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Dreh, a long noncoding RNA repressed by metformin, regulates glucose transport in C2C12 skeletal muscle cells

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ABSTRACT

Aims: The anti-hyperglycemic action of metformin on skeletal muscles is presently unclear. Long noncoding RNAs (lncRNAs) are implicated in multiple cellular functions. This study aims to explore the role of lncRNAs in the glucometabolic action of metformin on skeletal muscle cells.

Main methods: Metformin accumulation was assessed using [^{14}C]-metformin. A lncRNA array was used to investigate metformin-regulated lncRNAs in C2C12 skeletal muscle cells. Knockdown studies were applied to evaluate the function of lncRNA Dreh. A colorimetric assay was used for the measurement of medium glucose concentration; glucose transport was assessed using [^{3}H]-2-deoxyglucose; real-time PCR was used for RNA expression analysis, and western blotting was used to assess protein expression in myotubes. A Dreh overexpression plasmid was transfected into the cells.

Key findings: Metformin accumulated in C2C12 myotubes. Metformin reduced medium glucose concentration and repressed lncRNA Dreh expression in the myotubes. Knockdown of Dreh in the myotubes resulted in reduced glucose concentration in the culture medium, increased glucose transport, and increased levels of GLUT4 protein in the plasma membrane. Overexpression of Dreh attenuated the glucose-lowering effect of metformin in myotubes.

Significance: The glucoregulatory actions of metformin are mediated in part by a lncRNA, Dreh, in the skeletal muscle cells. Dreh is a novel regulator for glucose transport and could be a therapeutic target for diabetes.

1. Introduction

Metformin is the most commonly used oral anti-hyperglycemic drug; it is prescribed as a first-choice therapy for individuals with newly-diagnosed type 2 diabetes and as a combination therapy with other anti-diabetic drugs [1,2]. The glucose-lowering effects of metformin primarily occur in the liver by decreasing hepatic glucose production [3]. Metformin inhibits the function of complex I of the mitochondrial respiratory chain to suppress ATP production [4], which in turn activates AMP-activated protein kinase (AMPK) [5]. Furthermore, metformin decreases the production of cyclic AMP [6], inhibits the activity of mitochondrial glycerol-3-phosphate dehydrogenase [7] and fructose-1,6-bisphosphatase [8], and influences the redox state in hepatocytes [9]. Thus, the molecular mechanisms underlying the action of metformin in the liver have been intensively explored.

Although metformin acts mainly on the liver, it can act on skeletal muscle *in vivo* and *in vitro*. Metformin treatment of up to 2,000 mg/day in patients with type 2 diabetes for ten weeks significantly increased the activity and phosphorylation of AMPK in the vastus lateralis muscle [10]. In addition, a four-week metformin treatment of patients with type 2 diabetes increased forearm glucose uptake, mainly in the skeletal muscle [11]. Use of a hyperinsulinemic euglycemic clamp demonstrated increased glucose disposal rates after treatment with metformin doses of up to 2,000 mg/day for 8–12 months in patients with type 2 diabetes [12]. In rodent models, metformin treatment enhanced glucose uptake in skeletal muscles, such as the soleus, and increased glucose transporter 4 (GLUT4) expression in the plasma membrane of myocytes [13]. *In vitro*, metformin enhanced glucose transport in cardiomyocytes [14], rat L6 myotubes [15], and mouse C2C12 myotubes [16]. Metformin was reported to increase glucose transport due to increased

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expression of GLUT4 in the plasma membrane [14]. Furthermore, a kinetic study in humans using [^{11}C]-metformin has shown that labelled metformin accumulates extensively in the liver but gradually in muscles [17]. The collective evidence suggests that skeletal muscle could be a target of metformin to improve glucose sensitivity. However, the precise molecular mechanism of the action of metformin on skeletal muscle remains unclear.

Over the last decade, the development of sequencing technology has allowed the in-depth examination of the human genome and transcriptome. Such studies have revealed that approximately 70% of the human genome is actively transcribed, of which only 2% contains protein-coding genes [18]. The majority of the transcripts are non-coding RNAs (ncRNAs); these include microRNAs, small nucleolar RNAs, long ncRNAs (lncRNAs), and circular RNAs. Among these, lncRNAs are RNA transcripts longer than 200 nucleotides in length with no evident protein-coding potential. LncRNAs are emerging regulators of diverse biological processes, such as imprinting genomic loci, shaping chromosome conformation, regulating gene expression as molecular sponges, and regulating protein localization and enzymatic activity [19]. Because lncRNAs display a higher degree of tissue- and cell-type-specific expression profiles than protein-coding RNAs [20], emerging research regarding lncRNAs needs to be undertaken for every organ.

In this study, we aimed to explore the role of lncRNAs in the glucometabolic action of metformin on skeletal muscle cells.

2. Materials and methods

2.1. Cell culture and myotube differentiation

C2C12 mouse myoblasts were purchased from the American Type Culture Collection (ATCC, CRL-1772™, Manassas, VA). HuH-7 human hepatoma cells were provided from RIKEN Bio-Resource Center (Ibaraki, Japan). Both cell types were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose (DMEM, Sigma-Aldrich, St. Louis, MO) and 10% fetal bovine serum (Biowest, St. Louis, MO) at 37 °C in a humid atmosphere with 5% CO_2 and 95% air. In C2C12 cells, myotube differentiation was induced after the cells were grown to confluence by replacing the medium with that containing 5% horse serum (Invitrogen, Carlsbad, CA) according to the ATCC's instruction. Myotubes were used after five days of differentiation. Cell images were captured using an Eclipse TE2000-S inverted microscope (Nikon, Tokyo, Japan).

2.2. Metformin treatment

Metformin (1,1-Dimethybiguanide hydrochloride) was purchased from Sigma-Aldrich (D5035) and dissolved in DMEM. This solution was then sterilized by passing through a 0.2-μm filter. Next, the sterilized solution was immediately diluted to the final concentration indicated for each experiment with DMEM. DMEM was used as vehicle control. C2C12 myotubes were exposed to metformin for the analyses of medium glucose concentration and RNA and protein expression.

