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Supplemental Information

Serpina3c Regulates Adipogenesis by Modulating Insulin Growth Factor 1 and Integrin Signaling

Yoonjeong Choi, Hyeonjin Choi, Bo Kyung Yoon, Hyemin Lee, Jo Woon Seok, Hyo Jung Kim, and Jae-woo Kim

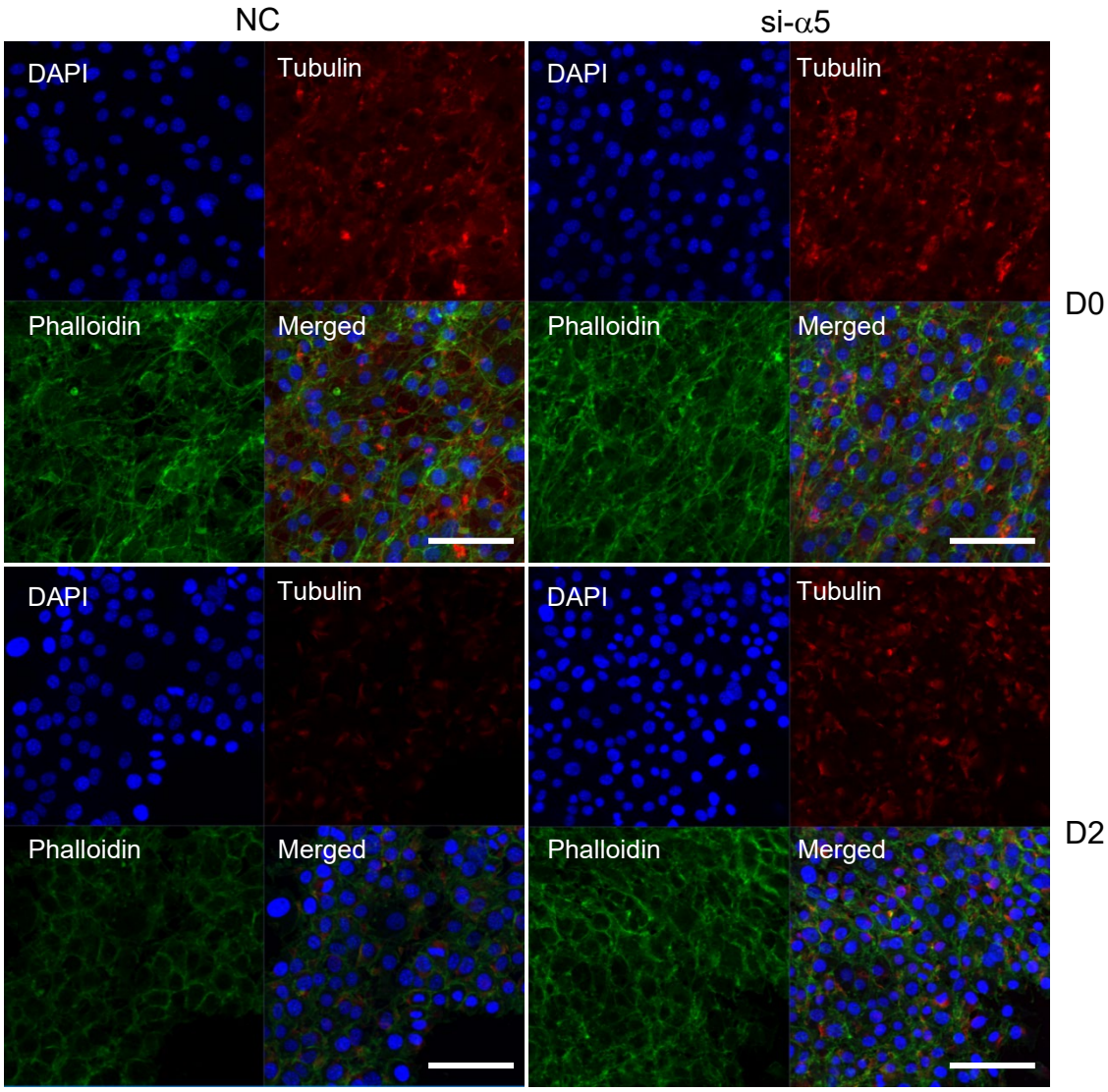

Figure S1. Integrin α5 knockdown does not change the cellular structure during early adipogenesis. Cells transfected with NC or si-Itga5 were induced to differentiate with MDI and fixed for immunocytochemistry against phalloidin or tubulin 48 h later. Scale bar = 50 μm.

