1. INTRODUCTION

1.1. RNA interference (RNAi)

1.1.1. Discovery of RNAi

Discovery of RNAi goes back to 1990 when Carolyn Napoli and Jorgenson tried to deepen the purple colour in petunias. In this attempt they wanted to check whether the *chalcone synthetase* gene (CHS) plays crucial role petunia coloration. Therefore a powerful promoter of cauliflower mosaic virus 35S to drive a chimeric CHS gene led to an unexpected result. Petunias were either totally white or pigment-patterned instead of intensive purple. This phenomenon was named "cosupression", in which both, the introduced gene and the homologous endogenous genes, were blocked on a post-transcriptional level (Napoli, Lemieux et al. 1990).

The "cosupression" was later obtained also in other plant species and was called "post-transcriptional gene silencing" (PTGS) (Hamilton and Baulcombe 1999) and in fungi such as *Nerospora crassa* "quelling" (Romano and Macino 1992).

The first evidence of RNAi acting in small animals came from Guo and Kemphues in the year 1992. The introduction of antisense ssRNA (single stranded RNA) shut down gene expression in the nematode *Caenorhabditis elegans* (Guo and Kemphues 1995). Surprisingly, administration of the sense strand RNA led to the same silencing effect, as they reported, and thus put the question how single stranded sense RNA can bind to mRNA and cause its degradation. Three years later this problem was clarified by Melo and Fire (Fire, Xu et al. 1998).

The injection of dsRNA (double stranded RNA) into *C. elegans* suppressed gene expression much more efficiently (Fire, Xu et al. 1998) than ssRNA demonstrated by Guo and Kemphues. The sense strand could achieve a silencing effect only when mixed with the antisense strand (Fire, Xu et al. 1998). Indeed, while preparing ssRNA with bacteriophage RNA polymerase, Guo and Kemphues may have contaminated the ssRNA resulting in dsRNA formation which efficiently blocked mRNA expression (Fire, Xu et al. 1998).

Beside this important scientific discovery for which they got the Nobel Price in 2006, Fire and Melo also showed silencing impact of RNAi in the progeny of a worm when dsRNA was introduced into its gut (Fire, Xu et al. 1998).
This novel post-transcriptional gene silencing mechanism opened new possibilities in the gene manipulation field. But still many questions remained unanswered at that time, like how the RNAi machinery processes its substrates.

1.1.2. RNAi Pathway

1.1.2.1. shRNA processing

Today it is well known that dsRNA introduced into cells is processed by two protein complexes, DICER and RISC (RNA-induced silencing complex), discovered by Hannon's group (Bernstein, Caudy et al. 2001). They performed digestion reactions using *Drosophila* cell extract and by high-speed centrifugation they showed two distinct enzymes involved in RNA degradation. First, DICER recognizes and splits dsRNA to short interference RNAs (siRNAs) which are further bound by another protein complex called RISC. RISC together with a subunit of the Argonaute protein family loads an antisense siRNA strand and acts on complementary mRNA causing its degradation (Figure 1) (Bernstein, Caudy et al. 2001; Martinez, Patkaniowska et al. 2002).
Argonaute proteins, identified by two domains, PAZ and PIWI (Martinez, Patkaniowska et al. 2002), are a highly conserved family across several species (Carmell, Xuan et al. 2002). The PAZ domain is responsible for binding of the target mRNA while the PIWI domain cleaves the bound transcript (Song, Smith et al. 2004). It has been shown that all four subfamilies Ago1, Ago2, Ago3 and Ago4 can bind siRNA, but only the Ago2 has RNAse catalytic activity shown by Liu and Carmell in 2004. Point mutations in the Ago2 PIWI domain clarified the crucial role of its highly conserved catalytic triad of three amino acids common to all RNAse H enzymes. The triad of three aspartates is corresponding to the cleavage activity of the "Slicer" (Liu, Carmell et al. 2004). To address the function of Ago2 *in vivo,* Ago2 null mice were

generated. Disruption of this gene in mice leads to several developmental abnormalities resulting in an embryonic-lethal phenotype (Liu, Carmell et al. 2004). Taken together, the RISC/Ago2 complex binds an siRNA, recognizes its target mRNA and cleaves the transcript (Figure 1). The question, how the RISC/Ago2 can distinguish the antisense strand from the sense of an siRNA, still remained unclear. Jayasena and colleagues performed thermodynamic analysis of sense (passenger) and antisense (incorporated or functional) siRNAs. Their results displayed lower thermodynamic stability of 5' antisense region which consequently enter the RISC in contrast to more stable 5' sense region (Khvorova, Reynolds et al. 2003). Moreover, it has been found that Ago2, as a crucial component of the RISC complex, also takes part in other protein complexes, like DICER (Chendrimada, Gregory et al. 2005; Gregory, Chendrimada et al. 2005). Its RNase catalytic activity was clearly shown but still its localisation within the cell remains to be determined.