2.3. Metformin accumulation in C2C12 myotubes

Metformin accumulation in C2C12 myotubes and HuH-7 hepatoma cells was evaluated by measuring [^{14}C]-metformin uptake. Cells were cultured in a 24-well culture plates and allowed to differentiate into myotubes. The myotubes were treated with 1 mM of metformin [995 μM of unlabeled metformin and 5 μM of [^{14}C]-metformin (ARC1738, American Radiolabeled Chemicals, St. Louis, MO)] in DMEM for indicated times. At each time point, the cells were immediately washed three times with ice-cold phosphate-buffered saline (PBS). The cells in each well were lysed with 200 μL of 0.5 M NaOH. The radioactivity was measured using Ultima Gold scintillation cocktail (PerkinElmer, Waltham, MA) and an LSC-6100 liquid scintillation counter (Aloka, Tokyo, Japan). Protein concentrations were measured in a colorimetric assay using a BCA protein assay kit (#23227, Pierce, Rockford, IL). Metformin uptake was expressed as counts per minutes (CPM) corrected by protein concentration of cell lysates.

2.4. Measurement of glucose concentration in culture medium

C2C12 myotubes were exposed to metformin and the cell culture medium was collected in each experiment. The glucose concentration in the medium was measured using a Glucose Assay Kit (GAGO-20, Sigma-Aldrich) according to the manufacturer's instructions, with a slight modification for application using a 96-well plate. Briefly, 50-μL of diluted (40X) sample was mixed with 100 μL of the assay reagent and incubated for 30 min at 37 °C. The colorimetric reaction was stopped with 100 μL of 12 N H_2SO_4. The absorbance was measured at a wavelength of 540 nm using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA). The glucose concentration of the medium was calculated based on the standard curve in each experiment.

2.5. Protein extraction and immunoblotting

Total cell lysates were extracted using a lysis buffer containing 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, 10% glycerol, and a protease inhibitor cocktail (cOmplete® ULTRA tablet, Roche Diagnostics GmbH, Mannheim, Germany). Plasma membrane proteins were extracted using a Minute Plasma membrane isolation and cell fractionation kit (SM-005, Invent Biotechnologies, Plymouth, MN) according to the manufacturer's instructions. Protein concentrations were measured via a colorimetric assay using a BCA protein assay kit (Pierce). Western blotting was performed as described previously [21]. In brief, equal amounts of extracted protein (10 μg per lane) were resolved on an SDS–PAGE gel followed by western blotting using antibodies against GLUT1 (sc-7903) and GLUT4 (sc-7938) purchased from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin (sc-47778, Santa Cruz Biotechnology) was used as a loading control for total cell lysates and N-cadherin (#610920, BD transduction Laboratory, San Jose, CA) was for plasma membrane proteins. Anti-Rabbit-HRP-conjugated (#7045S, Cell Signaling Technology, Danvers, MA) and anti-mouse-HRP-conjugated (sc-2005, Santa Cruz Biotechnology) antibodies were used as secondary antibodies. The bands were visualized with a Light Capture II system (ATTO Co., Tokyo, Japan) and their intensities were quantified using the ImageJ software (version 1.52e, National Institutes of Health, Bethesda, MD). Results were normalized using the expression levels of the β-actin or N-cadherin, and calculated as fold change over control.

2.6. Detection of lncRNA in metformin-treated C2C12 myotubes

C2C12 myotubes were treated with 1 mM of metformin or vehicle for 48 h. Total RNA was extracted using an miRNeasy mini kit (Qiagen, Hilden, Germany), and genomic DNA was eliminated via a DNase-on-column treatment with RNase-free DNase set (Qiagen), according to the manufacturer's instructions. The quantification and qualification of RNA was performed using a spectrophotometer (NanoDrop ND-1000, NanoDrop Technologies, Wilmington, DE). The purified total RNA was reverse transcribed to cDNA using an RT^2 First Strand Kit (#330401, Qiagen), according to the manufacturer's protocol. In each treated group, the same amount of cDNA sample was collected into one sample for pair comparison. A panel of 84 lncRNAs was assessed using RT^2 lncRNA PCR array Mouse lncFinder (LAMM_001ZC, Qiagen) and RT^2 SYBR Green ROX qPCR MasterMix (#330520, Qiagen) with StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). Amplification was determined using the comparative threshold cycle (C_T) method with accompanied StepOne™ Software version 2.2.2 (Applied Biosystems). For normalization and analysis of the results, the C_T values were uploaded into the RT^2 PCR Array data analysis web

portal at https://www.qiagen.com/dataanalysiscenter. LncRNAs were listed with at least 2-fold increase or decrease in expression in the metformin-treated group compared with the vehicle-treated group.

2.7. LncRNA and gene expression analyses

Total RNA was extracted from the cells treated in each experiment using TRIzol Reagents (Thermo Fisher Scientific, Waltham, MA) to evaluate the expression of lncRNAs and genes. Two micrograms of total RNA were reverse transcribed (RT) with mixed primers consisted of oligo(dT) and random hexamer into cDNA using the PrimeScript™ RT reagent Kit with genomic DNA eraser (RR047A, TaKaRa Bio Inc., Kusatsu, Japan) according to the manufacturer's instructions. Oligo $(dT)_{15}$ primers (#3805, Takara Bio Inc.) were used for transcription to assess the polyadenylation of the RNA transcripts. Quantitative polymerase chain reaction (qPCR) analysis was performed in a reaction volume of 20 μl per well with a StepOnePlus™ Real-Time PCR System (96-well format, Applied Biosystems). The TaqMan Gene Expression Assays (Applied Biosystems) were used to determine the expression of lncRNA Dreh (Down-Regulated Expression by Hepatitis B virus X protein, Dreh, Mm04411209_g1), myogenin (Mm00446194_m1), metastasis-associated lung adenocarcinoma transcript 1 (Malat1, Mm03947719_s1), GLUT1 (Mm00441480_m1), GLUT4 (Mm00436615_m1), β-actin (Mm00607939_s1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Mm99999915_g1). Amplification was determined using the comparative threshold cycle (C_T) method with accompanied StepOne™ Software version 2.2.2 (Applied Biosystems). Results were normalized by the expression levels of the β-actin and GAPDH. Fold-changes were calculated relative to the control. All experiments were performed at least in triplicate.

2.8. RNA stability assay

Actinomycin D (#A9415, Sigma-Aldrich), an inhibitor of DNA-dependent RNA polymerase, was employed to stop RNA transcription and assess RNA stability of Dreh after metformin treatment. C2C12 myotubes were treated with actinomycin D (10 μg/mL) in combination with either metformin (1 mM) or vehicle (medium only) at 0, 3, 6, 12, and 24 h. At the end of each time point, the expression levels of Dreh were measured by RT-qPCR as described above. Results were calculated as fold changes relative to the value at time 0 in each group then compared between the treatment groups at each time point.

