Compromised mitochondrial function in cortical bone osteocytes of long-lived growth hormone receptor null mice

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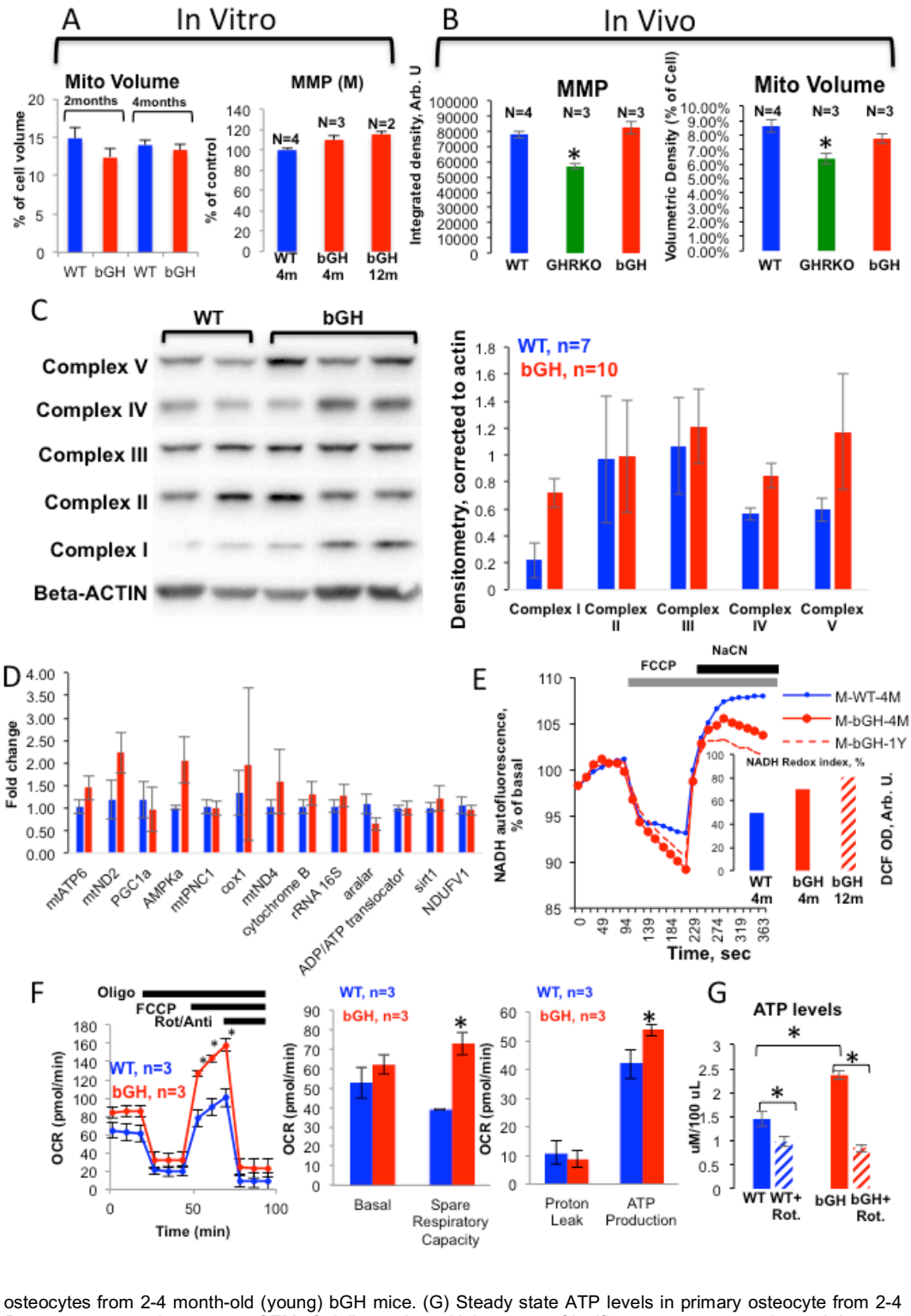
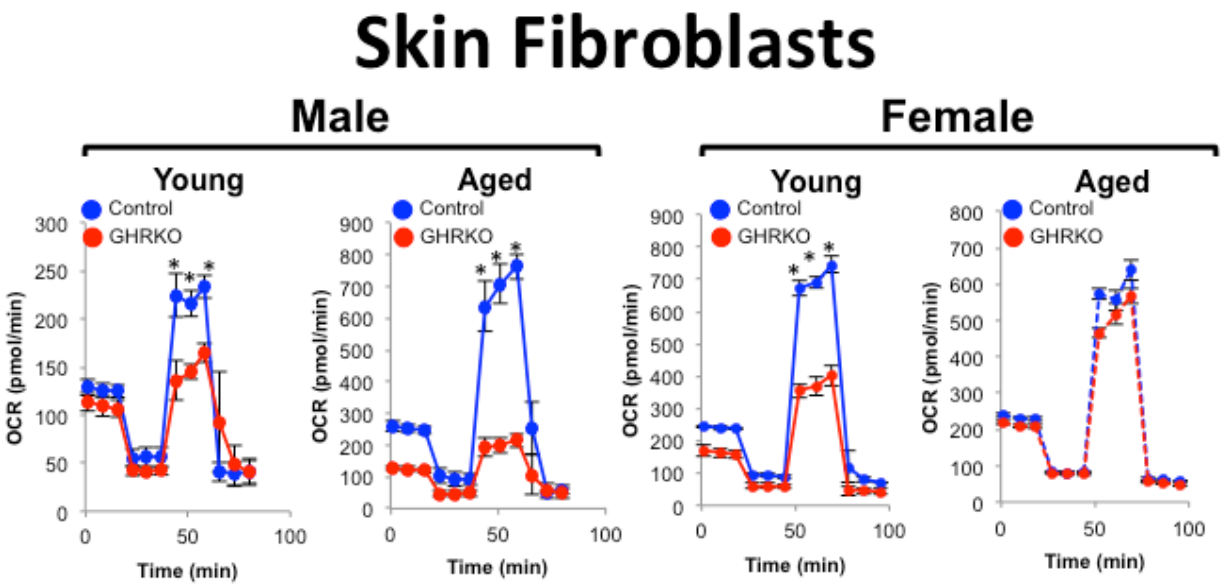
Supplementary information

