Supplementary

Postmenopausal osteoporosis is a musculoskeletal disease with a common genetic trait which responds to strength training: a translational intervention study

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Supplementary Methods

Microarray and data analyses

For mRNA expression profiling, 100 ng of total RNA was subjected to a cDNA synthesis and labeling kit (GeneChip HT One-Cycle; and GeneChip HT IVT), according to the manufacturer's protocol for whole-genome gene expression analysis (Affymetrix, Santa Clara, CA, USA). Labeled and fragmented single stranded cRNAs were hybridized to GeneChip Human Gene 1.0 ST Array (Affymetrix) covering 36,000 RefSeq transcripts, including 21,000 well-characterized human genes.

For mature miRNA expression profiling, 300 ng of total RNA was used for biotin labelling of miRNA by the Genisphere FlashTag HSR kit (Genisphere, Hatfield, PA), following the manufacturer's protocol. Labelled miRNAs were hybridized to the GeneChip miRNA 2.0 Array (Affymetrix).

Both array types were washed and stained using FS-450 fluidics station (Affymetrix). Signal intensities were detected by Hewlett Packard Gene Array Scanner 3000 7G (Hewlett Packard, Palo Alto, CA, USA). The scanned images were processed using AGCC (AffymetrixGeneChip Command Console) software. For gene expression analysis, the Affymetrix CEL files (containing probe intensities) were imported into Partek Genomics Suite software (Partek, Inc. MO, USA) for statistical analysis. Robust microarray analysis (RMA) yielding normalized log2 transformed signal intensities was applied for normalization (http://bip.weizmann.ac.il/toolbox/overview/Partek_Users_Guide.pdf). Gene transcripts with
maximal signal values of less than 5 (Log2 values) across all arrays were removed to filter for low and non-expressed genes, reducing the number of transcripts to 18811 transcripts. Differentially expressed transcripts between groups were identified using two-way ANOVA as implemented in Partek Genomics Suite.

Further bioinformatics analysis was conducted on the significant genes to identify functional implicatives by means of Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, CA, USA). Briefly, the data set containing gene identifiers and corresponding fold changes and p-values were uploaded into the web-delivered application and each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). The functional analysis identified the biological functions and/or diseases that were most significant to the data sets. Fisher’s exact test was performed to calculate a p value determining the probability that each biological function and/or disease assigned to the data set was due to chance alone. The data set was mined for significant pathways with the IPA library of canonical pathways and networks were generated by using IPA as graphical representations of the molecular relationships between genes and gene products.

**Immunohistochemistry**

Transverse sections of 8 μm thickness were cut at -22 °C in a microtome (CM3050, Leica Microsystems, Nussloch, Germany) and mounted on slides, air-dried over night before storage at -80 °C until further analyses. To quantify fiber type and fiber cross sectional area, sections was blocked for 30 min in room temperature with 1% BSA (bovine serum albumin; A4503, Sigma Aldrich, St. Louis, MO, USA) in 0,01 M PBS (P4417, Sigma Aldrich) with 0,05% Tween20 (P5927, Sigma Aldrich). Thereafter sections were incubated in primary antibodies against dystrophin (ab15277, Abcam, Cambridge, UK) and myosin heavy chain II (SC71, gift from Prof. S. Schiaffino), diluted in the blocking buffer (4 hours in room...
temperature). Thereafter sections were incubated in an appropriate secondary antibody solution (A11001 or A11012, Molecular Probes, Eugene, OR, USA). Muscle sections were then covered with coverslips and mounted with ProLong Gold Antifade Reagent with DAPI (P36935, Molecular Probes). Sections was washed 3 x 5 min in PBS-t between stages. Digital micrographs were obtained using a high-resolution camera (DP72, Olympus Corp., Tokyo, Japan) mounted on a microscope (BX61, Olympus Corp.) with a fluorescence light source (X-cite, 120PCQ, EXFO Photonic Solutions Inc., Mississauga, ON, Canada). Fiber type composition and cross sectional area was analyzed using TEMA software (Checkvision, Hadsund, Denmark). To visualise satellite cells, sections were analysed for immunoreactivity against NCAM/CD56 (monoclonal ab, ab9018, Abcam; 1:200) and laminin (rabbit polyclonal ab, Z0097, Dako; 1:500). AlexaFluor 488 and Alexa Fluor 594 (goat anti-mouse and goat anti-rabbit; Invitrogen-Molecular Probes, Eugene, Oregon, USA) were used as secondary antibodies. The sections were finally counterstained with DAPI (for nuclear staining) and mounted under coverslips (ProLong Gold Antifade Reagent with DAPI, P36935, Invitrogen-Molecular Probes). The number of satellite cells was counted as ring-like NCAM staining encircling at least 2/3 of a nucleus (DAPI staining) located inside the laminin staining and related to either fiber type I or II. A myonucleus was counted when 2/3 of the DAPI staining was located inside the dystrophin staining, and related to either fiber type I or II. As an indicator myonuclear domain muscle fiber CSA was divided on the number of myonuclei per fiber.