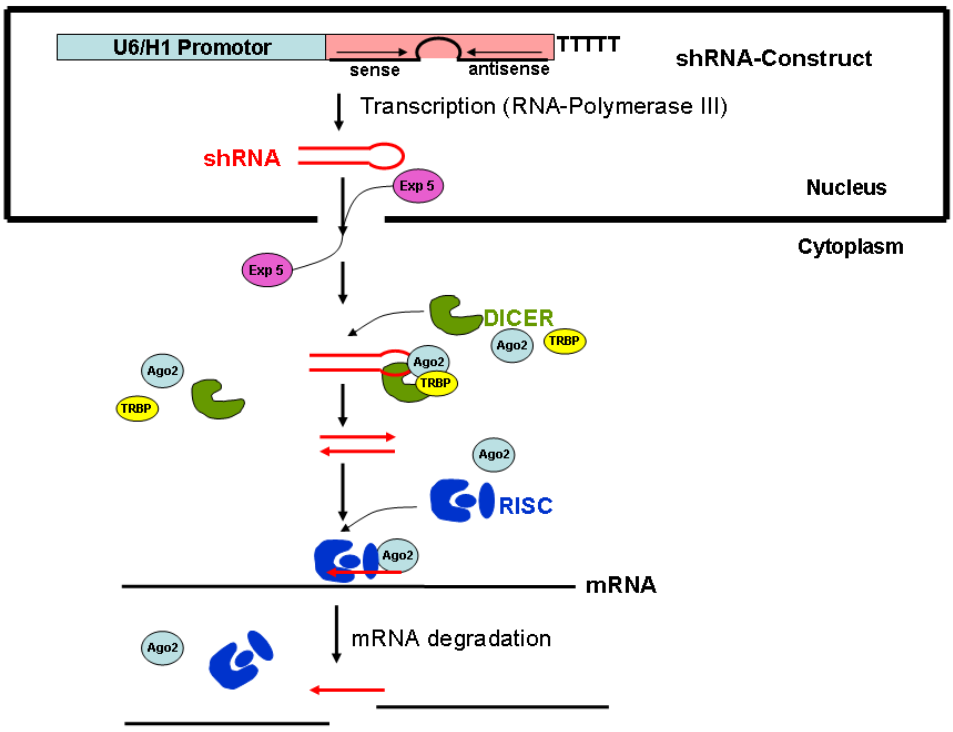

Figure 1: Model of RNAi pathway.

1.1.2.2. RNAi in the nucleus: microRNAs and shRNAs

The major part of the RNAi machinery is placed in the cytoplasm. But pathways of cellularly generated microRNAs or introduced shRNAs begin in the nucleus.
MicroRNAs are encoded by evolutionary highly conserved genes transcribed by RNA polymerase II first resulting in long primary microRNA transcripts (pri-microRNA) (Lee, Ahn et al. 2003; Lee, Nakahara et al. 2004). These pri-microRNAs are cleaved by endoribonuclease Drosha to shorter, ~ 70 nucleotides long microRNA precursors (pre-miRNA), which are further transported by the nuclear protein exportin-5 to the cytoplasm (Lee, Ahn et al. 2003; Yi, Qin et al. 2003; Lund, Guttinger et al. 2004). shRNAs are generated by RNA polymerase III and exported by exportin-5 to the cytoplasm (Figure 1). The fate of both, shRNA and microRNA, is continued by the same cytoplasmic RNAi machinery including DICER and Ago2/RISC as described above (Figure 1).

1.1.3. RNAi technology

1.1.3.1. Gene manipulation by RNAi

In the past few years an enormous progress has been achieved in the field of RNAi research. At the beginning, an application of this powerful genetic tool was limited to plants, flies, worms and mammalian cell culture. Interests in using RNAi for gene investigations in mammalians and also in the clinics have contributed to expansion of basic findings to gene modulation in higher organisms such as vertebrates.
Long before the discovery of RNAi, Hunter and co-workers (Hunter, Hunt et al. 1975) showed that the use of long dsRNAs provokes a strong cytotoxic response in mammalian cells. On the other hand, transfection of short dsRNA into cultured mammalian cells and embryos was found to result in a very efficient gene knockdown (Elbashir, Harborth et al. 2001; Elbashir, Harborth et al. 2002). Furthermore, successful gene suppression was shown using DNA plasmids carrying an shRNA cassette driven by RNA polymerase III promoters in cultured cells (Brummelkamp,

Bernards et al. 2002; Lee, Dohjima et al. 2002; Miyagishi and Taira 2002; Paddison, Caudy et al. 2002; Paddison, Caudy et al. 2002; Paul, Good et al. 2002; Sui, Soohoo et al. 2002). When targeting the same gene, siRNA turned out to be much more effective compared to antisense oligonucleotides (Miyagishi, Hayashi et al. 2003) and the effect was also of longer duration (Bertrand, Pottier et al. 2002). In further attempts short dsRNA molecules or plasmids containing a dsRNA sequence were injected into the tail-vein of mice which led to short term gene suppression in the liver (Lewis, Hagstrom et al. 2002; McCaffrey, Meuse et al. 2002).

At the end of 2002 scientists under Okabe's leadership published the first report showing a heritable shRNA knockdown of the EGFP gene in several tissue of EGFP transgenic mice and rats. These shRNA transgenic animals were made by pronuclear microinjection (Hasuwa, Kaseda et al. 2002).

Only two months later, Carmell et al. also reported about shRNA mediated gene knockdown in mice carried out by the manipulation of ES cells (Carmell, Zhang et al. 2003). Their study confirmed efficient gene silencing and successful germ line transmission to the F1 generation. But on the contrary to Okabe's report, Carmell and her colleagues pointed out that in their hands pronuclear injections of shRNA constructs never led to successful gene knockdown (Carmell, Zhang et al. 2003). Similar negative observations were reported also by Cao el al. in 2005. The main problem in their work was a low transmission ratio to the offspring (up to 13.9 %) and a low survival rate (15 %) of shRNA transgenic mice. They suggested that high shRNA expression may be toxic for embryonic development and thus leads to early fetal lethality (Cao, Hunter et al. 2005). Since then, ES cell based transgenesis was the most used way for successful production of shRNA transgenic mice. In this way efficient gene silencing and germline transmission of shRNA constructs were achieved with random (Saito, Yokota et al. 2005) and targeted (Seibler, Kuter-Luks et al. 2005) transgenesis.

