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A human *FSHB* transgene encoding the double N-glycosylation mutant (Asn⁷ Asn²⁴) FSH β subunit fails to rescue *Fshb* null mice

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Abstract

Follicle-stimulating hormone (FSH) is a gonadotrope-derived heterodimeric glycoprotein. Both the common α - and hormone-specific β subunits contain Asn-linked N-glycan chains. Recently, macro-heterogeneous FSH glycoforms consisting of β -subunits that differ in N-glycan number were identified in pituitaries of several species and subsequently the recombinant human FSH glycoforms biochemically characterized. Although chemical modification and *in vitro* site-directed mutagenesis studies defined the roles of N-glycans on gonadotropin subunits, *in vivo* functional analyses in a whole-animal setting are lacking. Here, we have generated transgenic mice with gonadotrope-specific expression of either an *HFSHB*^{WT} transgene that encodes human FSH β WT subunit or an *HFSHB*^{dgc} transgene that encodes a human FSH β ^{Asn7 24} double N-glycosylation site mutant subunit, and separately introduced these transgenes onto *Fshb* null background using a genetic rescue strategy. We demonstrate that the human FSH β ^{Asn7 24} double N-glycosylation site mutant subunit, unlike human FSH β WT subunit, inefficiently combines with the mouse α -subunit in pituitaries of *Fshb* null mice. FSH dimer containing this mutant FSH β subunit is inefficiently secreted with very low levels detectable in serum. *Fshb* null male mice expressing *HFSHB*^{dgc} transgene are fertile and exhibit testis tubule size and sperm number similar to those of *Fshb* null mice. *Fshb* null female mice expressing the mutant, but not WT human FSH β subunit-containing FSH dimer are infertile, demonstrate no evidence of estrus cycles, and many of the FSH-responsive genes remain suppressed in their ovaries. Thus, *HFSHB*^{dgc} unlike *HFSHB*^{WT} transgene does not rescue *Fshb* null mice. Our genetic approach provides direct *in vivo* evidence that N-linked glycans on FSH β subunit are essential for its efficient assembly with the α -subunit to form FSH heterodimer in pituitary. Our studies also reveal that N-glycans on FSH β subunit are

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The authors have nothing to disclose.

essential for FSH secretion and FSH *in vivo* bioactivity to regulate gonadal growth and physiology.

Keywords

Gonadotropin; Genetic rescue; N-glycosylation; Pituitary; Ovary; Testis

1. Introduction

FSH is a pituitary-derived heterodimeric glycoprotein hormone (Bousfield et al., 2006). It binds to G-protein coupled receptors on somatic cells of gonads and regulates both gametogenesis and steroidogenesis (Bousfield et al., 2006). FSH consists of a common α -subunit that is shared with two other glycoprotein hormones also produced within the pituitary, namely, LH and TSH (Bousfield et al., 2006). Additionally, the α -subunit is also present in human chorionic gonadotropin (hCG), a trophoblast-derived placental glycoprotein hormone (Bousfield et al., 2006). In specific cell types, the α -subunit is non-covalently associated with the specific β -subunit (FSH β or LH β or TSH β or hCG β) resulting in formation of a distinct heterodimeric hormone (FSH and LH in gonadotropes, TSH in thyrotropes and hCG in trophoblasts). A characteristic feature of this family of glycoprotein hormone subunits is the presence of N-linked oligosaccharides covalently attached to specific asparagine (Asn) residues (Bousfield et al., 2006; Baenziger and Green, 1988). Data obtained from several *in vitro* experiments involving both biochemical and site-directed mutagenesis approaches indicated that N-linked glycosylation of α - and β -subunits is critical for heterodimeric hormone assembly, rate of secretion, serum half-life and signal transduction in target cells (Bousfield et al., 2006; Baenziger and Green, 1988; Green et al., 1986; Matzuk and Boime, 1989).

Human (h) FSH consists of four co-translationally attached N-glycans, two each in the α - (at Asn⁵² and Asn⁷⁸) and β - (at Asn⁷ and Asn²⁴) subunits (Bousfield et al., 2006). A series of α -subunit (Asn⁵² or Asn⁷⁸ or at both Asn sites) and β -subunit (Asn⁷ or Asn²⁴ or at both Asn sites) N-glycosylation mutations in hFSH were previously constructed (Bousfield et al., 2006; Bishop et al., 1994; Flack et al., 1994a,b). These mutants were expressed in CHO and COS7 cells and the recombinant proteins tested in various *in vitro* binding and signal transduction assays (Bousfield et al., 2006; Flack et al., 1994a,b). These studies documented the roles of N-glycosylation on FSH. First, individual mutation of N-glycosylation sites on either FSH α - or β -subunits enhanced FSH-receptor binding affinity when rat Sertoli cell homogenate was used as a receptor source (Bousfield et al., 2006; Flack et al., 1994a,b). Second, mutations in individual N-glycosylation sites on β -subunit resulted in reduced signal transduction in rat granulosa cell cultures (Bousfield et al., 2006; Flack et al., 1994a,b). Finally, the second N-glycosylation site at Asn²⁴ in the FSH β -subunit was found critical for both receptor binding and signal transduction (Bousfield et al., 2006; Flack et al., 1994a,b).

