Nucleoredoxin promotes adipogenic differentiation through regulation of Wnt/β-catenin signaling

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Abstract Nucleoredoxin (NRX) is a member of the thioredoxin family of proteins that controls redox homeostasis in cell. Redox homeostasis is a well-known regulator of cell differentiation into various tissue types. We found that NRX expression levels were higher in white adipose tissue of obese ob/ob mice and increased in the early adipogenic stage of 3T3-L1 preadipocyte differentiation. Knockdown of NRX decreased differentiation of 3T3-L1 cells, whereas overexpression increased differentiation. Adipose tissue-specific NRX transgenic mice showed increases in adipocyte size as well as number compared with WT mice. We further confirmed that the Wingless/int-1 class (Wnt)/β-catenin pathway was also involved in NRX-promoted adipogenesis, consistent with a previous report showing NRX regulation of this pathway. Genes involved in lipid metabolism were downregulated, whereas inflammatory genes, including those encoding macrophage markers, were significantly upregulated, likely contributing to the obesity in Adipo-NRX mice. Our results therefore suggest that NRX acts as a novel proadipogenic factor and controls obesity in vivo.—Bahn, Y. J., K-P. Lee, S-M. Lee, J. Y. Choi, Y-S. Seo, and K-S. Kwon. Nucleoredoxin promotes adipogenic differentiation through regulation of Wnt/β-catenin signaling. J. Lipid Res. 2015. 56: 294–303.

Supplementary key words Wingless/int-1 class (Wnt) signaling • adipogenesis • obesity • inflammation

Adipose tissues play important roles in the regulation of whole-body energy homeostasis (1). Excess energy intake increases the number and size of fat cells (2). These obese adipose tissues facilitate chronic low-grade inflammation and insulin resistance, which result in severe obesity and diabetes (3, 4). Thus, understanding adipocyte development and adipogenesis could lay the groundwork for the development of efficient therapeutic strategies for preventing and treating metabolic disorders associated with obesity.

Adipogenesis is controlled by a balance of internal and external factors that either stimulate or repress adipogenic differentiation. In the early phase of adipogenic differentiation, CCAAT/enhancer binding protein (C/EBP) β and C/EBPα induce expression of C/EBPα and PPARγ, which are the principal adipogenic transcription factors that control the early differentiation of preadipocytes into lipid-accumulating fat cells (5, 6). In addition, PPARγ suppresses canonical Wingless/int-1 class (Wnt) signaling by accelerating proteasome-dependent degradation of β-catenin. Conversely, β-catenin, a transcriptional co-activator in the Wnt signaling pathway, blocks adipogenesis by repressing PPARγ and C/EBPβ (7, 8). A number of reports have suggested a relationship between Wnt signaling and adipogenesis (9, 10). Adipose tissue-specific expression of Wnt10b reduces adiposity and improves insulin sensitivity in the ob/ob obesity model (11). Although the extensive downregulation of β-catenin expression and its antadiapogenic effects during adipogenic differentiation have been characterized, less is known about the factors that modulate β-catenin activity during adipogenesis.

Wnt signaling is initiated upon binding of Wnt ligands to transmembrane Frizzled receptors. In the canonical Wnt signaling pathway, Frizzled receptors transduce signals through Dishevelled (Dvl) to inhibit glycogen synthase kinase 3 (GSK3β), resulting in hypophosphorylation and subsequent stabilization of β-catenin. Following nuclear translocation, active β-catenin binds to and coactivates members of the T-cell factor/lymphoid-enhancer factor

Abbreviations: Adipo-NRX mice, adipose tissue-specific NRX transgenic mice; C/EBF, CCAAT/enhancer binding protein; Dvl, Diskevelled; Fabp4, fatty acid binding protein 4; GGT, glucose tolerance test; ITT, insulin tolerance test; LSL-NRX mice, loxP-stop-loxP-NRX transgenic mice; NRX, nucleoredoxin; TRX, thioredoxin; WAT, white adipose tissue; Wnt, Wingless/int-1 class.

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family of transcription factors, leading to activation of target genes (12, 13).

Nucleoredoxin (NRX) is a member of the thioredoxin (TRX) family of proteins. TRX family proteins commonly possess a pair of redox-active, oxidation-sensitive cysteine residues in the catalytic center that are directly involved in the reduction of disulfide bonds in target proteins (14). Although NRX activity has been demonstrated in vitro assays, whether the TRX-related oxidoreductase activity of NRX plays a role in vivo is unknown (15). Previous studies have shown that NRX is a multifunction protein that regulates target proteins through its direct binding activity rather than its oxidoreductase activity (16, 17). Endogenous NRX protein is predominantly localized in the cytosol of cells, and its transcripts are widely expressed in all adult tissues (18). Knockout of the NRX gene in mouse embryos is perinatally lethal; NRX knockout embryos (day 18.5) are smaller than their WT littermates and exhibit craniofacial defects with short frontal regions (19). Interestingly, a genomic region around the mouse NRX gene is involved in type 1 and type 2 diabetes (20). However, a role for NRX in adipogenesis and obesity has not been reported.

