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Fine-scale genetic variation of follicle-stimulating hormone beta-subunit coding gene (*FSHB*) and its association with reproductive health

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Nagirnaja L, Rull K, Uusküla L, Hallast P, **Grigorova M**, Laan M. Genomics and genetics of gonadotropin beta-subunit genes: Unique *FSHB* and duplicated *LHB*/*CGB* loci. Mol Cell Endocrinol. 2010 Nov 25;329(1–2):4–16

II **Grigorova M**, Rull K, Laan M. Haplotype structure of *FSHB*, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. Ann Hum Genet. 2007 Jan; 71(Pt 1):18–28

III **Grigorova M**, Punab M, Ausmees K, Laan M. *FSHB* promoter polymorphism within evolutionary conserved element is associated with serum FSH level in men. Hum Reprod. 2008 Sep;23(9):2160–6

IV **Grigorova M**, Punab M, Poolamets O, Kelgo P, Ausmees K, Korrovits P, Vihljajev V, Laan M. Increased Prevalance of the –211 T allele of follicle stimulating hormone (FSH) beta subunit promoter polymorphism and lower serum FSH in infertile men. J Clin Endocrinol Metab. 2010 Jan;95(1):100–8.

V **Grigorova M**, Punab M, Zilaitiene B, Erenpreiss J, Ausmees K, Matulevicius V, Tsarev I, Jørgensen N, Laan M. Genetically Determined Dosage of Follicle-Stimulating Hormone (FSH) Affects Male Reproductive Parameters. J Clin Endocrinol Metab. 2011 Sep;96(6):E1534–E1541

Author's contributions:

Ref. I – contributed to the manuscript preparation

Ref. II, III, IV – participated in experimental design, performed *in silico* candidate gene regulatory region screening, conducted the experiments, analyzed the data, and contributed to the manuscript preparation

Ref V – participated in experimental design, conducted the experiments, analyzed the data, and wrote the first draft of the manuscript

LIST OF ABBREVIATIONS

AP-1	activator protein-1
AR	androgen receptor
BMI	body mass index
bp	basepair
BTB	blood-testis barrier
C11orf46	*chromosome 11 open reading frame 46* gene
cAMP	cyclic adenosine monophosphate
CEPH	Centre d′Étude du Polymorphisme Humain
CG	chorionic gonadotropin
CGA	*glycoprotein hormone alpha subunit* gene
CGB	*chorionic gonadotropin beta subunit* gene
CREB	cAMP regulatory element-binding protein
DNA	deoxyribonucleic acid
ECACC	The European Collection of Cell Cultures
eCG	equine chorionic gonadotropin
eLH	equine luteinizing hormone
ERK	extracellular signal-regulated protein kinase
FSH	follicle-stimulating hormone
FSHB	follicle-stimulating hormone beta subunit protein
FSHB	*follicle-stimulating hormone beta subunit* gene
FSHR	follicle-stimulating hormone receptor
FSHRKO	FSHR coding gene knockout
FSHβ	follicle-stimulating hormone beta subunit
FSHβKO	*follicle-stimulating hormone beta subunit* gene knockout
GalNAc	N-acetylgalactosamine
GnRH	gonadotropin-releasing hormone
GnRHR	GnRH receptor
GPCR	G protein-coupled receptor
GR	glucocorticoid receptor
GTH	gonadotropic hormone
hCG	human chorionic gonadotropin
HGDP-CEPH	Human Genome Diversity Panel- Centre d′Étude du Polymorphisme Humain
hpg	hypogonadal gonadotropin-deficient mice animal model
HPG	hypothalamic-pituitary-gonadal
HRE	hormone response element
HSD17B117	hydroxysteroid dehydrogenase
JNK	jun-N-terminal kinase
iL	intracellular loop
kb	kilobase
KCNA4	*potassium voltage-gated channel, shaker-related subfamily, member 4* gene
kDa	kilodalton

KS	Kallmann syndrome
LD	linkage disequilibrium
LH	luteinizing hormone
LHB	*luteinizing hormone beta subunit* gene
LHCGR	luteinizing hormone/chorionic gonadotropin receptor
LHβ	luteinizing hormone beta subunit
MAF	minor allele frequency
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NFY	nuclear transcription factor Y
Nur77	*orphan nuclear receptor* gene
OHSS	ovarian hyperstimulation syndrome
PCOS	polycystic ovary syndrome
pI	isoelectric point
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PR	progesterone receptor
PRE	progesterone response element
PROP1	*prophet of Pit1* gene
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SD	standard deviation
SF-1	steroidogenic factor-1
SNP	single nucleotide polymorphism
SRC	Rous sarcoma oncogene
STP	short time to pregnancy
TDS	testicular dysgenesis syndrome
TM	transmembrane domain
TSH	thyroid-stimulating hormone
TSHR	thyroid-stimulating hormone receptor
TSS	transcription start-site
UTR	untranslated region

INTRODUCTION

Together with luteinizing hormone (LH) and chorionic gonadotropin (CG), follicle-stimulating hormone (FSH) belongs to the gonadotropin hormones family. Multiple past and recent physiological and biochemical studies elucidated the essential roles of gonadotropins in reproductive processes in mammals (Pierce and Parsons 1981, Moyle and Campbell 1996). In human, FSH and LH produced by pituitary are involved in hypothalamic-pituitary-gonadal axis and are required for ovulation and spermatogenesis in females and males, respectively (reviewed in Edson *et al*. 2009, Weinbauer *et al*. 2010). Primate-specific CG secreted solely in placenta contributes profoundly to embryo implantation and maintenance of pregnancy (Moyle and Campbell 1996).

Gonadotropins are heterodimeric proteins composed of two subunits. The α-subunit is common in all gonadotropins, whereas β-subunit dictates receptor-binding specificity and unique function of the hormone. The genes encoding for FSH, LH and CG β-subunits have been suggested to have common evolutionary ancestry and belong to gonadotropin hormone β-subunit gene family. In human, follicle-stimulating hormone β-subunit is encoded by the *FSHB* gene (11p13), whereas the genes encoding for LH and hCG β-subunits are located in the cluster of duplicated genes (*LHB/CGB,* 19q13) consisting of one *LHB* gene and six *CGB* genes. Phylogenetic analyses have elucidated the high sequence conservation of the entire *FSHB* gene along with its upstream and downstream regions throughout the vertebrate lineage (Kumar *et al*. 2006). The high sequence conservation mirrors strong selective pressure acting towards the entire *FSHB* gene and its flanking sequences.

There has been growing interest towards the mapping of common genetic variants that contribute to human phenotypic diversity and prevalence of a disease. Despite the intense research on genetics of fertility and essential function of the FSH reproduction, there is limited number of studies describing the *FSHB* gene common sequence variation pattern (Liao *et al*. 1999, Lamminen *et al*. 2005). Possible contribution of the *FSHB* common genetic variants to the heterogeneity of the fertility phenotype remains largely unexplained and possibly underestimated. As yet, genome-wide association studies interrogating the genetic basis for variation in common human traits have not reported any *FSHB* common variant to be significantly associated with variation of human fertility phenotype (Hindorff *et al*. 2011). However, due to the stringent statistical corrections obligatory for large-scale studies, true genetic associations might have been overlooked. Comparison of the intra- and interspecies sequence diversity of biologically relevant genes, such as the *FSHB* gene, could allow functionally important genetic variants to be defined.

In this thesis, the literature review gives an overview of the evolutionary context, molecular structure, and function of the gonadotropin hormones. The second major part of the overview focuses on the genetics of gonadotropin subunits coding genes, the detailed genetic variation pattern of the human

follicle-stimulating hormone β-subunit coding gene (*FSHB*), and the regulation of the *FSHB* gene expression.

The experimental part of the current thesis investigates the worldwide fine-scale genetic variation pattern of the *FSHB* gene and possible association of the gene variants with female reproductive success. Additionally, this research includes *in silico* screening of the *FSHB* gene conserved flanking sequences resulted in the isolation of the putative gene expression-associated promoter polymorphism (–211 G/T, rs10835638) residing in transcription factor-binding site, Progesterone Response Element (PRE). This genetic variant was previously shown to represent functional regulatory locus associated with gene expression regulation (Webster *et al*. 1995, Hoogendoorn *et al*. 2003). In the framework of the current thesis, genetic association study is conducted to evaluate the association of the detected putative regulatory polymorphic position within the *FSHB* promoter with male reproductive parameters, in the Baltic cohort of young men (Estonians, Latvians and Lithuanians) and in the Estonian cohort of patients diagnosed with infertility.

1. REVIEW OF LITERATURE

1.1. Gonadotropin hormones

1.1.1. Evolutionary context of glycoprotein gonadotropins

The family of glycoprotein hormones includes thyroid stimulating hormone (TSH) and gonadotropin hormones: follicle-stimulating hormone (FSH), luteinizing hormone (LH) and chorionic gonadotropin (CG). Glycoprotein gonadotropins have the essential roles in reproductive processes and have been intensively studied in various animal species (Table 1). Gonadotropins are absent from the closest relatives to vertebrates, invertebrates marine chordate *Ciona intestinalis* and lancelets (e.g. *Branchiostoma sp.*), which represent the evolutionary lineage between vertebrates and invertebrates (Campbell *et al*. 2004, Holland *et al*. 2008, Kano 2010). However, in lancelets, a homolog for the thyrostimulin, a recently discovered glycoprotein synthesized by anterior pituitary also in vertebrates, has been identified (Nakabayashi *et al*. 2002, Campbell *et al*. 2004, Tando and Kubokawa 2009). The presence of a functional heterodimeric glycoprotein gonadotropin has been elucidated from the most primitive vertebrates, brown hagfish (*Paramyxine atami*) and sea lamprey (*Lampetra fluviatilis*) (Uchida *et al*. 2010, Sower *et al*. 2006). In all representatives of more complex vertebrates (Gnathostomes) including fishes, two gonadotropin hormones and TSH have been identified (Table 1; Schulz *et al*. 2001a, 2001b).

The accelerated evolution in mammals has led to further diversification of the gonadotropin hormones family giving rise to an additional hormone with novel function. The homolog for LH–CG–is produced in the placenta of primates and a few equines such as horse, donkey and zebra (Moyle and Campbell 1996). In equines, eLH and eCG have identical amino acid composition and differ in post-translational modification pattern. In primates, LH and CG represent separately encoded proteins with distinct patterns of expression and function.

Table 1. Gonadotropin hormones FSH, LH, CG, or their analogues identified in selected representatives of Chordates (phylum Chordata).

			FSH	LH	CG	References
Cephalochordates		amphioxus, lancelets	–	–	–	Holland *et al*. 2008 Hallböök *et al.* 2008
Urochordates		ascidian, sea squirt	–	–	–	reviewed in Campbell *et al*. 2004
Craniates						
Jawless vertebrates (agnathans)		sea lamprey, brown hagfish	GTH-like hormone		–	Uchida *et al*. 2010, Sower *et al*. 2006
Jawed vertebrates (gnathostomes)						
	teleost fish	chum salmon, sturgeon, Australian lungfish, zebrafish	GTH I	GTH II	–	Suzuki *et al*. 1988, Swanson *et al*. 1991, Laan *et al*. 2002, Quérat *et al*. 2000, 2004, Lin and Ge 2009
tetrapods	reptiles	snakes, lizards	FSH-like gonadotropin			Licht 1972a, Licht *et al*. 1979
		turtles, American alligator	+	+*	–	Licht and Papkoff 1974a, Licht 1972b, Licht *et al*. 1976
	amphibians	frogs, salamanders	+	+	–	Licht and Papkoff 1974b, Licht *et al*. 1975, Farmer *et al*. 1977
	birds	chicken, turkey	+	+	–	Stockell-Hartree and Cunningham 1969, Farmer *et al*. 1975
mammals	rodents	mouse rat	+	+	–	Harris and Jacobsohn 1952, Inoue and Kirosumi 1984
	equines	horse, donkey, zebra	+	eLH/eCG		Chopineau *et al*. 1999, Murphy and Martinuk 1991 Sherman *et al*. 1992
	primates	New World monkeys (e.g. marmoset, squirrel monkey)	+	–	+	Simula *et al*. 1995, Gromoll *et al*. 2003, Müller *et al*. 2004, Vasauskas *et al*. 2011
		Old World monkeys (e.g. macaques), great apes (chimpanzee, gorilla, orangutan), human	+	+	+	Maston and Ruvolo 2002, Hallast and Laan 2009

***** Green turtle (*Chelonia mydas*) LH shows an ability to interact with FSH receptor sites (*cross-reactivity*) and to stimulate physiological functions normally attributed to FSH.

1.1.2 Molecular basis of gonadotropins

1.1.2.1 Molecular structure of gonadotropins

Gonadotropins are members of the superfamily of cystine knot growth factors that includes also nerve growth factors, activins, transforming growth factor-β, and platelet-derived growth factor-β. Gonadotropins differ from other cystine knot growth factors in that both homodimers forming intact heterodimeric hormone are glycosylated (Sun and Davies, 1995).

Gonadotropin hormones are relatively large heterodimeric proteins each composed of α- and β-subunits that are joined by non-covalent hydrophobic and ionic interactions. α-subunit has identical amino acid sequence for each of the gonadotropins, whereas β-subunit confers a unique immunological and biological conformation and mediates the binding to the hormone's receptor (Pierce and Parsons, 1981).

Glycoprotein hormones exert their actions via the rhodopsin-like receptors belonging to the superfamily of G protein-coupled receptors (GPCRs) characterized by seven helices flanked by extracellular and intracellular domains. GPCRs family also include the β-adrenergic receptors, the rhodopsin receptor, the catecholamine receptors (Sprengel *et al*. 1990, Palczewski *et al*. 2000).

1.1.2.2 Molecular structure of follicle-stimulating hormone

Deglycosylated FSH has a molecular weight of 35 kDa and is comprised of 92 amino acid α-subunit and 111 amino acid β-subunit (Ryan *et al*. 1971, Fox *et al*. 2001). The α- and β-subunit are non-covalently associated forming an elongated slightly curved dimer (Figure 1). During dimer formation, C-terminal amino acids of the β-subunit wrap around the α-subunit and form a "seat-belt" structure important for the binding of the heterodimer to the receptor (Dias 2002, Moyle and Campbell 1996, Fan and Hendrickson 2005a, 2005b).

The α-subunit contains 10 cysteines involved in the forming of disulphide bonds within the subunit that is critical for its interaction with the β-subunit (Sato *et al*. 1997, Hiro'oka *et al*. 2000). In FSH β-subunit, three cysteine pairs (cysteines 3 and 51, 28 and 82, 32 and 84) form the cystine knot that is required for the subunit folding and heterodimer assembly (Figure 2, Fan and Hendrickson 2005b). Region between cysteines 88 and 104 has been shown to prevent binding to LH receptor, thus being responsible for the receptor binding specificity (Figure 2).

It has been established that FSH exists in circulation as a population of multiple glycoforms (isoforms) differing in carbohydrate chain composition, molecular weight and isoelectric point (pI) (Baenziger *et al*. 1988, Stockell-Hartree and Renwich 1992, Stanton *et al*. 1996). The functional heterodimeric FSH contains two N-linked glycosylation sites on the α-subunit (Asn52 and Asn78) and two carbohydrates on the β-subunit (Asn7 and Asn24) added post-translationally (Figure 1, Figure 2). The oligosaccharides that are attached to asparagine residues on the α- and β-subunits are major structural components comprising up to one third of the FSH mass (Dias 1996, Fox *et al*. 2001). It has been shown that the composition, structure and branching complexity of carbo-

hydrate chains attached to FSH αβ-heterodimer determine intracellular behaviour of the hormone (Barrios-De-Tomasi *et al*. 2002, Ulloa-Aguirre *et al*. 2003).

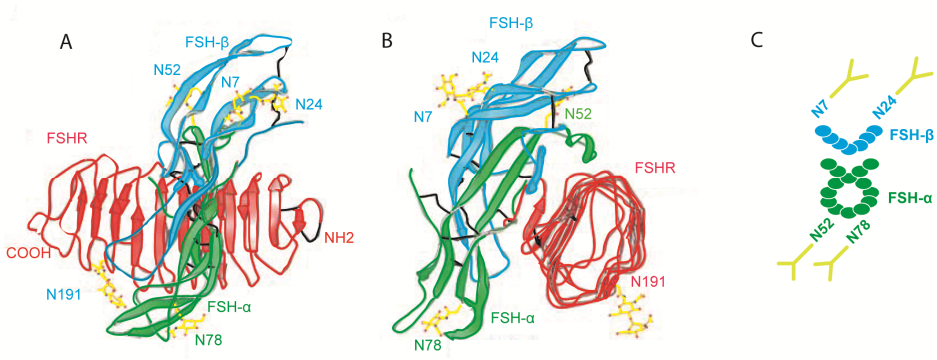


Figure 1. A, B Ribbon diagram of the human FSH bound to FSHR (Fan and Hendrickson 2005b). The structure is shown in two views related by a 90° rotation about the vertical axis. FSH α-chains and β-chains are in green and cyan, respectively. FSHR is in red. The observed N-linked carbohydrates at N52 and N78 of FSH-α, N7 and N24 of FSH-β, and N191 of FSHR are in yellow. Disulphide bonds are in black.
C Pattern of glycosylation of FSH subunits (Rozell and Okrainetz 2009).

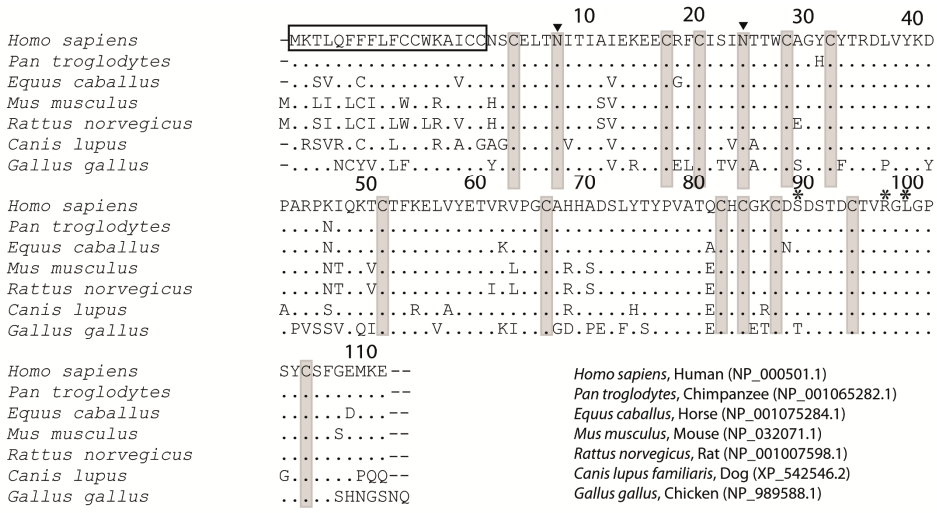


Figure 2. Amino acid sequences of the FSH β-subunits in selected vertebrate species. Positions are numbered according to mature FSH β-subunit peptide. Only those amino acid residues that are different from the human FSH β-subunit are shown. Human FSHB peptide signal sequence is *boxed*. Highly conserved cysteine (involved in disulfide bonds formation) and asparagine residues are marked by *shaded boxes*. N-linked glycosylation sites are marked by black inverted triangles. Residues implicated in binding specificity are marked by asterisks. Alignment performed with EBI ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

Human pituitary demonstrates two major FSH isoforms differing in glycosylation patterns, tetra-glycosylated and di-glycosylated FSH glycoforms. The former type has both α- and β-subunits oligosaccharides, whereas the latter possesses only α-subunit oligosaccharides (Ulloa-Aguirre *et al*. 1995, Walton *et al*. 2001). FSH α-subunit carbohydrates were shown to be essential for αβ-heterodimer stabilisation and signal transduction (Flack *et al*. 1994, Bishop *et al*. 1994, Bousfield *et al*. 2007). Non-glycosylated FSH β-subunit that doesn't have any attached carbohydrates comprised 60–65% and 50–60% of both healthy male and female (aged 21–43), and postmenopausal female (aged 71–77) total FSHβ produced in pituitary, respectively (Bousfield *et al*. 2007).

Glycosylated FSH β-subunits of tetra-glycosylated FSH isoform differ in their oligosaccharide chain structure. Oligosaccharides terminate with sialic acid and/or sulfonated *N*-acetylgalactosamine (GalNAc) that both are negatively charged. The amount of negatively charged residues has been shown to range from four to ten, whereas about 86% of FSH isoforms have 6–8 negatively charged groups (Wide *et al*. 2007). More acidic forms having increased sialyation and decreased sulfonation has been demonstrated to increase the half-life of the hormone in the circulation and reduce the bioactivity of the FSH at the target organ (Perlman *et al*. 2003, Wide *et al*. 2009). The amount of more acidic FSH isoforms has been found to be the highest in males and post-menopausal women, whereas less negatively charged, basic FSH isoforms, are more prevalent during midcycle and luteal phase of healthy women's menstrual cycle (Zambrano *et al*. 1995, Wide and Bakos 1993, Wide *et al*. 2007). Androgens have been shown to modulate the incorporation of sugar residues and favour the synthesis of acidic isoforms of FSH (Rulli *et al*. 1996).

