# 4. DISCUSSION

## 4.1. Transgenic rats with permanently expressed shRNA constructs

To study human diseases, the rat is one of the most used animal models in biomedical research. Because of its larger size compared to the mouse the rat is more suited for anatomical and physiological analyses. Furthermore, due to intellectual skills the rat is also very important and frequently used in behavioral investigations. Therefore, there was a great interest in the production of genetically modified rats in the last years. Until recently only forward genetic methods such as random mutagenesis, conventional transgenesis and serendipitous spontaneous mutations have been available in this species (Lazar, Moreno et al. 2005). In rats the most used gene manipulation strategy is standard pronuclear microinjection developed by several investigators nearly 30 years ago (Gordon, Scangos et al. 1980; Gordon and Ruddle 1981; Palmiter, Brinster et al. 1982). With a few exceptions this technology makes only gene over-expression possible but not gene down regulation which is crucial to study gene function. Gene targeting in rats has remained impossible because of the lack of pluripotent ES cell lines, which exist in mice. In order to overcome this problem, the goal of this work was to establish a new gene inactivation technology through pronuclear microinjection using RNA interference as a gene silencing tool.

In this study, the use of an shRNA against the EGFP gene, which was shown to be a well working silencer in mice and rats (Hasuwa, Kaseda et al. 2002), resulted in completely unsuccessful generation of transgenic rats by pronuclear injection. Among 128 genotyped newborns after injections, only a single founder was positive for the transgene insertion. However, this rat did not express any shRNA nor did it show any reduction in green fluorescence. Thus, less than 1% of newborns were positive for the transgene which was far under statistical average. In our facility, 3-10% of newborn rats of SD background are transgene positive after pronuclear microinjection (Popova, Krivokharchenko et al. 2004). Several factors can influence rat survival after microinjection procedure and thus contribute to low output of transgenesis. Superovulation protocols, embryo transfer, recipient's age, purification techniques of

DNA, the DNA concentration and other factors were constantly controlled in our microinjection facility. Several other constructs carrying different transgenes resulted in up to 12% of successful transgenesis in SD rats after injections (Popova, Bader et al. 2005). These data excluded technical reasons for the low percentage of shRNA transgenic founders in our study and pointed towards embryonic toxicity of shRNA.

The only evidence showing that shRNA works in transgenic rats after pronuclear microinjection was reported by Hasuwa and coworkers (Hasuwa, Kaseda et al. 2002). These authors describe almost complete attenuation of the EGFP gene in 11.5 days-old rat embryos but did not show shRNA expression in their transgenic rats. They also did not report any differences between transgenic lines, if there were any, nor any statistics such as survival rate of born embryos and germline transmission (Hasuwa, Kaseda et al. 2002). However, this report is in contradiction to the data presented in this study and data published by Carmell and coworkers reporting unsuccessful approaches towards the use of shRNA constructs for gene silencing in mice by pronuclear microinjection (Carmell, Zhang et al. 2003).

Furthermore, no germline transmission from the only generated transgenic founder to its progeny was found after breeding with a wild type rat. Again, this suggested that shRNA may somehow be toxic for embryonic development and thus leading to lethality before birth. I concluded that only non-transgenic fetuses or fetuses, in which shRNA was not active, could survive and be born. But, it still remained unclear, why expression of shRNA did not occur. In about 20% of all transgenic animals expression of a delivered transgene may be altered by other genes on the chromosome (Zhan, Liu et al. 2001). This may explain suppression of the integrated shRNA by neighboring sequences in the positive founder.

### 4.1.1. Toxicity of shRNA constructs

It was not clear whether embryonic death was caused by unspecific shRNA effects (Jackson and Linsley 2004) and could be triggered by any hairpin sequence, or whether lethality was caused particularly by the shRNA driven from the pRMCE-U6-shGFP construct. This question was further investigated using another sequence of shRNA against the same EGFP gene and another polymerase III promoter in the pU6shGFP-UbAsRed construct. Based on the lack of shRNA transgenic founders after injection of zygotes, unspecific effects of shRNA cassettes could not be analyzed in this work. Grimm and colleagues tested several hairpin constructs in mice and showed that death was caused only by high doses of delivered shRNA constructs independent of the target gene (Grimm, Streetz et al. 2006). Accordingly, absence of unspecific effects rising from shRNAs was shown also by other groups (Bridge, Pebernard et al. 2003; Fish and Kruithof 2004; Makinen, Koponen et al. 2006; Reynolds, Anderson et al. 2006).

