3. RESULTS

3.1. Generation of transgenic rats with ubiquitously active shRNA expression

To establish an shRNA transgenic rat model we first used EGFP overexpressing rats on an SD background, and targeted the EGFP gene with shRNA (Figure 8). The inactivation of such a non-essential EGFP transgene should not cause lethality in transgenic animals. Three DNA constructs, each containing an shRNA cassette against the EGFP gene under the control of the U6 promoter, were designed and microinjected into the pronucleus of zygotes of EGFP transgenic rats, as described in Popova et al. (Popova, Bader et al. 2005).

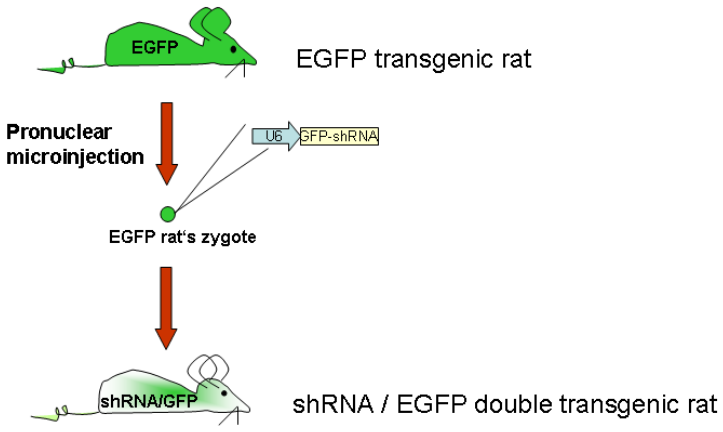


Figure 8: Generation of shRNA transgenic rats using pronuclear microinjection.

3.1.1. Generation of transgenic rats with pRMCE-U6-shGFP construct

As published by Okabe's group, an EGFP shRNA expressed from a DNA construct was a very efficient silencer in EGFP transgenic mice and rats (Hasuwa, Kaseda et al. 2002). For this reason the sequence of this published shRNA was used to design the pRMCE-U6-shGFP construct, in which the human polymerase III U6 promoter (Paddison, Caudy et al. 2002) drives transcription of the shRNA cassette (Figure 9). The chosen U6 promoter was shown to be active in several mice tissues (Seibler, Kuter-Luks et al. 2005).

Nevertheless, shRNA expression from the pRMCE-U6-shGFP construct was confirmed in a cell culture test using RPA (Figure 11).

Two sets of microinjections were performed. In the first set, the DNA fragment containing the shRNA cassette purified away from backbone plasmid (Figure 9) was separately injected at two concentrations 3 µg/mL and 0.3 µg/mL. In the second set of microinjections, the linearized vector pRMCE-U6-shGFP including backbone was delivered to the pronuclei at a concentration of 3 µg/mL (Table 7).

128 founders were born after the microinjection procedures (Table 7) and assayed for integration of any of the injected transgenes by PCR reaction, using U65 and U63 primers. Only one founder was found to carry the shRNA cassette after microinjection of the pRMCE-U6-shGFP including the backbone plasmid (Figure 10). As shown by RPA, shRNA was not expressed in this single positive founder (Figure 11).

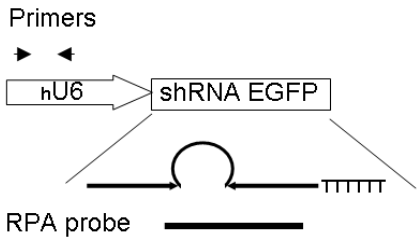


Figure 9: The pRMCE-U6-shGFP construct. Scheme indicates the backbone free fragment of the pRMCE-U6-shGFP construct harboring shRNA (21 mer) against the EGFP gene controlled by the human U6 promoter. Primers U65 and U63 specific for this promoter, as shown by arrows, were used for genotyping of born founders. RPA probe was designed to contain loop and antisense strand of the shRNA for the detection of its expression by RPA.

Table 7: In vivo development of rat embryos which were transferred to the foster mothers at zygote and 2-cell stages after microinjection with the pRMCE-U6-shGFP construct.

Injected substance	Total number of embryos transferred	No. of pregnant recipients, (%)	No. of embryos transferred to the pregnant recipients	No. of implan-tations on day 23*	Total number of offspring, (%)	No. of offspring per pregnant recipients	No. of transgen-ic offspring, (%)
pRMCE-U6-shGFP construct with backbone	200	11/16 (68.8)	140	0	50/200 (25.0)	50/140 (35.7)	1/50 (2.0)
pRMCE-U6-shGFP Insert	174	10/14 (71.4)	132	3	45/174 (25.9)	45/132 (34.1)	0/45
pRMCE-U6-shGFP Insert x10	148	7/13 (53.8)	77	8	33/148 (22.3)	33/77 (42.8)	0/33
Overall efficiency	**522**	**28/43 (65.1)**	**349**	**11**	**128/522 (24.5)**	**128/349 (36.7)**	**1/128**

* Number of implantations in fosters that became not pregnant and were killed to estimate the implantations

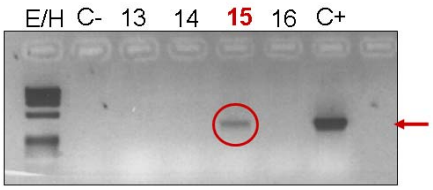


Figure 10: Genotyping of born founders. PCR analyses of rat founders were performed using primers U65 and U63. Amplified bands are shown with the red arrow. The single transgenic rat founder (nr. 15) of 128 newborn was born after microinjection of the pRMCE-U6-shGFP construct included the backbone plasmid. **E/H:** DNA marker λEcoRI/HindIII, **C-:** negative control of wild type rat, **13-16:** founders, **C+:** positive control.

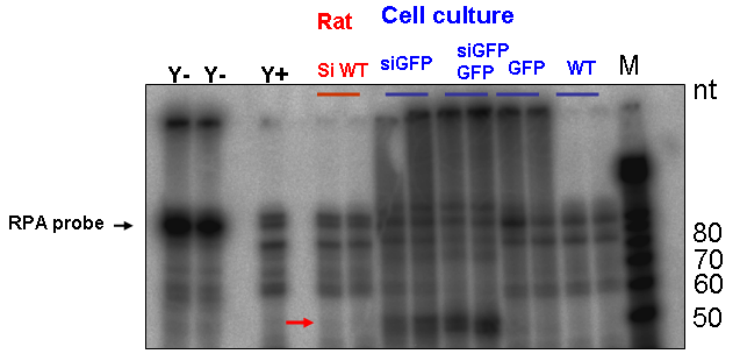


Figure 11: RPA analysis of shRNA expression in tissue culture and rat tails. Total RNA was isolated from the tails of the positive transgenic founder **(Rat Si)** and of a wild type **(WT)** rat as well as from COS7 cell culture transfected with either only pRMCE-U6-shGFP **(siGFP)** or only the pEGFP-N3 vector **(GFP)** or with both of them, as indicated. Wild type cells represent shRNA negative controls. RNA samples were hybridized with ^{32}P labeled RPA probe (the black arrow) for detection of shRNA (the red arrow). No shRNA expression was detected in transgenic rat founder **(Rat Si)**. **Y-:** yeast negative control, **Y+:** yeast positive control, **M:** RNA marker, **nt:** nucleotides.

3.1.1.1. Germline transmission

In order to examine germline transmission to progeny and whether inactive shRNA may become active in the F1 generation, the single transgenic founder (female) was bred with a wild type male rat. 18 born offspring were tested for insertion of pRMCE-U6-shGFP fragment into the genome. None of them carried the transgenic cassette, as shown by PCR using U65 and U63 primers.

3.1.2. Generation of transgenic rats with pU6shGFP-UbAsRed construct

Because unsuccessful production of shRNA transgenic rats using the pRMCE-U6-shGFP construct, a new construct, pU6shGFP-UbAsRed, was designed. Instead of the human U6 promoter the mouse homologue was employed to drive another EGFP shRNA downstream of which an AsRed protein controlled by the ubiquitin promoter was inserted (Figure 12). The AsRed protein was used as a marker for early check of the transgene integration. Both cassettes, shRNA and AsRed, were kindly provided by Prof. Dr. H. Reichardt from the University of Würzburg and subcloned to generate the pU6shGFP-UbAsRed vector. In cell culture the expression of shRNA from this vector was confirmed using RPA (Figure 13A). To confirm the silencing effect of the expressed shRNA, western blotting was performed from the same cell cultures (Figure 13B).

After microinjection of the pU6shGFP-UbAsRed construct into zygotes of EGFP transgenic rats 75 founders were examined by PCR using U6for and U6rev primers.

None of the born founders was positively confirmed for the transgene insertion.

Taken together, two different constructs, the pRMCE-U6-shGFP and pU6shGFP-UbAsRed with continuously active shRNA genes, did not give any expressing transgenic rat founders, of together 203 born. These results suggested the problem of shRNA toxicity and warranted other approaches which could prevent harmful effects of shRNA.

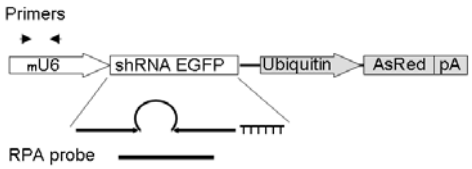


Figure 12: The pU6shGFP-UbAsRed construct. Scheme indicates the backbone free fragment of the bimodal pU6shGFP-UbAsRed construct harboring shRNA (19 mer) against the EGFP gene controlled by the mouse U6 promoter. Downstream of the shRNA cassette is an AsRed marker for red fluorescence driven by the RNA polymerase II promoter of ubiquitin. Primers U6for and U6rev specific for the mouse U6 promoter (indicated by arrows) were used for genotyping of born founders. RPA probe was designed to contain loop and antisense strand of the shRNA for detection of its expression by RPA.

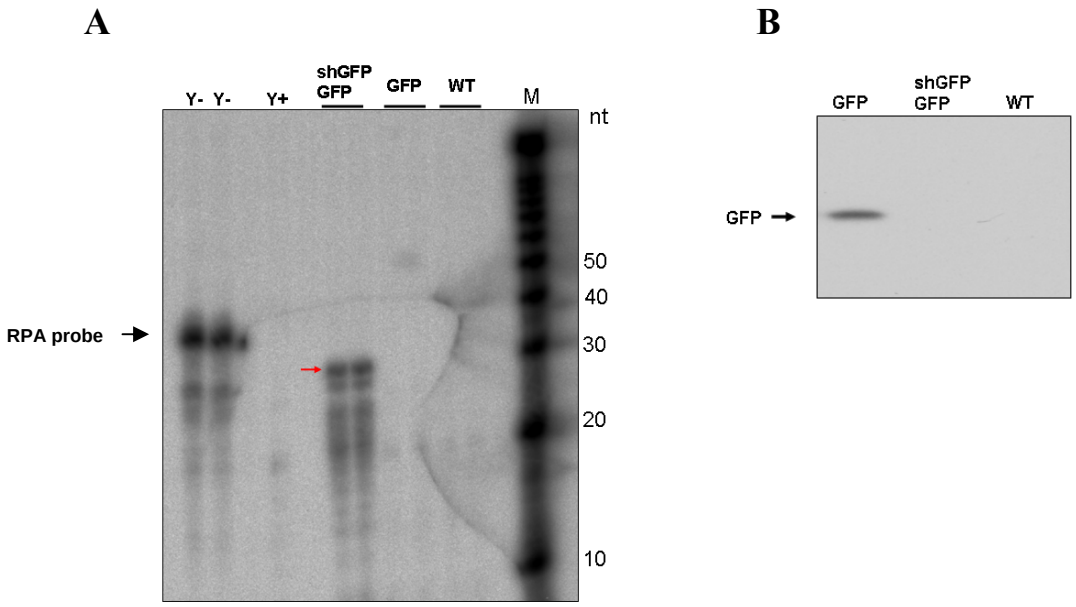


Figure 13: Tissue culture test of the pU6shGFP-UbAsRed construct. COS7 cells were transfected with either only pU6shGFP-UbAsRed **(shGFP)** or only the pEGFP-N3 vector **(GFP)** or with both of them. Non transfected wild type cells **(WT)** represent negative control. 48 hours after transfection cells were harvested for RNA and protein isolation. **(A) RPA analysis of shRNA expression.** 20 µg of total RNA was hybridized with ^{32}P labeled sense RPA probe indicated with the black arrow. The sense strand of the RPA probe recognized only anti-sense strand of transcribed shRNAs, as shown on the level of the red arrow. **(B) Western blot analysis of EGFP silencing.** 20 µg of protein lysate was loaded into 12% SDS-polyacrylamide gel. Blot was developed with anti-GFP antibody, as indicated.
Y-: yeast negative control, **Y+:** yeast positive control, **M:** RNA marker, **nt:** nucleotides.

