

Electronic supplementary material - methods

Human myocytes

AntagomiR experiment

For antagomiR transfection targeting miR-15b and miR-16, satellite cells were isolated from vastus lateralis muscle biopsies from five healthy donors (three males and two females) aged 59 ± 7 as previously described (1, 11). Proliferating myoblasts were seeded into 6-well plates. At day 3 with differentiation media (DMEM, high glucose, 2% horse serum) (Invitrogen), cells were transfected using Lipofectamine 2000 (Invitrogen) with 10 nmol/l antagomiRs (Exiqon, Vedbæk, Denmark) targeting miR-15b/miR-16 or with a non-targeting antagomiR (Exiqon). The simultaneous knockdown of miR-15b and miR-16 was performed to hinder a possible rescue effect that could occur when inhibiting them separately. An untreated sample and a Lipofectamine 2000 only sample were included as additional controls. Cells were incubated with the transfection reagents for 48 hours. Cells were harvested after 5-10 minutes incubation with 0, 1, 10, or 100 nmol/l insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark) in Trizol (Invitrogen) for RNA isolation or in lysis buffer (Cell signaling technology, Beverly, MA, USA) for protein isolation.

Short-term insulin experiment

Satellite cells were isolated from vastus lateralis muscle biopsies from five healthy donors (three males and two females) aged 59 ± 7 as previously described (1, 11). 10 nmol/l insulin (Actrapid, Novo Nordisk, Bagsværd, Denmark) was added to differentiated myocytes, when myotubes were formed (day 5 with low glucose (5.6 nmol/l) differentiation media) and cells were harvested in Trisol (Invitrogen, Carlsbad, CA, USA) for RNA isolation following 5, 10, 30, 60 or 120 minutes of incubation.

Rat protocol

Female Wistar rats were bred locally at a designated animal unit of the University of Cambridge (Cambridge, UK). Adult females weighing between 235 and 250 grams were fed *ad libitum* either a control diet (20% protein) n=11 or an isocaloric low protein (LP) (8% protein) diet n=11 (Arie Blok, Woerden, Netherlands) during gestation and lactation as described previously (28). Three days after birth, litter sizes were randomly standardized to four males and four females. At 21 days of age, male offspring were weaned onto a standard rat diet (LAD1; Special Diet Services, Witham,

UK). At three months of age, 22 males, one from each of the eleven control litters and one from each of the eleven LP litters was killed by a rising concentration of carbon dioxide. Vastus lateralis muscle biopsies were excised and snap frozen in liquid nitrogen before storing at -80°C for later use.

RNA isolation

Total RNA from human muscle biopsies were extracted with TRI reagent (Sigma-Aldrich, St. Louis, MO), from human cell cultures with Trizol (Invitrogen) and from rat muscle biopsies and L6 cells with mirVana total RNA isolation kit (Ambion, Applied Biosystems, Warrington, UK), all according to the manufacturers protocols.

LNA Arrays

For each of the discordant twins (n=22), 1 µg total RNA was labeled with Hy3 using the miRCURY labeling kit according to the manufacturer's instructions and hybridized to a miRCURY LNA miRNA array (Exiqon, Vedbaek, Denmark) representing version 9.2 of the Sanger miRBase using the HS-400-Pro microarray hybridization station (Tecan, Grödig, Austria). The 22 arrays were scanned using the ArrayWoRx white-light CCD-based scanner (Applied Precision, Issaquah, WA) and ImaGene 8.0 (BioDiscovery, El Segundo, CA) was used for image analysis including flagging of bad quality spots. The resulting text-files were normalized using normexp (20) and quantile normalization (21) in R (version 2.9.1). Updated annotation files representing miRBase version 14 were used and the 571 human miRNAs on the array were used in the downstream analysis.

Gene expression

ABI-PRISM 7900 Sequence Detection system (Applied Biosystems, Foster City, CA, USA (ABI)) was used for both miRNA and mRNA detection.

Human muscle biopsies – miRNA quantification; 1 µg of total RNA was reverse transcribed using Megaplex™ primer pools A v2.1. The product was loaded in duplicates and quantitative real-time PCR (qRT-PCR) was performed with TaqMan® miRNA assays for miR-15b, miR-16, miR-185, miR-494 and RNU48. The geometric mean of the three small non-coding reference RNAs (miR-185, miR-494 and RNU48) was used to normalize the expression of miR-15b and miR-16. miR-185 and miR-494 was chosen based on stability calculations (39). The SLqPCR package in R was used

to rank miRNAs from population 1 (n=22), and 16 additional skeletal muscle miRNA expression profiles from our laboratory, based on their stability. RNU48 is recommended as an internal control by ABI.

Human muscle biopsies – mRNA quantification; mRNA levels were quantified according to the manufacturer's directions using probes and primers obtained from ABI (Assay-on-demand). The following probes were used, *INSR* (Hs00169631_m1; ABI), *IRS-1* (Hs00178563_m1; ABI), *PI3KR1* (Hs00933163_m1; ABI) and Cyclophilin A (Hs99999904_m1; ABI). Cyclophilin A was used for normalization of the transcript quantities.

