Turun yliopisto
University of Turku

ASSAY DEVELOPMENT AND STUDY OF A MUTATION AFFECTING GONADOTROPIN ACTION

Ashutosh Trehan

TURUN YLIOPISTON JULKAISUJA – ANNALES UNIVERSITATIS TURKUENSIS
Sarja - ser. D osa - tom. 1246 | Medica - Odontologica | Turku 2016

University of Turku

Faculty of Medicine

Institute of Biomedicine

Department of Physiology

Turku Doctoral Programme of Molecular Medicine (TuDMM)

Supervised by

Ilpo Huhtaniemi, Professor
Department of Physiology,
Institute of Biomedicine,
University of Turku, Finland

and

Department of Surgery and Cancer,
Institute of Reproductive and Developmental Biology,
Hammersmith Campus,
Imperial College London
London, United Kingdom

Adolfo Rivero-Müller, PhD, Docent
Department of Physiology,
Institute of Biomedicine,
University of Turku, Finland

and

Faculty of Natural Sciences and Technology,
Åbo Akademi University
Turku, Finland

Reviewed by

Ulla Petäjä-Repo, PhD, Docent
Department of Anatomy and Cell Biology,
University of Oulu
Finland

Kari Keinänen, Professor
Department of Biosciences,
Division of Biochemistry and Biotechnology
University of Helsinki
Finland

Opponent

Craig McArdle, Professor
Labs. for Integrative Neuroscience & Endocrinology (LINE)
School of Clinical Sciences, University of Bristol
United Kingdom

Cover image: Ashutosh Trehan

The originality of this thesis has been checked in accordance with the University of Turku quality assurance system using the Turnitin OriginalityCheck service.

ISBN 978-951-29-6585-4 (PRINT)
ISBN 978-951-29-6586-1 (PDF)
ISSN 0355-9483 (Print)
ISSN 2343-3213 (Online)
Painosalama Oy - Turku, Finland 2016

ABSTRACT

Ashutosh Trehan

Assay development and study of a mutation affecting gonadotropin action

University of Turku, Faculty of Medicine, Institute of Biomedicine, Department of Physiology, Turku Doctoral Programme of Molecular Medicine (TuDMM), Turku, Finland

The gonadotropin action mediated via three glycoprotein hormones, luteinizing hormone (LH), chorionic gonadotropin (CG) and follicle-stimulating hormone (FSH) is paramount for sexual differentiation, pubertal development and reproductive functions. The gonadotropins bind to their respective receptors, luteinizing hormone/chorionic gonadotropin receptor (LHCGR) and follicle stimulating hormone receptor (FSHR), both belonging to G protein-coupled receptor (GPCR) family. The stimulation of gonadotropin receptors with their respective ligand leads, among other signaling pathways, to the production of cyclic 3',5' adenosine monophosphate (cAMP) through $G\alpha_S$-mediated adenylyl cyclase activation. Modeling of patient mutations or those created using site-directed mutagenesis has been instrumental in understanding structural-functional relationship of key residues affecting gonadotropin action. The current thesis characterizes a novel inactivating mutation in *LH beta* subunit of a patient as well as the development of two methods that were utilized for its molecular characterization. The first method, REPLACR-mutagenesis (Recombineering of Ends of linearized PLAsmids after PCR), is a one-step site-directed mutagenesis method, that utilizes *in vivo recombineering* for mutagenesis (deletions, additions and substitutions) in plasmid vectors. REPLACR-mutagenesis is an inexpensive alternative to commercial kits involving fewer steps and with similar efficiency. The second method, named CANDLES (Cyclic AMP iNdirect Detection by Light Emission from Sensor cells), was developed to monitor the kinetics of cAMP generation in cell cultures, without transfection of any real-time cAMP sensor, which is especially difficult in primary cell cultures. Finally, the *LH beta* mutation that causes a lysine (Lys20) deletion resulting in a hypogonadal phenotype in the patient was molecularly characterized with the above-mentioned methods.

Keywords: cAMP, mutagenesis, LHB, assay, recombineering, luteinizing hormone, luminescence, GPCR, glycoprotein hormone, hypogonadism

TIIVISTELMÄ

Ashutosh Trehan

Menetelmäkehitys ja gonadotropiinien toimintaan vaikuttava mutaatio

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Fysiologian oppiaine ja Turun molekyylilääketieteen tohtoriohjelma (TUDMM), Turku, Finland

Gonadotropiinivaikutus, jota välittää kolme hormonia, luteinisoiva hormoni (LH), follikkelia stimuloiva hormoni (FSH) ja istukkagonadotropiini (hCG), on keskeisen tärkeää sukupuolten erilaistumiselle, murrosiän aikaiselle kehitykselle sekä aikuisen lisääntymistoiminnoille. Gonadotropiinit sitoutuvat kohdesolujensa spesifisiin reseptoreihin, joita ovat luteinisoivan hormonin/istukkagonadotropiinin reseptori (LHCGR) ja follikkelia stimuloivan hormonin reseptori (FSHR); molemmat kuuluvat G-proteiineja sitovien reseptorien (GPCR) geeniperheeseen. Hormonin reseptoriin sitoutumisen aiheuttama stimulaatio saa aikaan syklisen adenosiini-3',5'-monofosfaatin (cAMP) muodostumiseen Gαs-proteiinin välityksellä ja adenylaattisyklaasientsyymin katalysoimana. Potilaista löydettyjen ja mutageneesillä tuotettujen mutaatioiden mallintaminen käyttäen kohdennettua mutageneesiä on ollut oleellisen tärkeää oppiessamme ymmärtämään molekyylien rakenne-funktio-yhteyksiä gonadotropiini-vaikutuksen yhteydessä. Tässä väitöskirjatyössä esitellään uusi potilaan LH:n beta-alayksikön inaktivoiva mutaatio sekä kuvataan kaksi uutta menetelmää sen molekyylitason karakterisoimiseksi. Ensimmäinen menetelmä, REPLACR-mutageneesi (Recombineering of Ends of linearised PLAsmids after PCR), on yksivaiheinen kohdennetun mutageneesin menetelmä, jossa käytetään rekombinaatio (recombineering) -menetelmää pistemutaatioiden (poistot, lisäykset, korvaukset) aikaansaamiseksi plasmidivektorissa. REPLACR-mutageneesi on huokea vaihtoehto kaupallisille reagenssisarjoille, ja niihin verrattuna siinä on vähemmän välivaiheita tehokkuuden pysyessä samanlaisena. Toinen menetelmä, nimeltään CANDLES (Cyclic AMP iNdirect Detection by Light Emission from Sensor cells), kehitettiin mittaamaan soluviljelyjen kineettistä cAMP-vastetta ilman tarvetta transfektoida soluja reaaliaikaisella cAMP-sensorilla, mikä on erityisen vaikeaa primaarisoluilla. Lopuksi karakterisoimme hypogonadiselta potilaalta löydetyn lysiini-20:n (Lys20) deleetiosta johtuvan LH:n beta-ketjun inaktivoivan mutaation aiheuttamat molekyylitason muutokset hormonin toiminnassa.

Avainsanat: cAMP, mutageneesi, LHB, menetelmä, rekombinaatio, luteinisoiva hormoni, luminesenssi, GPCR, glykoproteiinihormoni, hypogonadismi

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ABBREVIATIONS

AC	Adenylyl cyclase
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
β2AR	β2-adrenergic receptor
BAC	Bacterial artificial chromosome
bp	Base pair
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
cAMP	Cyclic-3',5'-adenosine monophosphate
CANDLES	Cyclic AMP iNdirect Detection by Light Emission from Sensor cells
CBX	Carbenoxolone
CFP	Cyan fluorescent protein
CG	Chorionic gonadotropin
CGA	Common glycoprotein alpha subunit
CRE	cAMP response element
CREB	cAMP response element-binding protein
CRY2	*Cryptochrome Circadian Clock 2*
Cx-32	Connexin-32
DMEM	Dulbecco's Modified Eagle Medium
EC_{50}	Half-maximal effective concentration
ECD	Extracellular domain
ELISA	Enzyme-linked immunosorbent assay
Epac	Exchange protein directly activated by cAMP
FRAP	Fluorescence recovery after photobleaching
FRET	Fluorescence resonance energy transfer
FSH	Follicle-stimulating hormone
FSHB	*FSH beta*
FSHR	Follicle-stimulating hormone receptor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GnRH	Gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GPHR	Glycoprotein hormone receptor
GS-293	HEK-293-GloSensor
hCG	Human chorionic gonadotropin
hCGB	*hCG beta*
HEK-293	Human embryonic kidney-293
HR	Homologous recombination
HTS	High throughput screening
IBMX	3-Isobutyl-1-methylxanthine
IVS	Intervening sequence

LB	Luria-Bertani
LH	Luteinizing Hormone
LHB	Luteinizing hormone beta subunit
LHCGR	Luteinizing hormone/chorionic gonadotropin receptor
LRR	Leucine rich repeats
LuRKO	LHCGR null
MEM	Minimal Essential Medium
NEB	New England Biolabs
NLS	Nuclear localization signal
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PKA	Protein kinase A
PRL	Prolactin
REPLACR	Recombineering of Ends of linearized PLAsmids after PCR
rLH	Recombinant human luteinizing hormone
RLU	Relative light units
SLIC	Sequence and ligation-independent cloning
SLICE	Seamless ligation cloning extract
TMD	(Seven) transmembrane domain
TSH	Thyroid-stimulating hormone
TSHB	*TSH beta*
TSHR	Thyroid-stimulating hormone receptor
WT	Wild-type
YFP	Yellow fluorescent protein

LIST OF ORIGINAL PUBLICATIONS

The PhD thesis is based on the following articles and submissions. All references to figures in the published papers and submitted manuscript henceforth will start with a Roman numeral (I, II or III). The original communications have been reproduced with the permission of copyright holders.

I. Ashutosh Trehan, Michał Kiełbus, Jakub Czapinski, Andrzej Stepulak, Ilpo Huhtaniemi, Adolfo Rivero-Müller (2016). REPLACR-mutagenesis, a one-step method for site-directed mutagenesis by recombineering. Sci Rep 6:19121.

II. Ashutosh Trehan, Emmi Rotgers, Eleanor T. Coffey, Ilpo Huhtaniemi, Adolfo Rivero-Müller (2014). CANDLES, an assay for monitoring GPCR induced cAMP generation in cell cultures. Cell communication and signaling: CCS 12:70

III. Iulia Potorac†, Adolfo Rivero-Müller†, Ashutosh Trehan†, Francois Pralong, Maria Cristina Burlacu, Jean-Jacques Menage, Michał Kiełbus, Albert Thiry, Krzysztof Jozwiak, Aicha Hafidi, Hernan Valdes-Socin, Anne-Simone Parent, Axelle Pintiaux, Ilpo Huhtaniemi§, Albert Beckers§, Adrian F. Daly§. A single amino acid deletion in the LHB gene causes intracellular retention of hormone and hypogonadism in a male patient.
† Equal Contribution
(Manuscript)

1 INTRODUCTION

G protein-coupled receptors (GPCRs) are known for their characteristic seven-transmembrane structure which can transduce the extracellular stimuli from a wide range of ligands (photons, ions, neurotransmitters, peptides, hormones etc.) to elicit specific intracellular signaling pathways in the cells (Fredriksson et al., 2003; Lagerstrom et al., 2008; Zhang et al., 2015). The sheer number of GPCRs (~800 in humans) along with their ligand diversity have made them the popular target for not only the currently available drugs but also for the development of next generation drugs (Drews, 2000; Hopkins et al., 2002; Jacobson, 2015). The breadth of data available today about the structure, signaling and pharmacology of GPCRs must be attributed to the development of methods, which along with genetic engineering, not only helped in dissecting GPCR structure - signaling interrelationships but also aided efforts for drug screening. Our understanding of the key residues involved in signal transduction was also aided by characterization of clinical and artificially-induced mutations (activating/inactivating) in different model systems such as cell cultures and genetically modified (knockouts and knock-ins) murine models (Munk et al., 2016; Ratner et al., 2014; Vassart et al., 2011).

The current thesis is focused on a subset of GPCRs called gonadotropin receptors, which comprises the luteinizing hormone/chorionic gonadotropin receptor (LHCGR) and follicle-stimulating hormone receptor (FSHR) (Cahoreau et al., 2015; Vassart et al., 2004). Many GPCRs, including gonadotropin receptors, upon receptor activation couple to a subset of G-proteins, namely $G\alpha_s$, that in turn activate adenylyl cyclase thereby catalyzing the production of cyclic-3',5'-adenosine monophosphate (cAMP) from adenosine triphosphate (ATP) (Katritch et al., 2013). Thus the activation of $G\alpha_s$-coupled GPCRs can be read by assays for detection and monitoring of cAMP production. In the presented PhD thesis, three approaches are utilized in understanding GPCR signaling; the first one involves development of an inexpensive and robust method of creating mutations in plasmid expression vectors, while the second one is focused on development of a method for monitoring cAMP production. Finally, the third one utilizes the two methods to characterize a novel patient mutation in luteinizing hormone beta (*LHB*) subunit that disrupts LHCGR signaling, thereby rendering a hypogonadal phenotype.

2 LITERATURE REVIEW

The literature review for the presented dissertation aims to address why the development of two methods, one for mutagenesis and the other for cAMP detection, was chosen in order to address the molecular characterization of a mutant gonadotropin.

2.1 Gonadotropins and their receptors: Structure and signaling

The term "gonadotropin" collectively refers to three hormones, luteinizing hormone (LH), chorionic gonadotropin (CG or hCG in case of humans) and follicle-stimulating hormone (FSH). Gonadotropins, along with thyroid-stimulating hormone (TSH) are relatively large hormones (~30 kDa) and are collectively referred to as glycoprotein hormones (Cahoreau et al., 2015; Szkudlinski, 2015). The carbohydrate chains that can constitute around 20-45 % of the total mass in glycoprotein hormones are essential for their solubility, half-life and functional activity, but not necessarily for receptor binding (Ryan et al., 1988; Sairam, 1989; Szkudlinski et al., 1995). Glycoprotein hormones are dimeric proteins, with two subunits, alpha (α) and beta (β), held together non-covalently to form a functional hormone. All glycoprotein hormones share a common alpha subunit (common glycoprotein alpha, CGA) while the beta subunits are specific for each hormone. The sequences of both alpha and beta subunits have been conserved during evolution, with common ancestors of both the genes being found in vertebrates as well as in invertebrates (Hsu et al., 2002; Li et al., 1998).

The key cysteine residues in both the subunits are also conserved, such that each subunit folds in the form of three loops that are joined by disulphide bridges to give rise to a conserved structural unit called a "cystine-knot", that is also found in other proteins such transforming growth factor beta family of proteins beside glycoprotein hormones (Alvarez et al., 2009; Vitt et al., 2001). The crystal structures of hCG and FSH later revealed that the cysteine-knots helps in the dimerization of the two subunits, where the beta subunit surrounds the alpha subunit like a seat-belt with an extended loop that latches the alpha subunit around a disulphide bond near the *C*-terminus of the beta-subunit (Fox et al., 2001; Lapthorn et al., 1994; Wu et al., 1994).

The action of the three glycoprotein hormones is mediated via the activation of their cognate receptors, LHCGR, FSHR and thyroid-stimulating hormone receptor (TSHR), respectively, that are together referred to as glycoprotein hormone receptors (GPHRs). GPHRs belong to the large family of rhodopsin-like GPCRs, also referred as Class A GPCRs (Fredriksson et al., 2003). Though GPHRs share a common seven transmembrane domain (TMD) structure with other members belonging to the GPCR family, they, in addition possess a large extracellular domain (ECD) for ligand binding and subsequent receptor activation (Ascoli et al., 2002; Jiang et al., 2012; Szkudlinski et al., 2002).

Structurally, the ECD is characterized by the presence of leucine rich repeats (LRR), in which multiple LRR are arranged in the form of a horseshoe. Amino acids side chains in the horseshoe form the ligand recognition/docking sites (Moyle et al., 2004). The ECD that is joined to the transmembrane domain by a flexible 'hinge' region, that has been shown to contain additional sites (such as sulfated tyrosine in FSHR) for ligand recognition (Bonomi et al., 2006; Jiang et al., 2012). Ligand binding in the ECD leads to conformational changes in the TMD that are transduced to specific signaling pathways via the intracellular domain through both *C*-terminal tail and intracellular loops of the TMD (Cahoreau et al., 2015; Vassart et al., 2004). Multiple pathways, both G protein-independent via the action of scaffolding proteins such as β-arrestins or G protein-dependent pathways are activated upon receptor stimulation in a cell and tissue specific manner (Ascoli et al., 2002; Kleinau et al., 2009; Oldham et al., 2008). A major pathway that is activated upon stimulation of GPHRs via their respective hormones induces production of the secondary messenger, cAMP (Figure 1) and is discussed in greater details in the following section.

GPHRs, being members of the GPCR family, recruit upon receptors stimulation heterotrimeric G proteins (Gα and Gβγ complex) to catalyze the exchange of guanosine diphosphate (GDP) with guanosine triphosphate (GTP) in the Gαs subunit. The GTP-bound Gαs can now dissociate from Gβγ to activate adenylyl cyclases (ACs). There are 10 different isoforms of mammalian ACs, nine of which are membrane bound and are activated by Gαs, while the tenth AC is not activated by Gαs but rather by bicarbonate ions and is found in a soluble form (Kamenetsky et al., 2006; Linder et al., 2003; Tresguerres et al., 2011). An activated AC utilizes adenosine triphosphate (ATP) as its

substrate to catalyze its conversion to cAMP (Linder, 2006). cAMP can thereafter alter the function of multiple downstream effectors by binding to protein kinase A [PKA] (Walsh et al., 1968).

