mann-Whitney's *U*-tests and *non* linear regressions were performed as appropriate. Differences were considered significant for $p<0.05$. Statistical analysis were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

4. Results

4.1 Analysis of cAMP production

The protocol for Leydig cells isolation was validated by the 3βHSD assay (Schumacher et al. 1978), revealing that the occuracy is more than 95% (Figure. 1).

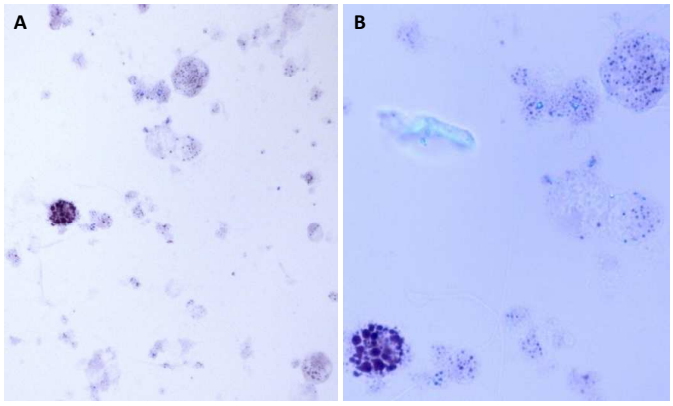

Figure 1. Detection of murine primary Leydig cells through 3β-HSD assay. Leydig cells suspension purified trought Percoll gradient centrifugation were positive to 3β-HSD staining by phase contrast microscopy (two different enlargements: **A** (10x) **B** (40x)).

cAMP production induced by 3 hours-hCG or -LH treatment of primary murine Leydig cells was analyzed, in the presence of 500 µM IBMX. The exposure of the cells to increasing hCG doses resulted in significantly different half maximal effective concentration (EC_{50}) value compared to that obtained by LH (hCG: EC_{50}=18.64±10.14 pM; LH: EC_{50}=192±53.96 pM; means±SEM; Mann-Whitney's *U*-test; p=0.0286; n=4), revealing approximately a 10-fold higher biopotency of hCG *versus* LH (Figure 2). However, the cAMP plateau is similar between cells treated by LH or hCG (100 nM).

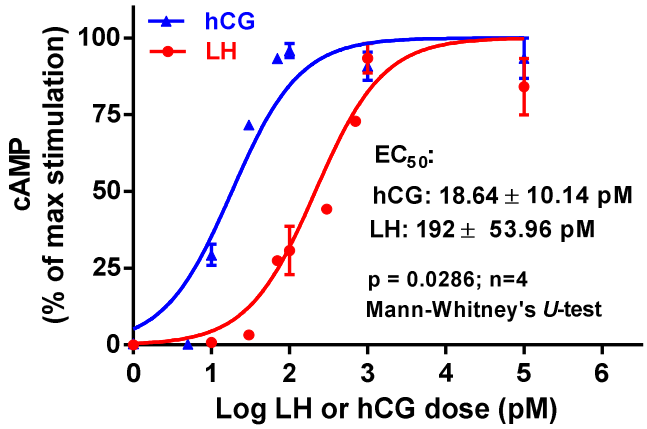

Figure 2. cAMP dose-response experiment. Murine primary Leydig cells were stimulated with increasing doses of hCG and LH in the presence of 500µM IBMX. Total cAMP was measured after 3 hours of incubation. All the results are represented as means±SEM in a logarithmic X-axis, then non-linear regressions were plotted. The EC50 values were compared by Mann–Whitney's *U*-test ($p<0.05$; n=4).

4.2 Evaluation of ERK and CREB phosporylation

Leydig cells were stimulated 15 minutes by increasing doses of LH or hCG. pCREB and pERK1/2 levels were evaluated by Western blotting (Figure 3) and semi-quantitatively measured (Figure 4).

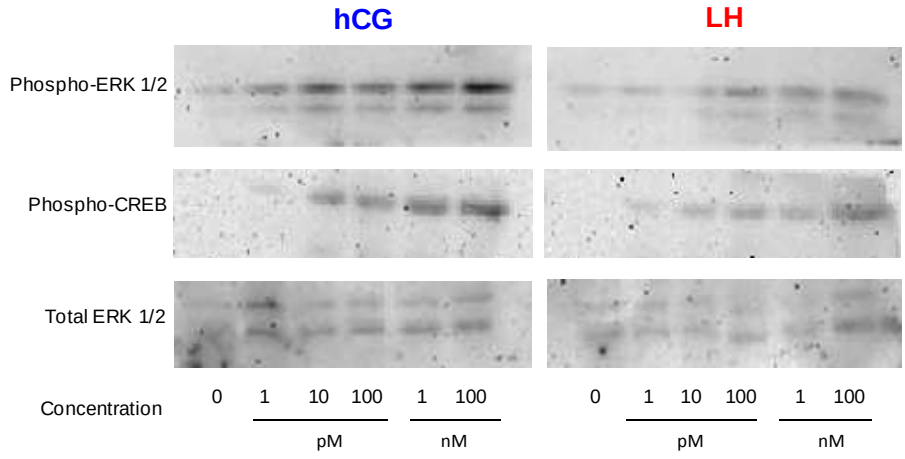

Figure 3. Dose-response experiment evaluating the maximal phospho-ERK1/2 and phospho-CREB activation in Leydig cells by Western Blotting. Leydig cells were stimulated for 15 minutes with increasing doses of hCG and LH (n=4).

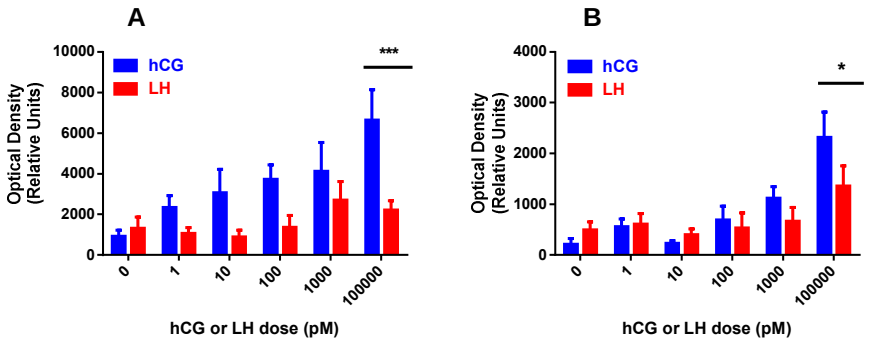

Figure 4. Densitometric analysis of pERK1/2 (A) and pCREB (B) western blotting signal in Leydig cells upon hCG or LH stimulation. pERK1/2 and pCREB signals were normalized using total ERK as loading control (§ = significant vs control; * = significant hCG *vs* LH; Mann-Whitney's *U*-test; $p<0.05$; n=4).

pCREB activation occurs by hCG but not by LH at the upper dose (100 nM) (Mann-Whitney's *U*-test; $p<0.05$; n=4). 100 nM hCG treatment resulted in higher pCREB activation than LH (Two-way Anova and Bonferroni post-test, $p<0.05$; n=4). Moreover, 100 nM hCG treatment induced pERK1/2 activation while LH resulted in no significant activation compared to the basal (Mann-Whitney's *U*-test; $p<0.05$; n=4). Also, pERK1/2 achieved higher levels of activation upon hCG *versus* LH treatment (Two-way Anova and Bonferroni post-test, $p<0.05$; n=4).

4.5 Gene expression analysis

The expression of target genes involved in steroidogenesis and cell cycle regulation was evaluated in mouse primary Leydig cells. Cells were maintained 12 hours in the presence of the hCG or LH $EC_{80,}$ as the lowest dose maximally activating cAMP (100 pM hCG; 1 nM LH). The expression of the steroidogenesis-related StAR (*STARD1*) gene, and of the cell cycle-related epidermal growth factor (EGF)-like factors amphiregulin (*AREG*) and epiregulin (*EREG*) was evaluated by real-time PCR analysis (Figure 5).

However, no significant differences in *STARD1*, *AREG* and *EREG* gene expression occurred between hCG and LH stimulation (Mann-Whitney's U-test; $p>0.05$; n=4).

