Synthesis and secretion of gonadotropins including structure-function correlates

George R. Bousfield · James A. Dias

Abstract The synthesis and secretion of the gonadotropic hormones involves coordination of signal transduction, gene expression, protein translation, post-translational folding and modification and finally secretion. The production of biologically active gonadotropin thus requires appropriately folded and glycosylated subunits that assemble to form the heterodimeric hormone. Here we overview recent literature on regulation of gonadotropin subunit gene expression and current understanding of the assembly and secretion of biologically active gonadotropic hormones. Finally, we discuss the therapeutic potential of understanding glycosylation function towards designing new forms of gonadotropins based on observations of physiologically relevant parameters such as age related glycosylation changes.

Keywords Transcription · FSHβ · LHβ · Common α subunit · GnRH

Abbreviations

ASGP-R	Asialo-glycoprotein receptor
cAMP	cyclic AMP
CG	chorionic gonadotropin
CRE	cAMP response element
CREB	CRE binding protein
EGR1	early growth response 1
ESI-MS	electrospray mass spectrometry
FSH	Follicle-stimulating hormone
FSHR	FSH receptor
Gn_2M_8	N-acetyl-glucosamine2 Mannose8
$Gn_2M_9Glc_1$	N-acetyl-glucosamine2 Mannose9 Glucose1
GnIH	gonadotropin inhibitory hormone
GnRH	gonadotropin releasing hormone
GPCR	G protein-coupled receptor
HEK293	human embryonic kidney 293 cell line
ICER	induciblecAMP early repressor
Isl-1	insulin gene enhancer protein
JNK1	Janus kinase 1
LH	luteinizing hormone
LHR	LH receptor
MALDI-TOF-MS	matrix-assisted laser desorption time-of-flight mass spectrometry
MEK1	mitogen activated protein kinase kinase 1
MGAT	N-Acetyl-glucosamine transferase
OST	oligosaccharyltransferase
PGF2α	prostaglandin F2α
PGI	prostaglandin I
PKC	protein kinase C
PLC	phospholipase C
SNARES	soluble N-ethylmaleimide-sensitive attachment protein receptor
ST3Gal	α2-3-sialyltransferase
ST6Gal	α2-6-sialyltransferase
TGFβ	transforming growth factor β
TSH	thyroid-stimulating hormone

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1 Introduction

The glycoprotein hormone family includes luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroid-stimulating hormone (TSH) and chorionic gonadotropin (CG) [1]. Production of these hormones occurs in either the pituitary (LH, FSH, TSH) [2] or by the chorion of a developing fetus (CG) [3, 4]. These hormones are heterodimeric, consisting of a common alpha subunit and a unique beta subunit (Fig. 1), and they are also glycosylated [5]. Therefore, their production requires coordination of transcriptional regulation of the subunit genes [6], translation of mRNA, post-translational processing and assembly, packaging and secretion.

2 Regulation of the gonadotropin genes

The canonical regulator of gonadotropin secretion is the hypothalamic decapeptide, GnRH, which can induce stimulus-secretion coupling as well as gene expression

[7]. However, it is clear now that many paths coalesce to achieve the complexity and disparate regulation of LH and FSH expression, a necessary first step to production and secretion. GnRH mediated gonadotropin secretion can be enhanced by the transcription factor Isl-1, which is elevated in response to GnRH as well as by activin and leptin stimulation, although the responses to activin and leptin were higher and more prolonged than that induced by GnRH. However, subsequent experiments indicated Isl-1 mainly is responsible for leptin-mediated FSHβ and LHβ production, not GnRH- or activin-mediated secretion, as knocking down Isl-1 expression had no significant effect on gonadotropin regulation by either GnRH or activin [8]. Despite this regulation, in marsupials, LHβ gene expression is relatively unchanged despite widely variable levels of LH secretion, whereas FSHβ gene expression covaries with level of FSH [9]. Thus, the secretion of LH appears to be induced, whereas the secretion of FSH is constitutive, as will be discussed below.

The release of GnRH from hypothalamic neurons is pulsatile [10]. Given the effect of GnRH as a releasing

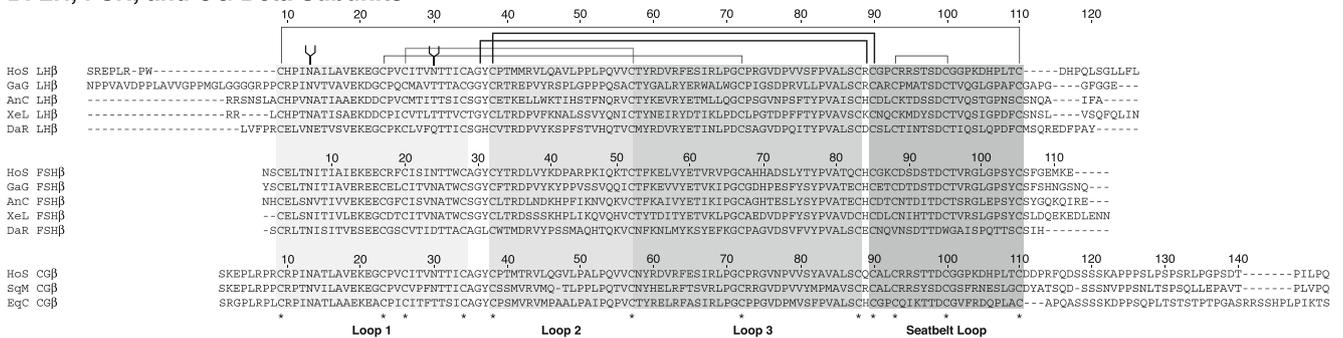## A. Alpha Subunits
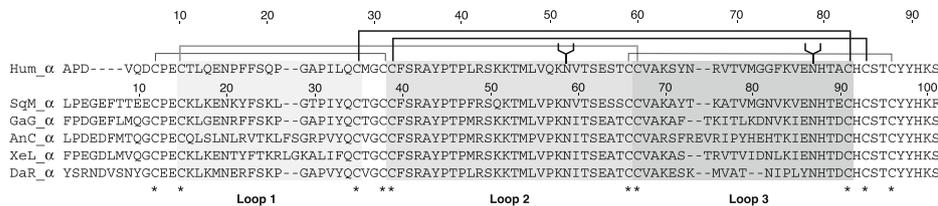
B. LH, FSH, and CG Beta Subunits