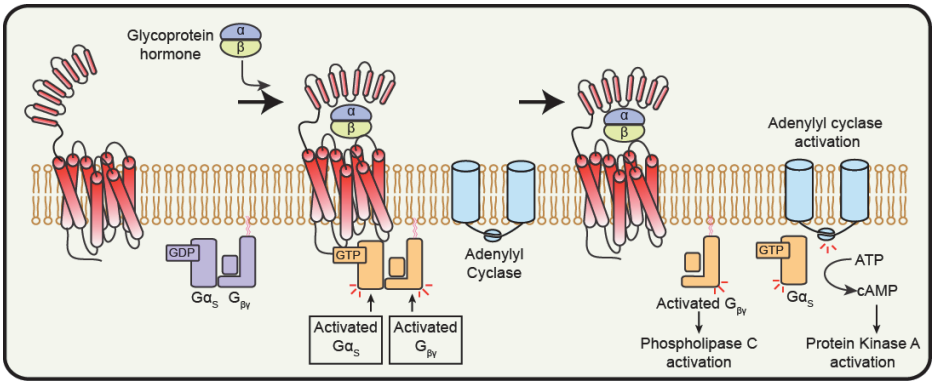

Figure 1. Glycoprotein hormone receptor signaling (also GPCR signaling in general). The activation of the receptor by dimeric glycoprotein hormone leads to adenylyl cyclase activation by $G\alpha_S$ while $G\beta\gamma$ activates phospholipase C for further downstream signaling.

PKA is a tetrameric protein with two regulatory subunits and two catalytic subunits. The binding of cAMP to PKA leads to the dissociation of its catalytic units from regulatory units and the former can phosphorylate multiple downstream effectors such as cAMP response element-binding protein (CREB) (Chin et al., 2002; Smith et al., 2011). CREB thereafter binds to the cAMP response element (CRE) sequence in the promoter region of various genes to regulate their expression (Mayr et al., 2001; Sands et al., 2008). Besides PKA, cAMP can also bind to exchange protein directly activated by cAMP (Epac) to activate small GTPases (de Rooij et al., 2000; Gloerich et al., 2010) or cAMP can bind to cyclic-nucleotide gated ion channels to regulate the transport of Na^+, K^+ or Ca^{+2} ions (Kaupp et al., 2002). However, cAMP content in the cells is also under the control of another set of proteins called phosphodiesterases (PDE) that catalyze the conversion of cAMP to adenosine monophosphate (AMP), thereby halting the cAMP-mediated signaling (Bender et al., 2006; Omori et al., 2007). Besides the activation of adenylyl cyclases via $G\alpha_S$, GPHRs have been shown to activate multiple other pathways to varying degrees, such as mitogen-activated protein kinases, increase in Ca^{+2} levels, phospholipase C activation, among others (Cameron et al., 1996; Grasso et al., 1993; Herrlich et al., 1996; Hirsch et al., 1996).

2.2 Gonadotropin secretion and physiology

Gonadotropin secretion and function is under the control of a combination of neural and endocrine tissues referred to as hypothalamic-pituitary-gonadal (HPG) axis that regulates the sexual development and reproductive physiology in humans, among other species (Jin et al., 2014; Peper et al., 2010). A subset of hypothalamic neurons found in arcuate nucleus releases the decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2), namely gonadotropin-releasing hormone [GnRH] (Baba et al., 1971; Schally et al., 1971). GnRH is then released via hypophyseal portal circulation to the anterior pituitary where it binds to GnRH receptor (GnRHR) in the gonadotropes. The gonadotropes upon stimulation with GnRH releases the two gonadotropins, LH and FSH, in the circulation (Bliss et al., 2010). However, another gonadotropin, hCG is secreted by placenta during pregnancy (Petraglia et al., 1995). The release of GnRH to stimulate the production of gonadotropins occurs in a pulsatile manner, which varies in amplitude and frequency (Maeda et al., 2010). Higher pulses of GnRH have been shown to preferentially cause production of LH while lower pulses of GnRH leads to FSH production. This is achieved by increasing the transcriptional rates of gonadotropin subunits, where a high pulse of GnRH increases both *CGA* and *LHB* gene transcription while a low GnRH pulse increase *FSHB* transcription (Dalkin et al., 1989; Haisenleder et al., 1991; Kaiser et al., 1997).

LH/hCG and FSH activate their cognate receptors, LHCGR and FSHR, to mediate a multitude of functions ranging from sexual differentiation prenatally to the development at puberty and maintenance in adulthood of the secondary sexual characteristics and fertility (Forest et al., 1976). The hCG-stimulated testosterone production by fetal Leydig cells causes stabilization of Wolffian ducts and their differentiation to internal and external male sex organs. The fetal Leydig cells are replaced at puberty by adult Leydig cells, and postnatally the action hCG is entirely taken over by pituitary LH (Forest et al., 1976). During early postnatal life, there is an increase in LH pulses caused by increased GnRH secretion and is referred to as mini-puberty (Waldhauser et al., 1981). In the period leading up to puberty, there is a quiescent period in which GnRH and subsequently gonadotropin secretion is greatly suppressed, though not completely absent (Wu et al., 1991). Subsequently at pubertal age, there is a steady increase in LH secretion, caused by reactivation of GnRH mediated gonadotropin secretion (Boyar et al., 1972). Upon reaching adult levels of

gonadotropins at puberty, LH action in males stimulates the production of testosterone upon LHCGR activation in Leydig cells while the action of FSH/FSHR in Sertoli cells (Figure 2), along with testosterone maintains spermatogenesis (Forest et al., 1976; Plant et al., 2001). However, with aging, a decrease in testosterone as well as spermatogenesis can be observed even though testicular function is still maintained throughout male adult life (Kaufman et al., 2005; Perheentupa et al., 2009).

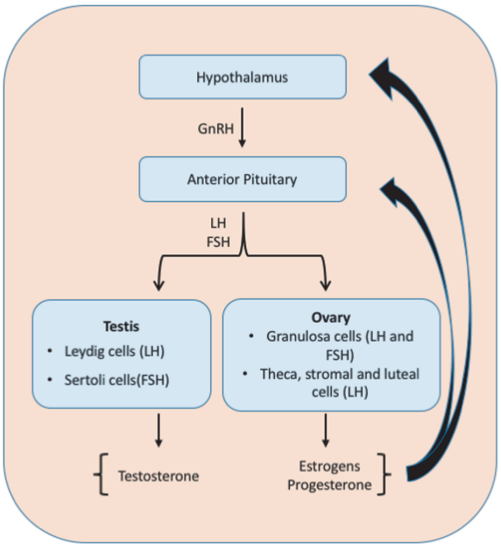

Figure 2. Regulation of gonadotropin secretion. Hypothalamic neurons secrete GnRH that binds to GnRHR in the gonadotropes in anterior pituitary thereby causing the release of gonadotropins (LH and FSH). Gonadotropins acts on their target cells in ovaries or testes leading to the production of steroids (estrogens, testosterone and progesterone) that provide a feedback either via hypothalamus or pituitary to control the release of GnRH and thereby gonadotropins.

The other fetal testicular hormone, Anti-Müllerian Hormone, cause involution of Müllerian ducts, giving rise to female sex organs (Huhtaniemi, 1994; Huhtaniemi et al., 1977). In females, the differentiation of the sex is independent of gonadotropin action. In adult females, FSH action leads to follicular development in the ovaries and the developed follicles synthesizes estrogens by aromatization of androgens. Androgen production is mediated by the action of LH in theca cells thereby providing substrate for estrogen production (Leung et al., 1980). The action of estrogen provides a negative feedback to limit the FSH production and causes subsequent increase in GnRH pulsatility in the late follicular phase for LH surge. LH acts through LHCGR in the

granulosa, theca and stromal cells and helps in the development and maturation of the oocyte. Ovulation and subsequent production of progesterone from corpus luteum are also triggered by the action of LH (Christensen et al., 2012).

Another level of control to regulate the secretion of gonadotropins occurs via the action of sex steroids in males and females (Chimento et al., 2014; Maggi et al., 2016). Testicular steroids have been known to provide a negative feedback resulting in a decrease in gonadotropin secretion as has been demonstrated in many indirect studies where castration was shown to be associated with an increased gonadotropin profile in different species (Damassa et al., 1976; Plant, 1982; Tilbrook et al., 1995). Recent studies have shown that the sex steroid feedback (negative or positive) either acts directly on the gonadotropes in the pituitary or via the action of androgen receptor and estrogen receptor in the hypothalamus, thus providing fine tuning of GnRH associated gonadotropin production (Bliss et al., 2010; Chimento et al., 2014; Maggi et al., 2016).

2.3 Clinical mutations affecting gonadotropin action

Given the role of gonadotropins and their receptors in the sexual differentiation, maintenance of reproductive functions and fertility, there is a very high selective pressure at the population level against mutations affecting gonadotropin function, and the mutations so far found in patients are rare. In most cases, such mutations are found in consanguineous families with known cases of infertility among its members. Broadly, the mutations found in beta subunits of gonadotropins (LH beta or FSH beta) are inactivating mutations while both inactivating and activating mutations have been reported for the gonadotropin receptors (LHCGR and FSHR). Since the work for this thesis is focused on the characterization of a LH beta mutation, the following section mainly describes mutations found in gonadotropin hormone subunits, without delving into mutations found in their receptors.

Any genetic mutations in CGA of glycoprotein hormones would lead to gross signaling alterations in all the pathways activated by LH/CG, FSH and TSH and would most likely be lethal in humans due to alteration of hCG function in pregnancy. However, *CGA* knockout mice are viable since mice do not express hCG and have a different

regulation during pregnancy (Kendall et al., 1995). There has, however, been one report of a somatic mutation in which the ectopically secreted CGA from a patient's carcinoma had Glu56Ala substitution, that could most likely be due unstable genetic background of the carcinoma (Nishimura et al., 1986).

Inactivating mutations in *LHB* gene have been studied in five reports (Table 1). The reported mutations caused functional LH deficiency resulting in hypogonadism with delayed puberty, low testosterone levels and arrested spermatogenesis, that could be treated with exogenous hCG. The first *LHB* mutation (GAC > GGC) in the male patient resulted in Gln54Arg substitution, such the resulting LH dimer was unable to activate LHCGR signaling, even though it was secreted normally and the heterodimer (LHB/CGA) could be detected by immunoassay (Axelrod et al., 1979; Weiss et al., 1992). The second *LHB* mutation (GGC > GAC) in the male patient, a Gly36Asp substitution, caused disruption of the cystine knot motif, thereby affecting its secretion and dimerization with CGA subunit (Valdes-Socin et al., 2004). The third LHB mutation caused a substitution (G→C) at the first nucleotide (+1) of intron 2 (also referred as intervening sequence at intron 2; IVS2) thereby disrupting the 5' splice site (IVS2+1G→C), and eventually the normal primary sequence of LHB and thus its secretion (Lofrano-Porto et al., 2007). The reported mutation (IVS2+1G→C) was found in three siblings (two males and one female). Although the male patients had a similar hypogonadal phenotype with delayed puberty as reported in previous cases of LHB mutation, the female patient underwent normal puberty but later presented with secondary amenorrhea and infertility.

The next reported LHB mutation caused deletion of three amino acids (His10-Ile12del), and was a peculiar case in which the secretion of LHB was reduced but the mutant LHB could dimerize with CGA and subsequently stimulate cAMP synthesis (Achard et al., 2009). This low basal activity of LHB resulted in normal pubertal development for both the affected siblings (male and female). The male patient had normal spermatogenesis even with low circulating LH while the female patient presented with secondary amenorrhea and infertility, indicating that low LH levels are insufficient to maintain ovarian function. The fifth report mentioned a compound heterozygous mutation in

LHB alleles in two siblings (female and male) with a deletion (Leu10-Leu13del) and a splice site mutation at the first nucleotide of intron 2 (IVS2+1 G→T), disrupting the synthesis and thus secretion of LHB (Basciani et al., 2012). In all the above-mentioned cases, the male patients had normal sexual differentiation during birth, demonstrating the importance of hCG for the first phase of sexual differentiation even in the absence of a functional LH. However, a functional LH is necessary for puberty and development of secondary sexual characteristics and mutations affecting LH function alter the normal sexual development as well as fertility, except in the case of (His10-Ile12del) where a low LH activity could maintain spermatogenesis and normal pubertal development in the male patient. In all the female patients with *LHB* mutations, pubertal development was normal indicating the production of LH independent androgen production by theca cells is sufficient for conversion to estrogens by the granulosa cells and hence normal pubertal development was achieved. However, ovulation is hindered in mutated LH leading to infertility in the affected females.

Table 1. Reported *LHB* mutations in patients. '++' indicates normal expression, '+' indicates reduced expression and '-' indicates no expression.

LH beta mutations	**Phenotype**	**Mutation**	**Expression (mRNA/ protein)**	**Dimerization**	**Reference**
Homozygous Gln54Arg	Hypogonadism, infertility	Missense	++	Yes	(Axelrod et al., 1979; Weiss et al., 1992)
Homozygous Gly36Asp	Hypogonadism, infertility	Missense	++	-	(Valdes-Socin et al., 2004)
Homozygous IVS2+1G>C	Hypogonadism and infertility in both sexes, female with normal puberty and secondary amenorrhea	Splice-site	+	-	(Lofrano-Porto et al., 2007)
Homozygous His10_Ile12del	Male: hypogonadism with preserved spermatogenesis; Female: normal puberty, secondary amenorrhea, infertility	Deletion	+	Yes	(Achard et al., 2009)
Compound heterozygous IVS2+1G>T and Leu10_Leu13del	Male:Hypogonadism and infertility; Female: Normal puberty and oligomenorrhea	Splice-site Deletion	+ +	- Unlikely	(Basciani et al., 2012)

Similarly, only few cases of *FSH beta* inactivating mutations have been reported (Table 2), where the female patients usually presented with primary amenorrhea, with elevated LH, low FSH and estrogen levels, with scant breast development (Clark et al., 2003; Kottler et al., 2010; Layman et al., 1997; Layman et al., 2002; Lindstedt et al., 1998; Matthews et al., 1993; Phillip et al., 1998). The first report of a woman with a frameshift mutation caused by the deletion of two nucleotides (TG) in the codon 61 of *FSHB* (Val61X) resulted in alteration of codons 61-86 followed by a premature stop codon. The resulting FSHB (Val61X) was shorter than WT (due to deletion of amino acids 88 -111) and thus lacked regions responsible for dimerization with CGA (Matthews et al., 1993). The patient presented with primary amenorrhea with undetectable FSH and high LH at the age of 27 and pregnancy was achieved only with exogenous FSH administration. A similar homozygous frameshift mutation (Val61X) was later found in a male patient who presented with low FSH and high LH, azoospermia and low testosterone indicating defects in androgen biosynthesis (Phillip et al., 1998). The likely effect of the Val61X mutation, as reported in the previous case, is in truncated biosynthesis of FSHB that is unable to dimerize with CGA and stimulate FSHR in Sertoli cells. Later on, a compound heterozygous mutation in *FSHB* was reported in a female patient in which one allele of *FSHB* coded for a Cys51Gly substitution while the other had the same frameshift mutation, Val61X (Layman et al., 1997). The Cys51Gly substitution altered the cysteine residue known to be involved in the formation of cystine knots, thereby affecting the dimerization with CGA. The secretion of both Cys51Gly and Val61X was affected drastically, as measured *in vitro* using Chinese hamster ovary cells. The female patient had similar hormonal profile as with previous patients having inactivating *FSHB* mutations, with low FSH, high LH and low estrogens.

Subsequently, a male patient encoding a Cys82Arg substitution in FSHB was reported (Lindstedt et al., 1998). The patient underwent normal puberty and virilization. Moreover, libido and sexual potency was also normal but was infertile since he presented with azoospermia. The hormonal profile showed undetectable FSH, with high LH and normal testosterone. The Cys82Arg substitution affected the cysteine residue responsible for disulphide bond formation in FSHB, which is essential for its tertiary structure and subsequent dimerization with CGA, thus affecting the FSH mediated Sertoli cell function.

In addition, FSHB mutations causing Tyr76X frameshift mutation has been reported in two females and one male (Berger et al., 2005; Layman et al., 2002; Lofrano-Porto et al., 2008) while another frameshift mutation (Ala79X) has been reported in a female patient with primary amenorrhea (Kottler et al., 2010). The phenotype of the patients and functional characterization of the mutations has been summarized in Table 2. The common theme with *FSHB* mutations indicate the importance of FSH in Sertoli cell function for spermatogenesis in males while FSH function is crucial for follicular development and production of estrogens in females.

Table 2. Reported patient mutations in *FSHB*. Normal expression (either at mRNA or protein level) is indicated by '++' while reduced expression is indicated by '+' and no expression is indicated by '-'.

FSH beta mutations	**Phenotype**	**Mutation**	**Expression (mRNA/ protein)**	**Dimerization**	**Reference**
Homozygous Val61X	Female: primary amenorrhea and infertility; Male: azoospermia and hypogonadism	Frameshift	+	Unlikely	(Matthews et al., 1993; Phillip et al., 1998)
Compound heterozygous Cys51Gly and Val61X	Primary amenorrhea and infertility	Missense and frameshift	-	Unlikely	(Layman et al., 1997)
Homozygous Cys82Arg	Azoospermia	Missense	+	Unlikely	(Lindstedt et al., 1998)
Homozygous Tyr76X	Female: primary amenorrhea and infertility; Male: normal puberty and azoospermia	Frameshift	+	Unlikely	(Berger et al., 2005; Layman et al., 2002; Lofrano-Porto et al., 2008)
Homozygous Ala79X	Primary amenorrhea, infertility	Frameshift	+	Unlikely	(Kottler et al., 2010)

All the patient mutations that were found for the gonadotropins, or other protein coding genes, in general are modeled in expression vectors in order to study their functional effects and the following section describes the current strategies for creation of mutations in expression vectors.

2.4 Mutagenesis strategies

As mentioned above, the analysis of key residues involved in signal transduction was deciphered by modeling either patient mutations or artificially induced mutations for structural and functional analysis. In most cases, the first step is the creation of an expression vector containing the DNA sequence with the desired mutation. Multiple expression vectors ranging from small plasmids to as large as bacterial artificial chromosomes (BACs) have been used for carrying the mutated DNA sequence of the gene of interest, depending upon the target model cell or organism for studying its effect. Usually there are three ways in which the effect of the mutation is studied. The first is by purifying the mutated protein for structural or *in vitro* reconstitution experiments. The second is by transfecting the expression vectors to assess any alterations in the signaling or physiology in cells. And finally the effect of a mutation can be studied by creating animal models in which the WT allele is usually replaced with the mutant allele. Since the creation of mutations or desired DNA sequences in expression or targeting vectors is almost always one of the very first steps for any molecular analysis, a multitude of methods have been created and commercialized for generation of mutations and cloning of DNA sequences. Although recent on-going efforts for mutagenesis are targeted towards genetic alterations at the genome level and in making the technology accessible and efficient (Ma et al., 2015; Wright et al., 2016), somehow the efforts for modifying small expression vectors such as plasmids have taken a backseat.