2.9. Silencing of Dreh expression

Predesigned small interfering RNAs (siRNAs) against Dreh (n443721, siDreh-1; n443723, siDreh-2) and negative control siRNA (#4390843, Silencer™ Select Negative control No.1 siRNA) were purchased from Thermo Fisher Scientific. Transfections into C2C12 myotubes were performed by lipofection using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions, with each siRNA at a final concentration of 10 nM. After 24 h of transfection, the culture medium was replaced with fresh medium containing 5% horse serum. The cells were examined at 48 h after transfection.

2.10. Glucose transport assay

Glucose transport in C2C12 myotubes was evaluated by measuring [^{3}H]-2-deoxyglucose uptake as described previously with minor modifications [22]. Myotubes easily came off the culture dish due to the many washing steps in this experiment. To avoid this occurrence, each well in a 12-well culture plate was coated with 1 mL of type I collagen (PSC-1-100-100, Nippi, Co. Ltd., Tokyo, Japan) solution at a concentration of 10 μg/mL in 5 mM acetate for 60 min. After washing the wells with sterilized PBS, C2C12 myoblasts were seeded, grown, and differentiated into myotubes. The myotubes were treated with siRNAs as described in the previous section. Then, the cells were washed with PBS and subsequently incubated with Krebs-Ringer-phosphate buffer containing 0.2% fatty-acid free bovine serum albumin (Sigma-Aldrich) for 30 min. The uptake of [^{3}H]-2-deoxyglucose was measured for an additional 30 min. The cells were lysed with 300 μL of 0.5 M NaOH per well. The radioactivity was measured using a scintillation cocktail (Ultima Gold) via a liquid scintillation counter (LSC-6100).

2.11. Stable transfection of Dreh in C2C12 myoblasts and effects of Dreh overexpression on metformin-induced glucose transport in C2C12 myotubes

The entire region of Dreh was amplified by PCR using KOD Fx Neo (#KFX-201, Toyobo, Osaka, Japan) with the mouse liver cDNA and a primer pair of 5′- AGGACTATAATCTAATGGTTTTTAGCAAGG -3' (sense) and 5′- CTTTTTATTTTATTGTACTTATAACCTTGG -3' (antisense). The product was subcloned into a pCAG-Hyg vector (#160-25611, Wako Pure Chemicals Industries, Ltd., Osaka, Japan) at the EcoRV site and confirmed by DNA sequencing, which resulted in the generation of the Dreh expression construct, pCAG-Hyg-Dreh. C2C12 cells were transfected with pCAG-Hyg-Dreh or pCAG-Hyg (mock) using Lipofectamine 3000 (Invitrogen) according to the manufacturer's instructions and screened for 10 days in the selection medium containing hygromycin B (300 μg/mL). For rescue experiments, stably transfected C2C12 myoblasts were differentiated into myotubes and used for the following experiments. The expression of Dreh and myogenin was confirmed by RT-qPCR, and the morphology was observed on a Nikon Eclipse TE2000-S inverted microscope. Glucose uptake was measured using [^{3}H]-2-deoxyglucose during the last 30 min of the treatment of metformin (1 mM) for 48 h. Glucose assay in medium was performed after the treatment of metformin (1 mM) for 48 h. To eliminate the effects of multiple experimental steps in an overexpression study on cellularity, relative reduction ratio of medium glucose concentration was calculated by subtracting the glucose concentration at the end of the experiment from the concentration at the beginning of the experiment (450 mg/dL). The values were corrected by protein concentration (Pierce) and the relative reduction ratio was calculated the values of metformin versus vehicle.

2.12. Statistical analysis

The results are expressed as means ± SEM. Two-tailed unpaired Student's *t*-tests were used for the comparison of two treatment groups. To compare more than two groups, an ANOVA was performed followed by Tukey's post-hoc test using PRISM software (version 7.0e, GraphPad Software Inc., San Diego, CA). Differences were considered significant if $p < 0.05$.

3. Results

3.1. Metformin accumulates in C2C12 myotubes

Myotube formation from C2C12 myoblasts was confirmed. Myoblast differentiation was successful (Fig. 1A and B). The C2C12 myotubes were treated with 1 mM metformin for 48 h and their morphology was examined by microscopy. No obvious change was seen between vehicle and metformin treatment (Fig. 1C, D). Metformin uptake in the C2C12 myotubes was assessed using [^{14}C]-metformin and compared with the uptake in HuH-7 human hepatoma cells. The accumulation of [^{14}C]-metformin in C2C12 myotubes was significantly lower than that in HuH-7 cells from 3 min to 4 h, but was significantly higher from 24 to 48 h (Fig. 1E). The data suggested that metformin accumulates in C2C12 myotubes for a longer period of time.

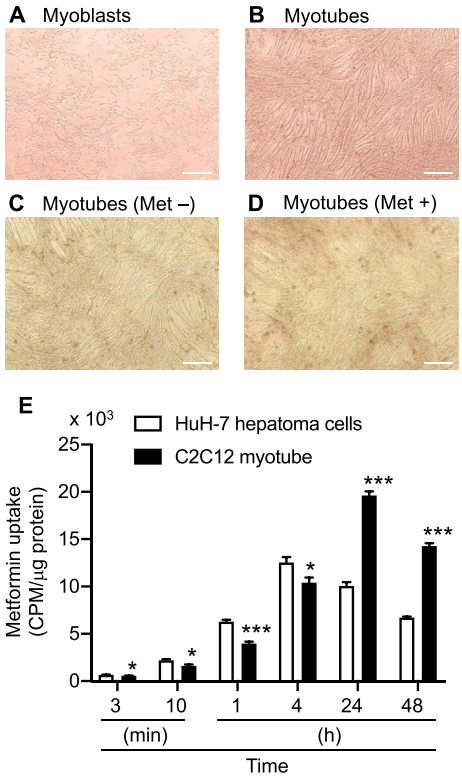

Fig. 1. Metformin accumulates in C2C12 myotubes. Cell images of C2C12 myoblasts (**A**), myotubes (**B**), myotubes treated with vehicle (**C**), and myotubes with 1 mM of metformin for 48 h (**D**) were captured using an inverted microscope (40 ×, *Scale bars*; 200 μm). (**E**) Uptake of [^{14}C]-metformin in HuH-7 hepatoma cells and C2C12 myotubes was expressed as counts per minutes (CPM) corrected by protein concentration (n = 6). Data are presented as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ vs. HuH-7 hepatoma cells.