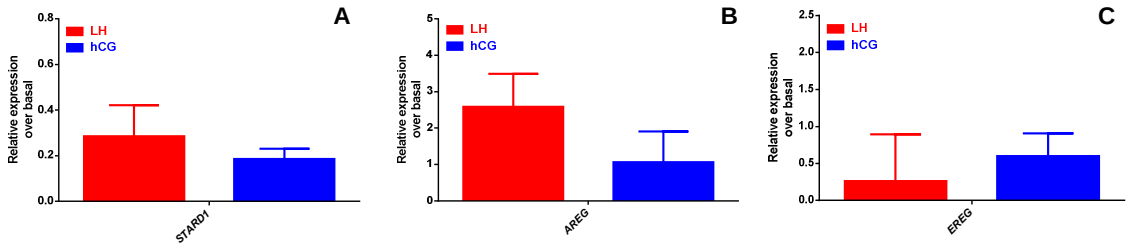

Figure 5. Evaluation of the gene expression induced in mouse primary Leydig cells by hCG or LH stimulation after 12 hours, performed by real-time PCR. Effects of the hCG or LH stimulation on *STARD1* gene expression (A). Increase in the gene expression of the EGF-like factors *AREG* and *EREG* induced by hCG and LH (B-C). In each stimulation, *HPRT* gene expression was used as normalizer (mean±SEM; Mann-Whitney's *U*-test; p>0.05; n=4).

4.3 Evaluation of testosterone production

Testosterone synthesis was measured in the media of murine primary Leydig cells stimulated 24 hours by increasing doses of hCG or LH. hCG and LH EC_{50} values calculated in dose-response experiment are not significantly different (hCG: EC_{50}=29.98±13.26 pM; LH: EC_{50}=61.87±17.60 pM; Mann-Whitney's *U*-test; p= 0.3429; n=4) (Figure 6).

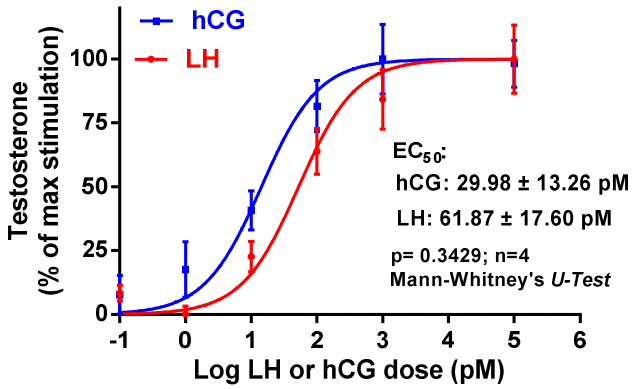

Figure 6. Evaluation of testosterone production upon hCG or LH stimulation. Murine primary Leydig cells were stimulated with increasing doses of hCG and LH in the presence of 500µM IBMX. Total testosterone was measured after 24 hours of incubation. All the results are represented as means±SEM in a logarithmic X-axis, then non-linear regressions were plotted. The EC50 values were compared by Mann–Whitney's *U*-test (p>0.05; n=4).

5. Discussion

We investigated the LH- and hCG-induced signaling in mouse primary Leydig cells *in vitro*. cAMP production, pERK1/2 and pCREB activation, gene expression and testosterone synthesis were evaluated.

We demonstrated that murine lhr mediates quantitatively different intracellular signaling upon hCG and LH treatment, supporting the *non* equivalence of the two hormones *in vitro*. Murine lhr shares approximately the 80% of aminoacid sequence identity with LHCGR (Figure 7) and human LH or hCG retain binding capability to both the receptors (Ascoli et al. 2002).

Figure 7. Aminoacid sequence alignment of human LHCGR and murine lhr. *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). * = aminoacidic residues fully conserved; **:** =conservation of strong groups; **.** = conservation of weak groups or no consensus.

Differences in hCG and LH binding to the human *versus* murine receptor remain to be explored. In clinical practice, hCG is used to induce testosterone synthesis in male infertile patients, although a clear comparison with LH was never performed in Leydig cells *in vitro*.

We first assessed how the gonadotropins elicit cAMP accumulation. Consistent with previous reports, we found that hCG has an about 10-fold higher biopotency than LH in murine Leydig cells, although inducing similar maximal response. This result confirms what previously described in human granulosa cells, where hCG treatment resulted in higher activation of the steroidogenic cAMP/PKA pathway compared to LH (Casarini et al. 2012). Moreover, long term stimulation of goat ovarian granulosa cells by hCG induced higher cAMP levels than LH which resulting in decreased cell number (Gupta et al. 2012). This suggests a link between steroidogenesis and cell proliferation. Also, in human granulosa cells, the high steroidogenic potential of hCG was increased in the presence of FSH (Casarini et al. 2015). Therefore both the murine lhr and the human LHCGR mediate higher cAMP accumulation by hCG than LH treatment.

Reflecting cAMP production, hCG mediates the activation of pCREB at higher levels than LH (Figure 3). At the 100 nM dose-point, cAMP achieves the plateau together with higher pCREB activation by hCG than LH treatment. This corroborates the higher steroidogenic activity of hCG compared to LH, due to the crucial role of pCREB activation in the downstream modulation of the expression of steroidogenesis-related genes (King et al. 2012). Surprisingly, LH triggers only slight pCREB activation compared to basal, even at the higher dose-point.

Moreover, we investigated the activation of the ERK1/2 pathway focusing on the ability of lhr to differentiate hCG and LH. In Leydig cells, pERK1/2 activation is involved in promoting both steroidogenesis (Martinelle et al. 2004; Stocco et al. 2005) and cell proliferation (Shiraishi and Ascoli, 2007). We demonstrate that Leydig cells stimulation by hCG resultes in higher pERK1/2 activation than LH, which, in turn, induces no significant ERK1/2 phosphorilation. pERK1/2 activation by hCG treatment was previously observed in the MA-10 mouse Leydig tumor cell line, naturally expressing the lhr and in primary immature rat Leydig cells (Shiraishi and Ascoli, 2007; Galet and Ascoli, 2008; Hirakawa and Ascoli, 2003; Tai and Ascoli, 2011). Since the activation of pERK1/2 is cAMP/PKA-dependent in these cell models, we could speculate that the high levels of ERK1/2

phosphorylation mediated by hCG is linked to its high potential in cAMP/PKA activation, which is higher than that of LH. This result is different to that previously obtained in human primary granulosa cells (Casarini et al. 2012), where LH treatment resulted in higher pERK1/2 activation than hCG. It suggests the existence of an LH-mediated, cAMP-independent mechanism of ERK1/2 phosphorylation intrinsic to the human LHCGR, which is not active for murine lhr. Interestingly, the effects of hCG were evaluated *in vivo* in genetically modified mice overexpressing hCG. They developed Leydig cell adenomas and encountered reproductive problems (Ahtiainen et al. 2005), reflecting that murine lhr weakly discriminates the qualitative binding of the two human gonadotropins, and resulting in higher hCG biopotency than LH.

No significant differences in *STARD1*, *AREG* and *EREG* expression occurred upon hCG and LH treatment of Leydig cells. Increased exopression of *STARD1* and other steroidogenesis-related genes occurred by hCG treatment in mouse Leydig cells (Matzkin et al. 2013), while cell cycle-related genes were never investigated. In primary human granulosa cells, both LH and hCG treatments resulted in the increase of *AREG* and *EREG* gene expression. In this case, LH induced higher *AREG* expression levels that hCG, reflecting the LH potential in promoting proliferative effects (Casarini et al. 2012). We speculate that the log-term (12 hours) stimulation of Leydig cells by gonadotropins did not allow to optimally detect downstream differences between hCG- and LH-induced changes of the gene expression in murine Leydig cells *in vitro*.

Moreover, we evaluated the LH- and hCG-induced steroidogenesis, in terms of testosterone synthesis. Genetically modified mice overexpressing high levels of hCG exhibited enhanced testicular steroidogenesis and highly elevated serum testosterone (Rulli et al. 2003), demonstrating that hCG retains the ability to promote steroid production in mice. In mouse primary Leydig cells *in vitro*, both gonadotropins stimulate testosterone production but, in contrast to the observed cAMP dose-response curves, no significant differences between hCG and LH EC_{50} were found (Figure 5). An *in vitro* study failed to demonstrate differential progesterone accumulation in primary granulosa cells continuously exposed to gonadotropins (Casarini et al. 2012). We could speculate that the continuous exposure of Leydig cells to hCG or LH induced similar downstream steroids synthesis, resulting in the long-term recovering of the differences in testosterone production.

6. Conclusions

Our results demonstrated that hCG, not naturally produced in mice, resulted in higher cAMP/PKA-dependent, lhr-mediated cell signaling activation in primary Leydig cells, compared to LH. The murine lhr, opposed to that observed for human LHCGR, mediates quantitatively but not qualitatively different signaling *in vitro*, probably due to the inability in differentiating the ligand-specific mediated pathways. Cell signaling pathways should be further investigated in the presence of specific inhibitors to dissect the hCG and LH activity in murine Leydig cells.