Here, we investigated the role of NRX in preadipocyte differentiation and the obesity phenotype using a 3T3-L1 preadipocyte differentiation system and adipose tissue-specific transgenic mice. We show that NRX mediates adipogenesis by modulating β-catenin activity in vitro and in vivo. Our findings suggest that NRX might act as a proadipogenic factor that is involved in adipocyte differentiation and aspects of the obesity phenotype.

MATERIALS AND METHODS

Generation of adipose tissue-specific NRX transgenic mice

The loxPstop-loxP-NRX transgenic mice (LSL-NRX mice) were obtained by microinjection and germ-line transmission of the transgenic construct (supplementary Fig. IA). The LSL-NRX mouse strain was backcrossed with the C57BL/6 strain for more than eight generations to create a uniform genetic background. The loxPstop-loxP-NRX transgenic mice (LSL-NRX mice) were produced by crossing LSL-NRX mice with adiponectin-Cre transgenic mice, generating mice with adiponectin-Cre transgenic mice, generating mice with adipose tissue-specific overexpression of hNRX. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of the Korea Research Institute of Bioscience and Biotechnology (KIRIBB).

Potential founder mice were genotyped by PCR analysis using genomic DNA isolated from mouse tail clips. Primers used for the detection of the transgenic product were 5′-TGC GAG ATT ACA CCA ACC TG-3′ and 5′-CCC ATA TGT CCT TGC GAG TG-3′.

Isolation and culturing of primary adipocytes

Primary adipocytes were isolated from epididymal fat pads using the collagenase method, as described previously (21). Briefly, freshly excised fat pads were minced and digested for 45 min to 1 h at 37°C in Krebs-Ringer bicarbonate (pH 7.4) containing 4% BSA and 1.5 mg/ml type I collagenase (Worthington, Lakewood, NJ). The digested tissue was filtered through a 300 μm nylon mesh to remove undigested tissue and centrifuged at 500 g for 5 min. The floating adipocyte fraction was removed, washed with buffer, and recentrifuged to isolate free adipocytes. The stromalvascular pellet was resuspended in erythrocyte lysis buffer (154 mM NH4Cl, 10 mM KHC03, and 1 mM EDTA), filtered through a 45 μm nylon mesh to remove endothelial cells, and pelleted at 500 g for 5 min. Enriched cells were cultured in a growth medium composed of DMEM, 20% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco-Invitrogen, Carlsbad, CA) at 37°C in a humidified 5% CO2 atmosphere.

Cell culture and adipogenic differentiation

The preadipocytes cell line 3T3-L1, derived from mouse embryo fibroblasts, was grown at 37°C in DMEM containing 10% heat-inactivated bovine calf serum (Gibco-Invitrogen), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified 5% CO2 atmosphere. 3T3-L1 cells were induced to differentiate into mature adipocytes according to the procedure of Student et al. (22), with minor modifications. Briefly, 2 days after reaching confluence (day 0), cells were placed in differentiation medium composed of DMEM, 10% fetal bovine serum, and MDI, a differentiation cocktail consisting of 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone, and 10 μg/ml insulin (Sigma, St. Louis, MO). The medium was replenished every other day.

Generation of stable 3T3-L1 cell lines

3T3-L1 cells stably expressing FLAG-tagged NRX were generated using a lentivirus-mediated infection system. FLAG is a short, hydrophilic fusion tag consisting of eight amino acids (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys). For expression of NRX, cDNA encoding FLAG-tagged NRX was cloned into the multicloning site of the green fluorescent protein (GFP)-tagged pLentiM1.4 vector. Lentiviruses were subsequently produced by transient cotransfecting HEK293T cells with pLP1, pLP2, and pVSV-G plasmid (Invitrogen, Carlsbad, CA) using Lipofectamine (Invitrogen). Forty-eight hours after transfection, supernatants containing lentiviral particles were collected and used to infect 3T3-L1 cells in the presence of 4 μg/ml polybrene. Infected cells were selected by incubation with 2 μg/ml puromycin for 2–3 weeks and used in experiments as indicated. NRX cDNA was kindly provided by Dr. Tasuku Honjo (Kyoto University, Japan) (15). An NRX mutant in which catalytic Cys205 and Cys208 residues were replaced with Ser (CS-NRX), provided by Dr. Sung-Kyu Ju, was constructed using site-directed mutagenesis.

