Inducible and reversible gene silencing by stable integration of an shRNA-encoding lentivirus in transgenic rats

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Currently, tools to generate loss-of-function mutations in rats are limited. Therefore, we have developed a lentiviral single-vector system for the temporal control of ubiquitous shRNA expression. Here, we report transgenic rats carrying an insulin receptor-specific shRNA transcribed from a regulatable promoter and identified by concomitant EGFP expression. In the absence of the inducer doxycycline (Dox), we observed no siRNA expression. However, Dox treatment at very low concentrations led to a rapid induction of the siRNA and ablation of INSR protein expression. As anticipated, blood glucose levels increased, whereas insulin signaling and glucose regulation were impaired. Importantly, this phenotype was reversible (i.e., discontinuation of Dox treatment led to INSR re-expression and remission of diabetes symptoms). The lentiviral system offers a simple tool for reversible gene ablation in the rat and can be used for other species that cannot be manipulated by conventional recombination techniques.

diabetes | transgenesis | RNAi

Gene targeting by homologous recombination in embryonic stem cells is a powerful tool to generate loss-of-function mutations in mice (1). Nevertheless, additional strategies to inhibit gene expression are desirable because this technique is not applicable to other species, is time- and cost-intensive, and is not reversible (2). RNA interference (RNAi) holds great promise as an experimental tool to achieve this goal. The method is based on the introduction of siRNAs into cells. After binding to the target transcript, the resulting dsRNA complex is incorporated into RNA-induced silencing complex (RISC), finally leading to mRNA degradation and inhibition of translation (3, 4). Experimentally, siRNAs can be introduced into cells by expressing a shRNA under the control of RNA polymerase III promoters, including H1 (5, 6). Incorporation of the shRNA cassette into viral vectors allows for inheritable gene knockdown in cells and even whole animals (7–9).

Lentiviruses have the unique ability to infect nondividing cells. Therefore, they have been used for the generation of transgenic models in various species, including overexpression and gene-silencing studies in mice (7–9) and rats (10–12). Temporal inactivation of genes employing inducible systems for the expression of shRNAs have been developed *in vitro* (13, 14). These systems are based on the expression of silencing RNAs by RNA polymerase III promoters containing operator sequences (tetO) of the *Escherichia coli* tetracycline resistance (tet) operon. Recently, a mouse model for tight control of RNAi was established (15), exploiting the reversible inhibition of gene transcription following binding of doxycycline (Dox) to the tet repressor (tetR). Using a similar approach, we developed a lentiviral single-vector system containing an H1-tetO-shRNA cassette and the codon-optimized tetR linked to EGFP.

Insulin resistance is a hallmark of type 2 diabetes mellitus, one of the most prevalent metabolic diseases of the Western world (16, 17). Impaired insulin signaling results in deregulation of glucose homeostasis and predisposes patients to obesity, vision impairment, and cardiovascular disease (18–20). To gain insight into the pathophysiology of insulin resistance and to exploit the advantages of the rat as a model organism, we used the recently developed lentiviral single-vector system to achieve an inducible and reversible knockdown of the insulin receptor (INSR). Here, we show that controlled ablation of the INSR in the rat results in the development of type II diabetes, which can be reverted upon shutdown of shRNA expression by withdrawing the inducer Dox.

Results

Design of a Lentiviral Single-Vector System for Inducible shRNA Expression. To generate inducible and reversible gene knockdown rats, we developed a lentiviral vector system designated FH1tUTG. For the regulation of the shRNA and identification of transgenic rats, we incorporated the codon-optimized tetR (15, 21) and EGFP downstream of the ubiquitin promoter. By placing the T2A peptide (22) between tetR and eGFP, we obtained reliable and independent expression of both protein products (Fig. 1*A*). Incorporated as a second vector component is an inducible shRNA cassette that comprises the H1 promoter with tetO (H1t) sequences inserted at the 3′ end of the TATA box (15) and a cloning site for the shRNA sequence (Fig. 1*A*). Constitutive expression of the tetR blocks shRNA transcription in the absence of the inducer Dox. Upon addition of Dox, the tetR is released, thereby initiating shRNA expression (Fig. 1*A*).

To test the new vector system, we inserted a shRNA directed against the INSR (15) under the control of the H1t promoter and incorporated the resulting cassette into the unique cloning site of FH1tUTG. Subsequently, we infected C2C12 mouse myoblast cells with FH1t(INSR)UTG lentiviral particles (23). Analysis of the transduced cells revealed constitutive EGFP expression and strong downregulation of the INSR (data not shown). Thus, the FH1tUTG vector is an efficient tool for silencing gene expression *in vivo*.