Interferon response induced by highly expressed shRNA in mice embryos was shown by Cao et al. after pronuclear microinjection (Cao, Hunter et al. 2005). Real time RT-PCR measurements of 2'5' oligoadenylate synthase 1 (OAS) in shRNA transgenic fetuses indicated up to 15 fold higher levels of OAS messenger RNAs compared to wild type fetuses. This clearly showed induction of IFN response, in spite of using short shRNA strands (19-21 bp) (Cao, Hunter et al. 2005). On the contrary, others demonstrated this induction only when longer dsRNA duplexes were used (Manche, Green et al. 1992; Fish and Kruithof 2004; Reynolds, Anderson et al. 2006). Furthermore, as suggested by Cao et al. highly expressed shRNA may be also responsible for the lack of germline transmission. Statistical analyses of their study showed only 15% survived F1 offspring from positive founders ($F_0$) while 38% of offspring died at birth and further 30% died during pregnancy. Next, little or inconsistent inhibition of gene expression appeared in liver, kidney and lung of survived transgenic offspring. This pointed towards toxicity of highly expressed shRNA or better to tolerance of lowly expressed hairpins in the surviving animals. Obviously fetuses with low shRNA expression escaped IFN response induction and

were able to survive in contrast to dead embryos probably highly expressing shRNAs (Cao, Hunter et al. 2005).

Recent investigations showed that intravenously delivered high doses of shRNA (25-mer) constructs cause toxicity in mice while low doses of 19-mer shRNA constructs gave an efficient and persistent knockdown for more than one year (Grimm, Streetz et al. 2006). These data are in accordance with studies made in cell culture (Fish and Kruithof 2004; Reynolds, Anderson et al. 2006). Cell death was triggered by the competition between highly expressed 25-mer shRNAs and endogenous microRNAs for intracellular processing (Grimm, Streetz et al. 2006). Maturation of cellular microRNA was shown to be impaired by abundant shRNA production. The authors also showed normal interferon α/β/γ levels in mice receiving high hairpin doses (Grimm, Streetz et al. 2006) which was contradictory to reports of other groups (Bridge, Pebernard et al. 2003; Fish and Kruithof 2004; Cao, Hunter et al. 2005; Reynolds, Anderson et al. 2006).

Together, there is increasing evidence for shRNA toxicity, such as induction of IFN and reduction of cell viability, both triggered either by long hairpin duplexes or high doses of shRNA expressing vectors when introduced into tissue cultures (Bridge, Pebernard et al. 2003; Fish and Kruithof 2004; Reynolds, Anderson et al. 2006) or *in vivo* (Cao, Hunter et al. 2005). However, in our study it was not possible to determine, how toxic the shRNA constructs employed in the rat embryos were, since no shRNA-expressing founders were born. The pRMCE-U6-shGFP and pU6shGFP-UbAsRed plasmids tested in cell culture did not show dying cells after transfection. In other words, tissue culture is very limited model and can not be compared to highly developed and complicated animal organisms. Therefore, in this thesis tissue culture was not used to analyze shRNA side effects *in vitro*, which may potentially appear *in vivo.* Besides this, IFN response induction is cell type – dependent and many tumor cells do not have the ability to induce IFN response (Stojdl, Lichty et al. 2000; Reynolds, Anderson et al. 2006). Short hairpins (19-mer) in high dose were shown not to trigger toxic effects in mice such as IFN response or overloading of RNAi machinery (Grimm, Streetz et al. 2006). If this is true, than 19 nt and 21 nt long hairpins from pRMCE-U6-shGFP and pU6shGFP-UbAsRed employed in this thesis were not expected to interfere with cellular microRNAs leading to embryo death even if expressed in high doses. However, it was technically impossible to find out, which

shRNA dose or which intensity of hairpin expression is suitable to achieve an effective but not toxic knockdown in rats. In this work an *in vivo* application of two different constructs harboring permanently active shRNA cassettes under non-inducible and thus non-controllable U6 promoters has been shown as a non-functional technology for rats, using standard pronuclear microinjection.