Recently, however, again mouse models expressing shRNA were published by two independent groups by the use of pronuclear microinjection (Peng, York et al. 2006; Xia, Zhou et al. 2006). Xia et al. used RNA polymerase II promoter to drive an shRNA - mimicking human microRNA mir-30a structure. An efficient gene knockdown (60 % - 90 %) in several organs and no detection of interferon response indicated a new progress in mouse gene manipulation.

The first rat model with knockdown of an endogenous gene was reported by Dann et al. Lentiviral delivery of DNA plasmids, containing an shRNA cassette, into fertilized rat eggs yielded a stable and heritable gene down regulation (Dann, Alvarado et al. 2006). The technological drawback was the lack of germ-line transmission due to the mosaic transgene integration pattern observed in several founders (Dann, Alvarado et al. 2006).
Recently, two scientific groups independently succeeded to establish reversible gene knockdown controlled by tetracycline in mice (Dickins, McJunkin et al. 2007; Seibler, Kleinridders et al. 2007). This method was shown to be a safe and effective tool of gene down regulation by shRNA or microRNA expression in mice (Dickins, McJunkin et al. 2007; Seibler, Kleinridders et al. 2007)

1.1.3.2. Problems in RNAi technology

Recently available RNAi animal models have also revealed problems of the RNAi technology. Above all, interferon response and microRNA pathway interruption are critical limitations for RNAi application *in vivo*.

Interferon response

Under normal growth conditions no dsRNA molecules exist or can survive longer in the cytoplasm of higher eukaryotic cells. Through evolution, mammalian cells developed a primary defence system against infections with viruses which mostly produce dsRNA in their host cells. Presence of a viral or introduced dsRNAs (longer than 30 bp) triggers an interferon (IFN) response (Manche, Green et al. 1992), of which the primary task is to suppress the virus invasion in mammalian cells (Cunnington and Naysmith 1975). Infected cells start to produce interferon α and β (IFN α and IFN β) which are released into the extracellular compartment (Pellegrini and Schindler 1993). Binding to their receptors (IFN α and IFN β receptors) initiate IFN signalling pathways leading to the activation of many transcription factors and up regulation of IFN-induced proteins like:

- dsRNA-activated protein kinase (PKR)
- 2'-5'-oligoadenylate synthetase (OAS)
- RNase L
- Toll-like receptor 3 (TLR3) -mediated dsRNA response

(Pellegrini and Schindler 1993; Patterson, Thomis et al. 1995).

An dsRNA-activated Ser/Thr protein kinase or PKR (Proud 1995; Clemens 1997) is located in the cytoplasm, unphosphorylated in an inactive form at low level (Hovanessian 1989; Samuel 1991). PKR was also found in the nucleus (Jimenez-Garcia, Green et al. 1993; Jeffrey, Kadereit et al. 1995; Kumar and Carmichael 1998). dsRNAs can bind to the dsRNA-binding domains of the PKR and induce its dimerization. This drives a conformational change and consequently its autophosphorylation (Manche, Green et al. 1992). Such activated PKRs can further phosphorylate several other substrates and transcription factors, all leading to gene silencing and translation inhibition with a common output - cell death by apoptosis (Gil and Esteban 2000; Wang and Carmichael 2004). It has been shown that the presence of siRNA in the cell can induce expression of IFN-related genes such as OAS, TLR3 and PKR (Bridge, Pebernard et al. 2003; Fish and Kruithof 2004; Cao, Hunter et al. 2005; Reynolds, Anderson et al. 2006).

RNAi pathway interruption

Several studies have been done to assay safety or toxicity of an introduced shRNA. In 2006 Dirk Grimm together with his colleagues described a crucial problem of an introduced shRNA in a cell. shRNA delivery based on adeno-associated virus type 8 (AAV8) via intravenous infusion into mice showed that high doses of shRNA or long hairpin molecules (more than 25 bp) may be toxic or even lethal for animals (Grimm, Streetz et al. 2006). To the opposite, low levels of short shRNA molecules (19mer) lead to an efficient and untoxic gene knockdown (Grimm, Streetz et al. 2006). Further analyses provided the insight that highly expressed shRNAs compete with endogenous microRNAs for their processing in the RNAi machinery. The RNAi pathway gets oversaturated and thus leads to cell apoptosis. This presumption has been proven by the overexpression of the nuclear exportin-5, which is shared by both,

shRNA and microRNA, for the translocation from the nucleus to the cytoplasm and which rescued the shRNA expressing cells (Grimm, Streetz et al. 2006).

Accordingly, John and colleagues showed that biogenesis of the liver specific microRNAs, miR-122, miR-16 and let-7a, are not interrupted when high doses of synthetic siRNAs (5 mg/kg) packaged in liposomal nanoparticles (Zimmermann, Lee et al. 2006; John, Constien et al. 2007) were injected into mice and hamsters (John, Constien et al. 2007).