RNA interference

NRX knockdown in 3T3-L1 cells was accomplished using shRNA against mouse NRX in pLKO.1-puro lentiviral vectors obtained from Sigma (clone ID NM_022483.3:1358s1c1 and NM_022483.3:452s3c1), according to the manufacturer’s protocol. Briefly, shRNA lentiviral particles were generated in HEK293T cells by transient transfection with pLP1, pLP2, pVSVG, and shRNA lentiviral vector or pLKO.1-scrambled (control) vector (SHC002V; Sigma) using Lipofectamine. Forty-eight hours after transfection, supernatants containing lentiviral particles were collected and used to infect 3T3-L1 cells in the presence of 4 μg/ml polybrene. Infected cells were selected by incubation with 2 μg/ml puromycin for 2–3 weeks and used in experiments as indicated. An shRNA-resistant NRX incapable of shNRX binding and subsequent NRX degradation was constructed by site-directed mutagenesis of the target region of shNRX. The construct was obtained by standard methods using...
the primers 5′-CTT TG A TGA ACT TC TGG C7G AAA AAC TC-3′ and 5′-TCA GGC TGG AGT TTT TGA GCA AGG AAC TGA TT-3′. Underlined regions indicate the sites that were mutated without changing amino acid residues.

RNA extraction and real-time PCR analysis
Total RNA was extracted using the Easy-Blue reagent (Intron Biotechnology, Korea), according to the manufacturer’s instructions, and treated with RNase-free DNase I (Takara, Shiga, Japan) to remove contaminating genomic DNA. cDNA was then synthesized from total RNA by RT using a DiaStar RT kit (Solgent, Korea). Quantitative RT-PCR analysis was performed using the Step One Plus real-time PCR system (Applied Biosystems, Waltham, MA) with the corresponding primers.

Immunoblotting and immunoprecipitation
Cells were lysed in a lysis buffer containing 20 mM HEPES (pH 7.2), 150 mM NaCl, 0.5% Triton X-100, 0.1 mM Na3VO4, 1 mM NaF, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride, and 5 mg/ml aprotinin (Sigma). Soluble proteins in cell lysates were separated by SDS-PAGE and analyzed by immunoblotting using anti-NRX (R&D Biosystems, Minneapolis, MN, AF5719), anti-PPAR (Cell Signaling Technology, Danvers, MA, #2430), anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, SC-7777), anti-Dvl-1 (Santa Cruz Biotechnology, SC-8025), anti-lamin A/C (Santa Cruz Biotechnology, SC-6215), anti-cyclin D1 (Cell Signaling Technology, 2922), anti-β-catenin (Cell Signaling Technology, 9562), anti-α-tubulin (Millipore, 05-829), anti-β-actin murine thymoma viral oncogene homology (AKT; Santa Cruz Biotechnology, SC-1618), anti-phospho-AKT (Cell Signaling Technology, 9271L), and anti-fatty acid binding protein 4 (FABP4; Cell Signaling Technology, 2120) antibodies. Dr. Chul-Ho Lee (KRIBB). For immunoprecipitation, lysates were incubated with anti-FLAG agarose (Sigma) or anti-NRX antibody at 4°C for several hours, after which the beads were washed three times with cell lysis buffer. The beads were resuspended in 1× SDS-PAGE sample buffer, and the eluted proteins were resolved by SDS-PAGE.

In vitro gene transfer
For Amaxa nucleofections, pellets containing 0.5–1.5 × 10⁶ 3T3-L1 cells were carefully resuspended in 100 µl of Nucleofector solution (Lonza, Allendale, NJ), mixed with 1–2 µg of plasmids, and subjected to nucleofection using T-30 Amaxa protocols. The cells were then gently transferred into a 6-well plate and cultured until analysis.

Luciferase assays and reagents
Luciferase activity was assessed using the Luciferase Assay system (Promega, Madison, WI), according to the manufacturer’s instructions. 3T3-L1 cells were transduced with TOPflash and FOPflash exogenous reporter constructs together with pCMV-β-galactosidase. Luciferase activity was normalized to β-galactosidase activity to adjust for transfection efficiency.

Oil Red O staining
Cultured cells were washed twice with PBS and fixed by incubating with 3.7% paraformaldehyde for 15 min at room temperature. The cells were then washed with distilled water and stained for 30 min with 0.3% filtered Oil Red O solution in 60% isopropanol. The stained cells were washed twice with distilled water and photomicrographed. Oil Red O staining was then quantified as described previously (23). Incorporated Oil Red O dye was extracted by adding absolute isopropanol to the stained cell-culture dish and shaking the dish for 30 min. Triplicate samples were read at 510 nm using an Ultrospec2000 Spectrophotometer (Pharmacia Biotech, Piscataway, NJ).