## 4.1.2. Transgenic rats with single copy integration of an shRNA construct

Taking into account all findings about the toxicity of highly expressed shRNA (Bridge, Pebernard et al. 2003; Fish and Kruithof 2004; Cao, Hunter et al. 2005; Grimm, Streetz et al. 2006; Reynolds, Anderson et al. 2006) which may explain the failure of shRNA technology in rats, the next step of this thesis was trying to reduce the amount of shRNA production in rats. Predictably, insertions in tandem repeat pattern normally observed in transgenic animals lead to high expression of shRNAs from the multiple inserted transgene fragments. To overcome such overproduction of hairpins, the use of a large Bacterial Artificial Chromosome (BAC) construct from the mouse locus *Rosa26* was chosen to prevent multi-copy integration sites of shRNA transgene into the rat genome after injection.

However, none of the 118 born founders was positive neither for the shRNA cassette in the *Rosa26* BACs nor for any *Rosa26* sequences after microinjection. This fact supports the hypothesis that even one copy of an shRNA construct is toxic for embryo development. Thus, the problem may be the used promoters. The RNA III polymerase system yields high rate of transcription with multiple rounds of initiation and an efficient and defined termination. Our work suggested that an abundant synthesis of shRNA molecules by the U6 promoter may exceed the limit of safe and non-toxic levels in embryos. RNA polymerase III promoters, U6 and H1, are most commonly used for shRNA transcription. An *in vitro* study, in which efficiency of U6 and H1 promoters was compared, demonstrated superiority of U6 upon H1 promoter (Makinen, Koponen et al. 2006). U6 was shown to achieve a stronger silencing effect than H1 promoter. But none of them led to induction of IFN response (Makinen, Koponen et al. 2006). Nevertheless, Cao and colleagues also stressed the importance

of promoter choice to drive shRNAs in mice. Percentage of positive liveborn mice was shown to be two fold higher when H1 promoter was employed to drive shRNAs compared to U6 promoter. The OAS gene, related to IFN response, was up regulated in fetuses carrying shRNA transgenes driven by the U6 promoter, while analyses testing the H1 promoter were not carried out (Cao, Hunter et al. 2005).

The GC rich region at the start positions (-2, -1, +1 and +2) of an U6 promoter was suggested to play an important role in IFN induction (Pebernard and Iggo 2004). When the natural GC rich region was exchanged with two AA nucleotides, expression of genes related to the IFN response was up-regulated, as shown in tissue culture. Indeed, in constructs employed in our study AA nucleotides were present at the transcription start site. This could theoretically have caused IFN response resulting in the loss of transgenic rat founders expressing shRNA in our study. But since the impact of such nucleotide exchange was confirmed only *in vitro* (Pebernard and Iggo 2004; Makinen, Koponen et al. 2006) and not *in vivo* it is difficult to extrapolate such findings to the non-working shRNA technology in rats described here.

### 4.1.3. *In vitro* analysis of shRNA toxicity in embryos

In order to investigate the impact of shRNA on survival of embryos at the early stage of development, the next goal of this study was to perform an *in vitro* study of embryos injected with shRNA constructs.

*In vitro* observations of EGFP rat embryos detected a single blastocyst which after injection of the pRMCE-U6-shGFP construct did not show green fluorescence in contrast to all other blastocysts. Due to its small size it was impossible to determine, neither by PCR nor by RPA, whether this silencing effect was indeed a consequence of the introduced shRNA construct, or whether it was rather an artifact. The survival rate of injected zygotes at the blastocyst stage scored up to 26 % and was in accordance to published data (Popova, Krivokharchenko et al. 2004). This result further suggested that shRNA may not harm embryos in preimplantation but in later developmental stages.

Accordingly, no toxic or lethal effects caused by expressed shRNA from the pU6shGFP-UbAsRed construct were detected in injected mice embryos, from zygote

until blastocyst stage when compared to control embryos receiving control plasmids. Active transcription of shRNA gene was expected to occur due to the fluorescence of the AsRed marker included in the same construct. This suggested again that any negative side effects of the introduced hairpin appears later in fetal development. However, embryos can not be cultured longer than until blastocyst stage *in vitro*. Therefore, long lasting analyses of embryonic development could not be carried out. On the other hand, an *in vivo* investigation for the same purpose would be complicated, animal and time consuming, and was therefore not performed in this thesis.