Together, these findings have pointed out that alterations in the cellular miRNA biogenesis are provoked by foreign long dsRNA molecules (longer than 21 nt) which interact early in the cellular RNAi pathway but not by shorter dsRNAs (John, Constien et al. 2007).

1.2. Diabetes Mellitus

1.2.1. Insulin

1.2.1.1. Insulin function

Insulin is a hormone biosynthesized from β cells which are clustered in islets of Langerhans and located in the pancreas. The insulin precursor preproinsulin is translocated to the endoplasmatic reticulum for post-transcriptional processing and then as mature and active insulin stored in secretory granules in the cytoplasm until its release is triggered. Insulin circulates throughout the blood stream and binds to the insulin receptor on the cell membrane. Signal transduction by an activated receptor induces the glucose transporter type 4 (GLUT 4) to take up glucose from the blood into the cell (Figure 2). Persistently increased levels of blood glucose hyperglycaemia, is caused by insufficient insulin excretion or also by impaired function of insulin receptors. This may lead to chronic diabetes mellitus (www.betacell.org).

1.2.1.2. Insulin receptor signaling

Insulin (Ins) is a ligand common for three receptors,

- insulin receptor (InsR)
- type 1 insulin-like growth factor receptor (Igf1r)
- InsR-related receptor (Irr),

of which all belong to the same subfamily of growth factor receptor tyrosine kinases (Ullrich, Bell et al. 1985; Ullrich, Gray et al. 1986; Shier and Watt 1989; Nakae, Kido et al. 2001).

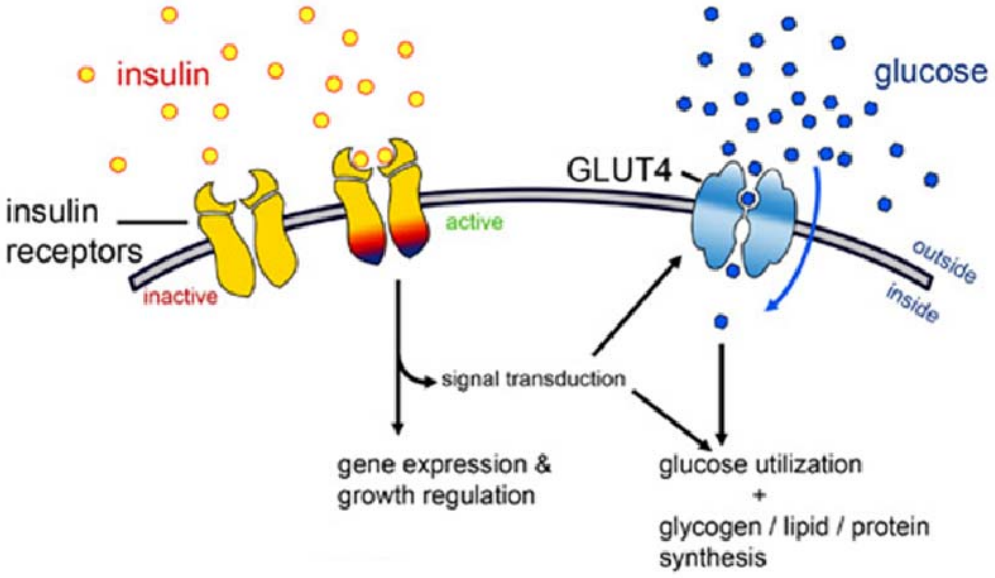

Figure 2: Scheme of insulin action and glucose uptake. Figure is taken from www.betacell.org.

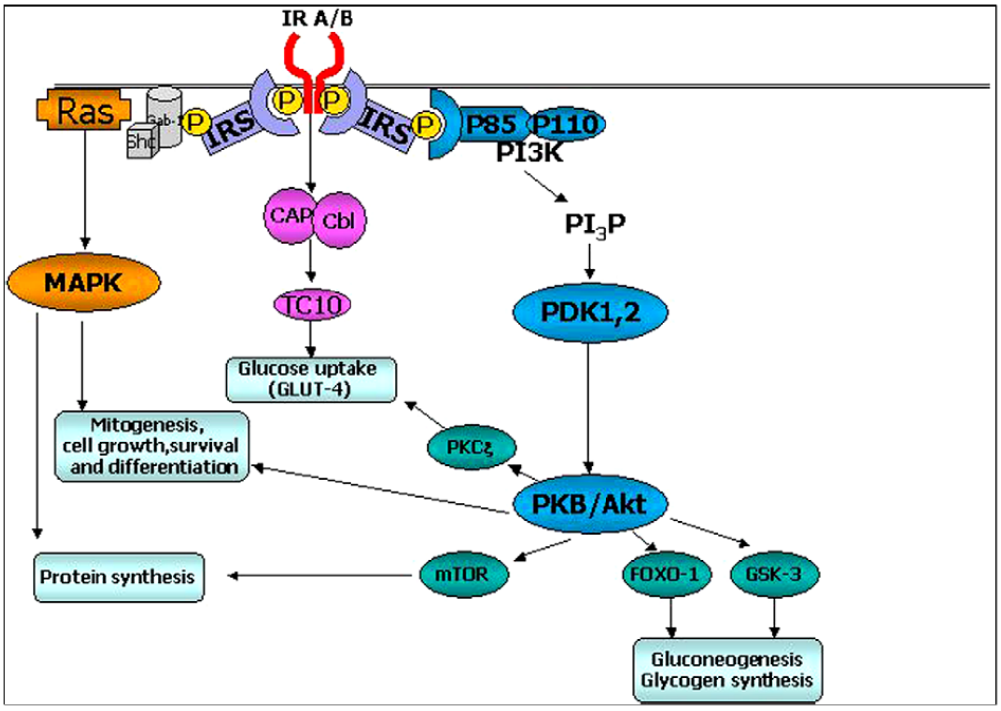

Figure 3: Model of intracellular insulin receptor signalling. Figure is taken from the *Leclercq, Da Silva Morais et al. (2007).*