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Chapter 3

Human luteinizing hormone (LH) and chorionic gonadotropin (CG) display biased agonism at the LH/CG receptor

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1. Abstract

Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) were considered biologically equivalent for decades because of their structural similarities and their binding to the same receptor: the lutropin/choriogonadotropin receptor (LH/CG-R). However, accumulating evidence suggest that LH/CG-R differentially react to the two hormones, triggering differential cAMP response, steroidogenesis, ERK and AKT activation as well as gene expression in granulosa cells. The mechanistic basis of these differential responses remains mostly unknown. Even though the effects of both hormones have been compared on the canonical Gs/cAMP/PKA pathway, their relative potencies and efficacies on β-arrestin- and Gq/PLC-dependent pathways are yet to be determined. Here, we compared the abilities of rhLH (Luveris®, Merck Serono) and rhCG (Ovidrelle®, Merck Serono) to elicit cAMP, inositol phosphate production, β-arrestin 2 activation and recruitment, as well as steroid production in two cell models: i) human embryonic kidney 293 (HEK293) cells transiently expressing hLH/CG-R; ii) mouse Leydig tumour cells (mLTC-1), endogenously expressing the murine LH receptor. For this, bioluminescence/fluorescence resonance energy transfer (BRET/FRET) technologies were used allowing quantitative analysis of rhCG/rhLH activities in real-time in living cells. Our data indicate that rhLH and rhCG differentially promote the different cell responses mediated by LH/CG-R, revealing interesting divergences in their potencies and efficacies. Our study provides clear evidence that LH/CG-R can discriminate the binding of the two hormones supporting the notion that endogenously expressed hCG and hLH could act as natural biased agonists *in vivo* during pregnancy.

2. Introduction

Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) are two heterodimeric glycoprotein hormones playing key roles in the reproductive system. Both LH and hCG are produced and circulate as mixtures of glycosylated isoforms which differ in the complexity of their carbohydrate side chains and, as a consequence, present different half-lives and bioactivities (Arey & Lopez, 2011; Bousfield & Dias, 2011). Both hCG and LH bind to luteinizing hormone/choriogonadotropin hormone receptor (LH/CG-R) which is mainly expressed in the ovary and testis where it regulates steroidogenesis (Ascoli et al, 2002). LH/CG-R belongs to a subgroup of class A (rhodopsin-like) GPCRs characterized by the presence of multiple leucine-rich repeats (LRRs) in their extracellular amino-terminal domain. This subgroup also includes the follicle stimulating hormone receptor (FSH-R), thyroid-stimulating hormone receptor (TSH-R) and the receptors for the peptidic hormone relaxin and INSL3 (RXFP1 and 2). LH/CG-R is known to mediate the canonical G protein-mediated signaling pathway through coupling to heterotrimeric $G_{\alpha s}$ proteins which activates adenylate cyclase, resulting in cAMP accumulation and activation of protein kinase A (PKA) as well as the exchange protein directly activated by cAMP (EPAC). This in turn triggers the activation of multiple downstream kinases that modulate the nuclear activity of cAMP response element-binding protein (CREB) and the expression of the genes involved in the physiological responses to these hormones. Besides, LH/CG-R was one of the first GPCRs shown to independently activate two G proteins, leading to both adenylyl cyclase and phospholipase C activation through functional coupling to $G_{\alpha s}$ and $G_{\alpha q}$ respectively (Gudermann et al, 1992a; Gudermann et al, 1992b). More recently, LH/CG-R has been reported to also engage a multiplicity of G protein-dependent and independent pathways (Ulloa-Aguirre et al, 2011), including β-arrestin-dependent pathways (Ayoub et al, 2015; Nakamura et al, 1999). For many years, β-arrestins have been considered exclusively as silencers of the GPCRs signaling, prompting ligand-induced receptor internalization and trafficking (Claing et al, 2002; Lefkowitz et al, 1998; Shenoy & Lefkowitz, 2003). Now it is well known that β-arrestins regulate GPCR signaling and trafficking and are able to engage G protein-independent signaling, the most studied of which being ERK1/2 pathway (Lefkowitz & Shenoy, 2005; Luttrell & Lefkowitz, 2002; Reiter et al, 2012; Reiter & Lefkowitz, 2006).

LH and hCG had been assumed to be equivalent for many years, even though distinct physiological (Khan-Sabir et al, 2008), molecular (Galet & Ascoli, 2005) and pharmacological (Grzesik et al, 2015; Grzesik et al, 2014) features were well known. However, similar to most other GPCRs, LH/CG-R is potentially susceptible to the phenomenon of biased signaling which implies that the binding of a given ligand can stabilize the receptor in an ensemble of activated conformations, thereby leading to selective modulation of downstream signaling pathways (Galandrin et al, 2007; Kenakin, 2007; Reiter et al, 2012; Violin & Lefkowitz, 2007). In line with this emerging concept, it has been recently proposed that LH and hCG produced as multiple glycosylated isoforms, could potentially trigger selective transduction mechanisms at the LH/CG-R (Arey & Lopez, 2011; Ulloa-Aguirre et al, 2011). Recent lines of evidence support this hypothesis, showing that, although their structures are similar and they shared the same receptor, LH and hCG elicit divergent signaling in several cell models (Choi & Smitz, 2014). A genomic deletion resulting in the complete absence of exon 10 of LH/CG-R found in a hypogonadic patient with Leydig cell hypoplasia type II (Gromoll et al, 2000) impaired LH, but not hCG, -induced cAMP production, without affecting ligand binding (Muller et al, 2003). More recently, it has been reported that, in human granulosa cells, hCG display higher potency on the cAMP/PKA pathway as well as steroidogenesis whereas LH is more potent on ERK1/2 and AKT phosphorylation as well as gene expression (Casarini et al, 2012). In goat ovarian granulosa cells, prolonged LH treatment promotes growth and proliferation whereas prolonged exposure to hCG leads to higher levels of cAMP and decreased proliferation (Gupta et al, 2012). In human granulosa cells treated with FSH, hCG is more potent on cAMP/PKA pathway and steroidogenesis while LH is predominantly a proliferative and anti-apoptotic factor through the activation of ERK1/2 and AKT pathways (Casarini et al, 2015).

Despite of these recent advances, the potential of hCG and LH to differentially activate $G_{\alpha s}$-, $G_{\alpha q}$- and β-arrestin-dependent pathways at the LH/CG-R has not been evaluated. In addition, the relative contributions of these transduction mechanisms to steroidogenesis are yet to be determined. In the present study, we used a series of BRET, TR-FRET and reporter assays to quantitatively assess the signaling induced by hCG and hLH both in real time and live cells. The canonical $G_{\alpha s}$/cAMP and $G_{\alpha q}$/PLC pathways as well as β arrestin 2 recruitment

were analyzed upon activation of transiently or endogenously expressed human LH/CG-R and mouse LHR, in HEK293 and immortalized murine Leydig cells, respectively.

3. Materials and Methods

3.1 Recombinant gonadotropins

Human recombinant LH (Luveris®, rhLH) and hCG (Ovidrel ®, rhCG) were kindly provided by Merck KGaA (Darmstadt, Germany).

3.2 Cell Culture and Transfection

HEK 293 cells were grown in complete DMEM medium supplemented with 10% (v/v) fetal bovine serum, 4.5 g/l glucose, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 1 mM glutamine (Invitrogen, Carlsbad, CA). mLTC-1 cells (ATCC CRL-2065, LCG Standards, Molsheim, France) were grown in complete RPMI medium supplemented with 10% (v/v) fetal bovine serum, 4.5 g/l glucose, 50µg/ml gentamicin, 10 units/ml penicillin and 10µg/ml streptomycin (Invitrogen, Carlsbad, CA). Transient transfections were performed by reverse transfection in 96-well plate using Metafectene PRO (Biontex, München, Germany) following the manufacturer's protocol. Briefly, 100 ng of total plasmids per well and 0.5 µl/well of Metafectene PRO were resuspended in serum-free medium and pre-incubated 5 minutes at room temperature separately. The two solutions were then mixed and incubated 20 minutes at room temperature. Cells (10^5 in 200 µl/well) in the corresponding complete medium were then incubated with the final plasmid-Metafectene PRO mix (50 µl/well) for two days before proceeding with the hormones stimulations.

3.3 Small interfering RNA transfection

The siRNA sequence 5'-AAAGCCUUCUGUGCUGAGAAC-3' was used to target mouse β-arrestin 1 (position 439-459 relative to the start codon) whereas sequence 5'-AAACCUGUGCCUUCCGCUAUG-3' was used to target mouse β-arrestin 2 (position 175-193 relative to the start codon) (Kovacs et al, 2008). One small RNA duplex with no silencing effect was used as a control (5'-UUCUCCGAACGUGUCACGU-3'). The siRNAs were synthesized by GE Healthcare Dharmacon (Velizy-Villacoublay, France). Early passage HEK293 cells at 30% confluency in 100 mm dishes were transiently transfected with GeneSilencer following the manufacturer's recommendations (Genlantis, San Diego, CA, USA).