Fig. 1 Primary structures of vertebrate gonadotropins. The mature amino acid sequences for the human gonadotropin subunits and equine LH/CGβ are shown along with those predicted by the SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) for the primary structures translated from nucleotide sequences. The amino acid sequences were obtained from NCBI and represent selected vertebrate species: mammalian (*Homo sapiens*), avian (*Gallus gallus*), reptilian (*Anolis carolinensis*), amphibian (*Xenopus laevis*), and fish (*Danio rerio*) of clinical or scientific significance. The *D. rerio* FSHβ sequence is suspect because of the lack of potential cystine noose disulfide bond. The horse (*Equus caballus*) and squirrel monkey (*Saimiri boliviensis boliviensis*) CGβ sequences were included to illustrate alternatives to the well known hCGβ primary structure and because they replace LHβ in their respective species. Sequence alignments were made using the NCBI tool, COBALT. The locations of N-glycans are indicated by the "tuning forks" above the glycosylation sites. Please note that while both FSHβ and hCGβ possess two N-glycans, most vertebrate LHβs are typically glycosylated at Asn^{13}, while human LHβ is glycosylated at Asn^{30}. The human CGb is also O-glycosylated at 4 Ser residues 121, 127, 132, and 138. Squirrel monkey CGβ is N-glycosylated at Asn^{30} and O-glycosylation remains to be determined experimentally, although they are expected to be absent. The three loops created by the cystine knot motifs and the β-subunit seat belt loop are shaded and labeled. Cys residues are highlighted by asterisks under subunit sequence. A. Alpha subunit sequences illustrating the difference in numbering between the 92-residue human and other vertebrate 96-98-residue α-subunit sequences. B. Beta subunit sequences grouped by hormone as follows: LHβ, FSHβ, and CGβ

hormone, one might expect that all secretagogues including GnRH would engender gene expression that would co-vary with pulse. When mouse pituitary gonadotrophs were stimulated with adenylate-cyclase-activating polypeptide there was no change in LHβ or FSHβ gene expression, although the common α subunit gene expression was induced [11]. Remarkably, perfusion of pulsatile doses of adenylate-cyclase-activating polypeptide engendered elevation of both the LHβ and FSHβ genes [11]. Although both LHβ and FSHβ gene expression was upregulated by pulsed administration of the peptide, high frequency pulses increased LHβ gene expression more than FSHβ gene expression whilst low frequency pulses had the opposite effect [11]. Thus, like GnRH, differential regulation of gonadotropin gene expression can be achieved by modulation of the signal frequency. The control of LHβ gene transcription by the transcription factor Early Growth Response 1 (EGR1) led to the hypothesis that the underpinning of the pulsed-dose differential regulation of the LHβ and FSHβ genes was EGR1. However, GnRH-induced JNK1 and MEK1 kinase activities, while essential, do not control EGR1 gene expression in a pulsed manner [12]. Another transcription factor, CREB, can bind to cAMP response elements on the FSHβ gene promoter, and could give rise to the differential pulse effect on gonadotropin gene expression. In concert with this, it has been demonstrated that high pulse frequency of GnRH does give rise to elevated ICER, which would bind to CRE on the FSHβ gene and dampen its transcription [13]. Thusly, the induction of a negative regulatory transcription factor could potentially provide a mechanism for the differential regulation of gonadotropin gene expression under low and high pulse frequency.

Although a mechanism for differential gonadotropin gene expression, which can posit transcriptional factor dampening of transcription is compelling, it also suggests that adenylate cyclase is not the only downstream effector of GnRH. In this regard, it has been shown quite elegantly that the prostaglandin biosynthetic pathway, and in particular PGF2α and PGI, can regulate not only LHβ and FSHβ gene transcription, but also the levels of GnRH receptor [14]. Such findings could have translational value for the adjuvant treatment of reproductive disease caused by gonadotropin imbalance and requiring differential pharmacologic intervention.

Since 1976 evidence has been provided, which showed the presence of the α subunit protein in non-pituitary and non-placental tissues [15]. Adding to the list, it was recently shown that HEK293 cells express high levels of α subunit mRNA (500 fold) and protein (600 fold) in response to elevation of cAMP caused by activation of transfected β-adrenergic receptor [16]. However, not only cAMP is involved because G_q-coupled receptor also induced expression of the α subunit. From these observations it is reasonable to suggest that the expression of the α subunit is not cell type dependent and the promoter likely embodies cyclic AMP response element (CRE) binding sites for CRE binding protein transcription factors. In addition, the control of this promoter can also be anticipated to respond to transcription factors that are regulated by intracellular levels of calcium.

Of course, every reproductive physiologist knows that castration gives rise to elevated gonadotropins in blood. Indeed, as ovarian function wanes in mid-life, and gonadal steroid production is decreased, FSH rises. Therefore, the long loop feedback of gonadal steroids on gonadotropin production was a harbinger of the potential for regulation of gonadotropin gene expression by gonadal steroids. However, the regulation of gonadotropin secretion by progesterone was unanticipated. Progesterone increases FSHβ gene expression but GnRH-induced LHβ gene expression is repressed [17, 18]. Moreover, whereas induction of FSH gene expression is due to progesterone receptor binding to the promoter of the FSHβ gene, in the case of LHβ gene regulation, progesterone receptor is not required [18]. Gonadal steroids also modulate neuronal firing and, therefore, secretion of gonadotropins. For example, during the estrous cycle, progesterone increases the locus coeruleus expression of fos related proteins and firing whereas in high estrogen expression is lower and lowered fos related antigen staining [19].

We would be remiss to not mention here that steroidal control of gonadotropin secretion can also be mediated by hypothalamic kisspeptin neurons controlling the release of GnRH [20]. Along these lines, it is also important to mention the hypothalamic factor, Gonadotropin Inhibitory Hormone (GnIH or RFRP-1,-2,-3 peptides), which inhibits release of gonadotropins mediated by GnRH [21]. Both types of neurons are emerging as positive and negative mediators of gonadal feedback, respectively, as well as environmental regulators of GnRH neurons [22]. The GnIH peptides, originally identified in avian species, have been found in mammalian species in recent years, and studies are in progress to confirm GnIH plays similar roles in mammalian reproduction [21]. Co-localization studies have shown 40–80% of GnRH neurons close to GnIH-positive fibers, implying a direct effect on GnRH secretion. Animal studies in rat, sheep, and cattle supported direct effects of GnIH on gonadotropes, however, a negative result in rats suggested species-specific differences in the mechanism of action for these peptides. A G protein-coupled receptor, GPCR147, for GnIH has recently been detected in human hypothalamic neurons and pituitary gonadotropes, suggesting clinical significance for GnRI peptides as adjuncts or alternatives to GnRH analogs [23].

During the 1970s a search for non-steroidal factors that regulate gonadotropin release led to the identification of

inhibin, which suppresses FSH secretion, and then serendipitously activin, which increases FSH secretion. Both inhibin and activin are members of the TGFβ superfamily which appear to preferentially control the secretion of FSH [6]. Activin acts as an autocrine/paracrine factor in the anterior pituitary, and stimulates FSHβ gene transcription, whereas inhibin, acting as an ovarian hormone, is inhibitory [24]. Interestingly, another TGFβ family member, bone morphogenetic protein-4, while having no effect on its own, can modify activin- as well as GnRH-mediated gonadotropin release by increasing mRNA of FSHβ [25].

