Supplementary Material

A human *FSHB* transgene encoding the double N-glycosylation mutant ($Asn^{7\Delta}$ $Asn^{24\Delta}$) FSHβ subunit fails to rescue *Fshb* null mice

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Running Title: Fshb null mice expressing an hFSHβ double N-glycosylation mutant subunit

1. Supplementary Materials and Methods

1.1. Transgene construction and production of transgenic mice

A human *FSHB* cDNA template was modified by site directed mutagenesis such that both the Asn^{7} (Asn-Ile-Thr) and Asn^{24} (Asn-Thr-Thr) N-glycosylation sites were destroyed by replacing the underlined Thr residues with Ala resdiues (Fox, Dias and Van Roey, 2001). This human *FSHB* double N-glycosylation mutant cDNA was cloned into a pBluescriptSK^{-} plasmid and the sequence verified. The 5' end was ligated downstream of a 4.7 kb ovine *Fshb* promoter that is gonadotrope-specific and functionally indistinguishable from the human *FSHB* promoter. The 3' end of the cDNA was ligated to a SV40 poly A sequence and the assembled transgene (*HFSHB*^{dgc}) was released from the vector backbone, column purified and microinjected into fertilized one-cell FVB x C57Bl/6 F1 hybrid mouse embryos and transferred into pseudopregnant foster female mice as described (Kumar, Larson, Wang et al., 2009). Transgene-positive founder mice were bred to wild-type (WT) control littermates and propagated up to the F2 generation to confirm stable integration and transmission of the transgene by PCR as described below. For comparison, transgenic mice expressing a gonadotrope-specific human *HFSHB*^{WT} transgene (10 kb) were used. These mice were generated as described before (Kumar, Low and Matzuk, 1998) and have been extensively characterized (Kumar et al., 1998,Wang, Larson, Jablonka-Shariff et al., 2014).

1.2. Screening of transgenic mice by genomic PCR assays

Genomic DNA was isolated from tail snips of mice by proteinase-K digestion overnight followed by alcohol precipitation as described (Wang et al., 2014). Specific oligonucleotide primers that bind to o*Fshb* promoter sequences and distinguish transgene-positive from wild-type mice were designed as described (Wang, Hastings, Miller et al., 2015) and used in a PCR reaction containing JumpStart Taq polymerase ready mix. An EZ load 100 bp DNA molecular ruler and PCR reactions were separated on 1.5 % agarose gels and the amplified transgene-specific 750bp

fragment visualized by ethidium bromide staining. PCR identification of human $FSHB^{WT}$ mice was done as exactly described before (Wang et al., 2014).

1.3. Genetic rescue of Fshb null mice

Adult transgenic mice expressing a gonadotrope-specific human FSHB wild-type ($HFSHB^{WT}$) or a double N-glycosylation site mutant ($HFSHB^{dgc}$) transgene (referred to as Tg^{+} in Fig. 1) were separately intercrossed with *Fshb* null mice in a two-step breeding scheme as described before (Kumar et al., 1998,Wang et al., 2014). Typically, *Fshb* null males (are fertile) and intercrossed with *Tg*+ females in Step1. The resultant $Fshb^{+/-}\ Tg^{+}$ sibling mice were subsequently intercrossed in Step 2 (Fig. 1) to produce the desired $Fshb^{-/-}\ Tg^{+}$ mice (rescue mice). $Fshb^{-/-}$ males were also occasionally intercrossed with $Fshb^{+/-}\ Tg^{+}$ females to increase the frequency of obtaining rescue mice. *Fshb* null mice were identified by PCR assays that distinguish the WT and mutant alleles as described before (Kumar et al., 1998,Wang et al., 2014).

1.4. RNA isolation, cDNA synthesis and Taqman real time qPCR assays

Total RNA was isolated from pituitaries and ovaries of at least 3 mice using RNeasy columns, DNaseI-digested and reverse transcribed using an oligo dT and Superscript III reverse transcriptase method as described (Wang et al., 2014,Wang et al., 2015). The cDNA samples were assayed in triplicate by Taqman real time quantitative PCR assays using custom synthesized or commercially available primer/probe combos (IDT). Relative expression levels of each gene were quantified using *Ppil1* as an internal control as described (Wang et al., 2014,Wang et al., 2015).