A variety of biochemical and structure-function studies indicated that the terminal sugars, such as sialic acid, contributes to micro-heterogeneity in FSH (Flack et al., 1994a,b; Ambao

et al., 2009; Anobile et al., 1998; Campo et al., 2007; Creus et al., 2001; Creus et al., 1996; Crowe et al., 1998; Loreti et al., 2009; Padmanabhan et al., 1988; Padmanabhan et al., 1999; Padmanabhan et al., 1992; Timossi et al., 2000; West et al., 2002). The resulting charge variants, called FSH isoforms, are secreted from the anterior pituitary in an estrus/menstrual cycle stage-specific manner in different species. The micro-heterogeneous FSH isoforms differ in their net charge and their relative abundance in serum reflects the ovarian cycle stage (Anobile et al., 1998; Creus et al., 1996; Loreti et al., 2009; Padmanabhan et al., 1988, 1992; West et al., 2002). A fully glycosylated FSH (FSH²⁴) has been the most abundant form expressed in mammalian expression systems *in vitro*. In more recent studies, Western blot analyses identified a novel macro-heterogeneity contributed by the absence of both (FSH¹⁵) or presence of either Asn²⁴ (FSH¹⁸) or Asn⁷ (FSH²¹) N-glycan chains in FSH β -subunit in pituitaries of several species (Bousfield et al., 2007; Davis et al., 2014; Walton et al., 2001). Based on the molecular mass of FSH β subunits, each containing different number of N-glycan chains, different FSH glycoforms have been named. For example, FSH¹⁵ contains no Asn-linked glycans on the FSH β -subunit with a molecular mass of 15 kDa; FSH¹⁸ and FSH²¹ each contain an FSH β subunit with one N-linked glycan with molecular mass 18 kDa and 21 kDa, respectively. Collectively, these FSH glycoforms are referred to as hypo-glycosylated FSH (Bousfield et al., 2007; Davis et al., 2014). Thus, a FSH heterodimer consisting of a α -subunit with two N-glycans units and a non-glycosylated FSH β -subunit lacking both the N-glycans (FSH¹⁵) was also identified (Bousfield et al., 2007; Davis et al., 2014; Walton et al., 2001).

Our group has recently reported the biochemical and cell biological properties of macro-heterogeneous FSH, hypoglycosylated FSH^{18/21} (mixture of both FSH¹⁸ and FSH²¹) and compared them *in vitro* to fully glycosylated FSH²⁴ (Bousfield et al., 2014a,b). However, the biological roles of hypoglycosylated FSH¹⁵ glycoform are unknown. Here, we report targeted expression of a human *FSHB* mutant transgene encoding an Asn^{7,24} double N-glycosylation site (Thr to Ala mutation) mutant FSH β subunit on an *Fshb* null genetic background. Using this genetic rescue strategy, we provide *in vivo* evidence that the double N-glycosylation site Asn^{7,24} mutant FSH β subunit is heterodimer assembly-incompetent. We also demonstrate that the corresponding heterodimeric FSH¹⁵ glycoform is secreted in very low levels into serum and fails to rescue *Fshb* null mice.

2. Materials and methods

2.1. Mice

All mice were maintained under 12-h dark, 12-h light cycles with food and water supplied *ad libitum*. All studies with mice were carried out in accordance with the Guide for the Care and Use of Laboratory Animals per the NIH instructions as adopted by the University of Kansas Medical Center and approved institutional protocols.

2.2. Other methods

All other methods are described in detail in the Supplementary material.

2.3. Statistical analysis

All the data were represented as Mean $\pm$ SEM. Statistical analyses, including Student's T-test and one-way ANOVA followed by Turkey's post-hoc test, were performed using PRISM software (GraphPad Software, Inc. La Jolla, CA). A P value < 0.05 was considered statistically significant.

3. Results

3.1. Generation of *Fshb* null mice harboring an *HFSHB^{dgc}* transgene

To test the biological roles of N-glycan chains on human FSH β , we first generated transgenic mice expressing an *HFSHB^{dgc}* mutant transgene (Fig. 1A) by pronuclear microinjections of fertilized one-cell mouse embryos. From a total of 22 viable pups produced from two independent microinjection experiments, we obtained 8 (8/22 = 36%) transgene positive mice as verified by genomic PCR assays on tail DNA samples (Fig. 1B) using specific primers in the *oFshb* promoter region as indicated (arrows in Fig. 1A). Five of the founders (3 males and 2 females) were mated to WT littermates from which we successfully established 3 independent lines (1 male and 1 female founder did not transmit the *HFSHB^{dgc}* transgene) designated #30, #60 and #220. We performed almost all of the experiments described herein with two lines #30 and #60. Next, we intercrossed F2 mice from two independent lines (Line #30 and Line #60) onto *Fshb* null genetic background and finally generated *Fshb*^{-/-} *HFSHB^{dgc}* mice in a 2-step breeding scheme (Fig. 1C) similar to that described earlier for generating *Fshb*^{-/-} *HFSHB^{WT}* rescue mice (Kumar et al., 1998; Wang et al., 2014).

3.2. Gonadotrope-specific expression of the *HFSHB^{dgc}* transgene on *Fshb*^{-/-} genetic background

The *oFshb* promoter (4.7 kb) has been extensively characterized previously and found to target various reporter genes to the pituitary gonadotrope lineage in transgenic mice (Huang et al., 2001; Pernasetti et al., 2003; Wang et al., 2016; Wu et al., 2004). To confirm that the *HFSHB^{dgc}* transgene was appropriately targeted to the pituitary, we measured transgene mRNA with respect to an endogenous control *Ppil1* mRNA in mouse pituitaries by Taqman real time qPCR assays using *HFSHB*-specific primers/probe combo. We found that *HFSHB^{dgc}* mRNA expression in pituitaries of both lines of adult *Fshb*^{-/-} *HFSHB^{dgc}* female mice was nearly identical to and not significantly different from that of the *HFSHB^{WT}* transgene encoded mRNA in pituitaries of *Fshb*^{-/-} *HFSHB^{WT}* mice (Fig. 2A; $P > 0.05$ by *T-Test*; $n = 3$ mice, each sample in triplicate).

