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A NOVEL METHOD TO INTERFERE WITH GENE EXPRESSION IN MICE USING LENTIVIRAL TRANSGENESIS

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TABLE OF CONTENTS

1. Summary 1

1. Zusammenfassung 2

2. Introduction 3

2.1. Nuclear receptor transcription factors 4

2.1.1. Structure of nuclear receptors 4

2.1.2. HNF4γ 5

2.2. RNA interference 8

2.2.1. siRNA and microRNAs (miRNA) 9

2.2.2. Selection of highly effective siRNA sequences 10

2.2.3. RISC function 11

2.2.4. Other silencing models 12

2.3. Lentiviral transgenesis 13

2.3.1. Lentiviruses 14

2.3.2. Lentiviral gene transfer vectors 15

2.3.3. Self-inactivating vectors 15

2.3.4. Vector elements 16

2.3.5. The lentiviral packaging system 16

2.3.6. Generation of transgenic animals by using lentiviruses 16

2.3.7. Induction of RNAi in transgenic mice generated by the use of lentiviral vectors 19

2.3.8. Aim of the project 20

3. Results 21

3.1. Analysis of HNF4γ expression 21

3.2. Selection of effective shRNA molecules against HNF4γ 25

3.3. Generation of transgenic mice using LentiLox 3.7 28

3.3.1 Copy number and segregation of the transgene 31

3.3.2. Transgene expression in LentiLox 3.7 F1 mice 32

3.3.3. Knockdown of HNF4γ by lentiviral siRNA 37

4. Discussion 40

4.1 Tissue distribution and expression of the HNF4γ gene 40

4.2. Selection of effective shRNA oligonucleotides against HNF4γ 41

4.3. Generation of transgenic mice using the LentiLox 3.7 lentiviral system 43

4.3.1. Analysis of F0 mice 43

4.3.2. Analysis of F1 mice 47

4.4. In vivo knockdown of HNF4γ gene expression 49

4.5 Conclusions 49

5. Materials and Methods 50

5.1. Equipment 50

5.2. Materials 50

5.3. Enzymes 51

5.4. Radioactivity 51

5.5. Special kits 51

5.6. Plasmids 52

5.7. Competent E. coli strains 52

5.8. Mouse strains 52

5.9. Buffers and solutions 52

5.9.1. Isolation and storage of DNA 52

5.9.2. Buffers for the alkaline lyses of bacteria and plasmid preparation 52

5.9.3. Buffers for genomic DNA preparations 53

5.9.4. Buffers for DNA electrophoresis 53

5.9.5. Medium for culturing of bacteria 53

5.9.6 Medium for culturing of 293T cells 54

5.10. Standard Molecular Biological Techniques 54

5.10.1 Plasmid Mini-prep DNA preparation 54

5.10.2. Genomic DNA preparation 54

5.10.2.1. DNA preparation for Southern blot 54

5.10.2.2. DNA preparation for genotyping by PCR 55

5.10.3. DNA electrophoresis 55

5.10.4. Southern blot 55

5.10.5. Random labelling of probes for Southern blot 56

5.10.6. Preparation of electro-competent E. coli cells 56
5.10.7. Immunohistochemistry protocol 57
5.10.8. Restriction analyses 58
5.10.9. Dephosphorylation of 5´ ends of dsDNA to prevent self-anniling of vector 59
5.10.10. Ligation of DNA fragments 59
5.10.11. PCR 59
5.11. Selection of effective shRNA molecules against HNF4γ 60
5.12. Oligonucleotide Design 60
5.12.1. Oligonucleotide format 60
5.12.2. Cloning of the shRNA 60
5.12.2.1. Annealing Procedure 60
5.12.2.2. Diluting of the ds oligonucleotides 61
5.12.2.3. Phosphorylation of the ds-oligonucleotides 61
5.12.2.4 Checking the integrity of the ds-oligonucleotides 62
5.12.2.5. Preparation of the pU6-empty vector for ligation 62
5.12.2.5.1. Digestion of pU6-empty vector by HpaI and XhoI 62
5.12.2.6. Dephosphorylation of the pU6-empty vector 63
5.12.2.7. Cloning the shRNA ds-oligonucleotides into pU6-empty vector 63
5.12.2.8. Analysis of the clones after ligation reaction 63
5.13. The GeneEraserTM Luciferase Suppression-Test System 64
5.13.1. pTarget-luc Cloning Strategy 64
5.13.2. Releasing of HNF4γ cDNA sequence from pBK-CMV- HNF4γ expression vector 64
5.13.3. Polishing the purified HNF4γ fragment 65
5.13.4 Cloning of HNF4γ cDNA sequence into pTarget-luc pre-digested vector 65
5.13.5 Identifying Transformants Containing the HNF4γ insert 66
5.13.5.1. A PCR strategy 66
5.13.6. Estimation of the efficiency of the different shRNA against HNF4γ 67
5.13.6.1. Transfection of 293T cells in 96 well plate format 67
5.13.6.2. Luciferase activity measurements and data processing 68
5.13.7. Re-cloning of U6-shRNA expression cassettes into pLentiLox 3.7 68
5.13.7.1. Releasing of U6-shRNA cassette 69
5.13.7.2 Preparation of pLentiLox 3.7 for cloning of the U6-shRNA cassettes 69

5.13.7.3. Dephosphorylation of pLentiLox 3.7 69
5.13.7.4. Cloning of U6-shRNA cassettes into LentiLox 3.7 69
5.14. Packaging of Lentivirus (lentiLox 3.7) 69
5.14.1. Plating of 293T cells 69
5.14.2. Transfection for package of the virus 70
5.15. Generation of RNAi- HNF4γ transgenic mice 70
5.15.1. Genotyping of the transgenic mice generated by LentiLox 3.7 lentivirus 75
5.15.2. Southern blot of DNA prepared from tails of LentiLox-HNF4γ mice 76
5.15.3. Determination of siRNA expression in LentiLox-HNF4γ mice 77
5.15.3.1. Isolation of total RNA for Northern blot hybridization 77
5.15.3.2. Electrophoresis of siRNA sample on denaturing acrylamide gel 77
5.15.3.3. Labelling the probe for Northern blot 78
5.15.3.4 Northern blot hybridization 78
5.15.4. Measurement of HNF4γ knockdown in LentiLox-HNF4γ mice by quantitative real-time PCR 78
5.15.4.1. Purification of the cDNA 79
5.15.4.2. Real-time PCR conditions 79
5.15.5. HNF4γ protein detection in intestine and pancreas 80
5.15.6. Glucose tolerance test 81
5.16. Fluorescence imaging 81
6. References 82
7. Acknowlegements 92

1. Summary

Transgenic animals are generated by injection of recombinant DNA sequences into fertilized oocytes. Here I applied a new methodology for the generation of transgenic knockdown mice using the LentiLox 3.7 lentivirus as a transfer vehicle bearing an U6-promoter dependent shRNA expression cassette. Lentiviruses are a sub-class of retroviruses that have the capability to infect non-dividing post-mitotic cells. Recently, in addition to their use to transform primary cells and established cell lines, lentiviruses have also been used to generate transgenic mice, pigs, cattle, rats and chickens. Thus, we hoped that lentiviral vectors containing U6/RNAi expression cassettes could serve as a fast and attractive alternative for the generation of mice with reduced expression of specific genes. As a model for this *in vivo* knockdown approach I chose the hepatocyte nuclear receptor 4 γ (HNF4γ) for which a knock out model was not available. Expression analyses of the HNF4γ gene demonstrated synthesis of the protein in the embryonic gut at day E16.5. In adult animals its expression is restricted predominantly to the differentiated, absorptive brush border cells of the small intestine (enterocytes) and to the cells of pancreatic islets (islets of Langerhans). In order to knockdown the HNF4γ gene, a panel of five shRNA hairpin sequences was selected by the public Sirna software and their activity was validated by transfection experiments in cell culture. After re-cloning of the U6/shRNA cassettes into the pLL 3.7 vector, infectious virus particles were generated and injected within the perivitelline space of one cell stage mouse embryos. 56% of the LentiLox 3.7 lentivirus founder mice were PCR-positive, however expression from the transgene was highly mosaic. The high mosaicism of F0 mice precluded their use for immediate expression analysis as it was hoped when the project was started. The high degree of mosaicism is also reflected by a low rate of germ line transmission. Only 6% of F1 mice expressed the indicator gene for EGFP as well as the shRNA transgene. Often expression was not ubiquitous probably reflecting the dependence of expression on the chromosomal integration site. A good correlation between EGFP activity and siRNA accumulation in organs of F1 mice was found as evidenced by Northern blot hybridisation. Despite the general low efficiency of transgenesis the down regulation of HNF4γ gene in one F1 line (A-I) reached 50% in the gut and 80% in pancreas proving that this targeted knockdown approach is working in living animals. .

1. Zusammenfassung

Transgene Mäuse werden durch Mikroinjektion von rekombinanter DNA in Oocyten erzeugt. In dieser Arbeit erzielte ich eine Geninaktivierung in transgenen Mäusen durch Verwendung des lentiviralen Expressionsvektors LentiLox 3.7, der eine durch den U6-Promotor kontrollierte Expressionskassette enthält. Lentiviren sind eine Unterklasse von Retroviren, die auch nicht in Zellteilung befindliche Zellen infizieren können. In letzter Zeit wurden lentivirale Vektoren, zusätzlich zu ihrer Verwendung in primären Zellen und permanenten Zellinien, auch zur Herstellung von transgenen Mäusen, Ratten, Schweinen, Rindern und Hühnern benutzt. Wir hofften, lentivirale Vektoren mit einer U6/RNAi-Expressionskassette könnten als attraktive schnelle Alternative für die Erzeugung von Mäusen mit einer reduzierten Expression spezifischer Gene verwendet werden unter der Vorstellung, dass die transgenen Mäuse dann ohne weiter Züchtung direkt analysiert werden könnten. Als Modell für dieses Vorgehen habe ich den hepatozytenspezifischen nukleären Faktor 4γ, HNF4γ, gewählt, da für dieses Gen kein knock out-Modell existierte. Expressionsanalysen zeigten, dass das HNF4γ-Protein im embryonalen Darm ab E 16.5 exprimiert wird. In adulten Tieren ist die Expression auf die differenzierten Bürstenepithelien (Enterozyten) des Dünndarms und auf Inselzellen im Pankreas (Langerhanssche Inseln) beschränkt. Zur Inaktivierung des HNF4γ-Gens wurden fünf unterschiedliche shRNA-Sequenzen mit Hilfe der allgemein zugänglichen Sirna-Software ausgesucht und deren Aktivität durch Transfektion in Zellkultur getestet. Nach Umklonierung der U6/shRNA-Kassette in LentiLox 3.7 wurden infektiöse Partikel erzeugt und in den perivitellaren Raum von Mausembryonen (Einzell-Stadium) injiziert. In 56% der Mäuse aus den injizierten Embryonen (F0) konnte durch PCR ein positiver Nachweis für das virale Transgen geführt werden, die Expression des Transgens war aber sehr mosaik-artig. Der ausgeprägt mosaike Expression in F0-Mäusen verhinderte ihre Verwendung für eine direkte Analyse von HNF4γ–Defizienz, wie es zu Beginn des Projektes erhofft wurde. Der hohe Grad von mosaiker Expression spiegelte sich auch in der geringen Rate von Keimbahntransmission wieder. Nur 6% der F1-Mäuse exprimierte das Indikator-Gen EGFP sowie shRNA. Häufig war die Expression nicht ubiquitär, was vermutlich eine Abhängigkeit der Expression des Transgens von der chromosomalen Integrationsstelle anzeigt. Northern-Hybridisierung zeigte aber eine gute Korrelation zwischen der Intensität

des EGFP-Signals und der Akkumulation der siRNA als Produkt der U6/shRNA-Expressionskassette. Trotz der allgemein niedrigen Effizienz der Transgenese war die Expression von HNF4γ-RNA in einer der F1-Linien (A-I) im Dünndarm um 50% und im Pankreas um 80% reduziert. Dies zeigt, dass die gewählte Methode zur Geninaktivierung prinzipiell auch in Tieren anwendbar ist, allerdings mit einer niedrigen Effizienz und ohne Zeitvorteil gegenüber der konventionellen knock out-Technologie.

2. Introduction

2. 1. Nuclear receptor transcription factors

Nuclear receptors function as ligand-activated transcription factors that regulate the expression of target genes and that are involved in the control of a diversity of cellular processes. Nuclear receptors are localized in the cytoplasm and/or nucleus. Their ligands are lipophilic molecules which either diffuse through the cell membrane or they are intracellular metabolites. Both types bind to their cognate receptors, thereby modulating their activity. The ligand-activated receptors then bind to DNA elements in the control region of target genes or modulate by protein-protein interaction the transcription of the target genes and thus transform extracellular and intracellular signals into a change of gene expression [1].
Currently, the human genome is reported to contain 48 members of the nuclear receptor family [2]. This super-family includes not only the typical endocrine receptors that mediate the actions of steroid and thyroid hormones as well as of fat-soluble vitamins A and D [1], but includes also the so-called orphan nuclear receptors, whose ligands, target genes, and physiological functions were initially unknown [3]. Many of the receptors are lipid sensors that response to cellular lipid levels and regulate metabolic activities or promote gene expression changes to protect cells from lipid overload [2].

2. 1. 1. Structure of nuclear receptors

The nuclear receptors have common structural features and are currently grouped into six different subfamilies [4]. They display a high degree of homology at the level of amino acid sequence which indicates similar functional principles. The nuclear receptors have a modular structure with autonomous functional domains. At the level of primary structure, five domains can be distinguished each with specific functions [4].
A typical nuclear receptor consists of the variable N-terminal A/B region, a highly conserved domain responsible for DNA binding (C region), D region with nuclear localization signal, the ligand binding and dimerization domain (domain E) and an F region (**Figure 1**). Domains responsible for trans-activation are found in the A/B region (activation function 1, AF-1), and in the E region (activation function 2, AF-2) [5].

2. 1. 2. HNF4γ

HNF4γ is a member of the nuclear receptor family the function of which is unknown, because a mutation of this gene has not been generated yet. HNF4γ is highly homologous to the previously characterized HNF4α; however their chromosomal localizations are distinct [6]. HNF4γ orthologues were identified in other organisms: DHNF4 (Drosophila HNF4) [7], XHNF4 (Xenopus HNF4β) [8]. The natural ligands for HNF4γ are fatty acids [9]. It was hypothesized that HNF4γ may have a role in regulation of an apoA-I/C-III/A-IV gene cluster expression in the intestine [10].

HNF4γ has the typical structure of nuclear receptors: the A/B domain contains transactivation activity, the C region contains two zink fingers responsible for DNA binding, the E ligand binding domain, the F region with regulatory functions (**Figure1**) [6].

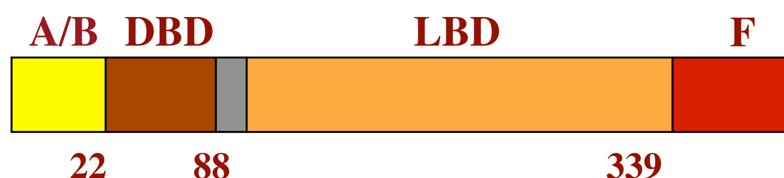

Figure 1. The protein structure of HNF4γ

Schematic representation of the HNF4γ protein: trans-activation domain (A/B); DNA binding domain (DBD); ligand binding domain (LBD); regulatory domain (F).

The structures of several orphan nuclear receptors, including human HNF4γ, peroxisome proliferators-activated receptor delta (PPARδ), retinoic acid-related orphan receptor β (RORβ), and a constitutively active mutant RXR were obtained as a complex with various naturally occurring lipids [11]. However, all of these nuclear factors have significant differences in their ligand binding pocket when compared with the HNF4γ binding pocket. The HNF4γ pocket is almost exclusively hydrophobic, with the exception of the conserved arginine that interacts directly with the fatty acid. Palmitic acid almost completely fills the HNF4γ binding pocket, leaving no room for additional bound water molecules. The HNF4γ structure showed the fatty acid with its acid head-group forming a hydrogen bond with a conserved arginine in helix 5. This prevents a direct interaction between the fatty acid and the AF2 helix [12]. The HNF4γ binding pocket is small (626A). The HNF4γ/α can only bind C14-18 saturated and monounsaturated fatty acids, with the ligand almost completely filling the pocket (**Figure 2**) [11].

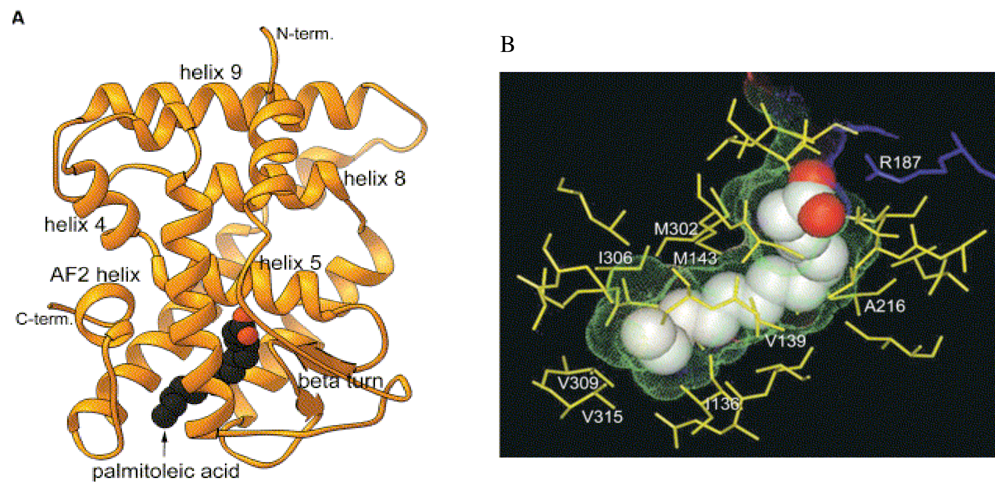

Figure 2: Structure of the HNF4γ ligand binding domain and the HNF4γ binding pocket

(A) Diagram of HNF4γ with bound palmitate. Palmitate has dark gray carbon atoms and red oxygen atoms.

(A) Model showing proposed interactions between palmitic acid and the HNF4γ binding pocket. HNF4γ atoms are yellow except for arginine 187 (blue). Palmitic acid has white carbons and red oxygens. The surface on the inside of the pocket is shown in green. C16-18 fatty acids fully occupy the pocket. (modified from G. Wisely; [11])

It was found that HNF4γ-bound fatty acid is sufficient to promote interaction with nuclear receptor co-activators. The attempts to measure HNF4γ ligand exchange with radiolabelled palmitic acid showed that either exchange does not occur or the rate is extremely slow under the conditions used. It can be speculated that the fatty acids undergo incorporation through a selection process during HNF4γ translation and folding and then are trapped in the fully folded protein. Moreover, the fatty acid does not behave like an exchangeable ligand, but rather as a structural cofactor for HNF4γ [11-12].

In co-transfection experiments in Hela and COS cells, HNF4γ was able to activate transcription, acting through binding sites that have been previously characterized as HNF4α binding sites for the apolipoprotein CIII and the TAT gene. mHNF4γ was able to activate transcription at low levels (50ng) and trans-activation reached a plateau when 250ng of the expression plasmid was used which was 3-fold above basal level (**Figure 3**) [6].

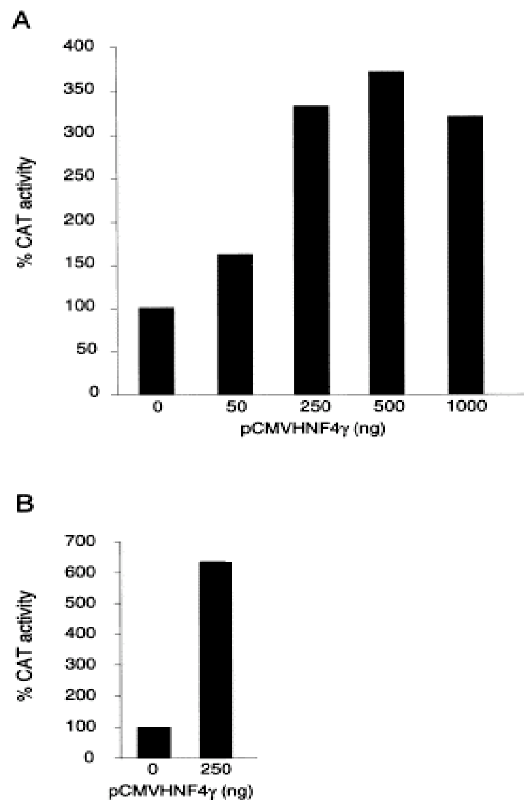

Figure 3: Transcriptional activation by mHNF4γ

(A) Increasing amounts (50-100ng) of expression vector containing the mHNF4γ cDNA (pCMVm HNF4γ) were co-transfected into HeLa cells with a CAT reporter construct containing a HNF4α recognition site from the apolipoprotein CIII gene promoter (pAPFIHIVCAT). The bars represent CAT activity, while CAT activity when no expression vector was transfected was used as a background control.

(A) mHNF4γ can function as a transcriptional activator through the tyrosine amino transferase (TAT) HNF4α binding sites. The reporter gene pBLCAT2xHNF4 contains an HNF4 binding site from the TAT gene as a dimer which was transfected into COS cells together with 250ng of the expression vector carrying the mHNF4γ cDNA (pCMVmHNF4γ) (Taraviras).

2. 2. RNA interference

RNA silencing or RNA interference [13] (RNAi) occurs in a wide variety of eukaryotic organisms [14,15]. It is mediated by dsRNA precursors that differ in length and origin. These dsRNA are rapidly processed into short RNA duplexes of 21 to 29 nucleotides, which then are incorporated into the RNA-induced silencing complex (RISC) leading to cleavage or translational repression of complementary, single-stranded RNAs, such as messenger or viral genomic RNAs [16].
According to their origin or function, three types of small RNA have been described: short interfering RNAs (siRNAs), repeat-associated short interfering RNAs (rasiRNAs) and microRNAs (miRNAs). In cells, dsRNAs are produced by RNA-templated RNA polymerization (from viruses) or by hybridization of overlapping transcripts (transgene arrays and transposons). Such dsRNAs give rise to siRNA or rasiRNAs, which guide mRNA degradation or/and chromatin modification. Otherwise, cellular transcripts that contain complementary or near-complementary 20- to 50-base-pair inverted repeats fold back to form dsRNA hairpins. These dsRNA are then processed into miRNAs that mediate translational repression and in rare cases mRNA degradation [16].
RNAi silencing was first recognized as an antiviral mechanism that protects organisms from RNA viruses [17], or which prevents the random integration of transposable elements. Later this perception was expanded by the finding that dsRNA can induce silencing in Drosophila [18], C. elegans [13,19, 20] and in other organisms that were otherwise not easily accessible to genetic analysis [21, 22]. Small RNAs were shown to be generated in plants and were found as products of the RNA silencing pathway [23]. The dsRNA-processing enzyme Dicer [24] was identified to produce these small RNAs, now called short interfering RNAs (siRNA). Synthetic RNAs engineered as Dicer products were shown to be capable to induce sequence-specific gene silencing in human cells without initiating non-specific gene silencing pathways [25]. A class of natural hairpin dsRNAs (miRNAs) [26, 27] was shown to be processed by Dicer [28-30] and to function together with RDE-I homologues [30], thereby linking the RNAi machinery to natural developmental gene regulatory mechanisms. More recently, the RNAi was also linked to chromatin regulation in yeast [31] and to chromosomal rearrangement during development of the somatic macronucleus in Tetrahymena [32].