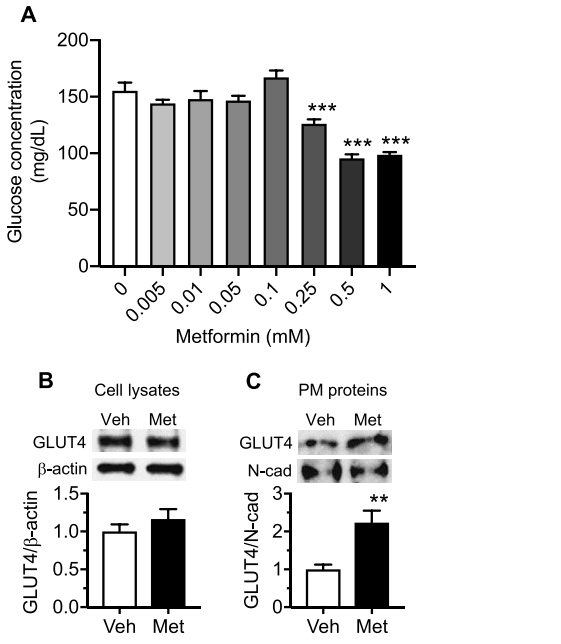

Fig. 2. Metformin reduces glucose concentration in the medium of C2C12 myotubes. (**A**) Glucose concentration of culture medium of the C2C12 myotubes treated with various concentrations of metformin for 48 h (n = 6). (**B**, **C**) Protein expression levels of GLUT4 in total cell lysates (**B**) or plasma membrane (PM) protein extracts (**C**) of the myotubes treated with vehicle or metformin. DMEM was used as vehicle control. β-actin and N-cadherin (N-cad) were used as the internal controls for total cell lysate proteins and PM proteins, respectively. Representative blots and protein quantifications are shown (n = 6). Data are presented as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. vehicle.

3.2. Metformin reduces the glucose concentration of the medium in C2C12 myotubes via increased GLUT4 levels on the plasma membrane

The glucose-lowering effect of metformin in C2C12 myotubes was confirmed. The glucose concentration of the medium of metformin-treated myotubes was significantly reduced at higher than or equal to 0.25 mM metformin (Fig. 2A). The protein expression of GLUT4 in the total cell lysates of cells treated with metformin was not altered (Fig. 2B), but its expression in the plasma membrane (PM) protein extracts was significantly increased after treatment with metformin compared with the vehicle (Fig. 2C).

3.3. Metformin represses Dreh expression in C2C12 myotubes at the transcriptional level

The expression profiles of lncRNAs in the myotubes treated with metformin were investigated. Among the 84 lncRNAs tested, metformin increased 13 lncRNAs and decreased 7 by more than 2-fold compared with vehicle (Table 1). Using RT-qPCR, it was confirmed that Dreh (NCBI Accession No. AK050349; UCSC ID uc008dfz), the most changed lncRNA by the array, was significantly decreased by metformin in dose- and time-dependent manners (Fig. 3A, B).

To evaluate the molecular mechanism of the Dreh reduction by metformin was investigated, specifically whether it has a poly(A) tail or not at the 3′end. GAPDH and Malat1 were used as positive controls for polyadenylated RNA and non-polyadenylated RNA, respectively [23]. The relative expression ratio shown in Fig. 3C indicated the possibility of polyadenylation in each transcript. The results showed that Dreh prefers to be polyadenylated at the 3′ end of the transcripts. We next considered the possibility that the Dreh stability is reduced by the effects of metformin. To test this, we examined whether or not metformin treatment affected the Dreh expression levels in the presence of actinomycin D (ActD), an inhibitor of DNA-dependent RNA polymerase. As shown in Fig. 3D, the decay in the actinomycin D plus metformin group (ActD + Met) was similar to that in the actinomycin D group (ActD). The estimated half-life in the presence and absence of the metformin treatment was 4.07 and 4.13 h, respectively. The results indicated that metformin treatment did not affect the decay rate of Dreh, suggesting that the metformin-mediated Dreh repression occurs at the transcriptional level.

The expression of lncRNA H19 was validated. The expression of H19 was significantly increased by metformin in a dose-dependent manner (Supplementary Fig. 1), as expected.

3.4. Dreh expression in the myogenesis of C2C12 cells

The expression profiles of Dreh in C2C12 cells were clarified because the biology of Dreh expression has not yet been elucidated. During myotube differentiation, the expression levels of Dreh were maintained, although they were temporally repressed (Fig. 4A). The induction of myogenin was used as a control for myotube formation (Fig. 4B).

3.5. Silencing of Dreh facilitates glucose uptake in C2C12 myotubes

The role of Dreh repression on glucose metabolism in C2C12 myotubes was explored because Dreh was the most changed lncRNA in the treatment of metformin. First, it was confirmed that the two specific siRNAs effectively knocked-down the Dreh transcripts (Fig. 5A) without affecting DNA and total RNA contents (Supplementary Figs. 2A and B). Second, medium glucose concentration of the Dreh-depleted myotubes was significantly reduced compared with vehicle control (Fig. 5B). siRNA-2 was used for subsequent experiments because more prominent

Table 1
Metformin regulated lncRNAs in C2C12 myotubes.