Regulation of chorionic gonadotropin expression is less well understood in part because no obvious placental signal has been identified. Chorionic gonadotropins have been found in only two groups of mammals, primates and equids. When only human and horse chorionic gonadotropins were well characterized, chorionic gonadotropin expression was apparently exclusively placental in humans, as a separate pituitary LH existed, whereas in the horse, pituitary LH and placental CG were found to be comprised of identical polypeptides. In the human, the CGβ gene appeared to be derived from an ancestral LHβ gene, which was located on the same chromosome and in the same gene cluster. In the horse it was not known if a similar gene duplication was followed by loss of one gene, as donkeys and zebras also appear to possess only a LH/CGβ-like gene. Recent studies of new world monkey CG may provide an example of a path from separate LH and CG β-subunit genes to a single LH/CGβ gene. The common marmoset (*Callithrix jacchus*) was found to possess both LH and CG β-subunit genes, however, only the CGβ gene was expressed in the pituitary [26]. As the structural gene appeared normal, the absence of expression probably reflects changes in regulatory elements. Two other new world monkeys, the squirrel monkey (*Saimiri sciureus*) and the owl monkey (*Aotus trivirgatus*) were also reported to express only the CGβ gene in the pituitary [27]. The potential for pituitary expression of CGβ exists even in humans, as the presence of pituitary-derived hCG was confirmed by its possession of pituitary-specific sulfated glycans [28]. The mechanisms for loss of pituitary expression of LHβ may emerge from studies of new world monkeys.

## 3 Regulation of gonadotropin assembly and secretion

It is clear that initiation of events that lead to gonadotropin gene expression rests at the level of secretagogues, and is modulated by several factors. The biosynthesis of the gonadotropin subunits can be considered in three steps. Translation involving the cytoplasm and the endoplasmic reticulum (ER), glycosylation and assembly taking place in the ER, glycan remodeling, hormone packaging and secretion taking place in the Golgi and secretogranins. Both subunits are glycosylated, and the timing of glycosylation relative to translation and transfer to the ER, as well as protein folding and assembly is still unclear. Only very early work is available on this topic. Rat LH subunits were reported to associate early in the secretory pathway based on endoglycosidase H sensitivity [29]. Ruddon's lab reported assembly of hCG subunits into heterodimer preparations possessing glycans ($Gn_2M_9Glc_1$ or Gn_2M_8 consistent with ER residency [30], as did Magner and Weintraub for TSH [31].

Since the glycoprotein hormones are heterodimeric, it could be anticipated that the expression patterns for both genes that encode the α and β subunits are coordinated, lest the production of either subunit be in vain without a partner to form the heterodimer. Alternatively, excess of one hormone can drive dimer formation. An early study showed that in placenta the ratio of hCGα:hCGβ synthesis is 1.7:1 in early pregnancy and 12:1 at term [32]. In the pituitary, this is difficult to evaluate because the α subunit synthesis is associated with three β-subunits for FSH, LH and TSH. Moreover, while hCGβ is often secreted as α free subunit, LHβ, FSHβ, and TSHβ are largely degraded intracellularly, presumably because of improper folding. Failure to associate with the β subunit results in aberrant glycosylation of the α subunit. In the pituitary, the major difference is the O-glycosylation of Thr^{39}, while in the placenta, increased N-glycan branching replaces predominantly hybrid and biantennary glycans with predominantly tri- and tetra-antennary glycans. Unlike studies which examine gene expression, using mRNA or promoter activation as readouts, an understanding of the structural and cell biology of gonadotropin secretion has relied upon readouts of secretion rates using pulse-chase analysis and formation of competent hormones often interpreted in light of mutational analysis.

Crystal structures of the gonadotropins and the FSH receptor provide a physical reference for such interpretations [33]. Figure 2 shows that the α and β subunits each with three loops, orient in a head to tail 2-1/1-2 manner and that the β subunit carboxy-terminal tail is wrapped around the α subunit [34]. A terminal cysteine residue forms a disulfide bond with the β-1 loop providing a latch for the so-called "seat belt". A nuance of the seat belt is that a portion of it is gathered into a small cystine noose, whose importance will become clear later in this paper. Although glycoprotein α and β subunits can assemble *in vitro*, the process is slow but can be accelerated by removal of the carbohydrate in the second loop of the α subunit. This observation squared with observations using pulse chase studies, and so it was surmised that *in vitro* the association of subunits relied on them to find a way for glycosylated loop 2 of the α subunit to slide under the seat belt (no small feat with carbohydrate attached), while *in vivo*, it was possible that formation of the seatbelt clasp occurred

Fig. 2 Three-dimensional structures for hCG and hFSH. The structures were derived from protein databank files 1HRB and 1FL7, respectively, [106] and the α-carbon backbones visualized using the software package iMol (http://www.pirx.com/iMol/). Diagrams were exported from iMol as encapsulated postscript files and structural elements labeled using the software package Adobe Illustrator. The subunits are associated in an anti-parallel manner with three Cys-knot loops at opposite ends: βL1-βL3-αL2 at one end and αL1-αL3-βL2 at the other. The receptor-binding elements are located on the right side of each model and consist of αL2, βL2, the α-subunit C-terminus, along with the seatbelt loop of which, the Cys-noose, featured in the subunit threading mechanism, is most readily visible
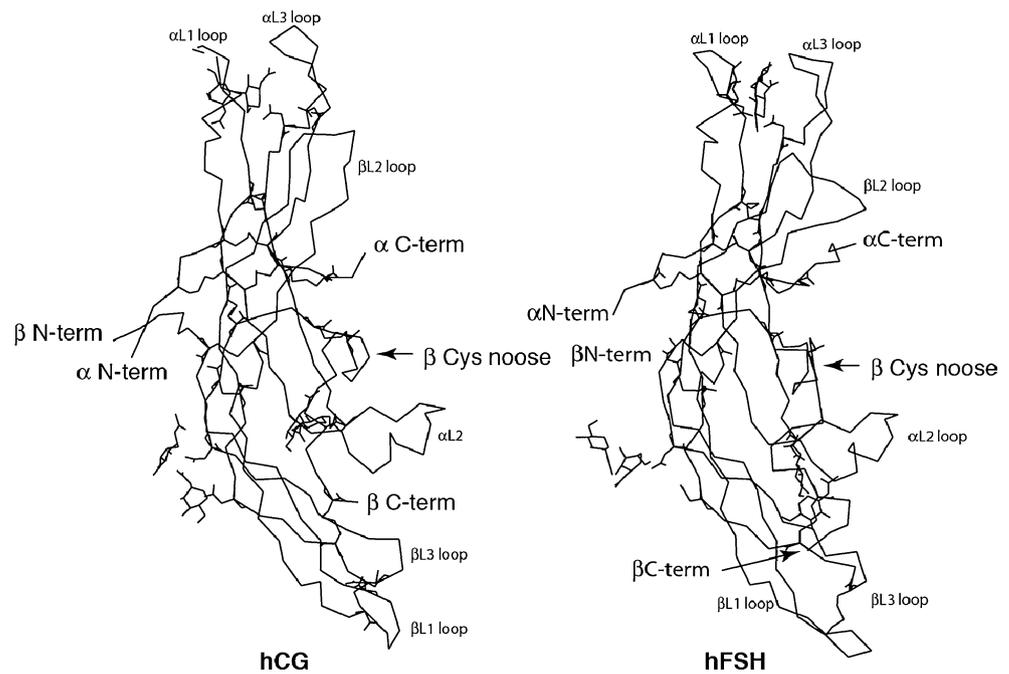

following protein folding and assembly of the subunits into the heterodimer.