2. 2. 1. siRNA and microRNAs (miRNA)

miRNAs are highly related to siRNAs. They are endogenous small RNAs that repress the expression of target genes. miRNAs differ from siRNA in their biogenesis, not in their functions [33-39]. Similar to siRNA, plant and animal miRNAs can direct cleavage of mRNA targets when both show extensive sequence complementarity [40-44], but repress mRNA translation when complementarity is less [45, 46]. The sequence of miRNA or of siRNA determine how effectively it might act by direct cleavage or by translational repression.

Both siRNAs and miRNAs can also trigger methylation of DNA of protein-coding genes that share sequence in plants [47, 48] and most likely in mammals [49, 50]. siRNA can direct the construction of repressive heterochromatin, namely centromeric heterochromatin [51-55]. As a class, miRNA and siRNA guide strands are functionally indistinguishable, but it is still not clear whether all miRNA and siRNA sequences are capable of effectively guiding all RNA silencing functions.

Natural miRNA are derived from long primary RNAs (pri-miRNAs) transcribed by RNA Polymerase II [56-58]. Animal viruses also can encode miRNA [59, 60]; these may be transcribed by RNA Polymerase II or III. Pri-miRNA contain stem-loop structures that harbour the miRNA [61]. In animals, the stem-loops are around 70nt long, and Drosha, a nuclear RNase III-type endonuclease, liberates the stem-loop from the primary transcript to yield a pre-miRNA [62]. The pair of cuts made by Drosha establishes one end of the miRNA [63][62]. In this process, Drosha participates with a double stranded RNA binding protein, called Pasha in flies and DGCR8 in humans [64-67]. The pre-miRNA liberated by Drosha is exported from the nucleus by the protein Exportin 5 [68-70], then cut by Dicer in the cytoplasm to produce the mature miRNA [28-30]. In some organisms, such as worms and man, the same Dicer that makes siRNA, processes pre-miRNA; in plants and flies, different Dicer paralogs make siRNA and miRNA [71].

Each miRNA or siRNA duplex can potentially yield two single-stranded 21nt RNAs capable of directing RNA silencing, but only one of them usually accumulates as mature siRNA (guide) in RISC complex (**Figure 4**). The passenger strand is usually destroyed[56].

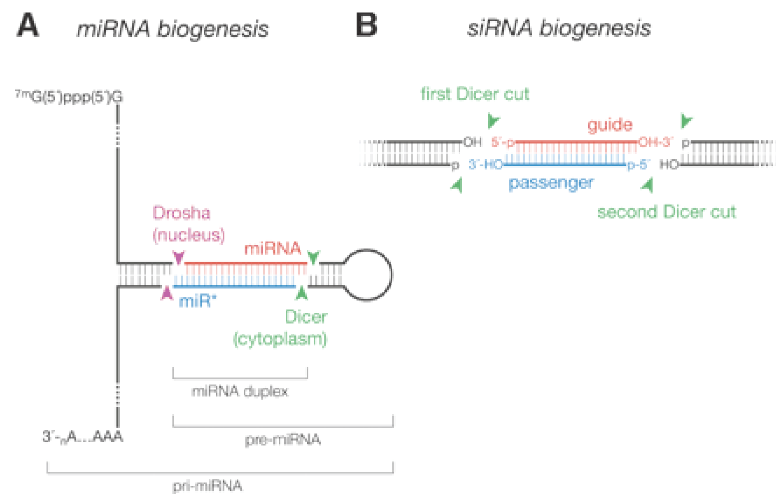

Figure 4: Small RNA biogenesis in animals

(A) miRNA are produced by the action of two RNase III ribonucleases. After their transcription by RNA pol II, primary miRNA (pri-miRNA) are cleaved in the nucleus by Drosha, which is in heterodimeric complex with the dsRNA-binding protein. Drosha cleavage generates the pre-miRNA, which binds Exportin 5 and is exported to the cytoplasm.

(A) Long dsRNA is a substrate for Dicer, but not for Drosha. Dicer must make two pairs of cuts to yield an siRNA duplex. Dicer is thought to preferentially initiate dsRNA cleavage at the end of dsRNA, which some times produces a phased string of siRNAs along the dsRNA. The RISC machinery selectively loads the guide strand into RISC; the passenger strand is degraded. (modified from Tomari [56])

2. 2. 2. Selection of highly effective siRNA sequences

Recently Khvorova and colleagues [72] used informatical methods to understand which siRNA strand enters the RISC as the guide and which is excluded and becomes passenger strand by comparing the thermodynamic profiles for hundreds of siRNAs- active and inactive. The thermodynamic signatures of siRNAs provide a graphical representation of how tightly base-paired one siRNA strand is to its complement at each position of the 19-bp helix that links the two strands (**Figure 5**). The local thermodynamic stability of nucleic acid helices is a function of the base pairs flanking an individual G:C or A:U base pair in an RNA helix. Thus, a "nearest-neighbour" analysis can predict the local stability at each position in a siRNA. Several reports provide so-called „design rules“ to distinguish functional from non-functional siRNA [73-75].

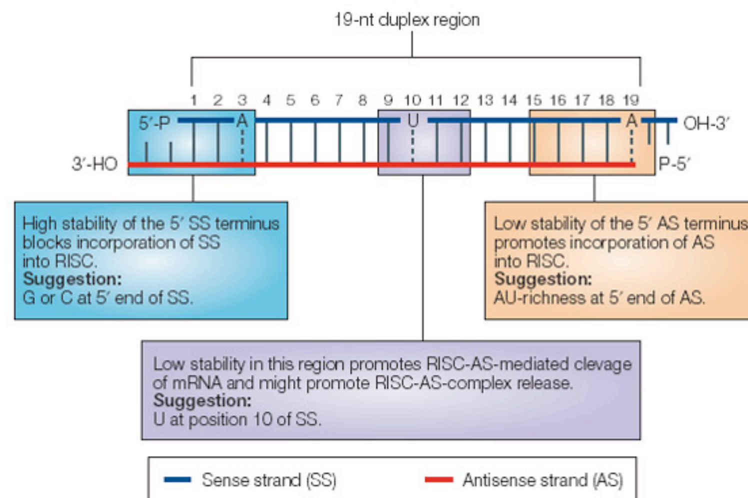

Figure 5: The generation of effective siRNA

A small interfering RNA (siRNA) is a 21-23nt dsRNA that contains: a 19-nt duplex region, symmetric 2-3-nt 3´ overhangs and 5´ -phosphate (P) and 3´ -hydroxyl (OH) groups. The effective siRNA has high stability at the 5´ terminus of the sense strand (blue box), lower stability at the 5´ antisense terminus (orange box) and the cleavage site (purple box). The sequence-specific preferences at the following positions on the sense strand are important: an A at position 19, an A at position 3, a U at position 10.

2. 2. 3. RISC function

Once loaded into RISC, the small RNAs guide at least three distinct modes of silencing. In the RNA pathway, small RNAs direct RISC to cut target RNAs. The cleavage by RISC requires Mg^{2+}, leaves 3´ hydroxy and 5´ phosphate termini, and is inhibited by phosphorothioates at the scissile phosphate, all hallmarks of a role for Mg^{2+} in the catalysis [76-78]. The cleavage is thought to be catalyzed by the Piwi domain of a subclass of Argonaute proteins [78][79]. This domain is a structural homolog of RNase H, a Mg^{2+} -dependent endoribonuclease that cleaves the RNA strand of RNA-DNA hybrids [80]. Unlike RNase H, each cleavage-competent RISC can break only one phosphodiester bond in its RNA target [81] (**Figure 6**). RISC is a multiple turnover enzyme; the siRNA guide strand incorporated localizes the RISC complex to the RNA target, the target is cleaved and the RISC complex is released intact and able to start a new round of cleavage [33, 76, 82].

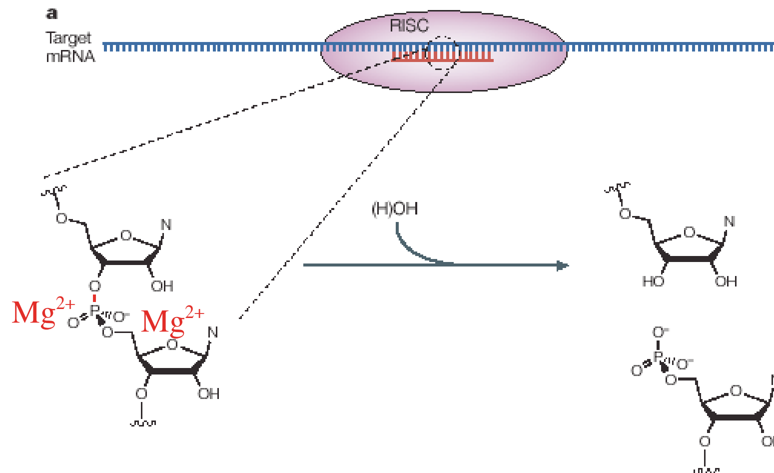

Figure 6: Chemical mechanism of RISC-catalysed target-mRNA cleavage

The RISC catalyses a phosphodiester hydrolysis reaction that generates 3´ -hydroxyl and 5´ -phosphate termini. The scissile bond is shown in red (modified from E. Sontheimer [79]).

The first position of a small RNA in RISC does not contribute to binding, as was first noted in studies designed to predict miRNA targets [83, 84]. Instead, bases 2-8 form a 5´ end of anti-sense strand (a "seed") that are important to pair with target candidate sequences. It was proposed that bases 2-8 of several miRNA were complementary to conserved sequence elements in the 3´ untranslated regions (UTRs) of post-transcriptionally repressed mRNAs in flies [85]. It is now clear that most of the binding energy that tethers RISC to a target RNA comes from bases in the 5´ half of the small RNA complementary to mRNA [46, 82]. This is a key difference between siRNA or miRNA and antisense oligonucleotides, where all bases contribute equally to specificity. In fact, complete pairing of the 3´ half of an miRNA or siRNA to its target RNA is not required for translational repression, provided that multiple small RNAs are bound to the target [46], nor for target mRNA destruction. This could mean that most active siRNAs will not only down-regulate their intended mRNA targets, but also could reduce expression of other mRNAs possessing partial complementary to the siRNA guide strand [86].

2. 2. 4. Other silencing models

In the translational repression pathway, small RNAs direct RISC to bind mRNA and repress its translation into protein, rather than cleaving it. Animal miRNAs typically mediate translational repression. In contrast, most plant miRNAs direct target cleavage. In all of the

well-studied examples of translational repression by animal miRNAs or siRNAs, translational repression is a response to the binding of multiple small RNA-programmed RISC complexes to the sites in the 3´ UTR of the mRNA target. The presence of multiple potential recognition sites in 3´ UTR sequences is a useful predictor of an mRNA for being regulated by miRNA [84]. The degree of complementarity between a small RNA and its RNA target helps to determine if the small RNA directs target cleavage or represses mRNA translation, because an A-form helix must be formed with the target RNA at the centre of an siRNA guide strand for cleavage to occur [82, 87]. In the absence of such a helix, translational repression occurs. Translational repression occurs at some step after translational initiation, since the distribution of ribosomes along the repressed mRNA is indistinguishable from the same mRNA undergoing active translation [45]. Ketting and Plasterk [88] propose that RISC somehow directs degradation of the nascent polypeptide, rather than simply stalling translational elongation. Both models are currently without experimental support. A recent evidence demonstrated that RISC complex guided by miRNA, brings the target mRNA in specialised cytoplasmic structures called P-bodies where the mRNA is degraded by the cellular RNA nucleases [89-91].
Small RNAs can also direct the formation of heterochromatin. The only complex thus far established for this pathway is the S. pompe RITS, a RISC-like complex that contains siRNA, an Argonaute protein (Ago1), the chromodomain protein Chp1, and Tas3, a protein of unknown function [54, 55]. How the RITS directs modification of histones to promote formation of heterochromatin is unknown. It is not known if the RITS binds the DNA of the targeted gene or binds nuclear RNA transcribed from targeted DNA sequences. dsRNA targeting promoter sequences also triggers transcriptional silencing in plants [92]. siRNA-directed transcriptional silencing via DNA metthylation was recently reported in animals [49].

2. 3. Lentiviral transgenesis

For the past two decades, pronuclear injection of DNA has been the most widely used method for generating transgenic mice. The cloned DNA is microinjected directly into the pronucleus of fertilized embryos. An important advantage of pronuclear injection is that it is not limited to certain species; it works nicely in mice and also has been used to produce transgenic farm animals like pigs. However, this technique has a low efficiency of

transgenesis. In mice, the transgene is integrated and expressed in not more than 4% of the animals generated from injected oocytes [93]. Because the production of transgenic mice by DNA microinjection is standardized, the losses during the injection is compensated by high-throughput production and screening.

The first viral transgenesis in mammals was done three decades ago. Janisch and Mintz employed a simian virus to transfer genetic material into mouse embryos [94]. Subsequently, Jaenisch reported the successful germ line integration of retroviral DNA in mice and its transmission into their offspring [95]. Later, other groups tried to replace viral genes by mammalian DNA in the virus to express it ectopically in animals. However, the genes cloned into these integrated retroviruses were not expressed in newborn mice. This block was not restricted to rodents, but it was also observed in cattle [96]. This transcriptional repression is thought to be mediated by both cis-acting de novo methylation of the integrated provirus and by cell-type-specific trans-acting transcriptional repressors. In addition, the major disadvantage of simple retroviral vectors is their inability to transduce non-dividing cells, which renders them extremely inefficient as an *in vivo* gene delivery system. In contrast with retroviruses, lentiviruses have the ability to infect also non-dividing cells [97, 98], because the lentiviral genome is actively transported into the nucleus [99, 100].

2. 3. 1. Lentiviruses

Lentiviruses belong to the large family of retroviruses [101]. The genus lentiviradea includes the human immunodeficiency viruses HIV-1 and HIV-2; the simian immunodeficiency virus, SIV; and nonprimate lentiviruses, such as the visna virus, equine infectious anemia virus (EIAV), feline and bovine immunodeficiency viruses (FIV and BIV) and some others. The mechanism by which lentiviruses infect non-dividing cells has not been completely elucidated. However, several studies have indicated that, by using the host cellular nuclear-import machinery, the *gag*, *vpr* and *pol* gene products can mediate an active transfer of HIV-1 pre-integration complexes into nuclei of non-dividing cells [102, 103]. In addition, lentiviruses carry at least three other genes, such as *tat* and *rev*, that contribute to the more complex life cycle of lentiviruses. After entry into the host cell, the viral RNA genome is reverse transcribed into DNA, which is integrated into the host genome (provirus) and serves as a template for production of progeny (virions). The

integration into the host cell genome is the basis for persistent infection and transmission of the integrated provirus to offspring after infection of germ cells (vertical transmission).

2. 3. 2. Lentiviral gene transfer vectors

Novel lentiviral vectors have been shown to work in many different settings, where gene transfer is needed, but was not achieved with vectors based on retroviruses [104]. The first lentiviral vectors were derived from HIV-1. Nowadays, an advanced third generation of HIV-based vector systems is available. Similar transfer vectors were developed from other lentiviruses [105, 106]. For reasons of biological safety, the typical lentiviral vector system carries the necessary minimal information for the formation of infectious particles is distributed on separate vectors and packaging cells. The transgene of interest is inserted between the LTRs.

2. 3. 3. Self-inactivating vectors

An important safety concern with lentiviral vectors was the possibility of insertional mutagenesis and activation of cellular proto-oncogenes. Thus, lentiviral vectors were designed in a way that viral regulatory elements within LTRs required for replication were deleted. The resulting vectors are known as self-inactivating (SIN) allowing the formation of infectious but non-replication competent viral particles [107]. It was also hoped that the SIN vectors may escape gene silencing, because it was assumed that active viral promoter sequences might recruit the host cellular silencing machinery to the integrated provirus.
Because the viral promoters were removed in the SIN vectors, other promoters had to be introduced to drive transgene expression. Most of the lentiviral vectors designed till now contain ubiquitously expressing promoters that allow broad transgene expression in almost all organs. Such promoters are the human cytomegalovirus immediate early promoter (CMV) [98], the promoter of the human phosphoglycerate kinase gene (PGK) [108], the human ubiquitin-C promoter [109] and the GAG promoter (a chicken beta-actin/CMV-compound promoter) [110]. By introduction of regulatable transcriptional elements, spatial and temporal regulation of the lentiviral transgene expression can be achieved. Nowadays, the most frequently used regulatable system is the bacterial tetracycline (Tet) resistance system.

2. 3. 4. Vector elements

A feature of lentiviral vectors is the incorporation of a Rev-responsive element (RRE) that enhances the production of unspliced vector RNA in the packaging cells. Thus, even complex transgenes containing introns and splicing signals can be included into the vector.
Incorporation of a central DNA flap, the polypurine tract (cPPT) of the *pol* gene, is another important cis acting factor. The cPPT enhances the import of the HIV-1 pre-integration complex into the nucleus of the infected cells [108].
The viral transduction efficiency was further enhanced by the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE) [111]. Insertion of the WPRE in the 3´ untranslated region of lentiviral vectors enhances transgene expression 5- to 8 fold in a promoter- and transgene-independent manner [111].

2. 3. 5. The lentiviral packaging system

The vectors carrying the above mentionned genes do not contain the lentiviral packing system. Therefore, the viral proteins *gag* and *pol* required for the production of lentiviral particles are provided in trans by packaging/producer cells. The Rev protein is also expressed in the packing cells to achieve sufficient expression of unspliced vector RNA and of the *gag* and *pol* genes, which carry RREs [112].
The *env* gene, which determines the tropism of retro and lentiviruses, has to be provided to obtain infectious particles. Similar to simple retroviruses, the tropism of lentiviral vectors can be changed by replacing the wild-type *env* gene with the envelope gene of another virus (Pseudotyping). The lentiviruses currently used are pseudotyped with the G protein of vesiculostomatitis virus (VSV-G) [104]. The advantages of these pseudotyped viruses (VSV-G) are the broad host range of VSV and the stabilization of the particles that can be concentrated by ultracentrifugation [104].

2. 3. 6. Generation of transgenic animals by using lentiviruses

A breakthrough for virus-mediated transgenesis was the switch in use of simple retroviruses to lentiviruses [109, 110]. Several groups introduced foreign genes into murine

preimplantation embryos at the zygote and morula stage using SIN vectors, generating transgenic mice. The transgene was expressed and further passed to the offspring (F1-generation) through the germ line. The F1 mice exhibited also transgene expression. By using different promoters, ubiquitous as well as tissue-specific expression was achieved [109]. In addition, different ways of infection were tested. The zona pellucida is a physical barrier that protects embryos from lentiviral infection. Therefore, either the zona pelucida has to be removed (denudation) or the virus has to be delivered into the perivitelline space by injection (subzonal injection). The draw back of the denudation was delayed embryonic development and reduced implantation rates [109]. Subzonal injection of the lentivirus was less invasive than DNA microinjection. Comparison between DNA microinjection and lentiviral transgenesis revealed a more than 8-fold increase in the number of transgens incorporated per embryo. If one considers only F1 progeny, lentiviral transgenesis was 4 fold more efficient than DNA microinjection.

Another way to generate transgenic mice by lentiviral infection is gene transfer into embryonic stem (ES) cells. Similar to the situation in preimplantation embryos, lentiviral vectors proved to be superior to retroviral vectors in infecting ES cells. Lentivirally-infected murine ES cells expressed the transgene during *in vitro* [113] and *in vivo* differentiation [110]. Injection of lentivirus-infected ES cells into blastocysts, followed by embryo transfer into fosters, resulted in the birth of chimeric animals that expressed the transgene in all cells derived from the transduced ES cells [110]. Embryos derived from crossing of chimeric mice expressed the transgene, indicating successful germ-line transmission.

The DNA microinjection is the most widely used technique for the production of transgenic mice. However, in farm animals the problem of low efficiencies is more pressing, because of the high production costs. Therefore, Hofmann [114] used a lentivirus that carries the gene encoding green fluorescent protein (GFP) to infect zygotes of pigs. The viral particles were injected into the subzonal space between the cytoplasmic membrane and zona pellucida. Subzonal injection had no significant effect on embryonic development. A transfer of 244 lentivirus-infected porcine embryos into six recipient pigs resulted in the birth of 46 piglets. The advantage of using GFP as the reporter lies in the easy identification of transgenic progeny. Of the piglets born, 32 (13% of the infected embryos) carried the transgene and 30 expressed it. In comparison to DNA microinjection, lentiviral transgenesis resulted in 27-fold higher yield in transgenic animals expressing the transgene per treated embryo. In all of the tissue analysed, including derivatives of three primary germ layers:

ectoderm (skin), endoderm (gut) and mesoderm (cardiac muscle), GFP was expressed. Furthermore, by incorporation of human keratin K14 promoter, Hofmann et al. were able to direct transgene expression exclusively to the skin of the pigs. They also showed that the transgene is present in germ line cells, which is the basis for its transmission to offspring.
Initial experiments using retroviral vectors resulted in a dramatic increase in transgenesis rates in cattle [96], however, retroviral transgenesis in cattle did not meet the high expectations [115]. The first experiments using lentiviruses to infect preimplantation embryos were not successful. By switching to lentiviral infection of bovine oocytes before in vitro fertilization, Hofmann et al. [116] generated lentiviral transgenic calves. All of the calves were transgenic and 100% of these animals expressed ubiquitously eGFP as detected by *in vivo* imaging and Western blotting.
The development of an efficient method for genetic modification of chickens had significant technical difficulties [117]. The earliest methods developed were based on the use of avian retroviruses and replication-defective vectors derived from reticuloendotheliosis virus [118]. Recently, an ALV replication-defective vector was used to produce transgenic birds at low frequency, probably because of host silencing of the viral sequences. Michael J.McGrew [119] investigated the efficiency with which lentiviral vectors could transduce the chicken germ line and examined the expression of introduced reporter transgenes. Ten founder cockerels transmitted the vector to between 4% and 45% of their offspring and stable transmission to the G2 generation was demonstrated. Analysis of expression of the reporter gene constructs in several transgenic lines showed a conserved expression profile between individuals that was maintained after the germ line transmission. These data showed that lentiviral vectors can be used to generate transgenic birds with an about 100-fold higher efficiency than with any previously published method, with no detectable silencing of transgene expression between generations.
For many applications in immunology, toxicology and cardiovascular research, the rat provides a more attractive model than the mouse. In addition, there several good rat models available for studding major human diseases such as hypertension, diabetes and multiple sclerosis. Unfortunately, the classical approach to generate transgenic rats by pronuclear injection is technically challenging. Therefore, the development of lentiviral vectors provided an attractive alternative to this technique. Janes van den Brandt et al [120] generated eGFP-transgenic rats using the lentiviral approach. Analysis of the founder generation demonstrated that 46% of the offspring had stably integrated the provirus into

the genome and of those 92% expressed eGFP in all blood-derived leukocytes. In contrast to their offspring, all founder rats were mosaic with regard to eGFP expression. The expression level of eGFP in the F1 generation is influenced by the site of proviral integration. A single copy of the transgene is sufficient for reliable detection by flow cytometry, irrespective of the leukocyte subtype. Adoptive transfer of purified CD4+ T-lymphocytes from transgenic rats and next re-isolation from various organs demonstrated that expression of the lentiviral transgene is maintained in a foreign host and allows for efficient tracking of transferred cells.