3.1.3. Generation of transgenic rats with pRosaBAC-shGFP construct

With the intention of protecting embryos against predicted toxic levels of shRNA after microinjection the next goal of this thesis was trying to avoid multi-copy integration of the shRNA transgene into the rat genome. To do this, a BAC with the mouse locus *Rosa26* was chosen to achieve single copy integration. BAC recombineering (described in 2.2.1.3.1.) (Figure 14), was used to generate a new pRosaBAC-shGFP construct (Figure 15). The EGFP shRNA and AsRed marker cassettes, were taken from the bimodal plasmid pU6shGFP-UbAsRed, shown in Figure 12. To this fragment containing both transgenic cassettes, long DNA arms of the mouse *Rosa26* locus were linked using BAC recombineering, as shown in Figure 14.

The resulting 30 kb long DNA fragment of the modified pRosaBAC-shGFP construct (Figure 14) was delivered into the pronucleus of fertilized eggs from EGFP transgenic rats.

118 newborns were genotyped using U6for and U6rev PCR primers to detect founders carrying the shRNA cassette. Again, none of all examined animals was positive for the shRNA transgene.

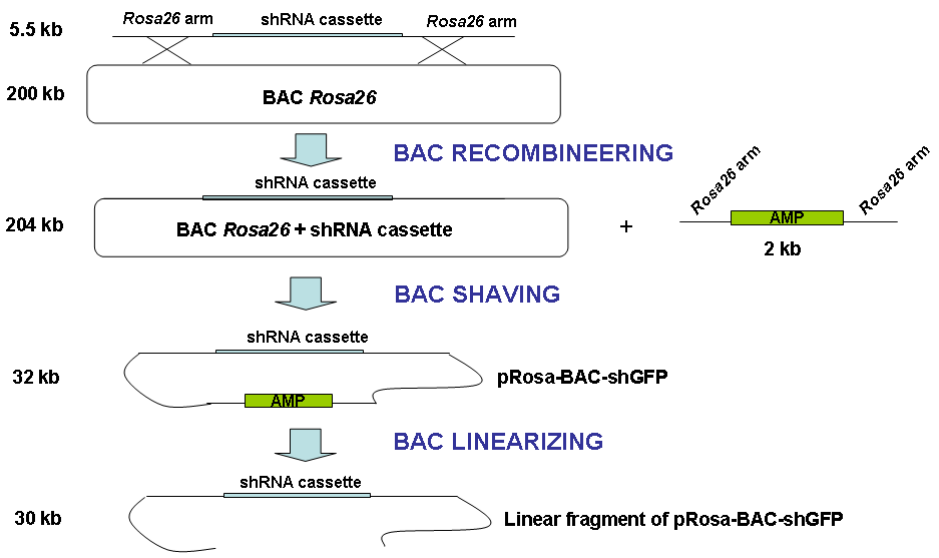


Figure 14: Bacterial Artificial Chromosome (BAC) recombineering. The 5.5 kb long DNA fragment of the pRosaBAC-shGFP construct carries arms of the mouse locus *Rosa26* (0.5 and 0.3 kb), shRNA cassette against the EGFP gene driven by U6 promoter and AsRed marker driven by the ubiquitin promoter as shown in Figure 15. This fragment was recombined with BAC genomic DNA harboring a sequence of the mouse locus *Rosa26* (200 kb), using BAC recombineering method as described in 2.2.1.3.1. The resulting 204 kb large BAC construct with homologously inserted shRNA cassette and AsRed marker was further shaved with a small DNA fragment (2 kb), containing an ampicillin resistance gene enclosed between *Rosa26* arms of 960 bp and 250 bp. In a third step, the final construct pRosa-BAC-shGFP (32 kb) was linearized with the SceI restriction enzyme for removal of the ampicillin resistance cassette and purified for microinjection.

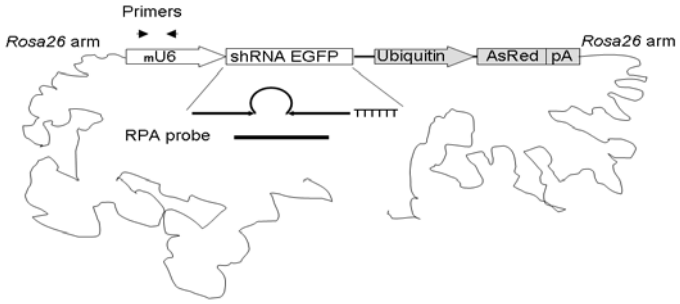


Figure 15: The pRosaBAC-shGFP construct. The 30 kb long linear DNA fragment of the pRosaBAC-shGFP construct contains left and right arms of the mouse *Rosa26* locus. *Rosa26* arms were linked to shRNA and AsRed cassettes, described in Figure 12, using BAC recombineering method, as summarized in Figure 14. An RPA probe was designed to bind to the loop and antisense strand of the hairpin. The 30 kb long DNA fragment of the pRosaBAC-shGFP was linearized by digestion with the SceI enzyme and purified for microinjection.

In order to test whether any fragment of the *Rosa26* arms was integrated into the genomic DNA of born founders further PCR analyses were carried out. PCR primers binding to the sequence of *Rosa26* at six different positions were used. No *Rosa26* DNA fragments were found to be integrated into the genome of tested founders.

Taking together, after microinjection of three different DNA constructs, all continuously expressing shRNAs against the EGFP gene, 312 founders were born and tested for integration of any of these transgenes. The final outcome was a single born transgenic rat, carrying shRNA cassette pRMCE-U6-shGFP, of which no expression of shRNA was detected. This result suggested that continuously expressed shRNA may be harmful for embryonic development and lead to lethality of transgenic embryos before birth. The only liveborn transgenic founder was probably rescued by complete shRNA suppression and thus escaped the lethal effects of expressed shRNA.

3.1.4. Analysis of shRNA toxicity in embryos

3.1.4.1. Rat embryos

Due to the extremely low percentage of positive born founders after pronuclear delivery of the pRMCE-U6-shGFP transgene vector, *in vitro* analyses were carried out (described in 2.2.5.1) to test for toxicity of shRNA constructs after pronuclear microinjection. Isolated zygotes of EGFP rats were microinjected with the pRMCE-U6-shGFP construct in two sets of injections as performed before with and without plasmid backbone (described in 3.1.1.) (Table 8). Observations at blastocyst stage revealed one embryo not showing any green fluorescence under the fluorescence microscope (Figure 16). The non-green blastocyst was supposed either to carry the shRNA transgene, the expression of which silenced the EGFP gene, or it might have lost EGFP expression by an independent mechanism.

These results indicated normal survival rate of rat embryos after microinjection (Table 8) (Popova, Krivokharchenko et al. 2004) and thus suggested that shRNA does not cause toxicity in embryos at early stages of development.

Table 8: In vitro development of rat microinjected zygotes.

Injected substance	No of zygotes, (%)			In vitro-development, (%)			Number of "non-green" blastocyst
	Injected	Survived	Cultured	Two-cell	Morula	Blastocyst	
pRMCE-U6-shGFP construct with backbone	144	121/144 (84.0)	121	100/121 (82.6)	75/121 (61.9)	26/121 (21.5)	1/26 (3.9)
pRMCE-U6-shGFP Insert	139	122/139 (87.8)	122	88/122 (72.1)	71/122 (58.2)	30/122 (24.6)	0/30 (0)
pRMCE-U6-shGFP Insert x10	101	89/101 (88.1)	89	56/89 (62.9)	42/89 (47.2)	24/89 (26.9)	0/24 (0)
No injection	-	-	72	70/72 (97.2)	64/72 (88.9)	37/72 (51.4)	-

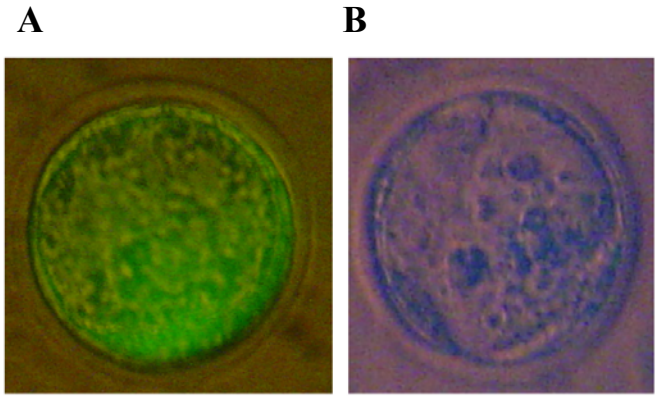


Figure 16: *In vitro* analysis of pronuclear transgenesis with the pRMCE-U6-shGFP construct. Zygotes isolated from pregnant EGFP transgenic female rats were microinjected with the pRMCE-U6-shGFP construct and cultured *in vitro* until blastocyst stage. Observations of EGFP down regulation were carried out under the fluorescence microscope. **(A):** control, a green non-injected blastocyst. **(B):** injected, non-green blastocyst (1 out of 26).

3.1.4.2. Mouse embryos

In order to continue the investigations of shRNA toxicity during early embryonic development *in vitro* studies in mouse embryos were carried out, using pU6shGFP-UbAsRed and pΔshRNA-UbAsRed constructs. The pΔshRNA-UbAsRed construct (Figure 17) was generated from the pU6shGFP-UbAsRed plasmid (Figure 12), of which the shRNA cassette was removed, and was employed as a control.

Each of the constructs, the pU6shGFP-UbAsRed and the pΔshRNA-UbAsRed, was separately microinjected into mouse zygotes (FVBN X Bl6) and cultivated *in vitro* until blastocyst stage, as described in 2.2.5.1.2. During the cultivation embryos were analysed for the AsRed expression under the fluorescence microscope at 2-cell, morula and blastocyst stages. In parallel, survival rate of these embryos was also determined. This attempt did not show any significant differences in survival rate (Table 9) and pronuclear transgenesis rate (Table 10) of injected embryos during *in vitro* cultivation (Figure 18).

This result suggested that shRNA expression does not toxically or lethally affect embryonic development at early developmental stages. On the other hand, no live-born shRNA transgenic rat could be generated suggesting that shRNA toxicity may appear in later phases of development.

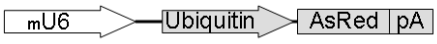


Figure 17: The pΔshRNA-UbAsRed construct. Scheme of backbone free pΔshRNA-UbAsRed construct indicates lack of shRNA cassette against the EGFP gene, which was removed from its original vector pU6shGFP-UbAsRed, described in Figure 12, using restriction enzyme PacI.

Table 9: In vitro development of mouse embryos (FVBN x Bl6), which were microinjected with one of the constructs, pU6shGFP-UbAsRed or pΔshRNA-UbAsRed.

Injected substance	No. of embryos	Survival rate after microinjection, (%)	In vitro development, (%)		
			2-cells	Morula	Blastocyst
pΔshRNA-UbAsRed (3 repeats)	164	136/164 (82.9)	119/136 (87.5)	80/136 (58.8)	52/124 (41.9)
pU6shGFP-UbAsRed (3 repeats)	104	86/104 (82.7)	75/86 (87.2)	56/86 (65.1)	36/75 (48.0)
Control (3 repeats)	66	-	65/66 (98.5)	53/66 (80.3)	48/66 (72.7)

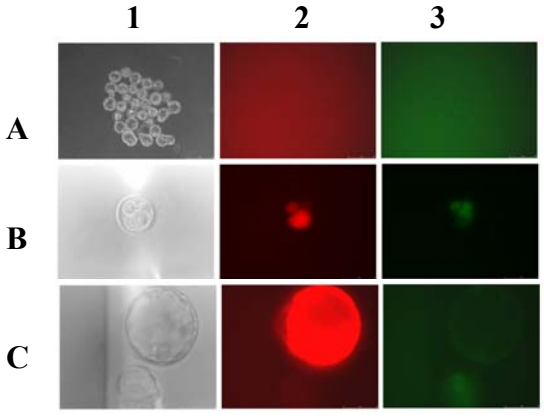


Figure 18: *In vitro* analysis of shRNA toxicity in mouse embryos (FVBN x Bl6). Zygotes (FVBn x Bl6) were microinjected with one of the constructs, pU6shGFP-UbAsRed or pΔshRNA-UbAsRed, and cultured *in vitro* until blastocyst stage. Microscopic observations for AsRed and GFP fluorescence were carried out to examine expression of AsRed marker in injected embryos. (**A)** non injected control embryos; (**B)** embryos injected with the pU6shGFP-UbAsRed construct; (**C)** embryos injected with the pΔshRNA-UbAsRed construct; (**1)** gray-phase contrast; **(2)** AsRed fluorescence; **(3)** GFP fluorescence.