The reasonable notion that *in vivo* seatbelt latching occurred following subunit association remained largely untested for 8 years after the crystal structure of hCG [35, 36] was published (Fig. 3). But shortly after the crystal structure of FSH [37] was published, confirming the seat belt motif as a general principle for the gonadotropins, subsequent studies were performed using mutagenesis and antibody binding which suggested that threading of the α subunit through the closed seat belt is the predominant pathway for assembly [38]. The conundrum of how this can occur was partially solved by a subsequent observation involving the cystine noose referred to above. It turns out that removal of the disulfide bond of this noose facilitates assembly of the subunits into the heterodimer, in effect letting out the gather of the seatbelt [39]. This mechanism would necessarily be underpinned by a redox-coupled system, but what this system is and whether it is regulated is not clear at this time. The compact nature of the GlcNAc$_2$Man$_9$Glc$_1$ glycan may also facilitate threading as, *in vitro* subunit association is most commonly studied using hCG and various mammalian LH α subunits possessing smaller, hybrid-type glycans (Fig. 4) attached to the αL2 Asn (residue 52 in humans, 56 in most mammals). In contrast, triantennary glycans, such as those attached to placental free α subunit [40], prevent subunit association, while elimination of this glycan facilitates subunit association [41, 42]. In fish, the mechanism of assembly appears to follow the less

efficient pathway where the subunits assemble first and then the seat belt wraps around the α subunit and is latched [43]. The current thinking on this pathway to assembly of the receptor binding competent heterodimer is as follows: Parts of the β subunit amino terminus, reduced cystine knot, and α subunit loops 1 and 3 dock; then the β subunit amino terminus and the oxidized cystine knot (gathered again into a noose) in complex with α subunit loop 2, form the stable heterodimer [44]. The reader is referred to these elegant and rather detailed studies for further information.

The secretion of the gonadotropic hormones has been studied by pulse chase methods, followed by immunoprecipitation of the heterodimeric hormone and analysis of the labeled subunits by gel electrophoresis. One of the first differences in secretion noted, actually came from a comparison of hCG and hLH, which are identical except for a β subunit carboxyterminal extension that is O-glycosylated in hCG and is absent in hLH. Using a cell line that exhibits apical/basal secretion, it was shown that hCG was secreted apically whereas LH was secreted basally [45, 46]. It has since been determined that the apical secretion of hCG is constitutive when compared to heterodimeric LH and that the carboxy terminal tail of hCGβ, which is not present in hLHβ, is necessary for the constitutive apical secretion [47]. When compared to LH, FSH was found to sort into both apical and basolateral secretory compartments, and in LH, the signal for basolateral secretion was found in the last seven amino acids of the β subunit [48]. The secretion of hFSH is constitutive, whereas LH secretion is induced by

A. Ruddon tethering model for subunit association
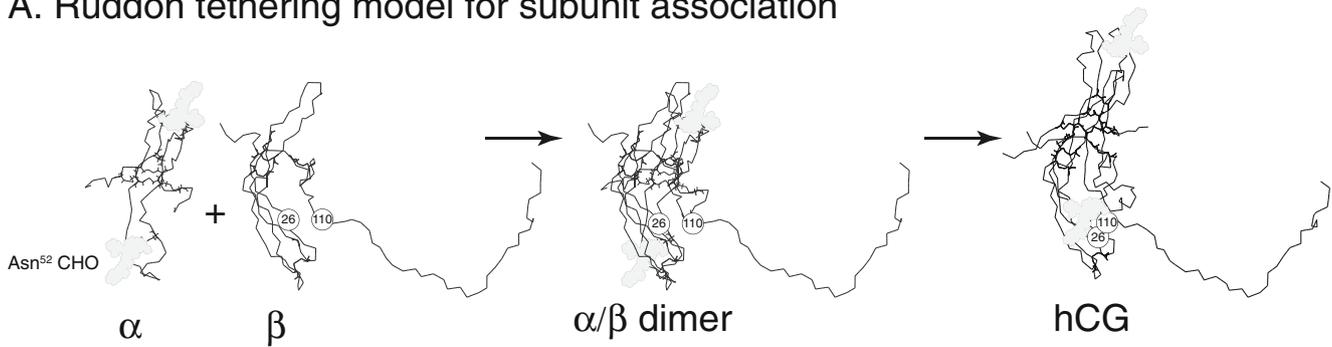
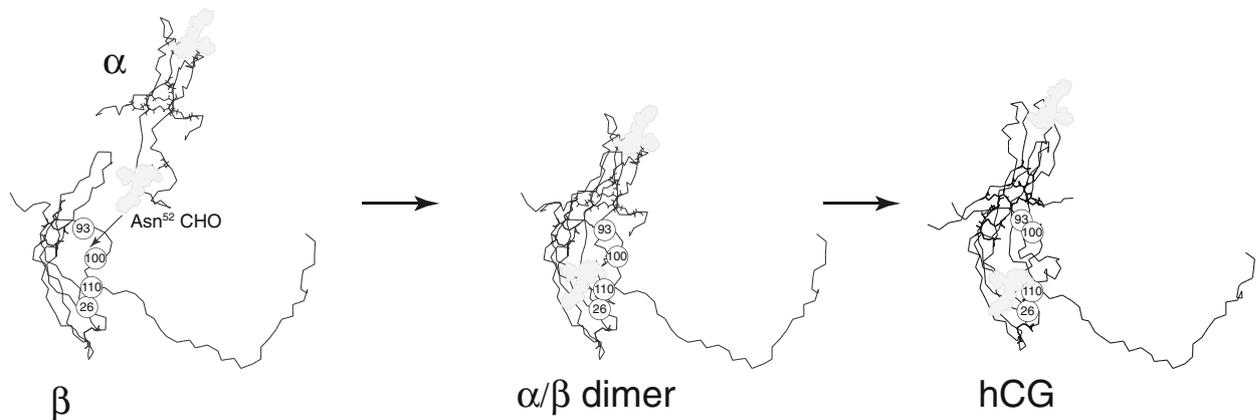## B. Moyle threading model for subunit association

Fig. 3 Mechanisms for hCG subunit association. A. Classic tethering mechanism proposed by Ruddon and colleagues [107]. The α subunit is folded and ready for association. Partially folded hCGβ gains dimerization competency when the 93–100 disulfide bond forms. Subunits dock, forming inactive α/β dimer. Active conformation forms and formation of 26–110 disulfide stabilizes functional hCG. B. Alternative threading mechanism proposed by Moyle and colleagues

[38, 39, 43, 44]. The hCGβ 26–100 seatbelt loop disulfide bond has formed, and the 93–100 disulfide opens, thereby enlarging the seatbelt loop. The α subunit with a compact glycan threads through the enlarged seatbelt loop, forming inactive α/β dimer. Active hCG conformation in αL2 is established and 93–100 disulfide bond reforms. To simplify the diagrams, only the α subunit N-glycans are indicated as outlines of the GlcNAc$_2$Man$_9$Glc$_1$ oligosaccharide

stimulation, and this difference is due to the hydrophobic stretch of amino acids (LSGLLFL) in the LH β-subunit tail [49]. In fact, FSH secretion could be switched from constitutive to regulated by fusing that sequence to the carboxyl terminal β-subunit tail of FSH [49, 50].