## 4.2. Transgenic rats with lentiviral shRNA constructs

Gene knockdown in transgenic rats was first shown by Dann et al. using lentiviral-mediated shRNA expression (Dann, Alvarado et al. 2006). This raised the question why transgenic rat production is possible with lentiviral transfection but not with pronuclear microinjection, as shown in this work. The advantage of lentivirus system is a higher transgenesis rate (>28%) in several species (Park 2007). This technology results in a very effective production of transgenic rats compared to pronuclear microinjection suffering from low survival rate and low number of born transgenic founders (3-10%) after microinjection (Popova, Krivokharchenko et al. 2004; Popova, Bader et al. 2005; Park 2007). Since viruses through long evolution adopted their invasion strategies to incorporate their genetic material into the genomic DNA of hosts, with an intention to survive and proliferate, they achieved an enormous efficiency of cell infection. Therefore, an application of viral vectors to manipulate the mammalian genome is a powerful tool for establishment of transgenic animals (Park 2007). In opposite to the viral natural cell penetration and genomic integration ability, the pronuclear microinjection is an artificial and mechanical process of delivery of foreign DNA. The plasma membrane of rat zygotes is very resistant against penetration and the pronuclear membrane is very sticky. Therefore, mechanical procedures in the rat zygote can easily damage the embryo leading to lysis. From this reason, particularly in rats even more than in other mammals, the pronuclear microinjection is difficult (Popova, Krivokharchenko et al. 2004).

On the other hand, an advantage of the conventional pronuclear microinjection approach is the short time necessary for the generation of transgenic lines. Founders generated by lentiviral-vectors are born with genetic mosaicism of transgene expression and multiple integration sites and must be further bred for several generations to yield genetically pure lines (Park 2007). Therefore, lentiviral transgenesis is more complicated, time and animal consuming.

Dann et al. did not comment on cellular toxicity caused by shRNA in their rat model if there was any detected. The low output of transgenic founders with efficient shRNA silencing and the extremely low germline transmission rate (Dann, Alvarado et al. 2006) somehow support the suggestion that only suppression or low activity of shRNA permits transgenic founders to overcome lethality caused by abundant hairpin production, as concluded from our work and the work by others (Cao, Hunter et al. 2005).

## 4.3. Transgenic rats with shRNA activated by a tetracycline inducible system

### 4.3.1. Successful transgenic rat generation with inducible and reversible gene knockdown in rats

The next goal in this study was to avoid the problem of permanent shRNA expression during embryogenesis. Driving the expression of an shRNA against the InsR with a tetracycline-activatable system allowed the generation of transgenic offspring by pronuclear microinjection at normal efficiencies. In the resulting lines the shRNA transgene was inherited by the rules of Mendel.

Until DOX was added to the drinking water, shRNA expression remained latent in the transgenic rats and blood glucose, as well as insulin levels, were equivalent to WT animals. After DOX treatment, shRNA expression was activated in several tissues of transgenic rats and led to variable inhibition of InsR synthesis dependent on the transgenic line and the tissue. In particular, the brain was partially refractory to the effects of DOX probably due to the blood-brain barrier. Studies in animals carrying active tetracycline inducible systems have gained comparable results in the past (Mansuy and Bujard 2000; Seibler, Kleinridders et al. 2007). Only little differences were found between Tet14 and Tet29 lines in inhibition of the InsR production and elevation of blood glucose as well as plasma insulin. Our findings provide evidence that TetR is ubiquitously present and keeps the shRNA expression latent in all tissues at all stages of development.

DOX withdrawal completely restored normal physiological conditions such as glucose levels and drinking consumption in transgenic rats. The time frame of reversibility using the tetracycline system is, however, dependent on the dose and the period of administration of DOX, as previously shown by several other scientists (Gossen and Bujard 1992; Furth, St Onge et al. 1994; Kistner, Gossen et al. 1996; Baron and Bujard 2000; Ohkawa and Taira 2000; Dickins, Hemann et al. 2005; Stegmeier, Hu et al. 2005; Dickins, McJunkin et al. 2007; Seibler, Kleinridders et al. 2007). Restoration of InsR expression and plasma insulin in knockdown rats after DOX withdrawal have not been tested in this study yet, but demonstrated to be

normalised in diabetic mice generated with the same construct (Seibler, Kleinridders et al. 2007).