Table 10: AsRed fluorescence in the injected preimplantation mouse embryos (FVBN x Bl6) developed *in vitro* after microinjection with one of the constructs, pU6shGFP-UbAsRed or pΔshRNA-UbAsRed.

Injected substance	No. of fluorescence positive embryos ("red" embryos)						
	1-cell	2-cells	4-cells	8-cells	Morula	Blastocyst	Total
pΔshRNA-UbAsRed	0/4 0/10 1/48 (after 3d in culture)	0/10	0/14	0/3	0/5	4/10 0/9 1/27	4/30 0/29 2/75 **6/134 (4.5%)**
pU6shGFP-UbAsRed	0/22	1/10 (after 3d in culture)	1/1 1/14 (after 3d in culture)	1/1 2/2 (after 3d in culture)	1/1 0/5 3/5 1/1	4/12	7/30 4/56 **11/86 (12.8%)**
Control							0/48

3.2. Generation of transgenic rats with inducible shRNA expression by tetracycline system

3.2.1. Generation of transgenic rats with pTetO-shInsR-TetR construct

3.2.1.1. Tissue culture test of pTetO-shInsR-TetR construct

The tetracycline inducible system applied in this project is summarized in Figure 19A. A bimodal DNA construct harboring a shRNA cassette against the insulin receptor (InsR) under the control of the H1 promoter with a tetO site and a tetracycline repressor (TetR) expression cassette driven by the CAGGS promoter (Figure 19B) was used to generate shRNA transgenic rats by pronuclear microinjection in zygotes.
First, this construct pTetO-shInsR-TetR (Figure 19B) was tested in cell culture. 24 hours after electroporation of COS7 cells with the pTetO-shInsR-TetR plasmid DOX induction was performed in two concentrations, 1 μg/mL and 5 μg/mL, per 10 cm dish for 48 hours. Western blotting analyses proved efficient down regulation of InsR protein in DOX treated cells containing the pTetO-shInsR-TetR construct (Figure 20A). RPA detected shRNA expression in pTetO-shInsR-TetR electroporated COS7 cells after DOX induction (Figure 20B). These results showed the silencing effect achieved with the pTetO-shInsR-TetR construct.

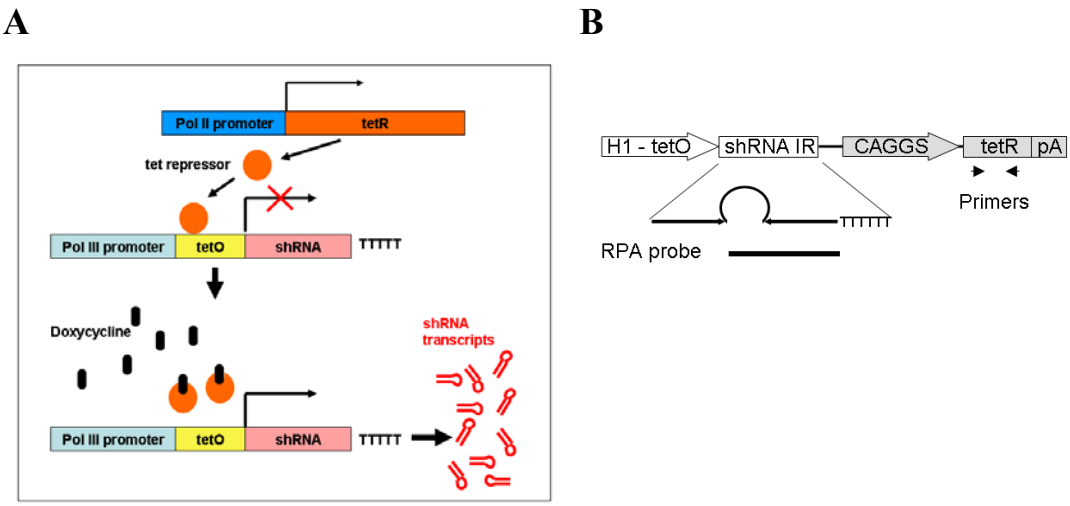


Figure 19: Tetracycline inducible system. (A) TetR, ubiquitously transcribed from RNA polymerase II promoter, binds to a tetO site integrated into the RNA polymerase III promoter. This binding inhibits transcription of shRNA. In the presence of Doxycycline, which binds TetR molecules, the RNA polymerase III promoter becomes released from the TetR. RNA polymerase III can transcribe shRNA molecules. **(B) The pTetO-shInsR-TetR construct.** The transgene construct contains two expression cassettes. One expresses shRNA against the insulin receptor (shInsR) under the control of the human H1 promoter carrying a tetracycline operator (tetO) sequence. The second cassette consists of a tetracycline repressor (tetR) cDNA driven by the CAGGS promoter. An RPA probe was designed to bind to the loop and antisense strand of the hairpin. Primers TetRfor and TetRback were used for genotyping of rats.

3.2.1.2. Generation of shRNA transgenic rat lines

In continuation of our attempt to establish shRNA technology with pronuclear microinjection in rats the pTetO-shInsR-TetR construct was injected into fertilized eggs of SD wild type rats. Two of 31 born founders, 8414 (female) and 8429 (male), were positive for the insertion of the pTetO-shInsR-TetR construct, as confirmed by PCR genotyping (Figure 21). Two lines of transgenic rats were established and named Tet14 and Tet29.

To test transgenic lines Tet14 and Tet29 for TetR and shRNA expression animals were treated with 2 mg/dL DOX in the drinking water with 10% sucrose for 4 days. By an RPA shRNA expression of both treated lines was confirmed in several tissues: muscle, liver, brown adipose tissue (BAT), white adipose tissue (WAT), kidney, heart and brain. No shRNAs were detectable in non treated transgenic rats (Figure 22).

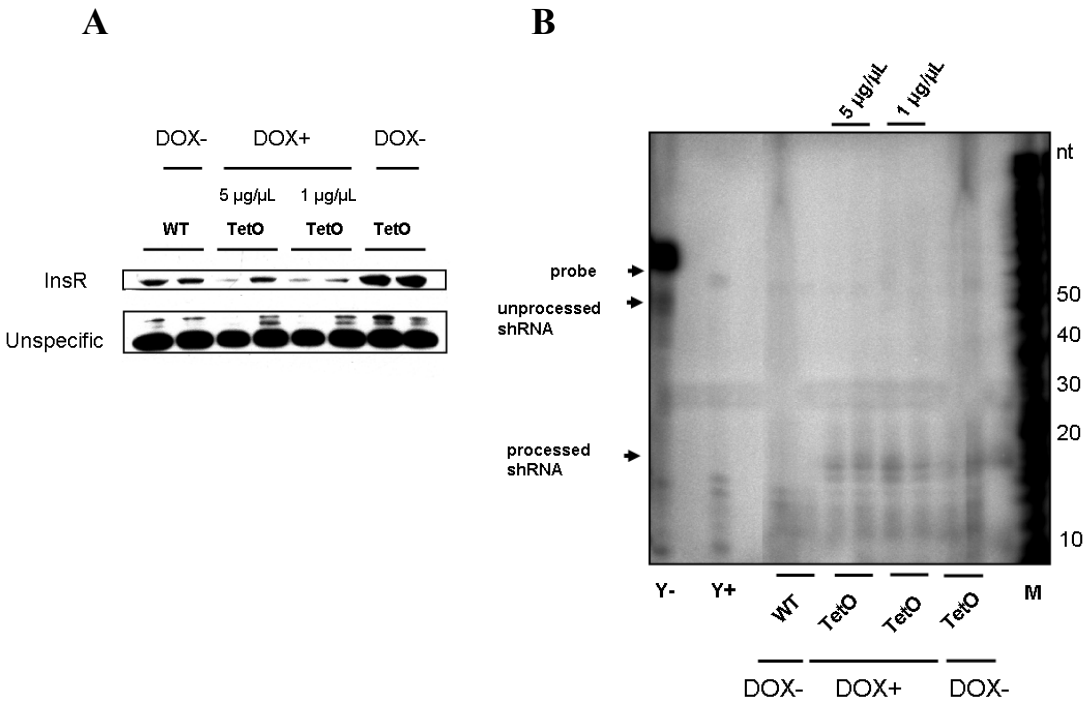


Figure 20: Tissue culture test of the pTetO-shInsR-TetR construct. The pTetO-shInsR-TetR construct was electroporated into COS7 cells. 24 hours after transfection cells were treated with DOX at two concentrations, as indicated. Two days later cells were harvested for protein and RNA extractions. **(A) Western blot**. 20 µg of protein lysate was loaded into 10% SDS-polyacrylamid gel. Blot was developed with anti-InsR antibody, as indicated. Unspecific bands were used as a control for equal loading. **(B) RPA assay.** 20 µg of total RNA was used for hybridisation reaction with ^{32}P labelled RPA probe. InsR shRNA is indicated with the arrow on the level of processed shRNA, as shown. **DOX:** Doxycycline; **TetO:** cells electroporated with the pTetO-shInsR-TetR construct; **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

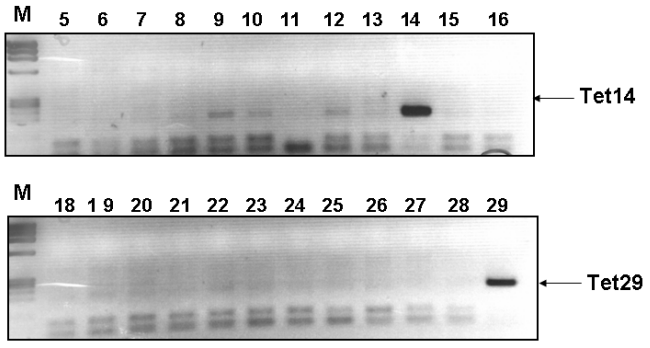
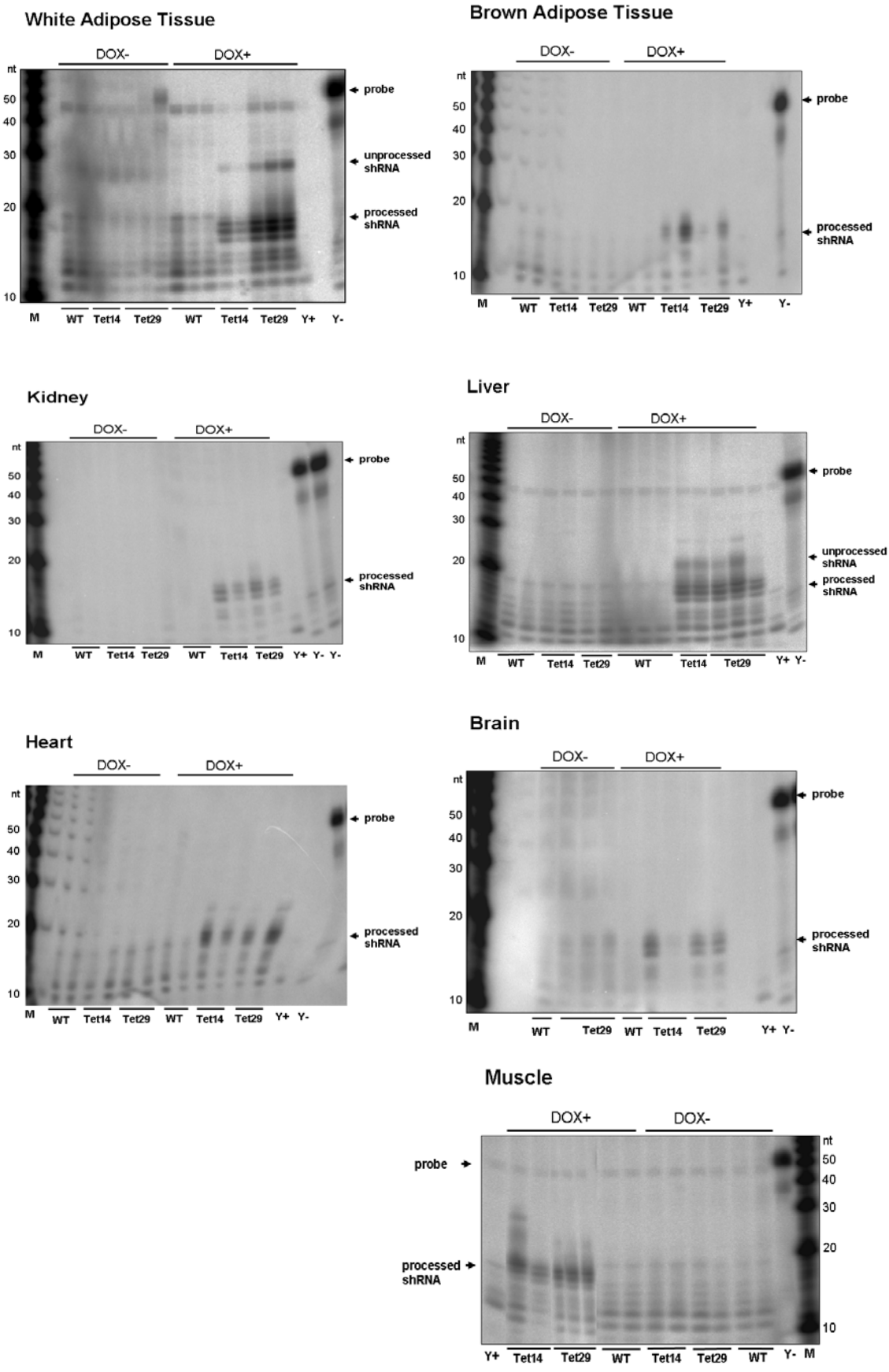


Figure 21: Genotyping of Tet14 and Tet29 born founders. After microinjection of the pTetO-shInsR-TetR construct 31 founders were born and genotyped by PCR. For PCR analyses genomic DNA of rat founders' tails was isolated and PCR reaction was performed using primers TetRfor and TetRback. Two founders with numbers 8414 and 8429 were positive. Amplified bands of positive founders are shown with arrows. **M:** Φ174 DNA/BsuRI (HaeIII) Marker; **numbers 5-29:** founders.