Generation of Inducible INSR Knockdown Rats. Using a highly concentrated FH1t(INSR)UTG lentivirus preparation, we generated transgenic Lewis rats, allowing for inducible silencing of

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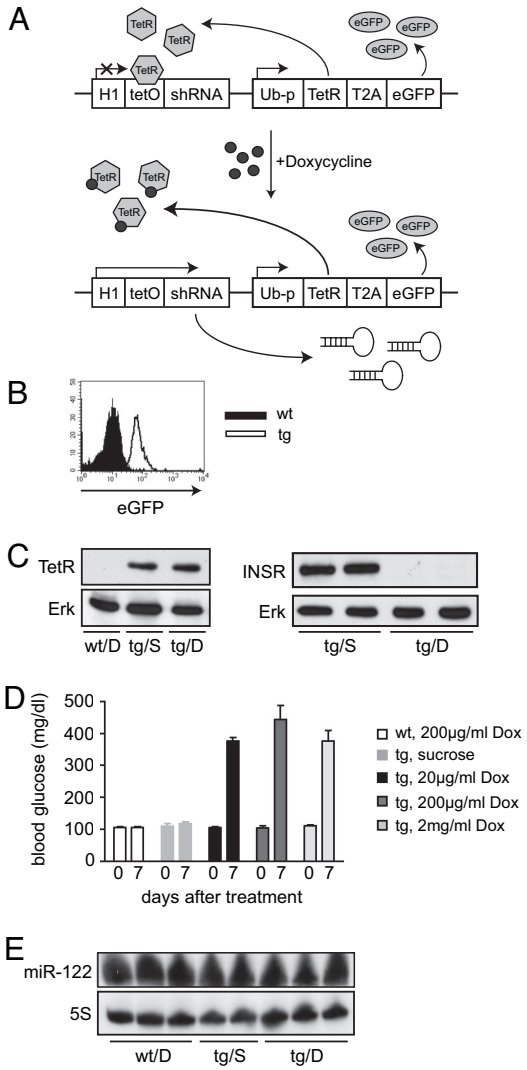

Fig. 1. Lentiviral system for inheritable gene knockdown. (*A*) Operating principle of the lentiviral single-vector system FH1tUTG. The vector contains one cassette consisting of a shRNA regulated by the H1 promoter with tetO and a second cassette consisting of the tetR linked to EGFP by the viral T2A peptide under the control of the ubiquitin C promoter (Ub-p). In the absence of Dox, the tetR binds to tetO and blocks shRNA transcription. After addition of Dox, the tetR is released, facilitating the onset of shRNA expression. EGFP is constitutively expressed under both conditions. (*B*) Peripheral blood leukocytes of one transgenic (tg) rat from the F1 generation of line 04 and a WT control were analyzed for EGFP expression by flow cytometry (gate: live CD45$^+$ cells). (C) WT and tg rats received either drinking water containing 200 μg/ml Dox and 1% sucrose (D) or only sucrose (S) as a vehicle for 7 days. Subsequently, the livers were analyzed by Western blot for protein levels of the tetR and INSR, with Erk as a control. (*D*) WT and tg rats of line 04 were treated with different doses of Dox for 7 days. Transgenic rats receiving only sucrose served as controls. Blood glucose levels were determined before (0 days) and after 7 days of continuous treatment. (*E*) Northern blot analysis of miR-122 and the 5S rRNA in the liver of WT and tg rats that had received either drinking water containing Dox and sucrose (D) or sucrose only (S) for 7 days.

the INSR in living animals. Five transgenic founders were identified based on constitutive eGFP expression, which were then backcrossed to WT Lewis rats. Three of them failed to give rise to transgenic offspring, whereas lines 04 and 87 were derived from the remaining two founder rats. Flow cytometric analyses of the peripheral blood confirmed that their F1 offspring expressed eGFP in all leukocyte lineages [Fig. 1*B* and supporting information (SI) Fig. S1*A*]. This confirms successful germline transmission and indicates that the transgene expression is stable and consistent.

Initially, we tested the basic features of the vector system *in vivo*. Transgenic rats were treated with Dox for 7 days, and the livers were analyzed by Western blot. WT rats treated with Dox as well as transgenic rats receiving vehicle only served as controls. Expression of the tetR was exclusively seen in transgenic rats, and its protein level in liver, muscle, and brain tissue was unaffected by the addition of Dox (Fig. 1*C* and data not shown). Importantly, INSR expression was undetectable following Dox administration in transgenic rats of line 04 (Fig. 1*C*) and was reduced to very low levels in line 87 (Fig. S1*B*). Thus, the successful generation of INSR knockdown rats was confirmed.