Forty-eight hours after transfection, cells were seeded into assay plates. All assays were performed three days after transfection.

3.4 BRET sensors

In order to measure cAMP response in real time in living cells, HEK293 cells were transiently transfected with two plasmids coding for hLH/CG-R (kindly provided by A. Ulloa-Aguirre, Universidad Nacional Autónoma de México, México) and the BRET-based cAMP sensor CAMYEL (kindly provided by L.I. Jiang, University of Texas, Texas, USA), respectively, whereas mLTC-1 cells, naturally expressing the endogenous lhr, were transfected with CAMYEL. The CAMYEL sensor, is composed by an inactive cytosolic mutant form of human Epac-1 fused with *Renilla* luciferase or Rluc (the donor) and the green fluorescent protein or GFP (the acceptor) (Ayoub et al, 2015; Jiang et al, 2007). Under basal condition, the proximity/orientation of Rluc and GFP within the sensor leads to high BRET signal. Upon rhCG/rhLH stimulation, when cAMP accumulation occurs, cAMP binding to Epac induces conformational change of Rluc-Epac-GFP sensor, resulting in dose-dependent decrease of BRET signals.

We assessed β-arrestin 2 recruitment by transiently transfecting HEK293 cells with hLH/CG-R C-terminally fused to the BRET donor Rluc (kindly provided by A. Hanyaloglu, Imperial College, London, UK) and with β-arrestin 2 N-terminally fused to the BRET acceptor yPET (kindly provided by M.G. Scott, Cochin Institute, Paris, France). Upon rhCG/rhLH stimulation, β-arrestin 2 translocates to the receptor, leading to crosstalk between Rluc and yPET, and as a consequence, to dose-dependent increases in BRET signals.

The conformational rearrangements elicited by β-arrestin 2 upon rhCG/rhLH exposure, was measured using β-arrestin 2 double brilliance BRET experiments. HEK293 cells were transiently transfected with hLH/CG-R and the β-arrestin 2 fused to both the BRET donor Rluc and the BRET acceptor RGFP (Rluc-β-arrestin 2-RGFP, kindly provided by R. Jockers, Cochin Institute, Paris, France)(Charest et al, 2005; Kamal et al, 2009). Changes in BRET signals caused by the different proximity/distance between the two sensors attached to β-arrestin 2 were monitored upon exposure to increasing doses of rhCG/rhLH.

3.5 Cell stimulations and BRET measurements

For the end-point dose-response experiments, medium was aspirated and cells were re-suspended in 40 µl/well of PBS 1X, HEPES 1mM. Cells were incubated for 30 minutes at 37°C in a total volume of 40 µl/well of PBS 1X, HEPES 1 mM containing or not increasing concentrations of rhCG/rhLH. BRET measurements were performed upon addition of 10 µl/well of 5 µM Coelenterzine h (Interchim, Montluçon, France), using Mithras LB 943 plate reader (Berthold Technologies GmbH & Co. Wildbad, Germany). For the real-time kinetic, cells were re-suspended in 60 µl/well of PBS 1X, HEPES 1 mM and, for cAMP kinetics, 200 µM IBMX. BRET measurement was immediately performed upon addition of 20 µl/well of EC_{50} concentrations of rhCG and rhLH as calculated in dose-response experiments, and 20 µl/well of 5 µM coelenterzine h.

3.6 Steroid measurements

Progesterone and testosterone levels were measured in mLTC-1 cells seeded in 48-well plates (10^5cells/well) in complete RPMI medium for three days. Cells were then re-suspended in serum-free RPMI for one hour and stimulated or not with increasing doses of rhCG and rhLH for 3 hours. Cell supernatants were collected and freezed. Progesterone production was measured with a home-made competitive ELISA assay. Briefly, a 96-wells plate was coated overnight at 4°C with a goat anti-mouse IgG antibody, 10ng/well (UP462140, Interchim, Montluçon, France). After three washes in PBS 1X containing 0.1% Tween 20, non-specific sites were saturated 1 hour with 200µl/ well of PBS-Tween supplemented with 0,2% BSA. Standard progesterone (Q2600, Steraloids) in PBS-Tween-BSA or mLTC-1 cells supernatants (25µl per well of 1:50 or 1:100 dilution) were then plated on the empty plate. Progesterone-11-Hemisuccinate-HRP (FX1630, Interchim, Montluçon, France) were added, together with 36ng/well of mouse anti-P4 antibody (7720-1420, AbD Serotec, Biogenesis, Interchim, Montluçon, France). The plate was incubated for 4 h at room temperature, washed and 100 µl/well of TMB Elisa substrate standard solution (UP664781 Interchim,Montluçon, France) was added and the mixture was incubated for 20 min at room temperature in the dark. The reaction was stopped with 2N H_2SO_4 and absorbance was measured at 450 nm using a Sunrise plate reader spectrophotometer (Tecan).

Testosterone levels were measured by time-resolved FRET using an HTRF Testosterone assay kit (CisBio Bioassays, Bagnol sur Ceze, France) following the manufacturer's protocol. Briefly, cells were seeded in 96-well plate (10^4 cells/well), starved overnight in serum-free medium and incubated 6 hours at 37°C in serum-free medium containing or not increasing concentrations of rhCG/rhLH. Then 10 µl of culture supernatant were transferred into 384-well and mixed with 10 µl of a mixture of HTRF mix containing an anti-Testosterone antibody and testosterone labeled with Terbium and d2 fluorophores, respectively. TR-FRET signals were detected using Mithras LB 943 plate reader (Berthold Technologies GmbH & Co. Wildbad, Germany).

3.7 Cre-dependent reporter assay

HEK293 cells were transiently transfected with hLH/CG-R and the pSomLuc plasmid expressing the firefly luciferase reporter gene under the control of the cAMP Responsive Element of the somatostatin promoter region (Troispoux et al, 1999). After 48-hours, cells were split into 96-well plates. The day after, cells were stimulated 6h with the agonist. Cells were then washed twice with ice-cold PBS and lysed in 200 µl of passive lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured using the luciferase assay system supplied by Promega. An aliquot (20 µl) of each sample was mixed with 50 µl of luciferase assay reagent and the emitted light was measured in Mithras LB 943 plate reader. Values were expressed in relative light units.

3.8 IP1 TR-FRET assay

Inositol phosphate 1 (IP1) accumulation was measured in HEK293 cells transiently transfected with hLH/CG-R or in mLTC-1 endogenously expressing lhr. IP1 levels were quantified with IP-One HTRF® assay kit (CisBio Bioassays, Bagnol sur Ceze, France), following the manufacturer's protocol. Briefly, cells were seeded in 384-well plate (10^4 cells/well) and incubated 30-45 minutes at 37°C in a total volume of 14 µl/well of stimulation buffer containing or not increasing concentrations of rhCG/rhLH. After adding HTRF mix containing an anti-IP1 antibody and IP1 labeled with both Terbium and d2 sensors respectively, TR-FRET signals were detected using Mithras LB 943 plate reader.

3.9 cAMP TR-FRET assay

Intracellular cAMP levels were measured using a homogeneous time-resolved fluorescence (HTRF®) cAMP dynamic 2 assay kit (CisBio Bioassays, Bagnol sur Cèze, France)(Norskov-Lauritsen et al, 2014). Forty-eight hours post-transfection cells were detached and seeded into white 384-well microplates with 5000 cells/well in 5 µl of stimulation buffer (PBS 1X, 200 µM IBMX, 5 mM HEPES, 0.1% BSA). For their stimulation, 5 µl/well of the stimulation buffer containing or not different doses of rhLH or rhCG were added. Then, cells were incubated for 30 minutes at 37°C, lysed by addition of 10 µl/well of conjugate-lysis buffer containing d2-labeled cAMP and Europium cryptate-labeled anti-cAMP antibody, both reconstituted according to the manufacturer's instructions. Plates were incubated for 1 h in the dark at room temperature and time-resolved fluorescence signals were measured at 620 and 665 nm respectively, 50 ms after excitation at 320 nm using a Mithras LB 943 plate reader.

3.10 Data analysis

BRET/FRET data were represented as Induced BRET Changes or Delta FRET by subtracting the 540 nm/480 nm or 665 nm/620 nm ratio respectively of the non-treated cells from the same ratio of cells stimulated with increasing doses of rhCG/rhLH. Steroid values were represented as % of maximal response. All the results were fitted following the appropriate nonlinear regression equations using Prism GraphPad software (San Diego, CA, USA). Mann-Whitney's *U*-tests and unpaired t-test were performed as appropriate. Differences were considered significant for $p<0.05$. Statistical analysis were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA).