2. 3. 7. Induction of RNAi in transgenic mice generated by the use of lentiviral vectors

RNAi is a post-transcriptional regulatory mechanism of gene expression which occurs, in a broad variety of eukaryotic organisms, as described above [16]. It also was recognized as an antiviral defence process that protects organisms from RNA viruses [17]. The development of siRNA expression systems that are based on transcription of short hairpin RNAs from expression plasmids containing Type III RNA polymerase (Pol III) promoters, such as the U6 [121] and H1 [122] RNA promoters, has broadened the use of RNAi dramatically. Lentiviral vectors carring an U6-driven siRNA was recently shown to induce efficient gene silencing in mice [123]. Infection of CD8-positive T cells with a lentiviral vector carrying siRNA directed against CD8 resulted in a more than 90% reduction of CD8 expression, showing the efficacy of lentiviral siRNA delivery. Infection of ES cells with such a RNAi lentivirus resulted in chimeric mice that exhibited 89% reduction in expression of target protein (CD8). Direct infection of single-cell embryos resulted in transgenic mice with a 88-94% decrease in CD8 expression in the F0-generation [123]. In addition, infection of GFP-transgenic zygotes with lentiviruses resulted in the suppression of GFP expression in the double-transgenic animals [124]. In another paper, Wange Lu et al. [125] succeeded to silence the Ryk receptor in transgenic mice expressing siRNA against it by a lentiviral vector system. The founder mice were mosaic but most of F1 animals died after birth, with survivors displaying a Ryk knockdown by 90%, defects in axon guidance and reduced body size in comparison to wt littermates.

2. 3. 8. Aim of the project

The goal of the project was to generate HNF4γ knockdown mice using a lentiviral vector system for expression of siRNA against the mRNA of that nuclear receptor. We hoped to achieve ubiquitous high expression of the U6/shRNA expression cassette. This knockdown approach to investigate gene function *in vivo* by using lentiviral transgenesis was considered as an attractive alternative to the classical knockouts. It was hoped to allow the production of a sufficient number of transgenic founder (F0) animals with a high degree of transgenesis within three months permitting functional and expression analysis of the targeted gene avoiding further breeding of the F0 animals. This method would be an ideal way for a quick gain of biological data. As the siRNA technology is quite new, a major issue was to determine the efficiency of transgenesis as well as to compare transgene expression and the dependence of gene knockdown from the sequence of the shRNA selected. As a model for *in vivo* knockdown I have chosen the hepatocyte nuclear receptor 4 γ (HNF4γ) for which no knockout mouse exists. HNF4γ is a member of the nuclear receptor family, and until recently it was considered as an orphan receptor. It is highly homologous to HNF4α; however its chromosomal localization is distinct and knowledge about its expression pattern and function is scarce. Therefore expression analysis was done, and the knockdown of this gene was performed in the hope to shed light on its role in development and physiology.

3. Results

3. 1. Analysis of HNF4γ expression

Since HNF4γ is a newly discovered nuclear receptor and since only limited information on its expression was available, I first looked by conventional RT-PCR which organs express HNF4γ. As seen in Figure 7 expression of HNF4γ gene is restricted to kidney, liver, intestine, pancreas and colon (**Figure 7**).

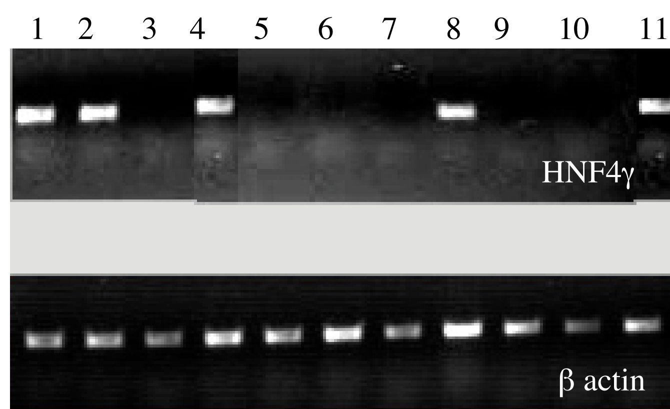

Figure 7: Expression analysis of HNF4γ mRNA by conventional RT-PCR

RT-PCR was performed on cDNA prepared from different organs: left to right; (1) liver, (2) kidney, (3) brain, (4) intestines, (5) skin, (6) muscle, (7) thymus, (8) pancreas, (9) lung, (10) spleen, (11) colon

The conventional RT-PCR is a semi-quantitative method and it does not allow a precise determination of the level of gene expression. Therefore, I performed quantitative real-time PCR. It has a sensitivity at least five orders of magnitude better than the best blotting procedures and a dynamic range of more than 10 orders of magnitude. This unsurpassable sensitivity and range has allowed to develop PCR into a powerful quantitative tool [126-128]. In a PCR reaction in principle the amount of a target DNA-template is doubled with every cycle, so ideally the reaction can be described by the exponential function $y=y_0\ 2^n$ where y is the quantity of target sequence at cycle n and y_0 is the initial quantity of template (**Figure 8**). But due to the fact that in a realistic PCR not every template is replicated in every cycle, a PCR is better described by the equation $y=y_0\ E^n$ where E is the so called efficiency of the reaction. The maximum efficiency E possible in PCR is 2 –

every PCR product is replicated every cycle. The minimum value is 1, corresponding to no amplification. For quantification a certain detection threshold, Y is set and depending on the starting amount of template and the efficiency of the reaction it needs a specific number of cycles N to reach this threshold (**Figure 8**).

$$Y = y_0 E^N .$$

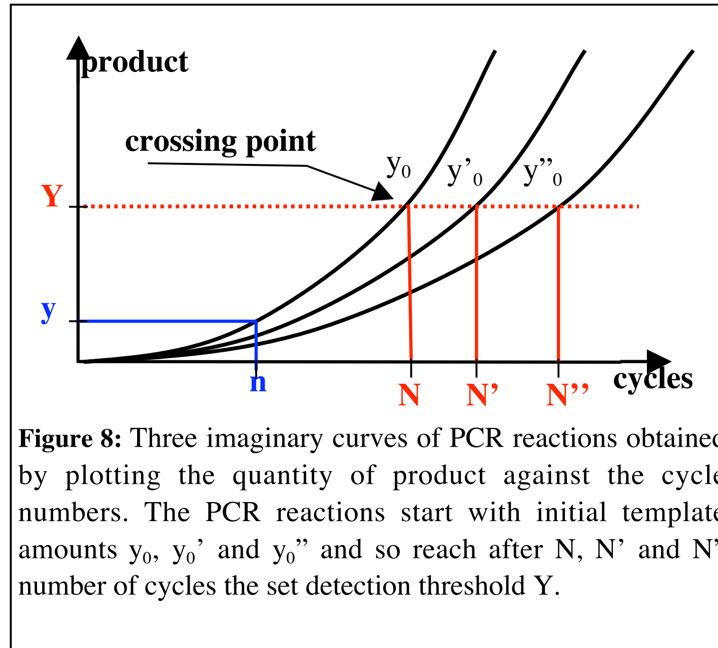

Figure 8: Three imaginary curves of PCR reactions obtained by plotting the quantity of product against the cycle numbers. The PCR reactions start with initial template amounts y_0, y_0' and y_0'' and so reach after N, N' and N'' number of cycles the set detection threshold Y.

The intersection of this detection threshold and the amplification curve represents the crossing point. The real-time PCR analysis for HNF4γ showed high mRNA levels in intestine and much lower level of expression in kidney, liver and pancreas (**Figure 9**).

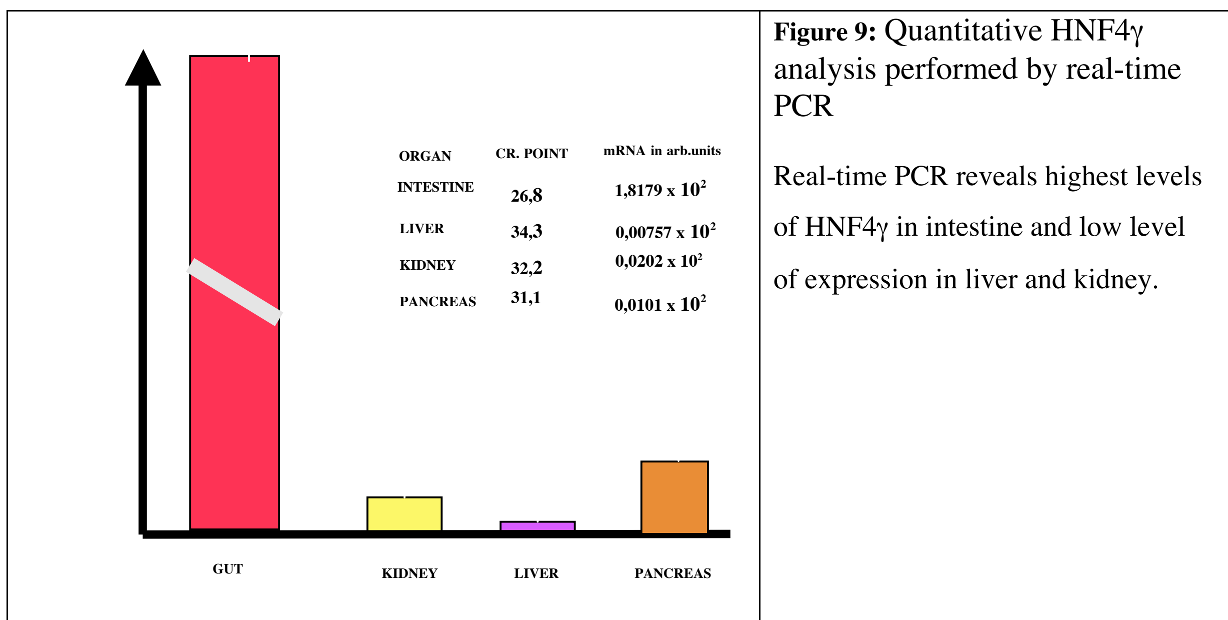

Figure 9: Quantitative HNF4γ analysis performed by real-time PCR

Real-time PCR reveals highest levels of HNF4γ in intestine and low level of expression in liver and kidney.

The lining of small intestine is a single-layered epithelium which covers the surface of the villi that project into the lumen, and it lines the crypts that descend into the underlying connective tissue. Dividing stem cells lie in the depths of the crypts. They generate four types of differentiated cells (1) absorptive cells (brush-border cells), with densely-packed microvilli on their surfaces to increase active surface area; (2) goblet cells, secreting mucus; (3) Paneth cells, forming part of the innate immune system; and (4) enteroendocrine cells secreting serotonin and peptide hormones that regulate the growth, proliferation and digestive activities of cells of the gut and other tissues. The absorptive cells travel upward from the stem-cell region and within 2-5 days they reach the tips of the villi, where they undergo initial stages of apoptosis and are finally discarded into the gut lumen.
In adult mouse intestine HNF4γ protein is restricted to mature brush-border cells of the villi. In comparison to the related HNF4α, HNF4γ cannot be seen in crypts (**Figure 10**).

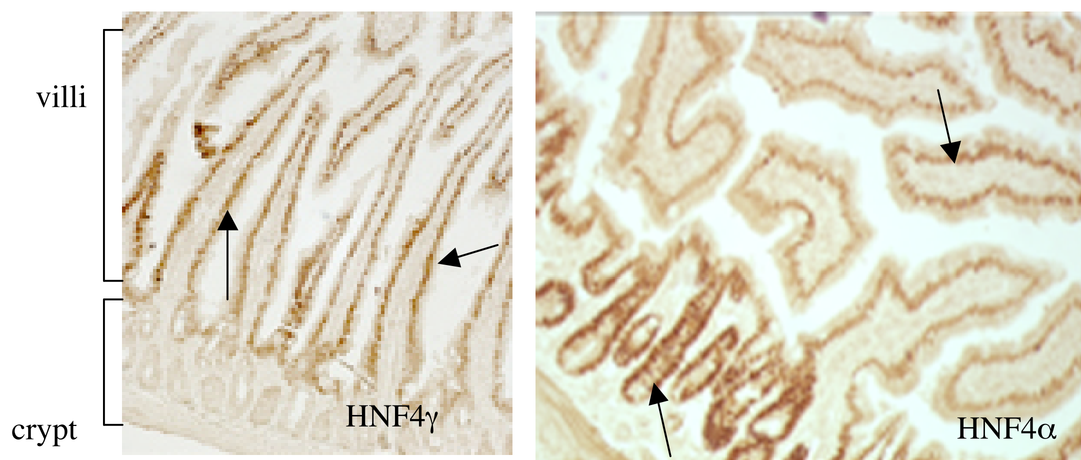

Figure 10: Expression of HNF4γ and HNF4α proteins in adult mouse intestine.
Immunohistochemical analysis reveals overlapping and distinct expression patterns of HNF4γ (left) and HNF4α (right) in adult mouse intestine.

HNF4γ showed differential expression along the gut with maximum levels in proximal intestine (duodenum) and low expression in colon (4 times less; data not shown). No HNF4γ transcript was detected in stomach.
During embryogenesis the gut derives from the endoderm. To identify whether HNF4γ expression occurs already in the developing intestine I performed immunohistochemical

analysis of intestinal tissue from day E16.5. The HNF4γ protein was detectable in the mature cells (brush-border cells) of the villi (**Figure 11**).

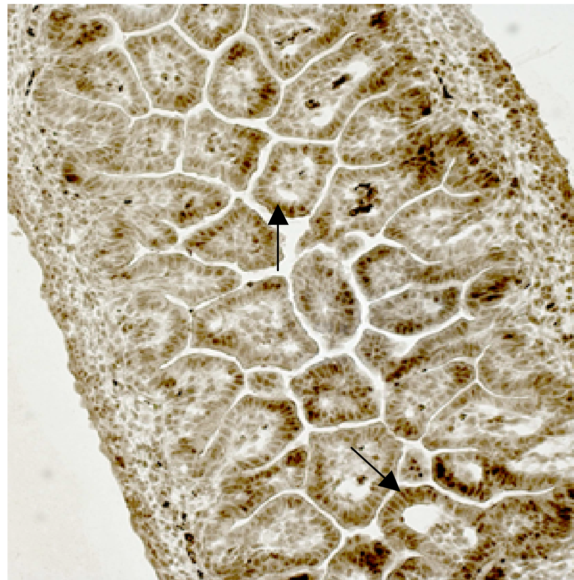

Figure 11: HNF4γ expression in the intestine of E16.5 mouse embryo.
Immunohistochemical analysis performed by a HNF4γ -specific antibody shows HNF4γ protein accumulation in enterocytes of villi (arrows).

The pancreas is the second organ where HNF4γ protein was detected. Pancreas is separated in exocrine and endocrine tissue. The exocrine pancreas is the bigger part which accounts for more than 99% of its mass. It consists of the cells that secrete bicarbonate to neutralize the acidity coming from the stomach and other cells that secrete digestive enzymes that work at neutral pH. The endocrine pancreas is represented by so called islands of Langerhans randomly distributed within exocrine tissue. The islands of Langerhans are built of β-cells secreting insulin (80% of the island cells), α-cells secreting glucagon and δ-cells with other functions. The immunohistochemical analysis demonstrated HNF4γ protein only in the islands (**Figure 12**). Because of the low sensitivity of the HNF4γ antibody it was not possible to determine in which cells HNF4γ protein is expressed.

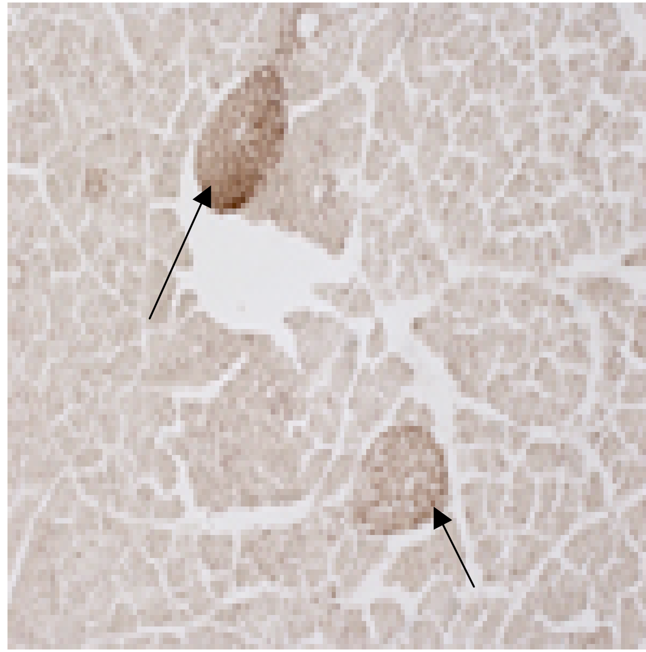

Figure 12: HNF4γ protein in the islands of Langerhans

Immunohistochemical analysis shows HNF4γ protein present in the islands of Langerhans.

3. 2. Selection of effective shRNA molecules against HNF4γ

Criteria for efficient siRNA based on thermodynamics have been determined allowing prediction for siRNA efficiency. For this purpose I used the published program Sirna stored on the Sfold server which website is freely available [129]. The program Sirna combines the most recent algorithms based on thermodynamics together with algorithms underlying RNA target accessibility. Based on this software I selected five oligonucleotides which I cloned into the mouse U6 promoter in the context of pBluescript II KS (+/-) backbone (**Figure 13**). The resulting constructs (pU6-shRNAs) can form a shuttle system allowing easy re-cloning of the whole U6-shRNA cassette into the pLentiLox 3.7 vector. The shRNA-615 was modified at position 18 of the sense strand from T to C in order to destroy a stop sequence for RNA polymerase III (**Figure 14**). The hairpin shRNA-615 will have an imperfect match between C and A at that position which could make this particular shRNA more efficient than what the Sirna program predicts, due to destabilization of the 5´-end of the antisense strand of mature siRNA. The shRNA-303 has a low score (score 7) and was selected as a non-functional shRNA against HNF4γ.

TARGET POSITION	Sequence	1	2	3	4	5	6	7	8	9	
615- 633	GUGAGGUCUUGCCAGAUUUTT AAAUCUGGCAAGACCUCACTT AA	9	1	7	1	47.4%	-3.0	5.1	-9.2	-38.8	shRNA-615
1320- 1338	GAUCCACUAACUGGGCAAATT UUUGCCCAGUUAGUGGAUCTT AA	12	5	5	2	47.4%	-11.0	3.0	-8.2	-39.4	shRNA-1320
303- 321	GUCAACUGUUUAUGUGCCATT UGGCACAUAAACAGUUGACTT GU	7	0	6	1	42.1%	0.1	-2.2	-7.4	-36.9	shRNA-303
1301- 1319	GCACCCACAUUUAUCUCAATT UUGAGAUAAAUGUGGGUGCTT AU	14	5	8	1	42.1%	-10.3	4.6	-9.4	-37.2	shRNA-1301
655- 673	GCACUGACAUAAAUAUUAATT UUAAUAUUUAUGUCAGUGCTT CA	15	4	9	2	26.3%	-9.0	6.7	-8.2	-30.7	shRNA-655

Figure 13: The sequence predicted by the Sirna program and efficiency of different shRNA hairpins against HNF4γ

The sense and antisense sequences of shRNA molecules selected by the Sirna program: (1) total score for siRNA dupex; (2) target accessibility score; (3) duplex feature score; (4) duplex thermodynamics score; (5) siRNA GC content; (6) antisense siRNA binding energy (kcal/mol); (7) differential stability of siRNA duplex ends (DSSE, in kcal/mol); (8) average internal stability at the cleavage site (AIS, in kcal/mol); (9) total stability of siRNA duplex (kcal/mol).

1 18

UGUGAGGUCUUGCCAGAUCU U U C A
UUACACUCCAGAACGGUCUAAA A A GAG

Figure 14: shRNA-615 modification

Predicted structure of shRNA-615 with the modification of the base at position 18 in the sense strand.

Since shRNA adds additional complexity in comparison to siRNA, as the processing enzyme Dicer is involved [130-132], I used the GeneEraser Luciferase Suppression-Test System (Stratagene) to test the candidate shRNA sequences**.** In this assay system, the sequences to be targeted by shRNA expression are inserted in the 3´ UTR of a luciferase gene. Following luciferase activity allowed selection of the most potent shRNA sequences. The vectors expressing shRNA (pU6-shRNA) were co-transfected into HEK293 cells at various amounts with the luciferase/HNF4γ fusion construct and an additional vector expressing Renilla luciferase serving as transfection control. Two days after transfection, expression of the luciferase was determined by measurement of luciferase activity (**Figure 15**).

knockdown efficiency of shRNAs

HNF4gamma expression

120
100
80
60
40
20
0

shRNA 615
shRNA 1320
shRNA 303
shRNA 1301
shRNA 655

100ng shRNA
1ng shRNA
10ng shRNA
100pg shRNA

Figure 15: Analysis of knockdown efficiency of shRNA sequences against HNF4γ by the GeneEraser Luciferase Suppression-Test System

The test was performed with 5 different shRNAs in a titration experiment: orange 100ng shRNA; yellow 10ng shRNA; green 1ng shRNA; dark green 100pg shRNA.

All U6-shRNA constructs showed dose-dependent silencing of the HNF4γ target sequence. By chance, four of the five shRNA performed equally well with a slight preference observed for shRNA-1301 and shRNA-655. This was in good agreement with the Sirna program prediction, since these two oligonucleotides have the highest total scores (15 and 14 respectively). The construct shRNA-303 with a total score 7 performed worst. According to the Sirna software, the sense strand (passenger strand) of the mature siRNA-303 should be incorporated preferentially in the RISC complex leading to an inefficient knockdown.

3. 3. Generation of transgenic mice using LentiLox 3.7

For the generation of HNF4γ knockdown mice I used the pLentiLox 3.7 vector to generate a lentiviral vector system that expresses shRNA synthesized from the U6 promoter [123]. This system is a third generation lentiviral system that contains a mutated 3´ LTR providing high biological safety (**Figure 16**).