Next, we optimized the protocol to induce silencing of the INSR to achieve metabolic deregulation and increased blood glucose levels, which are characteristic of the diabetic phenotype. Adding Dox at 20 μg/ml, 200 μg/ml, and 2 mg/ml to the drinking water of transgenic rats (line 04) led to a strong increase in blood glucose, reaching concentrations of around 400 mg/dl within 7 days (Fig. 1*D*). This is comparable to the degree of hyperglycemia observed in BB/OK rats, a model for spontaneous hereditary type 1 diabetes mellitus (*SI Text* and Fig. S2*A*). Transgenic control rats as well as WT animals had normal glucose levels (Fig. 1*D*). It is noteworthy that a similar increase in blood glucose levels was achieved by adding Dox at concentrations as low as 1 μg/ml, except that the onset of diabetes was retarded by approximately 1 week (Fig. S2*B*). Given that 20 μg/ml Dox allowed both strong and rapid induction of diabetes, we used this comparably low dose in all further experiments.

Although the offspring of both transgenic founders showed similar characteristics, INSR silencing in line 87 was less complete as compared with line 04 (Fig. S1*B*). In addition, the maximal blood glucose levels reached after Dox treatment were lower (Fig. S1*E*). Presumably, this is attributable to weaker expression of the transgene as indicated by the observed difference in eGFP fluorescence in leukocytes (Fig. S1*A*). Based on these findings, we chose line 04 for all subsequent analyses.

Specificity of the Vector System. To provide evidence for the specificity of the observed changes in INSR knockdown rats, we generated transgenic rats expressing an irrelevant shRNA. This was achieved by using the FH1t(shCon)UTG lentivirus, which is also based on our single-vector system. Transgene expression was induced by adding Dox to the drinking water at the highest concentration of 2 mg/ml for 14 days. Analysis of leukocytes for eGFP fluorescence by flow cytometry (data not shown) and siRNA expression by Northern blot (Fig. S1*C*) confirmed successful expression of the transgene. Importantly, INSR protein levels in the liver (Fig. S1*D*) and glucose concentrations in blood (Fig. S1*E*) remained unaltered in control-shRNA transgenic rats. Furthermore, no effect on the growth, behavior, or well-being of the rats was observed attributable to the transgene expression (data not shown). This indicates that the observed phenotype of the INSR knockdown rats was indeed specific, and therefore related to the induced expression of the INSR-specific siRNA.

It was previously reported that high siRNA levels might exert toxic effects because of an overloading of the cellular RNAi machinery (24). Therefore, we analyzed expression of endogenous miR-122 in liver before as well as after Dox treatment for 7 days. Importantly, the level of miR-122 was unaltered after treatment and indistinguishable from that of WT rats (Fig. 1*E*). Thus, effects observed in INSR knockdown rats are not caused by inhibition of the RNAi machinery.

Induction of shRNA Expression Leads to Organ-Specific and Reversible INSR Downregulation and Abolishes Insulin Signaling. Treatment of transgenic INSR knockdown rats for 7 days with 20 μg/ml Dox