SUPPLEMENTARY FIGURES:



Supplementary Figure 1: Mitochondrial function in cortical osteocytes from bGH mice. (A) Primary osteocytes from male mice were seeded (0.4×10^6 cells/ml) on collagen-coated glass plates and visualized using the 2000E Nikon Microscope Eclipse TE microscope at 60× magnification. Osteocyte mitochondrial volume/cell volume was determined from 3-dimensional images of z-stack volumes reconstructed using the Amira FEI software. Live cells were labeled with Calcein AM and mitochondria were labeled with tetramethylrhodamine, ethyl ester (TMRE) (n=20 cells/genotype/age). Osteocyte MMP was calculated from peak tetramethylrhodamine, ethyl ester (TMRE) intensity (n=20 cells/genotype/age). Data are presented as mean±SEM, and significance was accepted at $p<0.05$. (B) $\Delta\Psi_m$ was estimated in vivo using MPM from the integrated intensity density of TMRM injected mice for >20 osteocytes per mouse. "Active mitochondrial fraction" was calculated from the volumetric density for >20 osteocytes per mouse. Data are presented as mean ± SEM, and significance was accepted at $p<0.05$. (C) Protein levels of electron transport chain complexes (I-V) were determined from cell lysates of primary osteocytes by western immunoblotting using the Total OXPHOS cocktail. (D) Mitochondria-specific gene expression in primary osteocytes was determined using real-time polymerase chain reaction. (E) Cells were seeded (0.4×10^6 cells/ml) on collagen-coated glass plates and visualized using the 2000E Nikon Microscope Eclipse TE microscope at 20× magnification. Time lapse of NADH autofluorescence in osteocytes from male bGH mice (insert; NADH redox index). (F) Mitochondrial respiration in primary osteocytes from 2-4 month-old (young) bGH mice. (G) Steady state ATP levels in primary osteocyte from 2-4 month-old bGH mice W/O rotenone. Data are presented as mean ± SEM of triplicates in multiple assays. Significance was accepted at $p<0.05$.



Supplementary Figure 2: Mitochondrial respiration in primary fibroblast cultures from GHRKO and control mice. Primary fibroblasts were seeded (0.4 × 10^6 cells/ml) in triplicate. Oxygen consumption rate (OCR) was determined at basal conditions and upon the addition of oligomycin (1 μM), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) (2 μM), and rotenone/antimycin (0.5 μM). Data were normalized to cell number determined at the end of the assay. Data were calculated according to the manufacturer's algorithm and presented as mean±SEM of triplicate fibroblast cultures from 2-month-old (young) or 2-year-old (aged) male and female mice in multiple assays. Significance was accepted at $p<0.05$.