4. Results

4.1 cAMP Response

In our effort to compare the respective efficacies of rhCG and rhLH, we first assessed the ability of either hormone to elicit the accumulation of cAMP, the prototypical second messenger activated downstream of human LH/CG-R and murine lhr. For this purpose, HEK293 cells transiently co-expressing hLH/CG-R and the BRET-based cAMP sensor CAMYEL were used as previously reported (Ayoub et al, 2015). Changes in BRET signal were monitored after 30 minutes of incubation with increasing doses of rhCG and rhLH. As expected, both hormones showed very potent effects on this signaling pathway with EC_{50} values in the pM range (Figure 1A). Consistent with previous reports, the rhCG dose-response curve is significantly shifted towards the left compared to rhLH curve (Casarini et al, 2012). The EC_{50} of rhCG response was found approximately 30 times lower than that of rhLH (12.91 pM ±1.48 *versus* 378.6 pM ±1.2 respectively; Mann-Whitney's *U*-test; p=0.0079; n=5). The two hormones showed similar maximal responses (Mann-Whitney's *U*-test; p=0.22; n=5). Similar results were obtained in a mouse Leydig tumor cell line (mLTC-1), endogenously expressing the murine lhr. Therefore, mLTC1 cells were transfected only with CAMYEL sensor. Again, BRET measurements revealed a rhCG dose-response curve shifted towards the left compared to rhLH (Figure 2B). Specifically, the EC_{50} of rhCG was approximately 8 times lower than that of rhLH (66.81 pM ± 1.26 *versus* 589.00 pM ± 1.34 respectively; Mann-Whitney's *U*-test; p=0.0286; n=4). Both rhCG and rhLH promoted the same maximal responses (Mann-Whitney's *U*-test; p=0.6857; n=4). We also investigated cAMP accumulation in real-time kinetics for 30 minutes in both cell models. Cells were stimulated with doses corresponding to previously calculated rhCG and rhLH EC_{50} (Figure 1A and 1C). Our data clearly indicated that both rhCG and rhLH promote a rapid cAMP response with a plateau reached after about 5 and 10 minutes in mLTC-1 and HEK293 cells, respectively. The half-time values of the responses elicited by each hormone were calculated by nonlinear regression of the measurements made over the first 10 minutes. No significant difference was observed between rhCG/rhLH in term of the kinetics of cAMP accumulation in either the cell line (Figure 1E and 1F) (Mann-Whitney's *U*-test; p>0.05; n=4).

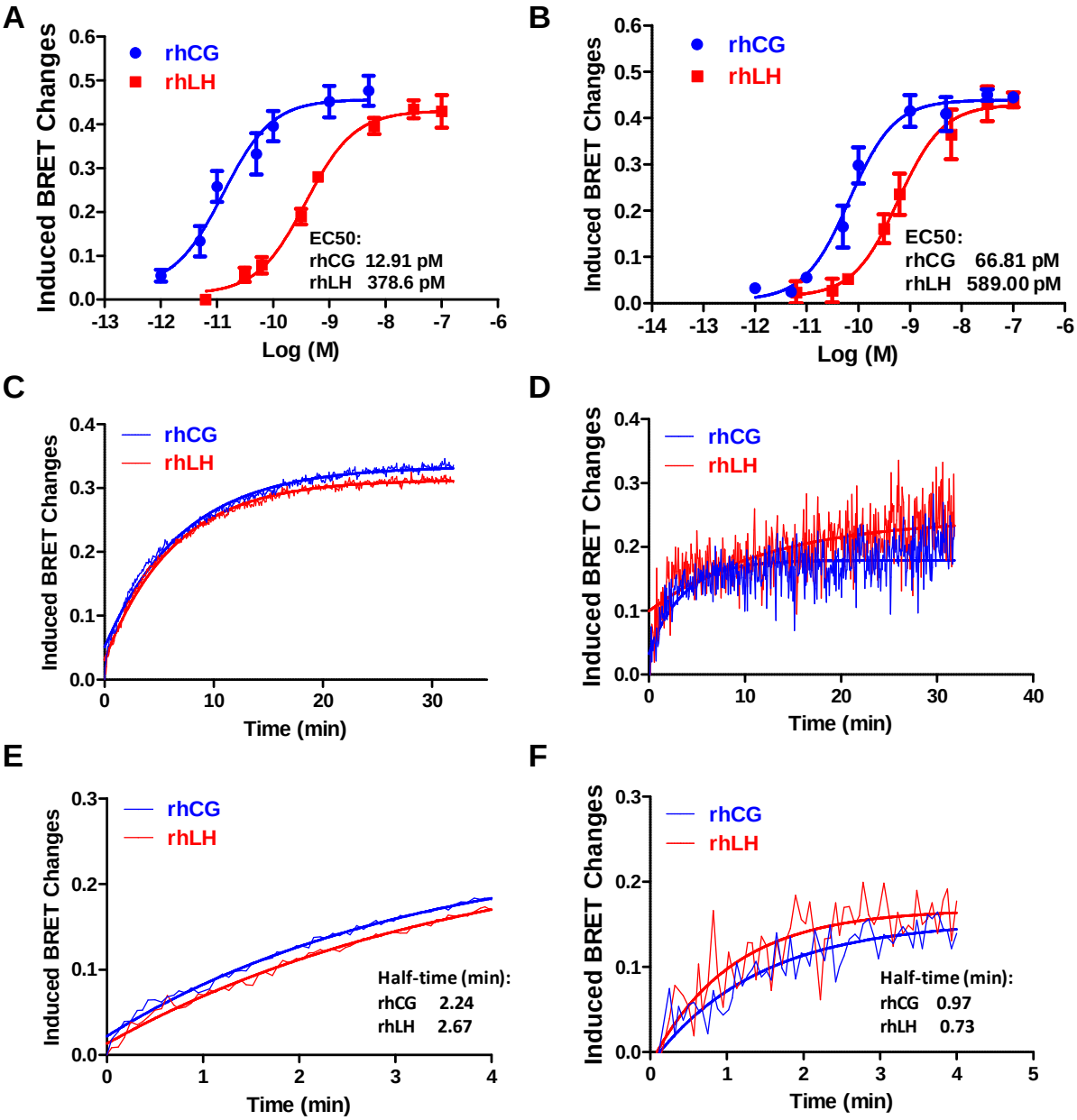

Figure 1. cAMP response induced by rhCG and rhLH in HEK293 cells transiently co-transfected with hLH/CG-R and CAMYEL sensor (A,C,E) and in mLTC1 cells transiently transfected with CAMYEL sensor alone (B, D, F). Cells were stimulated with rhCG and rhLH in a dose-response manner. cAMP accumulation was measured by BRET (Mann-Whitney's *U*-test; p>0.05; n=5) (**A**, **B**). Cells were stimulated with EC_{50} of rhCG and rhLH as calculated in (A, B) and the kinetics of cAMP accumulation was measured for 30 minutes in HEK293 and mLTC-1 cells respectively (**C**, **D**). Half-time of hormone-induced cAMP were evaluated in HEK293 and mLTC-1 cells respectively by non linear regression of the first 4 minutes of measurements (Mann-Whitney's *U*-test; p>0.05; n=4) (**E**, **F**). Experimental data are represented as means of 3 to 5 independent experiments.

4.2 β-arrestin 2 recruitment at the LH/CG-R

Increasing evidence suggest that β-arrestins play important roles not only in desensitization/internalization of GPCRs but also in their signaling (Lefkowitz & Shenoy, 2005; Luttrell & Lefkowitz, 2002; Reiter et al, 2012; Reiter & Lefkowitz, 2006). Here, we examined the recruitment of β-arrestin 2 upon exposure to increasing doses of rhCG/rhLH using BRET technology as previously reported (Ayoub et al, 2015). HEK293 cells were transiently transfected with Rluc-hLH/CG-R and yPET-β-arrestin 2 plasmids. Upon receptor activation, Rluc-hLH/CG-R recruits yPET-ß-arrestin 2 from the cytosol to the intracellular domains of the receptor. This interaction generates an increase in BRET signal proportional to the amount of receptor/β-arrestin complexes formed. We observed that, upon rhCG/rhLH stimulation, β-arrestin 2 was recruited to the receptor with EC_{50}s of 13.17 nM ±1.5 and 57.30 nM ±1.17 respectively (Figure 2A) (Mann-Whitney's *U*-test; p=0.0286; n=4). This finding is consistent with the fact that a higher receptor occupancy rate is needed to activate this intracellular event, compared to cAMP accumulation (Ayoub et al, 2015). Interestingly, our data reveal that rhLH exhibits partial agonistic activity compared to rhCG (i.e.: E_{max} rhLH: 0.14 ±0.008 *versus* E_{max} rhCG: 0.2 ±0.008; Mann-Whitney's *U*-test; p=0.0286; n=4). Then, real-time kinetics of HEK293 cells stimulated with EC_{50} concentrations of both rhCG and rhLH were monitored for 40 minutes (Figure 2B). No significant difference in recruitment half-times was measured between the two hormones (data not shown) (Mann-Whitney's *U*-test; p=0.1; n=3).

