Type I neuregulin1α is a novel local mediator to suppress hepatic gluconeogenesis in mice

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Neuregulin1 is an epidermal growth factor (EGF)-like domain-containing protein that has multiple isoforms and functions as a local mediator in the control of various cellular functions. Here we show that type I isoform of neuregulin1 with an α-type EGF-like domain (Nrg1α) is the major isoform in mouse liver and regulates hepatic glucose production. Forced expression of Nrg1α in mouse liver enhanced systemic glucose disposal and decreased hepatic glucose production with reduced fasting blood glucose levels. Nuclear forkhead box protein O1 (FoxO1) and its downstream targets, PEPCK and G6Pase, were suppressed in liver and isolated hepatocytes by Nrg1α overexpression. In contrast, silencing of Nrg1α enhanced glucose production with increased PEPCK and G6Pase expressions in cAMP/dexamethasone-stimulated hepatocytes. Mechanistically, the recombinant α-type EGF-like domain of NRG1α (rNRG1α) stimulated the ERBB3 signalling pathway in hepatocytes, resulting in decreased nuclear FoxO1 accumulation via activation of both the AKT and ERK pathways. In addition, acute treatment with rNRG1α also suppressed elevation of blood glucose levels after both glucose and pyruvate challenge. Although a liver-specific deletion of Nrg1 gene in mice showed little effect on systemic glucose metabolism, these results suggest that NRG1α have a novel regulatory function in hepatic gluconeogenesis by regulating the ERBB3-AKT/ERK-FoxO1 cascade.

De novo synthesis of glucose from non-carbohydrate substrates, such as lactate, glycerol, and alanine, in the liver, a process known as hepatic gluconeogenesis, is essential for maintaining blood glucose levels in a narrow range during starvation. The brain uses glucose as a favourable energy source even under low blood glucose levels; thus, sustained hepatic glucose production is required to circumvent life-threatening hypoglycaemia in fasting conditions. Aberrant activation of liver gluconeogenesis, in contrast, is strongly associated with hepatic insulin resistance and leads to abnormal increases in both fasting and postprandial blood glucose levels in diabetes mellitus. The coordinated regulatory mechanisms of hepatic glucose production have been extensively studied, and key hormones, such as insulin, glucagon, and glucocorticoids, have been shown to serve as crucial modulators in liver gluconeogenesis. In addition to these classical hormones, many extrahepatic organ-derived molecules, known as adipokines and myokines, have recently been reported to play important roles in the regulation of hepatic glucose production.1-2. The liver also participates in metabolic regulation by secreting several humoral factors, such as fetuin-A, fibroblast growth factor 21, and selenoprotein P, which modulate systemic glucose tolerance and insulin sensitivity without any apparent effects on liver gluconeogenesis.3-4. However, the importance of locally released proteins from the liver in hepatic glucose production is unclear.

Neuregulin1 (NRG1) is a growth factor that contains an epidermal growth factor (EGF)-like domain and consists of six major types with differing N-terminal regions. The various isoforms are generated due to either alternative splicing or use of 5′ flanking regulatory regions in humans.5. NRG1 is actively cleaved at the juxtamembrane region by type I transmembrane proteases, such as β-site amyloid precursor protein cleaving enzyme, releasing the extracellular portion of NRG1. This extracellular molecule contains a biologically active EGF-like domain and functions as a local mediator in either an autocrine or paracrine fashion. The cleaved ectodomain of NRG1 serves as a ligand for two ERBB receptor tyrosine kinases, ERBB3 and ERBB4. Consequently, it activates intracellular signalling cascades to regulate diverse biological processes involved in heart development, cancer,
NRG1 enhances systemic glucose disposal after glucose challenge by activating ERBB3-mediated AKT phosphorylation in rodents. In addition, NRG1 also shows the same blood glucose-lowering effects by stimulating ERBB3-AKT-FoxO1 signalling cascade and subsequently inhibiting hepatic gluconeogenesis in diabetic mice. These observations indicate that NRG1 has important roles in the regulation of systemic glucose metabolism by activating the ERBB3-mediated signalling pathways, although little information is available on which NRG1 isoforms are expressed in the liver and their functions as a local mediator of hepatic glucose metabolism.