1.1.2.3 Follicle-stimulating hormone receptor

Human follicle-stimulating hormone receptor (FSHR) shares 25–69% amino acid homology with luteinizing hormone/chorionic gonadotropin receptor (LHCGR) and thyroid-stimulating hormone receptor (TSHR), respectively. Transmembrane domains (TMs) of the glycoprotein hormone receptors display the highest homology (approx. 70%), whereas extracellular domains have lower homology (approx. 40%) (Simoni *et al*. 1997). It has been determined that receptor-ligand binding specificity is mediated by both the common α-subunit and extracellular domains of hormone-specific β-subunits (Dias and Van Roey 2001, Fan and Hendrickson 2005a).

The human FSHR consists of 695 amino acids, with the first 17 amino acid encoding a signal sequence (Gromoll *et al*. 1994, Simoni *et al*. 1997, Dias *et al*. 2002, Themmen and Huhtaniemi 2000, Fan and Hendrickson 2005b). Mammalian FSHRs demonstrate the highest amino acid homology in α-helical TMs (90%). Among species, extracellular NH_2-terminal domain and intracellular COOH-terminal segment sequence similarity ranges 80–85% (Simoni *et al*. 1997). Transmembrane helices are linked by intracellular loops (iLs). The latter structures have been shown to be crucial for G protein-coupling and signal transduction (Sairam and Babu 2007). Triple mutation introduced to iL3

affected FSHR structure and resulted in a complete inability to bind the receptor to G protein (Ulloa-Aguirre *et al*. 2007a). Large, glycosylated extracellular domain contains several leucine-rich repeats essential for ligand binding selectivity. The mutation of two amino acids in extracellular domain allows binding with LH in addition to FSH (Smits *et al*. 2003).

Hormone-receptor binding activates FSHR-bound heterodimeric G_s protein causing its dissociation into two molecules, the α-subunit and the β/γ-heterodimer. The α-subunit stimulates adenylyl cyclase that causes the elevation of intracellular cAMP, activation of protein kinase A (PKA), phosphorylation of transcriptional regulator elements including the cAMP regulatory element-binding protein (CREB), and transcriptional activation of specific genes (Means *et al*. 1976, Hunzicker-Dunn and Maizels 2006, Ulloa-Aguirre *et al*. 2007b). Additionally, cAMP-activated PKA acts on other downstream signalling pathways (e.g. mitogen-activated protein kinase (MAPK) and ERK1/2 cascades) involved in regulation of steroidogenesis, cell proliferation and survival (Pasapera *et al*. 2005).

FSH also activates cAMP-independent cellular signalling cascades, e.g. pathways involving protein kinase B (PKB), phosphoinositide 3-kinase (PI3K), Rous sarcoma oncogene (SRC) family tyrosine kinase (SFK), and other specific kinases (Gonzalez-Robayna *et al*. 2000, Brock *et al*. 2003, Wayne *et al*. 2007).

Additionally, FSH promotes phosphorylation or acetylation of histones associated with promoters of several FSH-target genes (e.g. inhibin-α, serum glucocorticoid kinase (SGK), c-FOS) causing chromosome remodelling and gene expression activation (Hunzicker-Dunn and Maizels 2006, and references therein).

1.1.3. Function and regulation of secretion of gonadotropins

1.1.3.1 The role of gonadotropins in hypothalamic-pituitary-gonadal axis

LH and FSH act synergistically to control the production of gametes and steroid hormones in both sexes. Pituitary secreted LH and FSH are measurable in the human pituitary gland as early as the 10th week of gestation and during the 12th week in peripheral blood.

In females, GnRH is secreted from the hypothalamus in a cyclical way leading to the pulsatile secretion of LH and FSH from anterior lobe of the pituitary gland, what maintains menstrual cycle. FSH targets ovarian follicle granulosa cells, stimulates the development of the ovarian follicle secretion of estradiol and progesterone. LH acts via receptors located on ovarian granulosa and theca cells and promotes the biosynthesis and secretion of androgen substrates that are converted to estradiol by granulosa cells (Figure 3). In males, GnRH causes the release of LH and FSH from the anterior pituitary, as in females. It was suggested that due to androgen exposure during embryonic development, different anatomical organization and functionality of hypothalamic GnRH system, and differential use of signal transduction pathways, the reproductive function of males demonstrates less responsiveness to GnRH pulsatility (Bliss *et al*. 2009, Bliss *et al*. 2010 and references therein). LH binds

to the receptors located on Leydig cells of the testicular interstitium and stimulates the production of testosterone that acts with FSH to regulate and maintain spermatogenesis.

The functional role of combined action of LH and FSH in gonadal development and reproduction was studied in mice deficient for GnRH (Mason *et al*. 1986) or for the common α-subunit of gonadotropins (Kendall *et al*. 1995, Stahl *et al*. 1999). Male and female mice, deficient for the α-subunit, demonstrated infertility, hypogonadism, and undetectable gonadal steroids level due to the absence of biologically active heterodimeric pituitary gonadotropins. GnRH-deficient mice never enter puberty and display a persistent hypogonadotropic-hypogonadal phenotype (Mason *et al*. 1986, Bouligand *et al*. 2010). Humans with naturally-occurring disease, Kallmann syndrome (KS), resulting from the impaired GnRH signalling due to disturbances in hypothalamic GnRH neurons development, are infertile and demonstrate hypogonadotropic hypogonadism (Bliss *et al*. 2010).

In males and females, FSH stimulates the production of gonadal inhibin, which has a negative feedback effect on the hypothalamus and pituitary (Figure 3). The sex steroids inhibit release of GnRH and therefore LH and FSH (negative feedback). However, at high levels estradiol has positive feedback effect causing an increase in LH secretion and ovulation (LH surge).

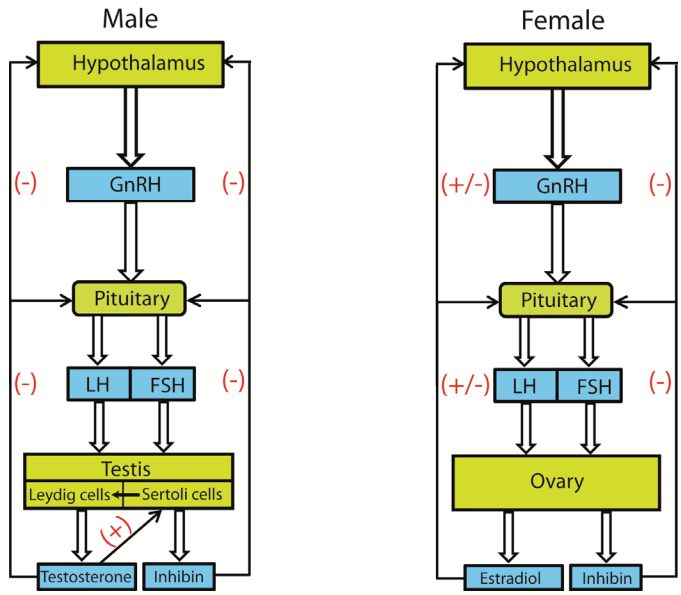


Figure 3. The hypothalamic-pituitary-gonadal (HPG) axis in males (*left panel*) and females (*right panel*). (-): negative feedback; (+): positive feedback

hCG is produced during pregnancy and its principal function is to prepare endometrium for the implantation and placentation, and to support early pregnancy (3–6 weeks of gestation) by promoting progesterone production in corpus luteal

cells (Moyle and Campbell 1996). hCG (which, being a LH analog, also stimulates testicular testosterone production) is required for masculinisation of the male fetus (Weinbauer *et al*. 2010).

1.1.3.2. The detailed function of FSH in males and females

FSH receptors are located on granulosa cells in females and on Sertoli cells in males. The necessity for FSH in females has been clearly established both in animal and human models. In female mammals, FSH stimulates the development of follicles that have resumed meiosis. During early stages of folliculogenesis, FSH is essential to prevent granulosa cells apoptosis and to stimulate granulosa cell proliferation, estradiol production and LH receptor expression (Chun *et al*. 1996, Richards 1994). Expression of several gene products including aromatase and inhibin-α have been shown to be induced by cAMP/PKA/CREB pathway in granulosa cells (Lambard *et al*. 2005, Andrieu *et al*. 2009). Granulosa cells respond to FSH by up-regulating estrogen-metabolizing aromatase CYP19A1 and 17-hydroxysteroid dehydrogenase (HSD17B1) resulting in increased estradiol synthesis. The estrogen rise via hypothalamic-pituitary feedback mechanisms leads to the release of LH (LH surge), terminating of preovulatory follicle growth, and initiation of ovulation (Edson *et al*. 2009).

FSH β-subunit as well as FSH receptor-deficient female mice phenotype is similar to human ovarian dysgenesis syndrome that have arisen due to a defective FSH-receptor signalling (Aittomäki *et al*. 1995, 1996). Female mice lacking FSH exhibited infertility, small ovaries, block in folliculogenesis, defects in granulosa cell proliferation, and suppressed aromatase secretion (Kumar *et al*. 1997, Abel *et al*. 2000, Huhtaniemi 2006). FSH β-subunit deficient mice ovaries contained all early developmental stages of follicles, responded to exogenous gonadotropins and produced oocytes (Kumar *et al*. 1997, Burns *et al*. 2001).

In contrast, FSH β-subunit deficient male mice were fertile, although they displayed decreased Sertoli cell number, reduced testes size, sperm count and motility (Kumar *et al*. 1997, Wreford *et al*. 2001). FSH acts as a primary mitogen on Sertoli cells stimulating the mitotic proliferation of immature Sertoli cells during fetal and early neonatal development. In the rat, suppression of FSH levels during this period of development resulted in a reduction of Sertoli cell numbers and thereby testicular size, and this effect was reversed by administration of FSH (Means *et al*. 1976, Orth *et al*. 1988). In addition to the direct effect on reproductive function via Sertoli cells, FSH regulates the production of different mitogens (e.g. growth factors, cytokines) that have stimulatory effect on Leydig cells growth, activity and survival (O'Shaughnessy *et al*. 1992, Matikainen *et al*. 1994, Baker *et al*. 2003). In adults, combined action of FSH and LH/testosterone is needed to support spermatogenesis and full fertility. Both in monkeys and adult human males, long-term immunization against FSH caused suppression of sperm production and testicular regression (Moudgal *et al*. 1992, Moudgal *et al*. 1997, Krishnamurthy *et al*. 2000). Treatment with recombinant FSH induces prepubertal testis growth in human prepubertal males exhibiting hypogonadotropic hypogonadism (Raivio *et al*. 2007).

In addition to the direct effect of FSH on fertility potential through FSH-specific receptors located on the plasma membrane of the Sertoli cells and granulosa cells, recent studies in the ovary have suggested that circulating pituitary gonadotropins (LH and FSH) and testosterone mediate the activity of hormonally sensitive micro-RNAs thereby having an effect on postranscriptional gene regulation (Fiedler *et al*. 2008, Yao *et al*. 2009). Furthermore, it was established that FSH plays a key role in the regulation of apoptotic pathways during spermatogenesis supporting survival of spermatogonia and sperm release (spermiation) in normal adult men (van Alphen *et al*. 1988, Ruwanpura *et al*. 2008)

Additionally, it was shown that FSH contributes to the genesis of peri-menopausal and early post-menopausal bone loss by either direct binding to FSH receptors on osteoclasts or mediating the production of bone-modulating factors by immune cells (Sun *et al*. 2006, Iqbal *et al*. 2006, Sun *et al*. 2010). However, other studies failed to detect any expression of FSH receptors in bone preparations and showed that elevated FSH rather increases bone mass in female mice (Allan *et al*. 2010). No direct effect of FSH on male bone mass was observed (Ritter *et al*. 2008).

1.1.3.3. Clinical conditions related to abnormal FSH production/level

The measurement of serum levels of FSH and LH in combination with testosterone is widely used in the diagnosis of development and reproduction disturbances. FSH level determination is primarily used for assessment of fertility potential and gonadal function. A normal serum FSH level suggests normal gonadal function.

An extremely low or undetectable serum FSH concentration both in males and females may mirror a congenital problem at the level of either hypothalamus or pituitary. Patients with isolated hypogonadotropic hypogonadism (IHH) and Kallmann syndrome (KS) exhibit hypogonadism due to disturbed hypothalamic secretion or action of GnRH resulting in impaired secretion of pituitary gonadotropins, FSH and LH (Behre *et al*. 2010). Other congenital pathophysiological conditions featuring low FSH concentrations include Prader-Willi syndrome, fertile eunuch syndrome, and combined pituitary hormone deficiency due to rare mutation in *PROP1* gene (Behre *et al*. 2010, Wu *et al*. 1998). Tumor of the pituitary gland may also be a cause of low serum FSH levels.

The margins of male and female physiologically normal FSH levels change throughout the life periods. Before and during puberty, the concentrations of serum FSH are comparable in males and females, and range 0–5.0 IU/mL and 0.3–10.0 IU/mL, respectively (Andersson *et al*. 1997). In adult males, normal concentration of serum FSH is ≤ 7 IU/L (following Ahda *et al.* 2005). However, one should consider other diagnostic indicators such as testes volume, sperm concentration and sex hormone ratios. High FSH levels in the presence of small (<6 mL) firm testes and azoospermia are characteristics of Klinefelter syndrome (47, XXY) (Simoni and Nieschlag 2010). Aging men exhibit the age-related changes in testicular spermatogenic function accompanied by a compensatory

increase (up to two-fold) in FSH level allowing for Sertoli cell function and spermatogenesis (Johnson 1989, Mahmoud *et al*. 2003, Rolf *et al*. 2010).

In women of reproductive age, normal serum FSH levels vary considerably during different times of the menstrual cycle (4–13 IU/L) being the highest during follicular phase and late luteal phase. Polycystic ovary syndrome (PCOS) characterized by subnormal FSH levels represents a disruption of cyclic ovarian function with unbalanced LH to FSH ratio and excessive androgen production (Knuth 2010, Goodarzi *et al*. 2011). Congenital developmental issues leading to elevated FSH levels include developmental failure or absence of one or both ovaries (ovarian agenesis) and Turner's syndrome (45, X). Women with normally induced perimenopause and menopause as well as patients exhibiting premature ovarian insufficiency have elevated FSH levels due to impaired ovarian response to the FSH-stimulation (Burger *et al*. 2008, Nelson 2009, Knuth 2010).

FSH-secreting pituitary gonadotroph tumors may also be a cause of elevated FSH level accompanied by hypopituitarism, headaches and visual changes both in females and males (Young *et al*. 1996, Chaidarun and Klibanski 2002). Gonadotrope adenomas (gonadotropinomas) primarily produce heterodimeric FSH but may also secrete high levels of α-subunit and free β-subunits. Since unassociated FSH subunits don't exhibit biological activity, adenomas secreting free subunits usually show no biological activity (Melmed 2008, Behre *et al*. 2010). A few reports of gonadotropinomas secreting intact FSH and LH describe various clinical fertility phenotypes including precocious puberty and increased sperm counts in 7-year-old boy along with increased testes volume, infertility and hypogonadism in adult males (Young *et al*. 1996, Heseltine *et al*. 1989, Zárate *et al*. 1986, Ambrosi *et al*. 1990). Women diagnosed with FSH-secreting adenomas present with ovarian hyperstimulation syndrome (OHSS), menstrual cycle disturbances, low LH levels, and either normal or elevated estradiol levels (Shimon *et al*. 2001, Roberts *et al*. 2005, Cooper *et al*. 2008, Baba *et al*. 2009, Gryngarten *et al*. 2010).

1.2. The genetics of gonadotropins (partially covered in Ref. I)

1.2.1. Gonadotropin α-subunit coding gene (*CGA*)

In human, the common α-subunit of glycoprotein gonadotropin hormones is encoded by a single gene (*CGA,* MIM 118850, 6q12–q21) spanning a region of 9603 basepairs. In some species of fish two types of α-subunit have been identified that are coded by different genes. The organization of both α-subunit coding genes was identified to be similar to the mammalian α-subunit coding genes (Itoh *et al*. 1990, Huang *et al*. 1992).

No human germ-line mutations in the *CGA* gene encoding for the common α-subunit have been described. One mutant form of the α-subunit was described that was ectopically secreted by a patient with carcinoma (Nishimura *et al*. 1986). The mutant form co-existed with functional form of α-subunit and had

one amino acid substitution in exon 3 (Glu56Ala). The mutation resulted in higher molecular weight and structural changes that lead to incapability of combining with at least LH β-subunit.

1.2.2. Follicle-stimulating hormone β-subunit coding gene

FSH β-subunit coding gene is highly conserved across species (Figure 4). Molecular phylogenetic analyses of mammalian *Fshb* genes revealed five regions of highly conserved sequence homology: the proximal 5' promoter region, exon 2, the 5' translated region of exon 3, and two regions at the 3' untranslated end of exon 3 that include putative polyadenylation and transcriptional termination signals (Figure 4, Kumar *et al*. 2006). Human *FSHB* gene genomic sequence shares 98% and 85% nucleotide similarity with chimpanzee (*Pan troglodytes*) and mouse (*Mus musculus*), respectively (Figure 4). Nucleotide identity with zebrafish (*Danio rerio*) is 55%.

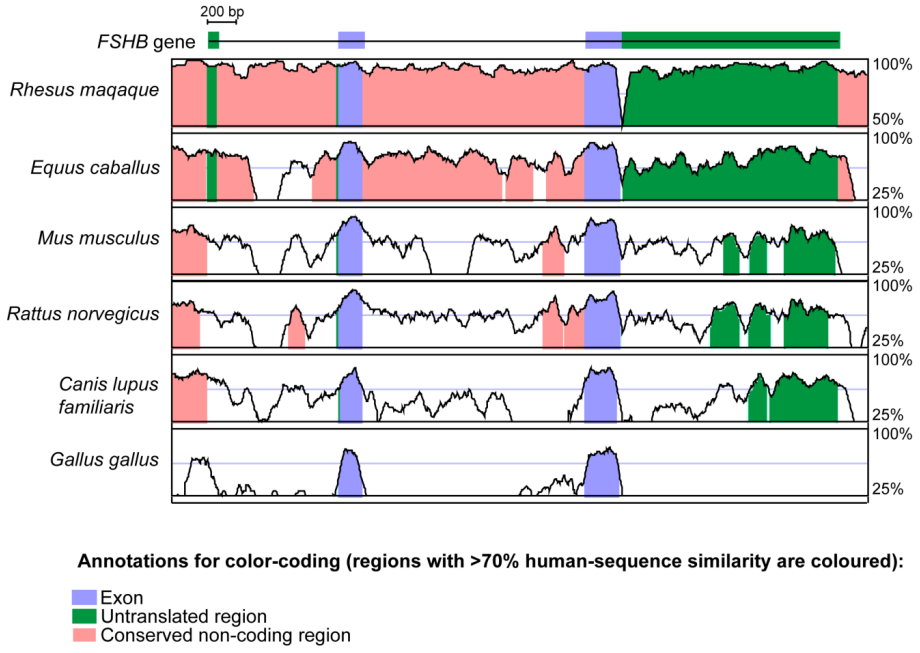


Figure 4. Comparative alignment of FSH β-subunit gene sequence from selected vertebrate species. Only regions with sequence similarity >70% are coloured. Reference sequence is human FSH β-subunit coding gene (GenBank genomic sequence accession number NC_000011.9). Horse, *Equus caballus*, NC_009150.2; Mouse, *Mus musculus*, NC_000068.6; Rat, *Rattus norvegicus*, NC_005102.2; Dog, *Canis lupus familiaris*, NC_006603.2; Chicken, *Gallus gallus*, NC_006092.2. Alignment and visualization were performed with Genome Vista tools (http://genome.lbl.gov/vista/index.shtml). The identity curve is calculated as a windowed-average identity score for the alignment. A 100 basepair-sized window is slid across the alignment and a score is calculated at each base in the coordinate sequence. Thus, the score for every point along the axis is the percentage of exact matches between the two alignments in a 100 bp-wide window centred on that point.

The genomic organization of the FSH β-subunit coding gene conforms to the pattern observed for other glycoprotein hormones β-subunit genes, and contains three exons and two introns (Figure 5). The similar exon-intron structure has been reported in mammals, birds and teleosts (Jameson *et al*. 1988, Gharib *et al*. 1989, Guzman *et al*. 1991, Kim *et al*. 1988, Kumar *et al*. 1995, Rosenfeld *et al*. 2001, Kawasaki *et al*. 2003).