To further confirm that the *HFSHB^{dgc}* transgene was expressed in gonadotropes, we performed dual immunofluorescence microscopy using specific antisera against FSH β and LH β on formalin-fixed pituitary tissue sections obtained from adult mice. As shown in Fig. 2B, many FSH β ⁺/LH β ⁺ dual-labeled gonadotropes were readily detectable in pituitary sections from *Fshb*^{-/-} *HFSHB^{dgc}* mice similar to those observed in sections from control normal (*Ctrl.*) and *Fshb*^{-/-} *HFSHB^{WT}* mice. As predicted, only LH β ⁺ gonadotropes were present in *Fshb* null mouse pituitary sections confirming the specificity of the FSH β antiserum that was used. Dual-immunolabeling with FSH β antiserum and antibodies against

each of the other anterior pituitary cell markers (TSH β , GH, PRL, ACTH) did not show any dual-labeled cells (data not shown) further indicating that the transgene is specifically targeted to only the gonadotrope lineage within the anterior pituitary.

The *HFSHB^{dgc}* transgene encodes a double N-glycosylation site mutant human FSH β subunit in which the N-glycosylation motif Thr⁹ and Thr²⁶ residues were mutated to Ala residues by site directed mutagenesis (Fox et al., 2001). The predicted molecular weight of this double N-glycosylation site mutant human FSH β subunit is 15–16 KDa. To determine the molecular mass of the *HFSHB^{dgc}* transgene-encoded double N-glycosylation mutant human FSH β subunit expressed *in vivo*, we performed Western blot analyses on pituitary extracts and probed the blots using an hFSH β -specific goat polyclonal antibody. In three independent lines (#30, #60 and # 220) extracts of *Fshb*^{-/-} *HFSHB^{dgc}* mouse pituitaries showed an immunoreactive band, representing the 16 KDa hFSH β subunit that was readily detectable (indicated as hFSH β * in Fig. 2C). In contrast, in *Fshb*^{-/-} *HFSHB^{WT}* mouse pituitary, we detected a fully glycosylated hFSH β 24 kDa subunit similar to recombinant hFSH (r-hFSH). A weakly immunoreactive band with a 24 kDa molecular mass was also detected in control normal mouse pituitary extract, consistent with limited cross-reactivity of the goat anti-human FSH β antibody with the mouse FSH β subunit (Fig. 2C). PNGase F treatment of the r-hFSH preparation and normal mouse pituitary extract followed by Western blot analysis revealed a 15–16 KDa immunoreactive de-glycosylated FSH β subunit identical to that observed in *Fshb*^{-/-} *HFSHB^{dgc}* mouse pituitary extracts (Fig. 2D, arrow b). Although a non-specific immunoreactive band was apparent in all the samples tested (indicated with an arrow in Fig. 2, indicated as ns in panels C and D), the absence of an immunoreactive FSH β 24 kDa band in *Fshb*^{-/-} mouse pituitary further validated the specificity of the goat polyclonal antibody used for Western blot analyses.

Gonadotropin subunits are evolutionarily conserved and interspecies hybrid heterodimers, i.e α -subunit from one species and β -subunit from the other, could be assembled both *in vitro* (Licht et al., 1976) and *in vivo* (Kumar et al., 1998; Wang et al., 2014; Licht et al., 1976). Therefore, we next tested whether the *HFSHB^{dgc}* transgene-encoded, double N-glycosylation site mutant human FSH β subunit assembled with the endogenous mouse α -subunit and formed an interspecies hybrid heterodimer in pituitaries of *Fshb*^{-/-} *HFSHB^{dgc}* mice. We readily identified the interspecies hybrid FSH dimer (mouse α -subunit and hFSH β wild-type subunit) in pituitary extracts of *Fshb*^{-/-} *HFSHB^{WT}* male and female mice prepared under non-denaturing conditions (Lane 4 in Fig. 2, panels E and F). In contrast, interspecies hybrid FSH dimers containing the mouse α -subunit and double N-glycosylation site mutant human FSH β subunit were weakly detectable in pituitary extracts of both lines of male and female *Fshb*^{-/-} *HFSHB^{dgc}* mice (Lanes 5 and 6 in Fig. 2, panels E and F). Recombinant hFSH served as a positive control and showed an immunoreactive band similar to that seen in pituitary extracts obtained from *Fshb*^{-/-} *HFSHB^{WT}* male and female mice (Lane 3 in Fig. 2, panels E and F), whereas pituitary extracts from control normal and *Fshb* null mice served as negative controls and did not show any immunoreactive bands corresponding to heterodimer (Lanes 1 and 2 in Fig. 2, panels E and F).

Furthermore, FSH content in pituitary extracts measured by a ¹²⁵I-hFSH radioimmunoassay was significantly higher in *Fshb*^{-/-} *HFSHB^{dgc}* mice as compared to *Fshb*^{-/-} controls (4.9

± 0.75 ng/pituitary vs. 0.6 ± 0.1 ng/pituitary; $n = 6$; $P < 0.01$ by *T*-test). Similarly, FSH bioactivity as determined by a radioreceptor assay using ^{125}I -hFSH tracer and rat testicular membranes showed a trend towards higher value in pituitary extracts from *Fshb*^{-/-} *HFSHB*^{dgc} mice but was not significantly different compared to that in *Fshb*^{-/-} mice (93 ± 13 ng/pituitary vs. 46 ± 12 ng/pituitary, $n = 6$; $P > 0.05$ by *T*-Test). In a separate assay, using CHO cells expressing hFSHRs, FSH content estimated was 1.4 ± 0.1 μg /pituitary in wild-type controls, 1.8 ± 0.4 μg /pituitary in case of *Fshb*^{-/-} mice, 5.4 ± 0.5 mg/pituitary in *Fshb*^{-/-} *HFSHB*^{WT} mice and undetectable in case of *HFSHB*^{dgc} mice. Collectively, all the above data indicate that the *HFSHB*^{dgc} transgene was appropriately targeted to the pituitary and expressed in gonadotropes, similar to the *HFSHB*^{WT} transgene. However, the double N-glycosylation site mutant hFSH β subunit, unlike hFSH β WT subunit, did not assemble well *in vivo* and formed inefficiently an interspecies hybrid heterodimer with the endogenous mouse α -subunit.