The following sections elaborate on two key mutagenesis strategies with different underlying principles. The most widely used methods for site-directed mutagenesis has relied on the application of polymerase chain reaction (PCR) for generation of DNA strands using mutagenesis primers, such as overlap extension PCR, Megaprimer PCR and Inverse PCR (Barik, 1996; Ho et al., 1989; Ling et al., 1997; Tseng et al., 2008). The mutagenesis primers usually contain the desired mutation (in case of additions or substitutions) or are designed to delete intervening sequences, in case of deletions. The PCR products are usually achieved in single or multiple reactions, depending upon size and number of desired mutations. The PCR products containing the mutation are in most cases subjected to phosphorylation and subsequent ligation steps for their circularization, with varying efficiencies. Recently, a commercial site-directed

mutagenesis method called QuikChange Site-directed mutagenesis (Agilent) has been developed for transformation of PCR products in bacteria for nick repair using endogenous bacterial DNA repair, that requires very few steps but can become quite expensive for multiple mutagenesis reactions. In addition, as will be discussed later, the dependence on bacterial endogenous machinery for nick repair in circular PCR products is not a very efficient process which usually results in very few bacterial colonies containing the desired mutation.

Since the length of DNA that can be amplified by PCR is limited (20-25 Kb with the most recent high fidelity polymerases), most methods for modifying larger pieces of DNA relied on the process of homologous recombination for DNA modifications, a method now called as *recombineering* (recombination-mediated genetic engineering). Homologous recombination (HR) is a natural process used by cells mainly to repair DNA lesions. HR is a multi-step process where the 3' end of one DNA strand invades a homologous DNA double helix and anneals to its complimentary DNA strand thus causing its 3' extension using native polymerases. HR has been used in yeast to directly delete genes by the transformation of PCR fragments containing homologous sequences using the endogenous recombination mechanism (Baudin et al., 1993). Such a transformation of linear PCR fragments in bacteria would immediately result in degradation of linear PCR products by the bacterial exonuclease RecBCD. The DNA modifications in yeast were then replaced with *in vivo* recombineering in bacteria because of the low yields of DNA and inherent genetic instability in yeast (Vrančić et al., 2008).

Recombineering relies on the application of recombination machinery from either of the bacteriophages, λ-phage encoding for Redα/Redβ proteins or the Rac prophage coding the RecE/RecT proteins (Figure 3). The bacteriophage recombination machinery is usually expressed in bacteria using an expression vector and the resulting bacteria are called recombineering bacteria. DNA fragments containing homologous sequences are then transformed in recombineering bacteria, where the bacteriophage recombination enzymes recombine the exogenous DNA fragments. Redα or RecE that are 5'-3' exonucleases bind to double stranded DNA breaks and help in generation of 3' single stranded overhangs (Cassuto et al., 1971; Little, 1967). These 3' overhangs are then bound by Redβ or RecT proteins, which are single strand binding proteins and

thereafter helps in strand invasion at a homologous DNA sequence (Karakousis et al., 1998; Kmiec et al., 1981). In addition, the Gam protein, also encoded by λ-phage, inhibits the exonuclease, RecBCD, present in *E. coli* to prevent the degradation of exogenous DNA strands (Karu et al., 1975; Murphy, 1991).

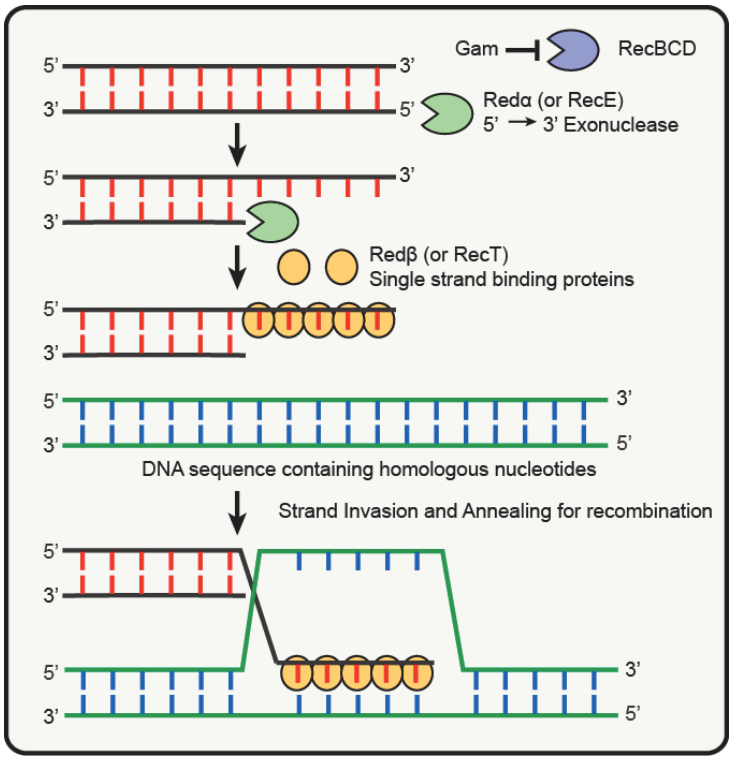

Figure 3. Principle of Recombineering (adapted from GeneBridges Manual for BAC modification kit #K0001). Double stranded DNA breaks are recognized by the exonuclease Redα (or RecE) for the generation of 3' overhangs that are subsequently protected from degradation by single strand binding protein, Redβ (or RecT). The 3' overhangs are aided by Redβ (or RecT) to find homologous sequences for strand invasion, annealing and subsequent recombination.

Initial studies using recombineering were done for gene replacements in *E. coli* chromosome, using relatively large homologous sequences of around 1 kb (Murphy, 1998; Murphy et al., 2000). Subsequent reports demonstrating the application of recombineering by using only 50-60 base pair (bp) homology further advanced the field, as it was easier to synthesize the homologous sequences in the PCR primers themselves (Muyrers et al., 2001; Zhang et al., 1998; Zhang et al., 2000).

Recombineering has been used in modification of BACs, plasmids, bacterial genomes and in generation of targeting constructs for knockout and knock-in models (Murphy, 1998; Muyrers et al., 1999; Rivero-Muller et al., 2010; Testa et al., 2003). The development of selection/counter-selection methods later-on solved the issue of selection of positive clones during recombineering (Rivero-Muller et al., 2007; Wang et al., 2014; Wang et al., 2009).

All the above-mentioned principles and protocols were developed for recombineering inside bacteria. Lately, the application of purified enzymes for *in vitro* recombination is gaining ground. Some of the popular ones include Gibson assembly, In-Fusion HD cloning, seamless ligation cloning extract (SLICE), sequence and ligation-independent cloning (SLIC), and GeneArt seamless cloning, among others (Li et al., 2007; Zhang et al., 2012). The underlying principle in these methods is *in vitro* recombination to join multiple DNA fragments with short (15-20 bp) homology at their ends. The purified enzymes, as sold by the commercial vendors, are not only expensive but also very few mutagenesis reactions are possible per kit. Moreover, the reactions conditions for enzyme activity are also strictly defined in their respective buffers. In contrast, *in vivo* recombineering simply involves transformation of DNA in bacteria with less stringent reaction conditions, thereby making it more robust, even for first-time users (in teaching courses). Lastly, the application of commercial methods with expensive enzymes is unnecessary for site-directed mutagenesis in plasmid vectors.

The following sections will explain once an expression vector containing the desired mutation has been created, in this case, for glycoprotein hormones that signal via cAMP production, which methods are currently available for detection of cAMP.

2.5 cAMP assays

cAMP as a molecule was first characterized in late 1950s as a molecule found in liver homogenates (Rall et al., 1958; Sutherland et al., 1958). Initial studies of cAMP production relied on determination of total cAMP content in the cells using competition-based assays, such as radioimmunoassay or using enzymatic assays (Butcher et al., 1965; Steiner et al., 1969). Subsequently the development of colorimetric or fluorescent cAMP immunoassays with increased sensitivity were safer to use than radioimmunoassay. A common theme among these assays is the process of lysing the cells to release its cAMP content to the lysis buffer. The cAMP in the lysis buffer is incubated with anti-cAMP antibody and an external cAMP (conjugated or labeled) competes with cAMP in the lysis buffer for antibody binding sites (Figure 4A). The final readout of the competition between labeled cAMP and unlabeled cellular cAMP can be based either on radioactivity, colorimetry, fluorescence or luminescence (Figure 4A).

The radio-labeled assays based on Flashplate technology (PerkinElmer) or scintillation proximity assays (Amersham Biosciences) uses [^{125}I]-labelled cAMP for competition with cAMP in the lysis buffer. Another cAMP assay developed by CisBio relies on time resolved fluorescence resonance energy transfer for cAMP detection (Degorce et al., 2009). Since the readout is ratiometric in this assay, the signal readout is not influenced by external factors such as changes in pH, temperature or presence of additional components in the lysis buffer. Some other assays utilizes enzyme fragment complementation technology by DiscoverX (Bradley et al., 2009) or proximity between labelled beads (ALPHAScreen, PerkinElmer) with differing chemistry to give a luminescent readout. Many of these assays have been miniaturized for high throughput screening (HTS) formats thereby aiding screening efforts for ligand detection (Gabriel et al., 2003; Williams, 2004). Although cAMP could be detected with these methods at very high sensitivity, the process of lysing the cells gives information about cAMP content in the cells at only one time-point. The determination of cAMP production at different time-points thereby requires a different set of samples that causes increased variation. Moreover, visualizing the compartmentalization of cAMP production is also not possible with competition-based assays.

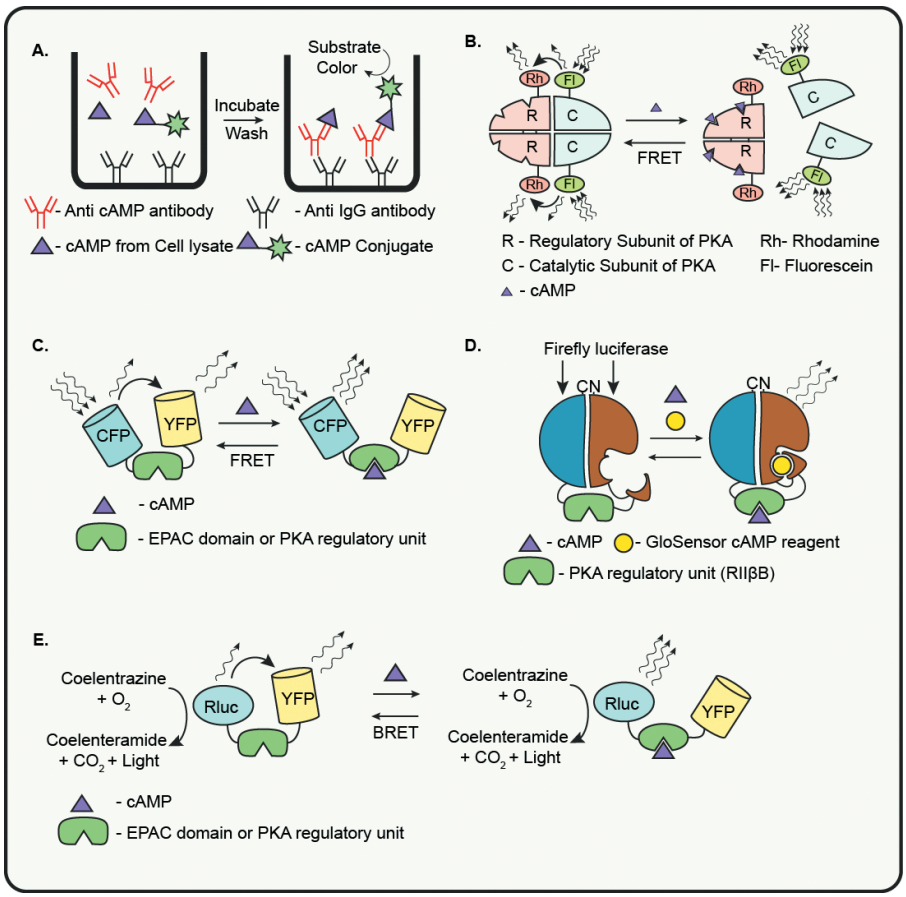

Figure 4. Principle of different cAMP assays [modified from (Trehan et al., 2014)] **A)** Competition-based assays rely on anti-cAMP antibodies for binding either to cAMP produced in the cells or conjugated-cAMP molecules that are externally added for either a colorimetric readout, as shown or a fluorescent/radiometric/luminescent readout. **B)** Design of FlCRhR sensor containing tetrameric PKA holoenzyme, where the regulatory (R) and catalytic (C) subunits are labeled with rhodamine and fluorescein, respectively. In the absence of cAMP, there is fluorescence resonance energy transfer (FRET) while the addition of cAMP leads to loss of FRET via dissociation of catalytic subunits. **C)** Unlike FlCRhR, unimolecular cAMP FRET sensors contain only one cAMP binding domain (from PKA or EPAC), where the cAMP binding leads to a conformational change in the sensor, thereby abolishing FRET between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). **D)** Design of a luminescent cAMP sensor, GloSensor-22F, where cAMP binding to the regulatory subunit of PKA, leads to conformational changes in the sensor, leading to a luminescent readout in the presence of its substrate (GloSensor cAMP reagent). **E)** A BRET cAMP sensor utilizes a chemiluminescent reaction as its donor for resonance energy transfer, unlike FRET where a fluorescent signal is used.

Subsequently, real-time cAMP sensors were developed for determination of cAMP kinetics in live cells. Most real-time cAMP sensors possessed two properties, first their ability to detect cAMP and second a continuous readout mechanism in live cells. The binding of cAMP to the real-time sensors was achieved by using cAMP binding domains from either Epac or PKA with additional domains for a fluorescent or luminescent readout. The fluorescent readout mainly relied on changes in fluorescent resonance energy transfer (FRET) for determination of cAMP variations in the cells. The first generation of real-time sensors, such as FlCRhR utilized purified PKA holoenzyme, where the two regulatory subunits (R_2) of PKA were labelled with rhodamine while the two catalytic subunits (C_2) were labelled with fluorescein (Adams et al., 1991). The labelled PKA holoenzyme (R_2C_2) in the absence of cAMP had FRET among fluorescein and rhodamine, whereas binding of cAMP led to dissociation of the catalytic and regulatory subunits thereby decreasing the FRET signal. Since the FlCRhR required first purification and then labelling with FRET pairs (rhodamine and fluorescein) and eventual microinjection of the sensor in the cells, the whole process was not only laborious but also technically challenging. Thereafter the next generation of sensors based on PKA were genetically encoded that could easily be transfected in the cells using routine DNA transfections (Lissandron et al., 2005; Zaccolo et al., 2000; Zhang et al., 2001).

One of the major advantages with FRET based sensors was not only real-time cAMP determination but also the ability to study localized cAMP signaling in the cells via microscopy. However, the application of the holoenzyme PKA also presented with some difficulties, most glaring of which was the functional activity of PKA itself upon overexpression of the sensor that can alter the signaling and expression of its endogenous downstream targets. Second, multiple transfections were required to transfect the catalytic and regulatory subunits tagged with fluorescent proteins, that were encoded in different plasmids. Moreover, stoichiometry of the subunits expressed by different plasmids also affected the final signal and was difficult to control consistently. In addition, the overexpression of sensors also caused cAMP buffering. This issue was later solved by the development of unimolecular sensors that only required a single transfection and also had modified PKA or Epac domains with no functional activity of their own in some cases (Klarenbeek et al., 2015; Nikolaev et al., 2004; van der Krogt et al., 2008). Moreover, unimolecular FRET sensors had quicker

response times since there was only one subunit for cAMP binding and unlike tetrameric PKA, unimolecular sensors also did not require association/dissociation of subunits to observe rapid changes in cAMP concentrations in the cell. Similarly, unimolecular sensors were also developed in which the final read-out was rather based on bioluminescence resonance energy transfer [BRET] (Jiang et al., 2007; Prinz et al., 2006). In contrast to FRET, the BRET involves the resonance energy transfer from donor luminescence (Renilla luciferase, RLuc) to acceptor fluorescence and thus no stimulation of cells with fluorescent light is needed, thereby eliminating photobleaching effects usually associated with FRET signal (Figure 4). Moreover, FRET signals via microscopy in many cases requires manual selection of the region of interest that can introduce a user bias and at the same time precludes easy processing of multiple samples for HTS applications. Though FRET signals can be read using plate readers in case of multiple samples, however, a luminescent readout is usually better suited for such applications.

Another cAMP sensor that directly provides a luminescent read-out upon cAMP binding without any ratiometric calculations is GloSensor-22F (and previous generation Glosensor-20F variant) from Promega (Binkowski et al., 2011). The GloSensor-22F variant has a cAMP binding domain from PKA regulatory subunit (RIIβB) between two fragments of *Photinus pyralis* luciferase, such that a conformational change upon cAMP binding allows a luminescence output in the presence of luciferase substrate, called GloSensor cAMP reagent (Figure 4D). The GloSensor-22F also has high dynamic range for cAMP detection (0.003-100 µM) and is suited for HTS screening applications, reviewed in (Paramonov et al., 2015).

Competition-based cAMP assays have been used by most researchers because of their high sensitivity for detection of cAMP using primary cell cultures from animal models or human samples. However, as stated earlier, cell lysis is a prerequisite for cAMP detection via competition-based assays, therefore cAMP content at only one time-point can be studied with one set of samples. Although, these assays can determine cAMP content at multiple time-points after cell stimulation but a different set of samples are required for each time-point. In order to determine a kinetic readout of cAMP produced in primary cell cultures, even real-time cAMP sensors have limited application since transfection of primary cells is highly inefficient with most methods, except viral

transductions. Moreover, any transfection of a real-time cAMP sensor in primary cells, however efficient, will still lead to a variable expression of the sensor in the cells and will therefore require cloning of primary cells for a stable and consistent sensor expression. But cloning primary cells for any antibiotic resistance gene will most likely be cytotoxic to a majority of the cells and the final cell clone, if made, will no longer be a true representative of the original primary cell culture and will have a limited number of cell divisions henceforth.