Human FSH β-subunit coding gene (*FSHB*, MIM 136530, genomic length 4262 bp) is located on chromosome 11p13 (Figure 5, Watkins *et al*. 1987). This genomic region is characterized as AT-rich and gene-poor region. The average GC-content in *FSHB* gene is approximately 36%, what is 20% lower compared to the average GC-content obtained from the analysis of 74 human genes (45%) (Crawford *et al*. 2004). *FSHB* GC-content varies from 43–52% in exonic regions to 30–33% in introns. The closest genes to *FSHB*, *C11orf46* and *KCNA4*, are located 89.3 kilobases downstream and 214 kilobases upstream of the *FSHB* gene, respectively.

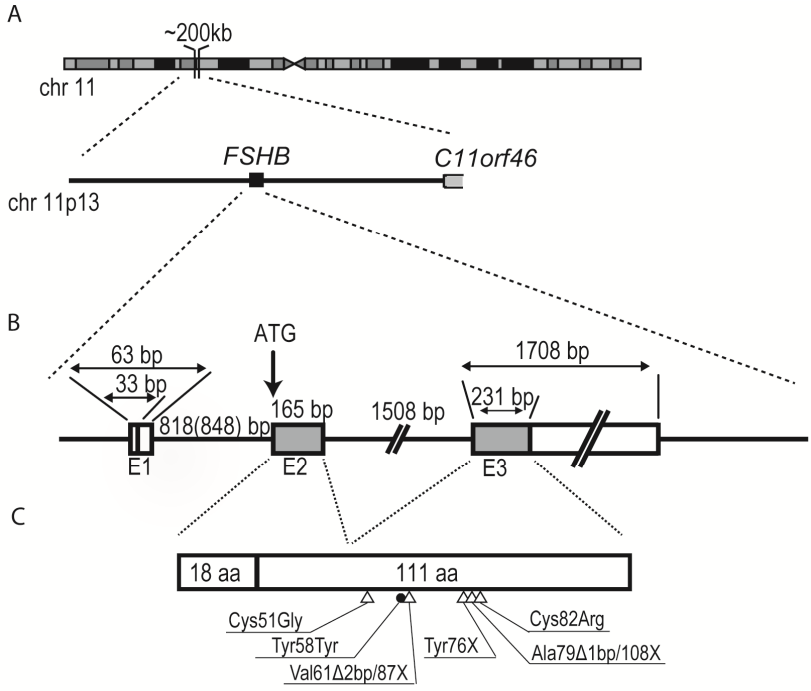


Figure 5. Schematic representation of the (**A**) genomic context, (**B**) structure of the *FSHB* gene and (**C**) FSH β-subunit protein. Grey and white boxes indicate translated and untranslated parts of exons E1-E3, respectively. FSH β-subunit protein non-synonymous changes causing clinical consequences are indicated with open triangles and polymorphism with a distinct phenotype is marked with black circle (detailed explanation see from text and Table 4).

Differently from other members of glycoprotein hormone β-subunit gene family, the *FSHB* gene structure allows alternative splicing and polyadenylation to produce four forms of *FSHB* mRNA (Jameson *et al*. 1988). Untranslated exon 1 contains an alternate splicing donor site which cause transcripts to have different lengths of 5' untranslated regions (5' UTR). It was demonstrated that approximately 35% of *FSHB* transcripts have 33 bases in their 5'UTR and 65% of transcripts have 63 bases in the 5'UTR. In addition to the differences in 5' UTR composition, there are also differences in the length of poly-A tail at the 3' untranslated region of the *FSHB* gene where four consensus polyadenylation signals (AAUAAA) were predicted. One polyadenylation signal coincides with the stop codon in exon 3, three additional sites is located 1–1.2 kilobases downstream of the stop codon resulting in longer 3' untranslated region (UTR) that is absent in other members of glycoprotein hormone β-subunits gene family (Figure 4, 5). Approximately 80% of transcripts have long poly-A tail as a result of the usage of the most distal polyadenylation signal, whereas 20% of transcripts have short or no poly-A tail (Jameson *et al*. 1988, Manjithaya and Dighe 2004). Different lengths of the *FSHB* mRNA transcripts may affect translational binding with the ribosome and, consequently, protein synthesis and also the stability of the transcripts within the gonadotrophs (Jameson *et al*. 1988, Stanton *et al*. 2000).

1.2.3. Genetic variation pattern of the human *FSHB* gene

1.2.3.1. Human *FSHB* gene common diversity

Although multiple animal and naturally occurring human models have indicated that FSH and FSH β-subunit gene are essential for full fertility, there are few studies on *FSHB* gene sequence inter- and intrapopulational genetic diversity as well as possible associations of common variants with phenotypic traits.

In the study focused on the proximal part (–489 bp relative to transcription start-site) of the promoter and translated area of the *FSHB* gene, three silent exonic and two intronic polymorphisms were identified (Lamminen *et al*. 2005). The rarity of polymorphisms in the *FSHB* gene region is consistent with central roles of *FSHB* gene in reproduction. In contrast to *FSHB*, in the genes coding for LH and hCG β-subunits (*LHB/CGB* gene cluster), the high density of polymorphic position was detected (e.g. *LHB* gene, 1541 bp, 12 SNPs).

Altogether, 27 nucleotide substitutions within the entire *FSHB* gene genomic sequence have been identified so far (Table 2, Table 3). Most of them localize to non-coding regions, introns and 3'-untranslated region of exon 3 (Table 2). Interestingly, no nucleotide substitutions have been mapped to *FSHB* exon 1. Three silent substitutions within the coding region of the gene have been identified (Table 3). Although synonymous changes do not affect protein sequence, they may co-exist with regulatory sequence variations. *FSHB* rs6169 located in exon 3 (+2623 bp from TSS, C/T, 58 Tyr ⇒ Tyr) has been suggested to be associated with polycystic ovarian syndrome (PCOS) on obese Chinese women (Liao *et al*. 1999, Tong *et al*. 2000). Allele frequency distribution of this

polymorphism showed population-specific differences ranging from 33.3% to 59.5% in Malays and Danish populations, respectively (Liao *et al*. 1999, Lamminen *et al*. 2005). Recently, in a large-scale gene association study focused on the genes related to steroid-hormone metabolism and breast cancer risk, *FSHB* rs6169 showed no association with susceptibility to the malignancy of mammary gland (Canzian *et al*. 2010). However, in another candidate gene association study, the same position was significantly associated with age at menarche (He *et al*. 2010).

Table 2. Known genetic variants within untranslated regions of the *FSHB* gene (intron 1, intron 2, exon 3 3'UTR) based on dbSNP Build 132 (http://www.ncbi.nlm.nih.gov/projects/SNP/). Nucleotide positions are defined according to the location of the transcription start-site in the genomic DNA sequence (NC_000011.9).

Location	rs number	Nucleotide substitution	Location	rs number	Nucleotide substitution
intron 1	613058	G342T	intron 2	78130864	A1778G
	118067137	A595G		77193389	C1792T
	35298877	A597T		34259552	T1847–
	34394185	A605T		74566831	A1870G
	550312	T627G		595496	T1988C
	611246	T756A		594982	C2121A
	111823008	T840C		112303897	A2176G
intron 2	35235566	G1047–		76152907	G2218T
	609896	T1079C		115600568	C2460T
	80319222	A1145C		506197	G3261A
	35536959	T1430G	exon 3 3'UTR	506306	G3305C
	34279061	A1476G		676349	A3420G
	34922768	C1600T		78946483	T3733G
	580646	T1706G		75464895	G3794A

–, deletion

1.2.3.2. *FSHB* gene mutations with clinical phenotype

Genetic alterations in the structure of FSH β-subunit have dramatical effects on fertility further supporting the critical roles of FSH. As yet, only five naturally occurring inactivating mutations of *FSHB* gene have been reported in three male and six female patients. Three mutations in exon 3–Val61Δ2bp/87X, Tyr76X, and Ala79Δ1bp/108X–lead to a premature stop codon and truncation of the FSH β-subunit protein (Figure 5, Table 3) (Matthews *et al*. 1993, Layman *et al*. 1997, Phillip *et al*. 1998, Berger *et al*. 2005, Lofrano-Porto *et al*. 2008, Kottler *et al*. 2010). Two other mutations identified in exon 3, Cys51Gly and Cys82Arg, result in a loss of a cysteine residue that prevent a formation of a cysteine knot structure within the β-subunit peptide (Figure 5, Table 3) (Layman *et al*. 1997, Lindstedt *et al*. 1998).

Patients homozygous for *FSHB* gene mutations exhibited absent or incomplete pubertal development and infertility due to the absence of heterodimeric FSH. Male and female patients demonstrated azoospermia and lack of follicular growth and differentiation, respectively. However, compound heterozygous mutation (Val61X/Cys51Gly) carrier's relatives that were heterozygous for the mutation Cys51Gly were fertile, indicating that a single normal copy/ allele of *FSHB* gene is sufficient to produce biologically active FSH (Layman *et al*. 1997).

One non-synonymous nucleotide substitution within *FSHB* exon 2 with unknown phenotypic consequence was identified (rs6170, G/T, Ser/Ile; Cargill *et al*. 1999).

Table 3. Non-synonymous genetic variants in the translated part of the *FSHB* gene. Nucleotide positions are defined according to the location of the transcription start site in the genomic DNA sequence (NC_000011.9). Amino acid positions are defined based on the sequence of mature protein (NP_000501.1). Non-synonymous inactivating mutations are indicated in bold.

Location	rs number	Nucleotide substitution	Codon substitution	Amino acid substitution	Reference
exon 2	6170	G946T	AGC ⇒ ATC	2 Ser ⇒ Ile	Cargill *et al*. 1999
exon 3	36206707	G2460A	AAG ⇒ AAA	40 Lys ⇒ Lys	Lamminen *et al*. 2005
	5030776	T2600G	TGT ⇒ GGT	**51 Cys ⇒ Gly**	Layman *et al*. 1997
	6169	T2623C	TAC ⇒ TAT	58 Tyr ⇒ Tyr	Liao *et al*. 1998
	5030646	TG2631–	GTG ⇒ G	**61 Val fs ⇒ 87 X**	Matthews *et al*. 1993, Layman *et al*. 1997, Phillip *et al*. 1998
	121909666	C2677A	TAC ⇒ TAA	**76 Tyr ⇒ 76 X**	Layman *et al*. 2002, Lofrano-Porto *et al*. 2008, Berger *et al*. 2005
	na	G2684–	GCC ⇒ CC	**79 Ala fs ⇒ 108 X**	Kottler *et al*. 2010
	5030777	T2693C	TGT ⇒ CGT	**82 Cys ⇒ Arg**	Lindstedt *et al*. 1998
	34365964	G2707A	AAG ⇒ AAA	86 Lys ⇒ Lys	Lamminen *et al*. 2005

–, deletion; X, translational stop-codon; fs, frameshift; na, rs# is not available in the database

1.2.4. The regulation of the *FSHB* gene expression

Follicle-stimulating hormone and luteinizing hormone are both produced by gonadotropic cells within the anterior lobe of pituitary gland. The production of gonadotropin common α-subunit and hormone-specific β-subunits (LHβ and FSHβ), and secretion of mature pituitary gonadotropins are controlled by

synchronized pulses of gonadotropin-releasing hormone (GnRH) produced by hypothalamic neurons (Pawson and McNeilly, 2005). Since α-subunit is produced in excess of gonadotropin β-subunits, the expression level of the latters is rate-limiting factor for pituitary gonadotropin hormones production kinetics (McNeilly *et al*. 2003).

It was determined that the secretion of α-subunit and LH β-subunit is the highest at GnRH pulse frequency 8 and 60 min, respectively, while lower pulse-frequency (pulse interval 2 hours) of GnRH results in a decline in LHβ and rise in FSHβ production (Haisenleder *et al*. 1991, Kaiser *et al*. 1995, Ferris and Shupnik 2006, Lim *et al*. 2009). GnRH acts via its receptor (GnRHR) located on gonadotroph membranes and causes activation of G proteins, release of calcium from intracellular stores, and increase in the activity of protein kinase C (PKC). As a result, three mitogen-activated protein kinase (MAPK) pathways are activated (ERK, jun-N-terminal kinase (JNK) and p38). Different activity combinations of these MAPK pathways result in differential activation of α-subunit, LHβ, and FSHβ coding genes expression (Vasilyev *et al*. 2002, Lim *et al*. 2009, Armstrong *et al*. 2010). MAP kinases activate different DNA *cis*-elements by phosphorylating their cognate transcription factors including activator protein-1 (AP-1) and cyclic AMP response element-binding protein (CREB) that are bound to GnRH responsive elements within the proximal part of gene promoter (Figure 6) (Wang *et al*. 2008, Ciccone *et al*. 2008, 2010; Bliss *et al*. 2010).

Additionally, epigenetic mechanisms of GnRH signalling have been suggested in embryonic gonadotroph precursor cells. Histone modifying enzymes, histone deacetylases (HDACs), occupy FSHβ coding gene promoter repressing expression. GnRH causes expression of *Nur77* gene product that phosphorylates and eliminates HDACs, thereby, inducing FSHβ gene expression (Lim *et al*. 2007).

Other *cis*-elements within FSHβ coding gene promoter were shown to bind steroidogenic factor-1 (SF-1), nuclear transcription factor Y (NFY), activin, steroid hormone receptors (Figure 6) (Thackray *et al*. 2006, Melamed 2010, Bernard *et al*. 2010, and references therein). Activin causes phosphorylation and binding of SMAD proteins to *Sma- and Mad-related protein-binding elements* (SBE-like 1 and SBE-like 2) within human *FSHB* promoter. This causes the induction of *FSHB* gene activity (Graham *et al* 1999, Bernard 2004, Suszko *et al*. 2005). Pituitary produced follistatin and gonadal inhibin both are able to repress the FSHβ coding gene expression by binding to activins and their receptors, respectively (Bernard *et al*. 2010 and references therein).

FSH β-subunit gene expression has been shown to be regulated by three steroid hormone receptors (glucocorticoid receptors, GR; progesterone receptor, PR, androgen receptor, AR) that bind to hormone response elements (HRE) located within FSH β-subunit gene promoter (Figure 6) (Burger *et al*. 2004, Thackray *et al*. 2006, 2010). Combination of steroid hormone with its receptor allows the phosphorylation and activation of the receptor, binding of the complex to HRE, and activation of transcriptional factors that alter gene

transcription. Single human polymorphic variant, rs10835638 G/T (–211 bp from TSS, Figure 6), located within one of the HREs, HRE3, has been shown to be responsible for differential regulation of human *FSHB* expression *in vitro* (Webster e*t al.* 1995, Hoogendoorn *et al*. 2003).

Ligand-independent mechanism of steroid hormone receptor activation has been also reported. Alternative mechanism involves peptide growth factors, cAMP-dependent protein kinase, and cyclins that phosphorylate and activate steroid hormone receptors independently of steroid hormone action (Power *et al*. 1991, Cenni and Picard 1999, Levine *et al*. 2001). Gonadal steroids exert their actions also at the level of hypothalamus, by the regulation of GnRH secretion (reviwed in Melamed 2010, Bernard *et al*. 2010).

Steroid hormones were also reported to modulate FSH β-subunit gene postranscriptionally by altering the stability of mRNA. In contrast to other members of the glycoprotein family, FSHβ mRNA has a long 3'UTR that was identified to regulate FSH β-subunit gene expression (Jameson *et al*. 1988, Kumar *et al*. 2006). Presence of long 3'UTR in bovine *Fshb* mRNA was shown to decrease association with translational ribosomal complex (Manjithaya and Dighe 2004). The phylogenetic analysis of FSH β-subunit coding gene 3'-flanking region revealed high level of conservation (up to 90%) among mammalian species (Figure 4). Gonadotroph-specific expression of human *FSHB* gene with truncated 3'-flanking sequence demonstrated the presence of genetic elements required for efficient gene expression (Kumar *et al*. 2006). Several AU-rich elements located within 3'UTR of FSH β-subunit encoding gene are capable of interacting with various *trans*-factors including steroid hormone receptors and ELAV family proteins (e.g. HuR protein) (Peng *et al*. 1998, Ing 2005). Additionally, AU-rich regions form complex stem-loop structures which may affect protein expression (Samaddar *et al*. 1999).

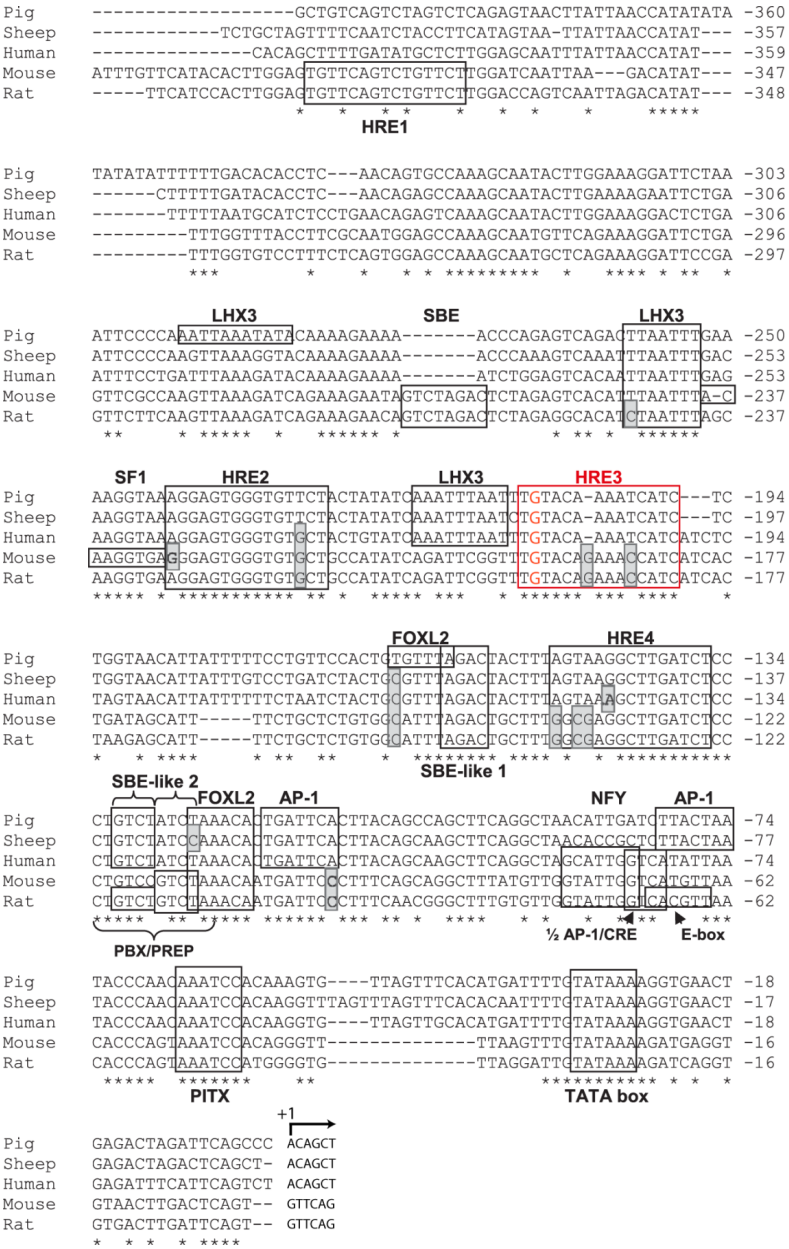


Figure 6. The alignment of proximal part (400 bp from the transcription start-site) of *Fshb/FSHB* promoters in pig, sheep, human, mouse, and rat. Transcription start-site is marked with horizontal arrow. The *Fshb/FSHB* gene promoter *cis*-elements (described in the text) are labeled and boxed. Nucleotides boxed in gray reflect differences from the experimentally defined *cis*-element. Hormone Response Element (HRE3) marked with red box and position –211 G were studied further in this thesis. Promoters were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Modified from Bernard *et al*. 2010.

2. AIMS OF THE PRESENT STUDY

The present thesis represents a focused research on the follicle-stimulating hormone β-subunit coding gene (*FSHB*). Specific aims of the study are as follows:

- to survey the fine-scale genetic variation patterns of the *FSHB* gene in human worldwide populations (Estonians, Czechs, Utah Mormons/ CEU, Mandenkalu, Han Chinese, Koreans)

- to determine the ancestral *FSHB* gene variant through comparative genomics approach using sequencing of the *FSHB* gene in great apes (chimpanzee, gorilla, orangutan)

- to conduct a pilot-study investigating whether the identified human *FSHB* gene haplotypes may have functional consequence on female reproductive health

- to perform *in silico* polymorphism screening in evolutionarily conserved 5'- and 3'-flanking regions of the *FSHB* gene in order to identify putative polymorphic regulatory variants

- to perform quantitative genetic association analyses between *FSHB* promoter polymorphism (–211 G/T, rs10835638) and male reproductive parameters in the population-based Baltic cohort of young men (Estonians, Latvians and Lithuanians) and Estonian cohort of males diagnosed with infertility.