With regard to LH secretion, lipid rafts were recently implicated in the mechanism for LH secretion when drugs known to disrupt lipid rafts were shown to stimulate LH secretion *in vitro* and *in vivo* [51]. The mechanism involved appears to be controversial, as SNARES, reportedly associated with rafts, are released, and in other systems either stimulate or inhibit protein secretion.

4 Terminal glycosyl modification and therapeutic gonadotropin preparations

One of the striking differences between FSH and LH, apart from their β subunits, is that the majority of carbohydrates on

FSH are terminated with sialic acid whereas the vast majority carbohydrates on LH are terminated in sulfate. In this regard, hLH glycosylation is an exception, as 28% of the glycans are terminated with sialic acid (Fig. 5) [52]. In the original model for glycoprotein clearance, sialic acid prevented recognition of the terminal galactose residues by Ashwell's lectin, the asialo-glycoprotein receptor (ASGP-R) [53], thus prolonging hFSH half-life in the blood. However, this was consistent only with α2–3 linked sialic acid residues found in hCG, which was used in the pioneering Ashwell experiments [54]. Interestingly, the ASGP-R did not appear to be involved in clearance of sialylated hCG, as saturating the receptor with desialylated glycoproteins had no effect on native hCG clearance, while slowing asialo-hCG clearance [55]. Subsequently, Baenziger and colleagues demonstrated that α2-6 linked sialic acid did not prevent galactose residue recognition by the ASGP-R (Fig. 6), which is relevant for hFSH and hLH, as they possesses both α2-3 and α2-6 linked sialic acid residues

Fig. 4 Models of N-glycans found attached to αLoop2 Asn[52] (human numbering). A. Top view of glycans found on hCGα under various conditions. B. Side view of the same glycans. The β(1–4)-linked Man residue that initiates branching is labeled in both views of each structure. C. Diagrams of glycan structures showing monosaccharide residues and linkages for glycans in A and B. Oligosaccharide models were built with the Woods Group (2005-2011) Glycam Web Carbohydrate Builder. Complex Carbohydrate Research Center, University of Georgia, Athens, GA. (http://www.glycam.com) Structure 1, high mannose glycan GlcNAc$_2$-Man$_9$Glc$_1$ found in ER on folding proteins; structure 2, typical urinary hCG-derived, hCGα Asn[52] hybrid-type N-glycan; structure 3, triantennary glycan, such as those associated with urinary free hCGα
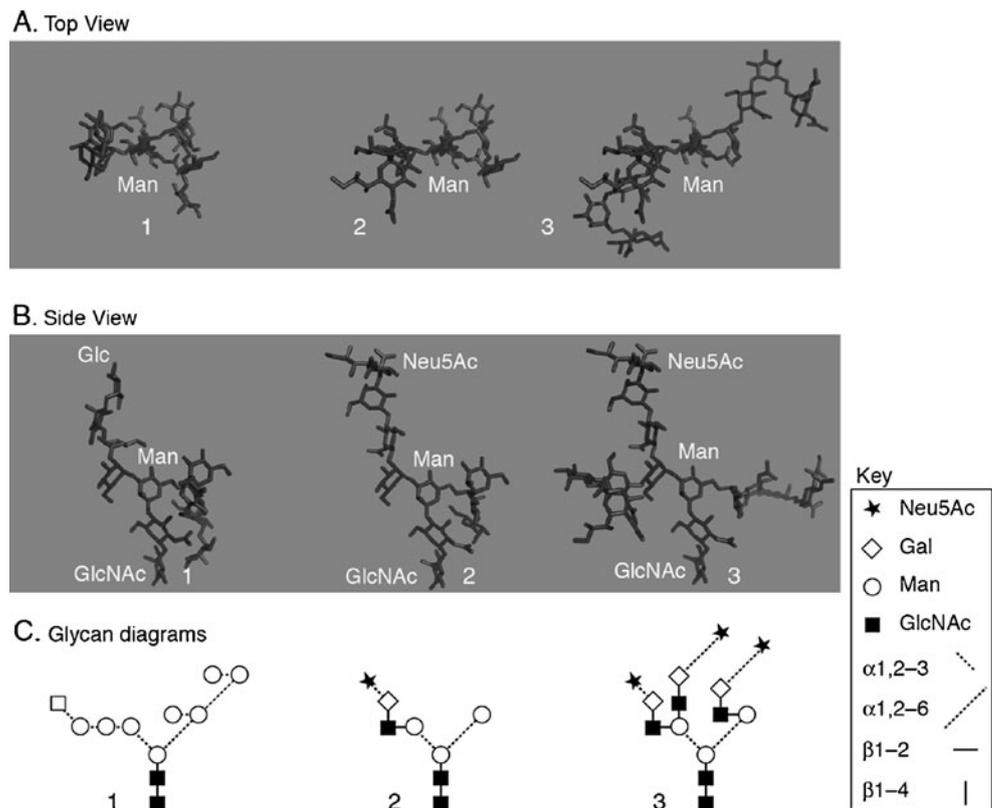
[56]. Moreover, in the female rat pituitary, estrogen feedback has been shown to regulate the expression of α2-3-sialyltransferase (ST3Gal III) [57]. In contrast, in the male α2-6-sialyltransferase (ST6Gal I) appears to be under
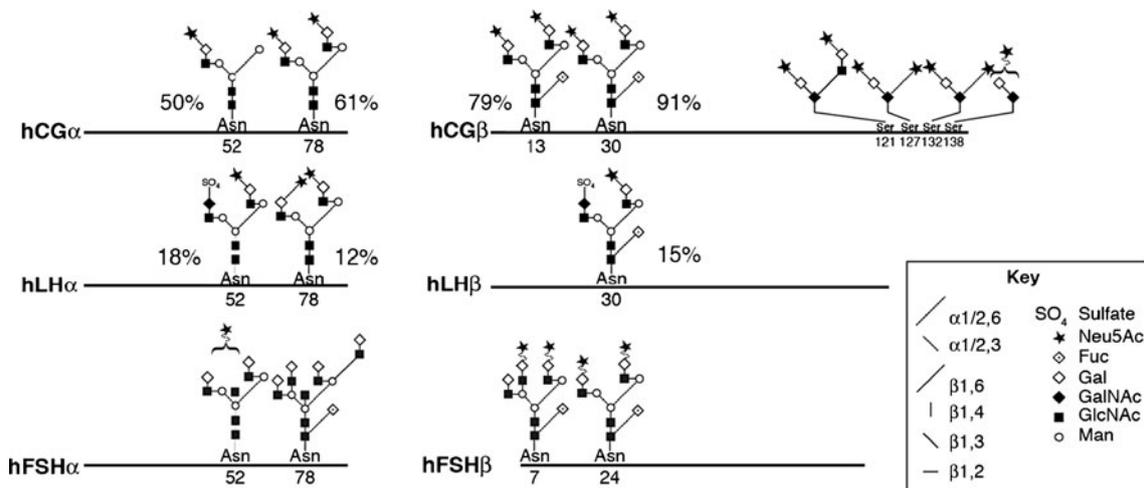