In the present study, we have demonstrated that the type I isoform of NRG1 with an α-type EGF-like domain (NRG1α) suppresses hepatic glucose production, in part, by activating both the AKT and ERK signalling cascades and subsequently inhibiting FoxO1-mediated gluconeogenic gene expression. These results suggest that NRG1α acts as a local regulator of liver gluconeogenesis in an autocrine/paracrine manner.

**Results**

Type I Nrg1α is a major isoform of Nrg1 in normal mouse liver. NRG1 has been reported to produce six N-terminal types of proteins with various isoforms using distinct promoters and alternative splicing. To identify which isoforms of Nrg1 are expressed in mouse liver, we performed a conventional PCR using specific primer sets and determined the sequences of the PCR products. Two distinct types of Nrg1, Type I Nrg1α and Type III Nrg1β, were predominantly detected in the liver by PCR (Fig. 1 and Supplementary Fig. S1). However, repeated sequencing of the latter isoform revealed that exon sequences encoding its N-terminal domain were not identical to those reported in the brain and lacked the final 70 nucleotides of exon 1, introducing premature stop codons in the EGF-like domain of Type III Nrg1β. As the EGF-like domain is required for biological functions via binding to its receptors, Type I NRG1α (designated NRG1α) is likely the only physiologically active NRG1 isoform in mouse liver.

NRG1 is proteolytically cleaved to locally release its extracellular region containing the EGF-like domain. We next determined whether the N-terminal ectodomain of hepatic NRG1α is released locally using isolated primary hepatocytes. Endogenous NRG1α protein was barely detectable in whole cell extracts but was found to be constitutively cleaved and released its N-terminal ectodomain (~45 kDa) in the concentrated conditioned medium of hepatocytes (Supplementary Fig. S2a). Consistent with this result, cleaved NRG1α fragment was enriched in the conditioned medium with small amounts of both full-length (~100 kDa) and cleaved (~45 kDa) form of NRG1α in the cell lysate when hepatocytes were infected with a Nrg1α-expressing adenovirus (Supplementary Fig. S2b). These results support the hypothesis that NRG1α acts as a local mediator in an autocrine and/or paracrine manner in liver.

Forced Nrg1α expression in liver improves systemic glucose disposal and decreases hepatic gluconeogenesis. To examine effects of hepatic NRG1α overexpression in regulating glucose metabolism, we injected plasmids encoding mouse Nrg1α tagged with a C-terminal Flag peptide into mouse livers three times every 6 days by hydrodynamic tail vein injection. Two days after the last injection, NRG1α protein was detected at ~100 kDa exclusively in the liver but not other tissues, such as skeletal muscle and epididymal adipose tissue using an anti-Flag antibody (Supplementary Fig. S3a). Consistent with this result, cleaved NRG1α fragment was predominantly observed in liver treated with the Nrg1α-expressing plasmid and not an empty vector using an anti-NRG1α antibody (Supplementary Fig. S3b). These results suggest that normal mouse liver shows limited expression of NRG1α at basal conditions, and that we have succeeded to overexpress NRG1α predominantly in the liver in the same manner to endogenously expressed NRG1α.

Systemic glucose disposal after oral glucose challenge (OGTT) was significantly enhanced in mice overexpressing Nrg1α compared to that of mice receiving the empty vector (Fig. 2a), whereas this treatment had little, if any, effect on insulin sensitivity in the peripheral tissues (Fig. 2b). To confirm the effects of hepatic NRG1α overexpression on peripheral insulin signalling, we measured the phosphorylation levels of AKT in response to insulin in skeletal muscle and epididymal adipose tissue. Overexpression of hepatic Nrg1α did not show any impacts on insulin-induced phosphorylation of AKT in these tissues (Supplementary Fig. S4). Forced expression of Nrg1α in the liver also attenuated hepatic gluconeogenic activity with decreased fasting glucose levels in pyruvate tolerance test (PTT) (Fig. 2c). NRG1 exerts its biological functions by binding to two single-transmembrane receptor tyrosine kinases, ERBB3 and ERBB4.