**Western blotting**

Total protein was extracted from muscle samples using a commercially available kit (T-PER® Tissue Protein Extraction Reagent, cat.no.78510, Thermo scientific, USA) according to the manufacturer’s procedures. Furthermore, 2% protease and phosphatase inhibitor
cocktail (Halt™ Protease and Phosphatase Inhibitor Cocktail, cat.no.78440, Thermo Scientific, USA) and 2% EDTA was added to the lysate dilution according to the manufacturer’s procedures. Since the above described method discards the cytoskeleton fraction of the homogenate, a second muscle sample was fractionated into a cytosol-, membrane-, cytoskeletal-, and nuclear fraction using a commercial available fractionation kit (ProteoExtract Subcellular Proteo Extraction Kit, Cat.no.539790, Calbiochem, EMD Biosciences, Germany) according to the manufacturer’s procedures. Protein concentrations were determined using a commercial kit (BioRad DC protein micro plate assay, Cat.no.0113, Cat.no.0114, Cat.no.0115, Bio-Rad, CA, USA), a filter photometer (Expert 96, ASYS Hitech, UK) and the provided software (Kim, ver. 5.45.0.1, Daniel Kittrich). γ-globulin was used as standard protein, ranging from 0.125 to 1.5 mg per ml. Protein standard curve and samples were analyzed in triplicates; standard curve: r²>0.9. Equal amount of protein (10-40 μg per well, depending on the fraction loaded) were loaded and separated by precast NuPAGE Novex 4-12% Bis-tris Midi gels (Cat. No NP0321, Invitrogen, USA) for 35-45 minutes at 200 volts in cold MES running buffer (NuPAGE MES SDS running buffer, cat. No. NP0002, Life technologies, Invitrogen, USA). All subcellular fractions and time points were routinely loaded on the same gel, to enable comparison. Separated proteins were transferred on to immune-blot PVDF membrane (Immuno-blot, Cat.no.162-0177, Bio-Rad, CA, USA), at 30 volts for 90 min in cold transfer buffer (NuPAGE transfer buffer, Cat.no.NP0006-1, Life technologies, USA). Whereas membranes used for HSP 70 and alpha B-crystallin were blocked over night at 4⁰C in a 5% fat free skimmed milk and 0.05% TBS-t solution (TBS, Cat.no.170-6435, Bio-Rad, CA, USA; Tween 20, Cat.no.437082Q, VWR International, PA, USA; Skim milk, Cat.no.1.15363, Merck, Germany). Membranes used for HSP60, COX IV, CS, and ubiquitination were blocked at room temperature (RT) for 2 hours. Blocked membranes were then incubated with monoclonal primary antibodies for 2 hours at RT, or
overnight at 4⁰C. Thereafter, membranes were incubated with secondary antibody (goat anti-
mouse, Cat.no.31430, Thermo Scientific/Pierce Biotechnology, IL, USA) diluted 1:30 000 or
1:2000 (anti-rabbit IgG, Cat.no. 7074, Cell Signaling) at RT for 1 hour. All antibodies were
diluted in a 1% fat free skimmed milk and 0.05% TBS-t solution. Between stages the
membranes were washed with 0.05% TBS-t. Protein bands were visualized, with HRP-
detection system (Super Signal West Dura Extended Duration Substrate, Cat.no.34076,
Thermo Scientific/Pierce Biotechnology, IL, USA). Chemiluminescence was measured using
a CCD image sensor (Kodak image station 2000R, Eastman Kodak Company, Rochester, NY,
USA) and band intensity was calculated using the Carestream molecular imaging software (v.
5.0.7.2.2, Carestream Health, New Haven, CT, USA). All samples were analyzed in
duplicates, and mean values were used for statistical analysis.
Supplementary Tables

Table S1. Cohorts of muscle donors

| Group         | Number of women | Bone status  | Intervention by heavy load strength training | Biopsy type |
|---------------|-----------------|--------------|---------------------------------------------|-------------|
| A Healthy I   | 18              | Normal BMD   | Yes                                        | Thigh muscle|
| B Osteoporotic I | 17             | Osteoporotic | Yes                                        | Thigh muscle|
| C Healthy II  | 12              | Normal BMD   | No                                         | Pelvic muscle|
| D Osteoporotic II | 12             | Osteoporotic | No                                         | Pelvic muscle|

Groups B, C and D were also donors of iliac bone biopsies and were part of total 84 donors with greatly varying BMD.