Fig. 5 Glycosylation patterns for human gonadotropins. Site-specific glycosylation patterns are shown for each hormone. The subunit polypeptides are indicated as solid bars, with the glycosylated Asn or Ser residues indicated by numbers below each bar. The most abundant glycan found at each site is shown along with its relative abundance, if known. The hCG and hLH glycans were determined by nmr [52, 108], therefore, monosaccharide sequence and linkage were unambiguous. The hFSH glycans were determined by glycopeptide mass spectrometry [62, 67] leading to ambiguities in branch location and sialic acid linkages, as indicated. The α-subunit glycans are shown without core fucose, as the abundance of this monosaccharide residue is found in 10% or fewer α-glycans. The β-subunit glycans are shown with core fucose, as 80–90% of β-glycans possess this residue. The O-glycan distribution is as determined by mass spectrometry of tryptic glycopeptides [97]. Glycan diagrams follow the Oxford Glycobiology Institute format [109] except that 1–6 and 2–6 bonds are indicated by a longer line to emphasize that the monosaccharide is attached to a potentially more flexible linkage via an exocyclic carbon atom, rather than to a ring carbon (see Key inset). Note that sialic acid is attached only via α2-3 linkages in hCG, but both α2-3 and α2-6 linkages are found in hLH and hFSH (although not shown for hFSH glycopeptides, it is well known from studies involving oligosaccharides)
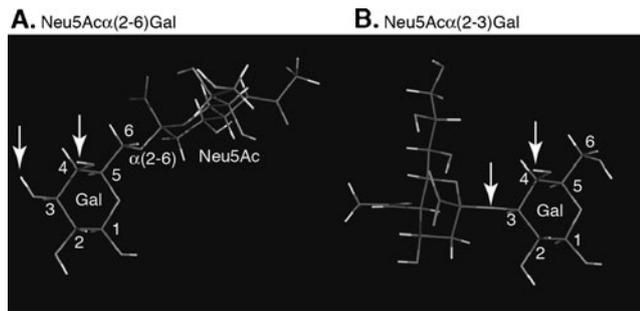
Fig. 6 Asialoglycoprotein ligands. Based on similarities between the mannose binding protein and ASGPR carbohydrate binding domains, the latter is expected to bind hydroxyls attached to Gal carbons 3 and 4 (arrow, see p 184 [110]). **A.** The α2-6-linked sialic acid does not block access to these. **B.** The α2-3-linked sialic acid blocks access to both hydroxyls by replacing the C-3 hydroxyl with a glycosidic bond and potentially steric hindrance of the C-4 hydroxyl

gonadal regulation, but not α2-3-sialyltransferase [58]. If gonadotrope expression of these enzymes is regulated by estrogen, then one potential mechanism for increasing the clearance rate of FSH may involve increasing the relative abundance of α2-6-linked sialic acid terminating FSH glycans as compared with α2-3-linked sialic acid. The sulfate on the other hand decreases the half life of LH providing for more fine control of blood levels in most species [59]. Although it seemed reasonable to hypothesize that the sulfation of terminal carbohydrate may in some way direct LH to the inducible secretory pathway, inhibition of sulfation did not shunt LH to the constitutive pathway [60]. One problem with this hypothesis is the fact that FSH from non-human mammals is decorated with 13–40% sulfated glycans [56, 61, 62]. Thus, human FSH represents a special case in that sulfated glycans represent less than 10% of the total glycan population [56, 62]. Moreover, hLH possesses 22–39% sialylated glycans at each glycosylation site, with sialic acid linked both α2-3 and α2-6. Human LH clearance more closely resembles that of hFSH than LH from other mammalian species because it ends up in the urine while more highly sulfated LH preparations, such as pLH, do not [63].

Pharmaceutical-grade recombinant human gonadotropins are expressed in Chinese hamster ovarian cells (Table 1), and their glycosylation reflects glycosyl transferase expression in that cell line. MALDI-TOF-MS and ESI-MS, as

well as MS/MS analysis of glycans derived from recombinant hLH, hFSH, and hCG preparations produced by EMD Serono, revealed only sialylated glycans associated with these gonadotropin preparations [64]. The most abundant glycans were biantennary, although lesser amounts of tri-, tetra-, and even penta-antennary glycans were also detected. For hCG and hLH, the natural forms of these hormones are largely decorated with biantennary glycans. For hLH, the absence of sulfate and α2-6-linked sialic acid [65] would appear to eliminate both liver clearance mechanisms, thereby prolonging hLH survival in the serum. Longer halflife would not seem to affect clinical use of hLH, as hCG, with an even longer halflife, is typically substituted for hLH to induce ovulation. Moreover, one clearance study in primates indicated only a modest reduction in recombinant hLH clearance as compared with pituitary hLH [66]. This result is consistent with an *in vivo* imaging analysis of the clearance of pLH, a highly sulfated gonadotropin preparation, in rats [63]. The labeled hormone was only transiently associated with the liver during the distribution phase, and clearance appeared to take place largely in the kidneys. For recombinant hFSH, the abundance of triantennary and tetraantennary glycans appears to be higher than observed in recombinant hLH and hCG preparations, but lower than in pituitary hFSH [64]. The distribution of glycans on the FSH molecule may be important for biological activity. Glycopeptide mass spectrometry, which potentially could simultaneously characterize all four FSH glycosylation sites, provided detailed information only for two sites, αAsn[52] and βAsn[7] [62, 67]. Limited data hinting at preferential attachment of highly branched glycans at αAsn[78] need confirmation [67].