Three intracellular signalling pathways are activated by the InsR when induced by the binding of insulin (reviewed in (Nandi, Kitamura et al. 2004; LeRoith and Gavrilova 2006; Leclercq, Da Silva Morais et al. 2007)) (Figure 3):

a) Irs (insulin receptor substrate) → Ras → MAPK (mitogen-activated protein kinase) pathway (Kido, Nakae et al. 2001) regulating mitogenic growth and cell differentiation effects (Leclercq, Da Silva Morais et al. 2007).
b) Irs→ PI3K (phosphatidylinositol 3-kinase) → phosphorylation of Ser/Thr kinases of AKT (Burgering and Coffer 1995) → phosphorylation of Gsk3 (glycogen synthase kinase 3) (Cross, Alessi et al. 1995) and Foxo transcription factors (Biggs, Meisenhelder et al. 1999; Brunet, Bonni et al. 1999; Nakae, Barr et al. 2000; Nandi, Kitamura et al. 2004) leading to activation of glycogen synthesis and inhibition of lipolysis.
c) Irs → activation of CAP/Cbl/Tc10 pathway regulating the glucose uptake via recycling of GLUT-4 (reviewed in (Leclercq, Da Silva Morais et al. 2007)).

1.2.2. Animal models for diabetes mellitus

Although failures in the insulin receptor have been known for years to cause hyperglycemia and insulin resistance leading to type II diabetes mellitus, still there is a huge world population suffering from its pathogenesis and complications. A long history in the field of diabetes research and several animal models (reviewed in (Rees and Alcolado 2005)) created to gain insights into the human disease have opened up new areas in drug discovery and development.

Human type I diabetes mellitus is known as disease with specific pancreatic β cell failure mainly caused by immune system mediated damage (Dahlquist 1998). To date, the most frequently used rat model for diabetes mellitus are streptozotocin treated wild type animals. Streptozotocin, isolated from *Streptomyces achromogenes*, damages β cells in pancreas and causes type I diabetes in rodents when injected (Bono 1976; Herold, Lenschow et al. 1997; Zuccollo, Navarro et al. 1999; Holstad and Sandler 2001; Mensah-Brown, Stosic Grujicic et al. 2002; Muller, Schott-Ohly et al. 2002; Yang, Chen et al. 2003).

Today, a few animal models, mainly mice, have been created to gain insights into the pathomechanisms of human type II diabetes mellitus (reviewed in (Chen and Wang 2005; Rees and Alcolado 2005)).
The existing rat models generated by selective breeding or by diet are described below.

The Goto Kakizaki (GK) rat

This model selectively bred from Wistar rats develops stable hyperglycaemia in adult life (Goto, Kakizaki et al. 1976). These rats have impaired insulin secretion and are insulin resistant. The number of islets is reduced at birth (Miralles and Portha 2001). This model is comparable to human diabetes in renal lesions (Janssen, Phillips et al. 1999), structural changes in peripheral nerves (Murakawa, Zhang et al. 2002) and abnormalities of the retina (Sone, Kawakami et al. 1997).

Psammomys obesus (the Israeli sand rat)

The Israeli rats become obese, insulin resistant and hyperglycaemic when fed with laboratory chow (Ziv, Shafrir et al. 1999). In their natural habitat they are vegetarians. In common with human type II diabetes they have hyperglycaemia, high demand for insulin secretion and insulin resistance. Impaired insulin production from β cells has been reported too (Cerasi, Kaiser et al. 1997).

The Otsuka Long-Evans Tokushima fatty (OLEFT) rat

The OLEFT rat was developed by selective breeding for glucose intolerance from the Long Evans rat colony (Kawano, Hirashima et al. 1992). This mildly obese model develops diabetes in its adulthood (male more likely than female) but it is not yet clear whether the genetic background, e.g., a null allele for the cholecystokinin A receptor regulating food intake, may play a role in their phenotype (Moralejo, Ogino et al. 1998; Moralejo, Wei et al. 1998; Kose, Moralejo et al. 2002).

The Zucker Diabetic Fatty (ZDF) rat

The ZDF rat line originates from inbreeding of a colony of Zucker rats which were established in the laboratory of Dr. Walter Shaw et Eli Lilly Research Laboratories in Indianapolis, USA (Chen and Wang 2005). The non-functional leptin receptor in homozygous male zucker rats (fa/fa) causes obesity leading to hyperlipidemia and hyperglycaemia. Hyperglycaemia appears at about 7 weeks of age and is triggered by insulin resistance followed by defect of pancreatic ß cells (Etgen and Oldham 2000; Zinker, Rondinone et al. 2002; Chen and Wang 2005). By 12 weeks, all obese male rats are fully diabetic and become an experimental model for type II diabetes. GLUT 4 is reduced by 25-55 % in adipose tissue, heart and skeletal muscle (Slieker, Sundell et al. 1992).