Table S2. Serum and urine biomarkers of all healthy and patients donors of iliac bone

|                     | Healthy (SD) | Osteoporotic (SD) | p-value |
|---------------------|--------------|--------------------|---------|
| Serum Vitamin K (ug/l) | 0.52 (0.25) | 0.452 (0.24)       | 0.4910  |
| Serum P-PTH (pmol/l)  | 4.05 (1.74)  | 5.13 (2.60)        | 0.0526  |
| Serum-Ca\(^{2+}\) corr. (mmol/l) | 1.24 (0.04) | 1.24 (0.04)       | 0.8180  |
| Serum-25(OH)\(_{2}\)D\(_{3}\) (nmol/l) | 80.71 (37.83) | 89.04 (45.04) | 0.4310  |
| Serum BGLAP (nmol/l)  | 1.30 (0.50)  | 1.58 (0.61)        | 0.0551  |
| Serum bone-spec ALP (u/l) | 3.63 (0.98) | 4.69 (2.17)       | 0.0099  |
| Urinary-NTx (mmol/l)  | 20.53 (9.75) | 28.85 (9.75)       | 0.0006  |
| Serum-1CTP (CTx) (ug/l) | 7.04 (2.58)  | 7.74 (2.34)        | 0.2840  |
| Urinary DPD (mM DPD/mM Cr) | 51.89 (23.65) | 75.16 (48.41) | 0.0155  |
| Serum-phosphate (mmol/l) | 1.14 (0.20)  | 1.18 (0.15)        | 0.3540  |

Parentheses show ±SD; PTH: parathyroid hormone; BGLAP: bone gla protein (ostecalcin); ALP: alkaline phosphatase; NTx: N-Terminal Telopeptide of Type I Collagen; 1CTP: Type I collagen degradation product; DPD: free deoxypyridinoline p-values were calculated using students t-test. The cohorts were pooled because their biomarkers were similar.
Table S3. Q-RT-PCR validation of Affymetrix data

| Gene Symbol | Affymetrix p-value | Fold change | qPCR p-value | Fold change |
|-------------|--------------------|-------------|--------------|-------------|
| ACTN3       | 0.0002             | -1.7        | 0.0059       | -1.9        |
| TUBA4A      | 0.0002             | -1.4        | 0.0296       | -1.2        |
| COL3A1      | 0.0087             | 1.5         | 0.0058       | 2.4         |
| FRZB        | 0.0136             | 1.5         | 0.0001       | 2.3         |
| IGF2        | 0.0008             | 1.3         | 0.0472       | 1.4         |

Table S4. Mean demographic characteristics of healthy and OP postmenopausal women donating iliac bone and pelvic muscle biopsies

|                      | Healthy (n=12) Mean (SD) | Osteoporotic (n=12) Mean (SD) | p-value |
|----------------------|--------------------------|--------------------------------|---------|
| Age (years)          | 67.5 (11.3)              | 67.3 (10.77)                   | 0.961   |
| Body mass (kg)       | 69.44 (9.56)             | 67.51 (15.29)                  | 0.726   |
| BMI (weight/height^2)| 25.1 (2.6)               | 24.9 (4.63)                    | 0.870   |
| Femoral neck (T-score) | -0.53 (1.17)         | -2.19 (1.24)                    | 0.004   |
| L1-L4 BMD (T-score)  | -0.45 (1.74)             | -2.71 (2.16)                    | 0.013   |
| Total body BMD (T-score) | -0.10 (1.62)       | -2.4 (1.64)                     | 0.005   |
| Lean mass (kg)       | 41.10 (4.29)             | 39.99 (5.44)                    | 0.617   |
| Fat mass (kg)        | 24.39 (6.17)             | 23.09 (9.28)                    | 0.717   |

p-values were calculated using students t-test.