Cell culture – mRNA quantification; 0.5 µg of total RNA was reverse-transcribed using cDNA high capacity kit (ABI) according to the manufacturer's protocol. cDNA samples were loaded in triplicates and qRT-PCR was performed with primers designed using Roche Universal probe library (Roche), *INSR* forward primer: 5'-gctggattattgctcaaagg, *INSR* reverse primer: 5'-tgagaattctcagactcgaatgg, *PI3KR1* forward primer: 5'-ggaatgttcaactctatacagaacaca, *PI3KR1* reverse primer: 5'- agtccacggagggtgtatca. 18S rRNA was used as endogenous control (ABI). The comparative delta CT method was used to calculate relative abundance of miRNA or mRNA levels between samples.

Rat muscle biopsies and L6 cells; TaqMan® individual miRNA assays for miR-15b, miR-16 and miR-185 were used as well as assays for Peroxisome proliferator-activated receptor (PPAR)-γ coactivator 1 alpha (*PGC-1a*) and Cytochrome c, somatic (*CYTC*). The expression of mir-15b and mir-16 were then normalized to the geometric mean of the housekeeping genes miR-185, *PGC-1a* and *CYTS* (none of which were influenced by maternal diet, insulin or glucose).

Protein expression

Muscle cell cultures - Meso-Scale discovery

Cell samples were harvested in protein lysis buffer (Cell signaling technology, Danvers, MA, USA). Phosphatase inhibitor cocktail 1 and 2 (Sigma Aldrich) and protease inhibitor complete mini (Roche) was added fresh to the buffer. Protein lysates were centrifuged at maximum speed for 1 h at 4°C, and the pellet was discarded. Protein concentration was measured using a colorimetric protein assay (Bio-Rad, Laboratories, Richmond, CA, USA.). *INSR* levels were measured according to the

manufacturer's protocol (Meso Scale Discovery, Rockville, USA). Briefly, 25 μ l protein lysate with a protein concentration of 0.46 μ g/ μ l was added to the wells of meso-scale discovery 96-well plates pre-coated with INSR antibody. Following standard ELISA incubation and washing procedures, INSR was detected by adding a detection antibody for INSR. The signal was captured using SI2400 Meso-scale discovery.

Muscle cell cultures - western blotting

In short, 6 μ g of skeletal muscle protein was separated on 10% BIS-TRIS gels and blotted to nitrocellulose membranes which were incubated overnight with primary antibodies against PIK3R1 (06-195, Millipore, Billerica, MA), phospho-Akt (Ser473) (#9271, Cell Signaling, Danvers, MA) or Akt (#9272, Cell Signaling). Membranes were stripped (Restore Western Blot Stripping Buffer, Pierce, Rockford, IL) between phospho-Akt and total Akt antibodies. The signal was detected with LumiGLO reagent (Cell Signaling), and the staining was visualized with a LAS-3000 Image-reader (Fujifilm, Tokyo, Japan). MultiGauge V2.0 software (Fujifilm) was used for the quantification. All membranes were subjected to Ponceau S staining (Sigma, St. Louis, MO) after blotting to validate equal loading. Protein content was expressed in arbitrary units relative to a standard (pool of 30 samples) loaded in duplicate on all gels. The intra-assay variation (calculated from the two standards on each gel) was below 10% for all antibodies.

Muscle biopsies – western blotting

Lysate was prepared as previously described (40). Protein content in the lysate was measured in a microtiter based assay using the bicinchoninic acid method (Pierce Chem. Comp., Ill, USA). Muscle content of various proteins was determined by SDS-PAGE and western blotting ("Criterion-gel system", BioRad, Denmark). Muscle lysate proteins were separated using 26 wells 7.5% or 10% Bis-tris gels (BioRad, Denmark), and transferred (semi-dry) to PVDF-membranes (Immobilion Transfer Membrane, Millipore A/S, Denmark). After blocking, the membranes were incubated with primary antibodies followed by incubation in horse radish peroxidase (HRP)-conjugated secondary antibody. Following detection with ECL+ (Amersham Pharmacia Biotech Limited, UK) and quantification using a CCD-image sensor and 1D software (Kodak Image Station, E440CF, Kodak, Rochester, NY), the protein content was finally expressed in arbitrary units relative to a skeletal muscle standard. The primary antibodies used were: anti-IRS-1 (#06-248), anti-PIK3R1 (#06-497) (from Upstate Biotechnology Inc (UBI)), MA). The INSR monoclonal CT3 antibody was raised

against the COOH-terminal of the INSR β -subunit and was a gift from Dr. Ken Siddle (Cambridge University, Cambridge, UK). The secondary antibodies used were: Goat-anti-rabbit HRP (P0448) and goat-anti-mouse HRP (P0447) (DAKO, Glostrup, DK).

All blots were performed using over night blocking (TBST+1% skim milk), followed by incubation in the appropriate primary antibody for 2 hours at RT (in TBST+1% skim milk). Analysis of assay linearity between signal and amount of protein loaded was verified for each of the antibodies used.