3.3. Double N-glycosylation mutant hFSH β subunit containing FSH dimer is secretion incompetent both *in vitro* and *in vivo*

Despite the poor assembly with the mouse α -subunit, the double N-glycosylation site mutant hFSH β containing interspecies hybrid FSH heterodimer was detectable in low abundance in the pituitary and thus it might be secreted in low levels into serum. To test this, we first evaluated secretion *in vitro* under short-term (6 h) and serum-free culture conditions using whole pituitaries from adult mice. Western blot analysis under denaturing conditions followed by densitometry quantification indicated that whereas $\sim 10\%$ – 14% of FSH β was readily detectable in medium compared to lysate (designated respectively as M and L in Fig. 3A) in case of control wild-type (WT) and *Fshb*^{-/-} *HFSHB*^{WT} mice, the FSH β signal was undetectable in medium in the case of *Fshb*^{-/-} *HFSHB*^{dgc} mice (Fig. 3A). Next, we measured serum FSH levels using a rat/mouse FSH RIA that we had previously validated for detecting mouse α /human FSH β interspecies hybrid FSH (Kumar et al., 1998; Wang et al., 2014). We found that serum FSH levels were significantly low in *Fshb*^{-/-} *HFSHB*^{dgc} mice compared to those in *Fshb*^{-/-} *HFSHB*^{WT} mice (Fig. 3, panels B and C) and were mostly undetectable as in serum samples from *Fshb* null mice.

One reason for low-level secretion of double N-glycosylation site mutant FSH β containing FSH dimer could be intracellular retention in the pituitary as a result of changes in N-glycosylation or defects in protein folding. Immunofluorescence followed by confocal microscopy analysis confirmed that the double N-glycosylation site mutant hFSH β subunit in pituitary sections of *Fshb*^{-/-} *HFSHB*^{dgc} mice was readily detectable in gonadotropes by the human FSH β -specific 4B monoclonal antibody. Moreover, when co-labeled with ER (Supplementary Fig. S1, panel A) and Golgi (Supplementary Fig. S1, panel B) resident protein markers, the mutant FSH β showed no intracellular retention in these protein trafficking organelles. An identical labeling pattern of FSH β and ER/Golgi markers was observed in pituitary sections from control and *Fshb*^{-/-} *HFSHB*^{WT} mice (Supplementary Fig. S1, panels A and B) that exhibited normal circulating serum levels of FSH. In addition, Taqman qPCR assays showed that expression of many of the gonadotrope-specific “hallmark” genes was not altered in pituitaries of *Fshb*^{-/-} *HFSHB*^{dgc} mice (Supplementary Fig. S2, panels A–F) and was not significantly different than that in pituitaries of control

(*Ctrl.*) and *Fshb*^{-/-} *HFSHB*^{WT} mice (*Fshb*^{-/-} *HFSHB*^{dgc} mice vs. *Ctrl.* or *Fshb*^{-/-} *HFSHB*^{WT} mice, $P > 0.05$; ONE-WAY ANOVA followed by Turkey's post-hoc test, $n = 3$ mice). Together, these data confirmed that an interspecies FSH heterodimer consisting of mouse α - and the double N-glycosylation site mutant hFSH β subunit was secretion incompetent, not as a result of aberrant gonadotrope marker gene expression or ER/Golgi retention, and was detectable in very low levels in serum of *Fshb*^{-/-} *HFSHB*^{dgc} mice.

3.4. *HFSHB*^{dgc} transgene does not rescue *Fshb* null male mice

Fshb null males demonstrate reduced testis size, accompanied by Sertoli and germ cell defects, but these null males are fertile (Wang et al., 2014; Kumar et al., 1997). Previously, we achieved genetic rescue of *Fshb* null mice by gonadotrope-targeted expression of a *HFSHB*^{WT} transgene and characterized the male reproductive phenotypes of these rescue mice (Kumar et al., 1997). To determine whether low levels of double N-glycosylation site mutant hFSH β subunit containing FSH dimer rescues *Fshb* null mice, we analyzed male reproductive phenotypes of *Fshb*^{-/-} *HFSHB*^{dgc} mice and compared them to those in *Fshb*^{-/-} *HFSHB*^{WT} mice. Gross testes weights were significantly lower in *Fshb*^{-/-} *HFSHB*^{dgc} adult male mice at 9 weeks of age compared to those in normal control and *Fshb*^{-/-} *HFSHB*^{WT} males (Fig. 4 A–B; $P < 0.05$; *T*-Test, $n = 6$) but were not different when compared to *Fshb*^{-/-} mice (Fig. 4 A–B; $P > 0.05$; *T*-Test, $n = 6$). Furthermore, epididymal sperm number (Fig. 4C), histological analysis of PAS/hematoxylin stained testes sections (Fig. 4D) followed by quantification of tubule diameter (Fig. 4E), and real time qPCR assays on testes RNA (Fig. 4F and G) to measure FSH-responsive marker genes indicated that testes phenotypes in *Fshb*^{-/-} *HFSHB*^{dgc} mice were all similar to those in *Fshb*^{-/-} male mice. But, these parameters were all significantly different when compared to normal control and *Fshb*^{-/-} *HFSHB*^{WT} male mice (Fig. 4 C–G). Thus, these data indicate that, whereas a *HFSHB*^{WT} transgene efficiently rescued, the *HFSHB*^{dgc} transgene failed to rescue *Fshb*^{-/-} mice. Since *Fshb* null males were fertile, there were no differences, as expected, in fertility performance (number of litters produced and litter size, i.e. pups per litter) across all genotypes of mice tested over a 6-month period of time (Fig. 4 H and I).