InsR downregulation by the shRNA was assayed in several tissues such as WAT, BAT, kidney, heart, skeletal muscle, liver and brain by western blot analysis, which detected an efficient gene silencing in both transgenic lines, Tet14 and Tet29, after treatment with 2 mg/mL DOX in 10% sucrose (Figure 23). To compare tissue specific InsR knockdown between both lines western blots of brain, heart, kidney, WAT and BAT were quantified. Quantification was carried out by the software Tina 2.08e and percentages of reduction of expression were calculated based on the band intensities. The extent of the silencing effects on the InsR was tissue and line specific (Table 11). TetR was expressed in all tissues of transgenic rats and remained unaffected by DOX treatment (Figure 23).


White Adipose Tissue
DOX-
DOX+
nt
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20
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probe
unprocessed shRNA
processed shRNA
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Tet14
Tet29
Y+
Y-
Brown Adipose Tissue
DOX-
DOX+
probe
processed shRNA
Kidney
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probe
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Y-
Liver
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probe
unprocessed shRNA
processed shRNA
Heart
DOX-
DOX+
probe
processed shRNA
Brain
DOX-
DOX+
probe
processed shRNA
Muscle
DOX+
DOX-
probe
processed shRNA

Figure 22: Expression of shRNA in DOX treated transgenic rats. Expression of shInsR was detected by RPA in 20µg of total RNA isolated from several tissues (as indicated) of wild-type (WT) and transgenic (Tet14 and Tet29) rats treated with DOX (2 mg/mL in 10% sucrose) for 4 days. RPA analyses were performed using a ^{32}P labelled RPA probe, as shown with the arrow. Expression of the hairpin is indicated with arrows on the level of processed shRNA. **DOX:** Doxycycline; **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

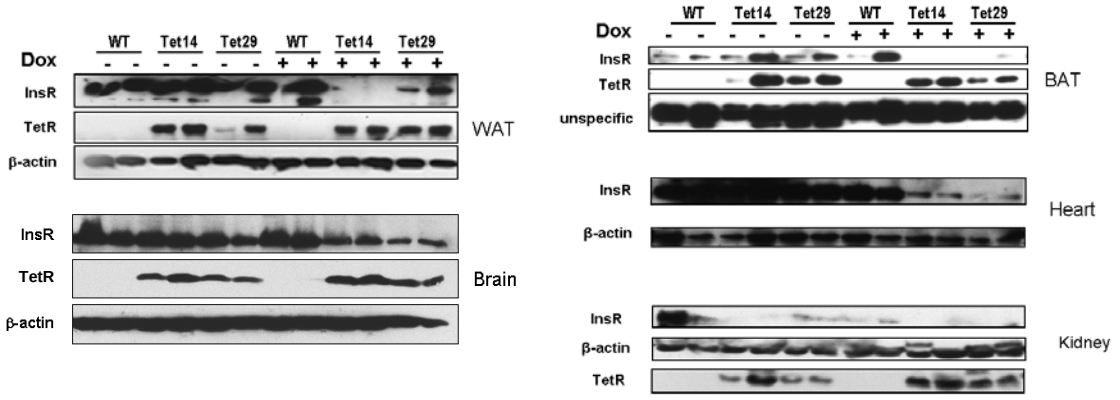


Figure 23: Expression of InsR and TetR in DOX treated rats. Treated rats received DOX at a concentration of 2 mg/mL in their drinking water containing 10% sucrose for 4 days while control rats did not receive any DOX. Expression of insulin receptor (InsR), tetracycline repressor (TetR), and ß-actin were detected by Western blot in 20 µg protein lysate of white adipose tissue (WAT), brown adipose tissue (BAT), kidney, heart and brain isolated from WT, Tet14, and Tet29 rats. **DOX:** Doxycycline**.**

Table 11: Reduction of the insulin receptor protein expression in Tet14 and Tet29 rats. WAT: white adipose tissue; BAT: brown adipose tissue.

	Tet14	Tet29
Brain	**31.6 %**	**38.9 %**
Heart	**62.3 %**	**72.9 %**
WAT	**89.5 %**	**76.3 %**
Kidney	**61.0 %**	**58.9 %**
BAT	**86.7 %**	**78.9 %**

3.2.1.2.1. Glucose and Insulin in shRNA transgenic rats

During acute DOX treatment (2 mg/mL in 10% sucrose) blood was taken from the tail vein of rats to measure blood glucose and plasma insulin. Drastic increases of these parameters were detected after three days of DOX treatment in Tet29 rats and one day later also in Tet14 rats (Figure 24). Blood glucose levels became 3 fold higher than in control animals (Figure 24A). Correspondingly, the plasma insulin level was enhanced more than 7 fold (Figure 24B). Body weight was markedly reduced in both Tet transgenic rat lines after 3 days of DOX treatment (Figure 24C).

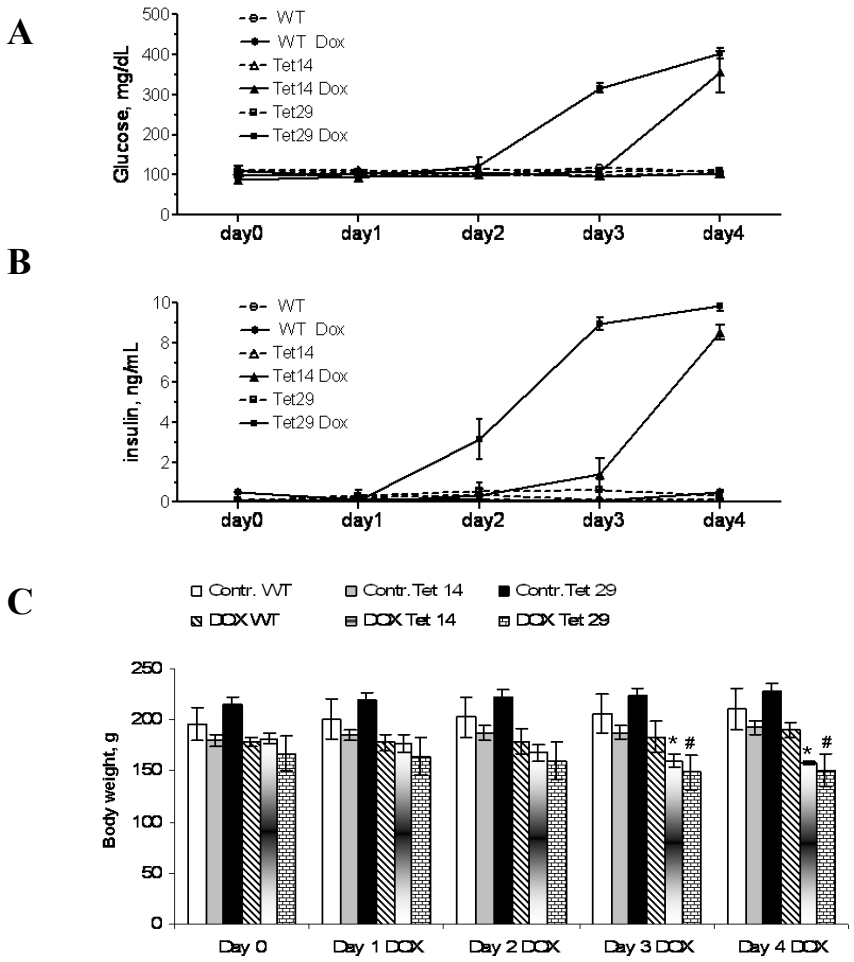


Figure 24: Levels of blood glucose, plasma insulin and body weight of rats. Blood glucose **(A)** and plasma insulin levels **(B)** were markedly increased in Tet14 and Tet29 transgenic rats after doxycyline treatment (DOX, 2 mg/mL in 10% sucrose for 4 days), while body weight **(C)** of the same rats was significantly reduced. Means ± SEM are shown. Statistical significance was confirmed using student's t-test, * $p<0.05$; # $p<0.05$ compared to the WT rats. (n=3).

3.2.1.2.2. Insulin signalling in shRNA transgenic rats

First, an insulin sensitivity test was performed to check whether glucose levels in the InsR knockdown rats can be affected by insulin injection. The blood glucose was measured before and 15 min after i.p. injection of insulin (10 U/kg) or saline as a control. Insulin led to a significant decrease in glucose level in both control groups, DOX treated and transgenic untreated animals, but not in the DOX treated transgenic rats (Figure 25A). These data suggested reduced signal transduction by the InsR in knockdown rats.

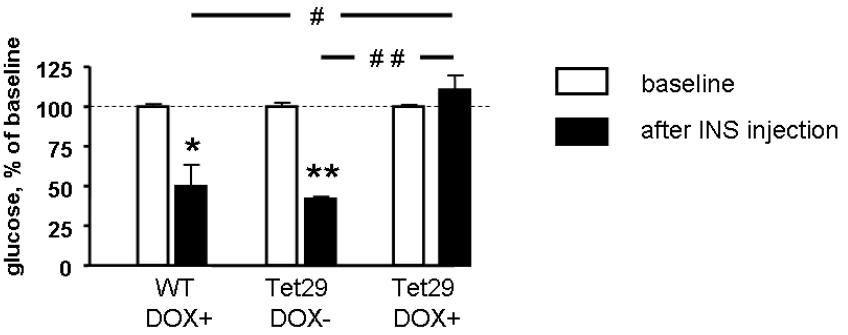
To further examine if intracellular signalling of the InsR is altered in rats acutely treated with insulin, the phosphorylation state of the AKT protein, a Ser/Thr kinase activated through the cascade of reactions initiated by the InsR after insulin binding, was analyzed. Western blotting analyses of proteins from WAT, BAT and skeletal muscle showed stronger phosphorylation of AKT after insulin injection in all control rats. In contrast, no or very weak AKT phosphorylation was seen in DOX treated transgenic rats (Figure 25B). This provided strong evidence for an efficient functional InsR inactivation achieved by DOX-induced shRNA expression.

3.2.1.2.3. Reversibility of knockdown

Next, it was tested whether the InsR knockdown was reversible. Three groups of Tet29 female rats were treated with different DOX doses (20 mg/kg, 2 mg/kg and 0.5 mg/kg) until glucose levels reached between 250 and 300 mg/dL in the treated transgenic rats. Thereafter DOX was withdrawn from drinking water. Despite cessation of the drug blood glucose increased further in all tested groups until reaching a plateau (350 mg/dL - 450 mg/dL) and, dependent on the given dose, stayed stable for 1-2 weeks. After that, the increased glucose levels slowly returned back to normal level in all examined groups (Figure 26A). In parallel, to test for a possible gender difference in the DOX response, a group of Tet29 male was examined with 20 mg/kg DOX in 1% sucrose, too. The pattern of blood glucose concentrations was similar as in females but the time of recovery to normal levels was longer for males (Figure 26A). In parallel to the blood glucose level, drinking level was increased dose-dependently

in all DOX treated transgenic rats and returned to normal level after drug withdrawal (Figure 26B).