3 AIMS

In order to characterize a patient mutation in *LHB*, two methods were needed; first a mutagenesis method for modeling of the mutation in an expression vector and second a cAMP detection method to study the functional effect of *LHB* mutation *in vitro*. Upon assessing the currently available methods for mutagenesis, we realized a scope for improvement in two key areas of mutagenesis. The first was the number of steps it takes to create mutations in plasmid expression vectors. The second was the total cost of mutagenesis that rapidly increases if multiple mutagenesis experiments are done routinely in the laboratory. Since the efficiencies of mutagenesis methods have been improving over the years to the extent that it has reached a saturation with most commercial alternatives, we decided any new method for mutagenesis should be at least as efficient as the currently available methods, if not more. The second aim was the development of a cAMP sensor cell line that can monitor cAMP production upon stimulation by gonadotropin receptors or $G\alpha_S$ mediated GPCR signaling in general.

In short, the current PhD thesis had the following aims:

1. Development of an inexpensive, quick and robust method for the mutagenesis of clinically relevant genes (REPLACR-mutagenesis).
2. Development of an assay to monitor GPCR induced cAMP kinetics in cell cultures (CANDLES Assay).
3. Clinical and molecular characterization of a novel luteinizing hormone beta subunit mutation from a patient, using the REPLACR and CANDLES methods.

4 MATERIALS AND METHODS

This section first describes the development of a site-directed mutagenesis protocol based on *in vivo recombineering*. The method first involved generation of linear PCR products using mutagenesis primers that were transformed in recombineering bacteria, where the ends of the PCR products were circularized and hence the method was named as REPLACR-mutagenesis (Recombineering of Ends of linearized PLAsmids after PCR). The general principle and primer design strategy is mentioned in Figure 5.

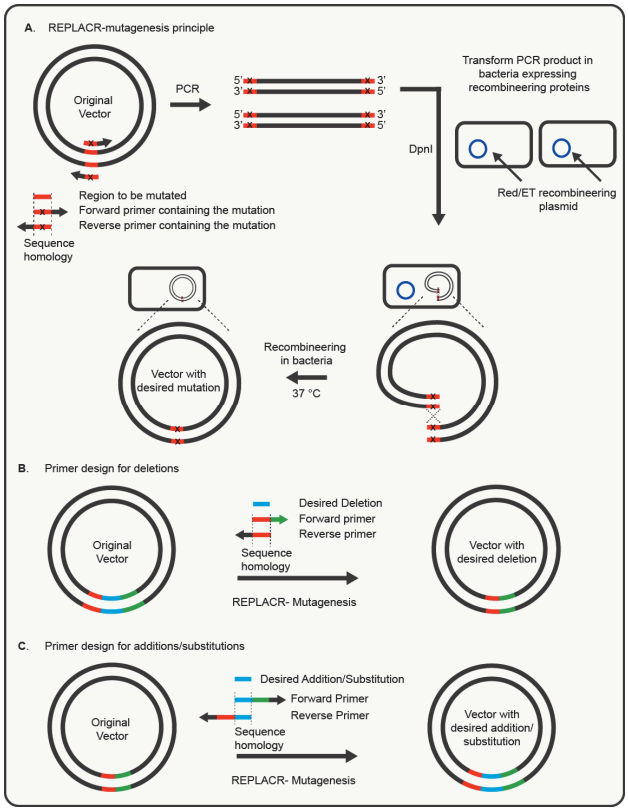

Figure 5. REPLACR-mutagenesis principle [modified from (Trehan et al., 2016)]. **A)** PCR products generated using mutagenesis primers are transformed in recombineering bacteria after *DpnI* digestion, where recombination at the ends of PCR products circularizes the linear PCR products to yield desired plasmids. The screening of resulting bacterial colonies is then carried out by colony PCR and sequencing. **B)** The forward primer for generating deletion contain the vector sequences on both sides of the deletion while the reverse primer contains homologous sequences to the 5'end of forward primer and vector sequences in the opposite direction. **C)** Mutagenesis primers for creating substitutions/additions contain homologous nucleotides (17 bp or more) incorporating the desired substitutions/additions at their 5' end in addition to vector sequences at their 3' end.

4.1 Methodology for REPLACR-mutagenesis development

4.1.1 Enzymes for PCR and mutagenesis

Biotools DNA polymerase was used for routine PCR while KOD-Xtreme hot-start DNA polymerase (Merck Millipore) was used in high-fidelity PCR for mutagenesis. Restriction endonucleases were purchased either from Promega (*ScaI*) or New England Biolabs (NEB; *DpnI* and *MfeI*).

4.1.2 Plasmids

Plasmids carrying cDNA of wild type (WT) human *β2-adrenergic receptor* (*β2AR*), *FSHR*, *LHCGR* and *Cryptochrome Circadian Clock 2* (*CRY2*) were used as templates for creating mutations (Jean-Alphonse et al., 2014; Kennedy et al., 2010; Rivero-Muller et al., 2010). The Red/ET recombineering plasmid (*pSC101BADgbaRecA*[tet]), henceforward referred to as Red/ET plasmid, was purchased from GeneBridges. A 24 kb *Wnt1* targeting vector (KOMP repository) was used as a proof-of-concept for editing larger complex plasmids using REPLACR-mutagenesis. Nuclear localization signal (NLS) from *pCRY2FL(deltaNLS)-mCherryN1* plasmid (Addgene, # 26871) was used for sub-cloning upon recombineering (Kennedy et al., 2010).

4.1.3 Electrocompetent recombineering bacteria

Electrocompetent bacteria were prepared according to GeneBridges manual. Briefly, *E. coli* (HS996) transformed with Red/ET plasmid were cultured overnight in 5 mL Luria-Bertani (LB) medium containing tetracycline (3 µg/mL) at 30 °C. The overnight bacterial culture was then transferred to 250 mL LB medium containing the same antibiotic (tetracycline) and was cultured for an additional 3h at 30 °C. *L*-arabinose (0.35 %) was then added to the culture and temperature was raised to 37 °C for induction of phase recombinases (RecA and Red γ, β and α) from the Red/ET plasmid for 1h. The bacterial culture was then centrifuged at 8000 *X g* for 15min at 4 °C and subsequently resuspended in distilled water (4 °C). The bacteria were again centrifuged and similarly resuspended in water (ice-cold), before a final wash with 10 % glycerol. After centrifugation (8000 *X g*, 15 min at 4 °C), the bacteria were suspended in 1-2 mL of 10 % glycerol and immediately snap frozen in liquid nitrogen (as 50 µl aliquots in microcentrifuge tubes) and subsequently stored at -80 °C for further use.

4.1.4 REPLACR-mutagenesis

REPLACR-mutagenesis involves the application of mutagenesis primers to generate linearized PCR products from plasmid templates and subsequent transformation of these PCR products in recombineering bacteria, as detailed in subsequent sections (Figure 5).

4.1.4.1 PCR for REPLACR-mutagenesis

PCR primer design for generating mutations (substitutions, additions or deletions) in plasmids has been outlined in Figure 5. The primer sequences for creating mutations in plasmids encoding cDNAs of *FSHR*, *β2AR*, *LHCGR* and *CRY2* are mentioned in I: Supplementary Table S5. The PCR conditions using a high-fidelity DNA polymerase (KOD-Xtreme) are stated in I: Supplementary Tables S7-S12. PCR products were ethanol-precipitated before *DpnI* digestion for removing template plasmid. The *DpnI* digested PCR products were ethanol-precipitated before transformation in recombineering bacteria. The PCR products (1-2 µl) could, however, be directly used for *DpnI* digestion without ethanol precipitation and the digested products can then be transformed directly into recombineering bacteria for faster processing.

4.1.4.2 Recombineering

Previously frozen, electrocompetent bacteria (50 µl) for recombineering were thawed on ice and 100 ng of PCR products were added. Electroporation was carried out at 1350 V (10 µF, 600 ohm) in a 1 mm-cuvette using an electroporator (Eppendorf 2510). LB medium (1 mL) was added to the bacteria followed by 1-2 h incubation in a shaker at 37 °C. Subsequently, bacteria were plated on LB-agar plates supplemented with necessary antibiotics. The bacterial colonies were first screened by colony PCR on agarose gels and subsequently verified by DNA sequencing at Turku Centre for Biotechnology, Finland. Primers used for sequencing are listed in I: Supplementary Table S6.

4.1.5 Efficiency of REPLACR-mutagenesis at varying homology lengths

Mutagenesis primers were designed to target a *ScaI* restriction site (AGTACT) in WT *LHCGR* plasmid (Rivero-Muller et al., 2010), by the addition of two nucleotides (AT) in the center of restriction site, such that the resulting sequence (AGTATACT) could not be digested by *ScaI*. The mutagenesis primers (forward and reverse) had varying homologous nucleotides, starting from 2 bp to 23 bp, with the homologous sequences in the primers being highlighted in I: Supplementary Table S1. The PCR products using mutagenesis primers were processed as per REPLACR-mutagenesis protocol (Figure 5). The bacterial colonies thus obtained were screened by colony PCR (PCR conditions in I: Supplementary Table S3). The forward and reverse primer sequences for colony PCR were AGGGTCCTGATTTGGCTGAT and TGGCATGTCTTAATCGCAGC, respectively.

4.1.6 GeneArt seamless cloning and Gibson Assembly

Two similar PCR products, obtained with mutagenesis primers having a 14 and 17 bp homologous nucleotides were used to obtain mutated *LHCGR* plasmids with commercial recombination-based mutagenesis and cloning kits (GeneArt and Gibson). PCR products (100 ng) were used for mutagenesis following the manufacturer's instructions. The bacterial colonies obtained were similarly screened by colony PCR (I: Supplementary Table S3) and subsequent *ScaI* digestion (I: Supplementary Figure S3 and Table S4).

4.2 Methodology for CANDLES Assay development

The following section describes the cell culture conditions, special assay medium formulations and how sensor cell lines were generated during the development of CANDLES assay (protocol summarized in Figure 6).

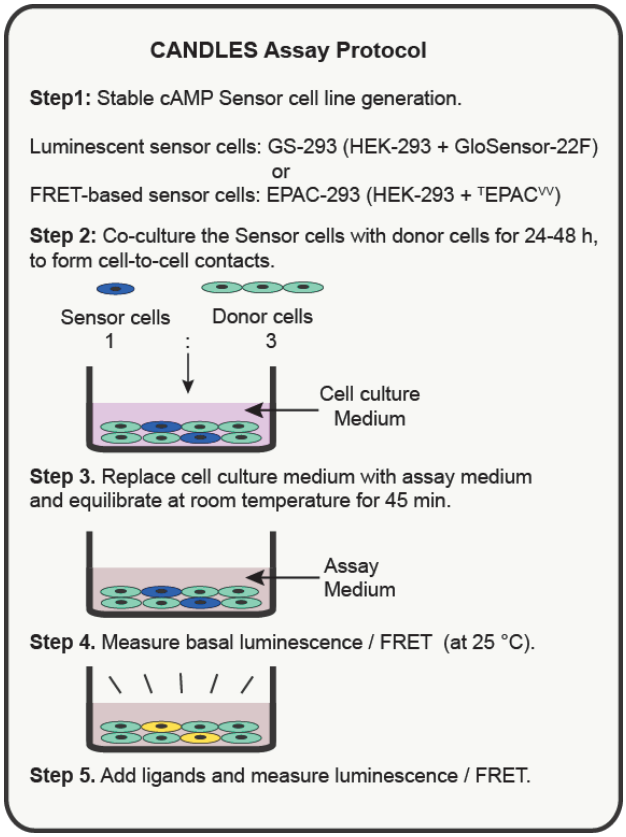

Figure 6. Quick protocol for CANDLES assay [modified from (Trehan et al., 2014)]. The first step involves the generation of a cAMP sensor cell line that must be co-cultured with test cells. Prior to the experiment, the cells must be equilibrated in assay medium for luminescence or FRET readout.

4.2.1 Cell culture

KK-1 cell line was previously developed in our laboratory, (Kananen et al., 1995) while human embryonic kidney-293 (HEK-293) cell line was obtained from American Type Culture Collection. HEK-293 cells stably expressing FSHR were generated (referred as FSHR-293). Cell lines were routinely cultured in humidified atmosphere at 37 °C with 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium (Gibco) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin and 10% fetal calf serum (PromoCell), henceforth called as DMEM complete medium. RPMI 1640 or McCoy's 5A media (Gibco) were also used in place of DMEM/F12 for cell culture in some cases as referred in the text.

4.2.2 Assay medium for measuring luminescence

The assay medium was prepared with DMEM/F12 (without antibiotics and fetal calf serum), CO_2-independent medium (Invitrogen), 3-Isobutyl-1-methylxanthine (IBMX; Sigma), bovine serum albumin (BSA; Gibco) and GloSensor cAMP reagent (Promega). The assay medium contained an equal ratio (1:1) of DMEM/F12 and CO_2-independent medium, along with 0.1% BSA, 100 µM IBMX and 2% GloSensor cAMP reagent. Prior to all luminescence measurements, a freshly prepared assay medium replaced the DMEM complete medium.

4.2.3 Luminescent cAMP-sensor cell line (GS-293) generation

HEK-293 cells were stably transfected with pGloSensor-22F cAMP plasmid (Promega) (Binkowski et al., 2011) using hygromycin (200 µg/mL) in DMEM complete media. The cells were subsequently seeded in 96-well plates and grown until confluence. The cell culture medium was replaced with assay medium (without IBMX) and cells were selected for maximum luminescent signal upon stimulation with 10 µM forskolin (LC laboratories, USA). The selected clones, those having maximal expression of the sensor, were again seeded in 96-well plates and a similar process of selection was repeated several times. The clones with maximal signal were finally selected for three properties 1) stable and low basal luminescence, 2) highest luminescence after 10 µM forskolin addition and, 3) stable luminescence with multiple passages. Finally, the best clone was used for all subsequent experiments and was called as HEK-293-GloSensor (GS-293).

4.2.4 FRET-based cAMP sensor cell line (EPAC-293) generation

HEK-293 cells were stably transfected with a FRET-based cAMP sensor ($^{T}EPAC^{VV}$), a kind gift by Prof. Kees Jalink (Klarenbeek et al., 2011), in G418 (400 µg/mL) selection medium. BD FACSAria III cell sorter was used to sort cells for the highest expression of sensor. The sorted cells were again selected similarly and the resulting cell line was called as EPAC-293.

4.2.5 CANDLES Assay methodology

Test cells (either primary cultures or established cell lines expressing the receptor to be studied) were co-cultured with sensor cells (GS-293) for 48 h in DMEM complete medium. After 48 h, cell culture medium was replaced with assay medium. Cells were kept in dark (wrapped in aluminum foil) for 45 min at room temperature. Cells were then transferred to either of the two plate readers (Victor or Ensight, Perkin-Elmer-Wallac) and kept at 25 °C for the next 15 min to equilibrate. Basal luminescence measurements were carried out for 5-10 min. Cells were then stimulated with respective ligands for the receptor under study and luminescence was measured using kinetic reads every few minutes for 1-2 h (Figure 6).

4.2.6 FRET methodology and analysis with EPAC-293 cells

A 48 h co-culture of FSHR-293 cells and EPAC-293 cells in 24-well plate was used for FRET analysis. For FRET, DMEM complete medium was replaced with DMEM/F12 medium (without phenol red) containing 0.1 % BSA and 100 µM IBMX. Cells were then kept at room temperature for 15 min, before being transferred to Synergy H1 plate reader (BioTek) at a constant temperature of 25 °C. The excitation wavelength for cells was 430/18 nm while emission was recorded at 480/18 nm and 528/18 nm. The ratio of fluorescent intensities at 480 nm and 528 nm was calculated to represent the FRET ratio. The FRET ratio at the beginning of the experiment was fixed to 1.

4.2.7 Analysis of gap junctions in cAMP transfer

A gap junction inhibitor, carbenoxolone disodium salt (CBX; Sigma), was used at different concentrations to study its effect on cAMP transfer in co-cultures of FSHR-293 and GS-293 cells. In addition, the effect of a 2-h CBX treatment on cell viability was also assessed by CellTiter AQueous non-radioactive cell proliferation assay

(Promega). A *C*-terminally mEmerald-tagged, human *Connexin-32* (*Cx32*) plasmid (Fort et al., 2011) was used to determine the effect of overexpression of Connexins in cAMP detection in co-cultures of GS-293 cells and FSHR-293 cells.

4.2.8 Colorimetric cAMP enzyme-linked immunosorbent assay (ELISA)

cAMP content in co-cultures of FSHR-293 and GS-293 was determined by a colorimetric cAMP ELISA kit (Cell Biolabs) using the manufacturer's protocol. Co-cultures (FSHR-293 and GS-293) were incubated with increasing concentrations of CBX (25, 50, 75 and 100 µM) in assay medium without cAMP GloSensor reagent for 1h. Cells were then stimulated with rFSH (200 mIU/mL) for 20 min before being lysed for analyzing cAMP content using ELISA kit. cAMP content was normalized to total protein content (using Bicinchoninic Acid protein assay kit, Thermo Scientific).

4.2.9 Half-maximal effective concentration (EC_{50}) comparison between CANDLES and commercial ELISA kit

EC_{50} values using the same colorimetric ELISA kit (Cell Biolabs) were calculated using FSHR-293 cells stimulated at increasing concentrations of rFSH (200 mIU/mL) for 20 min, with subsequent cell lysis to calculate cAMP production. However, for EC_{50} calculation using the CANDLES protocol, co-cultures of FSHR-293 and GS-293 cells were stimulated with increasing concentrations of rFSH, and luminescence values at 20 min after stimulation were used. In both cases, CANDLES and ELISA, normalization of cAMP values was done to percent maximal responses and subsequent curve fitting using a four-parameter logistic curve by PRISM 6 software to calculate EC_{50} values.