3.5. *HFSHB*^{dgc} transgene does not rescue *Fshb* null female mice

The complete absence of FSH results in hypoplastic ovaries and uteri, and a pre-antral stage block in folliculogenesis leading to infertility in females (Wang et al., 2014; Kumar et al., 1997). Gonadotrope-targeted expression of a *HFSHB*^{WT} transgene efficiently rescues and restores normal phenotypes in *Fshb* null female mice (Kumar et al., 1998; Wang et al., 2014). To determine if the *HFSHB*^{dgc} transgene rescues *Fshb* null females, female reproductive tracts were analyzed morphologically and histologically (Fig. 5). *Fshb* null females expressing the *HFSHB*^{dgc} transgene demonstrated hypoplastic uteri and ovaries similar to those observed in *Fshb* null females (Fig. 5A), and as reported earlier (Wang et al., 2014; Kumar et al., 1997). In contrast, uterine horn and ovarian morphology were indistinguishable in mice expressing the *HFSHB*^{WT} transgene compared to normal control mice at 9 weeks of age (Fig. 5A). Histological analysis indicated all stages of folliculogenesis including many secondary follicles (SF) and the presence of corpora lutea (CL) in ovarian sections obtained from control and *Fshb*^{-/-} *HFSHB*^{WT}, but not *Fshb*^{-/-} and *Fshb*^{-/-} *HFSHB*^{dgc} females at either 9 weeks (Fig. 5B) or 6 months of age (Supplementary

Fig. S3), indicating anestrus and anovulatory phenotypes only in the latter two mouse genotypes. When expression of FSH-responsive genes was measured by Taqman real time qPCR assays, we found that these genes were suppressed in the absence of FSH-mediated signaling in ovaries of *Fshb* null mice (Fig. 6). Only the *HFSHB^{WT}* but not the *HFSHB^{dgc}* transgene rescued the expression of these FSH-responsive genes to those levels observed in normal control mouse ovaries (Fig. 6 A–F). Immunolabeling of ovarian sections with antibodies against markers downstream of FSH-mediated signaling such as phospho-CREB (Fig. 7A) and phospho-PKA substrate (Fig. 7B) similarly indicated no rescue in *Fshb* null female mice expressing the *HFSHB^{dgc}* transgene compared to that in *Fshb* null female mice expressing the *HFSHB^{WT}* transgene. However, there were no differences in the immunolabeling pattern for phospho-Histone H3, a general mitotic marker in ovarian sections across all genotypes (Fig. 7C). Consistent with all the above female reproductive phenotypes, mating trials over a period of 6-months indicated that *Fshb* null female mice expressing the *HFSHB^{dgc}* transgene did not produce any litters, similar to *Fshb* null females (Fig. 7 D and E). In contrast, the breeding performance (i.e., litter number and size) of *Fshb* null female mice expressing the *HFSHB^{WT}* transgene was indistinguishable from that of control females (Fig. 7 D and E). Thus, these data confirm that the *HFSHB^{dgc}* transgene did not rescue *Fshb* null females similar to *Fshb* null males.

4. Discussion

The biological significance of Asn-linked N-glycans in glycoprotein hormones was originally established by chemical modification methods using reagents such as hydrogen fluoride and trifluoromethane sulfonic acid, or enzymatic treatment using glycosidase mixtures on purified hormones (Kalyan and Bahl, 1983; Manjunath and Sairam, 1982; Manjunath et al., 1982; Sairam, 1982; Sairam and Jiang, 1992; Sairam et al., 1986; Thotakura et al., 1990). Such de-glycosylated hormone preparations were pharmacologically tested *in vitro* or injected into rodents to evaluate serum half-life and bioactivities (Kalyan and Bahl, 1983; Manjunath and Sairam, 1982; Manjunath et al., 1982; Sairam, 1982; Sairam and Jiang, 1992; Sairam et al., 1986; Thotakura et al., 1990). However, it was not possible to pinpoint the contributions of individual N-glycans selectively by these general deglycosylation methods. Hence, subsequent studies focused on site-directed mutagenesis and transfection approaches using mostly CHO cell expression systems and defined the biological roles of individual N-glycan chains on glycoprotein hormone subunits (Matzuk and Boime, 1989; Flack et al., 1994a,b). One disadvantage noted with this approach was when mutagenesis of Asn residues (and thus loss of N-glycan chains) affected dimer assembly and secretion, bioactivity assessment was not possible.

In this manuscript, we have circumvented the problems associated with chemical modification and *in vitro* mutagenesis experiments by undertaking an *in vivo* genetic rescue approach. We initially produced transgenic mice with gonadotrope-targeted expression of either *HFSHB^{WT}* or *HFSHB^{dgc}* mutant transgenes and introduced these separately onto an *Fshb* null genetic background by intercrossing. This strategy permitted us to directly test the roles of N-glycans on transgene-encoded hFSH β subunit in the absence of endogenous mouse FSH β subunit (Fig. 8). The use of a cDNA construct and thus the lack of all regulatory elements on the *HFSHB^{dgc}* transgene was not an issue because full-length

HFSHB^{WT} transgene encompassing all elements was expressed at similar levels in the mouse pituitary (Fig. 2 A–C). This also suggests identical regulation of *HFSHB* and *oFshb* promoters in a mouse gonadotrope environment.