3. RESULTS

3.1. The fine-scale sequence diversity of the follicle-stimulating hormone β-subunit coding gene (*FSHB*) (Ref. I, II)

3.1.1. The variation pattern of the *FSHB* gene across populations originating from three continents

In order to determine the human population variation of the *FSHB* gene, I have studied six populations originated from Europe (Estonians, Czechs, Utah Mormons/CEU), Africa (Mandenkalu), and Asia (Han Chinese, Koreans) (Table 4). The *FSHB* gene genomic sequence (2909 bp) was re-sequenced in Estonians, Mandenkalu and Han Chinese. Czechs, Koreans and Utah Mormons/ CEU were studied by combining partial re-sequencing and Restriction Fragment Length Polymorphism (RFLP) analysis approaches (Table 4).

Table 4. Methodology, analyzed region of the *FSHB* gene, and population samples used in the study of the *FSHB* gene fine-scale variation.

Method	Analyzed region description	Population sample	No of individuals
full re-sequencing	2909 bp (+407...+3316 bp relative to TSS): 456 bp of intron 1, translated parts of exonic regions (exon 2, 165 bp; exon 3, 231 bp), intron 2 (1508 bp), 549 bp of exon 3 3'-UTR	Estonians (Europe)[a]	48
		Mandenkalu (Africa)[b]	24
		Han Chinese (Asia)[b]	25
combined approach	(i) partial re-sequencing (748 bp, +407…+1155 bp relative to TSS): 456 bp of intron 1, exon 2 (165 bp), 127 bp of exon 2, and	Czechs (Europe)[c]	50
		Korean (Asia)[d]	45
	(ii) RFLP analysis (rs594982, position +2121; rs6169, position +2623)	unrelated Utah Mormons/ CEU (Europe)[b,e]	30

TSS, transcription start-site

[a] Anonymous population-based cohort originated from the DNA bank of the Department of Biotechnology, Institute of Molecular and Cell Biology, Tartu University, Tartu, Estonia

[b] Samples were obtained from the HGDP-CEPH Human Genome Diversity Cell Line Panel (http://www.cephb.fr/HGDP-CEPH-Panel/ ; Cann *et al*. 2002).

[c] Samples shared by Dr. Viktor Kozich (Charles University First Faculty of Medicine, Institute of Metabolic Disease)

[d] Samples shared by Dr. Woo Chul Moon (Good-Gene Inc. Seoul, Korea)

[e] Unrelated Utah residents (USA) with Northern and Western European ancestry (http://www.cephb.fr/HGDP-CEPH-Panel/; Cann *et al*. 2002).

No non-synonymous changes have been identified within translated part of the *FSHB* gene in the analysed individuals. In total, 2909 bp re-sequenced region contained 12 SNPs (SNP density = 4 per kb): seven common polymorphisms (MAF>10%) spread in all populations and five rare singleton variants present on a single chromosome in a population (Table 5; Figure 7A; Supplementary Tables S2, S3 in Ref. II). Most identified polymorphisms were located within non-coding gene regions, two synonymous changes (T2623C, rs6169; G2707A, rs34365964) were found in translated part of exon 3 (Figure 7A). Additionally to common nucleotide changes, five rare nucleotide variants that were identified in only one population sample but absent in others have been identified. Exonic SNP rs34365964 detected in Estonian population in the current study has also been identified in heterozygote status in 8% of Finnish individuals (Lamminen *et al*. 2005). Intronic rs34279061 present in one Mandenkalu individual was further confirmed by the pilot-data from The 1000 Genomes Project (http://www.1000genomes.org/). Sequence variation data from the study was submitted to dbSNP under accession numbers ss49785048–ss49785060.

Table 5. Minor allele frequencies (MAF) of the *FSHB* gene SNPs identified in the current study.

Nucleotide change[a]	Location	dbSNP[b] rs no	Minor allele frequency (MAF, %)					
			Estonia (n=48)	Mandenka (n=24)	Han (n=25)	Korea (n=45)	Czech (n=50)	Utah/ CEU (n=30)
A597T	intron 1	35298877	–	–	S	–	–	–
A605T	intron 1	34394185	–	S	–	–	–	–
G627T	intron 1	550312	44.7	37.5	24.0	36.7	51.0	70.0
T756A	intron 1	611246	42.6	33.3	20.0	35.6	52.0	70.0
C1079T	intron 2	609896	44.7	37.5	18.0	32.2	52.0	70.0
T1430G	intron 2	35536959	–	–	S	na	na	na
A1476G	intron 2	34279061	–	S	–	na	na	na
G1706T	intron 2	580646	43.6	35.4	18.0	na	na	na
C1988T	intron 2	595496	44.7	37.5	20.0	na	na	na
C2121A	intron 2	594982	41.5	27.1	18.0	32.2	52.0	70.0
T2623C	exon 3	6169	45.7	16.7	18.0	25.6	50.0	70.0
G2707A	exon 3	34365964	S	–	–	na	na	na
G3261A*	exon 3 UTR	506197	na	na	na	na	na	na

[a] Relative to transcription start-site (GenBank Accession No. NC_000011.9, Appendix 3).
[b] dbSNP Build 132 (http://www.ncbi.nlm.nih.gov/snp)
*The position was identified in the Estonian, Mandenka and Han Chinese populations, but allele frequencies were not reliably determined.
S = polymorphism present in single individual; n = no of individuals; na = not analyzed; '–' = SNP not identified

Two estimators of nucleotide diversity have been calculated for Estonian, Mandenka and Han Chinese populations: (i) π, the direct estimate of per-site heterozygosity derived from the observed average pairwise sequence difference

among individuals and (ii) Watterson's θ, per-site heterozygosity based on the number of segregating sites. Among the studied populations, the *FSHB* gene nucleotide diversity (π) ranged from 0.00079 and 0.00123 (Table 6). *FSHB* gene overall diversity is comparable to human genome average based on the analysis of 1.42 million SNPs (π~0.00075) (Sachidanandam *et al*. 2001) and average per-site nucleotide diversity obtained from the re-sequencing of 74 genes (π=0.0083) (Crawford *et al*. 2004), but is up to six-fold lower when compared to the results of *LHB/CGB* genes (Hallast *et al*. 2005).

To investigate whether observed patterns of the *FSHB* gene diversity are consistent with the neutral model of molecular evolution (Kimura 1983), Tajima's D (D^T) (Tajima 1989), Fu and Li's D (D^{FL}), and Fu and Li's F (F^{FL}) (Fu and Li 1993) were calculated (Table 6). Tajima's D is the difference between π and θ estimates. In case of neutrality, π equals θ, and thus D^T statistic equals zero. Significant positive values of D^T and F^{FL} in Estonian and Mandenka populations (Estonians, D^T=3.22, F^{FL}=2.397; Mandenkalu, D^T=2.52; F^{FL}=1.938) signify excess of high-frequency SNPs and heterozygotes, indicating the possible balancing selection and/or population subdivision.

Table 6. *FSHB* nucleotide diversity parameters and neutrality tests.

	Estonians	Mandenkalu	Han Chinese
Sample size	47	24	25
Diversity estimates and neutrality tests			
π^1	0.00123	0.00109	0.00079
θ^2	0.00048	0.00056	0.00055
Tajima's D (D^T)	3.224**	2.523*	1.130
Fu and Li's D (D^{FL})	1.198	1.244	1.242
Fu and Li's F (F^{FL})	2.397*	1.938*	1.416

Estimation of per-site heterozygosity derived from
[1] the average pairwise sequence difference among individuals and
[2] the number of segregating sites *per* 1 bp (Watterson 1975)
Significance of the statistical test: * $p<0.05$, ** $p<0.01$

Pairwise linkage disequilibrium (LD) is the non-random association of alleles at two chromosomal loci. The amount of LD depends on the difference between observed and expected allele frequencies and is influenced by different factors including genetic linkage, selection, recombination and mutation rate, genetic drift, and population structure (Hudson *et al*. 2001, Pritchard and Przeworski 2001). Two types of statistics can be applied to measure the extent of LD between pairs of loci: (i) standardized coefficient of LD (D') (Lewontin 1964) and/or squared correlation coefficient r^2 (Hill and Robertson 1968), and (ii) statistical significance of the correlation coefficient (chi-square test, Fisher's exact test).

To examine patterns of LD within *FSHB* gene, squared correlation coefficient r^2 and significance of LD correlation coefficient r^2 for all pairwise comparisons of common polymorphisms (minor allele frequency, MAF > 0.1) was calculated using Arlequin 2.000 (Schneider *et al*. 2000) and Genepop 3.1d (Raymond and Rousset 1995). Squared correlation coefficient r^2 equals 0 if two alleles are inherited randomly. The LD coefficient is considered statistically significant if p-value is less than 0.05.

Allelic associations were significant throughout the gene for all studied populations (Estonians, $p<0.001$, $0.795<r^2<1$; Mandenkalu, $p<0.001$, $0.62<r^2<1$; Han Chinese, $p<0.001$, $0.53<r^2<1$). In order to study structure and frequencies of the *FSHB* gene haplotypes–"blocks" of alleles on a chromosome that are always inherited together–five SNPs (Figure 7A, B) were chosen to be analysed in additional populations (Table 4, Table 5). Two core *FSHB* gene haplotype variants (haplotype 1 and haplotype 2) composed of complementary nucleotides at all sites were identified (Figure 7B, C; Supplementary Tables S3, S4 in Ref. II). Among studied chromosomes (n=444), 96.6% of Utah, 96% of Czech, 92.6% of Estonia, 86% of Han Chinese, 79.2% of Mandenka, and 76% of Korea *FSHB* gene haplotypes were represented by two core haplotypes. Haplotype 1 was the prevalent haplotype for all populations except among Utah individuals. Haplotype 2 was enriched in populations of European origin (haplotype 2 frequency in Estonians, 39.4%; Czechs, 48.0%; CEU, 68.3%) compared to non-Europeans (Han Chinese, 14%; Mandenkalu, 16.7%; Koreans, 21%) (Figure 7C; Supplementary Table S4 in Ref. II).

3.1.2. Comparison of the *FSHB* gene sequence between human and great apes

In order to study the differences between human and great apes *FSHB* genomic sequences, respective genomic region in chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*) and orangutan (*Pongo pygmaeus*) was sequenced. The full sequences of great apes *FSHB* gene are deposited in NCBI GenBank (*P. troglodytes FSHB* Acc. No. DQ302103, *G. gorilla FSHB* DQ304480, *P. pygmaeus FSHB* DQ304481). The similarity scores between human and great apes *FSHB* gene genomic sequence ranged 97–99% and 95–98% within exonic and intronic regions, respectively (Table 7).

Table 7. Sequence similarity scores (%) between human (H, *Homo sapiens*), chimpanzee (C, *Pan troglodytes*), gorilla (G, *Gorilla gorilla*), and orangutan (O, *Pongo pygmaeus*) *FSHB* gene exonic (945 bp) and intronic (1964 bp) regions. Chimpanzee DNA was extracted from sperm material obtained from Tallinn Zoo, Estonia. The sources of orangutan and gorilla DNAs were primary cell lines AG12256 abd AG05151B, purchased from ECACC.

	species	H	C	G	O	
Exons	H	–	98	98	95	Introns
	C	99	–	97	96	
	G	98	98	–	96	
	O	98	97	97	–	

In total, 11 nucleotide differences in great apes coding region of the *FSHB* gene relative to that of human have been identified, three of which cause amino acid changes (Table 8). One difference from Lys to Asn (exon 3, amino acid 64) was present in all studied great apes species, Leu to Val change (exon 2, amino acid 4) in the signal peptide and Tyr to His change (exon 3, amino acid 49) were present in orangutan and chimpanzee, respectively.

Table 8. Nucleotide differences in *FSHB* gene coding region between human and great apes.

	Nucleotide difference[1]	Amino acid difference[2]	Signal (S) or mature (M) peptide	Exonic location
All studied great	2587 AAA→AAC	64 Lys(K)→Asn(N)	M	exon 3
apes species[3]	2728 GAT→GAC	111 Asp (D)→Asp(D)	M	exon 3
Chimpanzee	1032 TAC→CAC	49 Tyr(H)→His(H)	M	exon 2
(*Pan troglogytes*)	2641 CCC→CCT	82 Pro(P)→Pro(P)	M	exon 3
	2746 CTG→CTA	117 Leu(L)→Leu(L)	M	exon 3
Gorilla	2566 TAT→TAC	57 Tyr(Y)→Tyr(Y)	M	exon 3
(*Gorilla gorilla*)	2734 ACT→ACC	113 Thr(T)→Thr(T)	M	exon 3
Orangutan	897 CTC→GTC	4 Leu(L)→Val(V)	S	exon 2
(*Pongo pygmaeus*)	986 GAA→GAG	33 Glu(E)→Glu(E)	M	exon 2
	2734 ACT→ACC	113 Thr(T)→Thr(T)	M	exon 3

[1] Nucleotide positions are defined relative to transcription start-site on the human *FSHB* gene genomic DNA sequence (GenBank, NC_000011.9, Appendix 3)
[2] Amino acid positions are defined based on the sequence of human FSH β-subunit preprotein containing signal peptide (GenPept, NP_000501.1)
[3] Nucleotide differences present in chimpanzee, gorilla and orangutan.

Chimpanzee, gorilla and orangutan *FSHB* gene haplotype predicted from the positions of seven human common SNPs (Figure 7A) was identical among all studied species, except for a single basepair deletion in the orangutan gene for SNP rs611246 (Figure 7C; Table 2 in Ref. II). When compared to the human *FSHB* gene haplotypes, great apes *FSHB* haplotype is most similar to the second human *FSHB* core variant, haplotype 2, differing from the latter in two positions (rs594982 and rs6169) (Table 2 in Ref. II).

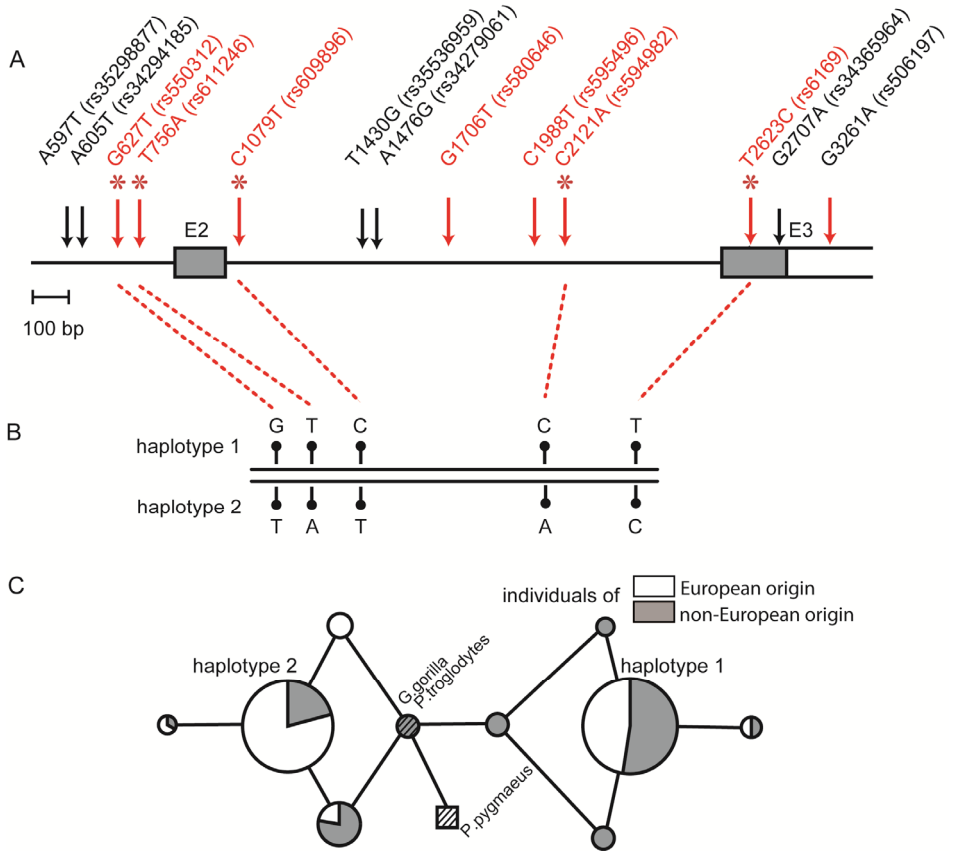


Figure 7. Identified variation pattern of the *FSHB* gene.

(**A**) Human polymorphic positions (*vertical arrows*) identified within re-sequenced human *FSHB* region (2909 bp) in Estonians, Mandenkalu and Han Chinese. Coding exons (E2, E3) are indicated by boxes and translated sequence is denoted by *grey* area. Positions are shown relative to *FSHB* transcription start-site: *red long arrows* mark common SNPs (MAF>10%) and *black short arrows* mark rare SNPs (MAF<10%). Five tag-SNPs (marked with *asterisks*) were chosen for genotyping the Korean, Czech and Utah Mormon/CEU samples. Allelic status of positions +627 (rs550312), +756 (rs611246) and +1079 (rs609896) was determined by re-sequencing approach; positions +2121 (rs594982) and +2623 (rs6169) were typed by RFLP analysis.

(**B**) Definition of *FSHB* gene core haplotypes (haplotype 1 and haplotype 2) based on the genotyping of five SNPs. The allelic composition of the two *FSHB* gene variants differs in each polymorphic position shown as black circles. Haplotype 1 and haplotype 2 correspond to the core haplotype 1 and core haplotype 13, respectively, in Ref. II (Table 2, Supplementary Material S4).

(**C**) Median-Joining (MJ) network for predicted *FSHB* haplotypes. Haplotypes have been constructed based on five SNPs typed for three European (Estonia, Czech, Utah Mormon/CEU), two Asian (Han Chinese, Korea), and African population (Mandenka). Relationships between predicted haplotypes were investigated using the Median-Joining (MJ) network algorithm (Bandelt *et al*. 1999) within NETWORK 4.0 software. The size of each node is proportional to the haplotype frequency in the total dataset. Branch

lengths represent one nucleotide substitution. The relative distribution of each haplotype among individuals of non-European and European origin is indicated by *grey* and *white*, respectively. *Banded FSHB* haplotype refers to the *FSHB* haplotype identical to chimpanzee (*Pan troglodytes*) and gorilla (*Gorilla gorilla*) gene variants. Orangutan (*Pongo pygmaeus*) had a deletion in the position +756 (rs611246), and orangutan's *FSHB* haplotype is denoted by *banded square*.

3.1.3. A pilot-study on possible association of *FSHB* variants with reproductive success

None of the *FSHB* gene polymorphisms identified in the study directly affects the composition and structure of the FSHB protein. However, the current study has determined that the *FSHB* gene has two genetic variants spread worldwide. The performed neutrality tests support the evidence that the gene has evolved under the influence of selection. FSH plays crucial role in maintaining of menstrual cycle in female allowing recruitment and growth of ovarian follicle. In order to explore the hypothesis that the *FSHB* core haplotypes might be associated with female fertility and reproductive health, a pilot-study for genetic association was performed. We asked whether the *FSHB* gene core haplotypes are distributed differently in the sample consisting of Estonian women who conceived naturally within three months after stopping contraception compared to random population-based Estonian women's cohort.

The *FSHB* gene was analysed in the sample of 48 Estonian women who had conceived within a period of three months after stopping contraception (Short Time to Pregnancy, STP-sample) (Table 9). The frequencies of homozygotes for haplotype 1 (HAP1/HAP1), heterozygotes (HAP1/HAP2) and homozygotes for haplotype 2 (HAP2/HAP2) were compared to those among unselected population sample of Estonian women (n=47) and unrelated Utah Mormon/ CEU individuals (n=30) (Table 9). The latter sample consisted of unrelated individuals from very fertile pedigrees, which were originally sampled to consist of four grandparents, the two parents and at least eight children (collected by the Centre d'Etude du Polymorphisme Humain (CEPH), Dausset *et al*. 1990). The frequency of HAP1/HAP1 carriers was 21.3% in STP-women and 27.7% in the random population sample, whereas the prevalence of HAP2/HAP2 was 34.0% in STP-group and 14.9% in the random population sample. HAP2/HAP2 homozygotes were overrepresented among Utah Mormon/CEU individuals (40%). Most extremely, among Utah Mormon/CEU individuals, no HAP1/ HAP1 homozygotes were identified.