The Nrg1α-induced inhibitory action on the hepatic glucose production was tightly associated with selective phosphorylation of ERBB3 in the liver overexpressing Nrg1α (Supplementary Fig. S5). In contrast, we hardly detected ERBB4 phosphorylation in these mice. These results suggest that NRG1α improves systemic glucose tolerance mainly by suppressing hepatic glucose production but not by increasing insulin sensitivity in the peripheral organs. Consistent with
this hypothesis, mRNA levels of the key gluconeogenic enzymes, Pepck and G6pase, and mitochondrial transporters for pyruvate, Mpc1 and Mpc2, were reduced in Nrg1α-overexpressing livers (Fig. 2d). We also confirmed the Nrg1α-mediated decrease in protein levels of PEPCK and G6Pase in the mice (Fig. 2e). FoxO1 and CREB are crucial transcription factors for hepatic glucose production under fasting conditions. Nrg1α reduced both nuclear and cytoplasmic amounts of FoxO1 with a marked decrease in ratio of nuclear to cytoplasmic FoxO1 (Fig. 2f). On the other hand, Nrg1α showed marginal effect on CREB phosphorylation. Taken together, these results suggest that NRG1α ameliorates systemic glucose tolerance by suppressing FoxO1-mediated gluconeogenesis in the liver.

**Nrg1α reduces glucose production with suppressed gluconeogenic gene induction in primary hepatocytes.** To further confirm the inhibitory effects of NRG1α on glucose production, we incubated isolated primary hepatocytes in medium containing gluconeogenic substrates and simultaneously stimulated the cells with cAMP and dexamethasone. This treatment modestly but significantly increased glucose output from the hepatocytes, but adenoiral-mediated Nrg1α overexpression reduced both basal and stimulated hepatic glucose production (Fig. 3a). Consistent with these results, the induction of PEPCK and G6Pase at their transcript and protein levels by the treatment with cAMP and dexamethasone was suppressed to a greater extent in hepatocytes overexpressing Nrg1α (Fig. 3b,c). Additionally, nuclear FoxO1 accumulation was reduced without any impacts on cytosolic FoxO1 in these cells (Fig. 3d).

We next examined if suppression of endogenous Nrg1 expression could conversely enhance glucose production in hepatocytes. Silencing Nrg1 expression by siRNA modestly but significantly increased glucose production with higher levels of both transcript and protein levels of PEPCK and G6Pase only when hepatocytes were stimulated with cAMP and dexamethasone (Fig. 3e,f,g,h), further strengthening the importance of endogenously expressed NRG1α on hepatic gluconeogenesis.
Figure 3. Hepatic glucose production is attenuated in isolated primary hepatocytes expressing Nrg1α.
(a) Glucose production of hepatocytes infected with the Nrg1α-expressing adenovirus. n = 4 per group.
*P < 0.05. (b) Quantification of Pepck and G6pase mRNA in Nrg1α-expressing hepatocytes stimulated with or without 8-bromo-cAMP and dexamethasone. n = 4 (without cAMP/Dex) and 6 (with cAMP/Dex) per group.
*P < 0.05. (c) Representative immunoblots of PEPCK and G6Pase in Nrg1α-expressing hepatocytes. The results of densitometric quantification are shown. n = 4 mice per group. *P < 0.05. (d) Representative immunoblots of FOXO1 and phospho-CREB in the nuclear fraction of Nrg1α-expressing hepatocytes. nFOXO1: nuclear FOXO1, cFOXO1: cytosolic FOXO1. The results of densitometric quantification are shown at the right. n = 4 mice per group. *P < 0.05. (e) Validation of siRNA for Nrg1 gene in hepatocytes. n = 6 per group. *P < 0.05. (f) Glucose production of hepatocytes silenced endogenous Nrg1α expression. n = 6 per group. *P < 0.05. (g) Quantification of Pepck and G6pase mRNA in Nrg1α-silencing hepatocytes stimulated with or without 8-bromo-cAMP and dexamethasone. n = 6 per group. *P < 0.05. (h) Representative immunoblots of PEPCK and G6Pase in Nrg1α-silencing hepatocytes. The results of densitometric quantification are shown. n = 4 mice per group. *P < 0.05.
ERK and AKT mediate NRG1α-induced inhibition of hepatic gluconeogenesis via ERBB3 phosphorylation. NRG1 undergoes proteolytic cleavage at the juxtamembrane region and subsequently releases its diffusible ectodomain, which has a biologically active EGF-like domain⁴. The ectodomain of NRG1 binds to either ERBB3 or ERBB4 to express its functions on target cells and tissues⁵. To assess the signalling cascade of NRG1α in the liver, we treated hepatocytes with bacterially expressed NRG1α recombinant protein (rNRG1α) that contains an α-type EGF-like domain. Transient treatment (30 min) of isolated hepatocytes with rNRG1α resulted in increased phosphorylation of ERBB3, but not ERBB1, in a dose-dependent manner (Fig. 4a). On the other hand, expression of both ERBB2 and ERBB4 was barely detectable in our experiments (data not shown).
rNRG1α also activated two ERBB receptor-associated downstream kinases, AKT and ERK, and increased FoxO1 phosphorylation (Fig. 4a).