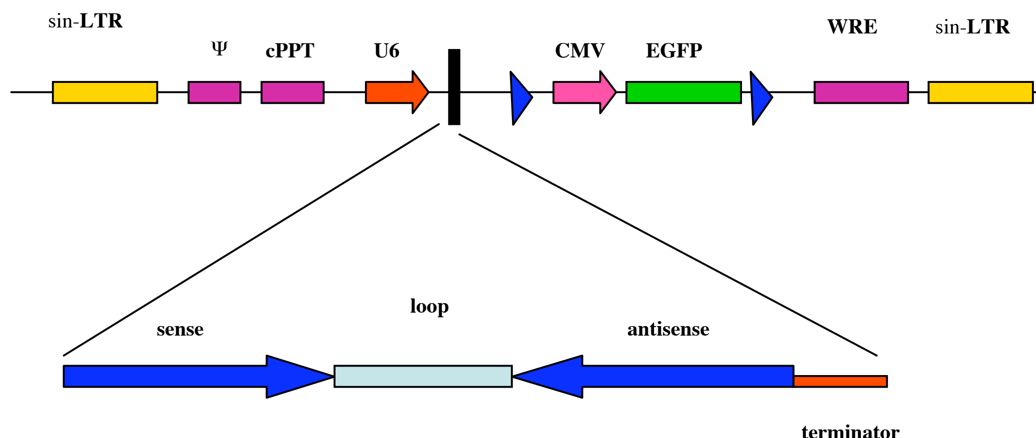

Figure 16: The LentiLox 3.7 vector system

The LentiLox 3.7 virus contains a CMV-driven EGFP reporter gene and an U6-based shRNA expression cassette.

The pLL 3.7 vector also contains a CMV-driven EGFP reporter gene the expression of which should allow to follow transgene transmission in mice. The U6-shRNA cassette was excised from the pU6-shRNA expression plasmids and cloned into the pLL 3.7 lentiviral vector.

To compare the efficiency of the knockdown in mice with the cellular assays *in vitro*, all five U6-shRNA expression cassettes were introduced into the pLentiLox 3.7 vector. Infectious virus particles were produced in 293HEK cells and concentrated by

ultracentrifugation to approximately 0,5-4 x10^9 infectious units/ml. Approximately 10 to 100pl of the concentrated virus particles were injected into the perivitelline space of mouse embryos at the 1-cell stage. After 24h, embryos were implanted into pseudo-pregnant foster mothers. To estimate efficiency of the lentiviral infection, embryos from the 4-cell stage and developmental day E3.5 were collected and analysed by confocal microscopy. The results showed mosaic expression of the reporter EGFP gene (**Figure 17**).

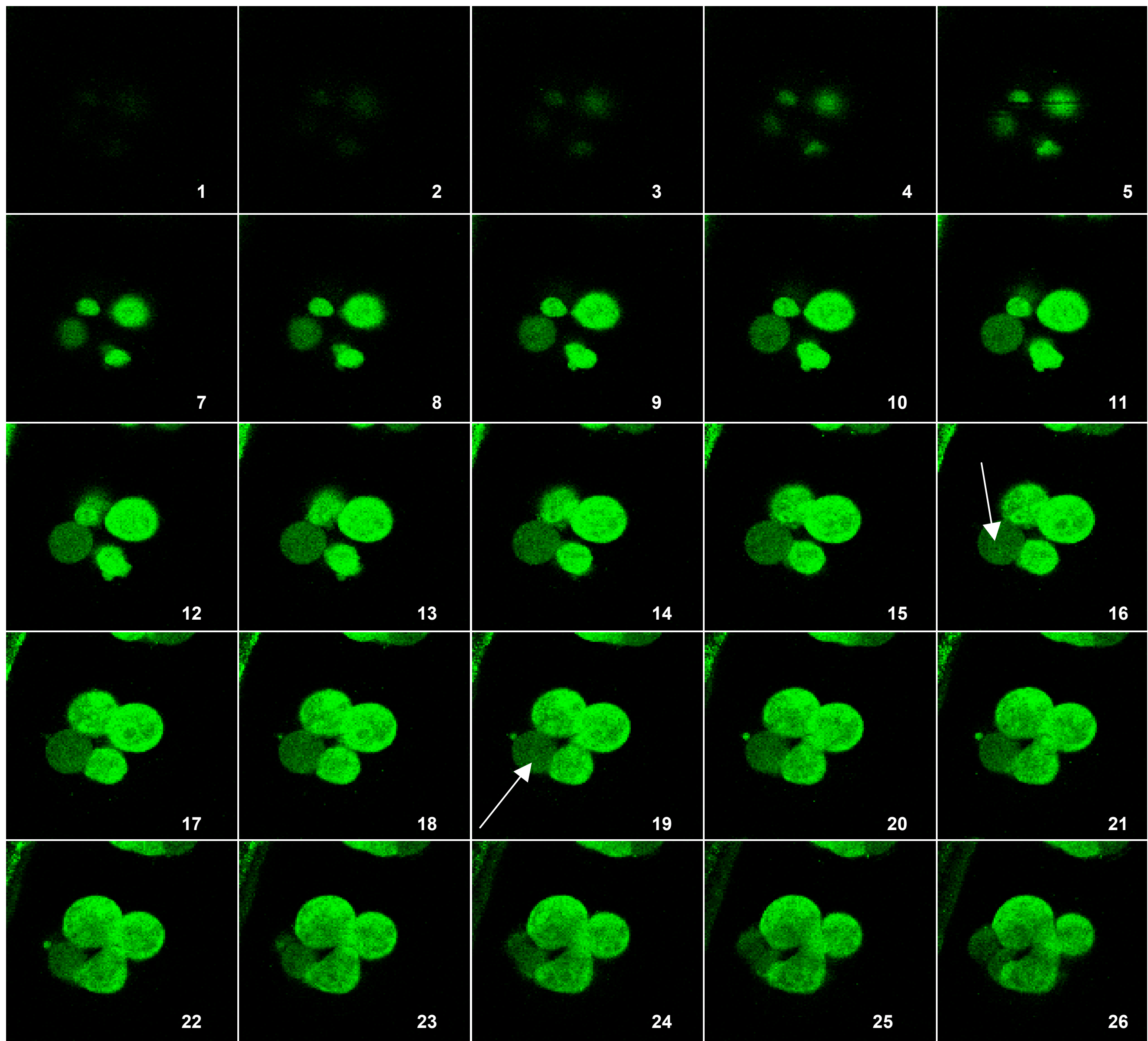

Figure 17. Differential expression of the reporter EGFP gene of the LentiLox 3.7 vector system.

The EGFP differential expression revealed by confocal microscopy from 4-cell embryos: The pictures were taken consecutively beginning from the top (1) and proceeding to the middle of the embryo (26)**.** Note that one of the cells has a weaker EGFP expression.

The mosaic expression of the EGFP gene was even more obvious in the blastocyst stage where the cells from the ICM (inner cell mass) showed different green signals (**Figure 18**).

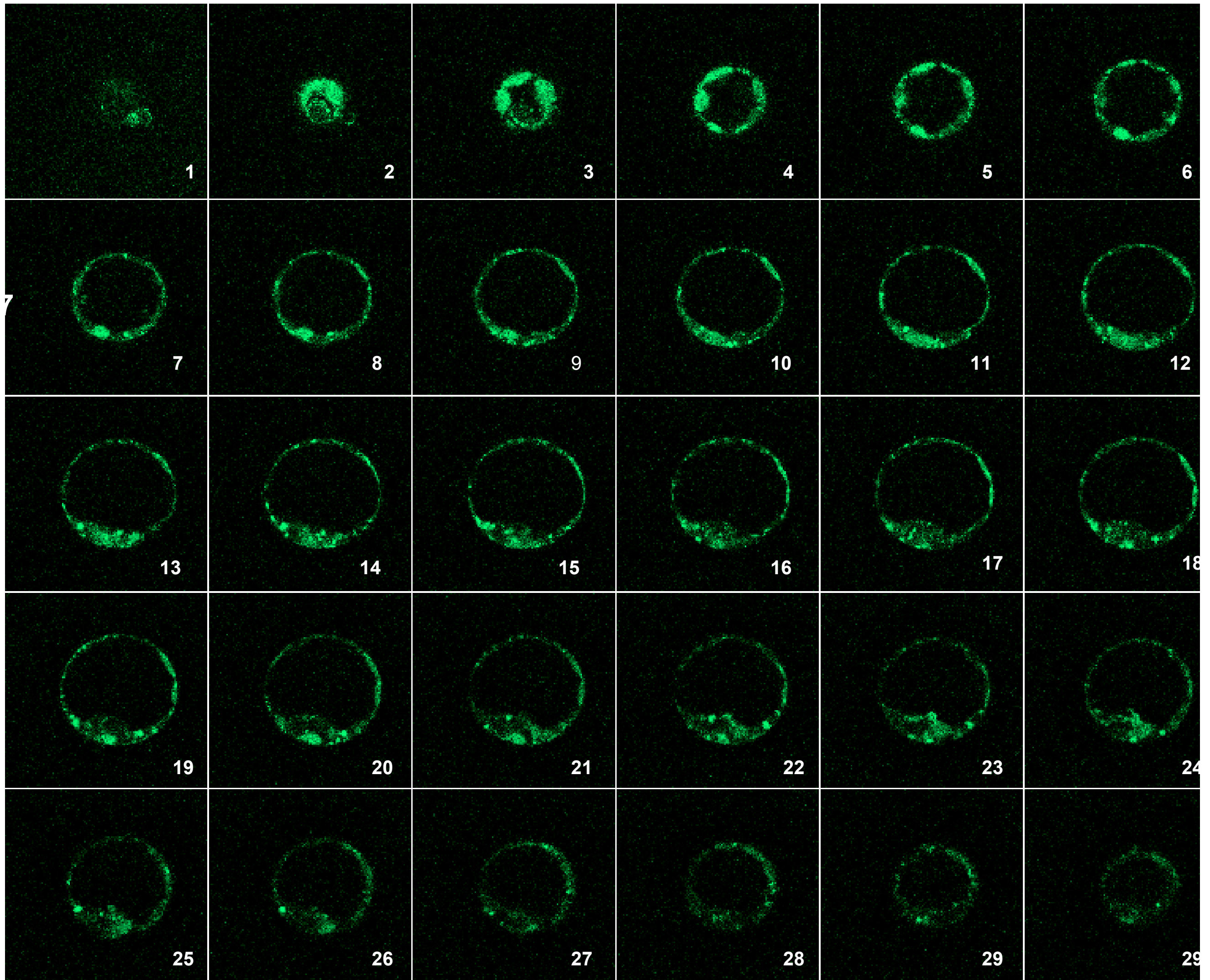

Figure 17. Mosaic expression of the lentiviral reporter EGFP gene in the ICM of early embryos.

The confocal microscopy from embryos E3.5 reveals mosaic expression of the reporter EGFP transgene in the cells from ICM. The pictures were taken consecutively beginning from the top (1) and proceeding to the bottom of the embryo (29).

From 1184 embryos transferred 206 founder mice (F0) were born. 117 F0 mice (56%) were positive for provirus integration as tested by tail PCR (**Table 1),** but only 52 founders showed visible, but mosaic EGFP expression in skin.

Constructs	Zygote transfers	F0 pups born	PCR-positive F0	PCR positiveF0, back crossed	F0 with germ line transmission/F0 giving green F1	F1 pups yielded	PCR-positive F1	Green F1
shRNA-615	156	20	14	11	3/1	125	8	2
shRNA-1320	224	29	24	14	12/0	125	39	0
shRNA-303	150	27	19	1	1/0	18	9	0
shRNA-1301	350	45	27	12	6/2	122	31	7
shRNA-655	304	85	33	14	8/5	163	45	24
total	**1184**	**206**	**117**	**52**	**30/8**	**553**	**132**	**33**

Table 1: Embryo viability, germ line transmission and transgene expression in mice generated by recombinant LL3.7 virus

The table presents the number of zygotes transferred, the yield of PCR positive and PCR negative founders and F1 mice, and the number of EGFP-expressing mice.

3. 3. 1. Copy number and segregation of the transgene

Southern blot analysis of F0 mice demonstrated the presence of multiple independent integration events ranging from 1 to 10 copies. The band intensity, however, varied, pointing to genetic mosaicism (**Figure 19**).

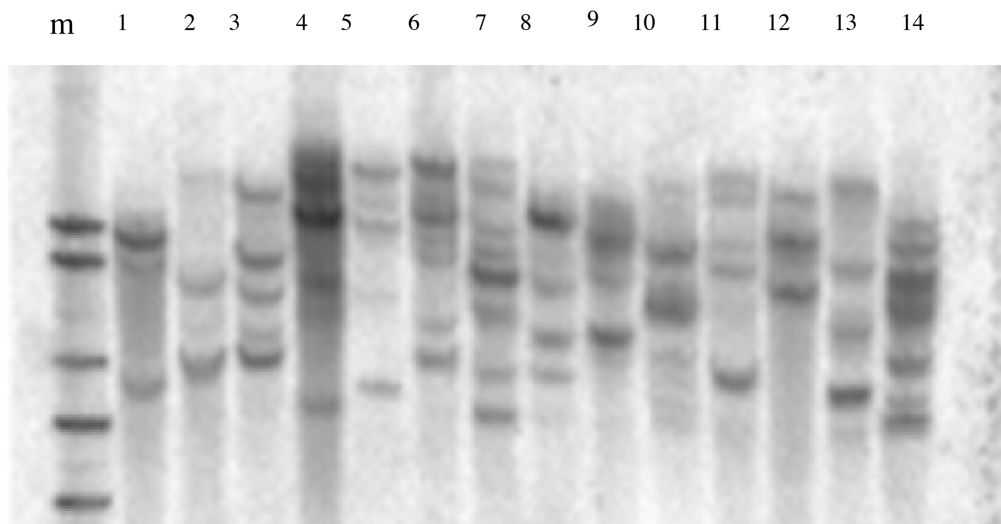

Figure 19: Transgene copy number and mosaicism in F0 mice generated by recombinant LL3.7 virus

Southern blot analysis of tail DNA prepared from founder mice reveals multiple virus integration events and mosaicism.

Mice that showed detectable spots of EGFP expression in skin were mated to C57Bl/6 wild type mice to generate a F1 progeny. 30 out of 52 founders transmitted the transgene as evidenced by PCR, but only 8 of those 30 founders gave rise to EGFP-positive F1 mice **(Table1).** Of 553 F1 mice, 421 were PCR- negative. Only 132 mice were positive by PCR, and of these only 33 mice expressed EGFP **(Table 1).** These EGFP-positive F1 mice had distinct integration sites, as evidenced by Southern blot analysis, and showed different levels of EGFP expression. The bands corresponding to the pro-viral integration sites were characteristic for each founder and segregated among the progeny (**Figure 20**).

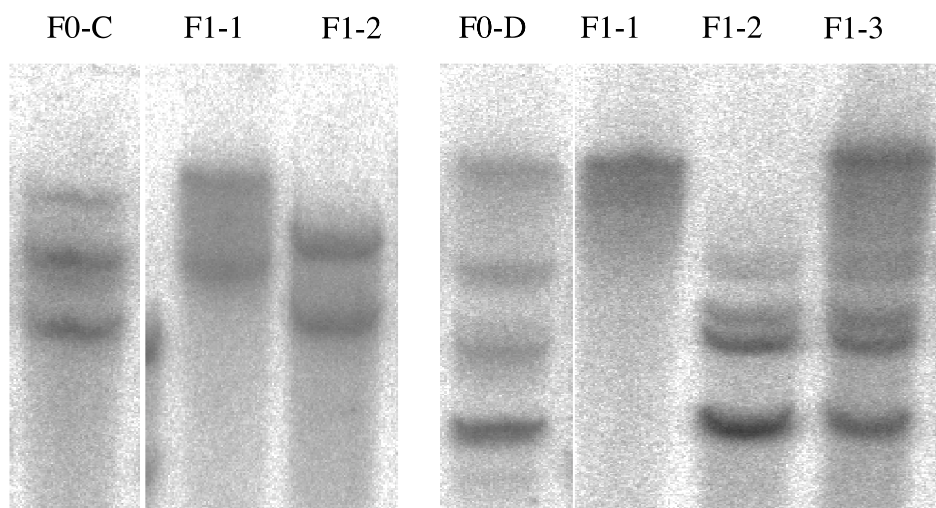

Figure 20: Segregation of the transgene among the F1 progeny
Southern blot analysis of founder C (left) and founder D (right) and transgene segregation in the F1 progeny.

3. 3. 2. Transgene expression in LentiLox 3.7 F1 mice

We next asked whether the EGFP reporter is ubiquitously expressed in the EGFP-positive F1 mice as expected from the properties of the CMV promoter. Therefore I analyzed EGFP fluorescence in F1 mice bred from two different founders (founder A and founder B) by inspection of the skin as well as by analysis of sections prepared from different organs.

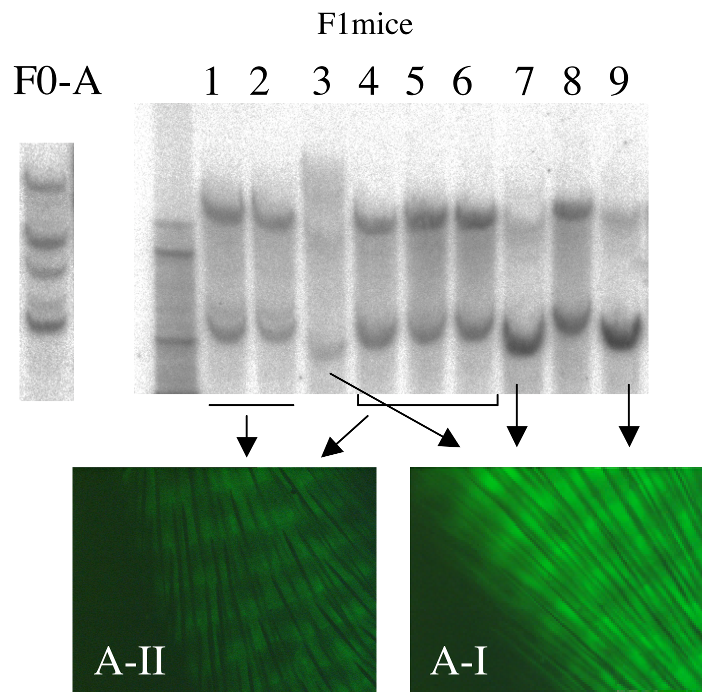

Figure 21: Copy number, segregation and site-dependent expression of the EGFP in transgenic F1 mice derived from founder A

Southern blot analysis of tail DNA prepared from founder A and segregation in F1. The level of EGFP expression was different between different F1 mice, depending of the site of provirus integration.

The founder A gave birth to two kinds of offspring named A-I and A-II, which had distinct provirus integration sites and in which a different extent of EGFP expression as observed by skin fluorescence (**Figure 21**). Some of the insertions found in tail DNA of the founders were not transmitted through the germ line. The A-I mice showed ubiquitous EGFP fluorescence (but with a weak signal in liver and spleen), but expression was variable with the strongest signals in skin, pancreas, intestines, muscle, kidney and heart (**Figure 22**).

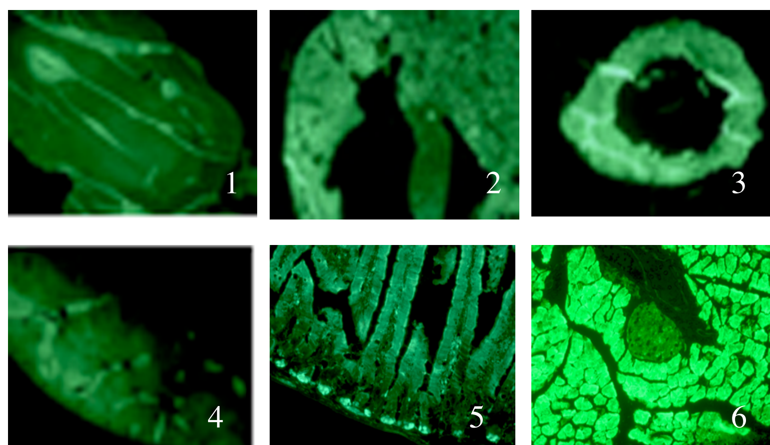

Figure 22: Tissue sections of organs from the A-I mouse

EGFP visualization of organ sections from F1 A-I mice; (1) brain, (2) kidney, (3) heart, (4) liver, (5) intestine, (6) pancreas.

In F1 A-II mice, EGFP was predominantly expressed in pancreas, heart, liver, kidney, but in general the EGFP signal was weaker (except the liver) than in A-I animals (**Figure 23**).

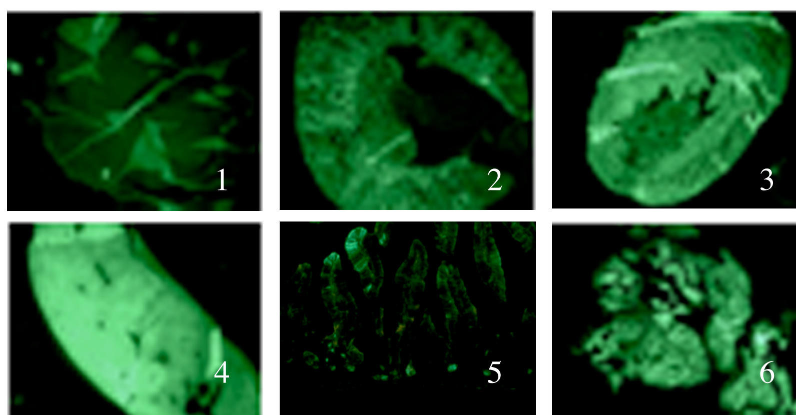

Figure 23: Tissue sections of organs from the A-II mouse

EGFP signal in organ sections from F1 A-II mice; (1) brain, (2) kidney, (3) heart, (4) liver, (5) intestines, (6) pancreas.

The founder B generated two different F1 mice (B-I and B-II), but in contrast to F1-A mice only B-I showed EGFP fluorescence in the skin (**Figure 24**).