There have been reports about inducible knockdown of genes in transgenic mice using the Cre-lox system (Ventura, Meissner et al. 2004; Coumoul, Shukla et al. 2005; Yu and McMahon 2006). While this technology allows tissue-specific gene silencing, it has the disadvantage to be not reversible. Furthermore, the extent of tetracycline-regulated gene knockdown can be titrated by the dose of DOX given to the animals, while the Cre-lox system can not. Thus the TetR-tetO regulated system is more versatile for conditional gene silencing. In mice, such conditional gene regulation has already been shown to be a very efficient genetic tool for gene silencing (Czauderna, Santel et al. 2003; Dickins, Hemann et al. 2005; Dickins, McJunkin et al. 2007; Seibler, Kleinridders et al. 2007).

## 4.3.2. Rat model of type II diabetes mellitus

In addition, to a novel strategy for loss-of-function experiments in rats, a new model of insulin resistance has been presented in this thesis. Consequently to the InsR down regulation, a cluster of phenotypes was observed that closely resemble human type II diabetes pathophysiology, including hyperglycaemia, hyperinsulinemia, loss of body weight, polyuria and proteinuria.

As herein shown, long lasted low-dose treatment resulted in chronic disease of type II diabetes mellitus including nephropathy. Progression of the disease was dependent on the DOX dose and age of rats. Lower body weight of younger animals led to an earlier onset of disease and also rapid increase of blood glucose values and drinking consumption compared to older animals. Slow but progressive development of symptoms in treated transgenic rats allowed survival for more than 6 weeks. Extremely low concentrations of DOX in the drinking water were sufficient for the induction of shRNA expression and the maintaince of InsR knockdown for a long period of time, not causing any side effects by DOX or shRNA.

Not many models of insulin resistance-associated type II diabetes have been described in rats, mainly due to the lack of gene targeting technologies in this species. The Zucker rat, Goto Kakizaki rat, Israeli sand rat and OLEFT rat are some of the few

examples, whose symptoms related to type II diabetes, appear spontaneously. The onset of complications in these rats is very variable depending on the strain, age, and gender (Chen and Wang 2005; Rees and Alcolado 2005). However, using the tetracycline inducible system to knockdown the InsR gene, a rat model of conditional insulin resistance, which is easy to be induced and well controlled by the dose of DOX, was generated.

## 4.3.3. Safety of the tetracycline activatable shRNA expression

Doxycycline, a derivate of tetracycline, is commonly used for a variety of infections. For human treatment daily doses of 1 to 6 mg/kg body weight are orally prescribed (www.smart-drugs.com). In this study, transgenic rats were treated with highest daily doses of 200-250 mg/kg body weight DOX for acute treatment, 0.7-30 mg/kg DOX in the reversible knockdown assay and only 1 mg/kg DOX per day in the long term treatment experiment. In this work no side effects were observed in WT rats receiving the antibiotic at the same doses as transgenic rats. Moreover, there is no evidence that orally given DOX in the doses required for induction of the tetracycline system causes any toxic effects in animal models (Gossen and Bujard 1992; Furth, St Onge et al. 1994; Kistner, Gossen et al. 1996; Mansuy and Bujard 2000; Ohkawa and Taira 2000; Czauderna, Santel et al. 2003; Dickins, Hemann et al. 2005; Dickins, McJunkin et al. 2007; Seibler, Kleinridders et al. 2007).

Elimination half-life of DOX varies from 11 to 23 hours (www.mold-survivor.com). Higher DOX doses induced stronger effects and longer duration of hyperglycaemia in treated rats after DOX withdrawal. A time delay of animals' recovery after DOX cessation was also shown in mice (Seibler, Kleinridders et al. 2007). As expected from pharmacokinetics, accumulation of DOX in the rat body was faster than its elimination. The elimination kinetics are very slow, probably due to the slow clearance of DOX from the circulation as has been reported before for animals carrying the tet-on system (Mansuy and Bujard 2000). Nevertheless, Böcker et al. demonstrated very fast elimination of DOX and tetracycline from the serum (Bocker, Warnke et al. 1984). However, rapid uptake and high binding of DOX to the liver,

kidney and lungs leads to non-homogenous distribution of the drug outside the plasma (www.mold-survivor.com; Bocker, Warnke et al. 1984). Slow drug clearance from bones and heart (Bocker, Warnke et al. 1984) may additionally contribute to prolonged drug elimination from the rat's body. The difference in absorption levels of DOX in different tissues probably contributes to variable knockdown detected in different organs of transgenic rats.