Symbol	Description	Reference Sequence	Fold-change
Up-regulated			
Fendrr	Foxf1 adjacent non-coding developmental regulatory RNA	ENSMUST00000181231	26.90
Uchl1os	Ubiquitin carboxy-terminal hydrolase L1, opposite strand	ENSMUST00000152002	9.86
Tunar Tcl1	upstream neural differentiation associated RNA	ENSMUST00000180458	6.38
Pldi	Polymorphic derived intron containing	ENSMUST00000036304	4.48
Sox2ot	SOX2 overlapping transcript (non-protein coding)	ENSMUST00000163261	3.91
Emx2os	Emx2 opposite strand/antisense transcript (non-protein coding)	ENSMUST00000136990	3.80
Neat1	Nuclear paraspeckle assembly transcript 1 (non-protein coding)	ENSMUST00000173672	3.38
Nespas	Neuroendocrine secretory protein antisense	ENSMUST00000143738	2.91
H19	H19 fetal liver mRNA	NR_001592	2.39
Nctc1	Non-coding transcript 1	ENSMUST00000123668	2.33
1810053B23Rik	RIKEN cDNA 1810053B23 gene	NR_040486	2.21
Pvt1	Plasmacytoma variant translocation 1	ENSMUST00000180432	2.16
Hottip	Hoxa distal transcript antisense RNA	ENSMUST00000141300	2.05
Down-regulated			
Dreh	Down-regulated in hepatocellular carcinoma	NR_105051	–290.93
G730013B05Rik	RIKEN cDNA G730013B05 gene	ENSMUST00000180509	–18.79
Pinc	Pregnancy induced noncoding RNA	NR_003202	–17.76
9530059O14Rik	RIKEN cDNA 9530059O14 gene	ENSMUST00000181107	–16.73
Gm17750	Predicted gene, 17750	ENSMUST00000182477	–13.19
Rian	RNA imprinted and accumulated in nucleus	ENSMUST00000180876	–12.35
Gm14005	Predicted gene 14005	ENSMUST00000125354	–2.14

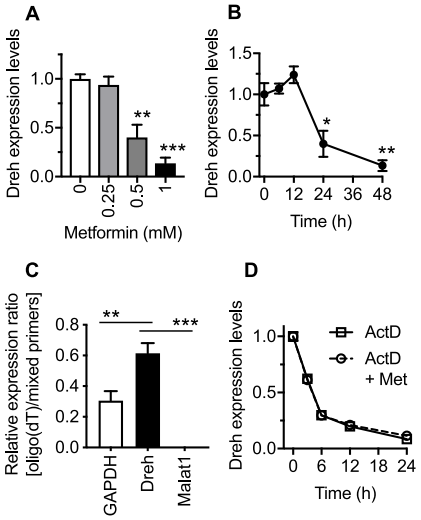

Fig. 3. Metformin represses lncRNA Dreh expression at the transcriptional level. (**A**) Dreh expression levels in C2C12 myotubes after treatment with various doses of metformin for 48 h measured using RT-qPCR (n = 4). (**B**) Dreh expression levels in C2C12 myotubes after treatment with 1 mM of metformin for 0–48 h measured using RT-qPCR (n = 4). (**C**) Polyadenylation at the 3′ end of the transcripts. Expression levels of GAPDH, Dreh, and Malat1 were determined by RT-qPCR in which the RT reaction was performed using either mixed primers consisting of oligo(dT) and random hexamer or oligo(dT) primers. Relative expression ratio was then calculated as the results of oligo(dT) primers divided by those of mixed primers (n = 6). GAPDH and Malat1 were used as positive controls for polyadenylated RNA and non-polyadenylated RNA, respectively. (**D**) Effect of metformin on the stability of Dreh transcripts in C2C12 myotubes treated with actinomycin D (10 μg/mL, ActD) or actinomycin D in combination with 1 mM metformin (ActD + Met). Expression levels of Dreh measured and compared at 0, 3, 6, 12, and 24 h using RT-qPCR (n = 6). There are no statistically significant differences between each comparison in the same time point. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs. vehicle (**A**), time 0 (**B**), or Dreh (**C**).

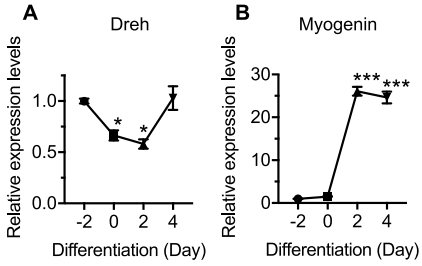

Fig. 4. Characterization of Dreh expression in C2C12 cells. Expression of (**A**) Dreh and (**B**) myogenin during differentiation from myoblasts to myotubes in C2C12 cells measured through RT-qPCR (n = 3). mRNA expression of myogenin was used as a control for myotube formation. Data are presented as mean ± SEM. $*p < 0.05$, $***p < 0.001$ vs. Day −2.

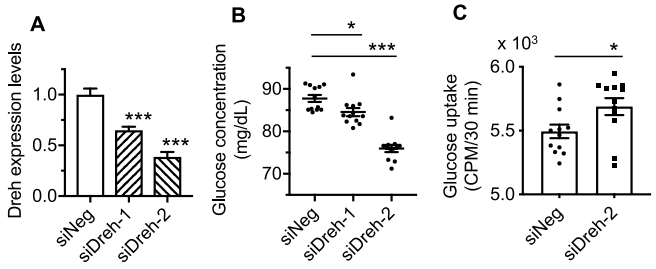

Fig. 5. Knockdown of Dreh expression facilitates glucose uptake in C2C12 myotubes. (**A**) Dreh expression in the C2C12 myotubes treated with siRNAs for Dreh (siDreh-1, siDreh-2) or negative control siRNA (siNeg) determined by RT-qPCR (n = 6). (**B**) Glucose concentration of the culture medium of the C2C12 myotubes treated with siRNAs as in (**A**) (n = 12). (**C**) Uptake of [^{3}H]-2-deoxyglucose in the C2C12 myotubes treated with siNeg or siDreh-2 (n = 12) by liquid scintillation counting, expressed as counts per minutes (CPM). $*p < 0.05$, $***p < 0.001$ vs. siNeg.