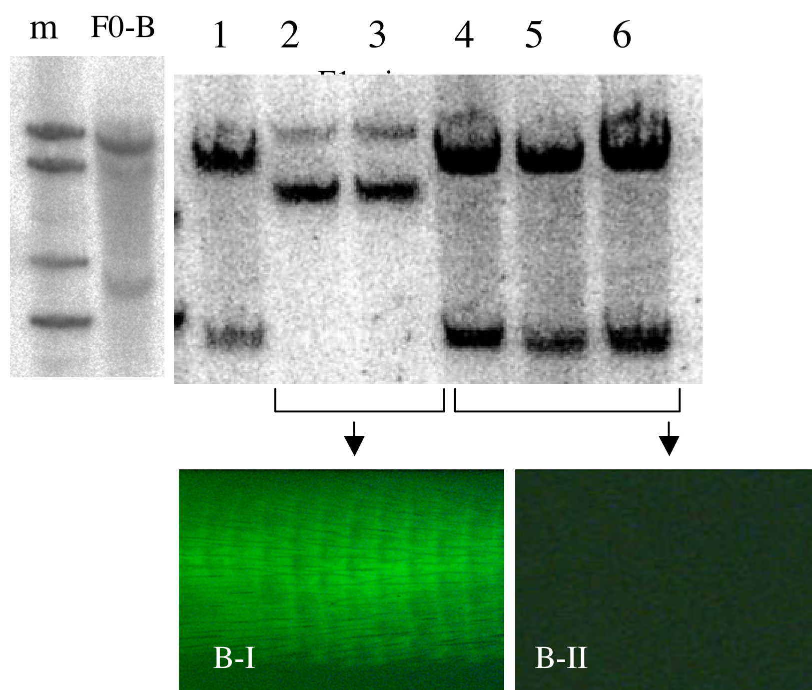

Figure 24: Copy number, segregation and integration site-dependent expression of the EGFP transgene in F1 mice derived from founder B

Southern blot analysis of tail DNA prepared from founder B and segregation of the transgene among the progeny. The EGFP expression is apparent only in B-I mice.

In the analysis of F1 B-I mice an EGFP signal was observed only in pancreas and in some cells of the base of intestinal crypts (**Figure 25**).

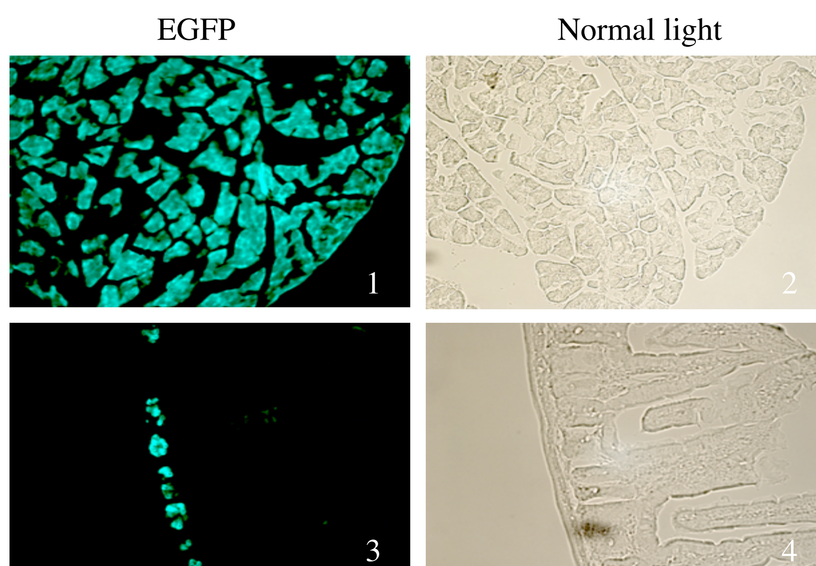

Figure 25: Sections of pancreas (1-2) and intestines (3-4) from B-I mice.

To answer the question whether EGFP expression correlated with the amount of shRNA transcribed from the U6 expression cassette, I performed Northern blot hybridization of total RNA isolated from intestine, pancreas (mice B-I and B-II) and from intestine, pancreas, liver, heart, kidney and brain (mice A-I and A-II) with ^{32}P-end labelled sense oligonucleotide probes.

Northern blot of F1-B mice showed the presence of mature siRNA in pancreas and small intestine with the highest levels in pancreas. Mice positive for virus integration, but without an EGFP signal (B-II), never showed detectable levels of siRNA transcribed from the transgenic U6 promoter although the signal from the endogenous U6 gene is clearly detectable (**Figure 26**).

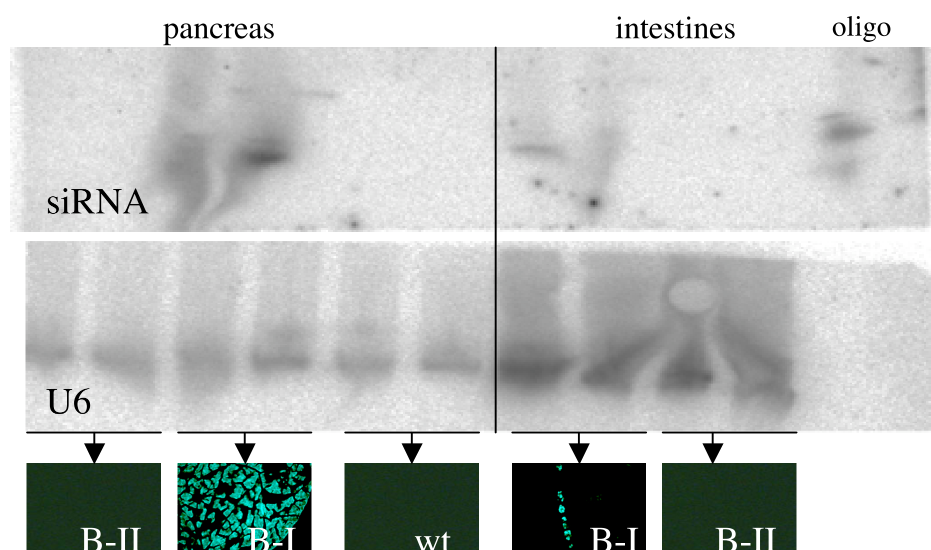

Figure 26: Correlation between EGFP and siRNA expression in lentivirus-positive F1-B mice

Northern blot hybridization for monitoring of siRNA expression with total RNA isolated from pancreas and intestine of F1-B mice (B-I, B-II and wild type control). The blot was re-hybridized with a ^{32}P-labelled U6 RNA oligonucleotide probe.

F1 mice (A-I and A-II) bred from founder A expressed mature siRNA. Mice A-I had a stronger siRNA signal in heart, intestine, brain, kidney and pancreas compared to A-II and undetectable siRNA in liver, correlating with EGFP expression as shown in **Figure 22.** Again, the signal of transgenic siRNA was weaker in comparison to the endogenous U6 gene (**Figure 27**).

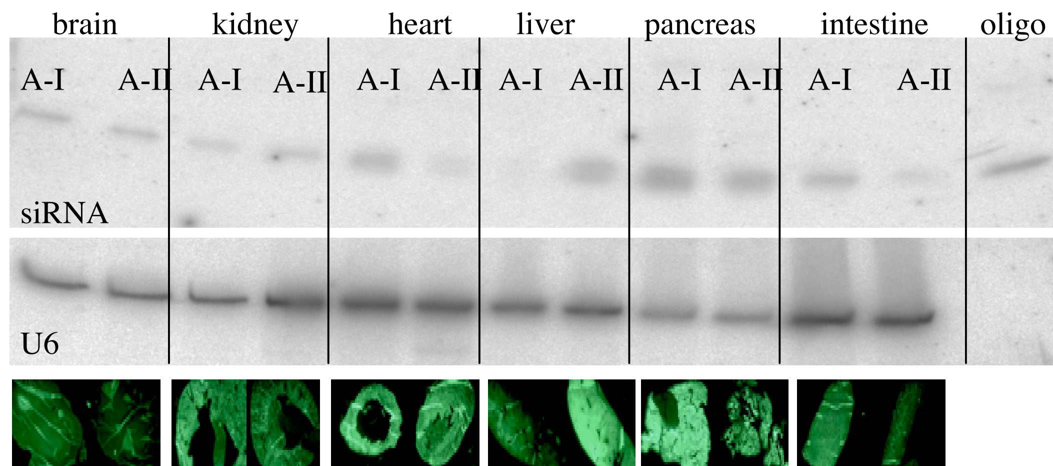

Figure 27: Correlation between EGFP and siRNA expression in lentivirus positive F1-A mice

Northern blot hybridization for monitoring of siRNA expression using total RNA isolated from different organs of mice A-I and A-II. The blot was re-hybridized with a ^{32}P-labelled U6 RNA oligonucleotide probe.

3. 3. 3. Knockdown of HNF4γ by lentiviral siRNA

As shown above, HNF4γ is expressed predominantly in the differentiated, absorptive brush border cells of the small intestine (enterocytes) and to a lower degree in the islets of Langerhans of the pancreas (**Figure 11**). To address the question whether HNF4γ is knocked-down in mice expressing siRNA directed against HNF4γ (A-I, A-II, B-I) the level of its messenger RNA was measured by qPCR in the two target tissues of transgenic mice and its wild type littermates. The F1 A-I, A-II and B-I mice (expressing shRNA-1301 and shRNA-655) showed a knock down of HNF4γ by 80% in pancreas and of about 50% in intestine (**Figure 27**).

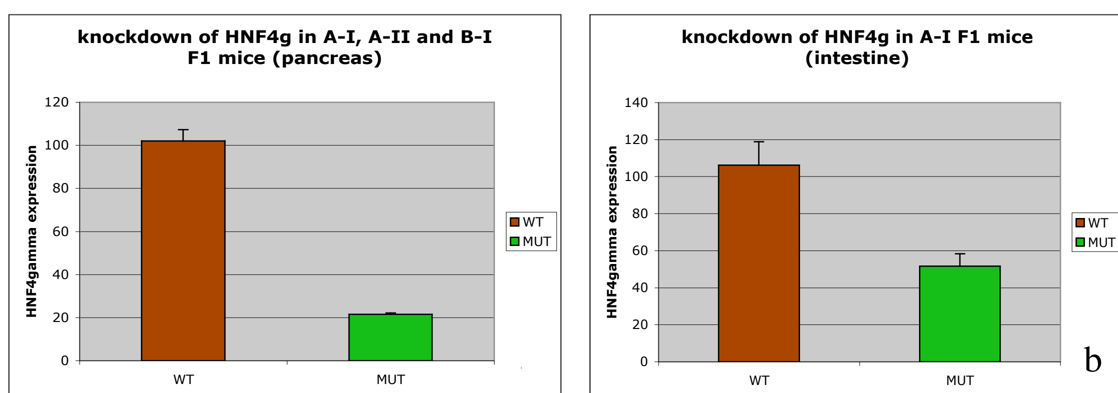

Figure 28: In vivo knockdown of HNF4γ gene expression

Knockdown measured by quantitative real-time PCR; (a) pancreas (A-I, A-II and B-I F1 mice) (b) intestine of A-I mice.

Only in the intestine of A-I mice a knockdown of about 50% was detectable **(Figure. 29),** but not in A-II and B-I animals.

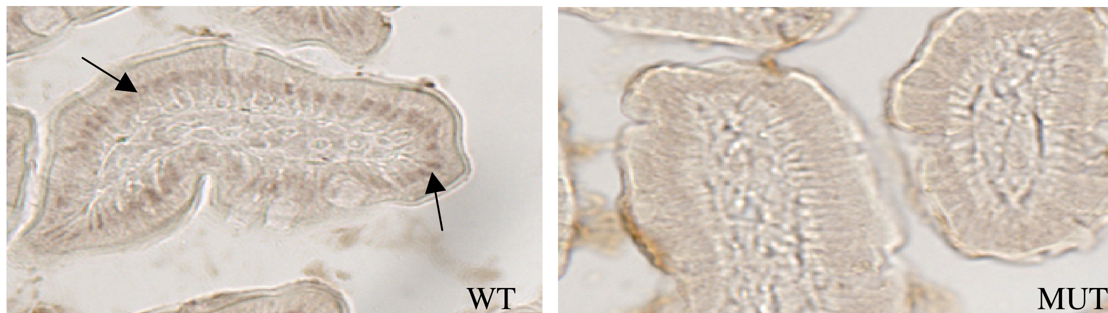

Figure 29: A knockdown of HNF4γ in the intestines of A-I mice

Immunohistochemical analysis demonstrated a reduced level of HNF4γ in the intestine of A-I mice.

The physiological role of HNF4γ is unknown. HNF4γ is highly related to nuclear receptor HNF4α, abundantly expressed in liver, kidney, intestine and to a lower degree in the islets of Langerhans in pancreas. A recent analysis of mice with a β cell-specific inactivation of HNF4α showed impaired glucose homeostasis in these animals [133].

To test the possibility that HNF4γ is also involved in regulation of glucose metabolism I performed a glucose tolerance test using A-I mice, which had an 80% HNF4γ knockdown in pancreas and wild type littermates as control (**Figure 30**).

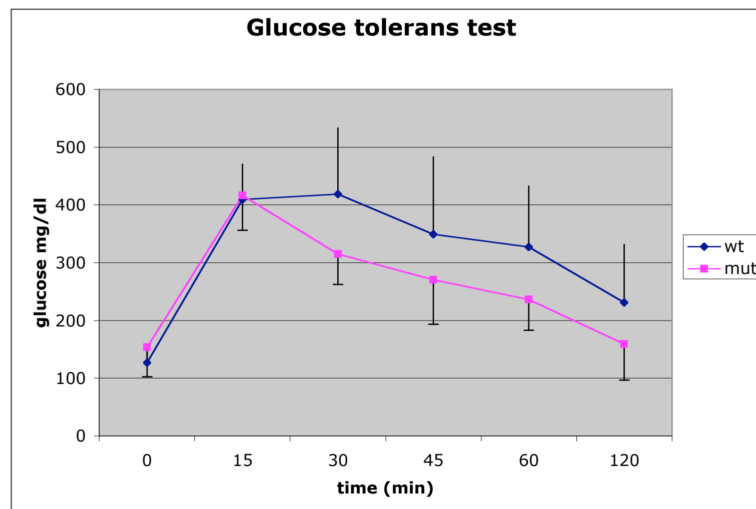

Figure 30: Glucose tolerance test using HNF4γ knockdown and wild type control mice

Since no significant difference in the glucose tolerance test was seen between HNF4γ knockdown and wild type mice, we have so far no evidence that HNF4γ is involved in glucose homeostasis.

4. Discussion

A new methodology for generation of transgenic mice using lentivirus-based vectors was employed to produce a knockdown of HNF4γ gene.
I have analyzed the expression pattern of HNF4γ gene, the transgenesis by the lentiviral vector LL 3.7 that expresses EGFP under control of the CMV promoter as reporter and different shRNA sequences directed against HNF4γ under control of the U6 promoter, the rate of germ line transmission, and transgene expression in founder and F1 mice.

4.1. Tissue distribution and expression of the HNF4γ gene

Since HNF4 γ is a recently discovered member of the nuclear receptor family I first wished to determine its tissue distribution. My analysis demonstrated that expression of HNF4γ is restricted to intestine and pancreas. Traces of mRNA were found also in liver and kidney at very low levels. Interestingly, in intestine HNF4γ showed a highly selective pattern of expression as it was detected only in the nuclei of brush-border cells (enterocytes). These cells are the most abundant, but short-lived cell population of intestine villi. After they exit from the crypt and migrate toward the tip of the villus they initiate a genetic program, which allows absorption of nutrients and water. A comparison with the closely related transcription factor HNF4α in intestine showed that this gene is expressed in both crypt and villus cells. Recently it was demonstrated that both nuclear receptors can bind the same hormone responsive elements on apoA-I/C-III/A-IV gene cluster [10] suggesting overlapping functions. Since, HNF4γ protein is found exclusively in the villi this suggested that HNF4γ and HNF4α might have separate functions. Mobility shift assays with nuclear extracts prepared from mouse intestinal villi identified HNF4γ as a nuclear receptor which is capable of binding to a new HRE found in the distal promoter of apoA-IV, thereby restricting the expression of apoA-IV gene only to enterocytes [10]. It is possible that the spatial expression of many gut-specific genes may be controlled by the HNF4γ/HNF4α ratio within crypt-to-villus axis *in vivo*. HNF4γ protein is already detectable in the embryo at day E16.5 suggesting for a potential role of this nuclear receptor in intestine development from the embryonic endoderm. Immunohistochemical analysis performed on pancreatic tissue demonstrated expression of HNF4γ protein in the cells of Langerhans islands.

Since there was no described knockout of HNF4γ, the knockdown approach to investigate gene function in vivo by using lentiviral transgenesis was considered as alternative possibility. The generation of knockout mice is time consuming and requires intensive breedings. Therefore there was the hope that lentiviral based transgenesis would allow the production of a sufficient number of highly transgenic founder mice. As the siRNA technology is a new tool it brings some uncertainty about the efficiency of the DNA constructs designed for the HNF4γ knockdown. It was therefore desirable to select shRNA sequences *in vitro* to reduce the number of mice to be generated.

4. 2. Selection of effective shRNA oligonucleotides against HNF4γ

For knockdown experiments *in vivo* it is highly desirable to first identify siRNA and shRNA sequences that are most effective in suppressing target gene expression. In principal, several siRNA/shRNA sequences can be chosen to attenuate expression of a given target gene. On the basis of the relatively small number of target genes that were successfully silenced, a set of empirical guidelines have been proposed for the design of siRNA [75] (**Figure 5**). One of them includes low stability of the 5´antisense terminus that promotes incorporation of the antisense strand (AS) into the RISC complex. A high stability of the 5´ RNA sense strand is required to prevent sense strand incorporation. Low stability of siRNA in the middle might facilitate the cleavage of passenger strand and to increase the processing rate of the RISC machinery [72]. Consistent with these criteria several softwares were designed in a way to predict the knockdown efficiency of candidate siRNA oligonucleotides [134-136]. The more advanced of them have an additional capacity to measure the thermodynamic stability around the target site as well as secondary structure of mRNA. Recent data on mRNA target accessibility demonstrated that the relative instability of mRNA around the siRNA target site represented as loops and mismatches was beneficial in achieving successful knockdown. Nevertheless, such software could be useful in the design of siRNA sequences that can be tested in cell culture experiments, but not with full confidence for shRNA, preferable species for stable knockdown *in vivo*. ShRNAs are more complex structures that resemble endogenous miRNA harbouring loop and stem sequences. Both miRNA and shRNA are transcribed by either RNA polymerase II (Pol II) or by Pol III, processed by Drosha/Pasha heterodimer and then transported to the cytoplasm by Exportin-5. In the cytoplasma shRNAs and miRNAs additionally are cleaved by the Dicer enzyme to generate

a mature siRNA which is incorporated into the RISC complex and guide the degradation of target mRNA (**Figure 31**).

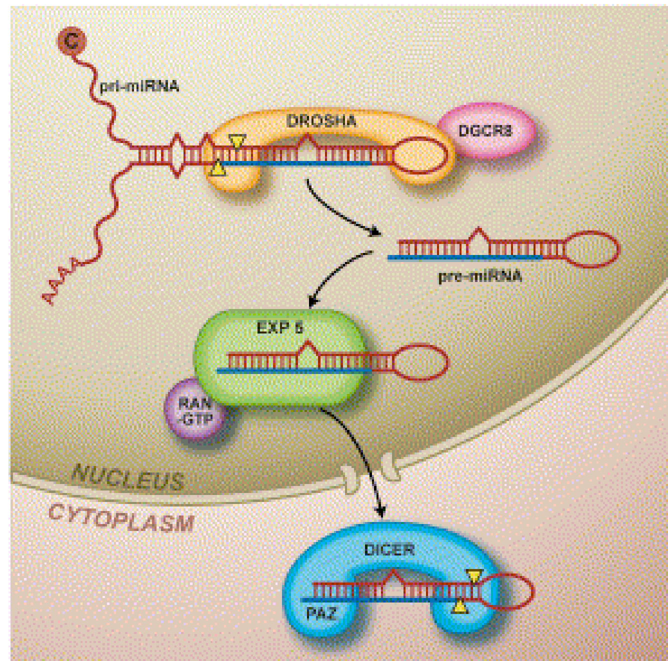

Figure 31: Key steps in miRNA biogenesis

The pri-miRNA are transcribed by RNA polymerase II and processed by the Drosha/Pasha (DGCR8) heterodimer to pre-miRNA. Then pre-miRNAs are transported to the cytoplasm where they undergo additional cleavage by the Dicer enzyme leading to mature miRNA. The shRNA are transcripts which resemble pre-miRNA in structure and biogenesis, but are expression products of exogenous DNA constructs bearing either Pol II or Pol III promoter. (Modified from B. Cullen).

Because of this structural complexity of shRNA and its downstream processing steps the use of computer programs for predicting to which extent a given shRNA will be efficient in achieving desirable knockdown is quite challenging. A preliminary experimental test for the efficiency of shRNA including panel of different shRNA is therefore highly desirable. For my experiments, I used the commercially available GeneEraser Luciferase Suppression-Test Sytem (Stratagene). In this system, the target DNA sequence is cloned within the 3´UTR of luciferase gene. By co-transfection of this construct with constructs expressing shRNA molecules it should be possible to select functional shRNA sequences. Efficiency was determined in a titration experiment. By a careful design including varying amounts of the plasmids it is possible to keep different vector-to-vector molar ratios. Moreover, if the

molar concentration for all tested pU6-shRNA constructs is the same, the state of the balance between shRNA and mRNA expression will be dependent only on the shRNA efficiency. A second advantage of the experimental test over the computer program is that available computer softwares do not consider changes in the shRNA such as mismatches, whereas the impact of this kind of modification on shRNA efficiency can be directly measured experimentally. For example shRNA-615 has a total score of 9, the estimation done by the Sirna program predicting either an inactive or at least a less active oligonucleotide. However, the substitution U to C at position 18 possibly avoided the stop signal for Pol III and improved the knockdown efficiency of the hairpin. In the case of shRNA-303, the performance shown by this oligonucleotide in cell culture experiment correlated well with predictions of the program. This molecule was selected as a negative/inefficient hairpin by the Sirna program and was used as reliability control in the test assay. The other shRNAs against HNF4γ achieved a nice knockdown in the test experiment and this result was consisted with the Sirna score prediction. One disadvantage of the test luciferase assay is that it is performed by co-transfection of several plasmids in cells (293T) that naturally do not express the HNF4γ target gene. This situation implicates an uncertainty about the knockdown efficiency in the mouse. A solution for such an obstacle will be that pre-selection of shRNAs either is done in a cell line expressing the endogenous target (if such a line is available) or is done using a construct allowing more cell-type independent shRNA-based knockdown. Unfortunately, I could not evaluate the shRNA potency *in vivo* and compare with the results obtained in the *in vitro* cell culture, because the number of mice expressing the shRNA was too low. It also turned out that siRNA expression was also dependent on the integration site, a confounding complication that was not considered when the work was started.