4.2.10 Primary cell culture of murine granulosa cells

Ovaries were dissected out from C57BL/6 female mice (25 day-old) and granulosa cells were extracted by follicular puncture method, as previously published (Burkart et al., 2006). Briefly, DMEM/F12 medium (without phenol red) containing 50 IU/mL penicillin and 50 µg/mL streptomycin was used to collect ovaries. Ovaries were then transferred for 30 min to DMEM/F12 medium containing 0.5M Sucrose and 10 mM EGTA (Sigma), followed by washing in fresh DMEM/F12 medium. Ovaries were then punctured with a 25G needle in 200 µl of DMEM/F12. For removing the cell aggregates, the samples were then filtered through a 100 µm cell strainer (BD

Biosciences) and the filtered samples were collected by centrifugation (100 *X g*, 10 min). Finally, the cells were resuspended in DMEM/F12 medium, containing 50 IU/mL penicillin and 50 µg/mL streptomycin, 10% fetal bovine serum-charcoal stripped (Sigma) and 1 X insulin, transferrin, selenium solution (ITS-G; Gibco) and subsequently seeded in appropriate plates for the CANDLES assay. Recombinant human luteinizing hormone (rLH; Organon) was used for stimulating granulosa cells.

4.2.11 Primary cell culture of rat cortical neurons

The isolation of primary cortical neurons from newborn Sprague-Dawley rats has been previously described (Bjorkblom et al., 2005). Briefly, poly-*D*-Lysine (50 µM) (Sigma) coated 24-well plates were used to culture the dissociated neurons (700,000 cells/cm^2) in Minimal Essential Medium (MEM), supplemented with 2 mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin, 33 mM *D*-glucose and 10 % bovine calf serum (HyClone). On the following day, the medium was supplemented with 2.5 µM cytosine β-*D*-arabinofuranoside (Sigma) to prevent the proliferation of non-neuronal cells. Cells were cultured at 37 °C in humidified chamber with 5 % CO_2, and then used for CANDLES assay after being in culture for 2-4 days *in vitro*. For neuronal stimulation, isoproterenol, epinephrine, glutamate or salbutamol (all from Sigma) were used.

4.3 Methodology for characterization of LH beta mutation

4.3.1 Mutation detection

Hormonal and genetic analysis among members of the kindred was done with informed consent. Genomic DNA was extracted from blood and a 1082 bp region coding for *LHB* was PCR amplified and sequenced in both directions from the patient and his first degree relatives. The *LHB* mutation, found in the patient, was also screened in a total of 31 members of the family.

4.3.2 Fluorescently-tagged cDNA expression vectors

The plasmid expression vector, called *AmCyan-P2A-mCherry*, was first generated in which the two fluorescent proteins, mCherry and AmCyan (with a nuclear localization signal, NLS) were separated by a 22 amino acid-long peptide, P2A. The expression of this vector yields a multicistronic mRNA containing sequences for translation of Amcyan, P2A and mCherry, such that Amcyan and P2A are first translated and a codon skipping step by the ribosome at the C-terminus of P2A yields a separate mCherry protein (Donnelly et al., 2001; Kim et al., 2011). Thus two separate protein moieties are achieved with very similar expression levels; mCherry that remains in the cytoplasm and AmCyan which is transported to the nucleus (III: Supplementary Figure S1). The full sequence of *AmCyan-P2A-mCherry* plasmid can be found in III: Supplementary Figure S2 and the plasmid is available via Addgene repository (Plasmid # 45350).

Subsequently, 500 bp gBlocks (IDT) coding for WT *LHB* and mutant *LHB* (Lys20del) were ordered and cloned as mCherry fusion proteins in *AmCyan-P2A-mCherry* vector using Gibson assembly (NEB). These constructs (*AmCyan-P2A-LHB-mCherry* and *AmCyan-P2A-LHB_Lys20del-mCherry*) thereby enables the LHB biosynthesis and transport to be followed via mCherry, whereas the overall expression of LHB can be followed via the expression of AmCyan that is produced by the same mRNA and polypeptide before the two proteins separated by P2A. Similarly, WT *TSH beta* (*TSHB*), *FSH beta* (*FSHB*) and *hCG beta* (*hCGB*) gBlocks were cloned as mCherry fusion proteins. The details of the *common glycoprotein alpha subunit* (*CGA*) plasmid have been previously published (Ahtiainen et al., 2010). The signal peptide of LHB was replaced with prolactin (PRL) signal peptide by using a gBlock coding for *PRL-LHB*

and similarly cloning it in the *AmCyan-P2A-mCherry* vector as a mCherry fusion protein. All cloned genes and their products are listed in III: Supplementary Figures S3 and S4, respectively.

4.3.3 Mutagenesis of beta subunits (LHB, FSHB, TSHB, hCGB)

REPLACR-mutagenesis was used to generate mutants using *Amcyan-P2A-mCherry* vector templates expressing WT *LHB*, *TSHB*, *FSHB* and *hCGB*. Mutagenesis primers are specified in III: Supplementary Tables S1 and S2. All the mutations were verified by sequencing.

4.3.4 Signal transduction (cAMP signaling)

HEK-293 cells were transiently transfected with plasmids coding for either beta subunits (*LHB*, *FSHB*, *TSHB* or *hCGB)* alone or co-transfected with the *CGA*. After 36h, HEK-293 cells were incubated in DMEM/F12, supplemented with 5% charcoal treated serum, for an additional 8 h. Subsequently, the medium was collected and frozen at -20 °C until further use. GS-293 sensor cells (developed for CANDLES assay) expressing either FSHR, LHCGR or TSHR were used to study the cAMP signaling upon receptor activation and are hereafter referred to as GS-FSHR, GS-LHCGR and GS-TSHR sensor cells, respectively. The medium collected before, was used for receptor stimulation in the sensor cells and cAMP production was monitored as a luminescent readout (relative luminescent units, RLU), which was expressed as area under curve (AUC) values. All experiments were done in triplicates and have been independently repeated at least thrice.

4.3.5 Confocal Microscopy

Zeiss LSM 510 and LSM 780 confocal microscopes were used for visualization of beta subunits (LHB, FSHB, TSHB and hCGB; WT and mutants) in HeLa cells. The laser lines at 543 nm and 458 nm were used for excitation of mCherry-tagged glycoprotein beta subunits (Emission: 578-696 nm) and AmCyan (Emission: 470-579 nm), respectively. Confocal stacks were exported as 3D volume renders using Imaris software. The contrast for WT FSHB, LHB and hCGB images was increased uniformly for better visualization of sparingly present beta subunits in the form of secretion

vesicles that are actively secreting their hormone subunits out of the cells (Ruddon et al., 1981).

4.3.6 Fluorescence Recovery After Photobleaching (FRAP)

Before bleaching a particular cytoplasmic region with 405 nm laser, a basal image of the cell was taken using 561 nm laser excitation (595/50 nm emission). Cells were bleached for 1-5 s and subsequent fluorescent recovery was monitored for 120 s (450 total scans; 512 X 512 pixels). The ratio of fluorescence at the bleached region to that at a reference region was used to calculate the normalized fluorescence, with minimum fluorescence set to zero.

4.3.7 Statistical Analysis

One-way ANOVA using GraphPad Prism 6 software was used to calculate p-values.

4.3.8 3D modelling

The crystal structure of hCG (1HRP.pdb) was used as a template for modeling the homologous LHB subunit by YASARA 11.11.2. package. Non-conserved residues between hCGB and LHB were replaced during the modelling.

4.3.9 Dimeric LHB/CGA and monomeric LHB measurements

For LHB detection, DELFIA hLH Spec kit (PerkinElmer) was used while for the detection of dimeric LHB/CGA, DELFIA hLH kit with secondary antibody from hFSH kit (for CGA recognition) was used.

5 RESULTS

5.1 Development of REPLACR-mutagenesis

5.1.1 Determination of an optimal homology length for REPLACR-mutagenesis

REPLACR-mutagenesis was designed on the assumption that it might be possible for the viral recombination machinery (RecA and Red α, β and γ) expressed in bacteria to circularize the ends of PCR products if both the ends possess enough homologous nucleotides. To test this assumption, a WT *LHCGR* plasmid (Rivero-Muller et al., 2010) was amplified using mutagenesis primers (I: Supplementary Table S1) such that the linearized PCR products had a range of homologous sequences at their ends (2 bp to 23 bp). The mutagenesis primers introduced a two base pair (AT) addition in the center of a *ScaI* restriction site (AGTACT). PCR products were transformed in recombineering bacteria and the bacterial colonies were screened by colony PCR and subsequent *ScaI* digestion. The mutated plasmid yielded 366 bp products which were not digested by *ScaI* while the WT background template yielded 364 bp products, which were digested by *ScaI* in 174 bp and 190 bp products (seen as a single band due to their similar size; I: Supplementary Figure S1).

The number of mutated plasmids over total number of bacterial colonies screened was used to calculate the efficiencies for different homology lengths (Figure 7A and I: Supplementary Table S2). With an increasing homology length at PCR ends from 5 bp up to 17 bp, there was an increase in efficiency of REPLACR-mutagenesis, reaching a maximum efficiency of 84 % (Figure 7A). However, a 2 bp homology yielded no positive mutant plasmids, probably because 2 base pairs are insufficient for proper recombination. In addition, primers with 20 bp and 23 bp homology resulted in incorrect PCR products possibly because the primers tested were only 23 bp in length, which favored primer-dimer formation over primer-template binding.

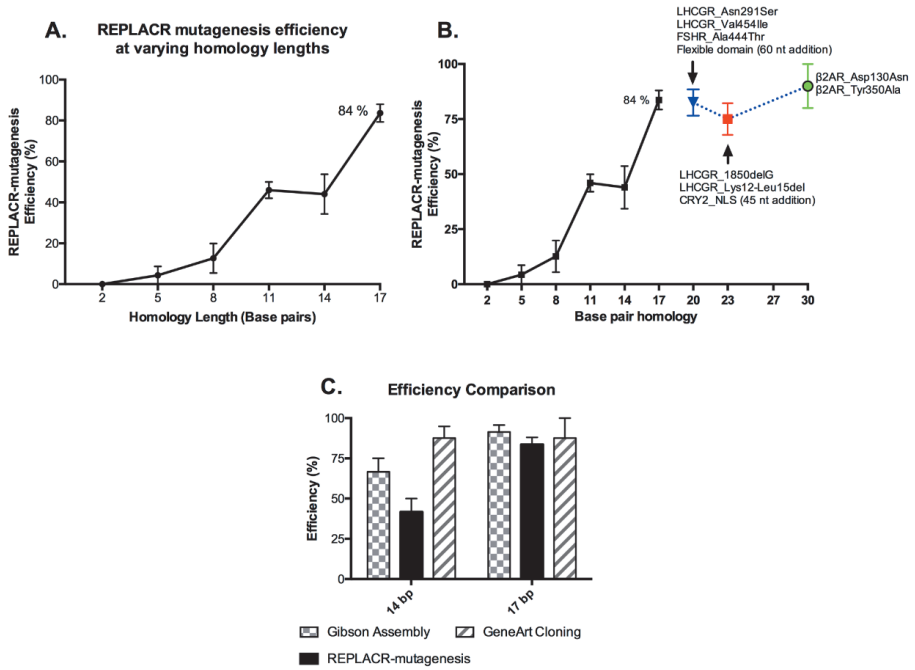

Figure 7. REPLACR-mutagenesis efficiency [modified from (Trehan et al., 2016)]
(A) An increase in REPLACR-mutagenesis efficiency can be seen with increasing homology at the ends of PCR products. (B) The efficiency of REPLACR-mutagenesis does not increase significantly with increasing homology beyond 17 bp. (C) The efficiency of REPLACR-mutagenesis is lowest with 14 bp homologous nucleotides while a 17 bp homology at PCR termini gave similar efficiencies for all the methods.

The homology length can be increased beyond 17 bp by using primers with extended 5' end containing longer homology regions and a 3' tail containing nucleotides (22-24) for primer-template binding. However, the mutants generated using more than 17 bp homology (20, 23 and 30 bp) resulted in no substantial increase in associated efficiencies (Figure 7B). As will be mentioned later in the text, five different mutations in the *LHB* gene were generated by REPLACR-mutagenesis with a 19 bp homology (III: Supplementary Table S1). The combined efficiency after screening the colonies was 88 % (data not shown), which is not statistically different from 84 % efficiency achieved with 17 bp homology. In conclusion, primers designed to yield 17 bp homology at PCR ends and containing an additional 3' sequence for efficient primer-template binding yielded best results for REPLACR-mutagenesis.

Finally, *E. coli* DH-10β (non-recombineering bacteria) transformed with PCR products with 11, 14 and 17 bp homology at their termini resulted in no colonies because of the absence of viral recombination machinery for circularizing linear PCR products.

5.1.2 REPLACR-mutagenesis efficiency comparison

Two commercial kits (GeneArt seamless cloning and Gibson assembly) were used for recombination using the same PCR products with 14 and 17 bp homology, since they yielded highest efficiencies with REPLACR-mutagenesis (Figure 7A). The bacterial colonies, thus obtained were similarly screened with colony PCR and *ScaI* digestion (I: Supplementary Figure S3) and the associated efficiencies were compared with REPLACR-mutagenesis (Figure 7C). Even though, for PCR products with 14 bp homology at their ends, REPLACR-mutagenesis was least efficient, a recommended 17 bp homology yielded very similar efficiencies for all methods (Figure 7C). A further comparison of the number of colonies obtained after recombination showed GeneArt seamless cloning to yield very few bacterial colonies as compared to other methods (I: Supplementary Table S4).

5.1.3 Substitutions

Either single nucleotide or double nucleotide substitutions were carried out in plasmids encoding WT *LHCGR*, *FSHR* or *β2AR* (Jean-Alphonse et al., 2014; Rivero-Muller et al., 2010). Eight different substitutions were generated (LHCGR_Asn291Ser, FSHR_Ala444Thr, LHCGR_Val454Ile, FSHR_ Gly70Ala, β2AR_Asp130Asn, β2AR_Asp79Asn, β2AR_Cys341Gly and β2AR_Tyr350Ala), some of which will be reported in future publications while others (β2AR mutations) were inactivating mutations. All substitutions were verified by sequencing (I: Supplementary Figure S4). I: Supplementary Tables S5 and S6 enlist the mutagenesis and sequencing primers, respectively.

5.1.4 Deletions

Before proceeding to larger deletions, we first tested the utility of REPLACR-mutagenesis in deleting one nucleotide in *LHCGR* cDNA (‘G’ at position 1850; hereafter referred to as 1850delG) to model such mutation found in a patient. The mutation was successfully generated and verified by sequencing (I: Supplementary

Figure S5). The functional test for the frameshift mutation (*LHCGR*_1850delG) has already been published (Rivero-Muller et al., 2015). We then proceeded to a larger deletion in the signal peptide of *LHCGR* gene (12 nucleotide deletion, referred to as LHCGR_Lys12-Leu15del). The sequencing results, verifying the deletion, are presented in I: Supplementary Figure S5. These mutations will be the subject of future work that is beyond this thesis.

Moreover, a massive deletion (144 kb) in a BAC clone of human *LHCGR* (RPCI-11-186L7) was also achieved using REPLACR-mutagenesis. This deletion was achieved in a single-step, as per REPLACR-mutagenesis protocol, that would have otherwise required multiple-steps using other methods (Testa et al., 2003). The resulting site of deletion was sequence verified (I: Supplementary Figure S5). The mutagenesis primers used, sequencing primers and the PCR conditions for the deletion are mentioned in I: Supplementary Tables S5, S6 and S7, respectively. The deletion was designed to exclude the origin of replication and chloramphenicol resistance gene. Since the resulting bacterial colonies containing the deletion could propagate in LB-medium (containing chloramphenicol), the deletion using REPLACR-mutagenesis maintained the backbone integrity.

5.1.5 Additions

We first tried a 27 nucleotide duplication in the *LHCGR* encoding plasmid, named as LHCGR_Leu10-Gln17Dup. However, most of the bacterial colonies obtained were negative, not only by REPLACR-mutagenesis protocol but also in case of Gibson assembly and GeneArt seamless cloning. This was perhaps due to incorrect PCR products when amplifying two identical sequences in tandem. The PCR conditions were modified for generation of either forward or reverse strands in two PCR reactions by using only one primer. The PCR products were then mixed, heated (95 °C) and allowed to anneal by slow cooling. Thereafter, the PCR products were similarly digested with *DpnI* and transformed in recombineering bacteria, as mentioned earlier for REPLACR-mutagenesis. The screened bacterial colonies were verified by sequencing for the right clone (I: Supplementary Figure S6).

A longer addition (45 nucleotides) containing a nuclear localization signal was made to a plasmid coding *CRY2* cDNA (Kennedy et al., 2010). Thereafter, REPLACR-mutagenesis was similarly used to add 60 nucleotides coding for a flexible domain (I: Supplementary Figure S6). The mutagenesis primers for additions are listed in I: Supplementary Table S5. Since it was costlier to order larger primers than generating synthetic DNA blocks for use with Gibson assembly, further additions using REPLACR-mutagenesis were not considered.

5.1.6 Application of REPLACR-mutagenesis for larger plasmids

REPLACR-mutagenesis was used to modify a *Wnt1* targeting vector (24 kb) by addition of two nucleotides (TG) at a *MfeI* restriction site (CAATTG). The original *Wnt1* targeting vector had three *MfeI* restriction sites, which upon *MfeI* digestion yields three bands (15.4, 6.4 and 1.8 Kb; I: Figure 3). The vector with one modified *MfeI* site (CAATGTTG), now had only two sites left, thus resulting in two bands upon *MfeI* digestion (15.4 and 8.2 Kb: I: Figure 3). The site of addition was sequence verified (I: Supplementary Figure S7). PCR conditions for the addition are mentioned in I: Supplementary Table S8. In addition, the integrity of origin of replication and kanamycin resistance gene was intact, since the mutated vector could propagate in LB medium conditioned with kanamycin. Thus, REPLACR-mutagenesis can be used to modify even larger plasmids.