4.3 Conformational changes within β-arrestin 2

Further, we investigated the impact of LH/CG-R activation by rhCG or rhLH on β-arrestin 2 conformation using a previously reported double brilliance β-arrestin 2 BRET sensor (Charest et al, 2005; Kamal et al, 2009). In this sensor, β-arrestin 2 is fused with both a BRET donor and an acceptor. As a consequence, any change in the BRET *ratio* reflects a conformational change in β-arrestin 2. Interestingly, we found that rhLH led to significantly higher and more potent BRET changes than rhCG (EC_{50}s of 0.24 µM ±1.16 and 2.39 µM ±1.14, respectively) (Mann-Whitney's *U*-test; $p<0.05$; n=3) (Figure 2C). Kinetics were also monitored over a 70 min period with no significant differences between the two hormones (half-times of 12.12 min for rhLH and 12.26 min for rhCG) (Mann-Whitney's *U*-test; $p>0.05$; n=3) (Figure 2D).

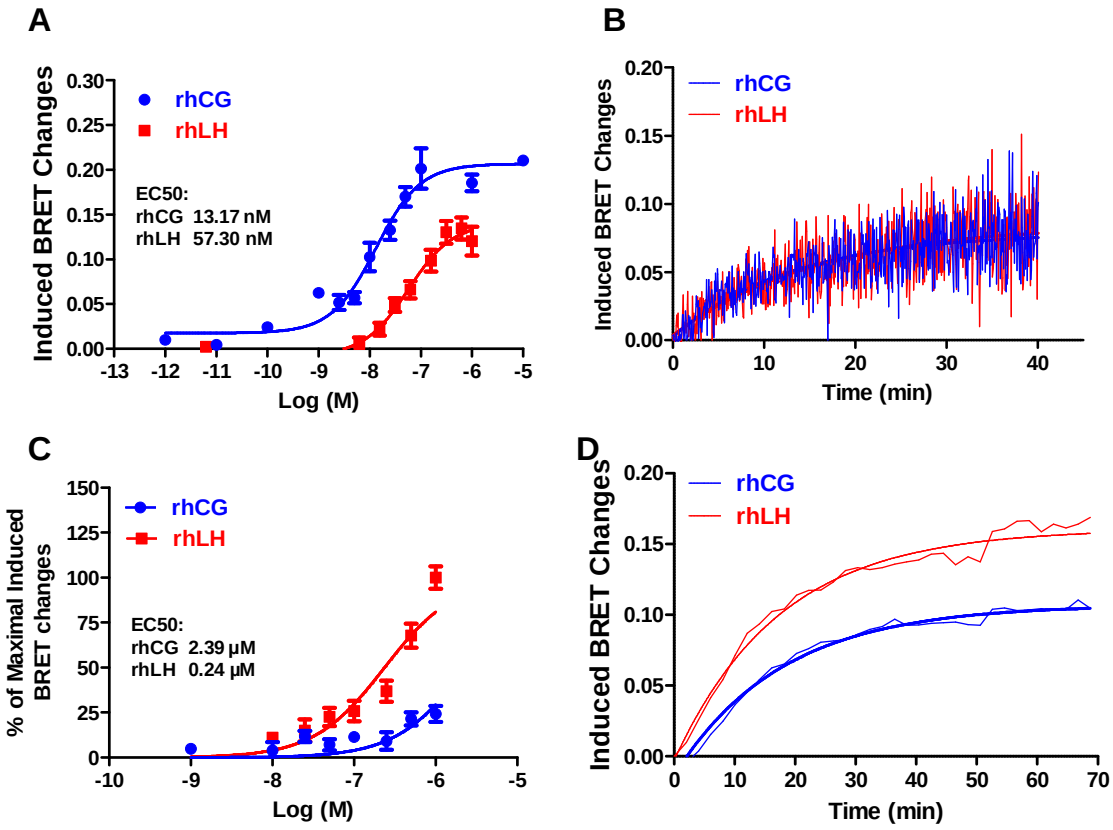

Figure 2. β arrestin-2 recruitment to LH/CG-R and β arrestin-2 conformational changes induced by rhCG and rhLH. β arrestin-2 recruitment induced by rhCG and rhLH in HEK293 cells transiently transfected with hLHCGR-Rluc8 and YPET-β arrestin 2. Cells were stimulated with rhCG and rhLH in a dose-response manner. β arrestin-2 recruitment was measured by BRET. Experimental data are represented as Mean ± SEM of 4 independent experiments (Mann-Whitney's *U*-test; $p<0.05$; n=4) (**A**). Cells were stimulated with prevously calculated EC_{50} of rhCG and rhLH. β arrestin-2 recruitment was measured in time course for 40 minutes by BRET (Mann-Whitney's *U*-test; $p>0.05$; n=4) (**B**). Conformation modifications of β arrestin-2 upon rhCG and rhLH exposure in HEK293 cells transiently transfected with hLHCGR and Rluc8-β arrestin-2-RGFP. Cells were stimulated with rhCG and rhLH in a dose-response manner. β arrestin-2 rearrengemets were measured by BRET. Experimental data are represented as Mean ± SEM of 3 independent experiments (Mann-Whitney's *U*-test; $p<0.05$; n=3) (**C**). Kinetics of b-arrestin-2 conformational changes. Cells were stimulated with prevously calculated EC_{50} of rhCG and rhLH. β arrestin-2 conformational changes were measured in time course for 70 minutes by BRET (Mann-Whitney's *U*-test; $p>0.05$; n=3) (**D**).

4.4 IP1 production

We then explored inositol phosphate production as an indicator of $G_{\alpha q}$/PLC signaling pathway activation. We used an HTRF assay to evaluate IP1 (a metabolite of IP3) levels upon rhCG/rhLH stimulation in both HEK293 and mLTC-1 cells. In HEK293, rhCG/rhLH exhibited different potencies, with EC_{50} approximately 140 times lower for rhCG than for rhLH (EC_{50} rhCG: 69.08 pM ±1.65; EC_{50} rhLH: 9.8 nM ±1.42) (Mann-Whitney's *U*-test;

p>0.05; n=2). (Figure 3A). rhLH was less potent, but led to a slightly higher maximal response. In mLTC-1, rhCG and rhLH displayed similar potencies (rhCG: 142 nM ± 2.15; rhLH: 210,3 nM ± 1,47) (Mann-Whitney's *U*-test; p>0.05; n=2) (Figure 4B) whereas rhLH elicited a higher maximal response than rhCG, even if not statistically significant due to the low number of replicates (Mann-Whitney's *U*-test; p>0.05; n=2).

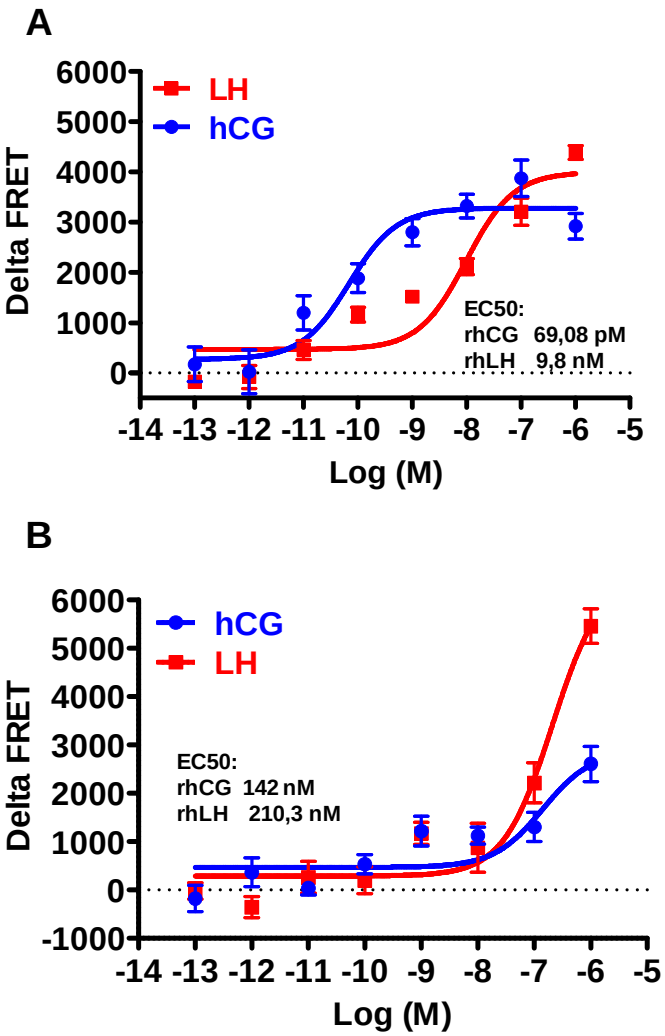

Figure 3. IP1 production induced by rhCG and rhLH in HEK293 cells transiently transfected with hLHCGR-WT (A) and in mLTC1 (B). Cells were stimulated with rhCG and rhLH in a dose-response manner. IP1 accumulation was measured by FRET. Experimental data are represented as Means±SEM of 2 independent experiments.