1.3. Conditional gene regulation

Complete gene deletion by gene targeting in ES cells results in entire protein deficit which often leads to lethality. Therefore, drug mediated gene inactivation strategies are becoming more and more important in genome research. Conditional gene regulation system, such as the Cre/loxP, the Flp/FRT, the Lac- and the Tet-system, permit reversible and tight control over transgene expression in a spatial and temporal manner (reviewed in (Gossen, Bonin et al. 1993; Gossen, Bonin et al. 1994; Lewandoski 2001; Gossen and Bujard 2002)). Moreover, such systems can also be applied in other species, not only in mice as the classical knock out. In recent years many groups have tried to develop or optimise new or already established gene induction systems to improve and extend their use in research and clinics (reviewed in (Gossen, Bonin et al. 1993; Gossen, Bonin et al. 1994; Lewandoski 2001; Gossen and Bujard 2002)).

1.3.1. Tetracycline activation system

However, the tetracycline resistance gene in *E. coli* is one of the most widely used binary systems for an artificial genetic "switch". The system is based on the *E. coli* tetracycline repressor (TetR) protein that specifically binds the *Tn*10-specific-tetracycline-resistance operator (tetO). This interaction between TetR and tetO negatively regulates the transcription of genes mediating tetracycline resistance in *E. coli* (Hillen and Wissmann 1989). However, when tetracycline is present in a cell than the TetR binds it and is released from the tetO. operator and transcription can occur (Hillen and Wissmann 1989). These properties of TetR and tetO are the basis for gene regulatory system employed in higher eukaryotic cells. Such a tetracycline-induced transcription modulation was first established in plants (Gatz and Quail 1988; Gatz, Kaiser et al. 1991) followed by the fungus *Dictyostelium discoideum* (Dingermann, Werner et al. 1992). Later, Wirtz and Clayton (1995) showed efficient gene repression by TetR in the protozoan parasite *Trypanosoa brucei (Wirtz and Clayton 1995)*.

Nevertheless, difficulties of this system appeared when employed in mammalian cells. In stably transfected cell lines the expression of TetR was not sufficient for tight transcription control (Bonin 1993). This problem was solved when Gossen and Bujard combined TetR with the C-terminal domain of VP16 from Herpes Simplex Virus (Gossen and Bujard 1992). VP16 is activating early viral gene transcription (Triezenberg, Kingsbury et al. 1988) and thus the fusion protein, *Tet*R-VP16, can serve as a transactivator that binds the tetO operator on the promoter and stimulates gene transcription (Gossen and Bujard 1992). In this case, the absence of tetracycline positively regulates gene transcription by the tetracycline transactivator (tTA) (Figure 4) which is opposite to the original *E. coli* TetR action.

Furthermore, a second system with tetracycline-based regulation of gene expression is in use these days. The first one, created by Gossen and Bujard and described above is known as "tet-off", whereas its modified version, "tet-on", is based on the reverse tTA (rtTA). The rtTA acts in an opposite manner and binds tetO DNA sequence only when an inducer is present and then activates gene transcription (Figure 4) (Gossen and Bujard 1992; Furth, St Onge et al. 1994; Gossen, Freundlieb et al. 1995). In this system it has been shown, that the tetracycline derivate doxycycline (DOX) is a very efficient effector at low doses that do not trigger any cytotoxic response (Gossen, Freundlieb et al. 1995; Baron and Bujard 2000).

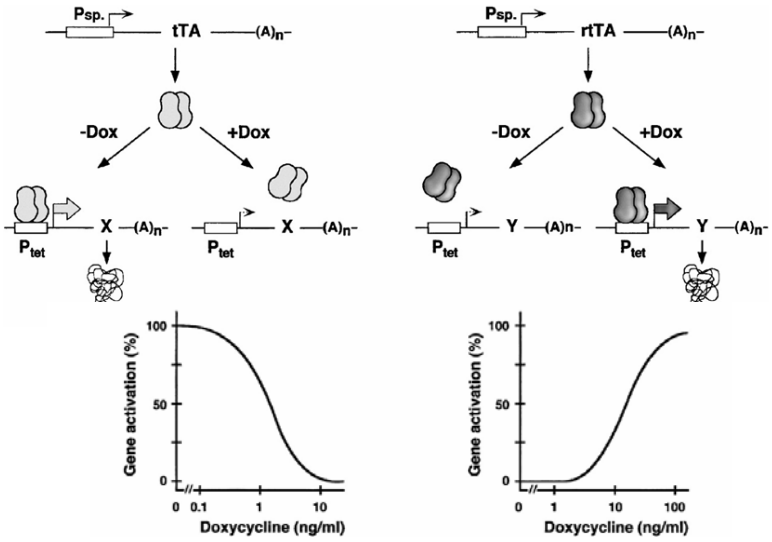

Figure 4: Scheme of the tetracycline activation systems, tTa ("tet off") and rtTA ("tet on"). Figure is taken from Gossen and Bujard (2002).

1.3.2. Inducible gene activation by mutated components of the RNA polymerase III system

Transcription of RNA molecules is directed by a DNA template and is catalyzed by RNA polymerases (pol) which together with several transcription factors (TF) bind to the initiation side of promoters and activate transcription. Higher eukaryotes have three different RNA polymerases in their nuclei distinguished by their functions. RNA pol I synthesizes pre-rRNAs, RNA pol II pre-mRNAs and some snRNAs while RNA pol III transcribes pre-tRNAs, small RNAs and some snRNAs.

