

Max-Delbrück-Center for Molecular Medicine, Berlin-Buch

Research Group Molecular Biology of Peptide Hormones  
Supervisor: Prof. Dr. Michael Bader

## **Gene knockdown in transgenic rats by shRNA technology**

### **DISSERTATION**

zur Erlangung des akademischen Grades  
**Doktor rerum naturalium (Dr. rer. nat.)**

eingereicht im Fachbereich Biologie, Chemie, Pharmazie  
der Freien Universität Berlin

vorgelegt von

**KATARINA KOTNIK**

aus Vitanje (Slovenien)

Berlin  
Juni 2008

Hiermit versichere ich, die vorliegende Dissertation selbständig und ohne unerlaubte Hilfe angefertigt zu haben.

Bei der Verfassung der Dissertation wurden keine anderen als die im Text aufgeführten Hilfsmittel verwendet.

Ein Promotionsverfahren zu einem früheren Zeitpunkt an einer anderen Hochschule oder bei einem anderen Fachbereich wurde nicht beantragt.

1. Gutachter: Prof. Dr. Rupert Mutzel

2. Gutachter: Prof. Dr. Michael Bader

Disputation am: 25. Juli 2008

# CONTENTS

<b>ACKNOWLEDGEMENTS</b>	<b>1</b>
<b>SUMMARY</b>	<b>2</b>
<b>ZUSAMMENFASSUNG</b>	<b>4</b>
<b>1. INTRODUCTION</b>	<b>6</b>
<b>1.1. RNA interference (RNAi)</b>	<b>6</b>
1.1.1. Discovery of RNAi	6
1.1.2. RNAi Pathway	7
1.1.2.1. shRNA processing	7
1.1.2.2. RNAi in the nucleus: microRNAs and shRNAs	9
1.1.3. RNAi technology	9
1.1.3.1. Gene manipulation by RNAi	9
1.1.3.2. Problems in RNAi technology	11
<b>1.2. Diabetes Mellitus</b>	<b>14</b>
1.2.1. Insulin	14
1.2.1.1. Insulin function	14
1.2.1.2. Insulin receptor signaling	14
1.2.2. Animal models for diabetes mellitus	16
<b>1.3. Conditional gene regulation</b>	<b>19</b>
1.3.1. Tetracycline activation system	19
1.3.2. Inducible gene activation by mutated components of the RNA polymerase III system	21
1.3.3. Conditional gene regulation by mutated Estrogen Receptor Ligand-Binding Domain	23
<b>1.4. Aims</b>	<b>25</b>
<b>2. MATERIALS AND METHODS</b>	<b>26</b>
<b>2.1. Materials</b>	<b>26</b>
2.1.1. Materials, substances and solutions	26
2.1.2. Kits, Enzymes and Markers	28
2.1.3. Lab instruments, machines and other material	29
2.1.4. Antibodies	30
2.1.5. Commercial Cloning Vectors	31
2.1.6. Oligonucleotides	31
<b>2.2. Methods</b>	<b>34</b>
2.2.1. DNA	34
2.2.1.1. Cloning of DNA	34
2.2.1.1.1. Polymerase Chain Reaction (PCR)	34
2.2.1.1.2. Reverse transcription	35
2.2.1.1.3. Restriction of DNA	35
2.2.1.1.4. Filling-up reaction	36

2.2.1.1.5. Annealing of oligonucleotides	36
2.2.1.1.6. Ligation of DNA fragments	36
2.2.1.1.7. TA-cloning	37
2.2.1.1.8. DNA extraction from agarose gel	37
2.2.1.2. DNA transformation in bacteria	37
2.2.1.2.1. Preparation of electro competent E.coli bacteria	37
2.2.1.2.2. Transformation of electro competent bacteria	38
2.2.1.2.3. DNA amplification in bacteria	39
2.2.1.2.4. Measurement of nucleic acids concentration	40
2.2.1.2.5. DNA sequencing	40
2.2.1.3. DNA recombineering in bacteria	41
2.2.1.3.1. BAC (Bacterial Artificial Chromosome) recombineering	41
2.2.2. RNA	42
2.2.2.1. RNA isolation	42
2.2.2.2. RNase Protection Assay	42
2.2.2.2.1. DNA probe design and digestion	42
2.2.2.2.2. Probe labeling	43
2.2.2.2.3. RNA marker labeling	43
2.2.2.2.4. RNA-RNA hybridization	44
2.2.3. Protein	45
2.2.3.1. Protein isolation	45
2.2.3.2. Measurement of protein concentration	46
2.2.3.3. Western blot	46
2.2.4. Cell culture	48
2.2.4.1. Culturing and transfection	48
2.2.4.2. Drug treatment of cells	49
2.2.5. Animals	50
2.2.5.1. Generation of transgenic rats	50
2.2.5.1.1. Superovulation and isolation of embryos	50
2.2.5.1.2. Embryo Culture	50
2.2.5.1.3. Preparation of DNA for microinjection	51
2.2.5.1.4. Microinjection of foreign DNA into the pronucleus	51
2.2.5.1.5. Embryo transfer	52
2.2.5.1.6. Fluorescence detection of expressed GFP and RFP in preimplantation embryos	52
2.2.5.2. Genotyping of transgenic rats	52
2.2.5.2.1. Isolation of genomic DNA from rats' tails	52
2.2.5.2.2. Genotyping	53
2.2.5.3. Animal treatment and phenotyping	54
2.2.5.3.1. Doxycycline treatment	54
2.2.5.3.2. Blood glucose test	54
2.2.5.3.3. Insulin test	55
2.2.5.3.4. Urine and albumin analysis	55