To further confirm the importance of these NRG1α-activated signalling cascades in suppressing hepatic gluconeogenic activity, we assessed whether pharmacological inhibition of the ERBB3-dependent PI3K-AKT and Raf-MEK-ERK pathways could reverse the rNRG1α-elicited suppression of Pepck and G6pase induction. As ERBB3 shows little, if any, kinase activity, ERBB3 requires dimerization with another ERBB receptor such as ERBB1 and ERBB2 to transmit its signals into cells17. We used lapatinib, a specific inhibitor for both ERBB1 and ERBB2, to evaluate the significance of ERBB3-mediated pathway in the NRG1α-evoked inhibition of hepatic gluconeogenesis. As expected, the pretreatment with lapatinib completely abolished both basal and rNRG1α-dependent ERBB3 phosphorylation (Fig. 4b). This is accompanied by a marked decrease in the phosphorylated forms of AKT, ERK, and FoxO1 (Fig. 4b). In addition, such a treatment totally restored the Nrg1α-induced suppression of expression of Pepck and G6pase and hepatic glucose production in hepatocytes stimulated with cAMP and dexamethasone (Fig. 4c,d). In addition, LY294002 (1 μM) and PD98059 (50 μM) completely inhibited both basal and the rNRG1α-elicited AKT and ERK phosphorylation, respectively (Supplementary Fig. S6). Both inhibitors at the same concentrations substantially but not completely abolished the inhibitory effects of Nrg1α on Pepck and G6pase induction (Fig. 4e).

**Acute rNRG1α treatment suppresses elevation of blood glucose levels in OGTT and PTT.** We next examined whether a short-term exposure (15 min before glucose and pyruvate challenge) to rNRG1α also shows its blood glucose-lowering effects. A single injection of rNRG1α successfully increased systemic glucose disposal and attenuated hepatic glucose production (Fig. 5a,b), suggesting that NRG1α has direct inhibitory effects on the regulation of glycolytic/gluconeogenic fluxes in the liver. These alterations were accompanied by activation of ERBB3-AKT/ERK-FoxO1 signalling cascade, but not any changes in PEPCK and G6Pase expression (Fig. 5c).

**A liver-specific deletion of Nrg1 gene shows little alterations on blood glucose levels after glucose and pyruvate challenge.** To determine effects of a loss of hepatic Nrg1 gene on systemic glucose metabolism, we generated liver-specific Nrg1 gene knockout mice (Nrg1KO) and subjected to OGTT and PTT. Unexpectedly and inconsistent with our in vitro results, Nrg1KO mice were found to display no apparent difference in fasting blood glucose levels compared to wild-type (WT) mice and elevate blood glucose levels after glucose and pyruvate challenge comparable to WT mice (Supplementary Fig. S7), suggesting little, if any, effect of hepatic Nrg1 on systemic glucose metabolism under non-stressed conditions.
Discussion

Glucogeneogenesis is a critical metabolic pathway in hepatic glucose homeostasis, especially in starvation conditions. Aberrant activation of glucogeneogenesis occurs due to hepatic insulin resistance and is a hallmark of diabetes mellitus. Various hormones, including insulin and glucagon, respond to alterations in nutritional conditions and contribute to the coordinated regulation of hepatic glucose production. As the liver is a major secretory organ, hepatocytes control their own glucose metabolism by releasing local mediators. However, information is limited on the biological significance of liver-derived humoral factors in hepatic glucose metabolism. In the present study, we demonstrated that Type I NRG1α is exclusively expressed among various NRG1 isoforms in mouse liver and may function as a local mediator to control hepatic glucogeneogenesis by releasing its N-terminal ectodomain extracellularly. The NRG1α-dependent inhibition of hepatic glucose production occurs by binding to ERBB3 and subsequently, in part, activating the ERK and AKT signalling pathways, resulting in decreased expression of key glucogeneogenic genes.