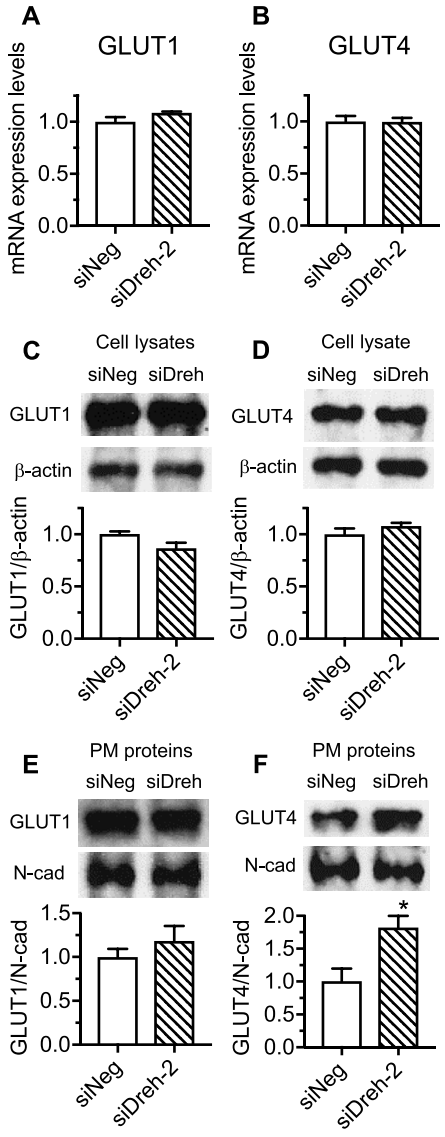

Fig. 6. Protein expression of GLUT4 increases in the plasma membrane (PM) of the Dreh-depleted myotubes. C2C12 myotubes were treated with negative control siRNA (siNeg) or Dreh-specific siRNA (siDreh-2). Gene expression levels of GLUT1 (**A**) and GLUT4 (**B**) in the myotubes by RT-qPCR. Protein expression levels of GLUT1 (**C**, **E**) and GLUT4 (**D**, **F**) in total cell lysates (**C**, **D**) or PM proteins (**E**, **F**) in the myotubes by western blotting. β-actin or N-cadherin (N-cad) were used for the internal control of total cell lysates or PM proteins, respectively. Representative blots and their quantifications are shown (n = 4). Data are presented as mean ± SEM. *$p < 0.05$ vs. siNeg.

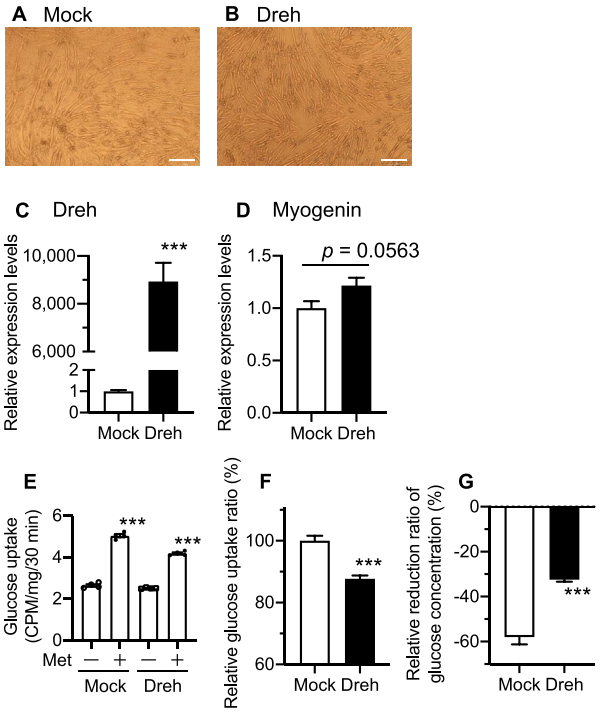

Fig. 7. Overexpression of Dreh attenuates metformin-induced glucose uptake in C2C12 myotubes. C2C12 myoblasts were stably transfected with Dreh-expressing vector (Dreh) or mock vector (pCAG-Hyg, Mock). Then, the cells were induced to form myotubes. Representative cell images of mock- (**A**) or Dreh-expressing myotubes (**B**) captured using an inverted microscope (100 × , *Scale bars*; 400 μm). The expression levels of Dreh (**C**) or myogenin (**D**) were determined in the mock- or Dreh-expressing myotubes by RT-qPCR (n = 6). (**E**) Uptake of [^{3}H]-2-deoxyglucose in the transfected C2C12 myotubes (Mock or Dreh) during treatment with vehicle (Veh) or metformin (1 mM, Met) was measured. The activity counts are expressed as counts per minutes (CPM) per mg of protein (n = 6). (**F**) By analyzing the data on (**E**), metformin-induced glucose uptake compared with vehicle (Met/Veh) in the Dreh or mock-expressing myotubes. (**G**) Relative reduction ratio of glucose concentration in the culture medium of Dreh or mock-expressing myotubes by the treatment of metformin (1 mM) for 48 h compared with vehicle (n = 6). ***$p < 0.001$ vs. mock (**C**, **F**, **G**) or the vehicle of mock (**E**).

effects were observed in the Fig. 5A. Third, glucose uptake in the Dreh-depleted myotubes using [^{3}H]-2-deoxyglucose significantly increased compared with vehicle control (Fig. 5C). These results indicate that the repression of Dreh expression facilitates glucose uptake in C2C12 myotubes.

3.6. Dreh depletion exhibits increased GLUT4 expression on the plasma membrane in myotubes

Next, the expression levels of glucose transporters, especially GLUT1 and GLUT4, in the Dreh-depleted myotubes were studied. The mRNA levels of transporters in myotubes treated with siNeg or siDreh-2 were not altered (Fig. 6A, B). The protein expression levels of both transporters in the total cell lysates of the myotubes were also not altered (Fig. 6C, D). GLUT4 but not GLUT1 expression was found to be significantly increased in Dreh-depleted myotubes (Fig. 6E, F) through further analysis of the plasma membrane proteins.

3.7. Overexpression of Dreh attenuates metformin-induced glucose uptake in myotubes

To validate the effects of Dreh on metformin-induced glucose uptake, stably Dreh- or mock-expressing myoblasts were established. The construct-expressing myoblasts were induced to differentiate into myotubes. The differentiation of both Dreh and mock-expressing cells was morphologically normal into myotubes under light microscopy (Fig. 7A and B). The expression levels of Dreh in the myotubes derived from Dreh-expressing myoblasts were significantly higher (8,932-fold) than mock transfected myotubes, suggesting successful overexpression of Dreh in the myotubes (Fig. 7C). The expression levels of myogenin, a myogenic marker, were also confirmed in both constructs-expressing myotubes (Fig. 7D). The expression level in the Dreh-overexpressing myotubes were slightly, but not significantly, higher than those in mock cells ($p = 0.0563$).