4. 3. Generation of transgenic mice using the LentiLox 3.7 lentiviral system

4. 3. 1 Analysis of F0 mice

To generate transgenic mice, LentiLox 3.7 lentiviral high titer preparations of 0,5-4 x 10^9 infection units/ml were microinjected within the perivitelline space of the zygote resulting in 56% of F0 mice carrying the transgene as determined by PCR. This number is rather high

in comparison to DNA incorporation after plasmid nuclear injection (4-5%). One possible reason is that the delivery of the transgenes by LL 3.7 lentivirus is less invasive resulting to better survival of the embryos. The LL 3.7 is a self-inactivating, replication-defective, third generation of lentiviruses which is pseudotyped with the G-protein of the vesiculostomatitis virus (VSV-G) [101]. This pseudotyping allows the virus to infect a broad range of cell types from diverse species. There is no need of mechanical delivery by needle of the provirus particles through the cell and nuclear membranes and thus increasing the chance that more embryos will not be injured and will continue to develop further in the next stage. Southern blot analyses of tail DNA from F0 mice showed 1 to 10 individual integration events and this number most likely is dependent on the virus titer applied. The variable intensity of some Southern bands indicated that F0 mice were genetically mosaic as it was already seen by EGFP expression in the skin in living animals or in other organs after dissection (data not shown). Only 52 founders of 117 (44%) showed a spotted EGFP expression on the skin (data not shown). An explanation for the high degree of mosaicism could be that integration of the virus into the host genome may take place only after the zygote has completed several rounds of divisions, thereby only a subset of the embryonic cells become transgenic [120] (**Figure 32**). This delay in lentivirus genome integration could be based on the nature of the lentiviruses themselves. These viruses have RNA as a molecule bearing the whole genetic information. Once the virus has entered the cell, its RNA is used as a template to generate complementary DNA chain (cDNA) which then is converted to double-strand DNA. The double-strand DNA forms a pre-integration complex together with the protein products of *gag*, *vpr* and *pol* genes, and this complex moves within the nucleus of the host cell. The virus enzyme integrase catalyzes integration of the invading virus DNA in the genome (provirus) that later serves as a template for progeny production (virions). The DNA integration also is the basis for transgene transmission to the progeny after germ cell infection.

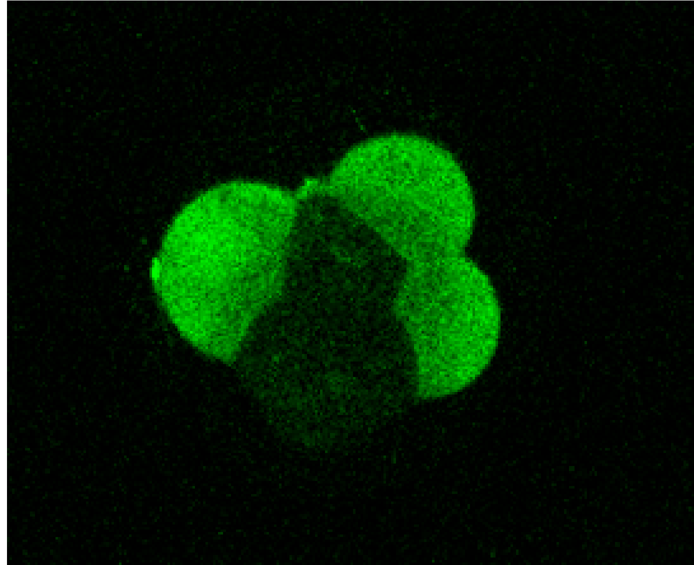

Figure 32: Mosaic expression of EGFP transgene in 8-cell stage mouse embryo

Confocal microscope picture revealed mosaicism in early embryo infected by LentiLox 3.7 virus. The infection possibly takes place after zygote makes several rounds of division.

Another reason for the mosaicism observed in F0 mice may be transgene silencing during embryo development. This argument comes from the fact that the majority of the F0 mice were positive for the lentiviral integration, but never expressed EGFP transgene. The transgenesis by lentiviruses was shown to escape epigenetic DNA modification in contrast to transgenesis mediated by retroviruses [110]. Recent data showed a high preference of murine leukaemia virus (MLV) and HIV-based vectors for integration into genes expressed actively[137, 138]. Our observations on embryo development *in vitro* demonstrated that indeed in most embryos at the morula stage EGFP was expressed at high level indicating lentivirus integration and expression, whereas at the more advanced blastocyste stage the EGFP expression either disappeared completely or became mosaic with respect of localization as well as of strength of expression. Specifically, only few cells within the inner cell mass still showed EGFP expression (**Figure 17**). This switch off of the EGFP reporter might coincide with the switch off of the early embryonic genes where the virus was integrated in. Because of the localization of the transgene within these genes, EGFP reporter and shRNA expression are strongly influenced by the silencing mechanism at that particular chromosomal place.

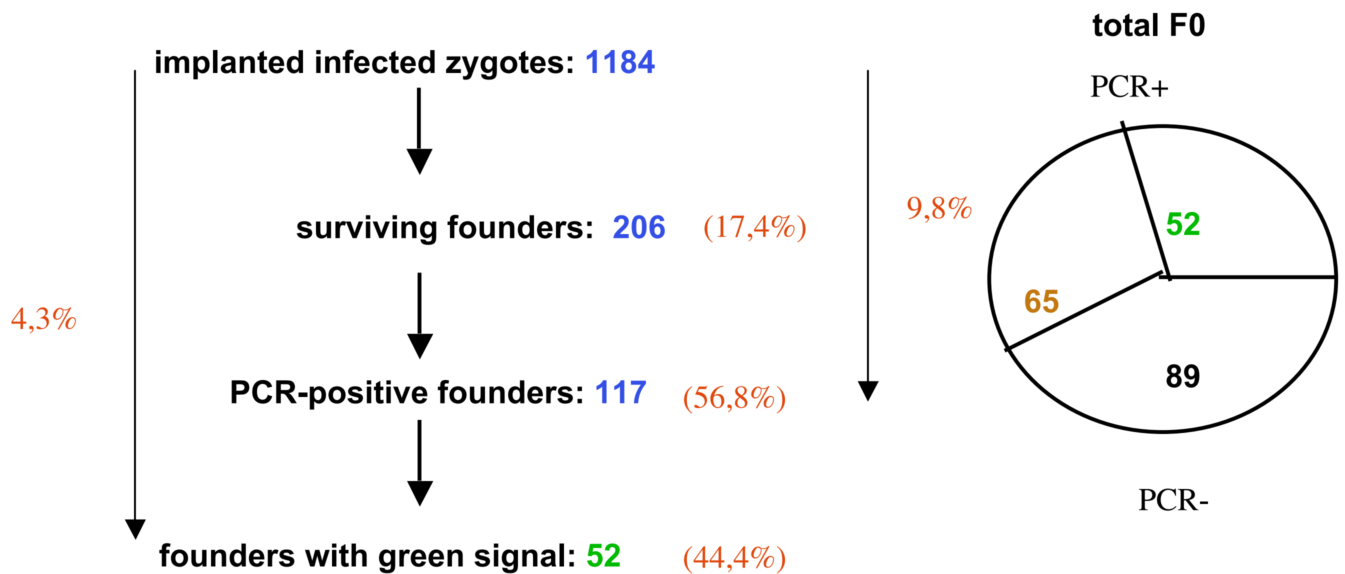

Figure 33: Efficiency of the infection by LentiLox 3.7 virus

The figure shows the total number of infected embryos, percentage of surviving founder mice and percentage of PCR+ founder mice.

The high degree of mosaicism seen in the founder mice (F0) was a disappointing observation, since we wished to analyze these animals without the need to breed further to produce the next generation.

In order to decrease the genetic mosaicism of F0 mice and make them useful for *in vivo* experiments a change in the protocol was tried. The zygotes were denuded by removal of the zona pelucida of the egg that could serve as a physical barrier to the lentivirus. The denuded embryos were incubated in high titer preparations of LentiLox in the hope that the opened surface of the mouse egg leads to uniform exposure of the embryonal cells and increased numbers of lentiviruses bound to the cell membrane, and consequently higher uptake. Our experience showed that denudation did not harm viability of the embryos and that they continue to develop normally (data not shown). However, this technique did not contribute to a decrease of mosaicism (data not shown). A possible explanation could be that irrespective of the higher chance to increase the virus number within the egg, all of the viruses will need again the same time for the formation of the pre-integration complex as well as for the DNA integration. As an alternative, a method that could delay the embryo development for example by reducing the incubation temperature may help lentivirus biogenesis. A new lentiviral vector that will carry a selectable marker gene will allow the

embryo to develop under a selective pressure. In these conditions only those embryonic cells will survive that express the selective marker in parallel with shRNA cassette. In an ideal case these cells will proliferate further contributing to the whole embryo with reduced expression of the target gene.

4. 3. 2 Analysis of F1 mice

The 52 EGFP-positive F0 mice were further mated with C57Bl/6 mice to determine the extent of germ line transmission. From 48 crosses, 553 F1 mice were generated (**Figure 34**). 30 mice F0 transmitted the transgene in the germ line. A minor fraction of F1 mice were transgenic with 2 or more integrations (132/24%) and showed transgene segregation as found by Southern blot (**Figures 20, 21 and 24**). This low degree of germ line transmission is correlated with the high degree of mosaicism seen in the founder mice.

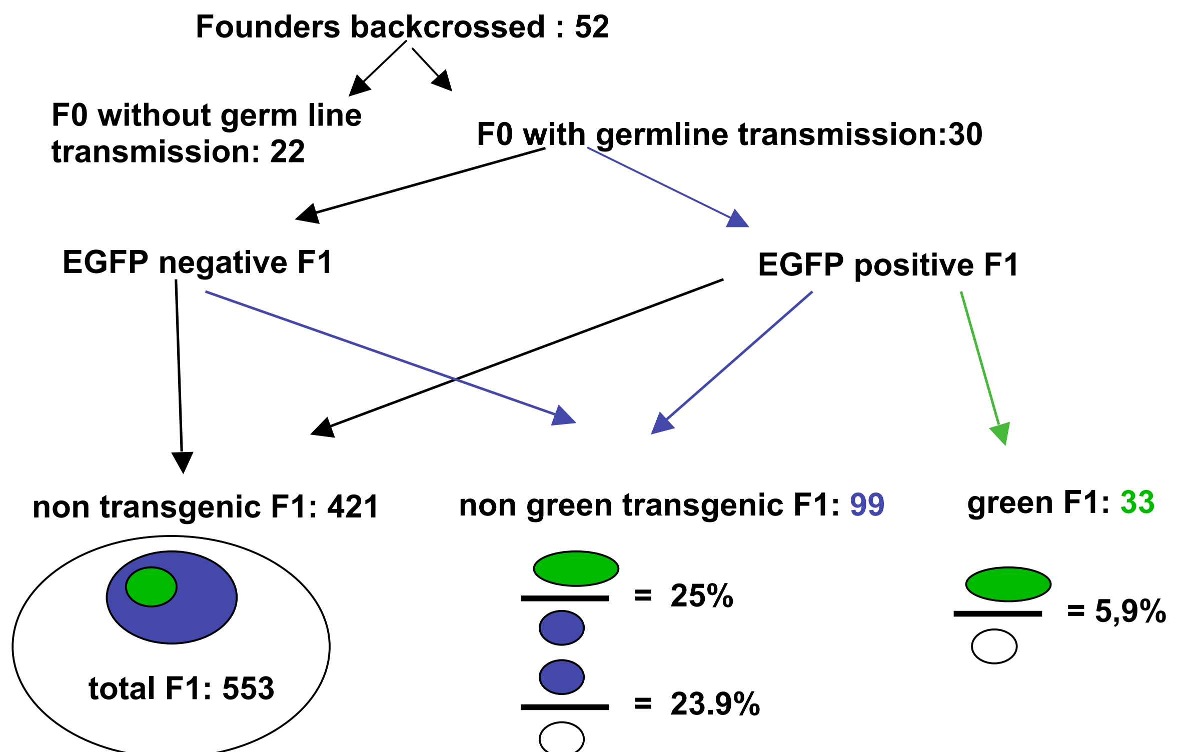

Figure 34: Low rate of germ line transmission due to mosaicism of F0 mice

The figure shows the number of F0 mice backcrossed, with and without germ line transmission, and the frequency of generated F1 animals.

An additional unexpected result was that only 25% (33) of the PCR-positive F1 mice expressed EGFP, and even in EGFP-positive F1 mice EGFP expression was not ubiquitous, indicating for epigenetic effects (silencing) during development, provirus integration into heterochromatin or into genes with low expression.
However, it was found that EGFP (CMV-dependent) and siRNA (Pol III-dependent) expression was highly correlated. Both promoters used are known to be active in all or almost all cells. The strong EGFP expression correlated with a similarly strong siRNA expression and vice versa (**Figure 26 and 27**). The expression pattern of both was quantitatively variable in the different F1 lines analyzed and in some lines tissue- and cell type-dependent. For example, F1 mice from the B-I line expressed EGFP in crypt cells of the intestine (**Figure 25**) and as was expected never showed knockdown of HNF4γ gene which expression is characteristic for enterocytes localized in the villi. A-I mice showed ubiquitous EGFP and siRNA expression and HNF4γ in both pancreas and intestine was attenuated. Interestingly, the expression of siRNA in all F1 mice was weaker than the expression of endogenous small U6 gene as evidenced by Northern blot (**Figure 26 and 27**). Since, both endogenous U6 and shRNA are driven by the same PolIII/U6 promoter one should expect the same or at least a similar level of expression. It is possible that lentivirus integration took place at a chromosomal site that does not fully support the expression of shRNA, because of either the lack of important enhancers or presence of silencing DNA elements. With the exception of the sequences coding for different shRNA, the integrated vector was identical allowing the conclusion that transgene expression (siRNA and EGFP) is most likely dependent on the integration site. If this finding can be extended to other lentiviral vector systems it would strongly limit the use of lentiviral vectors as gene shuttles.
As an experimental alternative, transgenic knockdown mice might be produced by using the BAC (bacterial artificial chromosome) technology. BACs are big DNA molecules that contain certain genes together with all their regulatory elements (promoter and enhancers). Therefore, by placing a shRNA cassette within the open reading frame of a given gene it would be possible to achieve a tissue and cell type knockdown effect. Since the BACs are huge molecules they can also allow site-independent expression of siRNA.
Recently it was also demonstrated that introduction of a single copy of shRNA cassette into appropriate genomic locus by homologous recombination in ES cells can lead to strong decrease in expression of the target gene [139].

4. 4. *In vivo* knockdown of HNF4γ gene expression

Among 10 green F1 mice analyzed a knockdown of HNF4γ levels in pancreas and intestine were found. The knockdown in intestine was 50%, which may lead to the lack of phenotype. The knockdown in pancreas was more pronounced (80%). Giving the fact that HNF4γ is highly related to HNF4α we asked for a role of HNF4γ in regulation of glucose metabolism. However, the results obtained from glucose tolerance tests showed no significant difference between the knockdown and control mice. A possible explanation could be that the reduced protein level of HNF4γ in pancreas is compensated by the induction of some alternative biochemical pathways regulated by other transcription factors from the same group.

4. 5. Conclusions

The use of the LentiLox 3.7 lentiviral delivery system [123] for the production of HNF4γ knockdown mice resulted in founder mice that were genetically mosaic and therefore not suitable for analysis without further breeding. The high degree of mosaicism was reflected in a low rate of germ line transmission. The number of transgene-positive F1 mice expressing EGFP and siRNA was unexpectedly low (6% of the total F1 number) despite of several provirus integrations. The low number of F1 expressing EGFP and shRNA is most likely due to epigenetic silencing during embryo development as well as integration site-dependent silencing, arguing that the lentiviral vector system used given its low efficiency is not suitable to replace conventional transgenesis by microinjection of DNA into the pro-nucleus.

5. Materials and Methods

5. 1. Equipment

PCR thermocyclers (MJ Research), LightCycler (Roche), heating blocks (Eppendorf), centrifuges and microcentrifuges (Heraeus, Sorvall, Megafuge, Beckman), fluorescent microscope (Nikon), digital cameras (Sony, Visitron Systems), binoculars (Nikon, Leica), cell counter (CASY1, Schärfe System), hybridization oven (Stuart Scientific), scintillation counter (Beckman), thermostat incubators (Labotect, Mytrom, Aqualytic), spectrophotometer (Beckman), luminometer (Luminoskan Ascent, Labsystems), power supplies (BioRad, Consort), gel UV photo documentation system (Biostar), electroporator (BioRad), waterbaths (GFL, Grant), UV crosslinker (Stratagene), micropipettes (Gilson), multi-channel pipettes, vortexes, agarose gel and PAGE minigel chambers, microwave oven, shakers, rotators, homogenizers.

5. 2. Materials

Reagents and consumables were bought from Stratagene, Bio-Rad, Boehringer Mannheim, Eppendorf, Falcon, Fluka, Invitrogen, Amersham, Sigma, Roche, Roth, Whatmann and others:

Agar; Agarose; Ampicillin; Blocking Reagent; Swine serum; Chloroform;
Diethylpyrocarbonate (DEPC); Dithiothreitol (DTT); dNTPs; EDTA; EGTA; Ethanol;
Ethidiumbromide; Formamide; G50 Microcolums; Glycerol; HEPES;
Hydrochloric acid; Hydrogen peroxide; Isoamyl alcohol; Isopropanol; Kanamicin; Luciferin; Lysolecithin; Magnesium chloride; Methanol; Mineral oil; Nitrocellulose membrane; Nonidet P-40 (NP-40); Paraformaldehyde (PFA); Phenol; Potassium chloride; Proteinase K; SDS; Sodium acetate; Sodium chloride; Sodium hydroxide; Spermidine trihydrochloride; Spermine tetrahydrochloride; ß-Mercaptoethanol (ß-ME); Triton X-100; TRIZOL Reagent; Sucrose; Trypsin; Tween-20; X-ray film; Yeast tRNA

5. 3. Enzymes

Enzymes were bought from:

Restriction enzymes	Roche Molecular Biochemicals, Mannheim (D)
	New England Biolabs, Schwalbach (D)
	Promega, Mannheim (D)
Klenow- Fragment	Roche Molecular Biochemicals, Mannheim (D)
Alkaline Phosphatase	Roche Molecular Biochemicals, Mannheim (D)
T4-DNA-Ligase	Roche Molecular Biochemicals, Mannheim (D)
T4-Polynucleotide kinase	Roche Molecular Biochemicals, Mannheim (D)
Pfu-DNA-Polymerase	Promega, Mannheim (D)
RNAse A	Qiagen, Hilden (D)
Proteinase K	Roth, Karlsruhe (D)

5. 4. Radioactivity

(α-^{32}P)dCTP (3000 Ci/mmol, 10mCi/ml), Fa. Amersham

(γ-^{32}P)ATP (3000 Ci/mmol, 10mCi/ml), Fa, Amersham

5. 5. Special kits

QIAprep spin mini-prep kit (Qiagen)

QIAGEN Plasmid Midi, Maxi

QIAex gel extraction kit (Qiagen)

QIAquick PCR purification kit (Qiagen)

Rneasy^{R} Mini Kit (50) (Qiagen)

SuperScript^{TM} First-Strand Synthesis System (Invitrogen)

Mm00660262_g1 (Hmbs) TaqMan Gene Expression Assay (Applied Biosystems)

Mm00443563_m1 (HNF4γ) TaqMan Gene Expression Assay (Applied Biosystems)

Platinum Quantitative PCR SuperMix-UDG (Invitrogen)

GeneEraser^{TM} Luciferase Suppression-Test System (Stratagene)

Dual Luciferase Reporter Assay kit (Promega)

VECTASTAIN ABC peroxidase system (Vector laboratories)

5. 6. Plasmids

pBluescriptII SK (Stratagene)

phRL-TK (Promega)

pTarget-luc (Stratagene)

pU6-shRNA plasmids (pBluescriptII SK based)

pd2EGFP (Clontech)

LentiLox 3.7 (pLL3.7) (Van Parijs Laboratory)

5. 7. Competent E. coli strains

XL-1 Blue

DH 10β (Invitrogen)

TOP 10 (Invitrogen)

XL10-Gold : Deficient in all known restriction systems. The strain is endonuclease-deficient (endA), and recombination deficient (recA). The Hte phenotype increases the transformation efficiency.

5. 8. Mouse strains

-C57BL/6 mice were obtained from CR Wiga, Germany

5. 9. Buffers and solutions

5. 9. 1. Isolation and storage of DNA

TE-buffer pH 8 (for plasmid-DNA)

10mM Tris/HCL pH 8.0
1mM EDTA, pH 8.0

TE-buffer pH 7.4 (for genomic DNA)

10mMTris/HCL pH 7.4
1mM EDTA, pH 8.0

5. 9. 2. Buffers for the alkaline lyses of bacteria and plasmid preparation

Buffer P1 (Resuspention buffer)	100 µg/ml RNAse A
	50 mM Tris/HCL pH 8.0

	10mM EDTA pH 8.0
Buffer P2 (Lysis buffer)	200 mM NaOH 1% SDS
Buffer P3	3 M Potassiumacetate pH 5.5

5. 9. 3. Buffers for genomic DNA preparations

NID Buffer	KCl 50mM Tris Hcl pH 8.3 10 mM $MgCl_2$ 2mM Gelatine 0,1 mg/mL NP 40 0,45% Tween 20 0,45%
Tail Buffer	Tris HCl 50 mM EDTA 100 mM NaCl 100 mM SDS 20%

5. 9. 4. Buffers for DNA electrophoresis

50x Tris-Acetate Buffer (TAE)	2M Tris 250 mM Na-Acetat 50 mM EDTA pH 8
6x Probe Buffer	0,25% Bromphenol blue 0,25% Xylene cyanol FF 15% Ficoll 400 in H_2O

DNA Ladder

Smart Ladder (Stratagene)	5µl per line

5. 9. 5. Medium for culturing of bacteria

LB (Luria/Bertani)	10g/l Trypton 5g/l Yeast extract 5g/l NaCl pH 7.0 with NaOH autoclaved

5. 9. 6. Medium for culturing of 293T cells

DMEM	10%FCS 1xGln (1:100 dilution) 1x HEPES (1:100 dilution)

5. 10. Standard Molecular Biological Techniques

5. 10. 1. Plasmid Mini-prep DNA preparation

1) Grow the bacteria in 2 ml LB medium overnight.
2) Pellet the bacteria at 5000 rpm, remove the supernatant and dissolve the bacteria in 250μl buffer P1.
3) Add 250μl of buffer P2 and mix by inversion and incubation for 5 minutes at room temperature.
4) Add 250μl buffer P3, mix and incubate on ice for 5 minutes.
5) Clear the supernatant by two rounds of centrifugation at 13,000 rpm for 5 minutes in a tabletop centrifuge. Each time transfer the supernatant to a new tube.
6) Precipitate DNA by adding 750μl isopropanol and centrifugation for 10 minutes at 13,000 rpm.
7) Wash the pellet by 70% ethanol, dry it and dissolve it in 50μl TE buffer.

5. 10. 2. Genomic DNA preparation

5. 10. 2. 1. DNA preparation for Southern blot

To achieve good results from Southern blot, at least 10-20 μg DNA are needed.

1) Cut 0,5 cm from the mouse tail and transfer it in Eppendorf tube.
2) Add 700 ml of tail buffer supplemented with 10 μl Proteinase K (10mg/ml).
3) Incubate over night at 56^0 C.
4) Mix gently and add 260 μl saturated 6 M NaCl. Mix on Eppendorf mixer.
5) Centrifuge at 13,000 rpm for 10 minutes.
6) Transfer the supernatant in a new Eppendorf tube and add the same volume phenol:chlorophorm (1:1) and shake gently on Eppendorf mixer for 2-3 minutes.