Transparent Methods

Animals. C57BL/6J male mice were maintained under a 12 h light/12 h dark cycle. A five mouse per cage maximum was followed. Five-wk-old mice were fed a high fat diet (Research Diets) or a normal chow diet (Dyets) for up to 8 or 16 wk. The composition of the HFD we used was 60 kcal% fat. All procedures were approved by the Committee on Animal Investigations of Yonsei University. All animal protocols were performed according to the National Institutes of Health guidelines and approved by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Cell culture and *in vitro* differentiation. 3T3-L1 preadipocytes were maintained at 37°C in Dulbecco's modified Eagle's medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, 8 μg/ml biotin, and 10% heat-inactivated calf serum at 37 °C in an atmosphere of 90% air and 10% CO2. To induce differentiation, 2-day postconfluent 3T3-L1 cells (designated day 0) were incubated in MDI medium, which consists of DMEM with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/ml of insulin, for 2 days. Cells were then cultured in DMEM containing 10% FBS and insulin for another 2 days, after which they were grown in DMEM containing 10% FBS. For preparation of CM, 2-day postconfluent 3T3-L1 cells were induced differentiation by MDI for 48 h, after which the used culture medium was harvested (Choi et al. 2014).

Oil Red O staining. Cells were washed with phosphate-buffered saline (PBS) and fixed with 4% formalin in PBS for 5 min. After rinsing with distilled water, the cells were stained for 1 h in a 60% filtered solution (vol/vol in distilled water) of Oil Red O stain (0.5 g Oil Red O/100 ml isopropanol) and rinsed twice with distilled water.

PCR analysis. Five micrograms of total RNA isolated with Trizol reagent was reverse transcribed with SuperScript II reverse transcriptase using random hexamer primers. The

primers (forward, reverse) used for PCR were as follows: *Serpina3n*, 5'-CACTG TGGTG GAGCT GAAGT-3', 5'-TGTGG ACCAC CTGAG AGACT-3'; *Serpina3c*, 5'-TGGCC TCCAT CAACA CTGAC-3', 5'-ATGGC TGAGC CTCTG TAGGA-3'; *Leptin*, 5'-TGG GGA GTT TTG TTC CAG TG-3', 5'- AGG GAC ATG AGC CTC TGA TT-3'; *AdipoQ*, 5'- AGA TGT GAA GGT GAG CCT CT -3', 5'- GGC TAT GGG TAG TTG CAG TC-3'; *Itga5* (integrin α5), 5'-ACGTC CTCCA GGATG TTTCTC-3', 5'-TGGGA CTTAA ACTCC AGTGG G-3'; *Itgab3* (integrin β3), 5'-GACAA CTCTG GGCCG CTC-3', 5'-CCTTC AGGTT ACATC GGGGT-3'; *Itga6* (integrin α6), 5'-GAGTG ACGGT GTTTC CCTCA-3', 5'-CCTTG TGATA GGTGG CATCG T-3'; *Gapdh*, 5'-ACCAC AGTCC ATGCC ATCAC-3', 5'-TCCAC CACCC TGTTG CTGTA-3'. Real-time quantitative PCR to detect *Serpina3c* from mouse tissues was performed using SYBR green master mix with an ABI PRISM 7300 RT-PCR system and the following primers (forward, reverse): *Serpina3c*, 5'-TCACA GCAGA CTTCC AGCAG-3', 5'-GTCAC GGGGA TTAAA GGGCA-3'; 18s rRNA (for normalization), 5'-GCAGG TGTTT GACAA CGGCA G-3', 5'-GATGA TGGAG TGTGG CACCG A-3'.

RNA sequencing. RNA was isolated as described above from confluent 3T3-L1 preadipocytes treated with MDI or CM harvested after 4 h and 24 h. RNA sequencing was performed by DNA Link (Seoul, South Korea).

Western blot analysis. Cells were washed with cold PBS and lysed in 1% sodium dodecyl sulfate and 60 mM Tris-HCl (pH 6.8). The cell lysates were heated and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis for Western blotting. Primary antibodies against C/EBPβ (Lee et al. 2009), C/EBPα(Lee et al. 2009), PPARγ, integrins (α5, α6, and phosphorylated β3) were from Santa Cruz Biotechnology (Dallas, TX), the FLAG antibody was from Sigma-Aldrich (St. Louis, MO), and antibodies against integrin β3, GAPDH, and total and phosphorylated forms of ERK, AKT, and GSKβ were from Cell Signaling (Danvers, MA). The Serpina3c antibody was from Antibodies-online (Aachen, Germany). Ponceau S staining was

used as a loading control alternative to GAPDH in precipitated media Sigma-Aldrich (St. Louis, MO). Targeted proteins were visualized via enhanced chemiluminescence.

Fluorescence-activated cell sorting. 3T3-L1 cells were trypsinized, centrifuged, washed with PBS, and fixed with 90% cold methanol. After washing with PBS, the cells were stained for 30 min in the dark with 50 μg/ml propidium iodide (in PBS with 100 μg/ml RNase A) prior to cell sorting with a FACS caliber flow cytometry system (BD Biosciences, Franklin Lakes, NJ) and analysis via ModeFit software.