4.5 Integrated responses

Next, we sought to measure read-outs located downstream in the signaling pathways. In HEK293 cells, we used a *cre*-dependent reporter gene, pSOMLuc, as an indicator of LH/CG-R-induced transcriptional activation. Consistent with cAMP response, both hormones showed potent activation of luciferase activity (Figure 4A). The EC_{50} of rhCG response was found approximately 6 times lower than that of rhLH (0.373 nM ±1.3 *versus* 2.152 nM ±1.25, respectively) (Unpaired t-test; p=0.0099; n=3). By contrast, the two hormones showed similar maximal responses. These observations are consistent with the cAMP data shown in Figure 1.

In mLTC-1 cells, endogenous progesterone and testosterone production was evaluated in a dose-response manner upon rhCG/rhLH stimulation. In progesterone assay, we found that the EC_{50} of rhCG (18.99 pM ±1.18) was approximately 17 times lower than the one of rhLH (323.3 pM ±1.18) (Unpaired t-test; p=0.0003; n=3) (Figure 4B). Interestingly, the maximal level of progesterone was significantly higher for rhCG compared to rhLH (E_{max} rhCG: 94.36% ±3.1; E_{max} rhLH: 41.46% ±4.1) (Unpaired t-test; p=0.0028; n=3). In this experimental setting, rhLH clearly behaved like a partial agonist for progesterone synthesis. The two dose-response curves show also a different Hill-Slope (rhCG: 2.42 ± 0.737; rhLH: 1.839 ± 0.485) (Unpaired t-test; p>0.05; n=3), suggesting a different kinetic of induction. In the case of testosterone, we also observed a higher potency of rhCG compared to rhLH, even if not statistically significant (rhCG: 0.26 pM ±1.78; rhLH: 17.52 pM ±1,93) (Unpaired t-test; p>0.05; n=3). Again, these observations are consistent with the cAMP and IP1 responses measured in HEK293 cells and to some extent to β-arrestin recruitment. We also noted that both hormones were clearly more potent at activating testosterone than progesterone, suggesting that distinct signaling pathways might control their production.

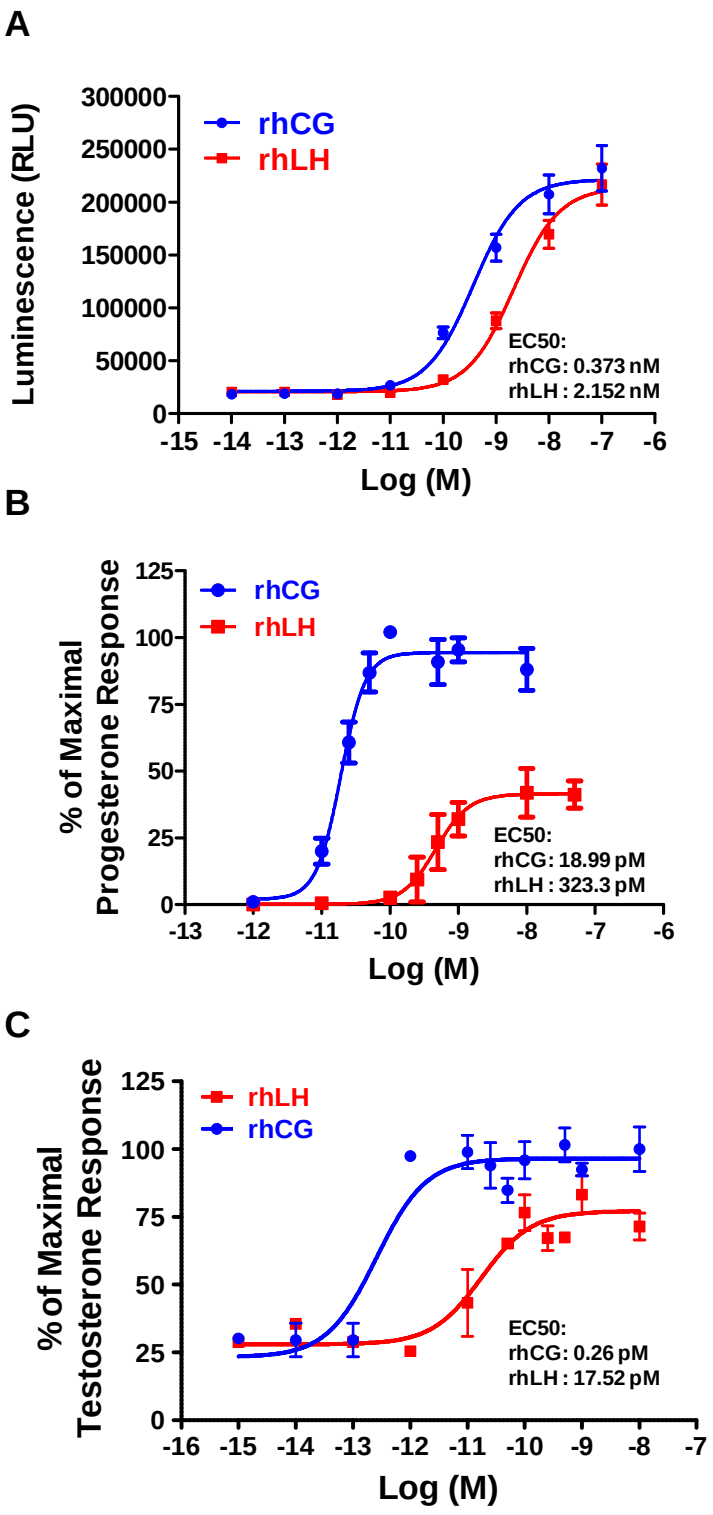

Figure 4. Cre-dependent transcription in HEK293 cells (A), progesterone (B) and testosterone (C) response in mLTC-1 cells induced by rhCG and rhLH. Cells were stimulated with rhCG and rhLH in a dose-response manner. Luciferase activity was measured as described in Materials and Methods (Unpaired t test; $p<0.05$; n=3) . Steroids production was measured by ELISA (progesterone) (Unpaired t test; $p<0.05$; n=3) or HTRF (testosterone) (Unpaired t test; $p>0.05$; n=3). Experimental data are represented as Mean ± SEM of 3 independent experiments.

4.6 β-arrestin-dependent steroid production

In order to explore the contribution of β-arrestin-dependent transduction to the control of steroidogenesis, siRNA-mediated depletion of endogenous β-arrestin 1 or β-arrestin 2 in mLTC-1 cells were carried out. Using Western blotting, we confirmed that siRNA transfection was indeed leading to the selective depletion of β-arrestin 1 or 2 compared to control siRNA (data not shown). Control, β-arrestin 1 or β-arrestin 2-depleted mLTC-1 cells were then exposed to increasing doses of either rhCG or rhLH and progesterone was measured through ELISA assays. Interestingly, we observed that both β-arrestin 1 and β-arrestin 2 depletion led to decreases in the maximal rhCG- and rhLH-induced progesterone production compared to control siRNA-transfected cells, even if not statistically significant (Mann-Whitney's *U*-test; p>0.05; n=3). These data suggest the implication of β-arrestins in LHR-mediated steroidogenesis.