Although none of the entire *FSHB* gene polymorphism/haplotype seems to directly affect FSHB protein, the second *FSHB* gene core variant, haplotype 2, was shown to be overrepresented among STP-sample as well as unrelated Utah Mormon/CEU individuals when compared to population-based Estonian women cohort. The enrichment of one of the gene variants might be a sign of the process of genetic hitchhiking by which functionally neutral allele/haplotype co-exists with a favourable regulatory variant (Barton 2000, Wall and Pritchard 2003). Additionally, the differences between Estonian sample and CEU individuals both originating from Europe may refer to possible effects of population history, for instance, population subdivision (Barton 2000). Further studies with larger sample sizes are needed to investigate the detail role of the *FSHB* variants in shaping reproductive phenotypic diversity and fertility in women.

Table 9. The distribution of homo- and heterozygous individuals for *FSHB* gene haplotypes in samples analyzed to investigate possible effects of *FSHB* haplotypes on reproductive function. The *FSHB* gene haplotypes were predicted based on *FSHB* gene positions rs594982 and rs6169.

Sample	no of individuals	HAP1/HAP1	HAP1/HAP2	HAP2/HAP2	Rare variants
[a] population-based Estonian cohort (Ref. II)	47	27.7% n=13	46.8% n=22	14.9% n=7	10.6% n=5
[b] STP-sample (Ref. II)	48	21.3% n=10	42.6% n=20	**34.0%** n=16	4.3% n=2
[c] unrelated Utah Mormon/CEU (Ref. II)	30	not identified	60.0% n=18	**40%** n=12	not identified

[a] Anonymous population-based female cohort representing general population. No phenotype data available (clinic: Tartu University Clinic, Tartu, Estonia; clinical coordinator: Dr. Kristiina Rull)

[b] Short Time to Pregnancy (STP) sample (see explanation in the text; clinic: Tartu University Clinic, Tartu, Estonia; clinical coordinator: Dr. Kristiina Rull)

[c] Unrelated Utah residents (USA) with Northern and Western European ancestry (Cann *et al*. 2002; http://www.cephb.fr/HGDP-CEPH-Panel/).

3.2. The putative regulatory loci in the *FSHB* gene flanking regions (Ref. III–V)

3.2.1. Screening of genetic variation in conserved 3'- and 5'- non-coding regions of *FSHB* gene

Transgenic studies by Kumar *et al*. (2006) had mapped the location of up- and downstream regions critical for efficient cell-specific *FSHB* gene expression to –350 bp of basal promoter region and +3142 bp following translational stop codon in exon 3 (+6060 bp from the transcription start-site (TSS). Phylogenetic analysis demonstrated regions of highly conserved sequences corresponding to these locations (Figure 4).

In the current study, *in silico* analysis has been performed to search the putative regulatory genetic elements/polymorphisms located within 3'- and 5'-untraslated regions that may co-segregate with *FSHB* core variants shown to be under balancing selection.

3.2.1.1 Variation pattern and potential regulatory units in conserved 3'-flanking region of *FSHB* gene (unpublished results)

A potential *cis*-acting gene regulatory unit, 296 bp long transposable element AluSg, spanning the region from +5771 bp to +6066 bp relative to the *FSHB* TSS was predicted within *FSHB* 3'-flanking region (Figure 8A) (RepeatMasker software, http://www.repeatmasker.org). Search for putative transcription factor binding sites resulted in identifying of Estrogen Response Element (ERE; from

+5848 to +5864 relative to TSS) within the transposable element AluSg (Figure 8A). The identified ERE is putative binding-site for estrogen receptor and may be involved in modulating of the expression of the *FSHB* gene (MatInspector 7.4.3 software, http://www.genomatix.de/).

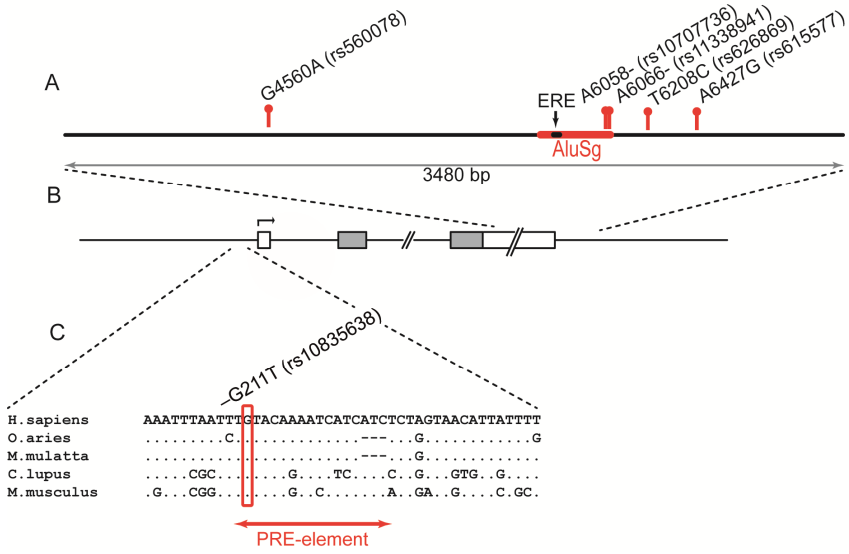


Figure 8. Localization of (A) downstream (3'-region) and (C) upstream (5'-region) putative regulatory areas within the human *FSHB* genomic region (B). Negative and positive numbers refer to positions relative to the transcriptional start-site, which is indicated by the arrow above exon 1. Untranslated and translated parts of exons (1, 2 and 3) are indicated by white and grey rectangles, respectively.

(A) Re-sequenced part of 3'-region of the *FSHB* gene. Identified polymorphic positions are marked with red circle-headed bars. The location of AluSg, transposable element found in forward orientation, was identified using RepeatMasker (http://www.repeatmasker.org) and marked with red horizontal line. The location of putative *cis*-acting regulatory element (Estrogen Response Element, ERE) within AluSg is shown and denoted by black arrow.

(B) Structure of the *FSHB* gene. Coding exons are indicated by boxes and translated sequence is denoted by *grey* area.

(C) A comparative alignment of the conserved 5'-upstream sequence element (from –212 to –197) shown to act as Progesterone Response Element (PRE) in ovine *Fshb* promoter (Webster *et al*. 1995). The sequences of corresponding promoter regions of five additional species including domestic sheep (*Ovis aries*, AH003774.1), rhesus monkey (*Macaca mulatta*, NC_007871.1), dog (*Canis lupus familiaris*, NC_006603.2), and mouse (*Mus musculus*, NC_000068.6) were downloaded from NCBI GenBank and used in the ClustalW multiple sequence alignment program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Bases that differ from the human sequence are shown. The location of PRE and human polymorphic position -211G/T are marked on the alignment with red horizontal arrow and red vertical box, respectively.

In order to screen the variation within the *FSHB* conserved 3'-flanking genomic sequence area, 3480 bp of 3'-flanking region of *FSHB* gene (from +3658 to +7138 bp relative to TSS) was re-sequenced in nine Estonian, six Korean and three Utah Mormon/CEU individuals randomly chosen from samples described in Table 4.

Within re-sequenced region, three single nucleotide substitutions (rs560078, rs626869, rs615577) and two deletion/insertion polymorphisms (rs10707736, rs11338941) were identified (Figure 8A). Deletion/insertion variants localized within 16 bp long poly(A) sequence and were omitted from the further analysis because genotypes were not reliably assigned.

Significant allelic associations among SNPs throughout the *FSHB* 3'-flanking re-sequenced area were found (Figure 9). Moreover, when five polymorphisms from the entire *FSHB* gene (rs550312, rs611246, rs609896, rs594982, rs6169) and 3'-flanking region polymorphisms (rs560078, rs626869, rs615577) were analyzed for LD using the measure r^2, the block of complete LD was found (Figure 9).

As none of the identified polymorphisms within *FSHB* gene 3'-flanking re-sequenced area have been found to localise within putative transcription factor binding sites, this gene region was not analysed further.

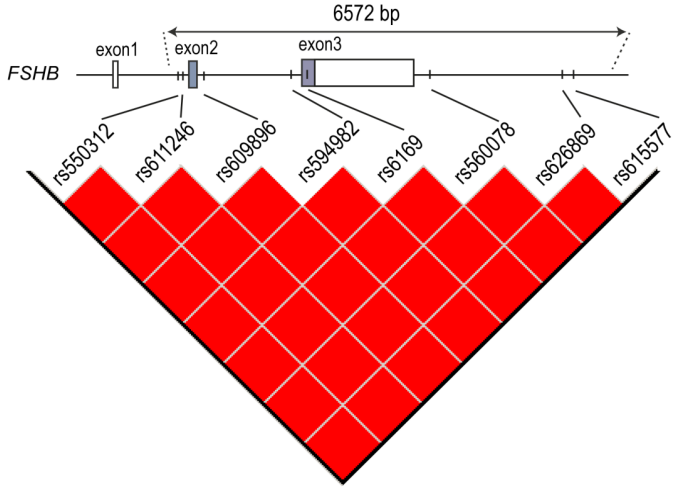


Figure 9. Linkage disequlibrium (LD) structure of the analyzed *FSHB* genomic locus spanning the *FSHB* gene (~3 kb) and 3'-downstream region (~3.5 kb) in nine Estonian, six Korean and three unrelated Utah Mormon/CEU individuals. The physical localization for analysed SNPs on the *FSHB* gene is shown. Red squares indicate that pairwise LD correlation coefficient, r^2, between all SNPs in the *FSHB* gene region is 1.00 meaning full LD. LD was estimated and LD plot was drawn with Haploview software (Barrett *et al*. 2005; http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview/).

3.2.2 Screening and variation pattern in *FSHB* gene evolutionary conserved promoter region

According to Kumar *et al*. (2006) and other studies on *FSHB* gene expression regulation, the proximal part of the gene promoter region containing multiple putative transcription factor binding sites is essential for efficient gene expression (Figure 4, Figure 6). In order to identify potential gene polymorphisms altering gene expression, screening of the human genome SNP database (dbSNP, NCBI, http://www.ncbi.nlm.nih.gov/) and manual inspection of previously reported regulatory sequence motifs within 350 bp upstream from transcriptional start-site were performed.

The database and literature search have resulted in the identification of an uncharacterised SNP (rs10835638, G/T) 211 bp upstream from the *FSHB* mRNA start-site (GenBank, NM_001018080.1). This SNP is located within a region which is conserved across mammals (Figure 8C) and is shown to act as Progesterone Response Element (PRE) capable of binding progesterone receptor (PR), thereby, regulating gene transcription. Moreover, functional studies conducted with ovine *Fshb* 5'-flanking region have shown that conserved G nucleotide at position –211 is one of critical positions for proper functioning of progesterone response element and efficient *FSHB* gene expression (Webster *et al*. 1995). Functional study on the human *FSHB* promoter activity showed that T allele in the *FSHB* gene promoter position –211 results in approximately 50% reduction in *FSHB* gene mRNA expression level (Hoogendoorn *et al*. 2003). Since concentration of the functional heterodimeric FSH has shown to correlate highly with intra-pituitary levels of *FSHB* mRNA transcripts (McNeilly *et al*. 2003), the position –211 G/T in *FSHB* promoter was targeted for further analyses of the association with human reproductive traits.

3.2.2.1 *FSHB* gene position -211 G/T alternative allele T is enriched in patients with male factor infertility

In order to study the effect of single nucleotide polymorphism at the *FSHB* gene position –211 G/T on male testicular and hormonal parameters, the position was genotyped for the cohorts of young men (Estonia, n=554; Latvia, n=277; Lithuania, n=223) and Estonian patients with male factor infertility (n=1029) (Table 10). Genotyping procedure is described in Ref. III.

Compared to Estonian young male cohort patients diagnosed with infertility exhibited a significant excess of both rs10835638 alternative allele T-homozygotes (1.1% *vrs* 2.4%) as well as GT-heterozygotes (22.4% *vrs* 25.1%) ($p<0.05$, Table 11). The difference in genotype frequencies remained borderline significant when patients with primary causes of infertility were excluded and only patients with idiopathic infertility were analysed ($p<0.1$).

Additionally, *FSHB* gene position –211 was genotyped in Latvian (n=277) and Lithuanian (n=223) cohorts of young men (Table 10). The distribution of 500 studied Latvian and Lithuanian individuals, on the basis of the *FSHB* gene promoter position –211, was 373 in GG (74.6%), 119 in GT (23.8%) and 8 (1.6%) in TT groups.

Table 10. Population samples used in the studies on *FSHB* rs10835638 association with male reproductive parameters.

Population	Clinic	Clinical coordinator	Description	Participants age (mean ± SD)	no of individuals	Reference
Estonia [a]	Centre of Andrology, University Clinic of Tartu	Margus Punab	population-based cohort of young men	19.2 ± 1.7	554	Ref. III–V
Estonia	Centre of Andrology, University Clinic of Tartu	Margus Punab	male partners of couples failing to conceive a child within one year (sperm concentration $<20x10^6$/mL)	31.7 ± 6.1	1029	Ref. IV
Latvia [a]	Riga Family and Sexual Problems Centre	Juris Erenpreiss	population-based cohort of young men	21.1 ± 1.9	277	Ref. V
Lithuania [a]	Institute of Endocrinology, Kaunas University of Medicine	Birute Zilaitiene	population-based cohort of young men	21.0 ± 1.8	223	Ref. V

[a] The Estonian, Latvian, and Lithuanian cohorts (Baltic cohort) of young men were recruited among the participants in a prospective study Environment and Reproductive Health (EU 6th FP project QLRT-2001-02911) in parallel at the three study centres (Tartu, Estonia; Riga, Latvia; Kaunas, Lithuania). The study was approved by the local Ethics Committees. Detailed principles of study group formation are described in Ref. III–V and in Punab *et al*. 2002.

Table 11. *FSHB* rs10835638 allele and genotype frequencies among Estonian male patients of infertile couples compared with the cohort of young Estonian men.

	n	Allele frequencies G (%)	T (%)	*p*-value[a]	Genotype frequencies G/G	G/T	T/T	*p*-value[b]
A) Population-based Baltic cohort (Ref. III,V)								
Estonians	554	87.7	12.3	0.042	76.5% n=423	22.4% n=125	1.1% n=6	0.043
Latvians	277	86.5	13.5		74.4% n=206	24.2% n=67	1.4% n=4	
Lithuanians	223	86.5	13.5		74.9% n=167	23.3% n=52	1.8% n=4	
pooled	1054	87.1	12.9	0.051	75.5% n=796	23.1% n=244	1.3% n=14	0.054
B) Estonian infertile male patients (Ref. IV)	1029	85.0	15.0		72.5% n=746	25.1% n=258	2.4% n=25	
Patients with idiopathic infertility	750	85.1	14.9		72.7% n=545	24.8% n=186	2.5% n=19	
Patients with causal factors for infertility[c]	279	84.9	15.1		72.0% n=201	25.8% n=72	2.2% n=6	

[a] *p*-value of $\chi 2$ test for allele frequencies in Estonian population-based cohort of men (Ref. III) or Baltic cohort of young men (Ref. V) vs. Estonian cohort of male infertility patients (Ref. IV)

[b] *p*-value of $\chi 2$ test for genotype frequencies in Estonian population-based cohort of men (Ref. III) or Baltic cohort of young men (Ref. V) vs. Estonian cohort of male infertility patients (Ref. IV)

[c] causal factors for infertility included cryptorchidism, chromosomal abnormalities, Y chromosome deletions, hypogonadotrophic hypogonadism, testicular diseases, sexual dysfunctions, androgen abuse, severe traumas and operation in genital area, chemo- and radiotherapy (see detailed description in Ref.IV).

3.2.2.2 *FSHB* gene position -211 G/T is associated with male serum FSH level

In order to find whether the *FSHB* gene promoter polymorphism -211 G/T is associated with male serum FSH level, marker-trait analysis by multiple linear regression with adjustment for appropriate confounders was performed. Multiple linear regression analysis revealed a strong association of rs10835638 alleles with serum FSH level both in the cohort of young Estonian men (GG>GT>TT, median FSH 2.98>2.46>2.06 IU/L, $p<0.005$; Table 1 in Ref. III) and in the cohort of male infertility patients (GG>GT>TT, median FSH 6.8>5.4>3.5 IU/L, $p<<0.001$; Table 2, 3 in Ref. IV). In both Estonian cohorts, subjects with TT and GT genotypes exhibited 30–50% and 16–35% lower serum FSH levels, respectively, than those in GG homozygotes (Table 1 in Ref. III; Table 2 in Ref. IV). The association of the SNP alleles with male serum FSH level remained significant when patients with causal factors for infertility were omitted from the analysis ($p<<0.001$; GG>GT>TT, 6.4>5.3>3.3 IU/L, Figure 10A).

Consistent with the results obtained from the analyses of both Estonian young men and Estonian infertility patients, in Baltic cohort of young men, the minor allele T of the rs10835638 was significantly associated with serum FSH level ($p<<0.001$). TT-homozygotes (median FSH 1.8 UI/L) had approximately 10% and 22% lower FSH levels than GT-heterozygotes (2.0 IU/L) and GG-homozygotes (2.3 IU/L), respectively (Table 12, Figure 10A; Table 2 in Ref. V).

Table 12. The results of statistical analyses performed to test the association between *FSHB* rs10835638 and male hormone levels and testicular parameters in Baltic young men cohort (n=1054) and Estonian patients diagnosed with idiopathic infertility (n=750).

FSHB rs10835638	Multiple linear regression and T-allele effect size			
	Baltic young men cohort (n=1054)		Estonian idiopathic infertility patients (n=750)	
	additive model p-value[a] (effect)	recessive model p-value[b] (effect)	additive model p-value[a] (effect)	recessive model p-value[b] (effect)
FSH (IU/L)	**1.11x10^{-6}** (–0.41)	7.08x10^{-2} (–0.59)	**1.4x10^{-7}** (–1.69)	**6.80x10^{-4}** (–3.08)
Inhibin B (pg/mL)[c]	**2.16x10^{-3}** (–14.67)	**1.30x10^{-2}** (–44.75)	8.36x10^{-1} (–1.29)	9.66x10^{-1} (–0.93)
LH (IU/L)	1.57x10^{-1} (0.15)	**2.25x10^{-2}** (1.07)	7.74x10^{-1} (–0.04)	7.61x10^{-1} (0.15)
Total testosterone (nmol/L)	**9.30x10^{-3}** (–1.46)	3.75x10^{-1} (–2.02)	2.17x10^{-1} (0.56)	6.56x10^{-1} (–0.63)
Estradiol (pmol/L)	8.19x10^{-1} (–0.35)	2.66x10^{-1} (7.20)	6.73x10^{-1} (0.92)	2.56x10^{-1} (8.06)
Total testes volume (mL)	**1.47x10^{-2}** (–1.64)	**1.19x10^{-4}** (–9.47)	2.31x10^{-1} (–0.93)	9.26x10^{-2} (–4.02)
Semen volume (mL)	6.35x10^{-1} (0.05)	2.60x10^{-1} (0.52)	8.07x10^{-1} (0.03)	4.37x10^{-1} (0.31)
Sperm concentration (10^6/mL)	3.14x10^{-1} (–4.11)	1.27x10^{-1} (–20.90)	1.77x10^{-1} (0.44)	9.26x10^{-1} (–0.09)
Total sperm count per ejaculate (x10^6)	5.41x10^{-1} (–12.54)	5.46x10^{-1} (–44.86)	1.38x10^{-1} (1.79)	8.38x10^{-1} (0.76)
Sperm A+B motility (%)	**4.1x10^{-2}** (2.23)	5.00x10^{-1} (3.00)	4.21x10^{-1} (–1.05)	6.24x10^{-2} (–6.95)
Morphologically normal sperm (%)	2.41x10^{-1} (0.45)	5.13x10^{-1} (–0.94)	5.63x10^{-1} (0.0)	7.36x10^{-1} (0.0)

Marker-trait association testing was performed using linear regression with

[a] additive model tested the effect of *FSHB* rs10835638 T-allele dosage and

[b] recessive model tested the effect of of TT-homozygosity.

P-value of multiple linear regression and effect size (unstandardized coefficient, B) of the *FSHB* rs10835638 T-allele dosage or T-allele homozygosity are shown.

[c] In Estonian infertility patients, inhibin B level values were available for 294 individuals.

In Baltic cohort of young men, tests were adjusted for age, BMI, smoking status and recruitment centre. Hormone measurements were additionally corrected for blood sampling hour, and semen parameters for abstinence time. In Estonian cohort of infertility patients, tests were adjusted for age, and semen parameters were additionally corrected for abstinence period.

Analyses were performed using PLINK version 1.07 (http://pngu.mgh.harvard.edu/purcell/plink/).