The focus on FSH isoforms for the last 38 years has emphasized terminal charged residues on the associated glycans because sulfate and sialic acid residues are negatively charged and the existence of charge heterogeneity is attributed to these residues. However, FSH glycosylation variations also include the absence of one or both N-glycans and branching of the glycans, as well as terminal sialylation or, to a lesser extent, sulfation. Thus, 90% of horse (e)FSHβ lacks Asn[7] glycans, while 13–54% of pituitary hFSH preparations lack both hFSHβ glycans [68]. The latter pattern may also exist in two other primate species, Rhesus macaque (*Macaca mulatta*) and Japanese

	Hormone	Trade Name	Manufacturer	Cell Line
Table 1 Recombinant gonado-tropin preparations	FSH	Gonal-f®	EMD Serono[a]	Chinese Hamster Ovary (CHO) cells
	FSH	Follistim®	Merck[b]	CHO cells
	LH	Luveris®	EMD Serono	CHO cells
[a] Formerly Serono	CG	Ovidrel®	EMD Serono	CHO cells
[b] Formerly Organon				

macaque (*M. fuscata*). While LH glycans are typically, hybrid or complex, biantennary [52], FSH largely lacks hybrid glycans, and possesses complex: biantennary, tri-aantennary, and tetraantennary glycans [56, 62, 69]. In hFSH, triantennary and tetraantennary glycans amplify heterogeneity as mono-, di-, and tri-sialyl variants of triantennary and tetraantennary glycans are found [62]. Terminal sialylation has been assumed to produce FSH isoforms, however, mass spectrometry of isoform glycans revealed a great deal of similarity in the glycan populations of FSH isoforms [67]. While two studies have examined expression of two sialyl transferases [57, 58], understanding sialylation is complicated by the large number of sialyl-transferase isoforms that exist in mammals. For example, there are 20 sialyltransferase genes in the mouse and human genomes [70]. These are arranged into four classes based on sialic acid linkage to the underlying glycan and substrate specificity [71]. The $\alpha2,6$-sialyltransferases ST6Gal I-II and the $\alpha2,3$-sialyltransferases ST3Gal III, IV, and VI are capable of attaching sialic acid to terminal Gal or GalNAc residues in gonadotropin N-glycans. While pituitary gona-dotropins possess both $\alpha2,3$- and $\alpha2,6$-linked sialic acid, placental hCG and recombinant gonadotropins produced in Chinese hamster ovarian cells possess only $\alpha2,3$-linked sialic acid. Recent developments in mass spectrometry procedures should facilitate identification of sialyltrans-ferases involved gonadotropin synthesis. Modification of sialylated glycans with 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride not only stabilizes sialylated glycans for analysis, but also distinguishes $\alpha2,3$ from $\alpha2,6$ linked sialic acid [72]. The relatively modest amounts of hormone needed,10 μg, makes it feasible to evaluate the effects of modifying sialyltransferase expres-sion on gonadotropin glycosylation. However, future \studies should also examine oligosaccharyltransferase, which initiates N-glycosylation, and the GlcNAc trans-ferases responsible for complex glycan branching.

5 Regulation of N-glycosylation and branching

N-glycosylation is initiated by an 8-subunit enzyme complex called oligosaccharyl transferase (OST) that transfers a preformed precursor oligosaccharide from dolichol pyrophos-phate to the nascent glycoprotein chain [73, 74]. Partially N-glycosylated forms of recombinant hCG and pituitary FSH have been reported, while LH and TSH appear to be completely glycosylated, as is the common α subunit. The all-or-none N-glycosylation of hFSHβ, appears to be the result of selective inhibition of OST activity, as the complementary hFSHα subunit is quantitatively glycosy-lated [75]. In yeast there are 9 genes for 8 OST subunits [76]. Mammalian equivalents of the yeast subunit genes have been

identified [74] as well as two isoforms of the catalytic subunit, STT3 [77]. Immunopurified yeast OST appears to be a dimer based on blue gel electrophoresis in which the major band was estimated to be a 500 kDa complex, while protein sequences predicted a 250 kDa molecular weight [78]. The yeast enzyme appears to be regulated by PKC [79], which is interesting because PKC is the primary signaling pathway activated by GnRH. The challenge is to understand how glycosylation of the FSHβ subunit is selectively inhibited, while permitting full glycosylation of the common α subunit, not to mention the LHβ subunit, within the same cellular compartment. One possible mechanism could involve the use of different OST isoforms.

Some lessons have been learned from studies in yeast. Two OST isoforms have been identified in yeast that differ in one subunit, Ost3p or Ost6p [80]. Mammalian homologs for both subunits are, N33 and IAP, respectively [81]. Mammalian OST isoforms also differ in the nature of the putative catalytic subunit STT3 [81]. STT3A appears to be involved in co-translational N-glycosylation, while STT3B appears to be involved in post-translational N-glycosylation [77]. Possible posttranslational glycosylation of FSH subunits might occur, as a low MW form was detected during pulse-chase expression of hFSH, that chased into the high MW form over time [82]. Thus, it may be possible to inhibit FSHβ glycosylation by knocking down STTB expression. Each of the yeast OST isoforms is associated with a specific translocon; OST possessing Ost3p with Sec61 and OST possessing Ost6p with Ssh1p [80]. Yeast translocons differ in the specificity of the signal peptides that they accept. Sec61 is necessary for cell viability and accepts a wider range of signal peptides while Ssh1p is less essential and accepts signal peptides with high hydrophobic character. Analysis of signal peptides for the common α subunit and hFSHβ with the DNA Star Protean software revealed a hydrophilic N-terminus in the first 8 residues of the common α subunit signal peptide and a hydrophobic N-terminus in the first 9 residues of the FSHβ signal peptide. This suggests a strategy of swapping leader sequences between the α and FSHβ subunits, however, a mammalian homolog of Ssh1p does not appear to exist. An uncharacterized homolog of Sec61 (accession number D3ZEH3) does exist in the rat that is the same size and is 93% identical to Sec61. In principle, mass spectrometry should permit analysis of partially glycosylated FSH variants, however, oligosaccharide heterogeneity, partic-ularly that associated with the common α subunit obscures FSHβ isoforms lacking one or more N-glycans [68].

N-glycosylation in the ER is a highly conserved process with defined intermediates involved in protein folding, exit of folded protein to the Golgi, and the retrograde transport of unfolded proteins to the cytoplasm for proteosome-mediated degradation [83]. Glycan diversity is subsequently generated in the Golgi, and glycan branching is a major