5.1.7 Effect of Red/ET plasmid on mutagenesis of plasmids with similar incompatibility

We wanted to test whether the recombineering bacteria expressing the Red/ET plasmid (with a pSC101 vector backbone) can be used to modify plasmids with similar incompatibility via REPLACR-mutagenesis. For testing, we used two Red/ET plasmids, one with a tetracycline resistance gene and the other with a hygromycin resistance gene (Rivero-Muller et al., 2007). Recombineering bacteria were first generated with the tetracycline resistance Red/ET plasmid. The mutagenesis primers were designed to delete 944 bases from the region coding for the temperature sensitive repressor (RepA) (I: Supplementary Table S5). The deletion was carried out in *Red/ET* plasmid expressing the hygromycin resistance gene and was verified via sequencing (I: Supplementary Figure S8). I: Supplementary Tables S6 and S9 enlists the sequencing

primers and PCR conditions for creating the deletion, respectively. The repressor (RepA) allowed the replication of original Red/ET plasmid at 30 °C only, while the mutated Red/ET plasmid with deleted repressor could be propagated at 37 °C as well. The mutagenesis of a plasmid with similar incompatibility was made possible because first, the enzymes for recombineering were already present in the bacteria, as mentioned earlier during their preparation and second, there was no selective pressure since the modified plasmid, unlike the original *Red/ET* plasmid, could be grown at 37 °C. However, the efficiency of REPLACR-mutagenesis was very low (33 %; 1/3 bacterial colonies were positive). Thus it is possible to modify plasmids with similar incompatibility to the *Red/ET* plasmid with REPLACR-mutagenesis but with lower efficiency and a different antibiotic resistance gene.

Thus, REPLACR-mutagenesis was used for multiple mutations, some of which have been already characterized (*LHCGR_1850delG*), while others will be characterized in the future articles. For the following section, REPLACR-mutagenesis was used for creating multiple mutations (as stated in the methods) in order to characterize the molecular biology of a patient mutation in the *LH beta* subunit.

5.2 Development of CANDLES Assay

5.2.1 Proof-of-concept for CANDLES assay

CANDLES assay was designed to monitor kinetic changes in cAMP content in the cells, especially in primary cells, without requiring any transfection with cAMP sensor encoding plasmids. We hypothesized that if a separate cAMP sensor cell line was generated and subsequently co-cultured with the test cells, we might detect the cAMP production from the test cells indirectly from co-cultured sensor cells (Figure 6). The cells producing cAMP were called *donor cells* (primary cells or cell lines) while the cells detecting cAMP were called *sensor cells*. HEK-293 cells with two kinds of real-time cAMP sensors were generated, one with a luminescent sensor (GloSensor-22F) and the other with a FRET-based sensor (^{T}EPAC^{VV}) and the sensor cells were called GS-293 and EPAC-293 cells, respectively. For donor cells, either HEK-293 cells that are stably transfected with FSHR or KK-1 cells that endogenously expresses LHCGR, were used.

For CANDLES setup, co-cultures of FSHR-293 with either of the sensor cells, GS-293 or EPAC-293, were stimulated with rFSH and the ability of sensor cells to detect cAMP produced in FSHR-293 was tested. Both GS-293 and EPAC-293 could detect the cAMP generated by the donor cells, establishing the proof-of-concept (Figure 8A and 8B). The luminescence values are expressed in relative light units (RLU). Since the production of cAMP via adenylyl cyclase and its degradation by phosphodiesterases is a dynamic process, we used a non-specific phosphodiesterase inhibitor, IBMX, in the assay medium to ensure the detection of cAMP by the sensor cells before it gets degraded by the donor cells themselves. In the absence of IBMX, the sensor cells were unable to detect cAMP generation by the donor cells (Figure 8A and 8B), thereby demonstrating the necessity of IBMX for CANDLES protocol. To observe the effect of IBMX alone, co-cultures without rFSH stimulation were used (Figure 8A and 8B). We then tested the best concentration of IBMX to be used in our assay. A 100 μM IBMX in the assay medium gave the best signal over background and was thus chosen for all further experiments (II: Supplementary Figure S1).

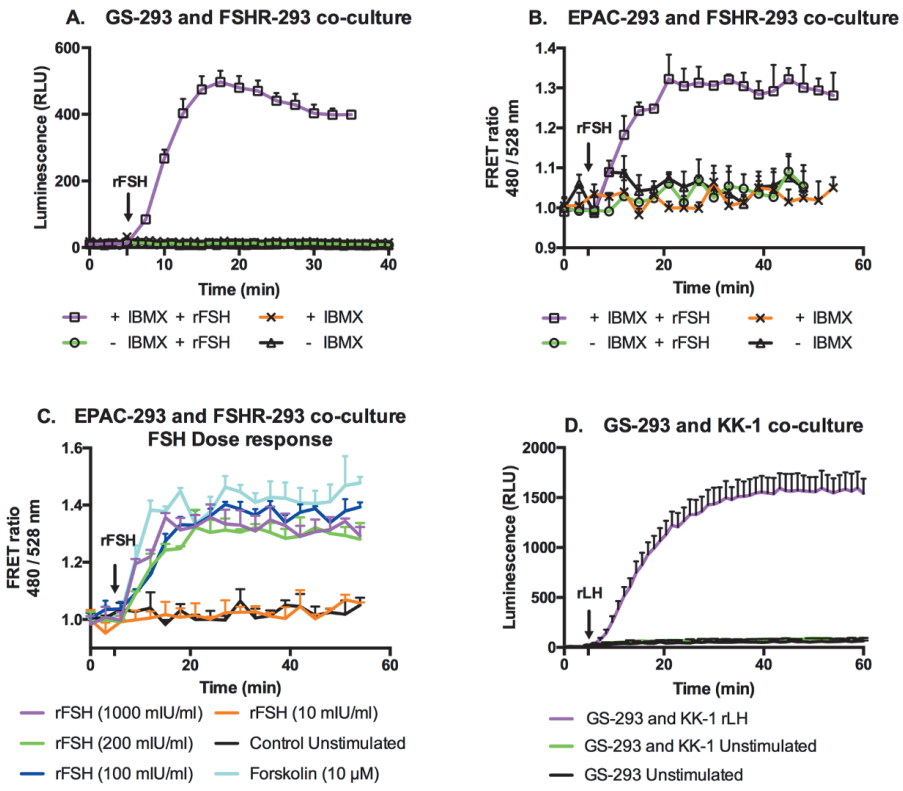

Figure 8. Proof-of-concept for CANDLES assay [modified from (Trehan et al., 2014)] (**A** and **B**) Sensor cells (GS-293 or EPAC-293) could detect cAMP production upon stimulation of FSHR-293 cells with FSH, only in the presence of IBMX. (**C**) EPAC-293 cells were unable to distinguish a dose dependent increase in cAMP production from FSHR-293 that were stimulated with increasing FSH concentrations. (**D**) Sensor cells (GS-293) were able to detect cAMP production from KK-1 cells following the stimulation of endogenous LHCGR by LH.

While the FRET sensor cells were able to detect cAMP produced by FSHR-stimulated donor cells, we could not see a dose-dependent increase in the FRET read-out with increasing concentrations of rFSH (Figure 8C). In addition, the luminescent signal, by its inherent nature, is neither amenable to photobleaching nor does it require additional normalization controls, in stark contrast to the fluorescent FRET signal. Thus, the luminescent sensor cells (GS-293) were chosen as the sensor cells of choice for further optimizations. In addition, the stimulation of endogenous LHCGR in KK-1 cells was also detected by co-cultured GS-293 cells (Figure 8D), providing strong evidence for the detection of cAMP from two different cell types (KK-1 and FSHR-293) and

receptors (LHCGR and FSHR). The reason for choosing KK-1 and FSHR-293 as donor cells while GS-293 as sensor cells was because KK-1 and FSHR-293 cells expresses the GPCRs being stimulated, while the sensor cells do not (II: Figure 3D and 3E).

5.2.2 Optimization of cell densities for donor and sensor cells

The optimal cell densities for maximal cAMP signal detection in co-cultures of sensor (GS-293) and donor cells (either KK-1 or FSHR-293) were first determined by increasing the number of donor cells, for a constant a number of sensor cells (GS-293). The ratio of sensor cells to donor cells was increased from 1:1 to 1:4 and the cAMP signal was monitored upon receptor (LHCGR or FSHR) stimulation. There was an increase in cAMP signal with increasing ratio of donor cells, however the increase in cAMP signal beyond a sensor to donor cell ratio of 1:3 was less pronounced (II: Figure 4A and 4B). Thus a ratio of 1:3 for sensor and donor cells can be considered a good starting point for using CANDLES assay. However, when we increased the number of sensor cells (GS-293), for a constant number of donor cells (FSHR-293), the cAMP signal actually decreased with increasing number of sensor cells (II: Figure 4C). This was possibly because as the number of sensor cells increases in a co-culture, the cell-cell contacts among sensor cells is favored over cell-cell contacts between sensor and donor cells, thereby diluting the amount of cAMP reaching individual sensor cells and thus decreasing the luminescent signal. Therefore, increasing the number of sensor cells does not necessarily increase the cAMP signal. For negative control, luminescence values of unstimulated co-cultures of GS-293 with either KK-1 or FSHR-293 cells were monitored.

5.2.3 Requirement of cell-cell contact for cAMP detection

All the experiments so far were done using co-cultures of sensor and donor cells, where the cells can freely form cell-to-cell contacts. To further investigate whether the decrease in luminescent signal, as observed in II: Figure 4C, was indeed due to a reduction in number of cell to cell contacts between sensor and donor cells, we tested the effect of complete physical separation of donor and sensor cells by using transwell permeable support wells (II: Figure 5A). The sensor cells (GS-293) were cultured alone in the transwell supports while FSHR-293 cells were cultured in the bottom of a separate 24-well plate. Prior to the experiment, the transwell chamber containing the

sensor cells were placed on top of the well containing FSHR-293 cells. This allows the two cell types to have the same cell culture medium but there is still a physical separation between the sensor and donor cells, thereby representing the setup with "No cell-cell contacts". In addition, both GS-293 and FSHR-293 cells were co-cultured in transwell supports, representing the "Cell-cell contact" setup. Cell culture medium was then replaced with assay medium (300 µL in the bottom well and 100 µL in the top transwell). Cells were then stimulated with rFSH and luminescence was recorded as before. The cAMP signal was detected only when there was cell-to-cell contact and the separation of sensor and donor cells completely abolished the detection of cAMP signal by the sensor cells (II: Figure 5B). Unstimulated GS-293 cells were used as negative control. The ability of transwell support to allow transfer of small molecules (via diffusion) or even large glycoprotein hormones was tested by stimulating co-cultures of GS-293 and FSHR-293, growing in transwell supports by stimulation with rFSH in either the bottom well or top transwell chamber. The transwell indeed allows even the movement of large glycoprotein hormones such as rFSH, as cAMP production was detected in both the cases (II: Figure 5C). The apparent higher signal when the co-cultures were stimulated in the upper transwell chamber as compared to bottom well is due to differing effective concentrations of rFSH (200 mIU/mL) in upper transwell (with only 100 µL assay medium) and bottom well (with 300 µL assay medium).

5.2.4 cAMP transfer is mediated by gap junctions

After establishing that cell-cell contact is essential for CANDLES assay, the possible role of gap junctions in mediating cAMP transfer from donor to sensor cells was investigated. Co-cultures of GS-293 and FSHR-293 were pre-incubated with a gap junction inhibitor, CBX, in the assay medium with varying concentrations for 1h. The production of cAMP was then monitored by stimulation with rFSH. There was a dose-dependent decrease in the luminescent signal with increasing concentrations of the CBX (II: Figure 6A). The highest concentration of CBX (100 µM) completely blocked the luminescent signal to the levels of negative control (unstimulated GS-293 sensor cells), while the lowest dose of CBX (25 µM) had no effect on the luminescent signal and was similar to the positive control (GS-293 and FSHR-293 co-cultures stimulated with rFSH, in the absence of CBX) (II: Figure 6A). The decrease in luminescent signal

with increasing doses of CBX could not be attributed to cytotoxicity since the presence or absence of CBX does not seem to affect cell viability (II: Figure 6B).

The next question was to address whether the drop in luminescence with increasing CBX doses (II: Figure 6A) was due to a block in cAMP transfer from donor to sensor cells or due to a decrease in cAMP production or due to the inhibition of the sensor. This was tested by calculating total cAMP content using ELISA in co-cultures of FSHR-293 and GS-293 cells (in 6-well plates), treated with similar doses of CBX (25-100 μM) for 1h and then stimulated with rFSH (200 IU/L) for 20 min. Cells were subsequently lysed and cAMP content was calculated using cAMP ELISA kit (Cell Biolabs) using the prescribed protocol. There was no decrease in cAMP production after rFSH stimulation in any of the CBX treated cultures (II: Figure 6C). For lower CBX doses (25 and 50 μM), cAMP content was very similar to the positive control (rFSH stimulated GS-293 and FSHR-293 co-culture without CBX treatment). However, for higher CBX doses (75 and 100 μM), cAMP content was higher than positive control (II: Figure 6C). This could most likely be attributed to a higher retention of cAMP inside the cells, since the cAMP transfer among cells and to the cell culture medium, that normally happens in physiological conditions, is blocked. Altogether, this suggests that the fall in luminescence with increasing CBX concentrations, as observed in II: Figure 6A, was most likely due to halted cAMP transfer from donor to sensor cells via gap junctions and not due to a decrease in cAMP production.

Since ELISA assay requires cell lysis and thereby the analysis of a single time-point (20 min after rFSH stimulation), we stably transfected GS-293 sensor cells with human FSHR, to generate GS-293-FSHR cells for monitoring the entire cAMP kinetics following rFSH stimulation after CBX treatment. GS-293-FSHR cells were similarly treated with increasing concentrations of CBX (25- 100 μM) for 1h in assay medium, without IBMX and then following luminescence upon rFSH stimulation (II: Figure 6D). cAMP generation following CBX treatment is very similar to the positive control (without CBX treatment), with higher CBX concentrations showing a moderate tendency to increase cAMP (II: Figure 6D). This again suggests that CBX treatment does not cause any decrease in cAMP production or inhibits the activity of cAMP sensor.

Finally, the effect of overexpression of Connexins, the molecular components of gap junctions, in co-cultures was tested. Increasing amounts of human Connexin-32 plasmid (*Cx32*) was transiently transfected in GS-293 and FSHR-293 co-cultures. pcDNA3.1 mock plasmid was used to keep the total amount of DNA transfected constant (0.75 µg DNA/well). Cx32 was fluorescently-tagged with mEmerald and its expression was verified under EVOS microscope (II: Supplementary Figure S3). Production of cAMP was then followed using CANDLES protocol. There was an increase in luminescence from sensor cells, which was significantly higher (*p*-value =0.0017) than the control (mock pcDNA control) after comparing the area under curve (AUC) values. The AUC values (± SEM) in arbitrary units for the samples *Cx32* (mock pcDNA control), *Cx32* (0.5 µg) and *Cx32* (0.75 µg) were 279000±16000, 358000±13000 and 384000±5400, respectively.
Altogether, gap junctions were found to be responsible for cAMP transfer from donor to the sensor cells.

5.2.5 Dose response curve using CANDLES and EC_{50} comparison

The ability of sensor (GS-293) cells to detect cAMP production from donor (FSHR-293) cells following multiple orders of stimulation with rFSH (0.01-1000 mIU/mL) was tested. This was done to test the dynamic range of the sensor cells for detecting differing cAMP production in the donor cells. GS-293 cells were able to detect differences in cAMP production following rFSH stimulation up to 10 mIU/mL on the lower range, below which the luminescence values were comparable to unstimulated negative control (II: Figure 7A). The same cAMP kinetics (luminescence; RLU) when expressed as area under curve (arbitrary units) for different rFSH stimulations are mentioned in the adjacent table in II: Figure 7A.

The EC_{50} values for CANDLES setup were determined from luminescence (RLU) values at a single time-point (20 min after rFSH stimulation). This was compared with EC_{50} values calculated using a traditional cAMP ELISA kit (Cell Biolabs) from FSHR-293 cells grown in 6-well plates and stimulated with similar concentrations of rFSH for 20 min and subsequently lysed for determining cAMP content. EC_{50} values calculated from a traditional cAMP ELISA kit (115.6 mIU/mL) were very similar to those calculated using CANDLES (123.6 mIU/mL) assay.

5.2.6 CANDLES assay using primary cell cultures

CANDLES assay was used to monitor cAMP production from two distinct primary cell cultures; rat cortical neurons and mouse granulosa cells. Rat cortical neurons were used as a blind test for glutamate and adrenergic receptors while mouse granulosa cells, that endogenously express LHCGR, were tested for cAMP production upon rLH stimulation. As expected, granulosa cells responded to rLH stimulation and cAMP production was monitored using GS-293 cells (II: Figure 8A). Cortical neurons, on the other hand, tested positive for adrenergic receptors following epinephrine stimulation while showing absence of glutamate receptors upon glutamate stimulation (II: Figure 8B). For negative controls, the same co-cultures of sensor cells (GS-293) with either granulosa cells or cortical neurons were used without any hormonal stimulation (II: Figure 8A and 8B).

In addition, rat cortical neurons were also used to stimulate adrenergic receptors with various agonists and their kinetics was compared using CANDLES assay (II: Figure 8C). Isoproterenol, epinephrine and salbutamol activate different subtypes of adrenergic receptors. Isoproterenol was used to stimulate β1 and β2 adrenergic receptors and epinephrine stimulated α1, α2, β1 and β2 subtypes non-selectively while salbutamol was more selective for β2 subtype, though it binds β1 subtype with a lower affinity as well (II: Figure 8C). The unstimulated co-culture of GS-293 with cortical neurons was used as a negative control. The sensor (GS-293) cells cultured alone were also stimulated by isoproterenol, epinephrine and salbutamol, thereby demonstrating the presence of adrenergic receptors in sensor cells themselves (II: Figure 8D). The unstimulated sensor cells were used for negative control. However, the stimulation of endogenous adrenergic receptors in GS-293 cells resulted in very low luminescence output as compared to co-culture of GS-293 with cortical neurons (different Y-axes scale in II: Figure 8C and 8D). Thus, it is still possible to use GS-293 as the sensor cells.

5.2.7 Adaptation of Sensor cells to different cell culture media

DMEM/F12 medium has so far been used to culture sensor cells (GS-293). However, there might arise cases where the donor cells are being grown in different cell culture medium, particularly important when culturing primarily cells that have no time to

adapt to new culture conditions. We therefore tested if the sensor cells could be adapted to different culture media to ensure proper growth conditions for the donor cells as well as to test the luminescence output. GS-293 cells were adapted in two of the most commonly used cell culture media, RPMI 1640 and McCoy's 5A.