Hematoxylin and eosin staining
Adipose tissue, muscle, and liver samples were fixed for 12–16 h at room temperature in 10% formalin (Sigma) and then embedded in paraffin. Five-micron sections cut at 50 µm intervals were mounted on charged glass slides, deparaffinized in xylene, and stained with hematoxylin and eosin (H and E). Adipocyte cross-sectional area was quantified for each adipocyte in each field using National Institutes of Health Image J software (http://rsb.info.nih.gov/ij/). To quantify cell number, adipocytes were isolated from 100 mg of adipose tissue by using 2 mg/ml collagenase type I-S (Sigma) digestion buffer and were counted using a cell counter (Logos Biosystems, Annandale, VA) (24).

Glucose and insulin tolerance test
Glucose tolerance test (GTT) was performed with 8 h fasted animals. After determination of fasted blood glucose levels, each animal was injected intraperitoneally with 20% glucose (1 g/kg). The insulin tolerance test (ITT) was performed, with an initial fasting for 4 h, and subsequent intraperitoneal injection of insulin (1 U/kg). In all tests, tail blood glucose levels were measured with a glucometer (Roche Diagnostics, Mannheim, Germany) at the indicated times after injection.

Immunohistochemistry
Formalin-fixed, paraffin-embedded sections of 5 µm were mounted on charged glass slides, deparaffinized in xylene, stained with hematoxylin, and processed for immunohistochemical detection of F4/80 according to standard immunoperoxidase procedure using VECTASTAIN Elite ABC kit (Vector Labs, Burlingame, CA) and anti-F4/80 antibody (Abcam, Cambridge, UK).

Statistical analysis
Quantitative data are presented as means ± SD unless indicated otherwise. Differences between means were evaluated using Student’s unpaired t-test. A P value < 0.05 was considered statistically significant.

RESULTS
NRX levels increase in the early stage of 3T3-L1 preadipocyte differentiation
We analyzed the expression of NRX during 3T3-L1 cells differentiation induced by a differentiation cocktail composed of IBMX, dexamethasone, and insulin. NRX transiently increased in the early stages of adipocyte differentiation at both mRNA and protein levels. Quantitative RT-PCR revealed that NRX mRNA reached a maximum 1 day after differentiation induction (Fig. 1A). Protein levels of NRX were proportional to mRNA levels (Fig. 1B). We next determined whether hyperglycemic conditions affected NRX levels in ob/ob mice. Interestingly, NRX protein was upregulated in WT of ob/ob mice compared with that in WT mice (Fig. 1C). These results suggest that increased expression of NRX may be associated with adipogenesis and, ultimately, obesity.
NRX overexpression leads to adipocyte hypertrophy combined with hyperplasia in mouse adipose tissue

To further investigate the function of NRX in adipogenesis in vivo, we generated LSL-NRX mice, then crossed these mice with adiponectin-Cre mice to generate Adipo-NRX mice. To validate the specific overexpression of NRX in adipose tissues, we analyzed NRX protein levels in several tissues isolated from Adipo-NRX and WT mice. As expected, overexpression of NRX was observed only in adipose tissues, including white and brown adipose tissue (supplementary Fig. 1B). To clarify activity of the adiponectin promoter, we checked expression levels of adiponectin during adipogenesis. We confirmed that the expression level of adiponectin was very low before induction of differentiation but gradually increased after induction of differentiation (supplementary Fig. 1C), which was consistent with a previous report (25). We also observed that expression of adiponectin promoter-driven NRX was induced at early differentiation stage in primary adipocytes isolated from Adipo-NRX mice (supplementary Fig. 1D).

Although there was a slight increase in body weight in Adipo-NRX mice compared with WT littermate controls, a comparison of organ weights revealed a notable increase in epididymal WAT in Adipo-NRX mice (Fig. 2A). Epididymal and perirenal fat masses were also significantly larger in Adipo-NRX mice than in WT mice (Fig. 2B, C). There was no difference in food intake between Adipo-NRX mice and WT littermate controls. An analysis of adipocyte cross-sectional areas showed that epididymal fat of Adipo-NRX mice contained hypertrophied adipocytes (Fig. 2D). A quantitative analysis revealed that adipose tissue expansion of Adipo-NRX mice was caused by an increase of adipocyte numbers (Fig. 2E) as well as an enlargement of adipocyte size, suggesting that hypertrophy was accompanied by hyperplasia in adipose tissue of Adipo-NRX mice.

The plasma analysis revealed that blood glucose and insulin levels were higher in Adipo-NRX mice than in WT mice (supplementary Table 1). We further assessed glucose homeostasis in WT and Adipo-NRX mice via a GTT and ITT. Adipo-NRX mice showed the tendency to glucose intolerance compared with WT mice (Fig. 2F) and exhibited impaired insulin tolerance that was associated with reduced insulin sensitivity in WAT but not in liver and skeletal muscle (Fig. 2G, H). Although the adipose tissue appeared lipid accumulation, there was no significant change in lipid deposition in liver and skeletal muscle (supplementary Fig. 2). These data demonstrate that adipocyte-specific NRX overexpression increases lipid accumulation in WAT, resulting in insulin resistance.