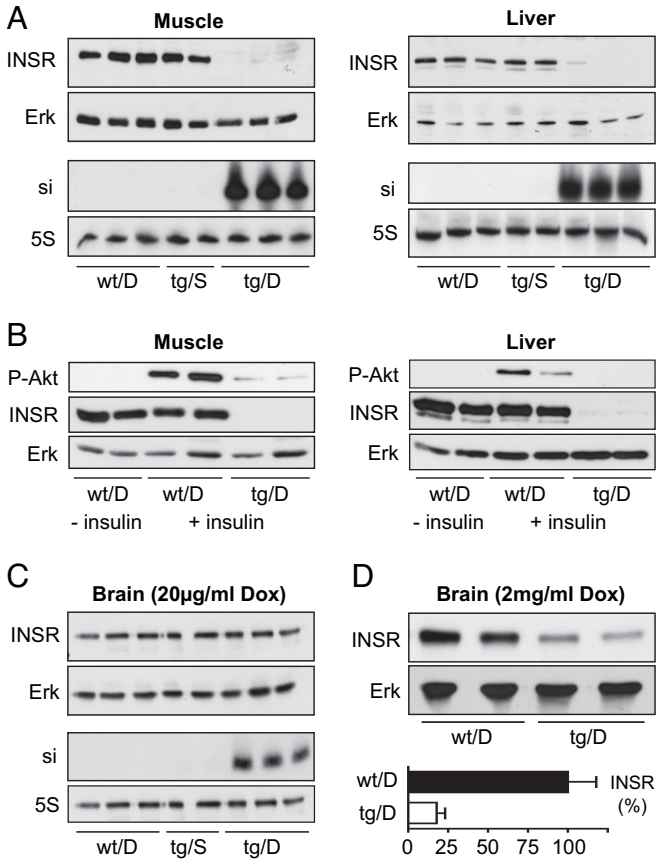

Fig. 2. Efficient silencing of the INSR and loss of insulin signaling in INSR knockdown rats. (A) WT and transgenic (tg) rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose (D) or sucrose alone (S) for 7 days, followed by analysis of muscle and liver tissue. (*Upper* [each organ]) Western blot analysis of the INSR and Erk as a control. (*Lower* [each organ]) Northern blot analysis of the INSR-specific siRNA (si) and the 5S rRNA as a control. (*B*) Insulin was injected into the inferior vena cava of anesthetized rats, and 5 min later, the tissues were harvested. Levels of phosphorylated Akt (P-Akt) as a measure of insulin signaling, with INSR and Erk as a control, were analyzed in muscle and liver by Western blot. (*C*) WT and tg rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose (D) or sucrose alone (S) for 7 days. (*Upper*) Western blot analysis of the INSR and Erk as a control. (*Lower*) Northern blot analysis of the INSR-specific siRNA (si) and the 5S rRNA as a control. (*D*) WT and tg rats received drinking water supplemented with 2 mg/ml Dox and 10% (w/v) sucrose (D) for 14 days, followed by Western blot analysis of the INSR and Erk as a control. Protein levels were quantified by densitometry based on two separate experiments, and the results are depicted as INSR relative to Erk expression. The average expression level in Dox-treated WT rats was set to 100% ($n = 4$).

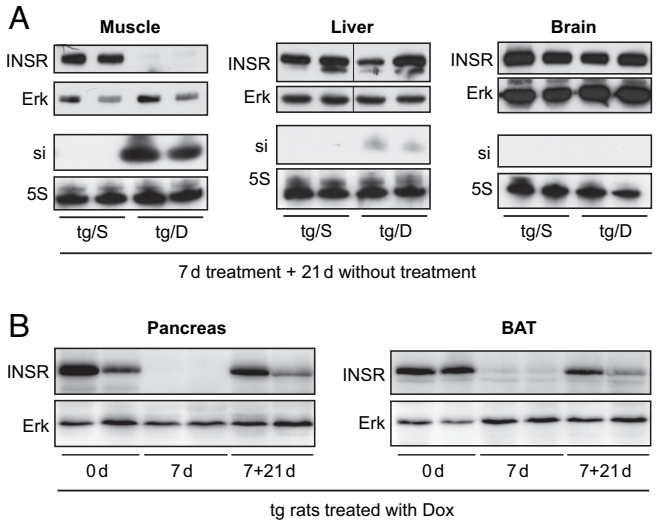

Fig. 3. Reversibility of INSR silencing in INSR knockdown rats. (*A*) Transgenic (tg) rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose (D) or sucrose alone (S) for 7 days, followed by 21 days of no treatment. On day 28, muscle, liver, and brain were analyzed. (*Upper*) Western blot analysis of the INSR and Erk as a control. (*Lower*) Northern blot analysis of the INSR-specific siRNA (si) and the 5S rRNA as a control. (*B*) Transgenic (tg) rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose for 7 days, followed by 21 days of no treatment. Pancreas and brown adipose tissue were analyzed by Western blot for INSR and Erk before Dox treatment (0 d), at termination of Dox treatment (7 d), and at the end of the observation period (7 + 21 d).

led to specific and strong siRNA expression accompanied by a downregulation of the INSR in muscle, liver, pancreas, kidney, and brown adipose tissue to almost undetectable levels (Figs. 2*A* and 3*B* and data not shown). We also detected siRNA in brain, but its expression was apparently too low under these conditions to induce significant downregulation of the INSR protein (Fig. 2*C*). However, following treatment with 2 mg/ml Dox for 14 days, a considerable degree of INSR silencing was also achieved in brain (Fig. 2*D*). Quantification revealed that protein expression was reduced to less than 20% of WT levels under these conditions (Fig. 2*D*). Importantly, no siRNA expression or INSR reduction was observed in vehicle-treated transgenic rats (Fig. 2 *A–C*). This confirms that the vector system is tight and allows for rapid and efficient gene silencing in peripheral tissues.

To confirm that loss of INSR expression also deregulates insulin signaling (18), we analyzed Akt phosphorylation in liver, muscle, and brown adipose tissue. In WT rats, injection of insulin led to a rapid increase in phosphorylated Akt levels, whereas INSR-ablated rats had a strong defect in insulin signaling (Figs. 2*B* and S3). Thus, the observed phenotype of the knockdown rats can be attributed to reduced INSR protein levels in insulin target tissues.