Although *HFSHB*^{dgc} transgene-encoded, double N-glycosylation site mutant FSH β subunit was expressed at high levels, it assembled poorly with the endogenous mouse α -subunit as assessed by non-denaturing electrophoresis followed by immunoblotting, and quantification of FSH content in pituitary lysates by human or mouse FSH RIAs. Thus, N-linked glycans covalently attached at Asn⁷ and Asn²⁴ residues on hFSH β subunit are essential *in vivo* for FSH dimer assembly. Our data are in agreement with biochemical studies using purified bovine glycoprotein hormone preparations in which differences in α -subunit glycosylation were similarly found and predicted to be important for dimer assembly (Arora et al., 1999; Arora et al., 2010). Moreover, recent molecular dynamics simulation studies have also indicated no evidence of misfolding of deglycosylated FSH β subunit in combining with the alpha subunit (Meher et al., 2015). Our data, however, are contradictory to *in vitro* studies using a heterologous CHO cell expression system in which Asn⁷/Asn²⁴ double mutant FSH β subunit-containing heterodimer immunoactivity was readily detected in gel filtration chromatograms (Flack et al., 1994a,b).

Although, previous studies indicated that interspecies hybrid glycoprotein hormones between purified α - and β -subunits obtained from different species readily formed (Licht et al., 1976; Burke et al., 1979; Licht et al., 1978; Licht et al., 1983), co-expression of mutant FSH β subunit with homologous α - subunit *in vitro* versus heterologous α - subunit *in vivo* could be one reason for the above discrepancy. An alternative approach to our strategy presented here would be to target in the future, expression of a mutant transgene encoding a single chain FSH analog in which double N-glycosylation site mutant human FSH β encoding cDNA is genetically fused to that encoding the human α – subunit (Ben-Menahem and Boime, 1996; Garcia-Campayo and Boime, 2001). However, such a strategy will bypass the subunit/heterodimer assembly step. If the resulting single chain hormone were processed similar to FSH heterodimer and gets secreted from pituitary, then this strategy would allow us to test the *in vivo* biological function of this novel double N-glycosylation site mutant FSH β – containing single chain FSH analog.

As a consequence of poor assembly, double N-glycosylation site mutant FSH β subunit-containing FSH dimer was inefficiently secreted. We confirmed this by RIA measurements of both whole pituitary organ culture medium samples and mouse serum samples. Impaired secretion was not due to dysregulated gonadotrope-specific gene expression, intracellular retention or “trapping” of either the mutant hFSH β subunit or FSH dimer containing this mutant subunit because both WT and mutant hFSH β subunits displayed identical patterns of localization by confocal microscopy. Consistent with this intracellular behavior and impaired secretion, *Fshb* null male and female mice expressing double N-glycosylation site mutant hFSH β -containing FSH dimer did not show any evidence of rescue. Testis and tubule size, as well as sperm number in *Fshb*^{−/−} *HFSHB*^{dgc} mutants were similar to those in *Fshb* null male mice. Similarly, female *Fshb*^{−/−} *HFSHB*^{dgc} mutants, like *Fshb* null mice were infertile, did not show any evidence of estrus cycles and their ovarian genes remained suppressed. Our previous (Kumar et al., 1998; Wang et al., 2014) and present studies also

confirmed that *Fshb*^{-/-} *HFSHB*^{WT} transgene encoding human FSH β subunit (with both N-glycosylation sites intact) fully rescues *Fshb* null mice. Both male and female *Fshb*^{-/-} *HFSHB*^{WT} mice exhibited phenotypes indistinguishable from those in control normal mice.

Micro-heterogeneity in glycoprotein hormones contributed by terminal sugar residues has been extensively studied (Creus et al., 2001, 1996; Loreti et al., 2013a,b). Over the past few years our group has identified and biochemically characterized several naturally occurring hypo-glycosylated FSH glycoforms resulting from macro-heterogeneity contributed by presence or absence of individual N-linked glycans on FSH β subunit (Bousfield et al., 2007; Davis et al., 2014; Walton et al., 2001; Bousfield et al., 2014a,b; Butnev et al., 2015). Previous mass spectrometry results misleadingly detected only deglycosylated FSH β in a FSH β preparation derived from hFSH²¹ glycoform in which only the 21 KDa FSH β band was detectable by Western blot (Walton et al., 2001). Thus, we initially believed hFSH β N-glycosylation occurred in an all-or-none manner. We were confident in the mass spectrometry results because when applied to a FSH β ²⁴ preparation possessing only the 24 KDa band in Western blot analysis, deglycosylated FSH β was also present. This led to molecular dynamics studies of hFSH¹⁵ models bearing various N-glycans (Meher et al., 2015), an unsuccessful attempt to express deglycosylated FSH β in bacteria, and the present manuscript to study transgenic FSH¹⁵ *in vivo*. Results reported herein provide the first evidence that loss of both N-glycans produced a 15 KDa FSH β band on Western blots.

Interestingly, the FSH¹⁵ glycoform (consisting of the double N- glycosylation site mutant FSH β subunit) was not the most abundant form identified in the above studies. This observation indicates that in addition to poor assembly, the double N-glycosylation site mutant hFSH β subunit containing FSH dimer may also be highly unstable in human pituitaries. Attempts to express the recombinant human FSH¹⁵ glycoform in pituitary-derived GH3 cells also consistently failed to produce much FSH dimer owing to perhaps poor assembly and stability of the dimer or FSH β subunit (Bousfield, G.R. et al., unpublished data). In contrast, hypo-glycosylated FSH²¹ (containing only one N-linked glycan chain on Asn⁷) was routinely detected in most human pituitaries (Bousfield et al., 2014a,b; Butnev et al., 2015), while FSH¹⁸ only became detectable in FSH²¹-enriched preparations. Both glycoforms were routinely expressed and have been functionally characterized *in vitro* (Bousfield et al., 2014a,b; Butnev et al., 2015). These recent data indicate that at least one N-linked glycan must be present on hFSH β subunit for proper FSH dimer assembly and secretion.