NRX overexpression leads to increased adipogenic differentiation

To evaluate whether overexpression of NRX facilitates adipogenic differentiation in cell culture system, we infected 3T3-L1 preadipocytes with a lentivirus expressing GFP-tagged mouse NRX or a control lentivirus. Stable overexpression of NRX was monitored by immunoblotting (Fig. 3A) and fluorescence imaging. 3T3-L1 preadipocytes stably overexpressing NRX showed increased accumulation of lipid droplets following induction of adipocyte differentiation compared with control cells (Fig. 3B, C). We confirmed that the adipogenic markers, PPARγ and FABP4, were also upregulated in NRX-overexpressing cells compared with control cells (Fig. 3D). To confirm that NRX overexpression positively regulates adipocyte differentiation, we next isolated and cultured primary adipocytes...
and Igax, markers of macrophage infiltration (Fig. 3G). Consistently, increased macrophage infiltration in WAT was shown by immunostaining using a macrophage surface marker, F4/80 (Fig. 3H). Obesity is associated with an overall increase in expression of several collagens that results in fibrotic state (28, 29). Then, we tested mRNA levels of several fibrosis-related genes, and found that Col1a1, Col3a1, Col6a1, and Elastin were upregulated (Fig. 3G) in WAT of Adipo-NRX. These suggest that adipocyte expansion is closely linked to inflammation and fibrosis in Adipo-NRX mice.

NRX knockdown attenuates adipogenic differentiation in 3T3-L1 preadipocytes

To test whether endogenous NRX influences adipogenesis, we examined the effect of NRX knockdown in 3T3-L1 preadipocytes. 3T3-L1 cells were infected with a lentivirus expressing a shRNA targeting NRX (NRX shRNA) or nontargeting (scrambled) shRNA. Depletion of endogenous NRX was confirmed by immunoblot analyses (Fig. 4A). Knockdown of NRX attenuated differentiation of 3T3-L1 preadipocytes into mature adipocytes, reducing accumulation of lipid droplets compared with control 3T3-L1 cells (Fig. 4B). Moreover, expression levels of the adipocyte markers, PPAR and FABP4, also were decreased in NRX-depleted cells compared with control cells (Fig. 4C). Next, to exclude the possibility of off-target effects of shRNA targeting...
Nucleoredoxin regulates adipogenesis through inhibition of GSK3β, leading to the stabilization and nuclear translocation of β-catenin (12). It has been reported that NRX regulates Wnt/β-catenin signaling by directly binding and inhibiting Dvl and thereby controls cell proliferation (16). Wnt/β-catenin signaling is also a central negative regulator of adipogenesis (7). Here, we found that NRX is involved in adipogenic differentiation, prompting us to examine whether NRX regulates adipogenesis through Wnt/β-catenin regulation. First, we tested the interaction between NRX and Dvl in adipocyte. Endogenous Dvl interacted with ectopically expressed WT NRX, but not with a cysteine-mutant NRX defective in thiol reducing activity, in 3T3-L1 preadipocytes (Fig. 5A).

Moreover, unlike WT NRX, ectopically expressed cysteine-mutant NRX did not increase 3T3-L1 differentiation (Fig. 5B). These results suggest that NRX controls adipogenesis through inhibition of GSK3β, leading to the stabilization and nuclear translocation of β-catenin (12).
lipid accumulation by 31.2%, while knockdown of Dvl together reduced lipid accumulation only by 10.5%. These results suggest that NRX regulates adipogenesis through inhibition of Dvl-β-catenin axis in adipocyte differentiation.

**DISCUSSION**

Our study demonstrated that NRX mRNA and protein were increased in the early stages of adipocyte differentiation and were also increased in WAT of ob/ob mice, a leptin-deficiency model of obesity. Using NRX-depleted and NRX-overexpressing 3T3-L1 preadipocytes, we showed that NRX is associated with adipogenic differentiation of 3T3-L1 cells. We found that differentiation of primary adipocytes from Adipo-NRX mice was increased in vitro, and epididymal and perirenal fat mass were increased in Adipo-NRX mice in vivo. These observations, taken together with our in vitro and in vivo adipogenesis data, thus uncover a novel role for NRX as a proadipogenic factor.