Up to this point, we have shown that the knockdown of INSR is indeed inducible; however, in principle, the vector construct would also be suitable for reverting knockdown of gene products. To test this characteristic *in vivo*, transgenic rats received Dox for 7 days followed by discontinuation of the treatment for another 21 days. At that point, siRNA expression had ceased (Fig. 3*A*), resulting in near-WT levels of INSR expression in liver, brain, pancreas, and brown adipose tissue (Fig. 3 *A* and *B*). In contrast, siRNA expression in muscle still persisted 21 days after cessation of Dox administration, preventing re-expression of the INSR protein (Fig. 3*A*). It is noteworthy that even another 9 weeks later, INSR expression in muscle still had not fully returned to WT levels (data not shown). Thus, the kinetics of inducibility and reversibility are partially tissue dependent.

INSR Silencing Leads to the Development of a Type 2 Diabetic Phenotype, Which Is Fully Reversible. To explore the physiological consequences of INSR silencing, rats received Dox for 7 days, after which treatment was withheld for another 21 days. Blood glucose levels were strongly elevated within a few days, reaching levels of around 400 mg/dl after 1 week (Fig. 4). Following removal of Dox, glucose levels consistently declined and had returned to basal levels after 3 weeks (Fig. 4). Concomitantly, water consumption increased in the course of Dox treatment and then normalized again after removal (Fig. 4). In all cases, WT littermates treated with Dox and transgenic rats receiving vehicle served as controls and did not show any alterations during the entire observation period (Fig. 4). In addition, INSR silencing in transgenic rats resulted in severe growth retardation that was

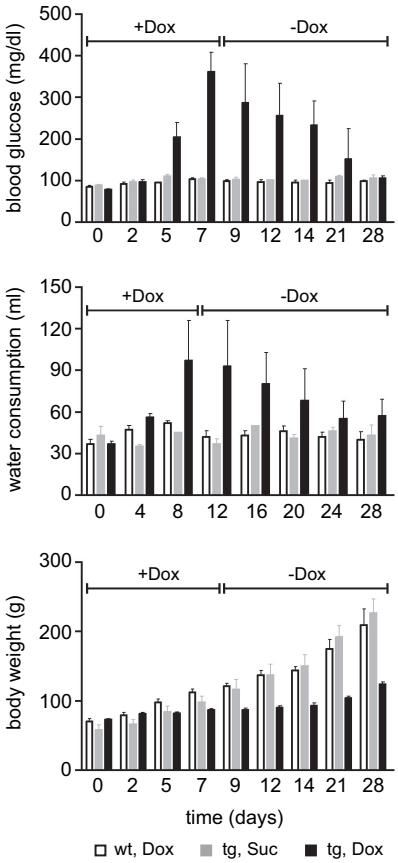

Fig. 4. Reversible induction of type 2 diabetes mellitus in INSR knockdown rats. WT and transgenic rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose (Suc) or Suc alone for 7 days (+Dox), followed by 21 days of no treatment (−Dox). Blood glucose levels (*Upper*), water consumption (*Middle*), and body weight (*Lower*) were determined at the indicated time points after commencement of Dox treatment.