SUPPLEMENTARY TABLE 1: Sequences of primers used for the quantitative polymerase chain reaction

Gene name	Forward (5' – 3')	Reverse (5' – 3')
DMP-1	GAAGAGAGGACGGGTGATTTG	CTGATGACTCACTGTTCGTGG
MEPE	GCCAGAGGGCCTCATGAAGAT	CTTCATTCGGCATTGGTGCCG
PHEX	GCTCATGTAAGGTGCAATTCC	GGCACCATTGACCCTAAATTG
FGF23	AGACCATCTACAGTGCCCTGA	GGCTGAAGTGAAGCGATCCAA
SOST	GACGCCAAAGATGTGTCCGAG	GTCTGTCAGGAAGCGGGTGTA
COX1	GGCATTGCACATCCATCCAC	GCGCATGAGTACTTCTCGGA
COX2	TTCAAAAGAAGTGCTGGAAAAGGT	GATCATCTCTACCTGAGTGTCTTT
RANKL	GCTCCGAGCTGGTGAAGAAA	CCCCAAAGTACGTCGCATCT
mt-ATP6	AATTACAGGCTTCCGACACAAAC	TGGAATTAGTGAAATTGGAGTTCCT
mt-PNC1	TACAGGTCGTTCGTGCAGAC	TGTCACTAAGCACGCTCTCC
mt-ND2	GGGCATGAGGAGGACTTAACCAAAC	TGAGGTTGAGTAGAGTGAGGGATGG
mt-ND4	CAGACCCCCTATCCACACCA	ATCCCTGCGTTTAGGCGTTC
GP38	AGATGGCTTGCCAGTAGTCAC	GCTCTTTAGGGCGAGAACCTT
rRNA 16S	TGGGGTGACCTCGGAGAATAA	GAAACCGACCTGGATTGCTC
Cytochrome B	TGTTCGCAGTCATAGCCACA	TGGGATGGCTGATAGGAGGT
Aralar	GAGGTGGACGGGGAGCATTA	CACCTCTCCATTTCCGCTCT
Sirt 1	GTAATGTGAGGAGTCAGCAC	TTGGACATTACCACGTCTGC
ANT	AGACTGCGTGGTTCGTATCC	TGTCCACACCACCCAGAAAG
Nrf2	CTCAGCATGATGGACTTGGAG	CACTTCTCGACTTACTCCAAGAT
SOD	CTGGCCAAGGGAGATGTTACA	GTCACGCTTGATAGCCTCCAG
Gpx	GTGGTGCTCGGTTTCCCGTGC	CCCGCCACCAGGTCGGACGTA
Cat2	GCTGAGAAGCCTAAGAACGC	GTCTCCTCAGCGGAGGCTGA
GLUT-1	TGTGCTCATGACCATCGC	AAGGCCACAAAGCCAAAGAT

SUPPLEMENTARY TABLE 2: Antibodies

Antibody	Company (Cat. #)	Dilution for western blot
Mito complex kit (total OXPHOS)	Abcam (ab110413)	1:250
LC3A/B	Cell Signaling (12741)	1:1000
α-Tubulin	Sigma (T6199)	1:5000
β-Actin	Cell Signaling (4970)	1:2000
GLUT-1	Cell Signaling (12939)	1:1000
GLUT-4	Biogenesis (4670-1704)	1:750
AMPKa	Cell Signaling (5831)	1:1000
AMPKb1/2	Cell Signaling (4150)	1:1000
Phosphorylated AMPKa	Cell Signaling (2535)	1:1000
Phosphorylated AMPKb	Cell Signaling (4186)	1:1000
Cleaved caspase-3 (CC3)	Cell Signaling (9664)	1:750

SUPPLEMENT METHOD:

Primary skin fibroblast cultures: Two cm of a mouse tail was collected at sacrifice, washed 2 times with 70% ethanol and rinsed with PBS. Tails were then transferred to Petri dishes (Falcon, 35-1007), minced to <1mm with sterile scalpel and incubated with collagenase (400 U/ml, Cat# 17101-015, Gibco) in DMEM (Cat# 11965-092, Gibco) for 20-24 hours at 37 ℃, 5% CO_2. The collagenase suspension of the treated tails was passed through cell strainer cap (Cat#352235, Falcon, Corning, NY) to remove cell clumps and centrifuged 5 minutes at 200 g to collect cells. Cell pellets were resuspended in 3ml DMEM medium supplied with 10% FCS (Cat# 26140, ThermoFisher), plated on T75 flasks and incubated at 37 ℃, 5% CO_2.