1.5. Pituitary organ cultures

Individual pituitaries from adult Tg^{+} male mice were collected intact into cold PBS and washed 3-4 times in culture medium (DMEM without serum and antibiotics) under sterile conditions. Each pituitary was later immersed in 250 μL of medium and incubated up to 5 h in a humidified incubator with 5 % CO_2 atmosphere at 37C. At the end of incubation, media were frozen and tissues were rinsed twice with cold PBS and the lysates prepared in RIPA buffer and

stored at -80C until further use. Both tissue lysates and media were subjected to Western blot analysis under non-denaturing conditions as described below.

1.6. Enzymatic deglycosylation by PNGase F treatment

Pituitary extracts (20 μg of total protein) from control mice or recombinant hFSH (10 μg) were first heat denatured at 95C for 10' and then incubated with PNGase F (NEB; at 1U/ 10 μg protein) in the presence of 10 % NP40 at 37C for 60'. Following enzyme treatment, the reaction mixture was heat denatured in 2X SDS-loading buffer and processed for Western blot analysis as described below.

1.7. Western blot analysis

Protein extracts from individual mouse pituitaries or concentrated media from organ cultures were prepared by homogenization in cold radioimmunoprecipitation assay buffer followed by centrifugation at 12,000 X g for 15' at 4C. Aliquots of the supernatants were used for protein estimation by the Bio-Rad's dye-binding method with BSA as a standard. Protein extracts were boiled with 2X sample loading buffer containing SDS/β–mercaptoethanol for 5 min. and separated on 12 % polyacrylamide gels. For non-denaturing electrophoresis, samples were mixed in 2X sample buffer containing no β-mercaptoethanol and directly loaded onto polyacrylamide gels without heat denaturation. The conditions for electrophoresis and transfer of proteins onto PVDF membranes were followed exactly as described (Wang et al., 2014,Wang, Graham, Hastings et al., 2015). Pre-stained molecular mass markers were run alongside for checking transfer efficiency. The membranes were blocked in 5 % non-fat dry milk, washed with TBS containing 0.1 % Tween-20 and incubated at room temperature with appropriate primary antibodies against: FSHβ (4B monoclonal antibody, a gift from Dr. Irving Boime, or mouse ascites fluid containing the monoclonal antibody against human FSHβ designated RFSH20) or anti-β-tubulin (Santa Cruz, sc9104,), washed and incubated with appropriate horse-radish peroxide-conjugated secondary antibodies. The antigen-antibody complexes were visualized using an enhanced chemiluminescence kit obtained from GE Healthcare.

1.8. Immunofluorescence assays and confocal microscopy

Pituitaries and ovaries from adult mice were collected and fixed in buffered formalin (pH = 7.4) overnight at 4C. The fixed tissues were processed, paraffin-embedded and 6 μm sections were cut onto glass slides as described (Wang et al., 2014,Wang et al., 2015,Wang et al., 2015). For immunofluorescence, sections were processed by serial immersion in xylene, followed by graded series of alcohol, washed in water, antigen-retrieved by heating in a microwave and incubated by blocking in 10 % normal goat serum as described (Wang et al., 2014,Wang et al., 2015,Wang et al., 2015). Pituitary sections were washed and incubated overnight at 4C with primary antibodies against LHβ and FSHβ, processed and further incubated with appropriate Alexa flurochrome-conjugated secondary antibodies, visualized by a Leica epifluorescence microscope and the images digitally captured as described (Wang et al., 2014,Wang et al., 2015,Wang et al., 2015). Ovarian sections were processed similarly and incubated with rabbit antibodies against p-CREB, p-PKA substrate and pHistone-H3 as described before (Wang et al., 2014). For confocal microscopy, pituitary sections were incubated with an antibody specific to FSHβ and either a FITC-conjugated ER- or Golgi marker and visualized by a Nikon TE200-U confocal microscope as described (Wang et al., 2014,Wang et al., 2015,Wang et al., 2015).

1.9. Gross and histological analyses

Female reproductive tracts from adult control, *Fshb* null and *Fshb* null mice carrying *HFSHB* transgenes were dissected, rinsed in cold PBS (pH 7.4) and photographed. Ovaries were later fixed in formalin or Bouin's reagent overnight at 4C. Fixed-tissues were processed, paraffin-embedded and 6 μm sections were cut and stained with periodic acid-Schiff's reagent (PAS)/hematoxylin as described (Wang et al., 2014,Wang et al., 2015,Wang et al., 2015). Stained sections were viewed with a DP 71 Olympus microscope and digitally photographed using the DP Controller software.