7) Centrifuge at 13,000 rpm for 10 minutes.
8) Transfer the supernatant in a new Eppendorf tube and add 0,7 volumes isopropanol. Mix the solution gently.
9) Centrifuge at 13,000 for 10 minutes.
10) Wash the DNA pellet with 500µl 70% ethanol.
11) Dry the DNA pellet for 5 minutes at room temperature and dissolve it in appropriate volume TE buffer.
12) Measure the concentration of the DNA.

5. 10. 2. 2. DNA preparation for genotyping by PCR

The DNA for PCR was prepared using short protocol with NID buffer.

1) Cut small piece of the mouse tail and transfer it in Eppendorf tube with NID buffer and Proteinase K (2-3µl of 10mg/ml).
2) Incubate the tails over night.
3) Shake solutions for 5 minutes on Eppendorf mixer and incubate them at 95^0 C shortly to inactivate Proteinase K.
4) Use 2µl of the tail solution for PCR.

5. 10. 3. DNA electrophoresis

0,6-2% agarose gels were used for the analysis of DNA fragments of 0,1-7 kb size. The gels were prepared using 1x TAE buffer containing 1µg/ml ethidium bromide; 1x TAE was used as a running buffer. Electrophoresis were performed at constant voltage for 30-60 minutes, and the DNA bands were visualised using UV-Transilluminator.

5. 10. 4. Southern blot

1) After the digest with an appropriate enzyme run the DNA electrophoresis on low voltage for 6 hours at least.
2) Stop the electrophoresis and expose the gel on UV-Transilluminator to confirm nice digestion of the genomic DNA and document the picture.
3) Incubate the gel by shaking in 0,25 N HCl for 10 minutes.
4) Incubate the gel in 0,4 N NaOH two times each for 20 minutes (denaturation).
5) Incubate the gel in 20x SSC for 5 minutes.
6) Build the blot;

Turn the gel up side-down on glass with bridges over 10x SSC buffer . Put the Biodyne B Transfere Membrane (Pall) onto the gel tightly and mound 6 rows of Whatman paper. Add additional paper and press for over night.

7) Dismantle the blot. Wash the membrane with 2x SSC for 15 minutes and crosslink it under UV illumination(1200 mJ/cm^2).
8) Pre-hybridise the membrane with church-Gilbert buffer (0,5M $NaHPO_4$ pH 7.2; 7% SDS; 1mM EDTA pH 8.0) for at least 1 hour at 65^0 C.
9) Incubate the membrane in church buffer with the radioactive probe over night at 65^0 C.
10) Wash the membrane first with Wash buffer I (40mM $NaHPO_4$ pH 7.2; 1mM EDTA pH 8.0; 5% SDS) and then with Wash buffer II (0,5M $NaHPO_4$ pH 7.2; 1mM EDTA pH 8.0; 1% SDS).
11) Dry shortly the membrane between paper lists and put it in cassette with Phospho-imager screen.
12) Incubate for 5-6 hours and document the signal.

5. 10. 5. Random labelling of probes for Southern blot

For the random labelling of the probe a Klenow enzyme was used.

1) Dilute the template DNA 2μl (DNA probe 200ng) in 11μl H_2O.
2) Incubate on 95^0 C for 5 minutes and then transfer the tube immediately on ice for 5 minutes.
3) Add Klenow enzyme (1U/μl)
4) Add (α-^{32}P) dCTP 5μl
5) Add 5μl mix of dNTPs without dCTP (5x OLB mix).
6) Mix gently and incubate for three hours at 37^0 C.
7) Purify the labelled probe using G-50 columns following the manufactures instructions (Amersham).
8) Measure the efficiency of the probe labelling by calculating the ratio CPM probe/CPM total.

5. 10. 6. Preparation of electro-competent E.coli cells

All the steps should be done as cold as possible, always on ice! The glass pipettes should be cooled once or twice by pipetting cold 10% glycerol up and down before pipetting cells.

1) Pick single colony from DH 10β, XL1 blue or TOP 10 E. coli bacterial cells and grow them in 50 ml LB medium without antibiotic.
2) Transfer 2 ml culture in new 50 ml LB medium and grow bacteria for additional 3 hours.
3) When the cells reach log phase (OD600= 0,4-0,5) pour into 50 ml falcon tubes and cool down on ice for 15 minutes.
4) Cool down the rotor for 10 minutes at 0^0 C by centrifuging at 4,000 rpm.
5) Spin the cells for 10 minutes at 4,000 rpm at 0^0 C.
6) Aspirate the supernatant and immediately put on ice, re-suspend cells in 50 ml of ice cold 10% glycerol. Repeat washing 2 times with 10% glycerol.
7) Pour away the supernatant and dry the tube on ice with Kleenex tissue.
8) Re-suspend the cells in the remained liquid (around 250μl).
9) Transfer 50μl of the cells into each pre-cooled Eppendorf tube and freeze in liquid N_2 or use directly for electroporation.

5. 10. 7. Immunohistochemistry protocol (cryo sections)

1) Sacrifice the mouse.
2) Take out the tissue of interest and cut it to small pieces (intestine).
3) Put the pieces intestine immediately in 50ml Falcon tube with 4% PFA/PBS.
4) Rotate the Falcon tube with the intestine for12 hours at 4^0 C.
5) Wash 1 time in PBS to remove remained drops of PFA.
6) Incubate the tissue in the same tube with 30% sucrose for 12 hours at 4^0 C.
7) Wash ones with PBS.
8) Embed the tissue in Tissue -tec and freeze it on dry ice.
9) Make the 5-7 microns thick sections on cryostat.
10) Store the sections at – 20^0 C or proceed according to the protocol.
11) Post-fix the sections into 4%PFA/PBS for 10 minutes on ice.
12) Wash 3 times for 5min each in PBS.
13) Cook the sections in citrate buffer (10x stock solution)

    2 minutes maximum power

    10 minutes 180 W
14) Cool down at RT for 30 min.

15) Wash with PBS for 5 minutes.
16) Incubate the sections in 1%H_2O_2 for 15-30 minutes (8,3ml 30% H_2O_2 + 125ml PBS,125ml MeOH).
17) Wash three times in PBS.
18) Surround the sections by Pad-pen.
19) Drop 40-50µl 5% NSS.
20) Incubate 30 min under humid conditions.
21) Aspirate the NSS and incubate with the first Ab in proper dilution (For HNF4g 1:500).
22) Leave the sections under humid conditions at 4^0 C for 12 hours.
23) Wash 3 times in PBS.
24) Drop the secondary Ab for 30 minutes (1:500 for HNF4γ and 1:900 for HNF4α dilution in PBS).
25) Wash 3 times in PBS.
26) Incubate with ABC (peroxidase) for 30 min.
27) Wash 3 times in PBS.
28) Perform staining reaction for several minutes.
29) Cover the sections and look them under a microscope.

VECTASTAIN ABC peroxidase system (Vector laboratories) 100µL A + 100µL B into 10ml PBS

Staining: Tris-Trizma; 0,74g/100ml H_2O; 40mg 3,3´ -diaminobenzidine (SIGMA) (DAB); 25µl 30%H_2O_2

5. 10. 8. Restriction analyses

To set up restriction reaction use 1U restriction enzyme per 1µg DNA.

Reagents	Amount
Restriction buffer (10x)	5µl
Rstriction enzyme 1U	1µl
DNA (1µg)	1µl
H_2O	13µl
Incubation time	1 hour at 37^0 C

5. 10. 9. Dephosphorylation of 5´ ends of dsDNA to prevent self-annealing of vector

Reagents	Protruding ends	Blunt ends
DNA	1 pmol	0,2 pmol
10x Dephosphorilation buffer	2 µl	2µl
Shrimp Alkaline Phosphatase	1U	1U
H_2O	Add up to 20µl	Add up to 20µl
Incubation	1 hour at 37 C	2 hours at 37 C

To stop, the reaction can be incubated for 15 minutes at 65^0 C.

5. 10. 10. Ligation of DNA fragments

Sticky ends:

If vector DNA and insert DNA are similar in length, a molar ratio of 1:3 (vector versus insert DNA) is recommended.

If vector and insert DNA are not similar in length, a molar ratio of 1:1 or 1:2 (vector versus insert DNA) is recommended.

Blunt ends: a molar ratio of vector DNA to insert DNA of 1:5 is recommended.

Reagents	Sticky ends	Blunt ends
DNA	Up to 1µg digested DNA	Up to 1µg digested DNA
10x ligation buffer	3µl	3µl
T4 DNA ligase	1-5 U	1-5 U
H_2O	Add up to 30µl	Add up to 30µl
Incubation	20^0 C for three hours	20^0 C for three hours

5. 10. 11. PCR

Reagents	Amount
10mM PCR Nucleotide Mix (each dNTP 200µM)	1µl
Upstream and downstream primer 0,1-0,6 µM each	1µl
Template DNA 0,1-0,25 µg	variable

H_2O	variable
Volume	25µl

The program for PCR is depends on the running application.

5. 11. Selection of effective shRNA molecules against HNF4γ

The selection of shRNA molecules against HNF4γ is described in section “Results”.

5. 12. Oligonucleotide Design

For the cloning of shRNA against HNF4γ pU6-empty was digested by Hpa I and Xho I restriction enzymes. The oligonucleotide design must incorporate a 5´ T in order to reconstitute the –1 nucleotide of U6.

5. 12. 1. Oligonucleotide format

Sense oligo: 5´ T-(GN18)-(TTCAAGAGA)-(18NC)-TTTTTTC
Antisense oligonucleotide: Complementary of the sense but with additional nucleotides at 5´ end to generate an Xho I overhang.
The loop sequence (TTCAAGAGA) is based upon Brummelkamp et al. [122].
The oligonucleotides were ordered from MWG and were purified.

5. 12. 2. Cloning of the shRNA

5. 12. 2. 1. Annealing Procedure

1) In a 1.5 sterile micro-centrifuge tube, set up the following annealing reaction at room temperature. The final concentration of the oligonucleotide mixture is 50µM.

Reagent	Amount
Top strand DNA oligo (500µM)	2µl
Bottom strand DNA oligo (500µM)	2µl
Annealing buffer: 100mM K-acetate; 30mM HEPES-KOH pH 7.4; 2mM Mg-acetate	16µl

2) Incubate the reaction at 95^0 C for 4 minutes on heated block.
3) Switch off the block and leave the probes to cool down till room temperature. The single-stranded oligonucleotides will anneal during this period.
4) Place the samples in a micro-centrifuge and centrifuge briefly (5 sec.).
5) Remove 1µl of the annealing mixture and dilute the ds oligonucleotides as described in **Diluting the ds oligonucleotides**.
6) Store the remainder of the 50µM ds oligonucleotide mixture at -20^0 C.

5. 12. 2. 2. Diluting of the ds oligonucleotides

To clone ds-oligonucleotides into pU6-empty vector, the ds oligonucleotides must be diluted and phosphorylated. The final concentration of the shRNA after phosphorylation will be 10nM.

1) Dilute the 50µM ds-oligonucleotide mixture (from annealing procedure) 100-fold into Dnase-free water (1µl of 50µM ds-oligonucleotide into 99µl of Dnase/Rnase-free water) to obtain a final concentration of 500nM. Vortex to mix.

5. 12. 2. 3. Phosphorylation of the ds-oligonucleotides

1) Set up the following reaction:

Reagent	Amount
Phosphorylation buffer 10x	5µl
Polynucleotide Kinase (10U/µl)	3µl
10mM ATP	5µl
ds oligo (500nM)	1µl
H_2O	36µl
Total volume	50µ

Once the oligonucleotides are diluted, there should be three stocks of annealed ds-oligonucleotides. Use each stock as follows:

-50µM ds-oligonucleotides (undiluted): Use this stock to prepare new diluted ds-oligonucleotides if existing stocks become denatured or cross-contaminated.

-500nM ds-oligonucleotides (100 fold dilution): Use this stock for gel analysis.

-10nM ds-oligonucleotides (5000-fold dilution): Use this stock for cloning.

5. 12. 2. 4. Checking the integrity of the ds-oligonucleotides

When using the diluted ds-oligonucleotide stock solutions (100 or 5000-fold diluted stocks), thaw the solutions on ice. Do not heat or allow the ds-oligonucleotide solutions to reach greater than room temperature as this causes the ds oligonucleotides to melt. The concentration of the oligos in the diluted solutions is not high enough to permit re-annealing and instead favours the formation of intra-molecular hairpin structures. These hairpins will not clone into pU6-empty vector.

The integrity of the annealed ds-oligonucleotides was checked using agarose gel electrophoresis.

5. 12. 2. 5. Preparation of the pU6-empty vector for ligation

For the cloning of shRNA into pU6-empty, the vector must be opened by Hpa I and Xho I enzymes in two separate restriction reactions. Usually a 10ng opened and dephosphorylated vector for single cloning reaction is enough. For the set up of the restriction reaction look in "Standard Molecular Biology techniques".

5. 12. 2. 5. 1. Digestion of pU6-empty vector by Hpa I and Xho I.

1) Digest 10μg DNA with Hpa I.
2) Incubate the reaction at 37^0 C for over night.
3) Purify the vector after the digest using QIAquick PCR Purification Kit Protocol Elute the Hpa I opened vector with 70μl EB buffer (Qiagen).
4) Set up the second digest by Xho I:
5) Incubate the reaction at 37^0 C for at least 6 hours.
6) Stop the reaction by putting it on the ice and prepare the 0.6-0.7% agarose gel.
7) Load the Xho I restriction reaction on the prepared gel and perform electrophoresis on low voltage (100V-150mA) to purify the opened vector. A small DNA fragment (30nt) will be released.
8) Extract the purified vector from the gel using the QIAquick Gel Extraction Kit Protocol.

9) Measure the concentration of the opened vector and perform analytical gel-electrophoresis.

5. 12. 2. 6. Dephosphorylation of the pU6-empty vector

To avoid vector selfligation a dephosphorylation of the opened pU6-empty vector is recommended. The amount of the vector should be between 0,2 to 1 pmol. For the set up of the dephosphorilation reaction look in “Standard Molecular Biology techniques”.

5. 12. 2. 7. Cloning the shRNA ds-oligonucleotides into pU6-empty vector

For optimal results is recommended an approximately a 10:1 or 15:1 molar ratio of ds-oligonucleotide insert: vector for ligation. The usual efficiency of the cloning should be more than 90%. For the set up of the ligation reaction look in “Standard Molecular Biology techniques”

5. 12. 2. 8. Analysis of the clones after ligation reaction

To check for positive clones an analytical digest by Xba I (upstream of the U6 cassette) and Xho I (downstream of the U6 cassette) should be performed. Digest 1μg plasmid with 1U of each enzyme. For the set up of the restriction reaction look in “Standard Molecular Biology techniques”.

1) Analyse the clones by gel-electrophoresis run on 2% agarose gel.

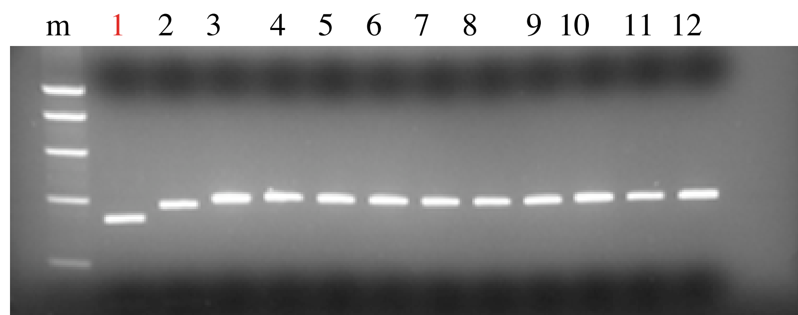

Figure 33: Analytical gel-electrophoresis of clones after cloning of shRNAs into pU6-empty

The positive clones are represented by slightly bigger size of the released fragment: (m) DNA Ladder; (1) negative control without shRNA cloned; (2-12) positive clones of pU6-shRNAs

The positive clones are sequenced by MWG-Comfort read option.

5. 13. The GeneEraser™ Luciferase Suppression-Test System

The HNF4γ coding sequence was cloned into the 3` un-translated region (3`UTR) of the luciferase gene. The 293T cells were transfected by the pTarget-luc-HNF4γ expression vector and the plasmids encoding different shRNA molecules against HNF4γ gene. After two days of culturing the luminescence of luciferase was measured. For better discrimination of the knockdown effect of shRNAs against HNF4γ, a dose-dependence was checked such that the concentration of pTarget-luc-HNFγ was kept constant (5ng/well) and that pU6-shRNA constructs were transfected in variable amounts (100ng/well; 10ng/well; 1ng;well; 100pg/well; 10pg/well; 1pg/well). For negative controls a pU6-empty and non-related pU6-p53 were used. To achieve normalization for transfection efficiency and for total DNA plasmid amount, the cells were also co-transfected with phRL-TK (expresses Renilla luciferase) and pBluescriptII SK (up to 300ng DNA). The experiment was performed in triplicates using 96 well plate format.

5. 13. 1. pTarget-luc Cloning Strategy

The target sequence was generated by digestion of construct containing the HNF4γ cDNA. The product is incubated with the pre-digested pTarget-luc vector, Srf I and T4 DNA ligase. Using the restriction enzyme in the ligation reaction (Srf I) is supposed to maintain a high-steady-state concentration of digested vector DNA. If the target sequence contains Srf I restriction site (5`-GCCCGGGC-3`), it will need to be mutated prior to clone into the pTarget-luc vector.

5. 13. 2. Releasing of HNF4γ cDNA sequence from pBK-CMV-HNF4γ expression vector

The HNF4γ cDNA fragment was released by restriction digest of pBK-CMV-HNF4γ expression vector by EcoR I and Xba I restriction enzymes. For the reaction were used 10µg DNA and 20U from each enzyme. For the set up of the restriction reaction look in "Standard Molecular Biology techniques".

1) Stop the reaction by putting on the ice and load it on 0,6% agarose gel to perform gel purification of the released HNF4γ cDNA sequence.
2) Gel-extraction of the HNF4γ coding sequence (QIAex gel extraction kit (Qiagen)).
3) Measure the concentration of HNF4γ cDNA fragment.

5. 13. 3. Polishing the purified HNF4γ fragment

Since HNF4γ was generated by restriction, which leaves protruded 5´ ends on the fragment, for successful cloning in the blunted pTarget-luc vector these ends have to be polished.

1) Set up the following reaction:

Reagent	Amount
HNF4γ cDNA fragment	10μl
10mM dNTP mix (2,5mM each)	1μl
Polishing buffer 10x	1,3μl
Pfu DNA polymerase (0,5U)	1μl

2) Mix the polishing reaction gently and add a 20μl mineral oil overlay.
3) Incubate the polishing reaction for 30 minutes at 72^0 C in a water bath.
4) Add an aliquot of the polishing HNF4γ fragment directly to the ligation reaction or store at 4^0 C for future use.

5. 13. 4. Cloning of HNF4γ cDNA sequence into pTarget-luc pre-digested vector

For optimal results a correct insert to vector ratio must be considered. The following equation suggested by Stratagene can be used:

X_ng of the fragment=(number of bp fragment)x(10ng of pTar-luc)/5695bp of pTarget cloning vector. For HNF4γ with 2,2kb fragment the optimal amount is 350ng.

1) Set up the following reaction:

Reagents	Amount
pTarget-luc cloning vector (10ng/μl)	1μl
PCR-Script buffer 10x	1μl
rATP 10mM	0,5μl

HNF4γ fragment	4μl
Srf I (5U/μl)	1μl
T4 DNA ligase (4U/μl)	1μl
H_2O	1,5μl

2) Mix the ligation reaction gently and incubate for 1 hour at 25^0 C.
3) Store the ligation reaction on ice until ready to perform the transformation into the XL10-Gold ultra-competent cells.
4) Transform the XL10-Gold ultra-competent cells according the manufacture recommendations.

5. 13. 5. Identifying Transformants Containing the HNF4γ insert

The presence and size of the target gene insert in the pTarget-luc vector may be determined by PCR amplification of DNA from individual colonies. In addition, a second PCR with a 3´ target-gene specific primer will identify colonies containing pTarget-luc vectors with a insert in the required sense orientation. Alternatively, the restriction digestion of isolated plasmid DNA can be done to determine the presence and orientation of the target gene.

5. 13. 5. 1. A PCR strategy

For the PCR strategy, a single colonies after transformation can be picked by a small sterile tip, and grow in 200μl LB media containing kanamicin for 4 hours. Use 2μl from the culture media and set up the PCR reactions (colony PCR).

-Primers for Insert Amplification from the pTarget-luc Vector

Primer	Nucleotide sequence (5´to 3´)
5´ primer	GAAAGGTCTTACCGGAAAACTCGAC
3´ primer	CAACAGATGGCTGGCAACTAGA

1) Set up the following PCR reaction as described in “Standard Molecular Biology techniques”.

2) Gently mix each reaction and overlay with one drop of mineral oil.
3) Perform PCR using the following cycling parameters:

Number of cycles	Temperature	Length of time
1 cycle	95^0 C	1 minute
40 cycles	95^0 C	15 seconds
	55^0 C	15 seconds
	72^0 C	1 minute per kb
1 cycle	72^0 C	5 minutes

4) Analyse the PCR products on 1% agarose gel by electrophoresis. A PCR product of 160 bp is generated from the vector without an insert. The presence and orientation of the target gene insert must be verified by nucleotide sequencing. The insert must be in the sense orientation.

5. 13. 6. Estimation of the efficiency of the different shRNA against HNF4γ

5. 13. 6. 1. Transfection of 293T cells in 96 well plate format

1) The day before transfection, trypsinize and count the cells, plate them 2-6x10^4 cells per well in 100µl of the appropriate complete growth medium without antibiotics.
2) For each well of cells, dilute 300ng of DNA into 25µl medium without serum (Opti-MEM I Medium) in 96-well, sterile micro-titer plates.
3) For each well of cells, dilute 1µl of FuGENE 6 transfection reagent (Roche) into 25µl Opti-MEM I Medium and incubate 5 minutes at room temperature. Once the FuGENE 6 is diluted, combine it with the DNA within 30 minutes.
4) Add 25µl of the diluted FuGENE 6 Reagent (from step 3) to each well containing diluted DNA (from step 2), mix gently, and incubate at room temperature for 20 min to allow DNA-FuGENE 6 Reagent complexes to form. The solution may appear cloudy.
5) Add the DNA-FuGENE 6 Reagent complexes (50µl) directly to each well of the plates containing cells and mix gently.

5. 13. 6. 2. Luciferase activity measurements and data processing

After transfection, the cells are cultured for 48 hours and then are subjected for lyses using the reagents from Promega.