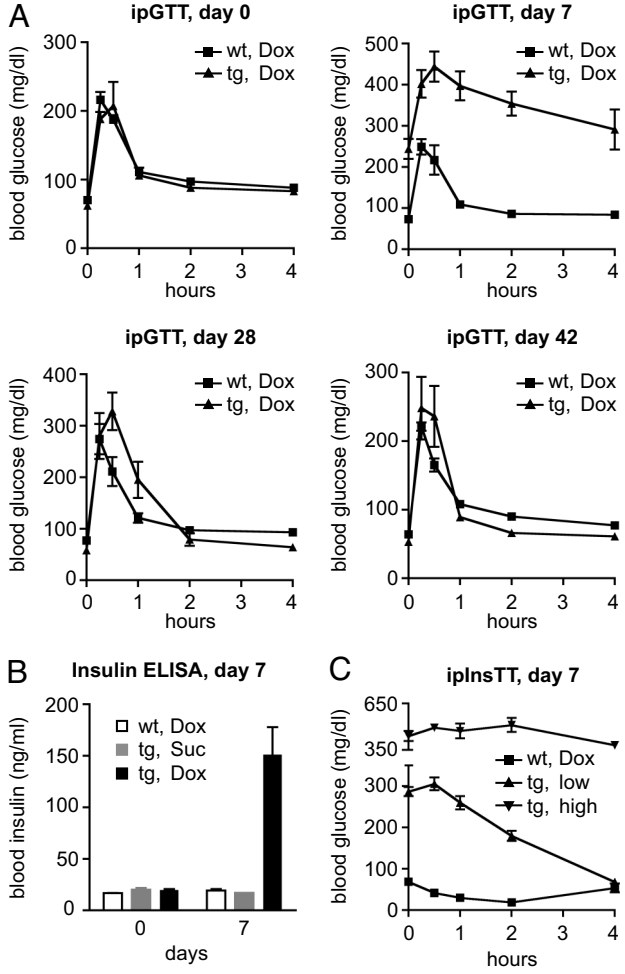

Fig. 5. Inducible and reversible impact of the INSR knockdown on glucose regulation and insulin homeostasis. (*A*) WT and transgenic (tg) rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose for 7 days, followed by 21 days (day 28) or 35 days (day 42) of no treatment. In addition, the rats were analyzed before the start of treatment (day 0). At all four time points, an i.p. glucose tolerance test (ipGTT) was performed and blood glucose levels were monitored over a period of 4 h post injection. (*B*) WT and tg rats received drinking water supplemented with 20 μg/ml Dox and 0.1% sucrose (Suc) or Suc alone for 7 days. Insulin serum levels were determined by ELISA before and after treatment. (*C*) WT and tg rats were treated with 20 μg/ml Dox (WT; D; and tg, low) or with 2 mg/ml Dox (tg, high). After 7 days, an ipInsTT was performed. Blood glucose levels were monitored over a 4-h period.

only slowly overcome after Dox removal (Fig. 4). We conclude that a physiological phenotype can be rapidly and reversibly induced in knockdown rats using this lentiviral vector system.

Inactivation of the INSR Reversibly Impairs Glucose Tolerance and Alters Insulin Serum Levels. Disturbed metabolic regulation caused by impaired insulin signaling can be studied using a glucose tolerance test. The i.p. injection of glucose resulted in a rapid increase in blood glucose levels, which was transient because of the release and activity of insulin, and therefore returned to basal levels within a few hours. Under basal conditions, no difference between WT and transgenic rats was observed, confirming the lack of any physiological effect of the transgene in the absence of Dox (Fig. 5*A*). However, downregulation of blood glucose levels was strongly attenuated in the knockdown rats after 7 days of Dox treatment (Fig. 5*A*). This indicates that insulin was no longer able to control blood glucose levels efficiently in Dox-treated transgenic rats because of the absence of INSR expression and insulin signaling in peripheral target tissues such as liver and muscle. Three weeks after discontinuing the treatment, glucose regulation was largely restored in the transgenic animals, and another 2 weeks later, it was indistinguishable from that of WT control rats (Fig. 5*A*).

As expected for animals lacking the INSR, insulin serum levels were strongly increased in transgenic rats following treatment with Dox for 7 days (Fig. 5*B*). In contrast, no effect was observed in control rats. To test whether INSR knockdown rats were still able to respond to exogenous insulin, we performed an insulin tolerance test. Transgenic rats as well as WT controls received Dox for 7 days. Subsequently, the rats were fasted and then injected with insulin, and blood glucose levels were followed over a 4-h period (Fig. 5*C*). In WT rats, glucose levels dropped within 2 h and subsequently normalized again. Transgenic rats treated with 20 μg/ml Dox exhibited a delayed decrease in blood glucose levels; however, despite this, they reached almost normal physiological levels after 4 h. In contrast, insulin was unable to reduce the hyperglycemia in transgenic rats having received 2 mg/ml Dox (Fig. 5*C*). This suggests that the loss of blood glucose control in INSR knockdown rats exerted by pharmacological doses of insulin is dependent on efficient INSR silencing in all tissues, including the brain.

Discussion

The KO technology has greatly advanced gene function studies in mice during the past 2 decades (1). However, disadvantages

still include the inaccessibility of other species to this method and the inability to revert gene inactivation. RNAi is an alternative to the KO technology and represents a powerful tool to manipulate gene expression in cells and even whole animals (7–9, 11, 12). Lentiviral vectors are particularly efficient tools for generating transgenic animals because they are able to infect nondividing cells and remain active throughout embryonic development (25, 26). The aim of this study was to develop a tool to create inducible and reversible loss-of-function mutations rapidly and reproducibly in rats. For convenient maintenance of the transgenic lines, we incorporated the EGFP gene into the vector. This was linked to a codon-optimized tetR (21) via the novel T2A peptide (22). In contrast to the use of internal ribosome entry site (IRES) sequences, the T2A peptide allowed both genes to be strongly expressed (Fig. 1 *B* and *C*). In addition, we incorporated a tetracycline-regulatable H1-shRNA cassette that had already been successfully used for the generation of mouse models (15). By adapting this system onto our lentiviral vector, we successfully silenced the INSR in transgenic rats.