Metformin-induced glucose transport was assessed in the Dreh- or mock-overexpressing myotubes using [^{3}H]-2-deoxyglucose. Metformin treatment significantly increased the glucose transport in both overexpressed myotubes (Fig. 7E). The results were analyzed in each construct-expressing myotubes (Fig. 7F). Overexpression of Dreh

significantly suppressed the metformin-induced glucose uptake by 12.3% compared to the mock myotubes during 30-min measurements (100.0 ± 1.6 vs. 87.7 ± 1.1%). Similarly, overexpression of Dreh attenuated the metformin-induced decrease of medium glucose levels by 44.0% compared to the mock-overexpressing myotubes for 48 h (−58.0 ± 3.2 vs. −32.5 ± 0.8%, Fig. 7G). These rescue studies suggested that Dreh is involved in the metformin-induced glucose transport in C2C12 myotubes.

4. Discussion

Metformin increases glucose transport in skeletal muscles *in vivo* [10–13] and *in vitro* [14–16], but its mechanisms have remained elusive. In this study, we searched for novel lncRNAs related to the glucometabolic action of metformin on skeletal muscle cells. As a result, metformin significantly repressed the expression of the lncRNA Dreh in C2C12 myotubes. In addition, the knockdown of Dreh facilitated glucose uptake by increasing the expression of GLUT4 on the plasma membrane of myotubes. Moreover, overexpression of Dreh significantly attenuated the metformin-induced glucose uptake. The collective results indicate that Dreh is a novel RNA regulator for glucose uptake and that it is involved in the glucose-lowering effect of metformin in myotubes.

Metformin is an unusual hydrophilic drug and its transport involves an active uptake process via organic cation transporters (OCT) [24]. OCT1 and OCT3 are expressed in isolated rat epitrochlearis and soleus muscles, and the transport of metformin is increased up to 60 min [25], suggesting that metformin rapidly accumulates in skeletal muscle. However, it has been unclear whether metformin can accumulate in C2C12 myotubes for a longer time. Here, we evaluated the uptake of metformin in C2C12 myotubes compared with HuH-7 hepatoma cells, a liver-derived cell line that expresses low levels of OCT1 and OCT3 [26]. Metformin gradually accumulated in C2C12 myotubes for 24–48 h. Thus, we exposed the myotubes to metformin in this study.

Metformin concentrations have differed significantly between *in vivo* and *in vitro* models. In clinical studies, many reports suggest that the plasma metformin concentrations are in the micro-molar range [27]. For example, the peak plasma concentration occurred in 2 h at 1.02 ± 0.34 μg/mL (approximately 7.9 μM) after a 0.5 g dose and 3.10 ± 0.93 μg/mL (approximately 24.0 μM) using a dose of 1.5 g in type 2 diabetes subjects [28]. On the other hand, relatively high concentrations of approximately 1 to 2 mM are widely used in *in vitro* studies. For instance, glucose uptake was increased by the treatment of C2C12 myotubes with 1 mM metformin for 16 h [16]. Similarly, increased glucose uptake and GLUT4 levels in the plasma membrane were achieved by the treatment of C2C12 myotubes with 1 mM metformin for 24 h [29]. Additionally, in rat L6 myotubes or cardiomyocytes, increased glucose uptake was observed in the presence of 1 to 10 mM metformin [15]. Consistent with these previous studies, we confirmed that mM concentrations of metformin lowered the medium glucose concentrations and increased GLUT4 levels in the plasma membrane in C2C12 myotubes. Although the concentrations we used are relatively higher than the therapeutic levels in the systemic circulation [30], mM levels are necessary for the glucometabolic action of metformin *in vitro*. This discrepancy may arise from the difference between human and rodents. Alternatively, gradual accumulation of metformin may be necessary to produce full activity, because improvement in hyperglycemia in patients with type 2 diabetes is seen only after several weeks of metformin treatment [30].

By searching for novel lncRNAs affected by metformin, we found that the expression of a long noncoding RNA, Dreh, was significantly suppressed by metformin. Several reports have mentioned the altered expression levels of lncRNAs in relation to the effects of metformin on various tissues and cells, such as pulmonary arteries tissues of pulmonary artery hypertension rats [31], cervical cancer cells [32], and the livers of non-alcoholic fatty liver disease mice [33]. To our knowledge, none of these reports documented Dreh. Thus, Dreh is a novel metformin-regulated lncRNA.

Dreh is composed of 727 nucleotides and was originally identified by Huang et al. as a down-regulated lncRNA in the liver of hepatitis B virus X protein transgenic mice [34]. The authors also identified a human ortholog of Dreh, which is termed DREH. These authors and those of another group have shown that the inhibition of Dreh/DREH promotes cell proliferation and migration in liver cell lines, whereas overexpression of Dreh/DREH inhibits the growth and metastasis of hepatocellular carcinoma cells *in vivo* [34]. Very recently, Ruan et al., reported that Dreh suppression is related to liver regeneration in rat hepatic progenitor cells [35]. These reports provide evidence that Dreh is involved in cell growth. Since we used the knockdown method to investigate the function of Dreh, one might think that the modulation of Dreh expression could affect cell growth in the myotubes. Therefore, we measured the total DNA and RNA contents as a substitute for the evaluation of cell numbers, because myotubes are fused cells and are impossible to enumerate. The finding that Dreh repression by its specific siRNAs did not alter the total DNA and RNA contents indicates that Dreh does not affect cell growth in C2C12 myotubes under a transient knockdown condition. This difference in cell growth between hepatic cells and myotubes under the Dreh-depleted conditions might be due to the cell type-specificity, which is consistent with the common understanding that lncRNA functions are more tissue-specific compared to protein-coding gene functions [20].

LncRNAs include polyadenylated and non-polyadenyalted transcripts in its 3′ end. Dreh can be a polyadenylated transcript confirmed by the transcription with oligo(dT) primers. This suggests that Dreh is transcribed by RNA polymerase II, similar to messenger RNA. Reduced Dreh expression by metformin can be due to either suppression of transcription or increase in degradation. By stopping transcription using actinomycin D, reduced Dreh expression by metformin was not due to enhanced degradation, suggesting that the mechanisms of metformin-induced Dreh repression occur at the transcriptional level. Metformin can modulate epigenetic enzymes such as histone acetyltransferases, class II histone deacetylases, and DNA methyltransferases [36] by activating AMP-kinase [5]. Therefore, the repression of Dreh transcription by metformin may be mediated through epigenetic mechanisms.