contributor. Branching is regulated by a family of GlcNAc transferases, which are characterized by the core mannosyl residue and linkage of the GlcNAc residue attached to the pentasaccharide core. All but the bisecting GlcNAc residue, which is attached $\beta(1–4)$ to the $\beta(1–4)$-linked Man residue, can be extended by other glycosyltransferases to yield the mature branches of the glycan. Based on the existence of largely hybrid and biantennary glycans attached to hCG and hLH, along with triantennary and tetraantennary glycans attached to hFSH, the following GlcNAc transferases modify gonadotropin glycans: MGAT1 (GlcNAc transferase I), MGAT2 (GlcNAc transferase II), MGAT3, (GlcNAc transferase III), MGAT4 (GlcNAc transferase IV), and MGAT5 (GlcNAc transferase V). LH and hCG glycans are products of GlcNAc transferase I alone, or both I and II, as only hybrid or biantennary glycans decorate these hormones. In the mouse and human genomes there are single genes for GlcNAc transferases I, II, and III, while there are 2 isoforms for both GlcNAc transferases IV and V. Loss of GlcNAc transferase I in the whole mouse is embryonic lethal [84] and oocyte-specific knock out leads to a reduced ovulation rate [85]. Loss of GlcNAc transferase II in mice is often embryonic lethal, but can be studied in some genetic backgrounds, which provide a model for the human disease congenital disorder of glycosylation type IIa [86]. Male mice with this disorder are sterile due to spermatogenic failure, while humans exhibit testicular atrophy. Loss of GlcNAc transferase III appears to inhibit growth factor signaling [87] and can counteract the metastatic properties of tumor cells expressing GlcNAc transferase V [88]. Both GlcNAc transferases IV and V are responsible for tri- and tetra-antennary glycan synthesis. These glycans appear to interact with galectins to prolong surface residence of membrane glycoproteins, which may be relevant to FSH, as recently galectin-1 was detected in porcine granulosa cells. Moreover, galectin-1 inhibited FSH-stimulated steroidogenesis in these cells [89]. GlcNAc transferase IV exists as two isoforms IVa and IVb. The former is normally expressed in the digestive system, while the latter is more broadly expressed. However, when the IVb gene was knocked out, compensatory expression of the IVa gene appeared to largely make up for the loss of the former [90]. Mice deficient in MGAT5A were reported in 2002, and exhibited susceptibility to autoimmune disease, reduced cancer progression, and a behavioral defect [91]. A year later the existence of a second isoform, MGAT5B was reported [92]. Expression of the former is widespread, while the latter is restricted to neural tissue and the testis. Thus, the full ramifications of this transferase have not yet been examined. Because contemporary mass spectrometry techniques can analyze glycans derived from as little as 10 μg recombinant gonadotropin, it is feasible to monitor the results of modifying GlcNAc transferase activities on the glycan populations associated with these hormones.

Hyperglycosylated hCG is one of several variants of this clinically and diagnostically valuable gonadotropin, and is associated with early pregnancy and cancer [4, 93]. Early pregnancy hyperglycosylated hCG is secreted by extravilous cytotrophoblast cells, and not by the syncyitiotrophoblast [94]. It appears to act in an autocrine/paracrine manner to promote cytotrophoblast invasion of the uterus [95]. Typically, hyperglycosylated hCG is identified in clinical studies by the binding of monoclonal antibody B152 that is dependent on the presence of a core type-2 O-glycan at Ser132 instead of the usual disialylated core type-1 glycan (Fig. 5, illustrates these glycan structures at positions 121 and 132, respectively, for normal pregnancy hCG). Reduced hyperglycosylated hCG is associated with increased risk for early pregnancy loss [96]. Glycosylation site-specific differences in glycan structure between tumor-derived hyperglycosylated hCG and normal pregnancy hCG β-subunits have been defined by mass spectrometry [97]. A site-specific increase in the abundance of tri-antennary glycans over bi-antennary glycans was observed at βAsn^{30}, but not βAsn^{13}. As Asn^{30} glycans are involved in signal transduction, this may have functional significance, since hyperglycosylated hCG has been shown to be impaired in its steroidogenic potency, yet promotes cell invasion by cytotrophoblasts [95]. On the other hand, the increased abundance of fucosylated glycans at Asn^{13}, may also influence function, as lentil lectin-bound TSH was reported to exhibit biased agonism, activating both cAMP and PLC pathways, while unbound TSH activated only the cAMP pathway [98]. Mass spectrometry also better defined the differences in O-glycosylation, revealing changes from core type 1 to core type 2 glycans at Ser132 and Ser138, as well as increased glycosylation site occupancy at Ser127 [97].

6 Glycosylation and therapeutic function

The design and implementation of glycoprotein hormones for therapeutic purposes in part relies on and is limited by their structural requirements for biosynthetic processes, in particular the regulation of glycosylation [99]. The heterogeneity of gonadotropin glycosylation has led to many theories about the relative role of differentially charged isoforms and concerns about their pharmacological properties [100–102]. Gonadotropin utility in humans and more recently in domestic animals [103] has encouraged thinking about strategies to enhance half-life and whether glycosylation plays roles in biological activity apart from circulatory half-life. Emerging strategies for homogenous glycosylation of the subunits could potentially allow for the preparation of completely homogenously glycosylated gonadotropins, which would be an improvement of the existing formulation [104]. Indeed the finding that glycosylation of the β subunit of

hFSH varies with age has prompted thinking that new forms of therapeutic gonadotropins should include more di-glycosylated as in young women [68]. Thus cycling young women have a greater proportion of heterodimeric FSH, which is not glycosylated at all on the β subunit, whereas menopausal women have a higher proportion of FSH which is glycosylated on all four sites [68]. It is reasonable to assume that there will be differences in biological activities of the tetra and di-glycosylated FSH because already there is evidence for biased agonism with other glycoform variants of hFSH [105].

7 Future directions

Current second generation gonadotropin preparations incorporate changes in the protein moieties to increase stability by expressing the α and β subunits as single-chain molecules and to increase survival in the circulation by addition of the hCGβ C-terminal peptide. The control of transcription of genes encoding proteins that are involved in the post-translational modifications of the gonadotropins, although more complicated, seems warranted. Little is known about how secreta-gogues such as GnRH or steroids affect their expression. Little is known how altering the activities of specific glycosyltrans-ferases will affect the relative abundance of a desired glycan at various glycosylation sites in the protein. For example, if GnTase IV is overexpressed, will there be an increase in triantennary glycans at all 4 sites in FSH? Will LH glycans become predominantly triantennary or will they remain largely biantennary? Oligosaccharide mass spectrometry can address the relative abundance for the total glycan population. Once desired glycan abundance increases, a more demanding approach will be needed to define glycosylation at individual sites. Here, mass matching of glycopeptides combined with detailed analysis of total oligosaccharide preparations would probably provide sufficient detail for developmental studies. If uniformly glycosylated gonadotropins could be prepared, then experiments to compare relative activities are readily performed and could be used to design more effective gonadotropin preparations for clinical use.

Overexpression of GnTases would be useful to make recombinant hyperglycosylated hCG, which would be valuable as a standard for diagnostic assays for early pregnancy loss. The literature largely describes tumor-associated hyperglycosylated hCG and glycosylation differences exist. Detailed characterization of early pregnancy hyperglycosylated hCG Replacement therapy with hyperglycosylated hCG might become possible when low levels of this hormone are detected, once obvious difficulties with administration of a paracrine factor are overcome. It is possible intrauterine rather than systemic administration would be effective. The potential to stimulate cell invasion would have to be carefully evaluated

and antagonists developed. Differences in glycosylation between young and menopausal women certainly suggests major differences in the post-translational modification pathways. Future studies should address whether di- and tetra-glycosylated hFSH are effective as isolated glycoforms. As physiological samples almost invariably contain both glycoforms, normal reproductive function may require the presence of both glycoforms. Preparation of individual glycoforms is necessary even if both are needed, as it would be possible to vary the ratios of the two forms and test their efficacy. For example, in IVF protocols, it might be helpful to vary the ratio of the two forms during the period of hyperstimulation to reflect the physiology of younger, more fertile women. Identification of the protein, which recognizes the seven amino acid sorting signal in hLH, and directs it to the induced secretion pathway would be of interest.

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