NRG1 produces multiple isoforms and exerts its diverse biological functions in an autocrine and/or paracrine manner in various organs. Among these isoforms, mouse liver predominantly expresses Type I NRG1α which is constitutively cleaved to release its N-terminal portion containing an α-type EGF-like domain extracellularly as a functional ligand for ERBB receptors. Although NRG1α can bind with and activate both ERBB3 and ERBB4 receptor on target cells, Type I NRG1α is likely to show a higher affinity to ERBB3 than ERBB4 in liver given that ERBB3 is exclusively phosphorylated in both liver overexpressing Type I NRG1α and primary hepatocytes exposed to rNRG1α. This might be explained by the differences in hepatic expression levels of ERBBs or Type I NRG1α-specific affinity for ERBB3. ERBB3 and ERBB1 are highly expressed in rat liver, whereas ERBB2 and ERBB4 expression are hardly detectable. Consistent with this report, we also found that ERBB3 protein is abundantly present in mouse liver, supporting the hypothesis that NRG1α preferentially recognizes and activates ERBB3 receptor in the liver. On the other hand, the latter hypothesis is also conceivable when considering that different type of NRGs exhibits distinct pattern of ERBB receptor activation. For example, Neuregulin4α, another member of the neuregulin family, has been reported to regulate hepatic lipogenesis by binding to hepatic ERBB4, but not ERBB3 receptor. In addition, variation in the short C-terminal region of the EGF-like domain, α and β type, in NRG1 also can affect binding capacity for and activation of ERBB receptors. NRG1β displays a higher binding ability for almost all ERBB receptors complexes containing either ERBB3 or ERBB4, whereas α type EGF-like domain of NRG1 can transmit its signal efficiently via binding exclusively with ERBB2/3 receptor complex. This is further supported by other reports showing that ERBB3 kinase activity is too weak to phosphorylate via formation of ERBB3 homodimers without any interaction with other ERBB receptors and small amounts of ERBB2 are sufficient to phosphorylate and activate ERBB3 receptor via a transient formation of ERBB2/3 receptor complex. Although we did not detect any expression of ERBB2 protein in isolated mouse hepatocytes and liver, trace amounts of ERBB2 seem to be involved in NRG1α-mediated ERBB3 activation and subsequent responses in the regulation of glucogeneogenesis in the liver. In fact, we found that the treatment with lapatinib successfully inhibits rNRG1α-elicited ERBB3 phosphorylation with a marked suppression of phosphorylation of its downstream molecules such as AKT, ERK, and FoxO1. In addition, such a treatment also restores expression of glucogeneogenic genes and hepatic glucose production in hepatocytes overexpressing Nrg1α. When considering that ERBB1 is barely phosphorylated in response to rNRG1α despite its ample expression, our present results suggest a crucial role of ERBB2/3 as functional receptor complex for Type I NRG1α in liver. Furthermore, an immunoglobulin-like domain of NRG1α, another structural characteristic of Type I NRG1α present in the N-terminal of the EGF-like domain, might compensate for the lower activity of NRG1α in liver given that ERBB1 is barely phosphorylated in response to rNRG1α. Consequently decreased hepatic glucose production finally occurs by binding to ERBB3-AKT signalling cascade, presumably leading to stimulated lactate production and consequently decreased hepatic glucose production. These could explain the acute effects of NRG1α on systemic glucose metabolism, a single injection of NRG1α into mice also shows its blood glucose-lowering effects after glucose and pyruvate challenge by stimulating the ERBB3-AKT signalling cascade without any changes in PEPCK and G6Pase protein levels. In our experiments, we also revealed that chronic Nrg1α overexpression in the liver improves systemic glucose disposal with reduced hepatic glucose production by suppressing expressions of key glucogeneogenic genes such as PEPCK and G6Pase. This is in line with a previous report showing enhanced systemic glucose clearance in diabetic mice by long-term exposure to NRG1α, although any direct evidence for the NRG1α-mediated suppression of liver glucogeneogenesis is not provided. Chronic Nrg1α overexpression in the liver decreases hepatic FoxO1 transcript levels (data not shown), suggesting a possible involvement of NRG1α-mediated transcriptional regulation of FoxO1 in the reduced hepatic glucogeneogenesis. However, considering that an intensive reduction of nuclear FoxO1 compared to cytosolic one occurs by Nrg1α, sustained activation of ERBB3-AKT-FoxO1 signalling cascade seems to play crucial roles in the NRG1α-evoked inhibition of hepatic glucose production in mouse liver. Consistent with this hypothesis, we found that Nrg1α attenuates not only expression of PEPCK and G6Pase but also glucose production in isolated hepatocytes with enhanced cytoplasmic sequestration of FoxO1 but not any changes in FoxO1 transcript levels (data not shown). In addition, silencing of hepatic Nrg1α conversely enhances glucose production with increased these glucogeneogenic enzymes expression in cAMP/dexamethasone-stimulated hepatocytes, further supporting the importance of NRG1α in the...
regulation of hepatic gluconeogenesis. Moreover, the ERBB3 signalling pathways stimulates not only the PI3K-AKT cascade but also Raf-MEK-ERK in the liver. Therefore, the modest, but significant, phosphorylation of ERK by NRG1α may cause phosphorylation and subsequent inactivation of FoxO1. Indeed, this hypothesis is further supported by our current findings that an ERK inhibitor partially but substantially abolishes Nrg1α-induced suppression of gluconeogenic enzyme expression. Taken together, these results strongly suggest that NRG1α inhibits hepatic glucose production, at least in part, in both an AKT- and ERK-dependent manner by suppressing FoxO1 activity.