1.10. Fertility assays

Mating trials between adult wild-type males at 42d of age and either *Fshb*$^{-/-}$ *HFSHB*^{WT} or

Fshb $^{-/-}$ *HFSHB*^{dgc} females at 42d of age (total 5 cages; one male and two females per cage) were monitored over a period of 4-6 months. The total number of litters and the number of pups produced per litter (litter size) during this period were recorded.

1.11. Radioimmunoassays

Radioimmunoassays (RIAs) were performed on pituitary extracts or serum samples by different assay methods. For measuring serum FSH, a rat/mouse RIA previously validated to detect interspecies hybrid FSH dimer (mouse α + Human FSHβ) was used at the University of Virginia Hormone Assay Core, Charlottesville, VA (Kumar et al., 1998,Wang et al., 2014). For measuring pituitary FSH content, a human FSH dimer-specific assay consisting of reagents obtained from the National Hormone and Pituitary Program, including a polyclonal AFP005 antibody and ^{125}I-hFSH tracer, were used as described (Walton, Nguyen, Butnev et al., 2001,Rosemberg, 1979). The sensitivity of the assay was 0.02 ng/tube cross reactivity with mouse FSH was 1.5 %. FSH content was expressed as either ng/ml serum or ng/pituitary.

1.12. Radioreceptor assays

Pituitary FSH content was also measured by a radio-receptor assay using rat testes homogenate (25 mg/tube) and ^{125}I-eFSH (2.5 ng/tube) as tracer. Human FSH reference preparation (AFP-7298; from Dr. A.F. Parlow and the National Hormone and Pituitary Program) was used for the calibration curve as described (Butnev, Gotschall, Baker et al., 1996).

2. Supplementary References

Butnev, V.Y., Gotschall, R.R., Baker, V.L., Moore, W.T. and Bousfield, G.R., 1996. Negative influence of O-linked oligosaccharides of high molecular weight equine chorionic gonadotropin on its luteinizing hormone and follicle-stimulating hormone receptor-binding activities, Endocrinology. 137, 2530-42.

Fox, K.M., Dias, J.A. and Van Roey, P., 2001. Three-dimensional structure of human follicle-stimulating hormone, Mol Endocrinol. 15, 378-89.

Kumar, T.R., Low, M.J. and Matzuk, M.M., 1998. Genetic rescue of follicle-stimulating hormone beta-deficient mice, Endocrinology. 139, 3289-95.

Kumar, T.R., Larson, M., Wang, H., McDermott, J. and Bronshteyn, I., 2009. Transgenic mouse technology: principles and methods, Methods Mol Biol. 590, 335-62.

Rosemberg, E., 1979. Immunoreactivity of standards and reference preparations used in the radioimmunoassay of follicle-stimulating and luteinizing hormones in serum, J Clin Endocrinol Metab. 48, 163-6.

Wang, H., Larson, M., Jablonka-Shariff, A., Pearl, C.A., Miller, W.L., Conn, P.M., Boime, I. and Kumar, T.R., 2014. Redirecting intracellular trafficking and the secretion pattern of FSH dramatically enhances ovarian function in mice, Proc Natl Acad Sci U S A. 111, 5735-40.

Wang, H., Graham, I., Hastings, R., Gunewardena, S., Brinkmeier, M.L., Conn, P.M., Camper, S.A. and Kumar, T.R., 2015. Gonadotrope-specific deletion of Dicer results in severely suppressed gonadotropins and fertility defects, J Biol Chem. 290, 2699-714.

Wang, H., Hastings, R., Miller, W.L. and Kumar, T.R., 2015. *Fshb-iCre* mice are efficient and specific Cre deleters for the gonadotrope lineage, Mol Cell Endocrinol. 419, 124-38.

Walton, W.J., Nguyen, V.T., Butnev, V.Y., Singh, V., Moore, W.T. and Bousfield, G.R., 2001. Characterization of human FSH isoforms reveals a nonglycosylated beta-subunit in addition to the conventional glycosylated beta-subunit, J Clin Endocrinol Metab. 86, 3675-85.