The toxicity of shRNA technology was further evaluated by IFN response and abnormal small RNA processing in the transgenic animals after shRNA induction. Exogenous shRNA expression has been shown to trigger toxic effects (Bridge, Pebernard et al. 2003; Fish and Kruithof 2004; Cao, Hunter et al. 2005), such as IFN response or impair maturation of endogenous microRNAs (Grimm, Streetz et al. 2006). In our model no alterations in PKR expression were found even after shRNA induction by high dose/acute administration or low dose/chronic treatment with DOX. This provided evidence for no IFN induction in our rats with shRNA expression. Absence of IFN response was also shown before by other investigators who manipulated the mouse genome with shRNA or microRNA constructs (Grimm, Streetz et al. 2006; Xia, Zhou et al. 2006).

mir-122 has been used as indicator for over-saturation of the endogenous RNAi machinery by siRNAs in the liver (Grimm, Streetz et al. 2006; Dickins, McJunkin et al. 2007; John, Constien et al. 2007). In this study, mir-122 processing was unchanged in animals after long term treatment with low DOX doses. Moreover, no liver damage was observed in our diabetic rats. It still remains to be determined, whether highly expressed shRNAs induced by high DOX doses would interfere with cellular microRNAs. Accordingly, it has been shown that at least liver cells may get necrotic, when too much shRNA molecules compete for the nuclear export by the karyopherin exportin-5 (Grimm, Streetz et al. 2006), which was shown to be shared by endogenous microRNAs and shRNAs (Yi, Qin et al. 2003; Kim 2004; Grimm, Streetz et al. 2006). These data support the conclusion that there are also no toxic side effects of shRNA induction at adult stage and confirm findings in mice, in which conditional shRNA expression has already been shown to be a very efficient tool for gene silencing without obvious toxicity (Dickins, McJunkin et al. 2007).

## 4.3.4. Importance and future prospects

The lack of gene targeting technology in rats has shifted many physiological studies to the mice, even though the rat is still the preferred model among physiologists. From now on, following the strategy described here, expression of any endogenous gene in the rat can be inhibited in any time schedule of choice.

Furthermore, the transgenic rats generated in this thesis represent an attractive alternative for further investigations on renal, cardiovascular, neurological, and other pathological complications triggered by type II diabetes. Novel therapeutic concepts targeting InsR signalling, such as kinase inhibitors (Engel, Hindie et al. 2006), can be evaluated in this model.

# 4.4. Development of a new conditional gene regulation system

## 4.4.1. *In vitro* gene activation by modified components of the RNA pol III system and tamoxifen

### *4.4.1.1. Transiently transfected COS7 cells*

In order to establish a novel conditional gene regulation strategy, *in vitro* studies were carried out using modified variants of the human U6 promoter and TBP protein. Initially, cotransfection of plasmids with mutated $U6_m$ promoter and mTBP protein were shown to induce transcription in transiently transfected COS7 cells. This result was in agreement with the work published by Meissner and Rothfels (Meissner, Rothfels et al. 2001). However, when the modified TBP protein was fused to a mutated estrogen receptor ($ER^{T2}$) ligand-binding domain (LBD) transcription was activated in the presence of the fusion protein, but was not regulated by tamoxifen treatment. The transduced COS7 cells highly over-expressed the mTBP-$ER^{T2}$ fusion protein due to the use of Lipofectamine 2000, being a very efficient DNA-delivery reagent, and the very active CMV promoter. Possibly, the over-production of chimeric proteins led to their nuclear import independent of ligand-binding due to insufficient binding by the endogenous heat shock proteins (Hsp). Hsp proteins have been shown to stabilize ER receptors in the cytoplasm and be essential for the ligand binding (Fliss, Benzeno et al. 2000). In addition, mTBP-$ER^{T2}$ may also be transported into the nucleus by some other mechanisms. This idea is supported by recent findings showing that the nuclear localisation signal of the ligand binding domain of steroid hormone receptors can be recognized also by other nuclear transporters, such as importin-7 and importin-8. These proteins do not require ligand binding for the translocation into the nucleus (Freedman and Yamamoto 2004; Pemberton and Paschal 2005). This may explain the tamoxifen independent nucleocytoplasmic shuttling of the mTBP-$ER^{T2}$.
Nevertheless, other researchers who demonstrated tight gene regulation using the same modified LBD domain of ER did not comment on similar difficulties of nuclear transport control (Feil, Wagner et al. 1997; Indra, Warot et al. 1999; Kuhbandner,