Our experimental model of *Fshb* null mice engineered to express desired human *FSHB* transgenes in a gonadotrope-specific manner offers a well-established genetic rescue platform (Kumar et al., 1998; Wang et al., 2014). Thus, it is feasible for us to systematically engineer and express different human FSH glycoformencoding transgenes on an *Fshb* null genetic background. Such *in vivo* studies are ongoing in our laboratory that will ultimately allow us to define the physiological significance of the existence of multiple FSH glycoforms in the pituitary and decode the precise biological roles of each FSH glycoform in a whole animal setting.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mce.2016.02.015>.

Abbreviations

ACTH	Adrenocorticotropin
cAMP	cyclin adenosine 5'-monophosphate
CHO	Chinese hamster ovary
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GH	Growth hormone
LH	Luteinizing hormone
PNGase F	Peptide -N-Glycosidase F
Ppil1	Peptidylprolyl isomerase-like 1
PKA	protein kinase-A
PVDF	Polyvinyl difluoride
PAS	Periodic acid Schiff's reagent
PRL	Prolactin
RIA	Radioimmuno assay
RRA	Radioreceptor assay
r-h	Recombinant human
TSH	Thyroid-stimulating hormone

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Fig. 1.

A transgene construct (A) consisting of a gonadotrope-specific ovine *Fshb* promoter driving the expression of a double N-glycosylation site mutant hFSH β subunit-encoding cDNA-SV40 polyA sequences was engineered and microinjected into one-cell mouse embryos to produce transgenic mice. Genomic PCR assay of tail DNA samples (B) using *oFshb* promoter-specific primers (indicated by arrows in A) amplified a 750 bp fragment that identified and distinguished founder mice carrying the transgene (+) from control wild-type (WT), transgene-negative mice. A two-step genetic rescue scheme (C) was employed to produce the desired *Fshb* null mice carrying the *HFSHB^{WT}* or *HFSHB^{dgc}* transgene (Tg^{+}).

Fig. 2.

Real time qPCR assay (A) shows nearly identical expression of *HFSHB* mRNA in pituitaries of *Fshb*^{-/-} mice expressing either a *HFSHB*^{WT} or *HFSHB*^{dgc} transgene. Two different lines #30 and #60, expressing the mutant transgene were developed. Dual label immunofluorescence visualization of pituitary sections (B) from adult mice confirmed gonadotrope-specific expression of *HFSHB*^{dgc} transgene similar to that of *HFSHB*^{WT} transgene (indicated by white arrowheads in merged images in the right panel in B). The absence of any green fluorescence in pituitary sections from *Fshb*^{-/-} mice confirmed the specificity of the FSH β antibody. The white bar in panel B represents 50 μ m. Western blot analysis of proteins under denaturing conditions probed with a goat anti-human FSH β -polyclonal antibody obtained from Santa Cruz Biotechnology, sc 7757 (C) shows a 15–17

KDa hFSH β ^{Asn7 Asn24} non-glycosylated subunit (denoted as hFSH β *) was abundantly expressed in pituitaries of three different lines of *HFSHB^{dgc}* transgenic mice (line # 220, line #30 and line #60) on an *Fshb* null genetic background. Black arrow indicates a non-specific band. Recombinant human (r-h) FSH β size-matched with that of *HFSHB^{WT}* transgene-encoded hFSH β subunit and weakly immunoreactive mouse (m) FSH β subunit detected in normal control mouse (*Ctrl.*) pituitary. Proteins from *Fshb*^{-/-} mouse pituitaries served as a negative control. PNGase F treatment followed by Western blot analysis of either r-hFSH or pituitary proteins from a normal control wild-type (*Ctrl.*) mouse showed an identical size deglycosylated FSH β subunit corresponding to that identified in an *Fshb*^{-/-} *HFSHB^{dgc}* mouse pituitary (D). Electrophoretic separation of proteins under non-denaturing conditions followed by Western blot analysis showed that in both male (E) and female (F) *Fshb*^{-/-} *HFSHB^{dgc}* mouse pituitaries, the non-glycosylated FSH β subunit, despite high level expression (C) was present as an interspecies hybrid (mouse α + human FSH β *) heterodimer (lanes 5 and 6 in panels E and F) at very low levels compared to the FSH heterodimer (mouse α + hFSH β) in pituitaries of *Fshb*^{-/-} *HFSHB^{WT}* mice (lane 4 in panels E and F). Pituitary proteins from control and *Fshb*^{-/-} mice (Lanes 1 and 2 in panels E and F) are used as negative controls and r-hFSH (Lane 3) as a positive control. Arrows in panel D denote: a = FSH β subunit, ns = non-specific band, b = deglycosylated FSH β subunit. Arrows in panel E and F indicate: a = FSH β subunit, b = double N-glycosylation site mutant FSH β subunit, c = uncombined/free FSH β subunit, d = uncombined double N-glycosylation site mutant FSH β subunit. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Western blot analysis (A) of proteins in lysates and medium fractions derived from whole pituitary organ cultures separated under non-reducing conditions and probed with 4B FSH monoclonal antibody, indicated that interspecies hybrid heterodimer containing the non-glycosylated hFSH β mutant subunit (in *Fshb*^{-/-} *HFSHB*^{dgc} mouse pituitary), was barely secreted into medium compared to the hFSH β WT subunit-containing interspecies hybrid heterodimer (in *Fshb*^{-/-} *HFSHB*^{WT} mouse pituitary) or mouse FSH heterodimer from control normal mouse pituitary. The black arrow indicates a non-specific band. *Fshb*^{-/-}