3. Supplementary Figures and Figure Legends

Supplementary Fig. S1

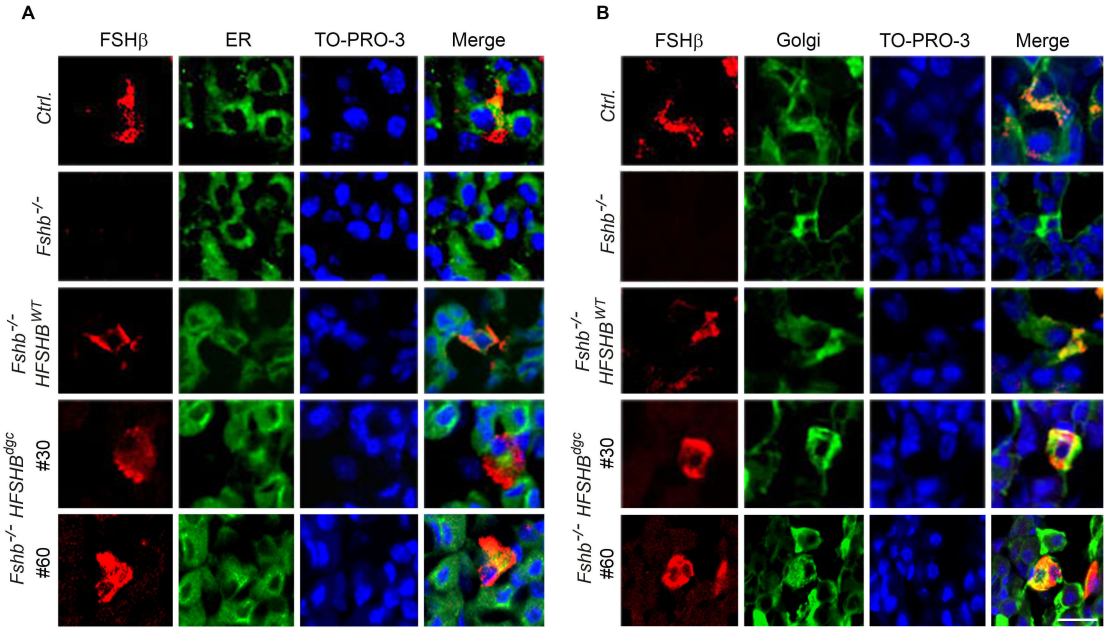

Fig. S1. Pituitary sections were triple-labeled with antibodies to FSHβ (visualized in red), TO-PRO-3 (nuclear dye staining visualized in blue) and either protein disulphide-isomerase (ER marker visualized in green in panel A) or FITC-WGA (Golgi marker visualized in green in panel B), respectively and visualized by confocal microscopy. The merged images were obtained by digitally combining the individual panels that showed identical patterns of co-localization of hFSHβ$^{\text{Asn7}\Delta\ \text{Asn24}\Delta}$ double mutant subunit with either ER or Golgi marker in two independent lines of *Fshb*$^{-/-}$ *HSFHB*^{dgc} mice, similar to the patterns observed with hFSHβ WT subunit in *Fshb*$^{-/-}$ *HSFHB*^{WT} mice or mFSHβ subunit in normal control mice (*Ctrl*). The absence of red fluorescence in *Fshb*$^{-/-}$ mouse pituitary sections confirmed the specificity of the antibody and revealed that only hFSHβ subunits were detected in pituitaries of transgenic mice on *Fshb* null genetic background. The white bar represents 20 μm