As the basic characteristics of Dreh in myogenesis have not been clarified, the expression profiles of Dreh during the myogenesis of C2C12 cells were confirmed. The expression levels of Dreh in C2C12 myotubes were similar to those in myoblasts. However, transient repression of Dreh expression was observed during myogenesis. Myotube differentiation from Dreh-overexpressed myoblasts was successful, although myogenin expression was slightly, but not significantly, increased in Dreh-overexpressed myotubes compared with mock-expressed myotubes. A recent study revealed that several lncRNAs are involved in muscle differentiation and regeneration [37]. Dreh may also have some roles in muscle characteristics. The precise effects of Dreh in myogenesis will be analyzed in the future.

We found that Dreh is a novel RNA regulator for glucose transport accompanied with the increase of GLUT4 on the plasma membrane in the myotubes. The functions of lncRNA are diverse [19,38]. Indeed, lncRNAs are involved in several biological processes including chromatin organization, transcriptional and post-transcriptional gene expression, and also act as structural scaffolds of nuclear domains [39]. Moreover, lncRNAs can form complexes with proteins to perform diverse structural and regulatory functions, including mRNA turn over, translation, protein stability, sponging of cytosolic factors, and modulation of signaling pathways [40]. The possible mechanisms by which Dreh repression increases GLUT4 expression in the plasma membrane could be mediated by the interaction between Dreh and specific molecules including RNAs and proteins. Thus, lncRNA has potential to act as a competing endogenous RNA by targeting specific miRNA [41]. We searched for miRNAs that potentially interact with Dreh using the

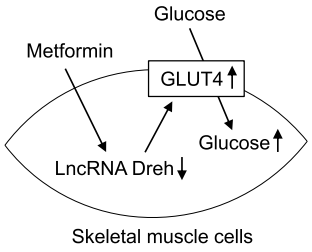

Fig. 8. A model showing that metformin reduces lncRNA Dreh expression, and Dreh silencing facilitates glucose uptake via increased GLUT4 expression on the cell surface in skeletal muscle cells. Dreh is involved, at least in part, in the glucose-lowering effect of metformin in the myotubes.

database, miRBase (http://www.mirbase.org/), and identified mir-717 as a likely candidate. However, our RT-qPCR did not detect the expression of mir-717 in C2C12 myotubes (data not shown), indicating that this miRNA does not bind to Dreh in this cell type. The possibility of other miRNAs interacting with Dreh cannot be ruled out. However, we prefer another possibility. A clue to investigate the mechanism arose from the previous finding that Dreh can combine with vimentin, an intermediate filament protein, to inhibit tumor metastasis of hepatocellular carcinoma [34]. In addition, the knockdown of vimentin expression exhibits a decline in GLUT4 expression in 3T3-L1 adipocytes [42]. Considering these reports, decreased Dreh expression may release the binding to vimentin, thereby increasing GLUT4 expression in the plasma membrane. Although those studies are performed in different types of tissues and cells, vimentin may be a candidate for increased GLUT4 expression induced by the repression of Dreh.

Interestingly, previous studies showed that increased GLUT4 expression in the plasma membrane is observed during metformin treatment [15,29], as well as by Dreh depletion. Concerning GLUT4 expression in the plasma membrane, an increase in the steady-state level of GLUT4 in this membrane is due to the stimulation of its mobilization for insertion into the plasma membrane (exocytosis) or reduction in its internalization from the surface and the return to intracellular stores (endocytosis) [43]. In this view, metformin stimulates glucose transport in myocytes by the reduction of GLUT4 endocytosis [14]. Many proteins are involved in the GLUT4 traffic such as Rab GTPase-activating proteins and downstream Rab GTPases along with the input of Rac1 and actin filaments, molecular motors, and membrane fusion regulators [44,45]. Dreh may interact with these traffic proteins to increase the protein expression of GLUT4 in the cell surface. To date, a few molecules, including protein kinase C [15,46], tiam-1 [16], and Src homology 2 domain-containing inositol-5-phoshatase 2 [47], have been reported to be involved in the glucose-lowering effect of metformin. Future research will examine the interaction of Dreh with these intracellular molecules.

Recently, a number of lncRNAs have been implicated in glucose metabolism and diabetes [48–50]. Many such lncRNAs are reported in cancer [51]. For example, knockdown of lncRNA ANRIL decreased the protein expression of GLUT1 in nasopharyngeal carcinoma cell lines [52], and increased expression of lncRNA NBR2 by the treatment of phenformin induced GLUT1 expression in kidney and breast cancer cell lines [53]. A recent report has shown that lncRNA H19 promotes muscle insulin sensitivity in part via the increased phosphorylation of AMPK [54]. In this study, we identified that metformin significantly increases H19 expression, suggesting that the upregulation of H19 may be an intermediate in the glucometabolic effects of metformin. Based on our results, Dreh is implicated as a new lncRNA in glucose metabolism.

There are a several limitations to this study. First, more precise mechanisms between repressed Dreh expression and increased GLUT4 expression in the plasma membrane need to be clarified. Second, the differences between mouse cells *in vitro* and human tissues *in vivo* need to be considered. Third, concentrations used in our study were supra-pharmacological in the millimolar range, which is above plasma concentrations reported in the systemic circulation at therapeutic levels [30]. These issues will be addressed in future studies.

Skeletal muscle is a promising target for the treatment of diabetes because it is the largest organ in non-obese subjects [55], the most important site of insulin- and exercise-stimulated glucose disposal [56], and the primary site of insulin resistance in type 2 diabetes [57]. Our data indicate that Dreh in muscle can be a target for the treatment of diabetes by the manipulation of its expression levels, such as antisense oligonucleotide therapeutics [58].

5. Conclusions

This study implicates Dreh as a novel glucose regulating lncRNA and also indicates that the glucoregulatory actions of metformin are mediated in part by Dreh repression in skeletal muscle cells (summarized in Fig. 8). In addition, Dreh could be a new target for the treatment of diabetes. Dreh was coined as an abbreviation for “Down-Regulated Expression by Hepatitis B Virus X Protein” [34]. Considering our findings, we propose that Dreh denotes “Down-Regulated Expression-related Hexose/Glucose Transport Enhancer by Metformin.”

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116906.

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Author contributions

Nobuhiko Takahashi: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing – Original Draft. Atsushi P. Kimura: Methodology, Investigation, Validation, Writing – Original Draft. Kai Otsuka: Investigation. Kazumasa Ohmura: Resources. Sumiyoshi Naito: Resources. Mika Yoshida: Resources. Masahiro Ieko: Supervision.

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