Adipose tissue expansion occurs through an enlargement in adipocyte size (hypertrophy) and/or an increase in adipocyte number (hyperplasia). We showed that Adipo-NRX mice exhibited hyperplasia in adipocytes, which is linked to enhanced adipogenic differentiation (32, 33), as well as hypertrophy (Fig. 2D, E). Adipo-NRX mice showed decreased expression of enzymes involved in lipolysis and fatty acid oxidation in WAT, while unchanged in lipogenic enzymes compared with WT mice. Decreased triacylglycerol catabolism is associated with the occurrence of prevalent metabolic diseases, such as obesity and type 2 diabetes (34–36). Hypertrophic adipocyte in our model is

by interacting with Dvl through a thiol-based mechanism. We further observed that endogenous NRX-Dvl binding was dissociated by Wnt3a treatment (Fig. 5C), suggesting that NRX controls adipogenesis via Wnt/β-catenin signaling. To test this hypothesis, we determined whether NRX controls the nuclear localization of β-catenin in 3T3-L1 cells and primary adipocytes isolated from Adipo-NRX mice. Nuclear β-catenin levels and transcriptional activity were increased in NRX-knockdown cells but were decreased in these cells following restoration of NRX expression (Fig. 5D). Enhancing NRX expression by increasing the amount of transfected plasmid induced a dose-dependent decrease in the levels of nuclear β-catenin and its downstream effector cyclin D1 in Wnt3a-treated 3T3-L1 cells (Fig. 5E), resulting in recovery from Wnt3a-mediated inhibition of adipogenesis (Fig. 5F, third panel). Consistent with the results obtained in 3T3-L1 preadipocytes (Fig. 5D, E), the expression of β-catenin target genes was decreased in WAT of Adipo-NRX mice (Fig. 5G).

Next, we examined whether the interaction of NRX with Dvl plays a role in adipogenesis. While single Dvl gene (Dvl-1, Dvl-2, or Dvl-3) knockdown showed no significant changes in adipocyte differentiation compared with control group (supplementary Fig. 3), triple knockdown of all Dvl genes enhanced differentiation of 3T3-L1 into mature adipocytes (Fig. 6A, B) accompanying with an elevated expression of FABP4, an adipocyte differentiation marker, compared with control (Fig. 6C). To test whether NRX role is dependent on Dvl, we checked the effect of NRX on adipogenesis in the presence or absence of three Dvls. We found that the Dvls knockdown suppressed reduced 3T3-L1 differentiation by NRX knockdown (Fig. 6D, E). NRX knockdown reduced lipid accumulation by 31.2%, while knockdown of Dvls together reduced lipid accumulation only by 10.5%. These results suggest that NRX regulates adipogenesis through inhibition of Dvl-β-catenin axis in adipocyte differentiation.
Nucleoredoxin regulates adipogenesis via modulation of β-catenin in 3T3-L1 cells. A: WT NRX interacts with Dvl and increases 3T3-L1 differentiation. A: 3T3-L1 cells were transfected with vectors expressing FLAG-tagged WT or mutant (Mut) NRX, lysed and immunoprecipitated with anti-FLAG antibody, and analyzed by immunoblotting with anti-Dvl antibody. B: After 8 days of incubation in differentiation media, cells were stained with Oil Red O dye. C: 3T3-L1 cell lysates were immunoprecipitated using anti-NRX antibodies after treatment with 200 ng/ml Wnt3a followed by immunoblotting, as indicated. D–F: NRX inhibits β-catenin activity and promotes recovery from Wnt3a-mediated inhibition of adipogenesis. D: Reporter gene (TOPflash) expression assays in NRX-knockdown 3T3-L1 cells after cotransfection of increasing amounts of resNRX. Nuclear (N) and cytosolic (C) fractions were analyzed by immunoblotting. Lamin A/C was used as a control for the nuclear fraction. E: 3T3-L1 cells transfected with vectors expressing FLAG-tagged NRX were treated with Wnt3a. Cytosolic and nuclear proteins were isolated for Western blot analysis of FLAG-NRX, β-catenin, lamin A/C, and cyclin D1. F: 3T3-L1 cells overexpressing NRX were induced to differentiate into adipocytes with Wnt3a for the first 2 days. After 8 days of differentiation, cells were stained with Oil Red O dye. Whole-cell lysates were prepared on day 8 for Western blot analysis of NRX, FABP4, and β-actin. G: β-Catenin target genes were examined in WAT from WT and Adipo-NRX mice by real-time quantitative RT-PCR (n = 3). Data are presented as mean fold-changes ± SD (*P < 0.05, **P < 0.01). MMP-7, matrix metalloproteinase-7.

likely caused by the decrease of triacylglycerol catabolism rather than an increase in triacylglycerol synthesis. However, the mechanism by which NRX regulates fatty acid catabolism remains to be determined.