A



B

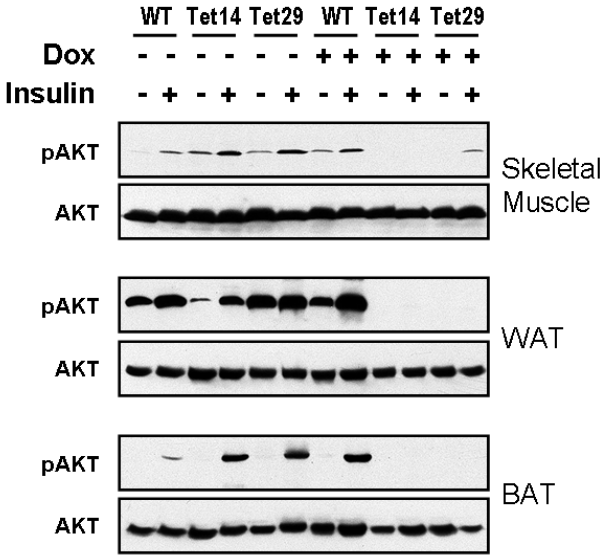
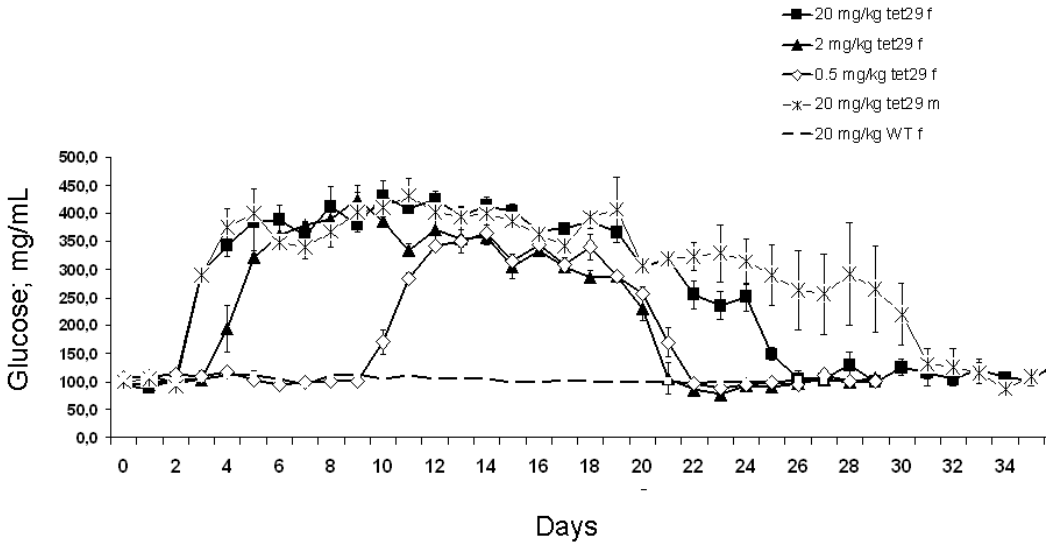


Figure 25: Insulin signalling in Tet14 and Tet29 transgenic rats. After acute doxycycline treatment (2 mg/mL DOX in 10% sucrose), WT and shRNA transgenic (Tet14, Tet29) rats were given an intraperitoneal bolus of 10 U insulin or saline and killed after 15 minutes. **(A)** Blood glucose from the tail vein of experimental rats was measured before (baseline) and 15 minutes after insulin or saline injection (after INS injection). For each column ±SEM (standard error of the mean) is indicated. Statistical significance was confirmed using the student's *t*-test (n=3), * $p<0.05$; ** $p<0.01$ compared to baseline; # $p<0.05$; ## $p<0.01$. **(B)** Skeletal muscle, white adipose tissue (WAT) and brown adipose tissue (BAT) were collected and homogenized in RIPA buffer for protein extraction. 20 µg of total protein lysate were then submitted to 10% SDS-PAGE gel and immunoblotted with antibodies against total AKT and phospho-AKT (Ser473-AKT) proteins. **DOX:** Doxycycline.

These data show that the tetracycline inducible system used in these rats for shRNA mediated gene knockdown is completely reversible after cessation of DOX. Whether other complications such as renal damage caused by diabetes were reversible too, was examined in further investigations described below.

A



B

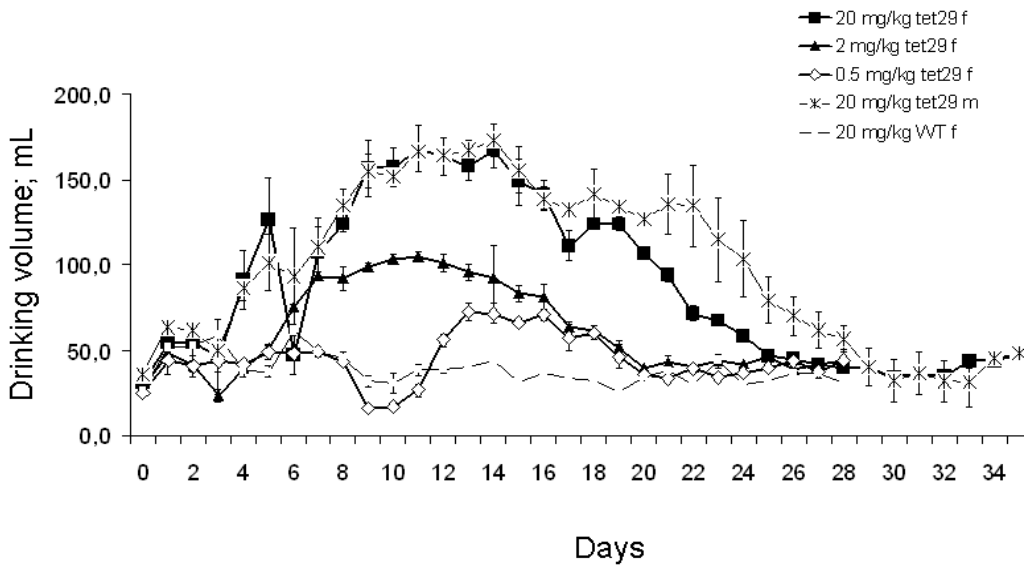


Figure 26: Reversibility of gene knockdown. The reversibility of insulin receptor knockdown was shown in three groups of Tet29 transgenic female rats and a group of Tet29 transgenic male rats, all treated with different doses of doxycycline in 1% sucrose as indicated. DOX treatment was stopped when blood glucose levels reached values between 250 and 300 mg/dL and the further development of blood glucose **(A)** and drinking consumption **(B)** were monitored. Means ± SEM are shown (n=3).

3.2.1.2.4. Long lasting knockdown leads to chronic diabetes type II model

In order to establish a novel chronic model of type II diabetes mellitus three groups of male Tet29 rats ("5→1", "5→2" and "young 5→1") were treated with low DOX doses in 1% sucrose for 7 weeks. The first two groups of rats were in the age of 3-5 months, while the third group of young rats "young 5→1" was 2 months old. Treatment begun with a 5 µg/mL dose of DOX and was given to all rats, until blood glucose levels reached 300 mg/dL. Thereafter this dose was changed to 1 µg/mL for the "5→1" and "young 5→1" groups and to 2 µg/mL for the "5→2" group with the subsequent lowering of the dose to 1 µg/mL after three days, to prevent further glucose increase. In all groups the 1 µg/mL dosage was maintained until the end of the study after 40 days. The long term treatment with these low DOX doses resulted in a moderate but statistically significant ($p < 0.05$) enhancement of the blood glucose levels (Figure 27A) and drinking volume (Figure 27B) in transgenic rats. Moreover, a slight progressive loss of body weight was observed in all chronically diabetic animals (Figure 27C). In the chronically treated rats a high expression of shRNA was also to detect (Figure 29A), as well as near complete down regulation of InsR in the heart (Figure 29B). ELISA measurements of plasma insulin showed drastic insulin elevation in the "5→1" rat group after 2 weeks of treatment (Figure 28).

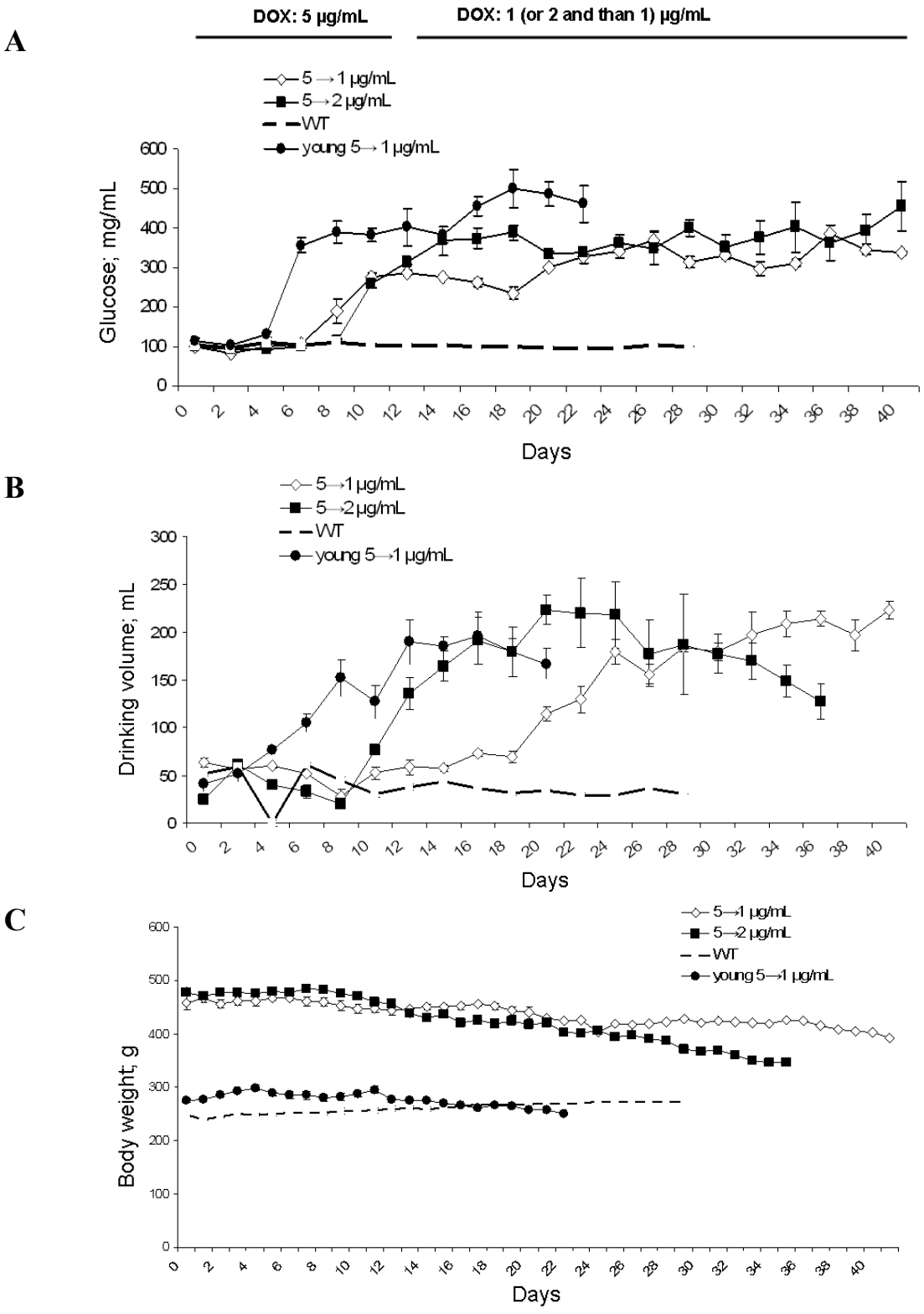


Figure 27: Chronic diabetes type II model in rats. A group of WT and three groups of Tet29 transgenic male rats ("5→1" and "5→2" and "young 5→1") were treated with 5 µg/mL of DOX in 10% sucrose to induce blood glucose increase up to nearly 300 mg/mL. Thereafter, the DOX dose was reduced to 1 µg/mL in two of the groups, the "5→1" and "young 5→1". The "5→2" group was first reduced to 2 µg/mL and after three days to 1 µg/mL of DOX. The lowest DOX dose of 1 µg/mL was maintained in all three groups until the end of the study. Blood glucose **(A)**, drinking volume **(B)** and body weight **(C)** were measured every second day, as indicated. Means ± SEM are shown (n=3).

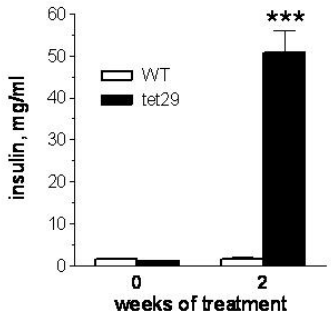


Figure 28: **Plasma insulin in rat model with chronic type II diabetes.** WT and Tet29 males were treated with low DOX doses, as described in Figure 27. WT and Tet29 ("5→1") groups were examined for plasma insulin level by ELISA. Measurements were carried out before and after two weeks of long term treatment. Means ± SEM are shown. Statistical significance was confirmed using student's t-test; *** $p<0.001$ compared to WT; (n=3).