A medium formulation containing 20 % RPMI 1640 and 80 % DMEM/F12 was used to culture GS-293 cells for the first week. The RPMI 1640 concentration was raised in increments of 20 % per week, up to 100 %. A similar adaptation protocol was followed for culturing GS-293 cells in 100 % McCoy's 5A medium. In addition, FSHR-293 donor cells were also adapted in both media (RPMI 1640 and McCoy's 5A). Finally, cAMP production in co-cultures of GS-293 and FSHR-293, adapted in two different media, was followed upon rFSH stimulation using CANDLES assay (II: Figure 9A and 9B). Cells adapted in both RPMI 1640 and McCoy's 5A media were able to generate and detect cAMP production (II: Figure 9A and 9B), demonstrating the adaptability of the sensor cells to the cell culture medium of choice, thereby ensuring optimal growth conditions for both sensor and donor cells. As negative controls, unstimulated GS-293 cells either cultured alone or in combination with FSHR-293 cells were used.

5.2.8 Distinction between cAMP accumulation using CANDLES assay and real-time cAMP content in cells

CANDLES assay necessitates the use of phosphodiesterase inhibitor, IBMX in order for cAMP production from donor cells to be detected by the sensor cells. Therefore, the process of cAMP degradation is halted to a great extent and the kinetics seen in CANDLES assay actually represents cAMP accumulation over time. This is in contrast to the usual process where cAMP production is followed by cAMP degradation by phosphodiesterases. For CANDLES assay, we used co-cultures of KK-1 (with native LHCGR expression) and GS-293. The luminescent readout following rLH stimulation was followed over time (II: Figure 10; left Y-axis). To follow instantaneous cAMP kinetics, we stably transfected GS-293 cells with LHCGR (thereby called GS-293-LHCGR cells) and stimulated with rLH in assay medium without IBMX (II: Figure 10; right Y-axis). cAMP kinetics shows a saturation after initial accumulation in CANDLES while cAMP degradation occurs in real-time using GS-293-LHCGR cells, without IBMX (II: Figure 10).

5.3 Clinical and molecular characterization of LH beta mutation

A male patient (20-year-old) of North-African descent was examined by our collaborators (see Paper III) for delayed puberty. The patient's phenotypic characterization, diagnosis and treatment was done at CHU de Liège, Université de Liège, where they found a mutation in the *LHB* gene. All subsequent molecular characterization of the mutation was done by our group.

5.3.1 Case report

Briefly, the phenotype of the patient was hypogonadal, with bilateral gynecomastia, tall stature (190 cm), micropenis, little androgen-dependent hair and low testicular volume of around 7 mL (III: Figure 1A and 1B). The hypogonadal phenotype was also corroborated via laboratory tests with low LH [0.4 IU/L reference range (RR): 2-10 IU/L], low testosterone [0.64 μg/L, RR: 2.5-10], high free alpha subunit [3.6, RR: 0.1-0.8 IU/L] and high FSH [19.6, RR: 1-8 IU/L]. Inhibin B level was normal [233.4, RR:105-439 ng/L]. Moreover, arrested spermatogenesis and relative hyperplasia of Sertoli cells was found in the testicular biopsy (III: Figure 1E). Azoospermia with no elongated sperms were found in the spermiogram where only spermatid-like cells were present.

The pituitary gland was normal as tested by MRI and the levels of FSH and LH rose to 38.1 IU/L and 2.6 IU/L, respectively upon GnRH stimulation (100 μg). The patient had normal male karyotype (46, XY) and had four more siblings (2 males and 2 females). At least two consanguineous marriages were present in the family (in four generations), with known cases of female infertility. One of the proband's sisters with her husband had undergone investigations due to infertility, where the husband had presented with anomalies in spermiogram whereas the wife had normal gonadotropin levels. The patient's second sister and one of his brothers had offspring, with consanguineous marriages. The youngest brother of the patient was prepubertal.

The patient was then treated with hCG (3000 IU twice per week) since LH deficiency was considered the main cause of hypogonadism. The treatment resulted in restoration of normal testosterone and FSH levels, where after 1 year, secondary male characteristics with improvements in gynecomastia and testicular volume (18 mL)

could be observed (III: Figure 1C and 1D). The spermiogram also showed an improvement (200,000/mL) where nearly 50 % spermatozoa showed normal shape and good mobility.

5.3.2 GnRH administration

Following hCG therapy for 14 months, the patient's treatment was stopped for two subsequent months. Thereafter, pulsatile GnRH (20 µg every 90 min; gonadorelin-HRF, Tramedico) was administered subcutaneously for 72 h using a pump (PANOMAT- Disetronic). Blood was collected every 12 h to assess levels of FSH, LH, free and total testosterone, sex-hormone binding globulin (SHBG) and free glycoprotein hormone alpha subunit (III: Supplementary Table S3). Both total testosterone and free testosterone values were almost double after 72 h GnRH administration as compared to basal values (III: Supplementary Table S3).

5.3.3 *LHB* Mutation

A non-frameshift deletion of three nucleotides coding for amino acid lysine 20 (Lys20del) in the mature LHB peptide (or Lysine 40 in the primary transcript) was found. The patient and his prepubertal brother had the mutation in a homozygous state, while his two sisters, his parents and a maternal uncle, including his two daughters, had the mutation in a heterozygous state (III: Figure 1F).

5.3.4 Signal transduction

GS-293 sensor cells that were generated for determination of cAMP kinetics in CANDLES assay were used for functional analysis of glycoprotein hormones (WT and mutants). All mutant constructs were created with REPLACR-mutagenesis. We first tested the ability of LHB (fused with mCherry) to activate LHCGR upon dimerization with CGA. The medium from HEK-293 cells expressing LHB (WT or mutant) and/or CGA was used to stimulate cAMP sensor cells (GS-LHCGR). WT LHB upon co-expression with CGA was able to activate LHCGR whereas LHB_Lys20del mutant was not able to activate the receptor (III: Figure 2A). As expected, neither of the LH subunits (LHB or CGA), when expressed alone, were able to activate the LHCGR (III: Figure 2A).

The localization and trafficking of LHB (WT and Lys20del mutant) was studied in under a Zeiss LSM 510 confocal microscope. The WT_LHB localization was seen scarcely in the cytoplasm in the form of concentrated vesicles indicating a quick secretion outside the cells whereas LHB_Lys20del mutant was heavily concentrated throughout the cytoplasm, indicating a probable defect in secretion of the mutant as compared to WT LHB (III: Figure 2B).

In order to understand more if the LHB_Lys20del was secreted outside the cells or was retained intracellularly, we analyzed cells expressing this mutant under a diffusion test. The principle is that proteins that are being transported/secreted efficiently will diffuse poorly in to the photobleached region, while proteins that are heavily retained intracellularly due to a secretion defect will recover quickly after photobleaching as new molecules will pass diffuse quickly to the bleached area. The diffusion test of LHB (WT and Lys20del) was done using fluorescence recovery after photobleaching (FRAP) (Cardarelli et al., 2012), where the efficiently secreted WT_LHB showed poor diffusion to a photobleached region, while the LHB_Lys20del, which was accumulated inside the cells, diffused quickly to the photo bleached region (III: Figure 2C). The expression levels of AmCyan (with nuclear localization) in both WT_LHB and LHB_Lys20del were similar, indicating normal expression of LHB (WT and mutant) from the plasmid vectors in both cases. Thus, the Lys20 deletion is either hindering the secretion of LHB or its dimerization with the alpha subunit or is simply unable to activate the receptor or a possible combination of these problems.

The Lys20 position that lies in a loop, as shown by 3D-modelling (III: Figure 3A), might play a role in proper folding of LHB. To test whether it was the presence of lysine (Lys20) that was responsible for proper folding and thus functioning of LHB, Lys20 position was mutated to either alanine, asparagine or arginine, thereby generating Lys20Ala, Lys20Asn and Lys20Arg mutants of LHB, respectively. In addition, we also tested whether the region of the mutation (Lys20), which is flanked by two glutamic acid residues (Glu), was important in LHB functional responses by deleting either of the two glutamic acids to generate LHB_Glu19del and LHB_Glu21del mutants. The functional cAMP responses of Lys20Ala, Lys20Arg and Lys20Asn mutants of LHB, when co-expressed with CGA, were very similar to WT_LHB (III: Figure 3C).

However, all the deletion mutants of LHB (Glu19del, Glu21del and Lys20del) had very low functional response (III: Figure 3C).

We then assessed if the Lys20 deletion was affecting the proper function of the signal peptide of LHB (first 20 amino acids of the primary transcript) by replacing it with signal peptide of prolactin (PRL-Lys20del), as PRL signal peptide has been previously shown to help in efficiently traffic even mutant receptors to the plasma membrane (Rivero-Muller et al., 2010). Both LHB_Lys20del and PRL-Lys20del had similar localization (not shown) and caused negligible receptor activation (III: Figure 3D).

The medium collected from HEK293 cells expressing WT_LHB or LHB_Lys20del, either in the presence or absence of CGA, was concentrated by Microcon centrifugal filters (10 kDa cut-off, Millipore). GS-LHCGR cells were stimulated with concentrated medium and an extensive stimulation with WT_LHCGR+CGA was seen, as expected (III: Supplementary Figure S5). Even though the response to the concentrated LHB_Lys20del+CGA fraction was much lower than to its WT counterpart, it was still higher than either the control medium or the CGA or beta subunits, when expressed alone (III: Supplementary Figure S5). The concentrations of LHB monomer or LHB+CGA heterodimers were determined in the concentrated medium fractions, where the concentration of WT_LHB (1042.7 IU/L) was 234-fold higher than the LHB_Lys20del (4.45 IU/L) mutant (III: Figure 4A). Surprisingly though, the ratio of heterodimers (LHB+CGA) to monomeric LHB was the same for Lys20del and WT fractions (III: Figure 4B). The WT_LHB+CGA fraction was diluted 234 times to equalize its concentration with LHB_Lys20del+CGA fraction and both the fractions upon stimulation of GS-LHCGR sensor cells, responded equally (III: Figure 4C). Therefore, it is highly likely that LHB_Lys20del mutant can indeed dimerize with CGA to cause a functional activation of its receptor (LHCGR) and the main effect of LHB_Lys20del mutation lies in the intracellular retention of the hormone that inhibits its secretion.

The importance of Lys20 position in beta subunits of all members of the glycoprotein hormone family (LHB, FSHB, hCGB and TSHB) was also studied by first aligning their sequences, using Clustal OMEGA program (III: Figure 5A) and then similarly generating respective deletion mutants corresponding to LHB_Lys20del, namely

FSHB_Lys14del, hCGB_Lys20del and TSHB_Arg13del as mCherry fusion products. The cellular localization of the deletion mutants (hCGB_Lys20del, TSHB_Arg13del and FSHB_Lys14del) is very similar to LHB_Lys20del mutant, being retained intracellularly in greater amounts as compared to their WT subunits, where distinct secretion vesicles, in much smaller quantities, can be seen (III: Figure 5B, 5C and 5D). Similarly, the deletion mutants of beta subunits of all glycoprotein hormones, when co-expressed with CGA, cause very weak receptor stimulation in comparison to WT beta subunits (III: Figure 5E).

6 DISCUSSION

The presented work in this thesis is focused on the development of two specific methods, named REPLACR-mutagenesis and CANDLES, which are not only applicable for studying gonadotropin signaling but also GPCR signaling, in general. REPLACR-mutagenesis aids in the creation of receptor mutants in a quick, inexpensive and robust manner while CANDLES is designed for monitoring Gαs coupled GPCR activation via cAMP production. Finally, using both methods, a novel patient mutation in luteinizing hormone beta subunit was characterized at molecular level, to provide mechanistic insight into the clinical diagnosis. The following sections discuss the main points of all three studies.

6.1 REPLACR-mutagenesis

REPLACR-mutagenesis was designed to reduce the number of steps necessary for mutating plasmid vectors, carrying the gene of interest, in a cost-effective and robust manner. The method relies on creating linearized PCR products using mutagenesis primers, such that the ends of the PCR products carry homologous sequences. The PCR products are subsequently transformed in bacteria expressing viral recombination proteins that can circularize the PCR products to yield mutated plasmids (See Figure 5 for principle). The crucial step in REPLACR-mutagenesis is thus the design of mutagenesis primers for PCR. Such primers for additions, deletions and substitutions in plasmid vectors must be designed using the general design guide, as illustrated in Figure 5. The primers must ensure that the final linearized PCR products have 17 bp homologous sequences at both ends for recombination in bacteria. Though a range of homologous nucleotides (both shorter and longer than 17 bp) were tested at the termini of PCR products, the highest efficiency of 84 % was achieved with a 17 bp homology (Figure 7). The mutagenesis primers should also contain non-homologous 3’ sequences for efficient primer-template binding, to avoid primer-primer self-complementarity which would favor formation of incorrect PCR products. The above-mentioned mutagenesis primers had 17 bp homologous sequences and an additional 6 bp non-homologous sequence to favor primer-template binding.

The mutagenesis primers for creating substitutions contain the substituted nucleotide(s) in the homology region itself, in addition to a 3' extension for template binding (Figure 5). However, the nucleotides for additions are placed at the 5' end of the mutagenesis primers (either one or both primers) while the 3' end contains sequences for template binding (20 or more nucleotides). The added nucleotides at the 5'end of the mutagenesis primers also contain the homologous sequences (17 bp). While one of the mutagenesis primers for creating deletions contains only the nucleotides adjacent to the desired deletion, the other primer has a 17 bp homology to the 5' end of the first primer and adjacent vector sequences in the other direction at its 3'end. Although the mutagenesis primers can be designed with more than 17 bp homology, it does not improve the efficiency of REPLACR-mutagenesis any further. Designing longer primers not only increases the cost of primer synthesis but can also increase secondary structure formation in the primers that can result in non-specific PCR products. The PCR products using mutagenesis primers are digested with *DpnI* to remove the original template plasmid and subsequently transformed in the recombineering bacteria.

During the preparation of electrocompetent bacteria for recombineering, the arabinose promoter controlling the expression of viral recombination proteins (Red γ, β, α and RecA) from Red/ET plasmid is induced by addition of *L*-Arabinose and these bacteria are then used for transformation of PCR products for recombineering. After transformation, the bacteria are grown at 37 °C because Red/ET plasmid contains a temperature sensitive repressor that allows its replication only at 30 °C. Therefore, the resulting bacterial colonies do not contain Red/ET plasmid but only the expected mutated plasmid. The mutagenesis of plasmids with similar incompatibility to Red/ET plasmid is also possible with REPLACR-mutagenesis, although with much lower efficiencies and only in cases where the Red/ET plasmid and the mutated plasmid contain different antibiotic resistance genes.

Multiple point substitutions were successfully generated using the REPLACR-mutagenesis. Similarly, the deletion of one nucleotide to a massive 144 kb deletion in a human BAC clone (containing *LHCGR*) was made by REPLACR-mutagenesis. Since REPLACR-mutagenesis only requires a single-step transformation of PCR products in recombineering bacteria, this greatly reduces the number of steps required, even for such large BAC deletions, thereby providing a major advantage over selection/counter-

selection based or restriction digestion based-methods (Li et al., 2013; Testa et al., 2003; Wang et al., 2014; Zhang et al., 2003). Moreover, REPLACR-mutagenesis was used to add 1-60 nucleotides in plasmid vectors. If more than 60 nucleotides need to be added, the construction of synthetic DNA blocks and assembly methods (Gibson, GeneArt, In-fusion) was found to be a cheaper alternative. The mutations thus far created were in both small (6-10 kb) and large (24 kb) plasmids vectors. All the mutagenesis experiments yielded a combined median efficiency of 75% (I: Table 1).

A variety of enzymes such as kinases (for 3' phosphorylation) and ligases (for nick repair) are typically used in traditional mutagenesis methods based on PCR, thereby also increasing the number of steps and cost required to form circular plasmids containing the desired mutation. Similarly, mutagenesis methods based on recombination, such as Gibson assembly and GeneArt seamless cloning, among others, also require multiple steps with expensive enzymes, where the PCR product is first typically purified for an *in vitro* incubation with purified enzymes for recombination and ligation, which precedes their transformation in bacteria. REPLACR-mutagenesis, on the other hand, does not require *in vitro* incubation with recombineering enzymes since circularization of the PCR products occurs directly in recombineering bacteria. Moreover, differences in the efficiency of REPLACR-mutagenesis with commercial alternatives (GeneArt and Gibson assembly) were negligible.

Although the primer design strategy in REPLACR-mutagenesis shares similarity to Quikchange site-directed mutagenesis, a commercial PCR-based mutagenesis method (Tseng et al., 2008), the underlying mechanism of mutagenesis is different. While the latter relies on bacterial DNA repair mechanism for nick repair in circular PCR products, REPLACR-mutagenesis employs the viral recombination machinery to circularize linear PCR products. In addition, a typical PCR reaction yields mostly linear products, with circular PCR products containing nicks in very low numbers, thereby making recombineering of linear PCR products in REPLACR-mutagenesis a far more efficient process. Some mutagenesis methods like "en passant mutagenesis" also employ recombineering in bacteria in one-step transformation but they require dual screening of bacterial colonies grown in different conditions by colony PCR, unlike the direct screening of bacterial colonies upon REPLACR-mutagenesis (Tischer et al., 2010).

One of the problems we faced was with a duplication which resulted in incorrect bacterial colonies. The correct duplication was obtained only after modifying the PCR conditions, where two PCR reactions were used for the generation of sense and antisense strands with either sense or antisense primer each and subsequent annealing of the two strands before transformation to recombineering bacteria. Since REPLACR-mutagenesis relies on PCR for mutagenesis, the amplification of larger vectors using PCR becomes the limiting factor, because high-fidelity polymerases currently available are recommended for up to 20-25 kb, with some generational improvement in polymerases happening over the years. However, most mutagenesis experiments with cDNAs in plasmid vectors hardly ever require more than 10-15 kb amplification. Thus REPLACR-mutagenesis should suffice for the majority of mutagenesis experiments.