Lipid accumulation increased exclusively in WAT of Adipo-NRX mice, with no change in liver and skeletal muscle compared with WT mice (supplementary Fig. 2). In plasma analysis, Adipo-NRX mice exhibited increased fasting insulin level accompanying with increased fasting glucose level, suggesting that pancreas function was normal (supplementary Table 1). Adipo-NRX mice showed the tendency to glucose intolerance in GTT analysis (Fig. 2F) and also showed mild insulin resistance as shown in impaired insulin tolerance and reduced insulin sensitivity in WAT but not in liver and skeletal muscle (Fig. 2G, H). Therefore, insulin resistance in our model is likely caused by excessive fat deposition in WAT.

Infiltration of adipose tissue by inflammatory cells has been described as a common feature of obesity (27). We also found increased expression of inflammatory gene and macrophage markers as well as dysregulation of adipokines in Adipo-NRX mice (Fig. 3G). Many rodent models of obesity are associated with chronic inflammation, and it is known that obesity-induced insulin resistance is strongly correlated with expression of inflammatory markers (37, 38). However, the molecular links between obesity and inflammation have not been completely elucidated. Adipo-NRX mice might be a potential model system for detailed mechanistic studies in the relationship between obesity and inflammation.

Wnt signaling is an important regulator of fate decisions in mesenchymal cells (31). In multipotent mesenchymal precursors in vitro, activation of Wnt/β-catenin signaling promotes osteoblastogenesis but inhibits adipogenesis (39). Given that NRX inhibits Wnt/β-catenin signaling (16), it is possible that NRX reciprocally modulates adipogenic and osteogenic differentiation. Activation of β-catenin in primary osteoblasts isolated from calvariae of NRX+/− embryos results in enhanced osteoblastic differentiation (19). However, there have been no reports of a role for NRX in adipogenesis. Here, we provide the first demonstration that NRX positively regulates adipogenesis by inhibiting the Wnt/β-catenin pathway. Wnt10b is a likely candidate for the endogenous Wnt that participates in adipogenesis. The expression level of Wnt10b, which is highly expressed in preadipocytes, declined upon initiation of differentiation (40), exhibiting a negative correlation with NRX expression (Fig. 1A). Wnt10b has been reported to attenuate the development of obesity in ob/ob mice by stabilizing β-catenin and subsequently inhibiting adipogenesis. Conversely, blocking Wnt signaling promotes adipogenesis.
and obesity (11). Based on our data, it is likely that NRX is involved in obesity in ob/ob mice through regulation of Wnt/β-catenin signaling.

Although current evidence suggests that β-catenin functions as a crucial regulator of adipogenesis (41), how its expression and activity are regulated during adipogenic differentiation, particularly the factors involved, remains largely unknown. During adipogenesis, there is a considerable reduction in total β-catenin protein as well as a marked decrease in nuclear β-catenin levels (8, 42). In addition to regulating β-catenin protein levels, we found that NRX directly interacted with and inhibited the Wnt/β-catenin effector molecule Dvl during adipogenesis. Both aspects of this negative regulation of Wnt/β-catenin signaling by NRX enabled induction of the adipogenic transcription factor PPARγ, which, in turn, downregulated β-catenin levels, ensuring complete terminal differentiation into mature adipocytes.

Our current findings show that the active site cysteine of NRX is required for proper inhibition of the Wnt/β-catenin pathway. However, we do not yet know how redox controls the inhibitory function of NRX in Wnt/β-catenin signaling, although we presume that thiol-disulfide exchange of the active site cysteine residues leads to a conformational change in the NRX molecule that modulates its inhibitory function. Further studies will be needed to confirm this.

In conclusion, our study provides the first evidence that NRX acts as a proadipogenic factor by regulating Wnt signaling and is associated with phenotypic manifestations of obesity. Therefore, we propose that modulating Wnt/β-catenin signaling by blocking NRX may ultimately prove to be an effective therapeutic strategy for managing obesity and metabolic disorders such as diabetes.

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REFERENCES

1. Spiegelman, B. M., and J. S. Flier. 2001. Obesity and the regulation of energy balance. Cell. 104: 531–545.
2. Kopelman, P. G. 2000. Obesity as a medical problem. Nature. 404: 635–643.
3. Wellen, K. E., and G. S. Hotamisligil. 2003. Obesity-induced inflammatory changes in adipose tissue. J. Clin. Invest. 112: 1785–1788.
4. Xu, H., G. T. Barnes, Q. Yang, G. Tan, D. Yang, C. J. Chou, J. Sole, A. Nichols, J. S. Ross, L. A. Tartaglia, et al. 2003. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J. Clin. Invest. 112: 1821–1830.
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5. Farmer, S. R. 2006. Transcriptional control of adipocyte formation. *Cell Metab.* 4:263–273.

6. Rosen, E. D., and B. M. Spiegelman. 2000. Molecular regulation of adipogenesis. *Annu. Rev. Cell Dev. Biol.* 16:145–171.