Significant difference has been highlighted: $p<0.1$, $\mathbf{p<0.05}$, $\mathbf{p<0.001}$

3.2.2.3 *FSHB* gene position -211 G/T is associated with other male quantitative reproductive parameters

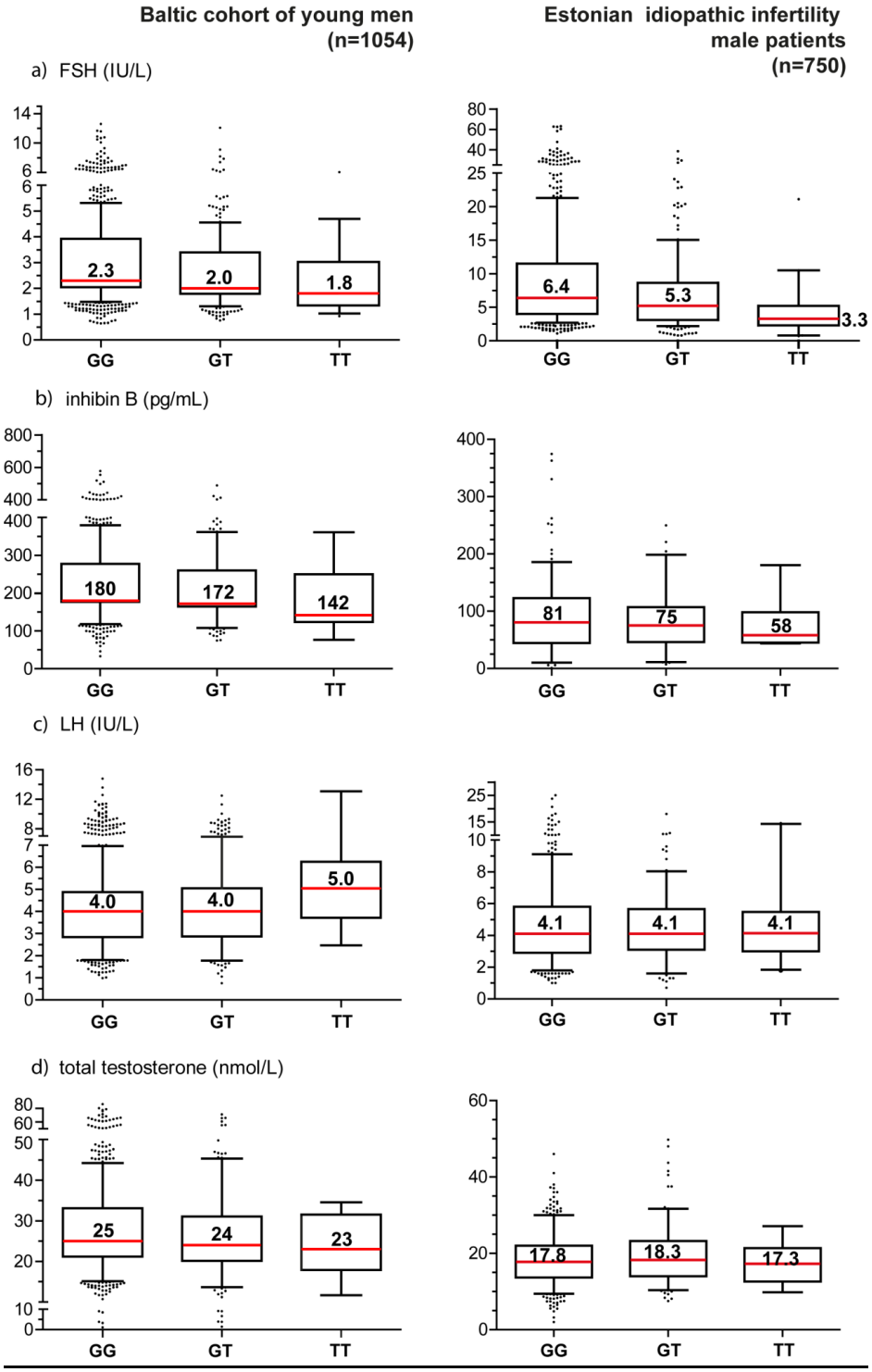
To characterize the detailed influence of *FSHB* promoter variants on male reproductive health, association of the *FSHB* gene position -211 alleles with several additional male hormonal (inhibin B, LH, total testosterone, estradiol) and testicular parameters (total testes volume, sperm concentration, total sperm count, semen volume, sperm forward (A+B) motility, sperm morphology) in Baltic cohort of young men (n=1054) and in Estonian cohort of idiopathic infertility patients (n=1029) was assessed (multiple linear regression with additive and recessive models with adjustment for confounders) (Table 12).

In Baltic cohort of young men, the dosage of the T-allele was significantly associated with reduced serum inhibin B level (linear regression additive model, $p=2.16x10^{-3}$, Table 12). Inhibin B levels in TT-homozygotes were approximately 20% and 5% lower than those in GG-homozygotes and GT-heterozygotes, respectively (GG>GT>GG, adjusted median 180>172>142 pg/mL; Figure 10B). Most probably due to restriction that inhibin B measurement data were available for 294 individuals only, in Estonian male patients diagnosed with idiopathic infertility, the association didn't reach the level of statistical significance. However, compared to GG-homozygotes, TT-homozygotes and GT-heterozygotes exhibited 28% and 7% lower inhibin B levels, respectively (median inhibin B pg/mL, GG>GT>TT, 80.6>75.2>58.2; Figure 10B; Table 3 in Ref. IV).

Additionally, serum LH and total testosterone levels were significantly associated with *FSHB* rs10835638 carrier status in Baltic cohort of young men (LH, recessive linear regression, $p=2.25x10^{-2}$; total testosterone, additive linear regression, $p=9.30x10^{-3}$) (Table 12). Compared to GG- and GT-genotype carriers, the TT-subjects had increase in serum LH (5.0 IU/L vrs. 4.0 IU/L), but decrease in total testosterone level (GG>GT>TT, 25>24>23 nmol/L) (Figure 10C, D). Neither serum LH nor total testosterone was affected by the carrier status of rs10835638 in idiopathic infertility patients (Figure 10C, D; Table 3 in Ref. IV). The lack of the association with LH and testosterone may be explained in two ways: (i) in infertile males, reproductive physiology and hormonal feedback mechanisms are disturbed in general, (ii) infertile patients exhibit high range and non-normal distribution of hormonal measurements.

Estradiol was slightly increased in TT-homozygotes compared to GG- and GT-individuals in either Baltic cohort of young men or Estonian idiopathic infertility patients (Baltic cohort of young men, GG~GT<TT, adjusted median 78~76<84 pmol/L; Estonian infertility patients, GG~GT<TT, median 88~87<98 pmol/L) (Table 12).

Hormonal parameters



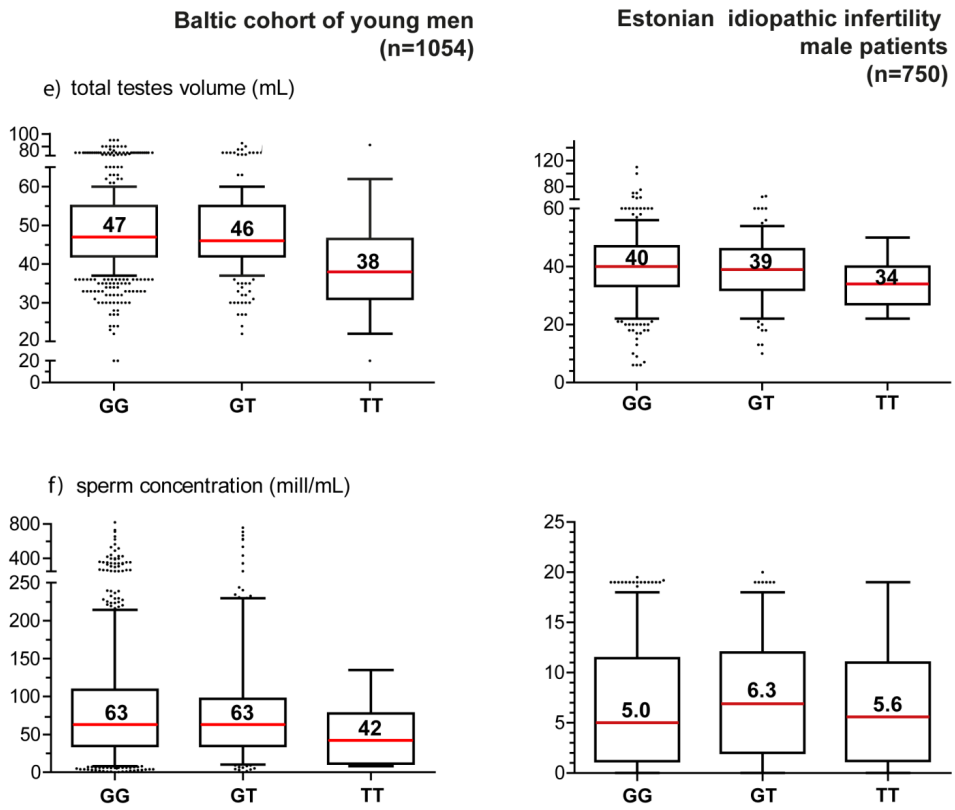


Figure 10. Box-and-whisker diagrams for the distribution of hormonal parameters: (a) serum FSH, (b) inhibin B, (c) serum LH, (d) total testosterone; and testicular and semen parameters: (e) total testes volume, (f) sperm concentration in the Baltic young men cohort (n=1054) (*left panel*) and Estonian male patients diagnosed with idiopathic infertility (n=750) (*right panel*). Individuals are subgrouped according to their *FSHB* promoter SNP rs10835638 genotype (Baltic young men cohort, GG/GT/TT, 796/244/14; Estonian male infertility patients, GG/GT/TT, 545/186/19). The boxes represent the 25th and 75th percentiles; the whiskers cover the 5th-95th percentiles of the data. Circles represent the outlier values. The median is either the value inside the box or the red line that bisects the boxes. For Baltic young men cohort, the confounder adjusted median values are shown (detailed description in Ref. V, Supplementary Text).

The effect of *FSHB* gene rs10835638 alleles on male testicular parameters was tested. The position rs10835638 TT-homozygotes demonstrated lower total testes volume in Baltic young men and Estonian idiopathic infertility patients (Baltic cohort, recessive linear regression, $p=1.47x10^{-2}$; Estonian infertility patients, $p=9.26x10^{-2}$). When compared to GG- and GT-genotype carriers, TT-homozygotes had 15–20% lower total testes volume than GG-homozygotes and GT-heterozygotes (Figure 10E, Table 2 in Ref. V; Table 3 in Ref. IV). Moreover, in Baltic cohort, sperm concentration was reduced in TT-individuals (adjusted median, 42 mln/mL) compared to GG- (63 mln/mL) and GT-individuals (63 mln/mL) (Figure 10F; Table 2 in Ref. V). Most probably due to high range of sperm concentration measurements and inclusion of cases with azoospermia, no significant association has been revealed in patients diagnosed with infertility (Figure 10E; Table 3 in Ref. IV).

4. DISCUSSION

4.1 The fine-scale variation of the *FSHB* gene and signatures of balancing selection

Genes that affect fertility and hormonal system are expected to be subject to strong selection owing to their direct effects on reproduction-related traits (Matzuk and Lamb 2008). Consistent with the essential function of the *FSHB* gene in reproduction, stringent purifying selection acts towards the changes in the translated region of the gene due to their deleterious effect. Confirming previous reports by Liao *et al*. 1999 and Lamminen *et al*. 2005, full re-sequencing of the *FSHB* gene in 96 individuals representing Europe (Estonia), Africa (Mandenka), and Asia (Han Chinese) did not uncover any non-synonymous changes. The majority of the *FSHB* SNPs were represented by common polymorphisms having worldwide occurrence. In contrast to the evolutionarily preserved *FSHB* gene, the highly diverse *LHB/CGB* gene cluster coding for luteinizing and chorionic hormone β-subunits was described as a region with dynamic genetic evolution (Hallast *et al*. 2005). Despite the *FSHB* gene and *LHB/CGB* cluster belong to the family of gonadotropin β-subunit genes, the *FSHB* gene exhibits approximately six-fold lower nucleotide diversity and stable genomic structure compared to the *LHB/CGB* gene locus exhibiting high number of population-specific variants (Hallast *et al*. 2005, Henke and Gromoll 2008). In *LHB/CGB* region, high level gene variation and active genome dynamics possibly generating unfavourable variants are compensated by multiple gene copies guarantying the required gene transcription dosage (Ref. I).

The results in Ref. II showed that *FSHB* exhibits strong linkage disequilibrium (LD) throughout the entire gene region and 3'-flanking area; and respective genomic region is presented by a pair of 'yin yang' core haplotypes spread worldwidely. The *FSHB* core variants are composed of at least eight consecutive SNPs that are inherited together (Figure 7, 9). 'Yin yang' haplotypes found in high frequencies among human populations have been suggested to enrich either (i) by chance under a neutral evolutionary model (Zhang *et al*. 2003), (ii) due to the events in population history such as admixture between two founder populations (Prugnolle *et al*. 2005, Curtis and Vine 2010), or (iii) due to the influence of balancing selection (Bamshad and Wooding 2003). In the case of the *FSHB* gene, the nearly equal allele frequencies of SNPs and the results of neutrality tests refuted the hypothesis on neutral evolution. Firstly, the demographic history of the entire human population, characterized by a series of population bottlenecks, may have shaped modern human genetic diversity of the *FSHB* gene leading to the enrichment of the second core haplotype in populations with European descent (Harpending and Rogers 2000, Amos and Hoffman 2010). However, all the studied populations share the two *FSHB* gene core haplotypes and demonstrate similar results for neutrality tests. Thus, the influence of the ancient balancing selection maintaining the simultaneous enrichment of two core haplotypes is more obvious. Interestingly, all examined

great apes species were homozygous for the gene variant more similar to the *FSHB* second core haplotype pinpointing the ancestral *FSHB* variant.

The balancing selection can arise when variation in phenotypic consequences, upon which it can act, is present (Bamshad and Wooding 2003, Crespi 2010). A classic example of balancing selection is found in the 5' *cis*-regulatory region of the *CCR5* gene, the co-receptor for HIV-1. At this locus, balancing selection created two major advantageous haplotype groups to maintain the defence against pathogens (Bamshad *et al*. 2002). Although relatively few single-gene studies for regions involved in fertility and reproduction have been performed so far, loci associated with medically relevant fertility phenotypes have been identified to be under the influence of balancing selection (reviewed in Crespi 2011). Similarly to the *FSHB* gene, the haplotype structure of *CGB8* gene promoter has been shown to be consistent with the scenario of balancing selection that might have triggered the enrichment of two divergent clusters of haplotypes possibly associated with different pregnancy success (Rull *et al*. 2008). As the *FSHB* gene haplotypes are composed of silent or non-coding polymorphisms, neither core variant affects the composition of the FSHB protein directly. Thus, balancing selection may be operating towards an upstream or downstream causative variant in tight linkage disequilibrium with the *FSHB* gene haplotypes.

4.2. The dosage of the follicle-stimulating hormone affects male reproductive parameters

4.2.1. Application of evolutionary genetics in studying gene functional domains

Phylogenetic analysis of mammalian and nonmammalian genomes has frequently been used to identify selectively constrained and functionally important sequences (Batzoglou *et al*. 2000, Dermitzakis *et al*. 2002, DeSilva *et al*. 2002, Woolfe *et al*. 2005). Comparison of the coding regions in human and mouse has revealed that exons in *H. sapiens* and *M. musculus* exhibit 85% identity at the nucleotide level. In contrast, introns tend to show lower similarity, approximately 35% (Makałowski *et al*. 1996, Batzoglou *et al*. 2000). In addition to protein coding regions, subsets of non-genic sequences that are not functionally transcribed have also been demonstrated to exhibit high conservation across vertebrate genomes. Evolutionary conservation has become widely used as a start-point for identifying putative genomic regions contributing to the regulation of gene expression (Dermitzakis *et al*. 2005, Drake *et al*. 2006, King *et al*. 2007). Initially, functionally conserved non-genic DNA sequences (CNGs) were defined as regions that are ≥70% identical over at least 100 bp of ungapped alignment of mouse and human sequences (Duret *et al*. 1993, Dermitzakis *et al*. 2002). Although the amount of sequence fulfilling this strict criterion is only about 1% of the human genome (Dermitzakis *et al*. 2005),

studies adopted this definition of CNGs have identified several biologically relevant non-coding loci associated with disease phenotypes, such as Gaucher disease (Blech-Hermoni *et al*. 2010) and cleft lip (Rahimov *et al*. 2008).

When the generation of alignment between species is not feasible, motif-finding methods with subsequent experimental annotation have also been used to isolate functional sequences, mostly in detection of transcription factor-binding sites (TFBSs) (Roth *et al* 1998, So *et al*. 2007, Gaffney *et al*. 2008). Combination of two approaches–evolutionary sequence conservation and biological relevance of the locus–revealed functional regulatory loci in the immune response gene, *interleukin-6* (*IL-6*). Two polymorphisms in the *IL-6* gene promoter located in close proximity to glucocorticoid response element (GRE) were shown to modify the expression of the gene and affect various downstream biochemical markers and disease phenotypes (Ferrari *et al*. 2003, Morgan *et al*. 2006). The conservation of the entire *Fshb*/*FSHB* gene genomic sequence along with upstream and downstream flanking sequences across the vertebrate lineage is a signature of strong natural selection acting towards both coding and non-coding regions of the gene (Figure 4, Kumar *et al*. 2006). The changes within the protein-coding region of the *FSHB* gene are selected against due to their drastic effect on reproduction. The proximal promoter region up to –350 bp relatively to the *FSHB* transcription start-site was shown to harbour multiple conserved TFBSs, *cis*-acting regulatory elements, capable of altering the expression of the *FSHB* gene (Figure 6, Bernard *et al*. 2010, Melamed 2010). The binding of the progesterone-progesterone receptor complex to the PRE sequence element (corresponds to –197…–212 bp from the human *FSHB* TSS) in the ovine *Fshb* gene promoter was shown to be capable of enhancing the gene expression (Webster *et al*. 1995). The position –211 G/T was shown to be critical in proper functioning of the PRE motif (Webster *et al*. 1995, Hoogendoorn *et al*. 2003), thus, representing a reasonable target locus for a study of the fitness effects of nucleotide substitutions within regulatory non-coding DNA.

4.2.2. The *FSHB* position –211 G/T: the association with serum FSH level and testes volume

This study has shown strong evidence for the association between the *FSHB* position –211 G/T and serum FSH level in the Baltic cohort of young men (Ref. V) and in the Estonian patients diagnosed with infertility (Ref. IV). Among –211 G/T genotype carriers, the declining gradient of the serum FSH measurements was observed (GG>GT>TT). The serum FSH level gradient among the *FSHB* –211 G/T genotype carriers is consistent with the results of a functional study on polymorphic proximal promoters of 170 human genes that revealed that alternative allele in the *FSHB* position –211 G/T results in the 46–50% reduction of the *FSHB* gene expression level (Hoodendoorn *et al*. 2003).

Considerable efforts have been undertaken to understand the relative importance of follicle-stimulating hormone and luteinizing hormone/testosterone for full male fertility. It is generally assumed that FSH plays essential role in regulation of quantitatively and qualitatively normal spermatogenesis in male mammals (Nieschlag *et al*. 1999, Plant and Marshall 2001, Weinbauer *et al*. 2010). Mice lacking FSH (FSHβKO) or FSH receptor (FSHRKO) showed that FSH is required for normal development of Sertoli cell and germ cell numbers as well as entry of spermatogonia into meiosis (Kumar *et al*. 1997, Abel *et al*. 2000, Krishnamurthy *et al*. 2000, Abel *et al*. 2008).

The sole target for the dimeric FSH molecule in male testes is Sertoli cell possessing cell membrane FSH receptors (FSHR). It is possible to distinguish roughly three time points, or developmental periods, of irreplaceable FSH action.

First, fetal FSH is the stimulator of immature Sertoli cells proliferation during male fetal and especially early neonatal development (Hansson *et al*. 1975, Singh and Handelsman 1996, Sharpe 2003, Baines *et al*. 2008, O'Shaughnessy *et al*. 2009). The exact role of the FSH during fetal development continues to be debated, since Sertoli cell number was shown to be normal during embryonal development in gonadotropin-releasing hormone deficient mice (*hpg*) (Baker and O'Shaughnessy 2001) and follicle-stimulating hormone receptor knockout mice (FSHRKO) (Johnston *et al*. 2004). However, in anencephalic human fetuses that exhibited the absence of hypothalamus and pituitary as well as in hypophysectomised rhesus monkey fetuses, the marked reduction in testes size was observed (Baker and Scrimgeour 1980, Gulyas *et al*. 1977). Suppression of the neonatal FSH concentration in rats reduces the final number of Sertoli cells by about 40% (Atanassova *et al*. 1999, Sharpe 1999). After birth, Sertoli cells expressing androgen receptors (ARs) also mediate androgen (testosterone) driven action required for meiotic and postmeiotic germ cell development (De Gendt *et al*. 2004, Tan *et al*. 2005, O'Shaughnessy *et al*. 2010a).