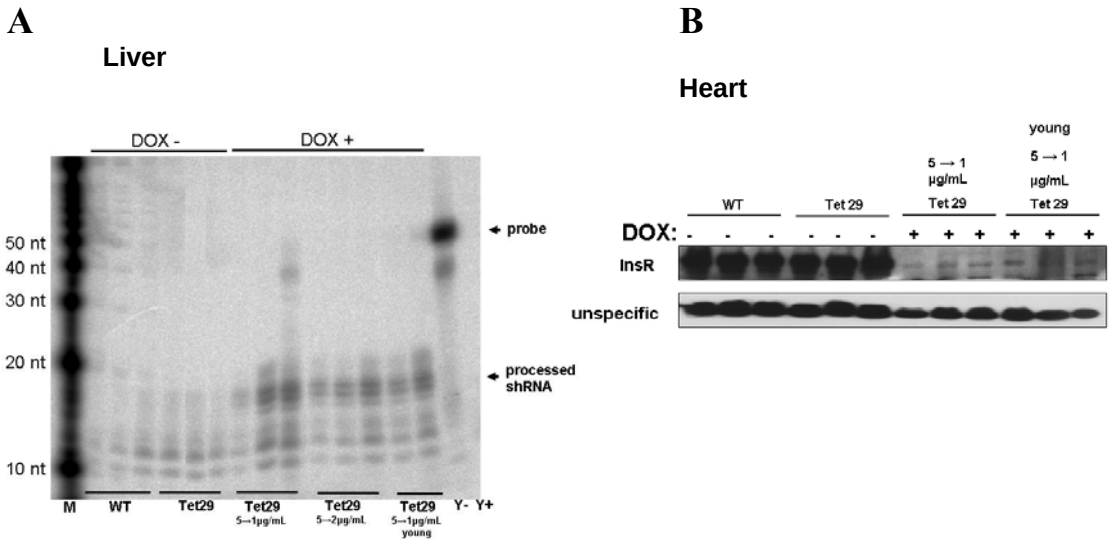


Figure 29: InsR and shRNA expression in rat model with chronic type II diabetes. After long term treatment with low doxycycline doses, as described in Figure 27, shRNA and InsR expressions were assayed in three groups of Tet29 rats, as indicated. **(A) RPA:** 20 µg of total RNA isolated from liver of WT and Tet29 rats was used to analyse expression of shRNA in treated transgenic rats. **(B) Western blot:** 20 µg of total protein lysate from heart of experimental rats was submitted to 10 % SDS-PAGE gel and immunoblotted with antibodies against InsR. Unspecific bands were used as a control for equal loading. **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

3.2.1.2.5. Renal damage

Chronic diabetes mellitus leads to permanent damage of different tissues including kidney, heart, vessels and retina. In order to test whether such pathologies appear in the presented chronic model, urine was collected to estimate the daily urinary output and albumin excretion. Measurements were carried out once weekly in the last 3 weeks of the study. These analyses showed significant polyuria of chronically treated Tet29 rats in the last weeks of the treatment (week 4, 5 and 6) compared to the non-treated Tet29 group (Figure 30A). This was in accordance to the drinking volume shown in Figure 27B. Furthermore, albumin excretion was markedly increased (Figure 30B). These analyses clearly confirmed the development of renal damage in the chronic rat model for type II diabetes mellitus, already after 5 weeks of low dose treatment with DOX.

To determine the renal damage of recovered Tet29 rats after DOX cessation the same set of tests for nephropathy were performed. Interestingly, in spite of normal blood glucose level, as shown in Figure 26, the total urine volume was significantly higher in treated compared to untreated animals (Figure 31A). Differently, albumin excretion was slightly, but not significantly, increased in the group with DOX withdrawal (Figure 31B).

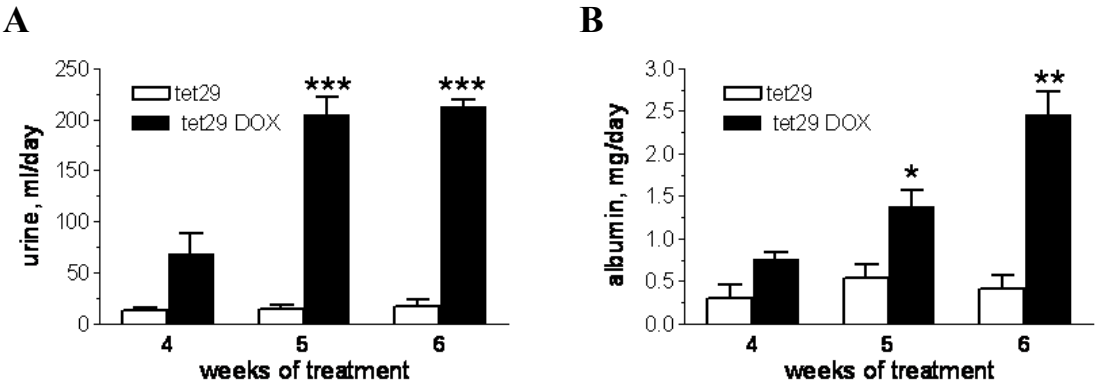


Figure 30: Polyuria and albuminuria in rat model with chronic type II diabetes. Tet29 transgenic rats were treated with low doxycycline (DOX) doses, as described in Figure 27. WT and Tet29 ("5→1") rats were kept in metabolic cages for one day (24h) per week and after 24 hours the volume of collected urine was determined. Urine volume (ml/day) (**A)** and albumin excretion **(B)** rates significantly increased in treated Tet29 transgenic rats, as shown. Means ± SEM are shown. Statistical significance was confirmed using student's t-test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$ compared to untreated rats; (n=3).

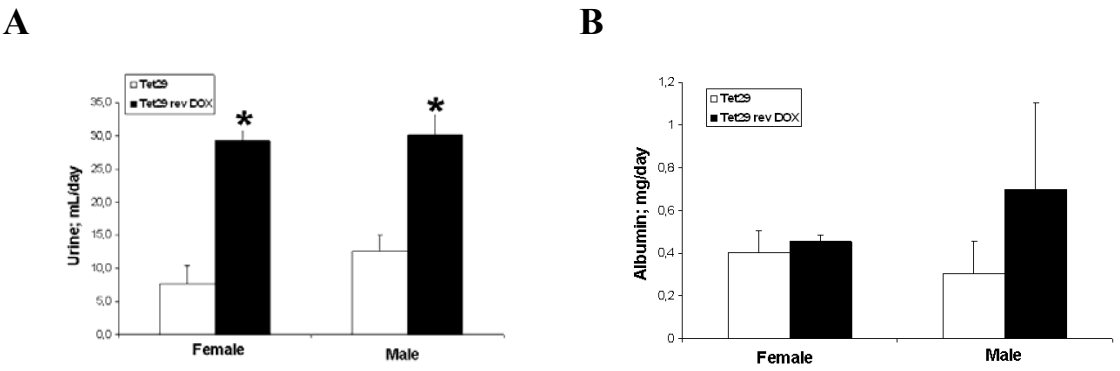


Figure 31: Polyuria and albuminuria in rats with reversible knockdown. Reversible knockdown in Tet29 rats was carried out with treatment of 20 mg/kg doxycycline (DOX) until blood glucose values reached between 250 and 300 mg/mL. Thereafter DOX treatment was stopped. Urinary volume **(A)** and albumin excretion **(B)** were determined when rats recovered from DOX treatment and showed normal blood glucose values for more than two weeks. Means ± SEM are shown. Statistical significance was confirmed using student's t-test, * $p<0.05$ compared to non-treated Tet29 rats; (n=3). **Tet29:** non-treated transgenic rats; **Tet29 rev DOX:** DOX treated transgenic rats after DOX withdrawal.

3.2.1.2.6. Lack of toxicity

The reversibility of the phenotype after DOX withdrawal already argued against a toxic effect of the shRNA expression. Nevertheless, it was tested whether shRNA expression triggers interferon (IFN) response in acute or chronically treated Tet29 rats. For this purpose Western blotting was used to detect PKR, an interferon-inducible Ser/Thr specific protein kinase. No PKR upregulation was detected in all tested tissues, such as BAT, WAT and kidney after acute high dose treatment with DOX (Figure 32A) as well as in the heart after chronic low dose treatment (Figure 32B).

Further analyses testing alteration in the biogenesis of natural microRNAs were carried out. Using RPA no alterations were observed in the expression of the endogenous mir122 in the liver of Tet29 transgenic rats after long term shRNA induction by low dose DOX treatment (Figure 33).

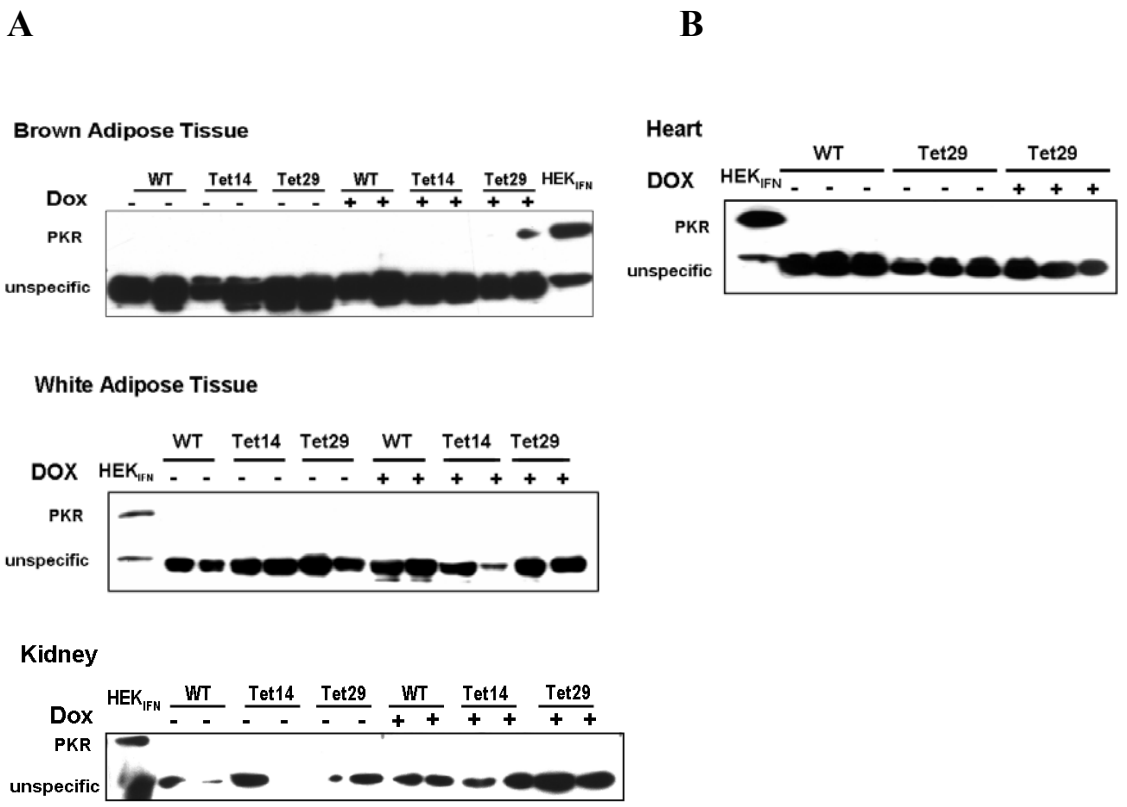


Figure 32: Western blot analyses for detection of IFN response induction. PKR expression was used as marker for interferon response in acutely **(A)**, as described in Figure 24, or chronically treated rats **(B)**, as described in Figure 27. PKR was detected by Western blot in 20 µg protein of brown adipose tissue, kidney and white adipose tissue of acutely DOX treated WT, Tet14, and Tet29 rats **(A)**, as well as in heart of chronically DOX treated Tet29 rats **(B)**. An unspecific band was used as loading control. **DOX:** Doxycycline; **HEK_{IFN}:** positive control for PKR expression, IFNα (10^6 for 24h) induced HEK cells.