Supplementary Fig. S2

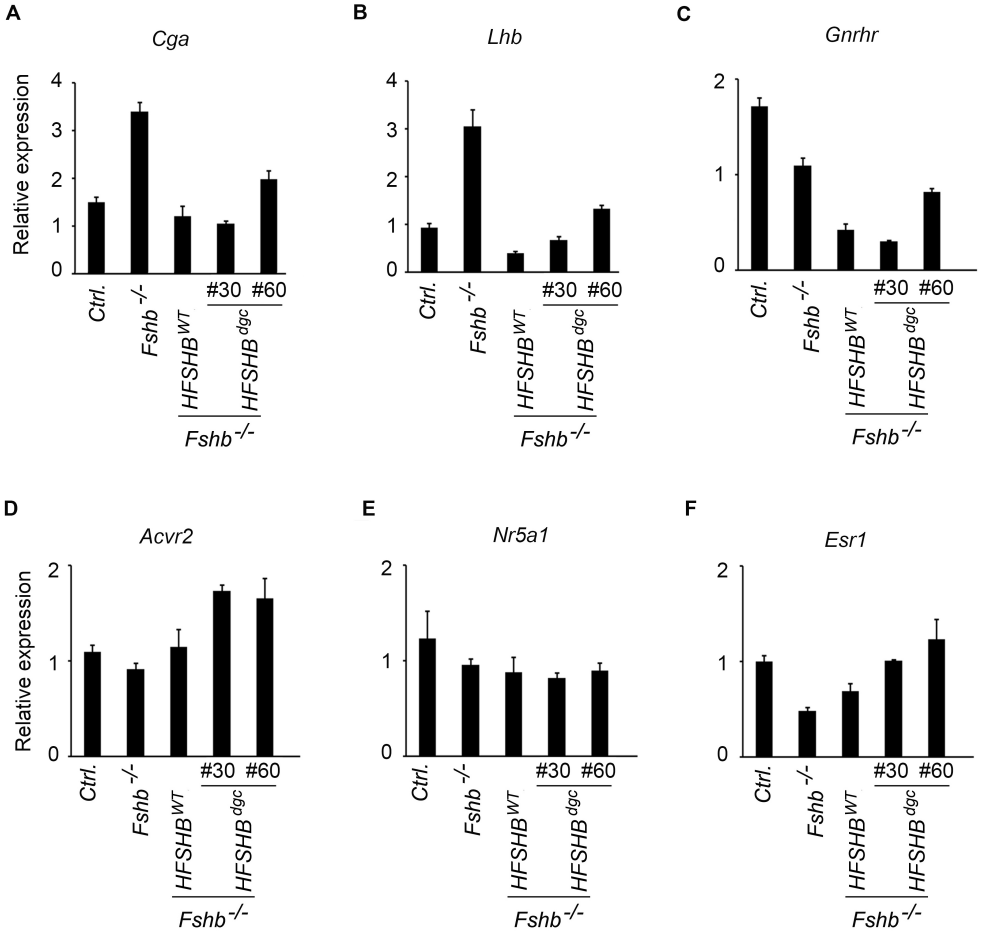

Fig. S2. Gonadotrope marker gene expression analysis by Taqman real time qPCR assays (A-E) showed no significant differences between *Fshb*$^{-/-}$ *HSFHB*^{dgc} mice and either control (*Ctrl*) or *Fshb*$^{-/-}$ *HSFHB*^{WT} mice; $P > 0.05$; pituitaries were obtained from 5 mice and each sample was tested in triplicate. Expression of the following genes was measured by qPCR assays - *Cga*: α-glycoprotein hormone, *Lhb*: luteinizing hormone β, *Gnrhr*: gonadotropin-releasing hormone receptor, *Acvr2*: activin receptor 2, Nr5a1: nuclear receptor subfamily 5, group A, member 1, also known as steroidogenic factor1, *Esr1*: estrogen receptor1 or α

Supplementary Fig. S3

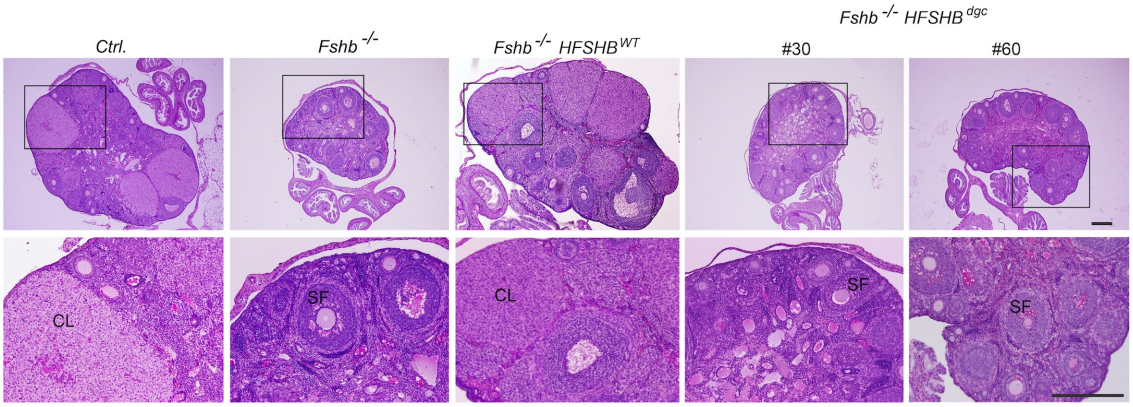

Fig. S3. The phenotypes described in Fig. 5 were not because of delayed reproductive maturation, because even at 6 months of age, there was no indication of either estrus cycles or a change in ovarian histology (top and enlarged panels at the bottom), which was similar to that observed at 6 weeks of age in *Fshb*$^{-/-}$ *HSFHB*^{dgc} mice. The bar represents 200 μm.