Secondly, the synergic action of the FSH and Leydig cells-produced testosterone is needed for the radical switch from an immature, proliferative state to a mature, non-proliferative state Sertoli cells during pubertal period (Tan *et al*. 2005, Willems *et al*. 2010). The functional maturation of Sertoli cells includes also the establishment of the blood-testis barrier (BTB), which in turn is connected with the process of formation of tight junctions between Sertoli cells during puberty. It was established that gonadotropin suppression results in disorganisation of tight junctions' proteins and loss of BTB function in rodent testis and the effect is reversible by FSH replacement (Tarulli *et al*. 2008, McCabe *et al*. 2010, Chui *et al*. 2010). Since each individual mature Sertoli cell is able to support a defined number of sperm (10 germ cells or 1.5 spermatozoa, Zhengwei *et al*. 1998), the finite size of Sertoli cells population determined during early stages of reproductive development–fetal, neonatal, and pubertal periods–is closely correlated to the capacity of the adult testes for sperm production. Consistently, Johnson *et al*. (1984) showed positive linear

correlation between number of Sertoli cells in adult human testis and dayly sperm production.

And thirdly, in adult male, FSH is involved in complex paracrine and endocrine regulatory control pathways, hypothalamic-pituitary-gonadal axis (Figure 3, Sharpe *et al*. 2003, Weinbauer *et al*. 2010).

Consistently with the role that plays FSH in the proliferation and functional maturation of Sertoli cells in the testes, the current study (Ref. III–V) demonstrated that homozygotes TT for *FSHB* gene promoter position –211 (rs10835638) having the genetically determined lowest levels of serum FSH display an approximately 20% reduction in testes volume both in the Baltic cohort of young men and in Estonian cohort of males diagnosed with idiopathic infertility. Thus, the alternative allele T in the regulatory position within *FSHB* promoter causing lower production of FSH is associated with reduction in testes volume, one of the most important parameters for estimating male reproductive capacity (Luetjens *et al*. 2005, Sakamoto *et al*. 2008, Nieschlag and Behre 2010). The evidence of the association with testes volume is consistent with the reduced testes size in the FSHβ-deficient mice and human males (Kumar *et al*. 1997, Phillip *et al*. 1998, Themmen and Huhtaniemi 2000). The testes volume in GT-heterozygotes was not affected, most probably due to possible involvement of compensatory mechanisms to maintain physiologically normal reproductive capacity.

4.2.3. The association with other secondary male reproductive parameters: long-term and short-term effects of reduced FSH production

An independent case report by Mantovani *et al*. (2003) has described a young male patient with normal virilization, azoospermia and isolated FSH-deficiency, who was homozygous for the T-allele of the *FSHB* –211 G/T polymorphism. This clinical case allows suggesting a hypothesis of possible contribution of this polymorphism to male infertility phenotype. The current study showed that the genetically inherited constituitevly diminished production of FSH affects a broad range of male reproductive parameters including sperm concentration and levels of inhibin B, testosterone and LH.

The declining gradient (GG>GT>TT) of inhibin B level and sperm concentration among *FSHB* promoter rs10835638 genotype groups in Baltic cohort of young men was observed. Inhibin B is a unique Sertoli-germ cells joint product that is secreted into male circulation upon the FSH-stimulation (Ramaswamy and Plant 2001, Weinbauer *et al*. 2010). Since serum FSH levels are not only determined by the testis but also influenced by hypothalamic function, inhibin B may offer a useful and direct serum marker describing gonadal functions (Anawalt *et al*. 1996, Meachem *et al*. 2001, Kumanov *et al*. 2006, Simoni and Nieschlag 2010).

The reduced levels of the inhibin B and sperm concentration in T-allele carriers may refer to the short-term effect of lower FSH secretion. Additionally, FSH-understimulated processes during male reproductive development may result in long-term effects including impaired functioning of the testes Sertoli cells (Sharpe *et al*. 2003). Quantitatively and qualitatively normal spermatogenesis is depending upon testis spermatogenic capacity built up during reproductive development as well as immediate effect of endocrine regulation (Weinbauer *et al*. 2010). In adult *hpg* mice, spermatogenesis was severely disrupted, and FSH treatment increased the germ cell number as well as spermatogonial and spermatocyte numbers by three- to fourfold (O'Shaughnessy *et al*. 2010b). Recently, it was established that in normal adult males FSH plays a key role in the regulation of apoptotic pathways during spermatogenesis supporting survival of spermatogonia and sperm release (spermiation) (Ruwanpura *et al*. 2008, 2010).

Additionally to the direct effect of FSH on the actions of Sertoli cells expressing FSH receptor, FSH has been shown to indirectly affect also testosterone-producing Leydig cell function. Although the gonadotropin-independent role for FSH receptors in FSHβ-deficient mice have been suggested (Baker *et al*. 2003), FSH-stimulated Sertoli cells produce various factors (e.g. proteins, cytokines, steroids, growth factors, modulators of cell division) that contribute to the induction of adult Leydig cell differentiation and functional maturation (O'Shaughnessy *et al*. 1992, Matikainen *et al*. 1994, Sriraman and Jagannadha Rao 2004, O'Shaughnessy *et al*. 2009 and references therein). Thus, it is intriguing that among Baltic young men, homozygotes for the minor allele had higher serum LH but lower testosterone level when compared to heterozygotes or homozygotes for the major allele. Reduced testosterone level in a combination with elevated serum LH level is used as sensitive marker of the impaired functioning of Leydig cells (Giagulli *et al*. 1988, Brennemann *et al*. 1997, Holm *et al*. 2003). The alternative T allele in *FSHB* promoter position –211 results in a lower level of mRNA expression and functional FSH formation during reproductive development what, in turn, is a cause of diminished activity of adult Leydig cells.

4.3. Implications and further development of the study

The carriers of the T-allele of the *FSHB* –211 G/T SNP represent a natural model for documenting short-term and long-term downstream effects of insufficient FSH action due to genetically inherited constitutively reduced hormone levels. Diminished production of the functional FSH results in changes in various complex networks of hormonal interactions regulating human fertility potential. The results of the current study showed the enrichment of the *FSHB* gene alternative allele T among Estonian cohort of infertility patients compared to the cohort of young men. This observation provides evidence for the possible contribution of this position to the complex phenotype of male infertility.

Impaired spermatogenesis is one of the first symptoms of testicular dysgenesis syndrome (TDS) (Skakkebaek *et al*. 2001) including also such traits as cryptorchidism, hypospadias, testicular germ cell cancer. Although TDS is most likely caused by a combination of environmental and life-style factors (Sharpe 2010), the genetic predisposition to this syndrome cannot be ruled out (Aschim *et al*. 2005). Additional studies are needed to be conducted to examine the detailed effect of the *FSHB* gene promoter position –211 G/T on various developmental issues in male.

It has been demonstrated that highly purified or recombinant FSH is an appropriate and valid treatment of oligozoospermic patients. However, only selected patient react positively on FSH therapy. FSH can improve seminal parameters in patients with FSH plasma levels in the normal range and testes tissue structure characterized by hypospermatogenesis without germ cell maturation disturbances (Baccetti *et al*. 2004, Paradisi *et al*. 2006, Foresta *et al*. 2000, 2008; Nieschlag and Kamischke 2010, Palomba *et al*. 2011). Determination of the genotype of the *FSHB* promoter polymorphism would allow the identification of the patients, whose primary cause of the infertility problems may be too low FSH production. Further investigations into the efficacy of FSH treatment on the basis of genetic polymorphisms are required to identify further parameters that are able to predict the seminal response to FSH treatment.

In the current thesis, a pilot-study for the association between the *FSHB* two divergent core haplotypes and female fertility revealed enrichment of the *FSHB* second core variant among 'short time to pregnancy' women (STP-sample) (Table 9; Ref. II). Noteworthy, the inclusion criterion for the Utah Mormon families' enrollment was high number of offspring, at least eight children (Dausset *et al*. 1990). The functional consequence of the *FSHB* core haplotypes remains to be elucidated. In future studies, confounding impacts of different covariates relating both partners such as data on anthropometric characteristics (*e.g.* age, body mass index) and covariates relating to both partners (smoking status, current alcohol consumption, frequency of intercourse *etc.*) are required (Joffe *et al*. 2005, Wise *et al*. 2011).

Time to pregnancy is a functional measure of biologic fertility depending on female ovary folliculogenic capacity and interplay between various molecular agents exerting their effects during fetal, pubertal and adult life periods. The adequate concentration of the FSH during female reproductive development may preprogram certain measures of women fertility potential in adulthood such as age at menarche, menstrual cycle length, time to pregnancy, ovarian aging, and age at natural menopause as well as response in IVF. Future research focusing on the effect of the *FSHB* gene promoter polymorphism –211 G/T on FSH level and female fertility parameters would provide new insights into the role and consequeces of differential ovarian stimulation by FSH.

SUMMARY AND CONCLUSIONS

The results of this thesis can be summarized as follows:

1. Follicle-stimulating hormone β-subunit gene (*FSHB*) coding for FSH β-subunit protein exhibits absence of common non-synonymous changes and excess of high frequency non-coding polymorphisms in six studied human populations (Estonians, Mandenkalu, Han Chinese, Koreans, Czechs, unrelated Utah Mormons/CEU). The respective genomic region is characterized by strong pairwise linkage disequilibrium throughout the entire *FSHB* gene sequence. Low genetic diversity and strong LD extends to the 3'-flanking region of the *FSHB* gene.

2. The human *FSHB* gene is represented by two core genetic variants (haplotype 1 and haplotype 2) spread worldwidely with population-specific frequencies, whereas the *FSHB* gene haplotype 2 is found to be enriched among populations of European origin (Estonians, Czechs and unrelated Utah Mormons/CEU). Core haplotypes differing in each polymorphic position ('yin-yang' haplotypes) and excess of polymorphisms with intermediate allele frequencies may indicate that the *FSHB* gene is under the influence of balancing selection.

3. Comparison of the translated parts of the *FSHB* gene in human and great apes (chimpanzee, gorilla, and orangutan) has revealed eleven and three nucleotide and amino acid differences, respectively. All the studied great apes are homozygous for the *FSHB* gene haplotype similar to the human *FSHB* gene second frequent variant (haplotype 2).

4. The results of a genetic association pilot-study on possible effect of the *FSHB* gene variants on female reproductive health has indicated significant difference in the distribution of the homozygotes for the haplotype 2 between random population-based cohort of Estonian women and Estonian females conceived naturally within three months after stopping contraception. The latter sample demonstrated the enrichment of the *FSHB* haplotype 2. Since the *FSHB* core variants consist of non-coding polymorphisms, genetic variants that distinguish the *FSHB* two core haplotypes may co-segregate with functional regulatory variants either upstream or downstream of the *FSHB* gene possibly contributing to the gene expression regulation.

5. *In silico* screening of the immediate 5'-flanking promoter region (–350 bp relative to transcription start-site) of the *FSHB* gene has resulted in the identification of previously uncharacterized putative regulatory polymorphic position –211 G/T (rs10835638). This SNP is located within Progesterone Response Element (PRE) capable of modulating *FSHB* gene expression.

6. When the allele and genotype frequencies of –211 G/T SNP were compared between the Baltic cohort of young men (Estonians, Latvians, Lithuanians; n=1054) and Estonian male patients diagnosed with infertility (n=1029), the frequency of the alternative allele T was higher in the latter. Thus, the *FSHB* promoter position –211 G/T may contribute to male infertility phenotype.

7. The quantitative genetic association studies on possible effects of the *FSHB* gene –211 G/T SNP allelic status on male reproductive parameters have provided strong evidence of significant association between –211 G/T carrier status and serum FSH level. Both in the Baltic cohort of young men and in the male patients diagnosed with idiopathic infertility, carriers of GT- and TT-genotypes exhibited 16–35% and 30–50% lower serum FSH levels, respectively, when compared to GG-genotype carriers.

8. Additionally, significant secondary associations between the *FSHB* promoter position and other male testicular and hormonal parameters including inhibin B, total testosterone, serum LH level, sperm concentration, and testes volume were identified. Carriers of the TT-genotype had approximately 20% lower total testicular volume when compared to GG- and GT- genotype carriers. These associations refer to the possible short-term and long-term downstream pleiotropic effects of lower serum FSH levels in men. Genetically determined lower FSH level during male embryonal, early natal, and pubertal development may affect different downstream reproductive parameters and contribute to the regulation of male reproductive potential.

Main outcome of the study:
The current study identified the first *FSHB* gene polymorphism that determines the level of male serum FSH and recapitulated the essential role of FSH in male reproductive processes.

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SUMMARY IN ESTONIAN

Folliikuleid-stimuleeriva hormooni beeta-alaühikut kodeeriva geeni (*FSHB*) järjestuse varieeruvus ja selle seos reproduktiivtervisega

Folliikuleid stimuleeriv hormoon ehk follitropiin (FSH), luteiniseeriv hormoon ehk lutropiin (LH) ja kooriongonadotropiin (hCG) on glükoproteiinsed gonadotropiinid, mis reguleerivad mõlema sugupoole sugurakkude teket ja küpsemist ning suguhormoonide tootmist. Igale gonadotropiinile on evolutsiooni käigus kinnistunud konservatiivne ning ajaliselt ja koeliselt spetsialiseerinud funktsioon. FSH ja LH tootmist ajuripatsi (hüpofüüsi) eessagara rakkudes reguleerib peamiselt vabastajahormoon (*Gonadotopin Releasing Hormone*, GnRH), mis eritub hüpotaalamuses. Suurel määral toimub regulatsioon tagasiside mehhanismil põhineva hüpotaalamuse, ajuripatsi ja gonaadide süsteemis hormoonide (peamiselt östrogeenid, testosteroon, progesteroon) abil, lisaks mõjutavad FSH ja LH tootmist ka muud parakriinsed ja endokriinsed tegurid. FSH seondub munasarja granuloosa rakkude membraanis paiknevate retseptoritega ja stimuleerib naistel munasarja folliikulite arenemist, küpsemist ja östrogeenide tootmist. LH põhjustab küpse folliikuli ovulatsiooni ja stimuleerib kollaskeha teeka rakkudes androgeenide tootmist. Meestel on FSH signaalirada oluline munandi Sertoli rakkude paljunemisel ja küpsemisel loote-, vastsündinu- ja puberteedieas; täiskasvanueas on FSH vajalik normaalseks spermatogeneesiks. LH tagab testosterooni tootmise munandi Leydigi rakkudes, mis on oluline nii spermatogeneesis kui ka suguorganite arengus. hCG toodavad platsenta süntsüütsiotrofoblasti rakud – seega hCG on lootelt pärinev rasedusspetsiifiline hormoon. HCG on vajalik viljastatud munaraku implantatsiooniks, immuuntolerantsi tekkeks ema ja loote vahel ning raseduse normaalseks kuluks.

Gonadotropiinide perekonna liikmed on heterodimeersed valgud, mis koosnevad kahest alaühikust. α-alaühik on kõikidel gonadotropiinidel identne, hormooni spetsiifilise toime ja retseptoriga seondumise määrab hormoonispetsiifiline β-alaühik. LH ja hCG β-alaühikuid kodeerivad geenid on väga kõrge DNA järjestuse sarnasusega ja paiknevad ühes geeniklastris19. kromosoomi pikas õlas (19q13). Geeniklastrisse kuulub üks *LHB* geen, mis kodeerib LH β-subühikut ning kuus *CGB* geeni, millest 4 kodeerivad hCG β-subühikut *(CGB, CGB5,7,* ja *8*). FSH β-subühikut kodeerib üks *FSHB* geen, mis on ligi neli tuhat aluspaari pikk ja paikneb 11. kromosoomis. Võrdleva genoomika analüüsi põhjal ilmneb, et teatud *FSHB* geeni piirkonnad: valku kodeeriv piirkond ning geeniga vahetult piirnevad mittekodeerivad piirkonnad, on liigiti väga kõrge DNA järjestuse sarnasusega viidates mainitud piirkondade tähtsale funktsioonile. Inimestel on seni tuvastatud ainult viis aminohapet muutvat mutatsiooni (kokku üheksa indiviidi), mis kõik põhjustavad olulisi muutusi valgustruktuurisning seonduvad viljatusega nii meestel kui ka naistel.

Vaatamata sellele, et FSH ja selle β-alaühikut kodeeriv geen *FSHB* on asendamatu reproduktsioonil, on seni vähe uuringuid, mis kirjeldavad *FSHB* geeni varieeruvust ja selle mõju inimese reproduktiivtervisele.

Doktoritöö kirjanduse ülevaade hõlmab gonadotropiinide evolutsiooni, molekulaarset struktuuri ja funktsioone. Järgmisena on tutvustatud gonadotropiinide alaühikuid kodeerivaid geene; FSH β-alaühikut kodeeriva geeni struktuuri ja varieeruvust; ning geeni ekpressiooni ehk avaldumise regulatsiooni.

Doktoritöö eksperimentaalses osas on püstitatud järgmised eesmärgid:

(i) iseloomustada *FSHB* geeni ülemaailmset varieeruvust (Euroopa, Aafrika ja Aasia populatsioonid)
(ii) tuvastada esivanemlik (šimpans, gorilla, orangutan) *FSHB* geeni variant;
(iii) leida *FSHB* geeni tuumhaplotüüpide võimalik seos naise viljakusega;
(iv) tuvastada polümorfsed variandid *FSHB* geeniga vahetult piirnevas evolutsiooniliselt konserveerunud mittekodeerivates piirkondades, mis võiksid potentsiaalselt mõjutada *FSHB* geeni avaldumise taset (*in silico* analüüs);
(v) leida seos *FSHB* geeni promootoralas paikneva regulatoorse ühenukleotiidse polümorfismi (–211 aluspaari transkriptsiooni alguskohast, G/T) ja seerumi FSH taseme vahel Baltimaade noorte meeste üldvalimis (Eesti, Läti, Leedu) ja viljatute Eesti meeste hulgas;
(vi) tuvastada assotsiatsioonid *FSHB* promootoralas paikneva polümorfismi ja FSH tasemega seotud reproduktiivsete parameetrite vahel.

Uurimistöö peamised tulemused on:

1. *FSHB* ülemaailmse varieeruvuse mustri uuringu tulemusena, mis hõlmas 222 indiviidi kuuest Euroopa, Aafrika ja Aasia populatsioonist (Eesti, Mandenka, Hiina Han, Korea, Tšehhi, *Utah* mormoonid ehk Euroopa päritoluga Ameerikas elavad indiviidid), ei tuvastatud ühtegi mittesünonüümset FSH valgu aminohappe muutust põhjustavat DNA muutust. Kõikides uuritud populatsioonides on levinud kõrge sagedusega ühenukleotiidsed polümorfismid, mis paiknevad valdavalt geeni mittekodeerivas piirkonnas ning mille alleelide vahel on täielik alleelne assotsiatsioon ehk aheldustasakaalutus (*Linkage Disequilibrium*, LD). Geenisisene LD ulatub ka *FSHB* geeni 3' ehk allavoolu piirkonda, moodustades kuni 6500 aluspaari pikkuseid tihedalt aheldunud alleelide blokke.
2. *FSHB* geen on esindatud kahe tuumhaplotüübiga, haplotüüp 1 ja haplotüüp 2, mis erinevad üksteisest igas polümorfses positsioonis (*yin yang* haplotüübid). Euroopa päritoluga populatsioonides on mõlemad haplotüübid levinud võrdse sagedusega mitte-Euroopa päritoluga populatsioonides esineb valdavalt *FSHB* geeni haplotüüp 1. Kaks *yin yang* haplotüüpi ning hulgaliselt sagedasi polümorfisme viitavad sellele, et antud genoomne piirkond on evolutsiooniliselt arenenud tasakaalustava valiku toimel.
3. Inimese ja inimahvide (šimpans, gorilla, orangutan) *FSHB* geeni võrdlusel ilmnes üksteist ühenukleotiidset erinevust, millest kolm põhjustavad erineva aminohappe esinemist valgu järjestuses. Inimahvidele on omane inimese *FSHB* geeni teisele tuumhaplotüübile sarnane haplotüüp. Kõik uuritud inimahvid on selle haplotüübi suhtes homosügoodid.