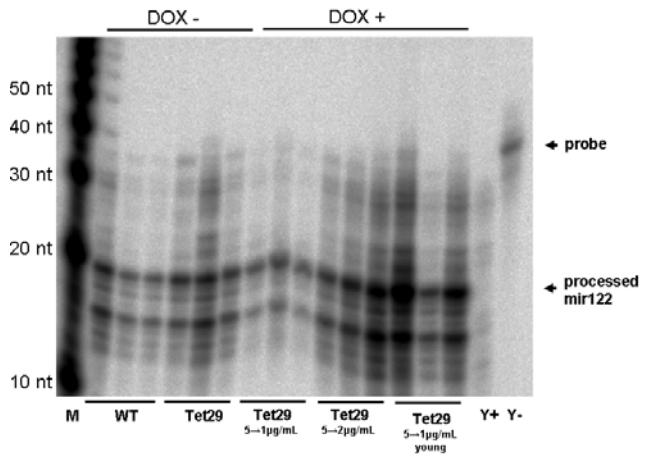


Figure 33: RPA analyses for detection of the endogenous microRNA, mir122. Tet29 rats were treated with low doxycycline (DOX) doses as described in Figure 27. 20 µg of total RNA from liver was used in an RPA for the detection of mir122. **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

3.3. Development of a new conditional gene regulation system

3.3.1. Activation of a modified U6 promoter by a mutated TATA Binding Protein (mTBP)

In order to establish a new inducible gene regulation system for shRNA expression initially two basic DNA constructs, pCMVmTBP and pU6mAdR, were assayed in tissue culture. The pCMVmTBP plasmid contains a modified TATA Binding Protein (mTBP) cDNA harbouring a substitution of three amino acids (Ile194 →Phe; Val203 →Thr and Leu205 →Valin) (Meissner, Rothfels et al. 2001) inserted downstream of the CMV promoter (Figure 34A). The second construct, pU6mAdR, (Figure 34B) consists of a short adenoviral sequence (72 bp) as marker controlled by a mutated U6 promoter bearing point mutations of two nucleotides in the TATA box (**TA**TAA→**AG**TAA) (Figure 6) (Meissner, Rothfels et al. 2001). The concept of how this basic system works is summarized in figure 35. COS7 cells transiently transfected with these constructs were monitored for expression of mTPB protein by Western blotting (Figure 36A). By RPA, the transcription of adenoviral RNAs was assayed and shown to occur only in the presence of the mTBP protein (Figure 36B).

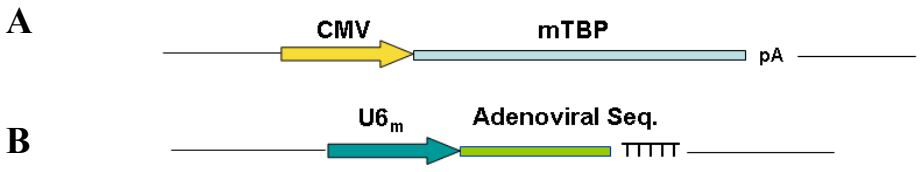


Figure 34: The pCMVmTBP construct is composed of the CMV pol II promoter driving a modified mTBP protein **(A). The pU6mAdR construct** carries as transcription marker an adenoviral sequence controlled by a modified $U6_m$ pol III promoter **(B)**.

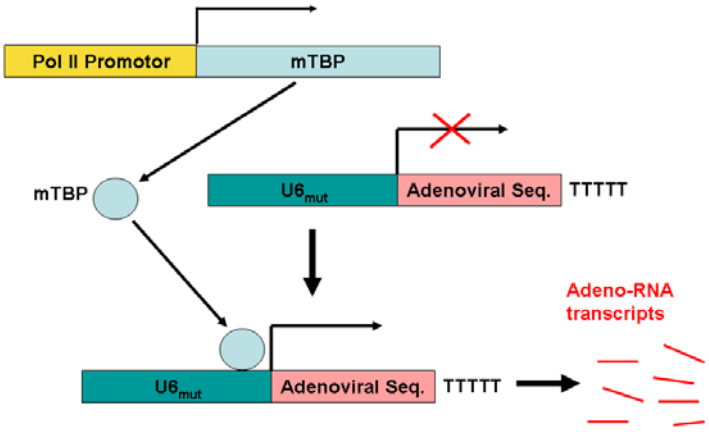


Figure 35: Gene regulation based on the modified mTBP protein and $U6_m$ promoter. Ubiquitously expressed mTBP protein has the ability to recognize the TATA box with point mutations in the $U6_m$ promoter as shown in the Figure 6. Interaction between the mTBP protein and the $U6_m$ promoter initiates the transcription of the adenoviral sequence.

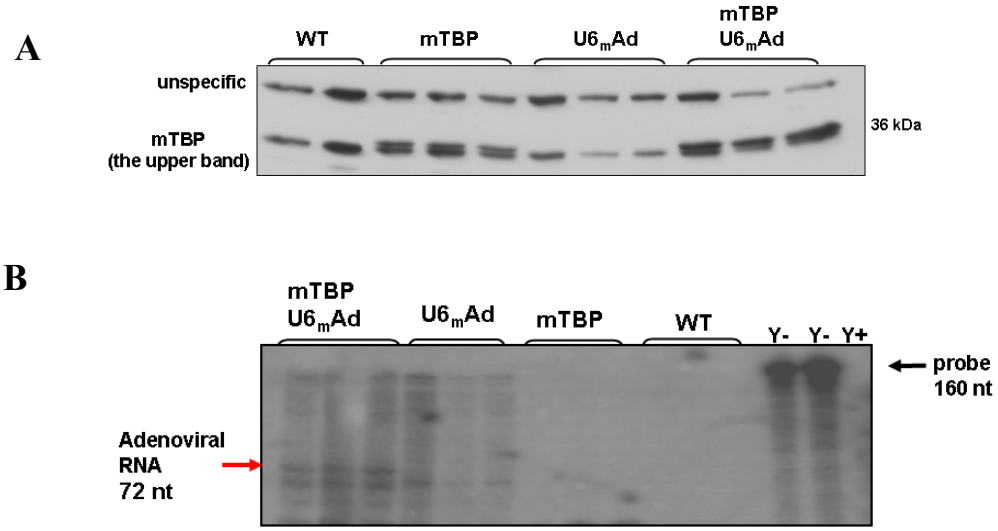


Figure 36: Tissue culture assay of the pCMVmTBP and $pU6_mAdR$ constructs. COS7 cells were transfected either with pCMVmTBP (mTBP), pU6mAdR ($U6_mAd$), or with both of the constructs. 24 hours later cells were harvested for protein and RNA isolation. 20 µg of the protein lysate was used in Western Blotting assays for the detection of the mTBP protein (36 kDa) expression using anti-Penta-His antibodies. An unspecific band was used as loading control **(A)**. By RPA, transcription of the adenoviral RNA (red arrow) was tested using 20 µg of total RNA hybridized with the RPA probe (160 nt long) **(B)**. **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

3.3.2. Tamoxifen-inducible activation of a modified U6 promoter by a chimeric mTBP-ER^{T2} protein

3.3.2.1. Tumor cells

In the next step the basic system of mTBP and $U6_m$ promoter was improved to become inducible by an outside stimulus, the addition of tamoxifen. For this purpose a chimeric protein was generated by the fusion of mTBP (Meissner, Rothfels et al. 2001) with the ligand binding domain of a modified estrogen receptor (ER^{T2}) (Kuhbandner, Brummer et al. 2000). Site-directed mutagenesis in the human ER^{T2} (Gly at 400 → Val; Met at 543 → Ala; Leu at 540 → Ala) has been shown to result in specific sensitivity to tamoxifen and to loss of response to endogenous estrogens (Kuhbandner, Brummer et al. 2000). This inducible system was intended to be used for the generation of shRNA (Figure 37).

The new pCMVmTBP-ER^{T2} construct was designed to express the chimeric protein, mTBP-ER^{T2}, driven by the CMV promoter (Figure 38).

COS7 cells transiently transfected with the pCMVmTBP-ER^{T2} and pU6mAdR constructs demonstrated expression of the fusion protein mTBP-ER^{T2}. Expectedly, synthesis of the mTBP-ER^{T2} was not affected by tamoxifen treatment (1 µM for 48 hours) (Figure 39A). As demonstrated by RPA the adenoviral RNA transcripts appeared only in the presence of the mTBP-ER^{T2} protein. However, transcription was not regulated by tamoxifen (Figure 39B).

Next, to test whether different tamoxifen doses can affect expression levels of the adenoviral marker, transfected cells were treated with different concentrations of tamoxifen for 48 hours: 0.01 µM, 0.1 µM, 1 µM, 10 µM and 100 µM. Synthesis of the mTBP-ER^{T2} protein remained unchanged (Figure 40A). The transcription of the adenoviral marker was not regulated by low doses of tamoxifen but was suppressed at high doses (Figure 40B). In addition, cell viability was diminished when high drug doses were applied (10 µM and 100 µM).

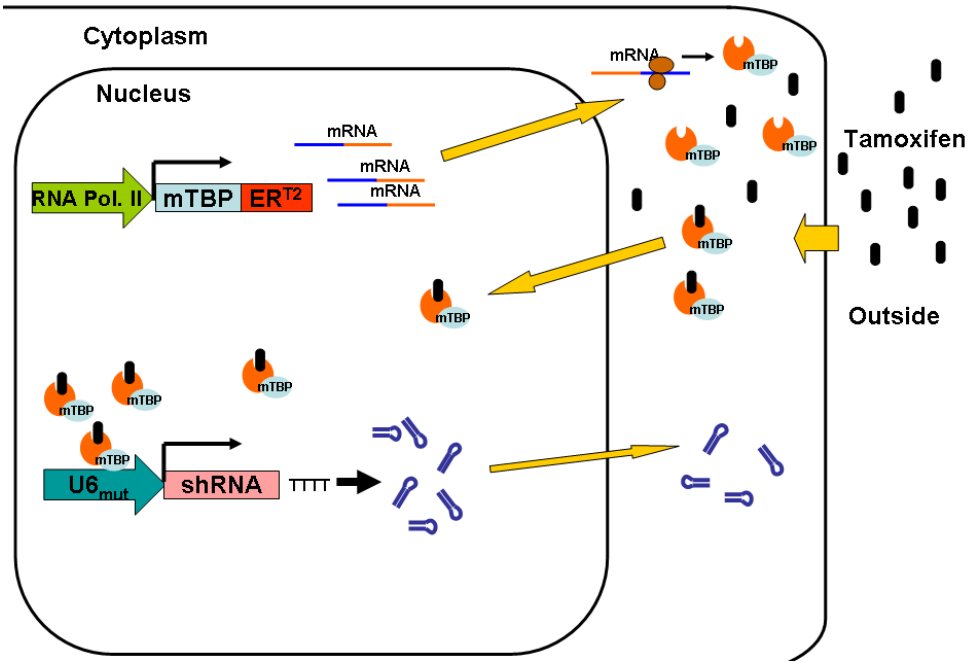


Figure 37: Conditional gene silencing by induction of shRNA with tamoxifen. Ubiquitously expressed mTBP-ER^{T2} chimeric protein is translocated into the nucleus only after tamoxifen binding. Transcription of shRNA is induced when modified mTBP protein recognizes the mutation in the TATA box of the U6$_m$ pol III promoter.

Figure 38: The pCMVmTBP-ER^{T2} construct bears the chimeric mTBP-ER^{T2} protein controlled by the CMV pol II promoter.

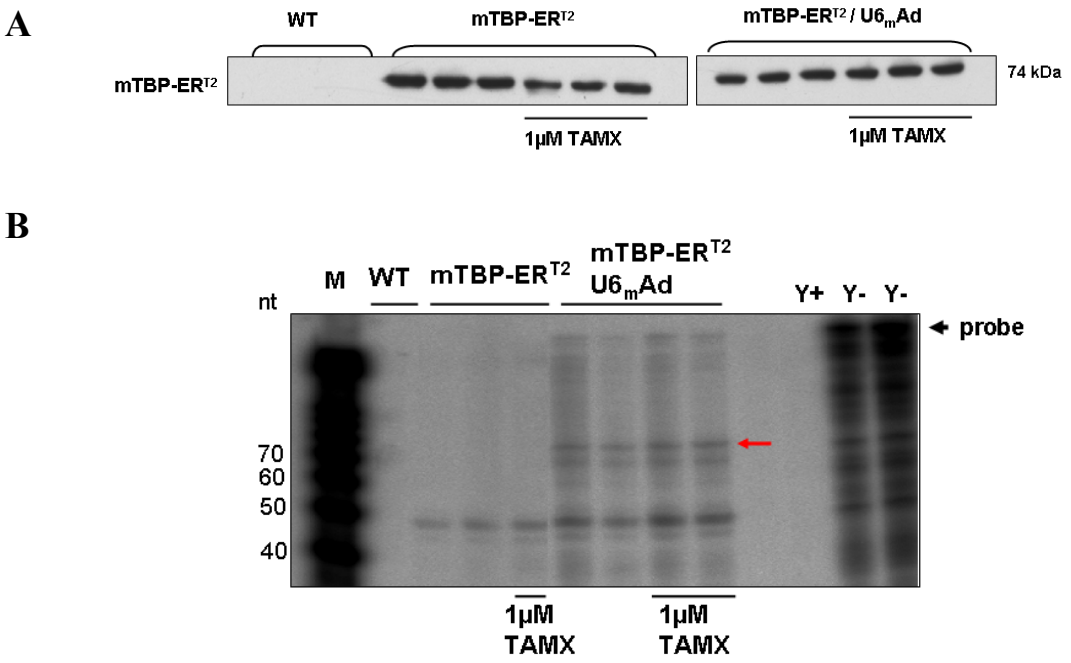


Figure 39: Cell culture assay of the pCMVmTBP-ER^{T2} and pU6$_m$AdR constructs. 24 hours after transfection of COS7 cells with either pCMVmTBP-ER^{T2} (signed as mTBP-ER^{T2}) or pU6$_m$AdR or with both of the constructs, cells were treated with 1 µM tamoxifen (TAMX) for 48 hours. Expression of the mTBP-ER^{T2} protein was detected by Western Blot of 20 µg of cell lysate using anti-Penta-His antibodies **(A)**. Activation of adenoviral RNA transcription (red arrow) was shown with 20 µg of total RNA by RPA **(B)**. **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

3.3.2.2. Stable cell lines

Trying to achieve low level expression of mTBP and mTBP-ER^{T2} proteins in cultured cells, two stable cell lines bearing either pCMVmTBP or pCMVmTBP-ER^{T2} constructs were established. As monitored by Western Blotting assays production of both proteins, mTBP or mTBP-ER^{T2}, was markedly lower in stable cell lines after G-418 selection when compared to transiently transfected cells (Figure 41). As observed by RPA, low expression of mTBP protein did not induce the transcription of the adenoviral RNA marker from the modified U6 promoter (Figure 42A). The same result was found for the mTBP-ER^{T2} protein, even after tamoxifen treatment (Figure 42B). These data suggested that low expression of both employed proteins, mTBP and mTBP-ER^{T2}, was not sufficient for gene activation from the U6 promoter with the point mutation in its TATA box.