Brummer et al. 2000) arguing against hormone-independent nuclear import (Freedman and Yamamoto 2004; Pemberton and Paschal 2005).

### *4.4.1.2. Stable cell lines expressing the fusion protein mTBP-ER$^{T2}$*

To overcome the limitation of protein over-production two stable COS7 cell lines of mTBP and mTBP-ER$^{T2}$ proteins were established. Constitutive expression of these proteins was markedly lower in comparison to the expression of transiently transfected cells. This low amount of both mutants was shown not to be sufficient to stimulate transcription of the adenoviral sequence from the modified U6$_m$ promoter. The fact that transcription was independent of tamoxifen suggested that the optimal production of the mTBP-ER$^{T2}$ protein to be regulatable by tamoxifen remains to be determined. Since the expression of the antibiotic selection marker was demonstrated to be not correlated with the expression of the transgene of interest (Alexopoulou, Couchman et al. 2008), selection pressure was not further optimized trying to increase the basic expression of both mutants in this study.

It would make sense to define the cellular localisation of these proteins by imunocytochemistry. Such experiments would probably clarify both: the localisation as well as density of the chosen protein inside of the cytoplasm and nucleus. It could further show whether or not nuclear shuttling of the mTBP-ER$^{T2}$ protein occurred in the stable cell lines after tamoxifen treatment. Imunocytochemical analyses were employed also by other groups to characterize the expression pattern of chimeric recombinases fused to a mutated ER-LBD domain (Brocard, Warot et al. 1997; Indra, Warot et al. 1999).

On the other hand, RPA may not be sensitive enough for the detection of low amounts of adenoviral RNA molecules. Therefore, it can not be completely excluded that the transcription based on the system described herein works at low concentrations of the mTBP-ER$^{T2}$ protein.

Another important factor may be the choice of the CMV promoter driving the mTBP-ER$^{T2}$ transgene. It has been shown that CMV promoter is often not suitable for the long term expression of transgenes and gives very poor transgene expression during selection of stable transfectants (Alexopoulou, Couchman et al. 2008). This evidence

suggests the use of alternative promoters to obtain sustained transgene expression. Whether the use of another promoter driving the mTBP and mTBP-ER$^{T2}$ proteins could lead to induction of the U6$_m$ promoter in stable cell lines, particularly after tamoxifen treatment, remains to be determined.

The lack of transcription induction at low levels of the mTBP and mTBP-ER$^{T2}$ mutants may also be explained by the distribution of human mTBP proteins between several endogenous promoters in COS7 cells. The mTBP mutants can be shared by the pol III and pol II transcription complexes (Meissner, Rothfels et al. 2001). Such interactions of remodelled TBP proteins were also shown in other species before (Strubin and Struhl 1992; Heard, Kiss et al. 1993) and may contribute to decreased specific binding to the U6$_m$ promoter and thus reduced transcription of the adenoviral RNA.

## 4.4.2. Expression of mTBP *in vivo*

Evidence for cell growth inhibition caused by the over-expression of TBP mutants *in vivo* (Ganster, Shen et al. 1993; Kou and Pugh 2004) led us to test whether the human mTBP protein could be toxic for rodents. Therefore, the generation of transgenic rats with this gene was carried out. Since the CMV promoter was demonstrated to be often silenced resulting in mosaic transgene expression in mice (Feil, Brocard et al. 1996; Brocard, Warot et al. 1997), the ubiquitin promoter was chosen to drive the modified TBP transgene. The embryo survival was very low after the microinjection procedure when compared to the published data (Popova, Bader et al. 2005) and no positive founder was born. This result proposed that human TBP mutants might interact with the intact pol II or pol III transcription machinery in the rat. From published data it is clear that transcription mechanisms based on TBP protein and TATA box components are highly conserved among different species i.e. human, plants and yeast (Strubin and Struhl 1992; Heard, Kiss et al. 1993). The authors demonstrated that interactions between transcription complex components of various organisms can lead to the induction of transcription *in vivo*. These data proposed that the human mTBP protein may react with the normal TATA box of rat genes. Consequently, the endogenous gene regulation might be impaired by the exogenous mTBP protein causing lethality