4. *FSHB* geeni tuumhaplotüüpide võimalikku seose leidmiseks naise viljakusega võrreldi haplotüüpide jaotuvust naiste juhuvalimis (n=47, puudub info viljakuse kohta) ja kiirelt rasestuvate viljakate (rasestumine toimus kolme kuu jooksul pärast rasedusest hoidumise lõpetamist = *short time to pregnancy* – STP) naiste hulgas (n=48) Eestis. Pilootuuringu tulemusena ilmnes, et kiirelt rasestuvate viljakate naiste hulgas leidus enam *FSHB* geeni teise tuumhaplotüübi kandjaid. Kuigi *FSHB* geeni tuumhaplotüüpide puhul on kodeeritav valk sarnase aminonappelise järjestusega, võivad tuumhaplotüüpide polümorfismid seonduda tugeva LD tõttu *FSHB* geenis ja sellega piirneval alal regulatoorse alaga, mis mõjutab geeni avaldumist.
5. *FSHB* geeni promootori (350 aluspaari enne geeni transkriptsiooni alguspunkti) *in silico* analüüsi tulemusena tuvastati potentsiaalne regulatoorne polümorfism –211 G/T (rs10835638). Lookus paikneb PRE-elemendis (*Progesterone Response Element*), mis toimib tranksriptsiooniteguri kinnituskohana ja reguleerib geeni avaldumist.
6. *FSHB* promootorpiirkonna polümorfismi G/T (positsioonis –211) alleelisageduste võrdlemisel Baltimaade noorte meeste kohordi (Eesti, Läti ja Leedu; n=1054) ja Eesti viljatute meeste kohordi (n=1029) vahel ilmnes, et minoorne alleel T esineb sagedamini viljatute meeste hulgas. T alleeli rikastumine viljatutel meestel võib viidata polümorfismi võimalikule seosele mehepoolse viljatuse kujunemises.
7. Geneetilise assotsiatsiooni analüüsi tulemusena ilmnes oluline seos *FSHB* promootori positsioonis –211 paikneva polümorfismi G ja T alleelide ning meeste seerumi FSH taseme vahel. Nii Baltimaade noorte meeste kui ka Eesti viljatute meeste hulgas, oli GT- ja TT-genotüüpide kandjatel vastavalt 16–35% ja 30–50% madalam vereseerumi FSH väärtus võrreldes GG-genotüüpi omavate indiviididega.
8. *FSHB* promootori positsioonis –211 paiknev G/T polümorfism seondub ka teiste hormonaalsete ja munandi funktsiooni näitavate parameetritega meestel: inhibiin B, testosteroon, seerumi LH tase, spermatosoidide kontsentratsioon ja munandite kogumaht. TT-genotüübi kandjatel on ligikaudu 20% väiksem munandite kogumaht võrreldes GG- ja GT-genotüüpide kandjatega.

Polümorfismi seosed mitme meeste reproduktiivtervise parameetriga viitavad madala FSH taseme võimalikele pika- ja lühiajalistele efektitele meeste reproduktiivpotentsiaalile. Geneetiliselt määratud madalam FSH tase looteeas, varajases lapse- ja puberteedieas võib mõjutada erinevaid etappe mehe reproduktiivse potentsiaali väljekujunemisel.

10–20% peredest kogevad mingil ajal oma elus probleeme soovitud laste saamisel. Umbes 50% juhtudel on paari viljatuse põhjuseks mehepoolsed tegurid. Üheks mehepoolse viljatuse põhjuseks on hormonaalsed häired ja neist tulenevad spermatogeneesi häired. On näidatud, et mitmetel juhtudel sõltub viljatuse ravi tulemus suures osas mehe FSH tasemest. *FSHB* geeni promotoorpiirkonna –211 positsioonis paikneva polümorfismi (rs10835638) minoorse

alleeli T kandvatel indiviididel on geneetiliselt määratletud madalam FSH tase ning sellest tulenevalt võib nende sooline areng loote- ja lapseeas olla mõjutatud pikema aja vältel. Täiskasvanueas võib sellistel meestel viljatuseprobleemide korral olla FSH ravi tõhusam ning spetsiifilisem lähtudes indiviidi pärilikust eelsoodumusest. *FSHB* geeni ülemaailmse varieeruvuse muster ning geeni promootorpiirkonnas paiknev oluline regulatoorne polümorfism, mis mõjutab seerumi FSH taset ja seondub mehepoolse viljatusega, loob tugeva eelduse töö jätkamiseks *FSHB* geeni varieeruvuse seose hindamiseks naise ja terviku paari reproduktiivpotentsiaaliga.

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APPENDIX

Appendix 1. List of SNPs and variation data identified in *FSHB* gene are deposited in dbSNP database under accession numbers:

211_FSHBprom	ss105106771
m292	ss49785048
m284	ss49785049
550312	ss49785050
611246	ss49785051
609896	ss49785052
543	ss49785053
589	ss49785054
580646	ss49785055
595496	ss49785056
594982	ss49785057
6169	ss49785058
1820	ss49785059
506197	ss49785060

Appendix 2. The full chimpanzee, gorilla and orangutan *FSHB* gene sequences from Ref. II are deposited in NCBI GenBank under accession numbers:

Pan troglodytes	DQ302103
Gorilla gorilla	DQ304480
Pongo pygmaeus	DQ304481

Appendix 3. Genomic DNA sequence of human *FSHB* gene (GenBank Acc. No. NC_000011.9). Exons are highlighted with grey, translated regions are underlined, location of translation start-site (ATG) and translation stop-site (TAA) is boxed.

ACAGCTCTTGCCAGGCAAGGCAGCCGACCACAGGTGAGTCTTGGCATCTACCGTTTTCAAGTGGTGACAG
CTACTTTTGAAATTACAGATTTGTCAGGACATGGAGGACAAAACTAGAGCTTCTCACTACTGTTGTGTAG
GAAATTTATGCTTGTCAACCTGGCTTGTAAAATATGGTTAATATAACGTAATCACTGTTAGCAAGTAACT
GACTTTATAGACCAATATGCCTCTCTTCTGAAATGGTCTTATTTTAAACAAATGTGAGCAAAAGAAAATA
TTTATGAGATTCTAAAAATGAAGACATAATTTTGTAGTATAGAATTTTCTTGGCCAGGAATGGTGGCTCA
TGCTTGTAATCCCAGCACTTTGGGAGGCCAAGGTCAGAGGATTGCTTGAGCCTGGAAGGTTGAAGATGCA
GTGATTCATGATTATACCACTGCACTCCAGCCTGGGCAACAGAGCAAGACCCTGTCTCAAGAAAAGAAAA
GAATTTTATTTTTCTTTTCAGACAAAAATAGACTTTAAAATAATAATGGAAGAACAAATATGATGATCAC
AATTATCAGAGTAATTACTTTATGACAGTCAGCAATAAGATTCTAATCTTTAAATATTCCTCTGCTTAAA
TCATTATATTGGAGTTTTGATCTATAATATATTCCCACCCTGACCCAAAAATTGAAGAAGGACAAGGAAA
AATGTTGTTCCAAGAAACAAAGATGTAAGTAAAAAGGCATAAGGAAGGAAAAAAAACTTTTGAAGCAAAA
TGTGATTGAGGAGGATGAGCAGACCAATTATTTTTGGTTTGGTCAGCTTACATAATGATTATCGTTCTTT
GGTTTCTCAGTTTCTAGTGGGCTTCATTGTTTGCTTCCCAGACCAGGATGAAGACACTCCAGTTTTTCTT
CCTTTTCTGTTGCTGGAAAGCAATCTGCTGCAATAGCTGTGAGCTGACCAACATCACCATTGCAATAGAG
AAAGAAGAATGTCGTTTCTGCATAAGCATCAACACCACTTGGTGTGCTGGCTACTGCTACACCAGGGTAG
GTACCATGTTTTGCTGGAAGCAAGGGTGTTGAAGGTCTGTATTAGGCCGGTTTCATTAGTTTCTACTTTA
TCAATATTTTATGTATTCTAAGTAACAGCCATGAGTCCTTTAGCCAAGACTGTCTGTGTTGTGATTGGGG
TTAATGACCACGATATCACTTAGATGTTTGGGCTTGGATTTGATTTGGGTAAATTTAGGAAAGCCTCAGA
TTTAATCTGATCAATTTGGTACTAGTCCAACTTTGCATCTACAGGGAAAAAGTATTTCTATGTTACGTTT
TTACACATAGAGAGATAAACATGGAAACATACATATATTTAATCATAAAGGACCTATAATATTCTCATAA
AGGCAATTTCTTTAACTGACACTACATCTTTGACACAAAATCACACCAAAATATGTCTCCAAGTCACATA
AAAACATAGACAGCCACTTAAAAAAATTGTCTTCCTGGCCCTACTAAATACAAATGCCAAAAAACAGCCT
GAGAACACAATCAATTCTTGCAGACTGTTAGAACAAAAATGAATCAGCAAACCCACTCCCTTCGTTATAG
CATTGAGAAAACCAAGACATAGAGGCATCAGTTGCTAGTCTGTGTTTGCAGTTTCCTTGCATTAATACAA
GTAGAGAAATAGTTTCCATGGTGCTTTCTTTTTTCTCTGCAGCACCCCTAATTATCTATGCAGAATTTCA
TTCTATAAACTAAAATTGAAAATGGCAACTTTTTAAATGAACGATACTTTATTTGACGGTAAATGAGTTT
GATCAAACTCCATTTATTACACAATTTATTGCACCTTCTTGGGATATACATTTGGTAGGATGATATTAAA
ATAAACAGAAGCCCCAATTTCTCTACGCAGTATAAATAATTTTTCCACTGGAAAGTGCTACTACAAATAA
TTTCTACCTGGATTAAAAATTCTTATATGCAAACTGCATATCCTTTGAAACTAGGAACCCTGCAAAGTAT
ACAGCTTTCAAGGGAGAAAAATGTCCACAAGGAGTTGGAATATTTAAAATCTTATGTTAGCCTTAGCAAA
CATGTTAACTTAAGCATTAAAATTTAAAATTATATTTTTGACCTTTTATAAATACTCAGGGCAGTGTA
TTTTAAAATATTTTTTCTGAGACATTGGATATCTTTGTTTATGGTTTGTTATTAATACAGCTTTCAATTA
AATATGAAAAGTCAACTTAAAATCCTGTCATGTTTTTCATCATTTTTCTATGCTAAAATTCAAAGTTCCT
TTATATTTTGAAAAATAGTTAATATTTTGATATAGCCATAGGAAGTAAGAAAAGAAATTACTTGTATTTT
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GGTAAATGTTAGAGCAAGCAGTATTCAATTTCTGTCTCATTTTGACTAAGCTAAATAGGAACTTCCACAA
TACCATAACCTAACTCTCTTCTTAAACTCCTCAGGATCTGGTGTATAAGGACCCAGCCAGGCCCAAAATC
CAGAAAACATGTACCTTCAAGGAACTGGTATACGAAACAGTGAGAGTGCCCGGCTGTGCTCACCATGCAG
ATTCCTTGTATACATACCCAGTGGCCACCCAGTGTCACTGTGGCAAGTGTGACAGCGACAGCACTGATTG
TACTGTGCGAGGCCTGGGGCCCAGCTACTGCTCCTTTGGTGAAATGAAAGAATAAAGATCAGTGGACATT
TCAGGCCACATACCCTTGTCCTGAAGGACCAAGATATTCAAAAAGTCTGTGTGTGTGCAATGTGCCCAGG
GGACAAACCACTGGATCAGGGGATTCAGACTCTACTGATCCCTGGTCTACTGGCAGAGGGAACTCTGGGA
ATTGAGAGTGCTGGGGGCCAGGACTCCATCATGATTCAGCTCTATATTCCTAGGTCTGATTTCATAAGGT
TTATTCAGTCTTAACTCACAGACTTGTGCCTGGTTTCTTCTTTAAAAATCTTAGAAATCTTCTCAGGCAA
TGCCTCTCTCTTAGGGGGAAACATAAGCCTAGAAGGAGGAAGCAGTAATGGGAGTGAGTGAAAGAACTAA
CTGCAGCAGTCTTCTGGTAGACTCTTGGGCCCTCTAGAGCAAGGTCAGCATCTTCAGCATTGTAGCGTCA
ATGCCTAGCACTCTGCCTGGAACTTAGAAACACAACAATGACTTCTTTAGATCAGAAAGGTCAAGGGTAG
AAAATACTGGAAGACGATGTTTGAGGTAAGCTGATGAGGCTGCCCGCAGCCACACCAGTCCCATGAAAGT
TAGTGGCATCAGTTCCACCTCGCCTTTTCTCCAGCACATGGAGTATTGAGACATGATGTATCTTTCTGAA
TTGTTTGGTACAGATGGGGAGTAACAGAGCTCAAGATTTCCAAGCTATTACTACCAAGCCTGTTAGTTAA
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TGATCACATGCTACTAGGCTCCTGCAAAACTCCTTTTTGAGATAAAGGGAAAAAACCAAACTATCTCACC
CTACCCTCCCTAGGATCCACTTCTTTGGAATGACAAAGGATTTGAAAGTAGGTTTGAAAGCAGTTTCAGC
AATTTAATAAATATAATTAATTTGTCTACAAATATATTTGTATAAATAAATAGCTCCTTTAGAAAGAATT
AGCCATGGGGGACGAGGGGAAACTGCTGTTTTCTAGGATCCTGTCTACATCAATCTTCTATTTTATCCAT
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TTTAAACGTGTTAACTGTCTTAGTTATGCACTCAGTTTCACACTCATATTGTTTAACTAATTTATTTAAA
TCTTATTTTTTTAATAAAGATGCTAGCCACCAGAGTCACAGGCTTGGATTGTTTTATGTACAAACAGATG
ACTTAGATATTCTGTATTTTATAATATTAGTGGAATGAAATCTTAAAATATAATTCCCAGTGTTTCTATA
AATATTACCTTTCCTTATCTTTGGAGATATTAAAAATAATTTTGTTGGATTTCTGAAGTGTTTTGTCACT
TAAATTTCCTGTCATTTTTTGAAGACATTTTCTGATGTAATTTGGG

PUBLICATIONS

CURRICULUM VITAE

Marina Grigorova

Date of Birth: 19.04.1982
Address: Department of Biotechnology,
Institute of Molecular and Cell Biology,
University of Tartu
Riia str. 23, 51010, Tartu, Estonia
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Education

1988–2000 Tartu Slaavi Gymnasium, *cum laude*
2000–2004 B.Sc in Biology, Chair of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu
2004–2006 M.Sc in Gene Technology, Chair of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu
2006–2011 Ph.D student in Molecular and Cell biology, Institute of Molecular and Cell Biology, University of Tartu

Professional employment

2005 technician, Chair of Biotechnology, Institute of Molecular and Cell Biology, University of Tartu
2010 specialist, Estonian Biocentre
2011– research scientist, Institute of Molecular and Cell Biology, University of Tartu

Scientific work and activity

In 2002, I started my research in Professor Maris Laan's group at the Institute of Molecular and Cell Biology, University of Tartu, Estonia. One of the focuses of the group is genetic basis of male and female fertility, reproductive genetics. My study focuses on a complex multigenic process of the establishment and regulation of the fertility of the organism. The dominant theme of my research relies on the worldwide variation of the follicle-stimulating hormone beta sub-unit gene (*FSHB*) and its association with male and female reproductive health.

Publications

1. **Grigorova M**, Rull K, Laan M. Haplotype structure of *FSHB*, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. Ann Hum Genet. 2007 71:18–28
2. Haller K, Salumets A, **Grigorova M**, Talja I, Salur L, Bene MC, Laan M, Uibo R. Putative predictors of antibodies against follicle-stimulating hormone in female infertility: a study based on in vitro fertilization patients. Am J Repr Imm. 2007 57:193–200
3. **Grigorova M**, Punab M, Ausmees K, Laan M. *FSHB* promoter polymorphism within evolutionary conserved element is associated with serum FSH level in men. Hum Reprod. 2008 23:2160–6
4. **Grigorova M**, Punab M, Poolamets O, Kelgo P, Ausmees K, Korrovits P, Vihljajev V, Laan M. Increased Prevalance of the –211 T allele of follicle stimulating hormone (FSH) beta subunit promoter polymorphism and lower serum FSH in infertile men. J Clin Endocrinol Metab. 2010 95:100–8.
5. Nagirnaja L, Rull K, Uusküla L, Hallast P, **Grigorova M**, Laan M. Genomics and genetics of gonadotropin beta-subunit genes: Unique *FSHB* and duplicated *LHB/CGB* loci. Mol Cell Endocrinol. 2010 329(1–2):4–16
6. **Grigorova M**, Punab M, Zilaitiene B, Erenpreiss J, Ausmees K, Matulevicius V, Tsarev I, Jørgensen N, Laan M. Genetically Determined Dosage of Follicle-Stimulating Hormone (FSH) Affects Male Reproductive Parameters. J Clin Endocrinol Metab. 2011 96:E1534–E1541

Fellowships

2005 Kristjan Jaak's stipend of Archimedes Academic Fund
2006 European Testis Workshop travel grant
2006 Estonian National Contest for Young Scientists at university level, III Prize for M.Sc thesis
2007 Estonian World Council, Inc. Margot M. and Herbert R. Linn stipend
2009 EcoGene fellowship 2010/2011, for young women in science (women returning from maternity leave)
2009 Tartu University Raefond Scholarship
2010 International Society of Andrology Young Investigator Travel Award (Genetics of Male Infertility symposium 2010)
2010 Artur Lind Scholarship by Estonian Genome Foundation

ELULOOKIRJELDUS

Marina Grigorova

Sünniaeg ja koht:	19.aprill1982, Tartu, Eesti
Aadress:	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, Biotehnoloogia õppetool, Riia 23, 51010, Tartu
E-post:	marina.grigorova@ut.ee

Haridus

1988–2000	Tartu Slaavi gümnaasium, kuldmedal
2000–2004	B.Sc bioloogia erialal, Biotehnoloogia õppetool, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool
2004–2006	M.Sc geenitehnoloogia erialal, Biotehnoloogia õppetool, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool
2006–	Doktoriõpe molekulaar- ja rakubioloogia erialal, Molekulaar- ja rakubioloogia instituut, Tartu Ülikool

Erialane teenistuskäik

2005	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, Biotehnoloogia õppetool, laborant
2010	Eesti Biokeskus, spetsialist
2011–	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut, inimese molekulaargeneetika teadur

Teadustegevus

Oma teadustöös olen fokuseerinud inimese reproduktiivfunktsiooniga seotud *Folliikuleid Stimuleeriva Hormooni beeta*-alaühikut kodeeriva geeni geneetilisele varieeruvusele ja selle seosele inimese reproduktiivtervisega.

Publikatsioonid:

1. **Grigorova M**, Rull K, Laan M. Haplotype structure of *FSHB*, the beta-subunit gene for fertility-associated follicle-stimulating hormone: possible influence of balancing selection. Ann Hum Genet. 2007 71:18–28
2. Haller K, Salumets A, **Grigorova M**, Talja I, Salur L, Bene MC, Laan M, Uibo R. Putative predictors of antibodies against follicle-stimulating hormone in female infertility: a study based on in vitro fertilization patients. Am J Repr Imm. 2007 57:193–200

3. **Grigorova M**, Punab M, Ausmees K, Laan M. FSHB promoter polymorphism within evolutionary conserved element is associated with serum FSH level in men. Hum Reprod. 2008 23(9):2160–6
4. **Grigorova M**, Punab M, Poolamets O, Kelgo P, Ausmees K, Korrovits P, Vihljajev V, Laan M. Increased Prevalance of the –211 T allele of follicle stimulating hormone (FSH) beta subunit promoter polymorphism and lower serum FSH in infertile men. J Clin Endocrinol Metab. 2010 95(1):100–8.
5. Nagirnaja L, Rull K, Uusküla L, Hallast P, **Grigorova M**, Laan M. Genomics and genetics of gonadotropin beta-subunit genes: Unique *FSHB* and duplicated *LHB*/*CGB* loci. Mol Cell Endocrinol. 2010 329(1–2):4–16
6. **Grigorova M**, Punab M, Zilaitiene B, Erenpreiss J, Ausmees K, Matulevicius V, Tsarev I, Jørgensen N, Laan M. Genetically Determined Dosage of Follicle-Stimulating Hormone (FSH) Affects Male Reproductive Parameters. J Clin Endocrinol Metab. 2011 96(6):E1534–E1541

Stipendiumid:

2005 Kristjan Jaagu stipendium
2006 XIV European Testis Workshop travel grant
2006 Eesti üliõpilaste teadustööde riiklik konkurss, III preemia magistriõppe üliõpilaste astmes
2007 Ülemaailmse Eesti Kesknõukogu (ÜEKN) Margot M. ja Herbert R. Linna stipendium
2009 Naisteadlase stipendium (EcoGene)
2010 International Society of Andrology Young Investigator Travel Award (Utah/Florence Symposium on Genetics of Male Infertility (USA), 2010)
2010 Artur Linnu nimeline stipendium

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