Our lentiviral vector system FH1tUTG combines several unique features: (*i*) All components necessary for inducible shRNA expression are present on one single vector, thereby circumventing the need to cross-breed several strains of animals. (*ii*) Constitutive expression of EGFP allows for easy identification of transgenic offspring and transcriptionally active proviral integrations as well as controlling for mosaicism. (*iii*) shRNA expression is initiated by the addition of Dox, thus circumventing continuous application as early as during pregnancy to prevent a potentially lethal knockdown phenotype. (*iv*) The system is completely tight, exhibiting no detectable gene inactivation in the absence of Dox. (*v*) Inducibility is very rapid, leading to an almost complete loss of the targeted gene product in most tissues within 7 days. (*vi*) The sensitivity of the system is high and allows gene inactivation at low Dox concentrations, thereby reducing the risk of side effects. (*vii*) Gene silencing is reversible. Soon after discontinuation of the treatment, shRNA expression ceases, the level of the target protein widely reverts to pretreatment levels, and the metabolic phenotype returns to normal within 3 weeks. Taken together, transgenic animals generated with our lentiviral vector fulfill most criteria that should apply to a system for inducible and reversible gene silencing and, most importantly, the lentiviral vector system can be successfully applied to rats and presumably any other species.

Nevertheless, we also identified two drawbacks of the new system. First, we observed limited accessibility of the brain to the induction of gene silencing. INSR protein levels were readily diminished in liver, muscle, pancreas, kidney, and brown adipose tissue within 7 days even at a concentration of 20 μg/ml Dox; however, this was not the case in brain. Here, a much higher concentration of 2 mg/ml Dox was required to achieve strong INSR silencing (>80%), which might be a consequence of the lower local Dox concentrations in brain (15, 27). This indicates that the system needs to be adapted to the individual aim of each study: high concentrations of Dox favor broad and strong gene inactivation, whereas low concentrations are required to revert the phenotype. Second, our study shows that the kinetics of reversibility differ between individual tissues. INSR levels in liver, brown adipose tissue, and pancreas were largely restored 21 days after discontinuation of Dox treatment; however, this was not the case in muscle tissue. Even after 12 weeks, INSR re-expression remained incomplete. Given the fact that Dox is very rapidly penetrating into tissues and its concentration is found to be highest in muscle, it is unsurprising that readsorption from this tissue might take longest (28).

Beyond the technical aspects, analysis of the INSR knockdown rats has shown that gene silencing allows for the generation of rat models to study human diseases that reflect the expected pathological features (17). Inactivation of the INSR induced pathology reminiscent of type 2 diabetes mellitus (i.e., blood glucose rose rapidly after the beginning of Dox treatment reaching diabetic levels of more than 400 mg/dl within 1 week). This is comparable to the hyperglycemia observed in BB/OK rats, an established model for spontaneous hereditary type 1 diabetes mellitus. Concomitant water consumption was increased, insulin serum levels were elevated, glucose tolerance was impaired, and growth was severely retarded. The fact that INSR expression at this stage was almost completely lost in liver and muscle but not in brain suggests that those features of diabetes depend on peripheral insulin actions. Importantly, the diabetic phenotype was fully reversed within 3 weeks after discontinuation of Dox treatment, which should allow the study of long-term effects of the disease even after its cure. In addition, our results suggest that insulin actions in liver suffice to correct diabetes despite the continued absence in muscle. Taken together, it should be possible to study different aspects of insulin resistance and the accompanying pathology by modulating the concentration of Dox and the time points of analysis in our model.

Collectively, we have developed a lentiviral single-vector system for inducible and reversible gene silencing in transgenic rats and presumably other species. Exemplified for the inactivation of the INSR, we could demonstrate the development and reversion of type 2 diabetes mellitus by the transient addition of Dox to the drinking water. We therefore believe that this technology holds great promise for the generation of additional animal models to study human diseases in various species, exploiting each of their individual advantages as model organisms.

Materials and Methods

Inducible Lentiviral shRNA Constructs. To clone the inducible lentiviral construct FH1tUTG, the previously reported FUGW vector (25) was modified by inserting the PCR-amplified H1-tetO-shIR5 cassette (15) into the PacI restriction site upstream of the ubiquitin promoter. The codon-optimized tetR sequence was amplified from pRMCE-tetO-htetRinv-IR5 (15) and linked with the eGFP cDNA through the T2A peptide (22) by overlap PCR. The fusion product was then cloned into the BamH1 and EcoRI restriction sites downstream of the ubiquitin promoter. The construct containing an INSR-specific shRNA sequence was designated FH1t(INSR)UTG.

To clone an inducible lentiviral construct with an irrelevant shRNA, two oligonucleotides corresponding to the respective stem-loop sequence were annealed and inserted into FH1tUTG—Oligo 1: 5′-tccc-CGGAGCAAGCTGAACGATATA-tttcaagaga-GTATATCGTTCAGCT-TGCTCCG-ttttttc-3′ and Oligo 2: 5′-tcgagaaaaaa-CGGAGCAAGCTGAACGATATAC-tctcttgaaa-TATATCG-TTCAGCTTGCTCCG-3′. This construct was designated FH1t(shCon)UTG.