In this work investigations were carried out using the pol III system. Therefore the following description is focused on the mechanism of RNA pol III.

Most genes transcribed by RNA pol III are classified in three groups of major promoter types: Type 1, Type 2 and Type 3. The classification is based on the type or position of cis-acting elements, which constitute their promoter (Figure 5) (Willis 1993).

Type 1 genes are characterized by three intragenic sequence elements: a 5' A block, an intermediate element and a 3' C block. Type 2 genes contain conserved A- and B-block elements. Cis-acting elements of type 1 and type 2 promoters are located downstream of the transcription start site. Transcription requires several transcription factors (TF) common for both promoter types: TFIIIC1, TFIIIC2, TFIIIBβ and RNA pol III. The type 1 promoter additionally requires TFIIIA (Yoshinaga, Boulanger et al. 1987; Teichmann and Seifart 1995; Wang and Roeder 1996; Paule and White 2000).

Type 3 genes are identified by three promoter elements located upstream of the transcription initiation site: a TATA-sequence (TATAAA), a proximal sequence element (PSE) and a distal sequence element (DSE) (Kunkel 1991). Transcription is initiated by PSE-binding protein (PBP) (Waldschmidt, Wanandi et al. 1991), TFIIIBα (Teichmann and Seifart 1995), TFIIIU (Oettel, Kober et al. 1998), RNA pol III and TATA-binding protein (TBP) (Simmen, Bernues et al. 1991; Waldschmidt, Wanandi et al. 1991), which in this case does not include transcriptional activation function (TAFs) complex as in the pol II system. A similarity between pol II and type 3 pol III promoters occurs in the TATA-box sequence which is present in both systems and regulated by the same TBP protein.

As demonstrated by Strubin and Struhl (Strubin and Struhl 1992), site-directed-mutations in the TATA-box (TATAAA → TGTAAA) of pol II promoters markedly reduces the binding capacity of TBP in yeast. Substitution of three amino acids in the highly conserved C-terminal domain of TBP (Ile at 194 → Phe; Val at 203 → Thr; Leu at 205→ Val) was shown again to allow the interaction between the mutated DNA and TBP. Furthermore, also human TBP-mutants were shown to have the ability to interact with wild type or modified TATA box in yeast and plants (Strubin and Struhl 1992), but not in humans. Finding another TATA box nucleotide substitution (TATAAA → **AG**TAAA) in human U6 promoter, which was recognized by the modified human TBP protein, allowed Meissner et al. to develop a new gene regulatory system using the human RNA pol III system (Figure 6) (Meissner, Rothfels et al. 2001).

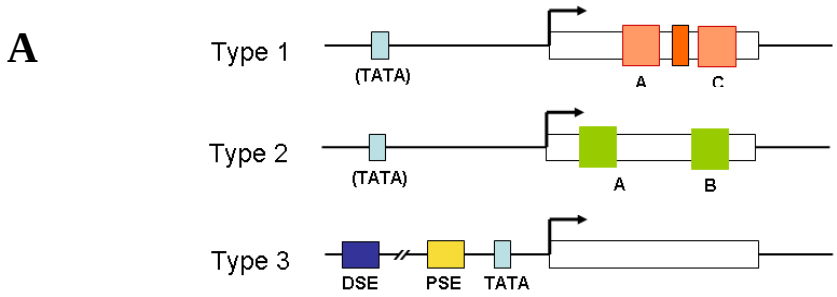

Figure 5: Major promoter types of RNA pol III system (Willis 1993). **DSE:** Distal Sequence Element; **PSE:** Proximal Sequence Element; **A, B and C:** intragenic sequence elements of Type 1 and Type 2 pol III promoters.

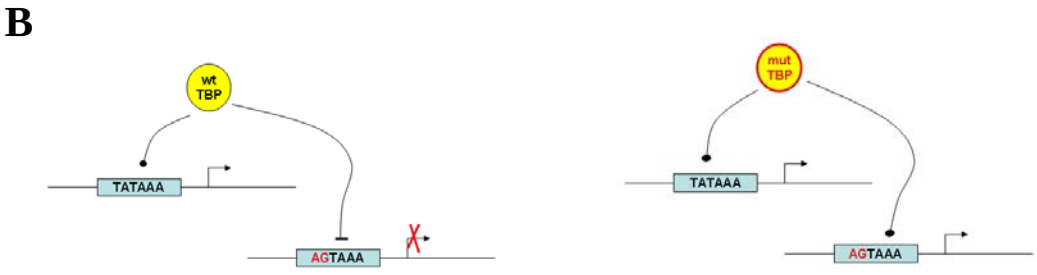

Figure 6: Transcription activation from the intact (TATAAA) or modified (AGTAAA) TATA box of human pol III promoter by human wild type and mutated TBP proteins. TBP: TATA-Binding Protein.