Table S5. Muscle morphology parameters in osteoporotic and healthy postmenopausal women before the training intervention (untrained state)

|                      | Type I fibers | Type II fibers |
|----------------------|---------------|---------------|
|                      | Osteoporotic Mean (SD) | Healthy Mean (SD) | p-value | Osteoporotic Mean (SD) | Healthy Mean (SD) | p-value |
| Fiber type distribution (%) | 51 (12) | 43 (18) | <0.01 | 49 (12) | 57 (18) | <0.01 |
| Fiber cross sectional area (μm^2) | 4046 (862) | 4295 (1234) | 0.60 | 2729 (801) | 2948 (478) | 0.50 |
| Number of nuclei per fiber | 1.9 (0.4) | 2.2 (0.2) | 0.17 | 1.8 (0.3) | 2.0 (0.2) | 0.12 |
| Myonuclear domain | 2162 (606) | 1968 (366) | 0.43 | 1523 (431) | 1476 (290) | 0.79 |
| Number of satellite cells per fiber | 0.04 (0.02) | 0.04 (0.02) | 0.91 | 0.02 (0.02) | 0.02 (0.01) | 0.91 |

Type II fibers are Type IIA and IIX fibers. Differences were evaluated by students T-test.
Table S6. The eighteen most significantly (p<0.01) regulated microRNAs after strength training in osteoporotic women

| Probeset ID         | p-value | Fold-change (after vs. before) |
|---------------------|---------|--------------------------------|
| hsa-mir-378         | 0.0004  | -1.15                          |
| hsa-mir-21          | 0.0011  | 1.92                           |
| hsa-mir-660         | 0.0019  | 1.46                           |
| hsa-mir-29c         | 0.0035  | -1.30                          |
| hsa-mir-23b         | 0.0036  | 1.13                           |
| hsa-mir-500         | 0.0054  | 1.41                           |
| hsa-mir-99b         | 0.0055  | 1.18                           |
| hsa-mir-23a         | 0.0056  | 1.24                           |
| hsa-mir-532         | 0.0056  | 1.40                           |
| hsa-mir-339         | 0.0058  | -1.17                          |
| hsa-mir-130b        | 0.0058  | 1.27                           |
| hsa-mir-432         | 0.0064  | 2.00                           |
| hsa-miR-652         | 0.0067  | -1.26                          |
| hsa-miR-532-3p      | 0.0072  | 1.41                           |
| hsa-miR-362-5p      | 0.0075  | 1.33                           |
| hsa-miR-501-3p      | 0.0075  | 1.37                           |
| hsa-miR-140-3p      | 0.0078  | -1.13                          |
| hsa-miR-378c        | 0.0088  | -1.10                          |

miRNAs with mean signal values < 32 both before and after training were filtered out. P-values were calculated by repeated measures ANOVA for osteoporosis patients before and after exercise intervention.
Table S7. Ingenuity Pathway Analysis of affected muscle mature miRNAs or mRNAs in patients after intervention

| Top Networks - Physiological System Development and Function | P-value  | molecules |
|------------------------------------------------------------|----------|-----------|
| 1 Organismal Development                                    | 3.6E-02 – 2.0E-10 | 27        |
| 2 Cardiovascular System Development and Function            | 3.3E-02 – 5.4E-05 | 17        |
| 3 Organ Morphology                                           | 3.6E-02 – 5.4E-05 | 13        |
| 4 Skeletal and Muscular System Development and Function      | 3.6E-02 – 5.4E-05 | 15        |
| 5 Tissue Morphology                                          | 3.6E-02 – 5.4E-05 | 18        |

All muscle mRNAs and miRNAs that were changed upon intervention (q<0.1) were included.

Table S8. Nine skeletal muscle genes differentially expressed in patients after strength training and strongly associated with BMD compared to experimental data

| Affymetrix ID | Genes in dataset | Effect on bone formation as predicted from global bone transcriptional profiling | Fold change in muscle upon heavy load strength training in patients | Effect on bone formation as predicted from the literature (References) |
|---------------|------------------|--------------------------------------------------------------------------------|------------------------------------------------------------------|------------------------------------------------------------------|
| 8057506       | FRZB             | Decreased                                                                      | 1.438                                                            | Decreased(1)                                                      |
| 8115327       | SPARC            | Increased                                                                      | 1.416                                                            | Increased(2)                                                     |
| 7937772       | IGF2             | Increased                                                                      | 1.298                                                            | Increased(3)                                                     |
| 7965410       | DCN              | Increased                                                                      | 1.294                                                            | Increased(4)                                                     |
| 7961514       | MGP              | Increased                                                                      | 1.282                                                            | Increased(5)                                                     |
| 7965873       | IGF1             | Increased                                                                      | 1.267                                                            | Increased(3, 6-9)                                                |
| 7936673       | RGS10            | Decreased                                                                      | 1.261                                                            | Decreased(10)                                                   |
| 7919815       | CTSK             | Decreased                                                                      | 1.234                                                            | Decreased(11)                                                   |
| 8103922       | CASP3            | Increased                                                                      | 1.205                                                            | Increased(12)                                                   |

Predicted effects on bone formation from global bone transcriptional profiling(13) (column 3) as compared to experimentally documented results from the literature (column 5).
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