Virus Production and Transduction of Cells. Lentiviral particles were produced by transfecting HEK293T cells seeded in 6-cm dishes with 10 μg of vector DNA together with three helper plasmids (5 μg of pMDL-RRE, 2.5 μg of pRSV-REV, and 3 μg of pVSV-g) using $CaPO_4$ precipitation according to published protocols (10). The virus-containing cell culture supernatants were collected 48 h after transfection, passed through a 0.45-μm filter, and concentrated by centrifugation in a swinging bucket for 90 min at 83,000 *g*. The virus pellet was subsequently resusupended in 30 μl of PBS/0.1% BSA and titrated by serial dilutions on HeLa cells. Viral titers were typically around 2×10^8 Transduction Units/ml.

Generation of Transgenic Rats. Transgenesis was performed according to our established protocol (10). The lentiviral concentrate (10–100 pico liter) was injected into the perivitelline space of fertilized single-cell rat embryos obtained from superovulated Lewis (Lew/Crl) female rats (Charles River). The injected zygotes were cultured overnight; the next morning, two-cell stage embryos were transferred into the oviduct of pseudopregnant Crl/CD female rats. All experiments were conducted in accordance with accepted standards of humane animal care and approved by the responsible authorities of Bavaria (*Regierung von Unterfranken*, *Würzburg*) and Lower Saxonia (*Nds. Landesamt für Verbraucherschutz und Lebensmittelsicherheit*, *Braunschweig*).

Dox Treatment. Tap water supplemented with 2 mg/ml Dox (Sigma D-9891) and 10% (w/v) sucrose, 200 μg/ml Dox and 1% sucrose, or 1–20 μg/ml Dox and 0.1% sucrose was freshly prepared every second day, protected from light, and provided to the rats as drinking water.

Protein Isolation and Western Blot Analysis. Tissue extracts were obtained following cell lysis in a buffer containing 1% Nonidet P-40, 0.1% SDS, and 0.5% sodium deoxycholate in PBS. A protease inhibitor mixture was added before extraction (Roche). Protein concentrations were determined in accordance with the manufacturer's instructions (BioRad).

The lysates were fractionated on a SDS-PAGE gel and blotted for 2 h at 70 mA using a semidry device. Western blot analysis was performed by antibody incubation in PBS containing 0.1% Tween and 5% (w/v) milk powder according to standard protocols. The following primary antibodies were used in this study: anti-INSR (C-19; Santa Cruz, 1:200 dilution), tet02 (Mobitec; 1:500 dilution), ERK (C-14; Santa Cruz, 1:1,000 dilution), and pAkt (Cell Signaling Technology, 1:1,000 dilution). Goat anti-rabbit IgG-HRP and goat anti-mouse IgG-HRP (Dianova) were used as secondary antibodies, and ECL reagent (Pierce) was used for developing.

RNA Isolation and Northern Blot Analysis. RNA was isolated in TRIzol reagent (Invitrogen) from homogenized tissue samples according to the manufacturer's protocol.

For Northern blot analysis, 15 μg of RNA was fractionated on a denaturizing polyacrylamide gel and blotted onto a nylon membrane at 35 mA for 90 min. RNA was cross-linked to the membrane using UV light and incubated at 80 °C for 30 min. The membrane was incubated for 2 h in 10 ml of prehybridization solution (Clontech) and detected using radioactively labeled oligonucleotide probes directed against the antisense strand of the INSR-specific shRNA (gaccagacccgaagatttct), control shRNA (CGGAGCAAGCTGAACGATATA), miR-122 (aaacaccattgtcacactcca), or 5S rRNA (tcctgcaattcacattaattctcgcagctagc). Ten units of T4-polynucleotide-kinase and 10 μCi of [^{32}P]-ATP (10 μCi/μl) were used for oligonucleotide labeling.

i.p. Glucose and Insulin Tolerance Tests. Rats were fasted for 16 h overnight before both tests. For the i.p. glucose tolerance test, the rats were injected i.p. with 2 g of glucose per kg of body weight, and for the i.p. insulin tolerance test, they were injected i.p. with 0.5 IU of human insulin per kg of body weight. Blood samples were taken from the tail, and blood glucose levels were determined using a commercial device.

Insulin Signaling. The rats were fasted for 16 h overnight and anesthetized using isoflurane. Eighty international units of human insulin per kg of body weight was injected into the inferior vena cava, followed by the collection of tissues 5 min later. Controls were injected with saline only. Protein extracts were analyzed by Western blot for Akt phosphorylation.

Insulin Measurement. Insulin levels in the serum were determined using an ELISA specific for rat insulin according to the manufacturer's instructions (EIA-2943; DRG Instruments GmbH).

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