1.3.3. Conditional gene regulation by mutated Estrogen Receptor Ligand-Binding Domain

Estrogen receptors (ER) represent a family of nuclear hormone receptors which activate gene transcription after binding of a hormonal ligand such as 17β-estradiol (E2) (Evans 1988; Beato 1989; Ham and Parker 1989). In the absence of a hormone ER receptors are associated with a heat shock protein (Hsp90) in the cytoplasm (Catelli, Binart et al. 1985; Redeuilh, Moncharmont et al. 1987; Denis, Poellinger et al. 1988; Howard and Distelhorst 1988; Chambraud, Berry et al. 1990; Schlatter, Howard et al. 1992). Such inactive ER-Hsp90 oligomeric complexes dissociate in the presence of endogenous ligands (E2) which bind to ER receptors with high affinity. ER-ligand dimmers translocate into the nucleus, bind to the DNA, and activate gene transcription (Figure 7) (Notides, Lerner et al. 1981; Kumar and Chambon 1988; Skafar 1991). The nuclear import of the ligand-activated ER receptor is carried out by the nuclear protein transport machinery through the nuclear pore complex (NPC). This machinery recognizes all the proteins bearing a nuclear localization signal (NLS) and import them into the nucleus (Kohler, Haller et al. 1999; Pemberton and Paschal 2005).

ER is characterized by two regions called transcriptional activation function (TAF) domains (Tora, White et al. 1989). The DNA binding domain (DBD) or TAF-1 region on the NH2-terminus is responsible for the binding to the DNA. TAF-2 or also named the ligand binding domain (LBD) on the C-terminus interacts with E2 (Kumar, Green et al. 1987; Webster, Green et al. 1988; Lees, Fawell et al. 1989).

The ER-LBD domain has been used as a powerful tool for gene manipulation in cultured cells and mice. For this purpose the domain has been changed by site-directed mutagenesis to achieve specific sensitivity to synthetic steroids such as 4-hydroxytamoxifen (OHT) or ICI 182,780 (ICI) (Metzger, Clifford et al. 1995; Feil, Brocard et al. 1996; Feil, Wagner et al. 1997; Indra, Warot et al. 1999; Kuhbandner, Brummer et al. 2000). At the same time, such modified ER-LBD domain became insensitive to the natural ligand E2. Such an ER-LBD mutant fused to the Cre-recombinase protein can successfully control gene expression in a temporal manner *in vitro* as well as *in vivo* (Feil, Brocard et al. 1996; Brocard, Warot et al. 1997; Feil,

Wagner et al. 1997). The drawback of the very first established ligand-dependent Cre-recombinases was the high drug concentration required for activation of the ER-LBD domain. Consecutively, the recombination by chimeric Cre-ER mutants was not complete in a chosen cell population –even not at high doses of the ligand (Feil, Brocard et al. 1996; Schwenk, Kuhn et al. 1998; Indra, Warot et al. 1999). To overcome these problems and to eliminate side effects of high tamoxifen concentrations, Feil et al. (Feil, Wagner et al. 1997) generated new ER-LBD mutants. These ER-LBD domains combined with Cre recombinase (CreER^{T1} and CreER^{T2}) were shown to be highly sensitive to nanomolar concentrations of OHT and ICI (Feil, Wagner et al. 1997; Indra, Warot et al. 1999).

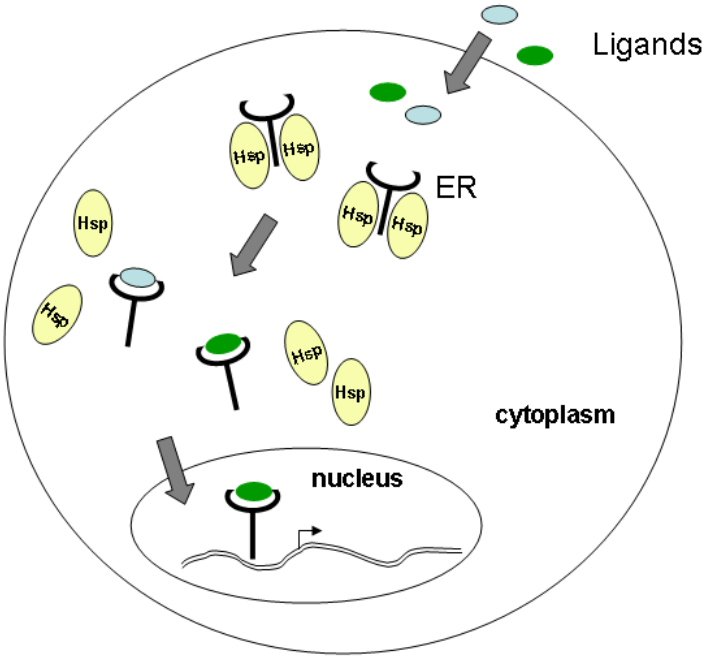

Figure 7: Translocation of the estrogen receptor (ER) into the nucleus after ligand-binding.

1.4. Aims

The rat is the preferred animal model in several areas of research including cardiovascular and neural biology. However, due to the lack of germline competent embryonic stem cells in this species and, thus, of gene targeting technology, the rat has lost grounds compared to the mouse as experimental animal model in the last two decades. Therefore, the main goal of this thesis was the development of a new gene manipulation strategy mediated by RNA interference. In order to establish shRNA technology using pronuclear microinjection for constitutive and inducible gene silencing in rats the following experimental program was adopted:

- Generation of shRNA transgenic rats using ubiquitously expressed hairpin-constructs against the EGFP gene.
- *In vitro* analyses of possible embryo-toxicity of shRNAs.
- Generation of shRNA transgenic rats using a tetracycline activation system for inducible knockdown of the insulin receptor gene by shRNA.
- Analyses of insulin receptor knockdown transgenic rats as a novel rat model for type II diabetes mellitus.
- Development of a new conditional gene activation system using modified components of the RNA polymerase III system and a mutated ligand binding domain of ER.