6.2 CANDLES Assay

The development of assays to screen ligands, mutant variants of GPCRs, as well as to study different aspects of GPCR signaling led to a paradigm shift in our understanding of GPCRs. The understanding of GPCR signaling evolved from a simple heterotrimeric G protein activation to the current facets in GPCR oligomerization, biased agonism and G protein-independent signaling (Ferre, 2015; Ji et al., 2004; Rivero-Muller et al., 2010; Tilley, 2011). Even though many aspects of GPCR signaling have been studied in cell culture models using transient/stable transfections of GPCR encoding plasmids, they do not necessarily represent a physiological state, since the GPCR is often being overexpressed in the highly unstable genetic background of immortalized cell lines. A better approach to study GPCR signaling would be the application of primary cells directly from animal models such as transgenic and knockout mice, or even of primary cells from freshly isolated tissues or biopsies from patients, to guarantee that the cells are as close to their physiological state as possible. However, using the current generation of methods we found two major bottlenecks to monitor the GPCR-evoked activation of the cAMP signaling pathway, especially using primary cells. First, immunoassay methods are unable to monitor cAMP kinetics from the same cell samples, as they require cell lysis for determination of cAMP content. The only way to determine kinetics is to use different cell samples for various time-points, thereby

increasing the variability. Second, the transfection of primary cells with real-time cAMP sensors (luminescent or fluorescent) is a highly inefficient process with most methods, except for viral transduction, and even with a high transfection efficiency the variable expression levels of the sensor and cellular heterogeneity of the primary cells cause large variations in the signal readout.

CANDLES assay was designed to circumvent these problems by the introduction of separate cAMP sensor cell lines, GS-293 or EPAC-293, which upon co-culture with either primary cells or established cell lines (donor cells), could kinetically monitor cAMP production over time, following GPCR activation (see Figure 6 for principle). GS-293 cells express a luminescent cAMP sensor (GloSensor-22F) while EPAC-203 express a FRET based cAMP sensor (^{T}EPAC^{VV}). The co-cultures of sensor cells (GS-293/EPAC-293) with donor cells (FSHR-293) were used for establishing the initial proof-of-concept, in which the cAMP production following FSHR stimulation with its ligand, rFSH, was successfully followed over time (Figure 8). Similarly, the production of cAMP in co-cultures of sensor cells (GS-293) with KK-1 donor cells, endogenously expressing LHCGR, was detected as an additional proof-of-concept using different receptor and cell types. It was also established that the presence of a phosphodiesterase inhibitor (IBMX) is essential for the assay, to prevent degradation of the cAMP produced in the donor cells by endogenous phosphodiesterases before it can be detected by the co-cultured sensor cells (Figure 8). The readout of CANDLES assay, due to the presence of IBMX in the assay medium, thus represents cAMP accumulation over time rather than instantaneous cAMP concentration present in the cells, since the process of degradation of cAMP (via phosphodiesterases) is greatly reduced.

Thereafter, it was determined whether an increase in cAMP production in the donor cells also correspond to an increase in the signal readout from the sensor cells (either luminescence or FRET readout). This was determined by stimulating the co-cultures of donor cells (FSHR-293) and either of the sensor cells, EPAC-293 or GS-293, with increasing concentrations of rFSH. Although the differences with increasing rFSH stimulation were detected by GS-293 cells with an increasing luminescence output, the FRET ratios with EPAC-293 sensor cells were very similar even with increasing doses of rFSH. In addition, FRET signal was not only affected by photobleaching but also required additional controls for normalization along with more sophisticated

instruments (microscopes/plate reader), which was not the case with luminescent readout. Thus, GS-293 cells were chosen over EPAC-293 for all further experiments and optimizations.

During the optimization of cell seeding densities it was found that the donor cells must be seeded at three-fold higher density than the sensor cells in the co-cultures for best signal output. However, an increase in the number of sensor cells did not necessarily increases the signal output from donor cell stimulation, but it rather decreased the luminescence output possibly due to reduction in cell-cell contacts between sensor and donor cells, and subsequent dilution of cAMP reaching individual sensor cells. The essential role of cell-to-cell contacts between donor (FSHR-293) and sensor (GS-293) cells was also substantiated by their physical separation by using transwell chambers, where the sensor cells could not detect cAMP generation upon donor cell stimulation.

Upon further investigation of the molecular components crucial in cell-to-cell contacts, gap junction channels were found to be mediating cAMP transfer from donor to sensor cells. Multiple observations led to this conclusion: carbenoxolone (CBX), a gap junction inhibitor, caused a dose-dependent reduction in the luminescent readout in GS-293 and FSHR-293 co-cultures, upon rFSH stimulation (II: Figure 6A). Next, cell viability was not affected upon CBX treatment, thereby eliminating the possibility of cytotoxicity for the reduction in signal readout. Subsequently, we explored the possibility of a reduction in cAMP production by the donor cells to account for decreased signal readout from sensor cells upon CBX treatment. However, CBX treatment did not cause a reduction in cAMP production from the donor cells. Finally, the overexpression of *connexin-32*, the molecular components of gap junctions, in FSHR-293 and GS-293 co-culture resulted in increased luminescent signal read-out after stimulation with rFSH. Altogether, the role of gap junctions in cAMP transfer using CANDLES assay was thus established. The observed role of gap junction channels is also consistent with the inherent property of cell-cell communication via gap junction channels (Giepmans, 2004; Herve et al., 2012; Sosinsky et al., 2005), which allow the movement of small metabolites and molecules (less than 1 kDa), including cAMP among neighboring cells (Bevans et al., 1998; Kam et al., 1998; Lawrence et al., 1978; Ponsioen et al., 2007).

Thereafter, the CANDLES assay was successfully employed in detecting cAMP production from primary cultures of mouse granulosa cells and rat cortical neurons. Even subtle kinetic differences in cAMP production, occurring for a short duration, when adrenergic receptors were stimulated with various agonists, could be differentiated using the CANDLES assay (II: Figure 8C). Competition-based cAMP immunoassays (ELISA) are usually unable to detect these quick changes in cAMP production. Altogether, there was no need to transfect primary cells with real-time cAMP sensors in order to monitor the cAMP kinetics after receptor stimulation with the CANDLES protocol, thus representing a more physiological response from the endogenous receptors. Moreover, since the same primary cells were used for cAMP detection at all time-points, the sample variability at all kinetic time-points was greatly reduced, which is in contrast to competition-based cAMP assays. The specificity of the CANDLES assay was ascertained with a dose-dependent increase in cAMP production following rFSH stimulation of GS-293 and FSHR-293 co-cultures.

CANDLES assay is best suited for relative measurements of cAMP among different samples and controls but not for determining absolute cAMP content in the cells, where immunoassays still offer the best sensitivity but can only measure one time-point per sample. However, we found very similar EC_{50} values for the CANDLES assay and a commercial cAMP ELISA kit. Another general limitation of luminescence based assays, including CANDLES assay, is the need of equilibration of cells in the assay medium containing the luminescent substrate, which only allows multiple stimulations in the same medium since changing assay medium between different stimulations will also change the baseline. In addition, all possible combinations of donor and sensor cell types might not form cell-to-cell contacts and thus the choice of cell types must be determined empirically. However, different cell types, even from different species (mouse: KK-1 and granulosa cells; rat: cortical neurons; human: HEK-293) expressing different receptors (LHCGR, FSHR and adrenergic receptors) responded well with the CANDLES assay.

Next, a novel patient mutation in *LHB* was characterized using both REPLACR-mutagenesis and cAMP sensor cells generated for CANDLES assay.

6.3 Characterization of LH beta mutation

An inactivating mutation in the *LHB* gene of a male patient was characterized at the clinical and molecular level. The homozygous mutation was found to be a deletion of 40th codon of *LHB* such that the resulting amino acid, lysine, was deleted in the primary LHB peptide. The deletion corresponds to the 20th amino acid (Lys20del) in the mature peptide - the signal peptide (coding for the first 20 amino acids) is cleaved off during its biosynthesis (Ascoli et al., 2002). The conformational changes caused by the deletion of lysine (Lys20del) caused intracellular retention of the LHB subunit, thereby hindering its secretion to a great extent. The LHB_Lys20del mutant was however capable of dimerization with CGA and could also stimulate LHCGR, though the secretion was minimal as compared to WT LHB. However, under physiological conditions, the minimal secretion of LHB_Lys20del mutant in the patient would be inconsequential for LHCGR stimulation.

LHCGR is stimulated by two gonadotropins, LH and hCG. While hCG stimulates LHCGR during pregnancy and in fetal life, LH takes over the function in the post-natal and non-pregnant stages. The stimulation of LHCGR in Leydig cells (fetal/adult) leads to the production of androgens that are responsible not only for male sexual differentiation but also for the development of secondary sexual characteristics and reproductive function (Themmen et al., 2000). Thus mutations in LHCGR usually have a more pronounced effect since the entire LHCGR signaling right from prenatal stage is affected while mutations affecting LH alone are rescued in the fetus by hCG function.

Since LHB and CGA subunits together form the functional heterodimeric luteinizing hormone (LH) that can stimulate its cognate receptor, LHCGR, the male patient carrying LHB_Lys20del mutant was devoid of functional LHCGR signaling, resulting in a hypogonadal phenotype. The LHB_Lys20del mutation discussed here is a rare inactivating mutation, since only five mutations of the *LHB* gene (Table 1) have thus far been described (Achard et al., 2009; Basciani et al., 2012; Lofrano-Porto et al., 2007; Valdes-Socin et al., 2004; Weiss et al., 1992). The previously reported *LHB* mutations had an expected hypogonadal phenotype with delayed puberty in males while the female patients despite having normal secondary sexual characteristics, presented with anovulation, oligomenorrhea (or secondary amenorrhea) and infertility (Achard et al.,

2009; Basciani et al., 2012; Lofrano-Porto et al., 2007). The previously reported male patients with mutated *LHB* had low testosterone levels due to isolated LH deficiency. When treated with exogenous hCG they responded with increased testosterone production, but were unresponsive to GnRH. In the presented case with LHB_Lys20del mutation, prolonged GnRH treatment was however, able to increase the testosterone production slightly, which also corroborates with the *in vitro* analyses, where LHB_Lys20del overexpression could activate LHCGR, though much less than WT LHB. If secreted Lys20del/CGA heterodimers were concentrated to the same levels of WT LH, the responses were identical.

As revealed by the hCG crystal structure, the two subunits (alpha and beta) contain cysteine-knot motifs and 3 elongated loops. The heterodimer is stabilized by wrapping of the *C*-terminal segment of beta subunit around alpha subunit similar to a seat-belt and subsequent latching via disulphide bridge made by Cysteine 26 (and Cysteine 110) of beta subunit that resides in β1-loop (Lapthorn et al., 1994). Although LH has not been crystallized, its tertiary structure is likely to be very similar to that of hCG due to their very high (80%) homology (Vassart et al., 2004). Through structural modeling, the LHB_Lys20 deletion was shown to reside in the β1-loop which is a part of the seat-belt latch and five residues before Cysteine 26. The Lys20 deletion seems not to affect the dimerization and functional activity of the dimer (with CGA) but only secretion of the beta subunit.

The presence of a hydrophilic residue (Lys20) was shown to be of very little relevance, as it could be easily substituted for an uncharged (Asp), hydrophobic (Ala) or another hydrophilic (Arg) residue, without any change in its functional activity. However, deletions in the same region (Glu19, Lys20 and Glu21) resulted in negligible activation of LHCGR in all cases thus demonstrating their importance for folding and secretion of the peptides. The intracellular retention of LHB_Lys20del mutant was visualized with confocal microscopy which was in contrast to the WT_LHB that gets actively secreted and therefore shows far less intracellular accumulation in the form of secretion vesicles. The effect of similar deletion (corresponding to LHB_Lys20 position) in beta subunits of other glycoprotein hormones (hCGB, TSHB and FSHB) was also tested, where similar lack of receptor activation due to intracellular retention of the deletion mutants was found.

Finally, the treatment of our patient with hCG caused restoration of secondary sexual characteristics and qualitative improvement in spermatogenesis. Moreover, clinical intervention in case of the patient's prepubertal brother (with homozygous mutation) can also be started.

7 SUMMARY

The presented thesis was focused on the development of two crucial assays to characterize not only gonadotropin signaling in general but also in modeling of clinical mutations. Since the function of key residues in any protein/receptor, including GPCRs, is usually characterized by site-directed mutagenesis, a method, named REPLACR-mutagenesis, was designed to quickly create mutations in cDNAs of the desired genes in plasmid expression vectors. Different site-directed mutations (additions, deletions or substitutions) were generated, where PCR products obtained with mutagenesis primers were transformed in a single-step in the recombineering bacteria to yield mutated plasmid vectors in a cost-effective manner. We see the application of REPLACR-mutagenesis not only in quickly creating mutations for experienced molecular biologists but also in teaching purposes, where first-time users can quickly accomplish their mutagenesis experiments using a simple PCR and a bacterial transformation. In addition, the gonadotropin receptors belong to the family of GPCRs that couple mainly via $G\alpha_s$ to activate the cAMP signaling cascade, among other pathways, the thesis work also dealt with the development of an assay, named CANDLES, to monitor the kinetics of cAMP production in cell culture model systems (cell lines or primary cells). The main advantage of CANDLES resides in its ability to use the same cells for the entire cAMP kinetics, without the need for transfections, especially in difficult to transfect primary cell cultures. Finally, a novel mutation in LH beta subunit of a male patient was characterized, that caused a hypogonadal phenotype. The *LHB* mutation (Lys20 deletion) caused an intracellular retention of the peptide that leads to a functional LH deficiency in the patient that was treated with exogenous hCG. Future studies must continue looking for alternative therapies such as the use of pharmaco-chaperones to correctly fold and possibly help in secretion of such misfolded proteins, that are either degraded or are retained intracellularly.

8 ACKNOWLEDGEMENTS

The study was carried out at the Department of Physiology, Institute of Biomedicine, University of Turku, during the years 2010-2016.

I foremost wish to thank both my supervisors Prof. Ilpo Huhtaniemi and Docent Adolfo-Rivero-Müller for introducing me to the world of GPCR biology. The sheer innovative approach to science as I have seen in Adolfo kept me motivated and led to the development of methods that could be useful for a larger audience. In addition, Ilpo's guidance over the years helped me in teaching how to do science by designing experiments that yield specific answers to the problems rather than only creating more questions.

I would also like to thank all the co-authors and collaborators for sharing their expertise and contributions in the published articles and submitted manuscript; Emmi Rotgers, Eleanor T Coffey, Michał Kiełbus, Jakub Czapinski, Andrzej Stepulak, Krzysztof Jozwiak, Francois Pralong, Aicha Hafidi, Jean-Jacques Menage, Albert Beckers and Adrian F. Daly.

I would also like to thank Docent Ulla Petäjä-Repo and Prof. Kari Keinänen for reviewing my thesis. The recommendations of my PhD thesis advisory committee members Prof. Kid Tornqvist, Prof. Mika Scheinin, along with my supervisors were of great help in monitoring the progress of my projects over the years and suggesting necessary changes for their successful completion.

Besides I would like to thank the all the group leaders in Physiology for creating a stimulating environment, where collaboration and helping others is the norm; Prof. Jorma Toppari, Prof. Matti Poutanen, Prof. Harri Niinikoski, Prof. Manuel Tena-Sempere, Docent Jukka Kero, Docent Marko Kallio, Assistant Prof. Nafis Rahman and especially Assistant Prof. Noora Kotaja for being always ready to read and comment on so many of my applications over the years, including TuBS application.

All the experiments conducted at Department of Physiology were seamlessly made possible because of the coordination by our lab manager Dr. Tuula Hämäläinen along

with all the technical help from Johanna Järvi, Minna Lindroth, Anneli Vesa, Minna Kangasperko, Taina Kirjonen, Taija Poikkipuoli, Katri Hovirinta, Erica Nyman, Marja-Riita Kajaala, Leena Karlsson, Heidi Liljenbäck, Anu Salminen, Jonna Palmu, Mona Niiranen, Merja Leppiaho, Nina Messner, Sanna Viertomanner, Pauliina Toivonen, Heli Niittymäki and Marko Tirri. I cannot even count how many reasonable and unreasonable requests I have made for reagents at haphazard timings, and every time I was helped by someone.

All these years I have greatly enjoyed the friendship and countless discussions with my colleagues, Tiina Lehtiniemi, Michael Awosanya, Mahesh Tambe and Ram Prakash Yadav, and all the people I have shared my office (aka children's room): Sofia-Maria Pruikkonen, Konrad Patyra, Sathyavathy Anandan, Hanna Korhonen, Holger Jäschke, Michał Brouze, Stefania Musilli, Viktoria Kramer, Opeyemi Olotu, Marion Adam, Alessia Visintin, Eric Karu, Emma Kutvonen, Meeri Jännäri, Titta Kivikoski and Laura Kätkänaho.

A special thanks to all the current and past students and senior researchers for making Department of Physiology such as pleasant place to work: Sheyla Cisneros Montalvo, Karin Söstar, Matteo Da Ros, Oliver Meikar, Ida Björkgren, Marcin Chrusciel, Henriette Undeutsch, Wiwat Rodprasert, Freja Hartman, Milena Dorosko, Janne Hakkarainen, Heli Jokela, Matias Knuuttila, Heidi Kemiläinen, Mari Lehti, Hanna Heikelä, Gabriela Martínez Chacón, Natalia Gurvits, Christoffer Löf, Valeriy Paramonov, Henna Joki, Anna Eggert, Jaakko Koskenniemi, Annika Adamsson, Petra Sipilä, Mirja Nurmio, Fuping Zhang, Leena Strauss, Pirjo Pakarinen, Helena Virtanen, Sergey Sadov, Niina Saarinen-Aaltonen, Antti Perheentupa, Riikka Huhtaniemi, Taija Saloniemi-Heininen, Jenni Mäki-Jouppila, Tiina Lahtela, Arttu Junnila, Lili Niinimäki, Laura Mathe, Kati Asp, Kalle Rytkönen, Suvi Ruohonen and Aleksi Hakkarainen.

I wish to thank Prof. Olli Lassila and Nina Widberg for all the scientific and non-scientific events that were moderated by TuBS and now TuDMM, along with all the travel grants that made it possible to present my work at various meetings and learn new techniques at various courses.

Finally, my parents (Anshu Trehan and Ajay Kumar Trehan) and my brother (Pinaki Trehan) have always been a constant source of encouragement, even though they still do not know what exactly I do. I could not have made it this far without their love and affection and this should at least force them to go through my thesis despite their business and arts background.

This work has been supported by Academy of Finland, Sigrid Juselius Foundation, University of Turku/Faculty of Medicine, Turku Doctoral Programme of Biomedical Sciences (TuBS) and Turku Doctoral Programme of Molecular Medicine (TuDMM).

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