Although a liver-specific deletion of Nrg1 gene did not evoke any alterations in systemic glucose disposal after glucose and pyruvate challenge, our data provide an evidence that Type 1 NRG1α might act as a local mediator to control gluconeogenesis by activating ERBB3 signalling cascade and consequently inactivating FoxO1-mediated gene expression in the liver. Hepatic NRG1α expression has been reported to be induced in diabetic rats, suggesting the pathological importance of NRG1α in the regulation of liver glucose metabolism in insulin resistant conditions. Although further investigation is needed to examine its pathological significance, our findings indicate that NRG1α may have therapeutic potential in reducing hepatic glucose production in diabetes mellitus.

Methods

Animals and treatments. C57BL/6j male mice (5–6 weeks old) were obtained from CLEA Japan (Tokyo, Japan). Liver-specific Nrg1 knockout mice (male, 5–6 weeks old) were generated by crossing Nrg1 mutant mice (Acc. No. CDB0743K; http://www.cslst.riken.jp/arg/mutant%20mice%20list.html) and Alb-Cre transgenic mice. These mice were maintained on a 12/12 h light/dark cycle with free access to water and chow (Sankyo Labo Service, Tokyo, Japan). All experiments were conducted in accordance with the Waseda University Animal Welfare Guidelines and were approved by the Animal Experimentation Committee of the Waseda University (Protocol number: 2015-A083a). Mice received 30 μg of either pCAGGS alone or a mouse Nrg1α-expressing vector intravenously by hydrodynamic tail vein injection. The mice were injected three times every 6 days. Two days after the last injection, mice were subjected to further analyses. OGTT (2 mg/g body weight), PTT (2 mg/g body weight, intraperitoneal injection), and insulin tolerance test (ITT; 0.75 IU/Kg body weight, intraperitoneal injection) were performed on mice fasted for 16h, 24h, and 6h, respectively. Blood glucose levels were measured using an automatic glucose monitor (Accu-Chek, Roche Diagnostics, Basel, Switzerland). In some experiments, recombinant mouse NRG1α (rNRG1α) protein (100 ng/g body weight) was intraperitoneally administered to mice 15 min before performing the OGTT and PTT.

PCR and quantitative PCR (qPCR). Complementary DNA was synthesized using total RNA extracted from the liver and isolated hepatocytes with a GoScript™ Reverse transcription system (Promega, Fitchburg, WI, USA), as described previously. A conventional PCR was performed using specific primer sets (Supplemental Table 1) and Q5 High-Fidelity DNA Polymerase (New England BioLabs, Ipswich, MA, USA) to determine which isoforms of Nrg1α were expressed in mouse liver. mRNA levels were measured using either GoTaq qPCR Master Mix (Promega) or FastStart TaqMan® Probes Master (Thermo Fisher Scientific, Waltham, MA, USA). TaqMan gene expression assays (Thermo Fisher Scientific) and sequences of primers and probe are listed in Supplemental Table 1. Gene expression levels were normalized to 18S rRNA as an internal control.