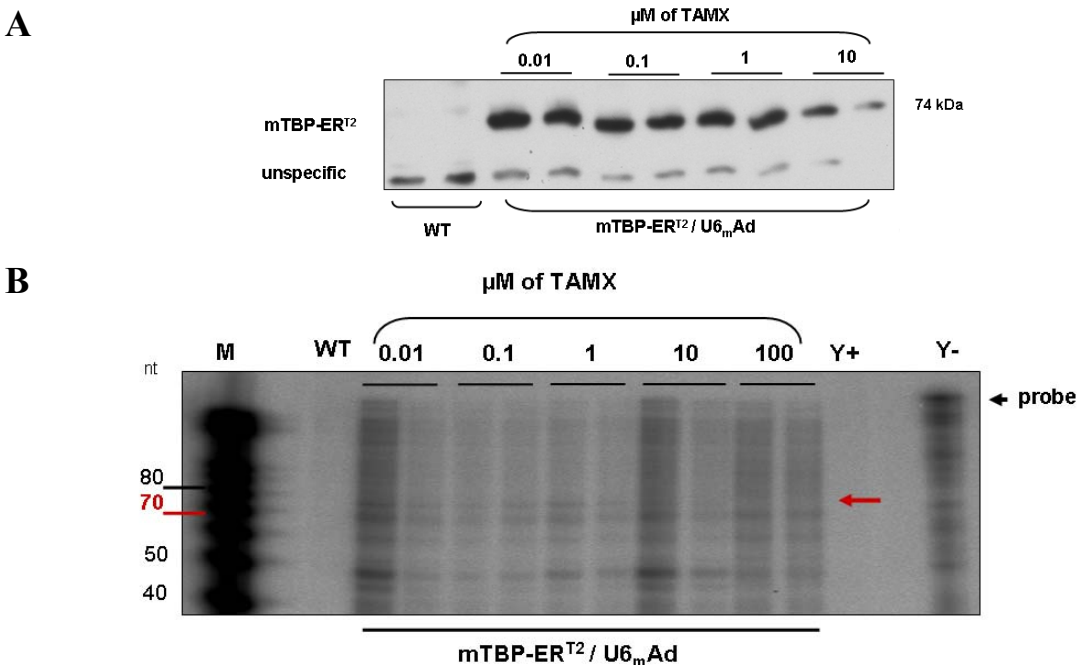


Figure 40: Activation of RNA transcription at different tamoxifen concentrations in COS7 cells. Cells transfected with the pCMVmTBP-ER^{T2} and pU6$_m$AdR constructs were stimulated at different molarities of tamoxifen (TAMX) for 48 h, as indicated. The expression of the mTBP-ER^{T2} protein was assayed by Western Blot using anti-Penta-His antibodies **(A)**. The transcription of the adenoviral RNA (red arrow) was detected by RPA **(B)**. **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

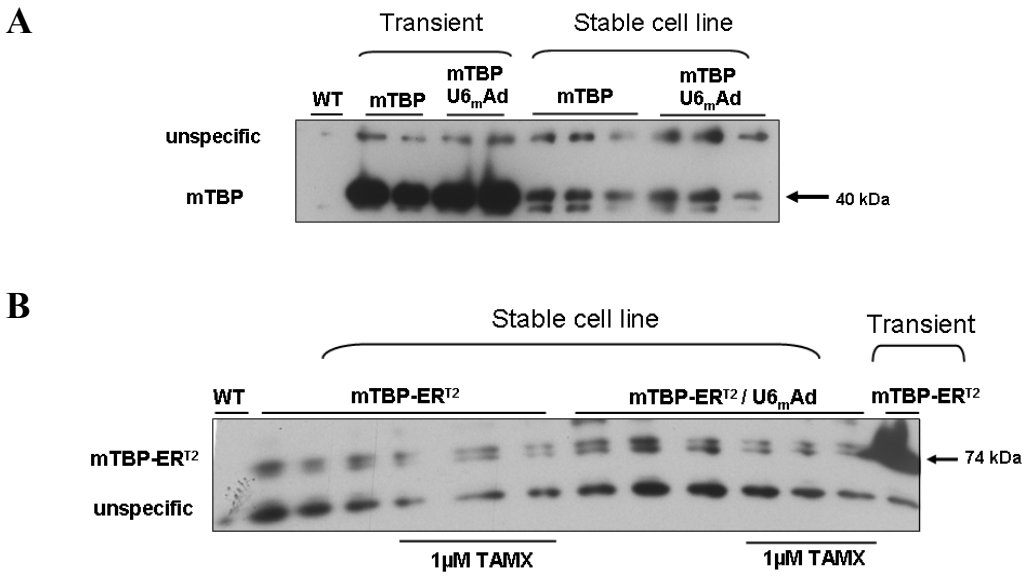


Figure 41: Expression of the mTBP and mTBP-ER^{T2} proteins in stable lines of COS7 cells. COS7 cells were transfected with pCMVmTBP and/or pCMVmTBP-ER^{T2} and/or pU6$_m$AdR constructs, as indicated. To establish stable cell lines for the mTBP and mTBP-ER^{T2} proteins transfected cells were grown under G418 antibiotic selection for 3-4 weeks. Detection of both proteins, mTBP **(A)** and mTBP-ER^{T2} **(B)**, was performed by Western Blotting of 20 µg of protein lysate using anti-Penta-His antibodies. Comparison between stable and transient protein expression was performed in the same blots.

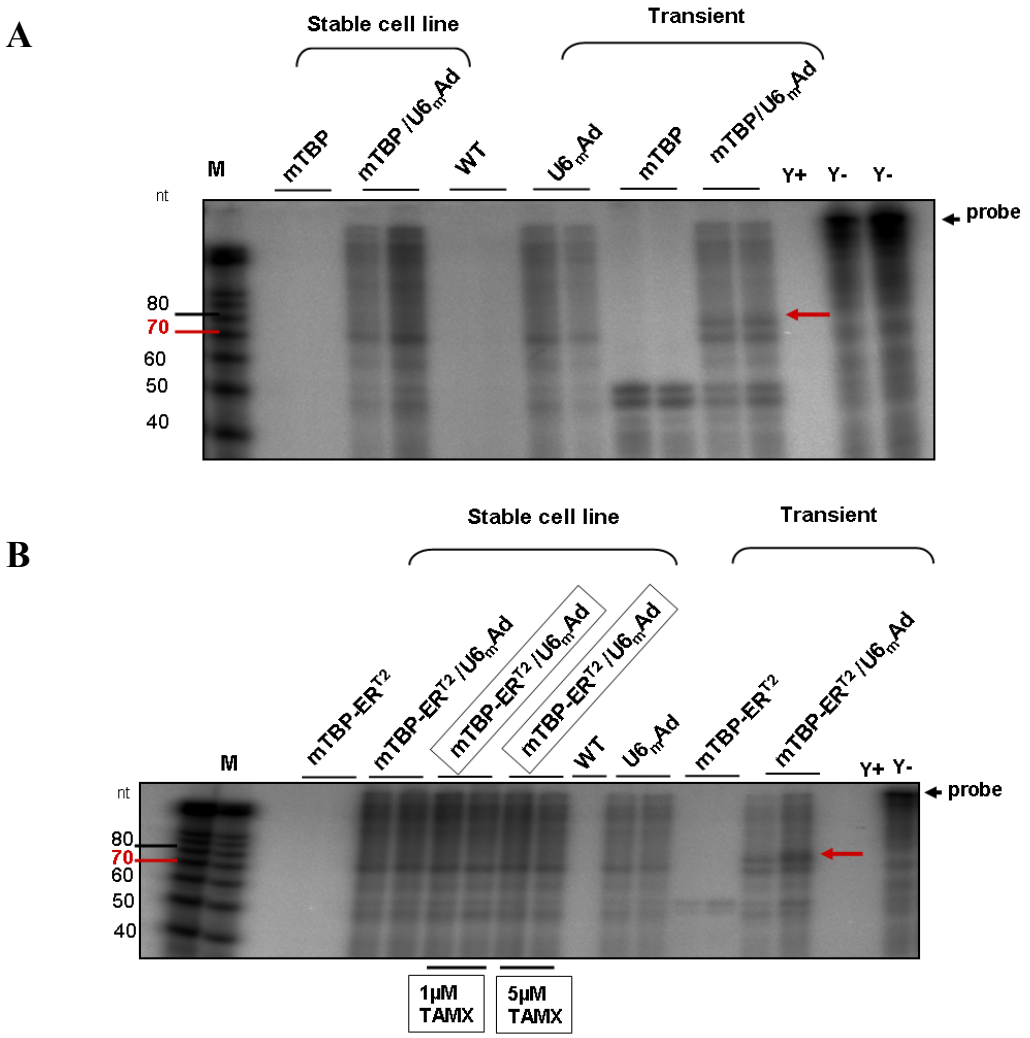


Figure 42: Induction of RNA transcription in stable COS7 cells. Stable cell lines of the mTBP and mTBP-ER^{T2} proteins were established as described in Figure 41. Induction of transcription of the adenoviral sequence by the modified mTBP protein **(A)** and by the chimeric mTBP-ER^{T2} protein in the presence or absence of tamoxifen (TAMX) **(B)** was assayed in corresponding stable cell lines using RPA. Transient expression was employed as a positive control. **M:** RNA Decade marker; **Y+:** yeast positive control; **Y-:** yeast negative control; **nt:** nucleotides.

3.3.3. Generation of mTBP-transgenic rat model

The main goal of developing a new conditional gene manipulation strategy using tamoxifen and the chimeric mTBP-ER^{T2} protein was to apply this system *in vivo*. To determine whether the modified TBP protein may cause any side effects in rats, mTBP-transgenic rats were generated. For this purpose, the pUb-mTBP plasmid bearing the mTBP cassette driven by the ubiquitin promoter (Figure 43) was generated and microinjected into the pronucleus of fertilized eggs of SD rats. Replacement of the CMV promoter with the ubiquitin promoter was carried out to achieve ubiquitous but not too strong expression of the mTBP protein. Survival rate of embryos after pronuclear injection using this construct was very low (Table 12). No offspring of only 16 born (Table 12) was found to be positive for the pUb-mTBP transgene, as detected by PCR genotyping. This result suggested toxicity of the modified TBP protein when expressed *in vivo*. For this reason the inducible shRNA system was not further developed in this thesis.

Figure 43: The pUb-mTBP construct contains the modified mTBP protein driven by the ubiquitin promoter.

Table 12: Overall efficiency of rat transgenesis using pUb-mTBP construct.

Injected DNA	Donor strain	Total no. of injected zygotes	Total no. of zygotes transferred	No. of zygotes transferred to the pregnant recipients	No. (%) of pregnant recipients	Survival rate of embryos, (%)		No. of died pups (%)	No. of transg. pups, (%)
						Total	Per pregnant recipients		
pUb-mTBP	♀ SD x ♂ SD	200	137	72	6/14 (42.9)	16/137 (11.7)	16/72 (22.2)	2/16 (12.5)	0/16 (0)