7. Ross, S. E., N. Hemati, K. A. Longo, C. N. Bennett, P. C. Lucas, R. L. Erickson, and O. A. MacDougall. 2004. Inhibition of adipogenesis by Wnt signaling. *Science* 299:959–963.

8. Moldes, M., Y. Zuo, R. F. Morrison, D. Silva, B. H. Park, J. Liu, and S. R. Farmer. 2003. Peroxisome-proliferator-activated receptor gamma suppresses Wnt/beta-catenin signalling during adipogenesis. *Biochem. J.* 376:607–613.

9. Grant, S. F., G. Thorleifsson, I. Reynisdottir, R. Benediktsson, S. E. Ross, N. Hemati, K. A. Longo, C. N. Bennett, P. C. Lucas, M. R. Opp, and O. A. MacDougall. 2004. Wnt10b inhibits development of white and brown adipose tissues. *J. Biol. Chem.* 279:35590–35599.

10. Wright, W. S., K. A. Longo, V. W. Dolinsky, I. Gerin, S. Kang, C. N. Bennett, S. H. Chiang, C. F. Burant, et al. 2007. Wnt10b inhibits obesity in ob/ob and agouti mice. *Diabetes.* 56:295–303.

11. Kurooka, H., K. Kato, S. Minoguchi, Y. Takahashi, J. Ikeda, S. Habu, N. Osawa, A. M. Buchberg, K. Moriwaki, H. Shisa, et al. 1997. Cloning and characterization of the nucleoredoxin gene that encodes a novel nuclear protein related to thioredoxin. *Genomics.* 39:351–359.

12. Behrens, J. B. A., J. Jerchow, M. Wurtele, J. Grimm, C. Asbrand, R. Kennell, J. A., and O. A. MacDougald. 2005. Wnt signaling inhibits development of white and brown adipose tissues. *Cell Metab.* 9:1483–1487.

13. Teo, R., R. Mohrlen, G. Picken, W. A. Muller, and U. Frank. 2006. An evolutionary conserved role of Wnt signaling in stem cell fate decision. *Dev. Biol.* 299:91–99.

14. Wang, Q. A., P. E. Scherer, and R. K. Gupta. 2014. Improved methodologies for the study of adipocyte biology: insights gained and opportunities ahead. *J. Lipid Res.* 55:605–624.

15. Clevy, H. 2006. Wnt/beta-catenin signaling in development and disease. *Cell.* 127:469–480.

16. Langin, D., A. Dicker, G. Tavernier, J. Hoffstedt, A. Mairal, M. Ryden, E. Arner, A. Sicard, C. M. Jenkins, N. Viguerie, et al. 2005. Adipocyte lipases and defect of lipolysis in human obesity. *Diabetes.* 54:3190–3197.

17. Zechner, R., P. C. Kienesberger, G. Haemmerle, R. Zimmermann, and A. Lass. 2009. Adipose triglyceride lipase and the lipolytic catabolism of cellular fat stores. *J. Lipid Res.* 50:3–21.

18. Jocken, J. W., D. Langin, E. Smit, W. H. Saris, C. Vallee, G. B. Hul, C. Holm, P. Arner, and E. E. Blaak. 2007. Adipose triglyceride lipase and hormone-sensitive lipase protein expression is decreased in the obese insulin-resistant state. *J. Clin. Endocrinol. Metab.* 92:2292–2299.

19. Blackburn, P., J. P. Despres, B. Lamarche, A. Tremblay, J. Bergeron, I. Lemicieux, and C. Couillard. 2006. Postprandial variations of plasma inflammatory markers in abdominally obese men. *Obesity (Silver Spring).* 14:1747–1754.

20. Aror-Wisniewsky, J. J., J. W. F. Jorgensen, C. Poutou, F. Darakhshan, D. Farias, A. Basdevant, A. Aisa, P. C. MacDougall, and M. Clement. 2009. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. *J. Clin. Endocrinol. Metab.* 94:4619–4623.

21. Cawthorn, W. P., A. J. Bree, Y. Yao, B. Du, N. Hemati, G. Martinez-Santibanez, and O. A. MacDougall. 2012. Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a beta-catenin-dependent mechanism. *Bone.* 50:477–489.

22. Bennett, C. N., S. E. Ross, K. A. Longo, L. Bajnok, N. Hemati, K. W. Johnson, S. D. Harrison, and O. A. MacDougall. 2002. Regulation of Wnt signaling during adipogenesis. *J. Biol. Chem.* 277:30998–31004.

23. Ramírez-Zacarías, J. L., F. Castro-Munozledo, and W. Kuri-Harcuch. 1992. Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil red O. *Histochemistry.* 97:493–497.