mouse pituitary lysate and r-hFSH served as negative and positive controls, respectively. Expression of β -tubulin was used as an internal control and is shown in the blot at the bottom of panel A. L: lysate; M: medium; hFSH β^* : non-glycosylated hFSH $\beta^{\text{Asn7 Asn24}}$ double site mutant subunit. RIAs on serum samples from adult male (B) and female (C) mice revealed very low levels of non-glycosylated hFSH $\beta^{\text{Asn7 Asn24}}$ double site mutant subunit-containing FSH dimer in two lines (Line #30 and Line #60) of *Fshb*^{-/-} *HSFHB*^{dgc} mice compared to that in normal control (*Ctrl.*) or *Fshb*^{-/-} *HSFHB*^{WT} mice expressing hFSH β WT subunit. In panels B and C: * $P < 0.01$ compared to *Ctrl.* or *Fshb*^{-/-} *HSFHB*^{WT} groups; ** $P < 0.01$ compared to *Fshb*^{-/-} group.

Fig. 4.

The *HFSHB*^{dgc} transgene, in contrast to the *HFSHB*^{WT} transgene, did not rescue *Fshb* null male mice as illustrated by gross testicular morphology (A) and size (B), quantitation of epididymal sperm (C), PAS/hematoxylin-stained testis histology (D), tubule diameter measurements (E) and FSH-responsive gene expression analysis by real time qPCR assays (F and G). Breeding performance over a period of 6 months was assessed in terms of number of litters produced (H) and litter size, i.e. number of pups per litter (I) is independent of FSH and *Fshb* genotype tested. *P < 0.01 versus *Ctrl.* or *HFSHB*^{WT} groups; ** P < 0.05

versus *Fshb*^{-/-} group. *Fshr*: follicle-stimulating hormone receptor, *Lhr*: luteinizing hormone receptor.

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Fig. 5.

Gross morphology (A) and PAS-hematoxylin stained ovarian histology (B) showed that *HFSHB*^{dgc} transgene unlike the *HFSHB*^{WT} transgene did not rescue *Fshb*^{-/-} females. Multiple developing secondary follicles and corpora lutea are readily seen in ovarian sections obtained from control (*Ctrl.*) and *Fshb*^{-/-} *HFSHB*^{WT} mice but not in those from *Fshb*^{-/-} or *Fshb*^{-/-} *HFSHB*^{dgc} mice. Boxed areas in top panel in B are enlarged and shown below. SF: secondary follicle; CL: corpus luteum. The bar in panel B represents 200 μm.

Fig. 6.

Ovarian gene expression measured by real time qPCR assays was indicated in panels A–F. The results showed many of the FSH-responsive genes suppressed in *Fshb*^{-/-} mice remained the same in *Fshb*^{-/-} *HFSHB*^{dgc} mice. *HFSHB*^{WT} transgene restored the expression levels of many genes similar to that observed in ovaries of control (*Ctrl.*) mice. Each assay was performed on four cDNA samples and each sample run in triplicate. * P < 0.01 versus *Ctrl.* or *Fshb*^{-/-} *HFSHB*^{dgc} groups; ** versus *Fshb*^{-/-} group. qPCR assays to detect the expression of the following genes were used- *Fshr*: follicle-stimulating hormone-receptor, *Cyp19a1*: aromatase, *Inhbb*: activin beta b, *Kcnj8*: potassium inwardly-rectifying channel subfamily J member 8, *S100a6*: S100calcium binding protein A6 (calcyclin), *Lhr*: luteinizing hormone receptor.

Fig. 7.

Immunolabeling of ovaries obtained from mice at 21d of age showed that phospho-CREB (A) and phospho-PKA substrate (B) levels did not differ between *Fshb*^{-/-} and *Fshb*^{-/-} *HFSHB*^{dgc} mice indicating no rescue in contrast to that observed in *Fshb*^{-/-} *HFSHB*^{WT} mice. However, there were no differences in mitotically active phospho-Histone H3⁺ cells (C) in ovarian sections obtained from all genotypes of mice. *Fshb*^{-/-} *HFSHB*^{dgc} female mice, similar to *Fshb*^{-/-} females were infertile as indicated by mating trials that resulted in production of no pups in a 6 month-period. In contrast, the breeding performance of *Fshb*^{-/-}

HSFHB^{WT} mice was identical to that observed in control mice (D and E) indicating full-functional rescue by the *HSFHB*^{WT} transgene.

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Genotype	FSH dimer composition	# of N-linked glycans			FSH dimer assembly	FSH secretion
		α	β	Total in FSH dimer		
<i>Fshb</i> ^{-/-} <i>HFSHB</i> ^{WT}		2	2	4	Efficient	Efficient
<i>Fshb</i> ^{-/-} <i>HFSHB</i> ^{dgc}		2	0	2	Inefficient	Inefficient

Fig. 8.

Comparison of *Fshb*^{-/-} *HFSHB*^{WT} and *Fshb*^{-/-} *HFSHB*^{dgc} mice, their interspecies hybrid FSH heterodimer composition, number of N-linked glycans, FSH dimer assembly and secretion characteristics. Genetic rescue of *Fshb*^{-/-} mice was achieved with *HFSHB*^{WT}, but not *HFSHB*^{dgc} transgene, confirming that N-linked glycans of hFSH β subunit are essential for FSH heterodimer assembly and secretion *in vivo*. The positions of Asn-linked N-glycans on FSH α (Asn52 and Asn78) – and β -subunits (Asn7 and Asn24) are indicated.