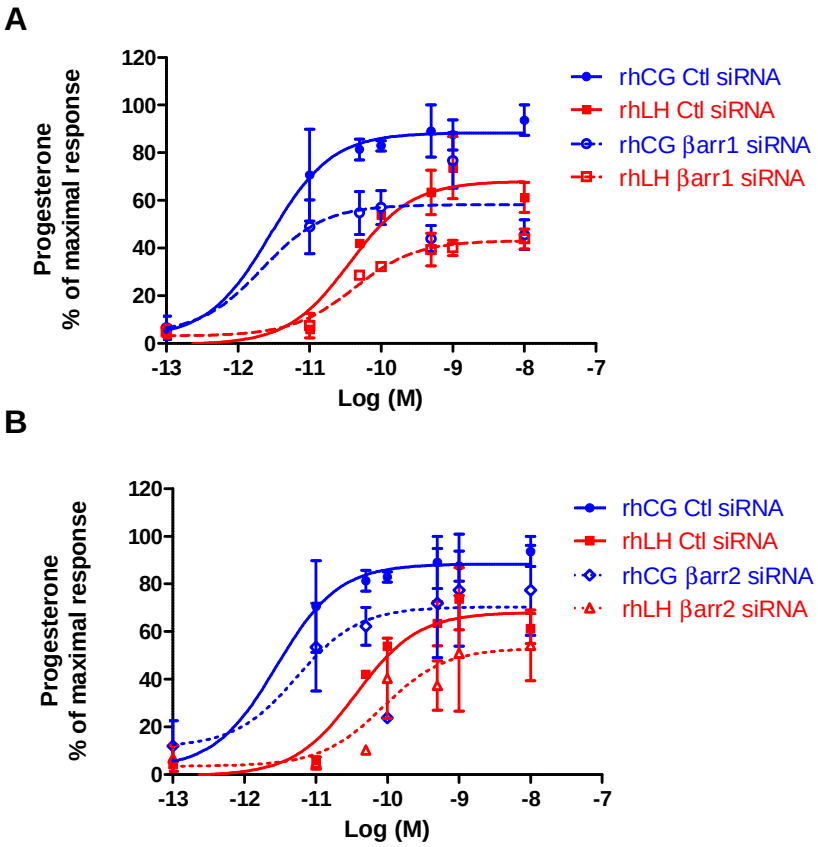

Figure 5. Progesterone production after siRNA β Arrestin 1-2 transfection in mLTC-1 cells. mLTC1 cells were transiently transfected with control and β-arrestin 1 siRNAs **(A)** or control and β-arrestin 2 siRNAs **(B)**. Dose-dependent accumulation of progesterone upon exposure to increasing doses of rhCG/rhLH was measured using ELISA assay. The maximal Progesterone response obtained with control siRNA was arbitrary chosen as 100%. Data represent the mean ± SEM from three individual experiments (Mann-Whitney's *U*-test; p>0.05; n=3).

5. Discussion

In the present work, we investigated the differential activity exerted by rhLH and rhCG upon binding to their common receptor, LH/CG-R in human and lhr in mice. The attention was focused on comparing the contributions of the G protein- ($G_{\alpha s}$ and $G_{\alpha q}$) and β-arrestin-dependent transduction mechanisms, both known to operate at the level of the receptor, to the modulation of steroidogenesis. Altogether, our results support the concept that hCG and hLH could be natural biased agonists capable of selectively fine-tune the activity of LH/CG-R *in vivo*. Our data are consistent with previous studies reporting that hCG is more potent than LH on cAMP accumulation and steroid hormone production in Cos7 and granulosa cells (Casarini et al, 2012). Moreover , LH- and hCG-induced signaling is known to be differently modulated by exon 10 deletion in LH/CG-R, which results in structural and spatial rearrangements at the hinge region of the receptor (Muller et al, 2003). In the presence of this deletion, LH signaling is impaired while hCG signaling remain unchanged, suggesting divergences between hCG - and LH - receptor interactions and actions on the hinge region. Recently, hCG and LH were shown to interact differently with the hinge region of the receptor (Grzesik et al, 2015). Further, only hCG is capable of inducing both cis- and trans-activation of human LH/CG-R (Grzesik et al, 2014). In the present study, we found the same difference in potency between the two gonadotropins in HEK293 cells and in mLTC-1 murine Leydig cells. Importantly, our quantitative pharmacological profiling also revealed striking peculiarities when comparing the maximal responses elicited by the two gonadotropins on the different readouts: even though identical maximal cAMP responses were reached with either gonadotropin, LH clearly led to significantly weaker maximal responses than hCG when progesterone and testosterone production were measured. In other word, LH is full agonist for cAMP, as well as for *cre*-dependent transcriptional response in LH/CG-R expressing HEK293 cells, whereas the same hormone is partial agonist for steroid production. This observation strongly suggests that hCG and LH exert biased agonism when binding at their common receptor.

We observed further evidences of biased actions of the two hormones when we characterized IP1 and β-arrestin responses. First, we were able to measure clear dose-dependent IP1 responses, a hallmark of $G_{\alpha q}$/PLC pathway activation, in our two cellular models with either hormone.

This is consistent with previous findings that LH/CG-R couples not only to $G_{\alpha s}$ but also to $G_{\alpha q}$ (Gudermann et al, 1992a; Gudermann et al, 1992b; Jonas et al, 2015). Even though different groups failed to detect $G_{\alpha q}$/PLC pathway activation upon hCG stimulation with high concentration (Ascoli et al, 1989; Rebois & Patel, 1985), this was likely due to a very low expression of the endogenous receptor (Zhu et al, 1994) and/or the low sensitivity of the assays used to evaluate the signal. Here, we examined IP1 production by time resolved FRET, in both HEK293 and mLTC-1 cells, comparing, for the first time, rhCG and rhLH responsiveness and again, we found clear differences between the two hormones. In HEK293 expressing LH/CG-R, rhCG was found approximately 140 times more potent than rhLH for IP1 production but LH led to slightly higher maximal response. In mLTC-1, the two hormones were equipotent with hCG clearly eliciting a partial maximal response compared to LH. Consistent with the fact that $G_{\alpha s}$ coupling represent the primary transduction mechanism, the EC50s measured for IP1 production were higher that the EC50s obtained for cAMP, particularly in mLTC-1 cells, indicating that higher receptor occupancy must be reached to recruit $G_{\alpha q}$ than $G_{\alpha s}$. Importantly, the IP1 data strengthen the notion that hCG and LH could be natural biased agonists at the same receptor.

We further explored the pharmacological profiles of the two hormones by assessing their respective abilities to activate β-arrestin-dependent transduction at the LH-CG-R. In line with our recent study, we found β-arrestin 2 to be recruited to LH/CG-R upon hCG exposure with a potency right-shifted by approximately 3 logs compared to cAMP response (Ayoub et al, 2015). Noteworthy, we found that LH was 5 times less potent but, more importantly, that it led to a partial maximal recruitment compared to hCG. We also observed that both hormones differently affected the conformation of β-arrestin 2 as assessed in double brilliance BRET assay (Charest et al, 2005; Kamal et al, 2009). In this assay, hCG elicited less induced BRET than LH, a result consistent with the idea that the two hormones could be biased. In this conceptual framework, hCG would be more efficacious than LH at stabilizing peculiar conformation, or set of conformations, of activated LH/CG-Rs that are capable of recruiting β-arrestins.

With these data in hands, we observed that the differential efficacies and potencies of the two hormones for progesterone and testosterone production paralleled the differences measured upstream, at the cAMP and β-arrestin levels. We therefore hypothesized that β-

arrestin-dependent transduction could be involved in the control of the balance between progesterone and testosterone production. Supporting this view, we demonstrated that depletion of endogenous β-arrestin 1 or β-arrestin 2 indeed led to significantly reduced progesterone responsiveness to both hormones.

Our data also reveal that hCG and LH are inversely efficacious at inducing $G_{\alpha q}$- or β-arrestin-dependent transduction. Given that these coupling occur in similar range of receptor occupancy, an attractive explanation is that β-arrestin and $G_{\alpha q}$ compete for receptor coupling. LH being partial agonist for β-arrestin recruitment, it leaves more active receptor to couple with $G_{\alpha q}$ than with hCG which recruits β-arrestins to a higher proportion of occupied receptors. Further studies will be necessary to explore this possibility.

6. Conclusions

Altogether, our results give support to the concept of biased agonism exerted by hCG and hLH and bear the notion that LH/CG-R can discriminate the binding of the two hormones, thereby triggering different transduction mechanisms hence intracellular responses. The fact that both hormones naturally coexist during pregnancy raises intriguing prospects. The use of these hormones in medically assisted procreation could also be impacted by the present findings.

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Conclusions

Our results provide clear evidence of the hCG and LH *non* equivalency *in vitro*, in both primary cells and transfected LHCGR-expressing cell lines. LHCGR, differently from lhr, can qualitatively discriminates the binding of the two hormones, thereby triggering different intracellular signaling pathways. Biased agonism exerted by hCG and LH was found, revealing ligand-dependent preferential G-protein and β-arrestins signaling.

Given the limitations of the *in vitro* model as a simplistic system to reproduce the *in vivo* dynamics, these results highlight the different bioactivity of the two gonadotropins, giving the basis for further investigations *in vivo*, and prompting their distinct use in clinical assistance.

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