Recombinant NRG1α protein fragment preparation. A partially purified rNRG1α fragment from His171 to Arg242 of the mouse NRG1α protein was prepared using pCold I (TaKaRa Bio, Shiga, Japan) and HisTALON gravity columns (TaKaRa Bio).

Western blotting. Western blotting analysis was performed using 20–100 μg of each nuclear extracts, cytoplasmic extracts, or whole extracts of mouse livers and isolated hepatocytes, as described previously. The conditioned medium of isolated hepatocytes was also analysed. Antibodies against the following proteins were used: AKT, phospho-AKT (Ser473), CREB, phospho-CREB (Ser133), ERBB1, phospho-ERBB1 (Tyr1068), ERBB3, phospho-ERBB3 (Tyr1289), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), FoxO1, phospho-FoxO1 (Ser256) (Cell Signaling Technology, Danvers, MA, USA), NRG1α (R&D Systems, Minneapolis, MN, USA), Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-Tubulin and Flag (Sigma, St. Louis, MO, USA). Polyclonal antibody against mouse G6Pase and PEPCK was originally established by injecting the corresponding peptides (G6Pase; aa344–357, PEPCK; aa587–600) into guinea pigs (TaKaRa Bio).

Cell culture, adenovirus infection, and rNRG1α treatment. Primary mouse hepatocytes were cultured in William’s medium E (Thermo Fisher Scientific) supplemented with 10% foetal bovine serum at 37°C in 5% CO2. Cells were treated with either an adenovirus (ViraPower Adenoviral Gateway Expression Kit, Thermo Fisher Scientific) expressing mouse Nrg1α or siRNA (Supplemental Table 1). Integrative DNA Technologies KK, Tokyo, Japan) targeting for mouse Nrg1 24 h before stimulation with 500 μM 8-bromo-cAMP (Cayman Chemical, Ann Arbor, MI, USA) and 100 nM dexamethasone (Sigma) for 3 h. Viruses encoding mouse Nrg1α or siRNA (Supplemental Table 1, Integrated DNA Technologies KK, Tokyo, Japan) targeting for mouse Nrg1 were used: AKT, phospho-AKT (Ser473), CREB, phospho-CREB (Ser133), ERBB1, phospho-ERBB1 (Tyr1068), ERBB3, phospho-ERBB3 (Tyr1289), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), FoxO1, phospho-FoxO1 (Ser256) (Cell Signaling Technology, Danvers, MA, USA), NRG1α (R&D Systems, Minneapolis, MN, USA), Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA), α-Tubulin and Flag (Sigma, St. Louis, MO, USA). Polyclonal antibody against mouse G6Pase and PEPCK was originally established by injecting the corresponding peptides (G6Pase; aa344–357, PEPCK; aa587–600) into guinea pigs (TaKaRa Bio).

Glucose production assay. Primary mouse hepatocytes were incubated for 6 h in KRB buffer (119 mM NaCl, 5 mM KCl, 2.6 mM KH2PO4, 2.6 mM MgSO4, 2 mM CaCl2, 24.6 mM NaHCO3, and 10 mM HEPES, pH 7.4) supplemented with 0.5% BSA and gluconeogenic substrates (10 mM sodium lactate and 5 mM sodium pyruvate)
in the presence or absence of 500 μM 8-bromo-cAMP and 100 nM dexamethasone. The conditioned medium was collected to measure glucose concentrations using Accu-Chek. Glucose production was normalized to the total hepatocyte protein concentrations.

**Statistical analyses.** Results are expressed as the mean ± SEM. Statistical analyses were performed using Mann-Whitney U tests and one-way ANOVAs, followed by Tukey-Kramer tests for all experiments. P values < 0.05 were considered to be significant.

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**Author Contributions**

T.A., Y.O., K.S. and Y.A. performed the experiments. All authors analysed the data. K.S., S.S., M.K. and T.S. contributed to discussions. S.A. provided Nrg1 mutant mice. N.G. and T.A. wrote the paper. N.G. supervised the project. All authors reviewed the manuscript.
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