Cell count assay. 3T3-L1 cells cultured in 12-well plates (1.5×10^5 cells/well), incubated for 24 h and synchronized to quiescence by serum starvation for 12 h. At indicated time points, the cells were trypsinized and counted with an ADAM automated cell counter (NanoEnTek, Seoul, South Korea) according to the manufacturer's instructions (Kim et al. 2016).

RNA interference. 3T3-L1 preadipocytes were transfected with validated double-stranded stealth mouse short interfering RNA (siRNA) oligonucleotides from Santa Cruz Biotechnology 18–24 h after they were plated in 35-mm dishes: mouse *Serpina3c*, 60 nM sc-153358 (set of 2 siRNAs); *Serpina3n*, 60 nM sc-153364 (set of 3 siRNAs); *Itga5*, 100 nM sc-35687 (set of 3 siRNAs). Control cells were transfected with oligonucleotides with comparable GC contents (60 nM SN-1013; Bioneer, Daejeon, South Korea). Transfections were performed in OPTI-MEM medium using Lipofectamine RNAiMAX (Invitrogen of Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's protocol. The medium was replaced the next day with fresh Dulbecco's modified Eagle's medium containing 10% calf serum. Differentiation was induced 24 h later.

Preparation of nuclear extracts. Cells were washed twice with cold PBS, centrifuged, and lysed in hypotonic lysis buffer (20 mM Tris-Cl [pH 8.0], 10 mM NaCl, 3 mM $MgCl_2$, 1 mM

dithiothreitol, 1 mM sodium orthovanadate, 30 mM β-glycerophosphate) containing 10% NP-40 (IGEPAL CA-630) with a Dounce homogenizer. The lysates were centrifuged and the nuclear pellet was resuspended in nuclear storage buffer (40% glycerol, 50 mM Tris-Cl [pH 8.0], 3 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM β-glycerophosphate). The nuclear lysate was centrifuged and the pellet and cell nuclei were resuspended in NUN buffer (0.3 M NaCl, 1 M urea, 1% NP-40, 25 mM HEPES [pH 7.6], 1 mM dithiothreitol, 1 mM sodium orthovanadate, 30 mM β-glycerophosphate) before a final centrifugation. The supernatants were transferred to new tubes with 10% glycerol (final concentration).

Immunocytochemistry. 3T3-L1 cells were transfected with siRNAs and differentiated on coverslips in 6-well plates. After 24 h, the cells were washed with PBS, fixed for 10 min in 3.7% formaldehyde, and incubated for 1 h in blocking solution (PBS containing 5% bovine serum albumin). Cells were then incubated in blocking solution with GSK3β antibody (1:100; Cell Signaling) for 1 h and then with anti-rabbit IgG-fluorescein isothiocyanate for 2 h. 4',6-Diamidino-2-phenylindole was used to stain nuclei, and the cells were imaged with a confocal laser scanning microscope (Olympus FV1000; Olympus Corp, Tokyo, Japan).

Serpina3c overexpression. Cells were transiently transfected with 0.5 μg pcDNA3.0-Serpina3c-FLAG encoding full-length Serpina3c with a C-terminal FLAG tag via electroporation with a OneDrop MicroPorator MP-100 (Digital Bio, Seoul, South Korea) to maximize the transfection efficiency. The cells were trypsinized, washed with 1× PBS, and resuspended in 10 μl of resuspension buffer R with 0.5 μg of plasmid at a concentration of 200,000 cells per pipette. The cells were then microporated at 1,300 V, with two consecutive 20-ms pulses. Following microporation, the cells were seeded in 35-mm cell culture dishes and placed at 37 °C in a 10% CO2-humidified atmosphere (Kim et al. 2016). The cells were then plated in 35-mm cell culture dishes.

Cathepsin G activity assays. 3T3-L1 cells transfected with siRNAs were harvested from 60-mm dishes 24 h after differentiation was induced. Cathepsin G activity was measured by a Colorimetric Cathepsin G Activity Assay kit (Abcam, Cambridge, United Kingdom) according to the manufacturer's protocol. For cathepsin G inhibitor treatment, confluent cultures of 3T3-L1 cells seeded (2×10^4 cells) in 60-mm dishes were treated with MDI and 0, 5, or 10 μM cathepsin G inhibitor (Abcam). After 48 h, total RNA was isolated as described above for real-time PCR analysis.

Statistical Analysis. All results are expressed as means ± standard deviations. Groups were compared by using unpaired Student's *t* tests.

Data and Software Availability. The accession numbers for the 3T3-L1 RNA-seq using MDI and CM is GEO: GSE144130. Expression levels of *SERPINA3* in human adipose tissue, which is a human ortholog gene of *SERPINA3C*, were obtained from a public repository (GEO: GDS5056 and GDS 1480). Expression levels of *SERPINA3* were compared among groups by Student's *t* tests using R version 3.6.0 software (R Development Core Team, Vienna, Austria).

Supplemetal Reference

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