<b>3. RESULTS</b>	<b>56</b>
<b>3.1. Generation of transgenic rats with ubiquitously active shRNA expression</b>	<b>56</b>
3.1.1. Generation of transgenic rats with pRMCE-U6-shGFP construct	57
3.1.1.1. Germline transmission	59
3.1.1.2. Generation of transgenic rats with pU6shGFP-UbAsRed construct	60
3.1.1.3. Generation of transgenic rats with pRosaBAC-shGFP construct	61
3.1.1.4. Analysis of shRNA toxicity in embryos	64
3.1.1.4.1. Rat embryos	64
3.1.1.4.2. Mouse embryos	65
<b>3.2. Generation of transgenic rats with inducible shRNA expression by tetracycline system</b>	<b>68</b>
3.2.1. Generation of transgenic rats with pTetO-shInsR-TetR construct	68
3.2.1.1. Tissue culture test of pTetO-shInsR-TetR construct	68
3.2.1.2. Generation of shRNA transgenic rat lines	69
3.2.1.2.1. Glucose and Insulin in shRNA transgenic rats	74
3.2.1.2.2. Insulin signalling in shRNA transgenic rats	75
3.2.1.2.3. Reversibility of knockdown	75
3.2.1.2.4. Long lasting knockdown leads to chronic diabetes type II model	78
3.2.1.2.5. Renal damage	81
3.2.1.2.6. Lack of toxicity	82
<b>3.3. Development of a new conditional gene regulation system</b>	<b>84</b>
3.3.1. Activation of a modified U6 promoter by a mutated TATA Binding Protein (mTBP)	84
3.3.2. Tamoxifen-inducible activation of a modified U6 promoter by a chimeric mTBP-ER <sup>T2</sup> protein	86
3.3.2.1. Tumour cells	86
3.3.2.2. Stable cell lines	88
3.3.3. Generation of mTBP-transgenic rat model	91
<b>4. DISCUSSION</b>	<b>92</b>
<b>4.1. Transgenic rats with permanently expressed shRNA constructs</b>	<b>92</b>
4.1.1. Toxicity of shRNA constructs	94
4.1.2. Transgenic rats with single copy integration of an shRNA construct	96
4.1.3. <i>In vitro</i> analysis of shRNA toxicity in embryos	97
<b>4.2. Transgenic rats with lentiviral shRNA constructs</b>	<b>98</b>
<b>4.3. Transgenic rats with shRNA activated by a tetracycline inducible System</b>	<b>100</b>
4.3.1. Successful transgenic rat generation with inducible and reversible gene knockdown in rats	100
4.3.2. Rat model of type II diabetes mellitus	101
4.3.3. Safety of the tetracycline activatable shRNA expression	102
4.3.4. Importance and future prospects	104
<b>4.4. Development of a new conditional gene regulation system</b>	<b>105</b>
4.4.1. <i>In vitro</i> gene activation by modified components of the RNA pol III system and tamoxifen	105
4.4.1.1. Transiently transfected COS7 cells	105

4.4.1.2. Stable cell lines expressing the fusion protein mTBP-ER <sup>T2</sup>	106
4.4.2. Expression of mTBP <i>in vivo</i>	107
4.4.3. Future prospects	108
<b>5. BIBLIOGRAPHY</b>	<b>110</b>
<b>Abbreviations</b>	<b>120</b>
<b>Curriculum Vitae</b>	<b>123</b>
<b>Publications</b>	<b>124</b>
<b>Scientific Meetings</b>	<b>125</b>