of the embryos. This may explain why embryonic lethality after pronuclear microinjection of a TBP construct was higher than usual (Popova, Bader et al. 2005).
Furthermore, it was shown that the dimerization of TBP proteins plays an important role in transcription regulation (Vanathi, Mishra et al. 2003; Kou and Pugh 2004). It is not clear whether TBP mutants employed in this study have an ability to form dimmers when not bound to the DNA. Site directed mutations in the highly conserved C-terminal domain of the human mTBP protein could theoretically abolish dimmer-forming. Therefore, it can not be exclude that inability of the mTBP mutants to form dimmers may cause toxic effects on embryo and cell viability as shown in yeasts (Kou and Pugh 2004).
The study by Meissner and Rothfels (Meissner, Rothfels et al. 2001) noted that expression of the same mTBP mutant used herein did not affect the viability of transfected cells. This result is in agreement with the *in vitro* data provided in this study, but not sufficient to predict effects of the mTBP acting *in vivo*.
Nevertheless, the creation of mTBP-ER$^{T2}$ transgenic rats may be possible since the tightly regulated translocation of the mTBP-ER$^{T2}$ protein achieved by tamoxifen treatment may not lead to any interruption of the cellular gene regulation by remodelled TBP protein *in vivo.*

## 4.4.3. Future prospects

The herein presented approach towards novel conditional gene regulation was intended to be used for gene silencing based on shRNA expression. In the first part of this thesis it has been shown that ubiquitous and permanent expression of shRNA is lethal for embryos. However, conditional shRNA expression was demonstrated to be a possible option for gene downregulation in this species. Therefore the goal of this study was to provide a novel inducible strategy that could be used for RNAi mediated gene knockdown.
As shown in this project, induction of gene expression from the modified U6 promoter was achieved by mTBP and mTBP-ER$^{T2}$ fusion protein. However, to establish the system to be controllable by external stimuli i.e. addition of tamoxifen, further optimisations must be carried out. In particular, an appropriate promoter of the

mTBP-ER$^{T2}$ transgene must be defined to replace the less suitable CMV promoter that may be silenced (Feil, Brocard et al. 1996; Brocard, Warot et al. 1997). Next, mTBP-ER$^{T2}$ transgenic rats must be generated in order to examine possible toxic effects of the chimeric protein in these animals. It was shown before, that expression of the ER$^{T2}$ and other ER mutants, when fused to the Cre recombinase, do not trigger any side effects *in vivo* (Feil, Brocard et al. 1996; Brocard, Warot et al. 1997; Feil, Wagner et al. 1997; Indra, Warot et al. 1999; Kuhbandner, Brummer et al. 2000). This fact raises the hope that the here presented mTBP-ER$^{T2}$ fusion product should not be harmful for rats as well. Furthermore, the tamoxifen doses required for the activation of Cre-ER$^{T2}$ mediated recombination are extremely low (Feil, Wagner et al. 1997; Indra, Warot et al. 1999; Kuhbandner, Brummer et al. 2000) and even higher doses (1 mg/mouse), when other ER-LBD mutants were employed, did not trigger any harmful effects in rodents (Feil, Brocard et al. 1996; Brocard, Warot et al. 1997; Indra, Warot et al. 1999). Nevertheless, Danielian et al. (Danielian, Muccino et al. 1998) demonstrated that tamoxifen causes embryo lethality during pregnancy when high concentrations (more than 1 mg/mouse) were administrated. Thus, nanomolar amounts of this drug which are completely sufficient for activation of the ER$^{T2}$ domain should not be harmful.

In conclusion, this system based on a modified pol III transcription mechanism including the ER$^{T2}$-LBD mutant could be used to establish a conditional post-transcriptional gene silencing method in animal models, when proven to be safe *in vivo*.