1) Dilute the passive lyses buffer 5 times freshly with distilled water in a container that accommodates a multi-channel pipette.
2) Remove medium from cells and add to each well 50μl of 1x passive lyses buffer, place the culture plate on an orbital shaker and shake for 15-20 minutes at room temperature and then frozen and thawed. 1-2 freeze/thaw cycles are sufficient to increase the total activity in the lysates, because of more complete lyses.
3) Prepare luciferase Assay reagent II (LAR II) and Stop & Glo ragent (S/G) according the product information of the Dual Luciferase Reporter Assay kit from Promega. The LAR II and S/G can be further diluted 5 times without significant effect on the results. Therefore, prepare the working LAR II and S/G buffers by diluting 5x with distilled water in a final volume sufficient for the number of wells to be measured (number of wells x 50μl + 0,5ml. This extra 0,5ml is to load the injector).
4) Transfer 20μl from each cell lysate sample to the measuring plate and load the Fluroscan Ascent FL (Labsystems) with all buffers.
5) Run the measurement and save all data in Microsoft Excel format.
6) Process the data by calculating the rate between firefly (encoded by pTarget-luc-HNF4γ) and Renilla (encoded by phRL-TK) luciferase activites and then the average and standard deviation among the triplicates.

5. 13. 7. Re-cloning of U6-shRNA expression cassettes into pLentiLox 3.7

The tested constructs with U6-shRNA cassettes form a shuttle system that could be easily re-cloned in pLentiLox 3.7 used to generate infectious virus particles.

5. 13. 7. 1. Releasing of U6-shRNA cassette

The U6-shRNA cassette was released by double digestion with Xba I and Xho I restriction enzymes. For the set up of the restriction reaction look in “Standard Molecular Biology techniques”.

1) Incubate the reaction at 37^0 C for three hours.
2) Purify the released U6-shRNA cassette through 0,8% agarose gel-electrophoresis.
3) Extract the U6-shRNA fragment from the gel (QIAquick Gel Extraction Kit) and measure its concentration on UV spectrophotometer.

5. 13. 7. 2. Preparation of pLentiLox 3.7 for cloning of the U6-shRNA cassette

To clone U6-shRNA cassette into pLentiLox vector, the vector must be opened by Xba I - Xho I and dephosphorilated. !0µg DNA was used for the digestion. For the set up of the restriction reaction look in “Standard Molecular Biology techniques”.

1) Incubate the reaction for at least for 6 hours at 37^0 C.
2) Purify the opened vector on 0,7% agarose gel and extract the DNA fragment by (QIAquick Gel Extraction Kit).
3) Measure the concentration of the open pLentiLox 3.7 vector.

5. 13. 7. 3. Dephosphorylation of pLentiLox.3,7

For the reaction a 1pmol DNA was used.. For the set up of the dephosphorilation reaction look in “Standard Molecular Biology techniques”.

5. 13. 7. 4. Cloning of U6-shRNA cassettes into LentiLox 3.7

For optimal cloning the molar ratio (insert:vector) should be 3:1. For the set up of the ligase reaction look in “Standard Molecular Biology techniques”.

1) Incubate the reaction at 20^0 C for three hours and then put it on ice.
2) Transform competent DH10β bacterial cells with 1µl from the ligation reaction.
3) Plate the transformed bacteria on LB agar medium supplemented by Amp and grow over night to get single colonies.

4) Prepare plasmid DNA from single colonies (Mini-prep) and analyse them by analytical digestion with Xba I - Xho I restriction.

5. 14. Packaging of Lentivirus (LentiLox 3.7)

All steps were done in a S2 facility.

5. 14. 1. Plating of 293T cell

1) Cell were cultured in the tissue culture plate (15cm x 20mm) at starting density of 1 million in 20ml.
2) Transfer the 293T cells from plate into flask (175 cm^2) at seeding density about 6-10 millions in 20ml medium the day before transfection.

5. 14. 2. Transfection for package of the virus

1) Change the medium of 293T cells in the flask with 18ml of fresh medium 1 hour before transfection.
2) Mix the plasmids complex with 100µl $CaCl_2$ (2,5M) and ddH_2O up to 1ml.
3) Add 20µl of Chloroquine (10-25µM) to the cells.
4) During the bubbling of the calcium-plasmids complex, a 2x HBSS was drop-wise added into the complex for the formation of homogenous fine precipitation of DNA-calciumphosphate complex. The mixture then was further bubbled for additional 10 sec.
5) Add the complex into the flask and incubate the treated cells over night for transfection.
6) After 16-20 hours, the medium was changed with fresh 10ml to collect the infectious particles. An another batch of 293T cells were plated in 12 well-plate at density of 5 x 10^4/ml with 1 ml in each well for measurement of the virus titer at the next day.
7) After 24 hours the conditioned medium of the cells was transferred into Falcon tubes and centrifuged at 2000 rpm. The supernatant was filtrated through 0,45µm membrane. The remained cells were re-suspended in 5 ml PBS and measured on FACS machine. Usually over 90% of living cells give positive signal.

-Composition of the reagents:

HBSS in use: 100mM HEPES-Na pH 7,11; 280 mM NaCl; 1,5 mM Na_2HPO_4

For 2^0 generation of Lentiviral vector: 40μg vector (pLentiLox 3.7); 26μg Packaging construct (pCMVdeltaR8.91); 14μg ENV plasmid (pMD2.G)

For 3^0 generation of Lentiviral vector: 40μg vector (pLentiLox 3.7); 26μg Packaging construct (pMDLg/pRRE); 14μg ENV plasmid (pMD2.G); 10μg REV plasmid (pRSVrev)

5. 15. Generation of RNAi- HNF4γ transgenic mice

Engineered lentiviral particles are microinjected directly into the perivitelline space of mouse embryos 0.5 days after fertilization. The viral particles are comprised of a self-inactivating viral vector containing a gene of interest and a promoter, as well as a marker gene. The particles also contain reverse transcriptase to catalyze the incorporation of the vector sequence into the genome and the viral particle itself has a glycoprotein coat that mediates its adherence to the embryo. In some percentage of cases, the viral vector incorporates into the genome of the one-celled embryo, carrying the gene of interest with it. Once integrated, the viral sequences cannot be replicated, due to a deletion in the requisite sequence. Embryos are incubated at 37°C overnight. Two-cell embryos are implanted into the oviduct of pseudopregnant female mice the following day. Pups resulting from this procedure are genotyped to test for the presence of the transgene.

-Superovulation and mating of the donor female mice

1) Three days prior to lenti-viral injections, administer 0.1 ml PMS by intraperitoneal injection to female donor mice (C57BL/6 or FVB).

2) Forty-seven hours after the PMS injection, administer 0.1 ml hCG by IP injection and pair the females with stud males.

3) Check for plugs the following morning.

-Medium preparation

1) The afternoon prior to injection day, set up two 60 mm culture dish (Corning catalogue #25382-381) with four 50 μl microdrops of KSOM (Cell and Molecular Technology, catalogue #MR-101-D) covered with oil, and one 35 mm dishes (Corning catalogue # 25382-348) with three 50 μl microdrops covered with oil. Label each dish with your name and the date, then place into the CO2 incubator. The medium is incubated overnight to allow the temperature, and more importantly, the pH to equilibrate.

3) Just prior to egg harvest on the morning of the injections, add 4 ml of FHM (Cell and Molecular Technology catalogue #MR-025-D) to a 15 ml conical tube containing 2.6 mg of hyaluronidase (aliquots stored in the –20 freezer). Invert gently to prevent foam, do not shake.

-Embryo collection

1) On the morning of injection, sacrifice female donors in groups of five or less, using cervical dislocation.
2) Collect oviducts and place them into a drop of FHM/hyaluronidase on the bottom of a 60 mm culture dish.
3) Make another FHM/hyaluronidase drop in the same dish.
4) Using two pairs of forceps, gently tear open only those oviducts that have an obviously swollen ampulla, releasing the egg/cumulus cell bunch into the drop.
5) The remaining oviducts can be flushed using the FHM/hyaluronidase mixture, a 1 cc syringe and a 32 gauge flushing needle.
6) Transfer the embryos to the 60 mm dish of four drops of KOSM, set out the night before. Rinse the embryos through the drops until all debris are removed.
7) On Mondays, set aside five embryos as controls. They should remain unmanipulated in the incubator to ensure that the media, incubator etc. are adequate. In most cases, all five embryos should develop into blastocysts by Friday. Results are recorded in a log.

-Viral loading

1) Viral aliquots provided by the investigator are kept at –80 C. Take one aliquot of 5-10 ?l and thaw within a 50 ml conical tube filled with ice. Keep the conical tube in an ice bucket. Any direct contact with the virus should be done under the hood while double gloved.
2) If dilution is necessary, this should be performed in the hood. The viral titer should be 5 $x10^8$ to 5×10^9. If high titer prep is too viscous, it may be diluted 1:1 or 1:2 with ice-cold PBS.
3) Complete a project record sheet, filling in the viral vector, the gene construct, the date of injection, the date of preparation, the viral preparer, the injector, and the project number and replicate number.

4) In the hood, back-load the injection needle using a long, sterile micropipette tip. Attach the needle to the needle-holder and, with the needle-holder in the hood, press the flush button on the injector until the media fills the tip of the needle.
5) Attach the needle-holder with the loaded needle to the micromanipulator and then exterior gloves prior to continuing with the injection procedure.

-Setting up the microscope

1) Turn on the nitrogen tank
2) Turn on the microinjector (Green switch)
3) Place a set of eggs into a drop of oil-covered FHM in the cooled (4C) injection dish (~30)
Note: Time of egg exposure to FHM (including harvest time) should be no more than one hour.
4) Place the injection dish in the center of the microscope stage.
5) Focus the microscope at the lowest power on the embryos.
6) Place a holding pipette onto the opposite side of the scope, (e.g., if you are right handed, the holding pipette should be on the left and the injection needle on the right).
7) Lower both pipettes using the course controls into the injection dish.
8) Maneuver needle and holding pipette so that they are parallel to each other and visible at the lowest magnification.

-Injections

1) Use the holding pipette to stabilize eggs for injection.
2) Focus on the zona pellucida at the highest power, and inject into the perivitelline space with adequate volume to see an obvious swelling of the perivitelline space.
3) After ~30 eggs have been injected, place eggs into the 35 mm dish with three drops of warmed KSOM: Use the first two drops for rinsing to remove residual injection media.
4) Incubate all injected eggs overnight.
5) Unused virus, the injection needles, and the injection dishes should be discarded in biohazardous waste. The needle holders, the injection dish tray, and all pipetmen used should be placed in the hood under UV light.

-Oviduct implant surgeries

1) The next morning count the number of one cell and two cell embryos. The ratio should be 80/20, two cell to one cell; an indication that the virus was at an optimal concentration. If the ratio is lower than 80:20, then the virus should be diluted further at the next injection.

2) Transfer the two-cell embryos bilaterally into the oviducts of a 0.5 day pregnant recipient (See SOP for oviduct embryo transfer).

3) A maximum of 26-30 two cells should be placed into one recipient.

References; [109, 123]

-Oviduct Transfer Protocol

Purpose: To transfer mouse 2-8 cell embryos into 0.5 day pseudopregnant recipient females for any of several procedures; embryo transfer rederivation, subsequent to in vitro fertilization, and following pronuclear injections and cryopreservation recovery.

1) Pipette three microdrops (~50 μl) of FHM or M2 media on the bottom of a 30 mm petri dish.

2) Cover the media with embryo-tested mineral oil and leave at room temperature.

3) After weighing a recipient female, inject the calculated dose of Avertin intraperitoneally.

4) The caudal dorsal area of the anesthetized female is shaved, then scrubbed using povidone iodine solution or scrub alternately with 70% alcohol on cotton tipped swabs (repeat two times).

5) Place the prepared pseudopregnant female under the dissecting microscope, ventral side down.

6) Surgical instruments should be either autoclaved or bead-sterilized. Using a small scissors and watchmaker forceps, make a 5 mm skin incision. Separate the skin from the body wall by blunt dissection using scissors tips. Make an incision through the body wall avoiding nerves and large blood vessels. Manipulate the incision until the white fat pad surrounding the ovaries is visible. Grasp the fat pad with a dull forceps and pull it through the incision.

7) Position the ovary for easy access to the oviduct.

8) Pipette enough embryos for one recipient into a microdrop of FHM.

9) Load a transfer pipette (the tip diameter should be only slightly larger than the embryos) with oil from transfer dish by using mouth suction. Oil level should be close to the

beginning of the widest part of the pipette. Next, draw a 2-5 mm air bubble into the pipette, then media with embryos. Carefully press pipette barrel into the clay on the dissecting microscope until ready to transfer the embryos.

10) Gently tear open the bursa surrounding the oviduct with two pair of forceps allowing access to the infundibulum, which is the opening to the oviduct. In case of bleeding, use a sterile cotton swab to gently blot.

11) Once the infundibulum can be visualized, slide the tip of the loaded pipette in and blow into the pipette until the air bubble is visible within the oviduct.

12) Check the pipette tip underneath the microscope to make sure the embryos have been transferred.

13) Gently replace the ovary/fat pad and uterus into the abdominal cavity.

14) Close the body wall with one or two simple interrupted sutures of 5.0 silk. The skin is apposed with one or two sterile surgical clips.

15) The transfer can be done either uni- or bi-laterally. The pups are expected on day 20 following surgery.

5. 15. 1. Genotyping of the transgenic mice generated by LentiLox 3.7 lentivirus

The genotyping of LentiLox generated mice was done by multiplex PCR using gene-specific primers for EGFP and appropriate primers giving amplification of the genomic DNA fragment (encompassing exon 1 from HNF4γ gene) for ensuring the correct PCR conditions. In addition some of the mice could be genotyped by observing EGFP fluorescence in the skin.

-Primers used for genotyping of LentiLox-HNF4γ mice

Primers	Nucleotide sequence (5´- 3´)
EGFP_N3 (f)	TAAACGGCCACAAGTTCAGC
EGFP_N3 ®	CGGCCATGATATAGACGTTG
519_F	TGTGGTGACAGAGCAACAGG
520_R	GACCCAGGATGTATGGACCTT

-PCR conditions:

Number of cycles	Temperature	Lenght of time
1 cycle	95^0 C	1 minute
40 cycles	95^0 C	15 sec
	57^0 C	15 sec
	72^0 C	30 sec
1 cycle	72^0 C	5 minutes

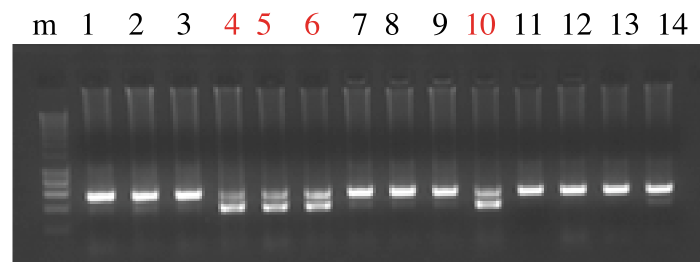

Figure 33: Genotyping of LentiLox-HNF4γ mice

(m) DNA Ladder; mice (1, 2, 3, 7, 8, 9, 11, 12, 13, 14) are wt; mice (4, 5, 6 and 10) are positive for lentivirus integration.

5. 15. 2. Southern blot of DNA prepared from tails of LentiLox-HNF4γ mice

Southern blot analyses were done with tail DNA (25µg), digested by EcoR I and subjected for electrophoresis in 1% agarose gel and blotted onto Biodyne B Transfere Membrane (Pall). Hybridization was performed with a ^{32}P random labeled by Klenow enzyme 1482bp EGFP internal probe, released from pLL3.7 by Xho I-EcoR I digestion (For detailed protocol of Southern blot look in „Standard Molecular biology techniques“).

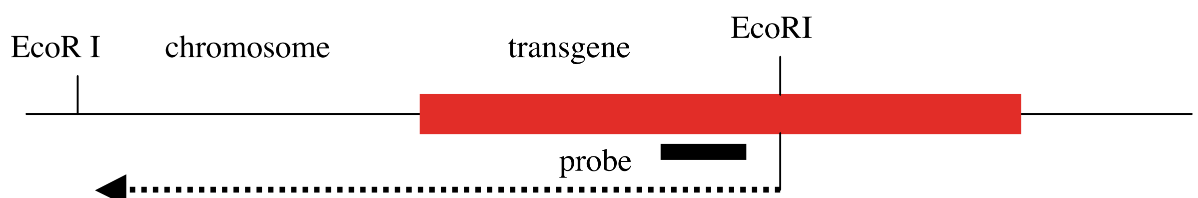

Figure 34: Southern blot strategy employed for LentiLox-HNF4γ mice

5. 15. 3. Determination of siRNA expression in LentiLox-HNF4γ mice

The siRNA expression in different organs of LentiLox-HNF4γ mice was determined by Northern blot hybridization. The protocol used includes slight modifications for siRNA detection.

5. 15. 3. 1. Isolation of total RNA for Northern blot hybridization

1) Dissect the mouse and take out the organs.
2) Transfer the organ tissue in RLT buffer (Qiagen) supplemented by βMESH in plastic 50ml Falcon tube. The ratio in the mass solution : tissue must be at least 10:1. As alternative the tissue could be stored for future use in RNA^{later} buffer (Qiagen). Homogenize the tissue by homogenizer immediately.
3) Transfer the homogenate to a fresh polypropylene tube and sequentially add 0,1 ml of 2M sodium acetate (pH 4.0), 1ml of phenol, and 0,2 ml chloroform-isoamyl alcohol per ml of the homogenate. After addition of each reagent, cap the tube and mix the contents by inversion.
4) Vortex the homogenate vigorously for 10 seconds. Incubate the tube for 15 minutes on ice to permit complete dissociation of nucleoprotein complexes.
5) Centrifuge the tube at 9000 rpm for 20 minutes at 4^0 C, and then transfer the upper aqueous phase containing the extracted RNA to a fresh tube.
6) Add an equal volume of isopropanol to the extracted RNA. Mix the solution well and allow the RNA to precipitate for 1 hour or more at – 20^0 C.
7) Collect the precipitated RNA by centrifugation at 9000rpm for 30 minutes at 40 C.
8) Wash the pellet by 80% EtOH and dissolve it in appropriate volume of DEPC treated H_2O.
9) Measure the concentration of the RNA.

5. 15. 3. 2. Electrophoresis of RNA sample on denaturing acrylamide gel

1) Prepare a 15% denaturing acrylamide gel with 7 M urea in 1x TBE within 20x20 cm mold.
2) Pre-run the gel for 15-20 min at low voltage.
3) Dissolve the RNA (50μg) in 80% formamide containing buffer with dye.
4) Load the RNA on the gel and perform the electrophoresis at 500V -35 mA.

5) After 2 hours run dismantle the chamber, transfer the gel on Whatman paper and covered it with Hydrobond-N+ nitrocellulose membrane.
6) Perform electro transfer in special chamber filled with 0,5x TBE under 250 mA (30-37 V) for about 4 hours.
7) Briefly dry the membrane and fixed it under UV crosslink (auto cross, 1200).

5. 15. 3. 3. Labelling the probe for Northern blot

Hybridization was performed whit 5´ end ^{32}P labelled 19 mer (sense) probe specific for each siRNA. For normalization of the blot a sense 5´^{32}P-labelled oligonucleotide for endogenous U6 gene was used.

1) Set up the labelling reaction:

Reagents	Amount
T4 kinase buffer 10x (Roche)	5µl
T4-PNK (1U/µl)	2µl
Oligo sense probe	2,5µl (5pmol)
γ-^{32}P ATP	3µl
H_2O	37,5µl

5. 15. 3. 4. Northern blot hybridization

The membrane was pre-hybridized in 6 x SSC, 10x Denhardt`s solution and 0,5% SDS at 42^0 C for 6 hours and hybridized with the probe in 6x SSC and 0,1% SDS over night. At the next day, the membrane was washed several times first with 6x SSC (3x10 min), then with 2x SSC/0,1% SDS (3x10 min) at 37^0 C. To reveal the signal from siRNA, the membrane was exposed on Phospho Imager screen for 2 hours.

5. 15. 4. Measurement of HNF4γ knockdown in LentiLox-HNF4γ mice by quantitive real-time PCR

For determination of HNF4γ mRNA in pancreas and intestine, RNA was isolated by RNeasy Mini Kit 50 (Qiagen) following the manufactures instructions. A 2µg RNA was reverse transcribed in cDNA by SuperScript™ First-Strand Synthesis System and Oligo

$(dT)_{12\text{-}18}$ primer (Invitrogen). The cDNA was purified using QIAquick PCR Purification Kit (Qiagen) with slight modification of the protocol.

5. 15. 4. 1. Purification of the cDNA

1) Add 500µl Buffer PB to the cDNA sample and mix.
2) Apply the cDNA mixture to the MinElute column and centrifuge for 1 minute at 13,000 rpm in a conventional tabletop micro-centrifuge.
3) Discard the flow-through. Place the MinElute column into the same collection tube.
4) Add 750µl Buffer PE to the MinElute column into the same collection tube and centrifuge for 1 minute.
5) Discard the flow-through. Place the MinElute column back into the same collection tube.
6) Add 500µl 80% ethanol to the MinElute column and centrifuge for two minutes.
7) Discard the flow-through. Place the MinElute column back into the same collection tube.
8) Open the column caps and place in a microfuge with the cap opposite the direction of the rotation of the rotor to avoid breaking the cap off. Centrifuge for 5 minutes.
9) Place the MinElute column into a clean, labeled, 1,5mL microfuge tube.
10) To elute cDNA, add 50µl Buffer EB to the centre of the column membrane. Incubate at room temperature for 2 minutes. Centrifuge for 2 minutes. Discard column and save the 50µl eluted cDNA.

5. 15. 4. 2. Real-time PCR conditions

The analyses were done with Mm00443563_m1 (HNF4γ), Mm00660262_g1 (Hmbs) TaqMan Gene Expression Assays (Applied Biosystems) and Platinum Quantitative PCR SuperMix-UDG (Invitrogen) onto Light Cycler machine (Roche). The mRNA/cDNA HNF4γ abundance was calculated relative to the expression of a house-keeping gene HMBS.

1) Set up the following reaction.

Reagents	Amount
PCR Super mix-UDG (Invitrogen)	10µl
Bovine serum albumin (10%)	1µl
Taq Platinum (5U/µl)	0,12µl
Gene Assay (ABS)	1µl
$MgCl_2$ (50mM)	1µl
cDNA (1:2 dilution)	7µl

2) Cycling program:

Number of cycles	Temperature	Lenght of time
1 cycle	50^0 C	2 minutes
1 cycle	95^0 C	2 minutes
45 cycles	94^0 C	5 seconds
	60^0 C	20 s (single acquire)

5. 15. 5. HNF4γ protein detection in intestine and pancreas

The HNF4γ/α proteins in intestine and pancreas were detected by immunochistochemistry using specific antibody on cryo sections. The protocol is described in the section „Standard Molecular Biological techniques".

-Antibodies

Molecule/Epitope	Sort of antibody	Company	Working solution
HNF4γ	goat polyclonal IgG	Santa Cruz cat#sc-6558	1:500
HNF4α	goat polyclonal IgG	Santa Cruz cat#sc-6556	1:900

5. 15. 6. Glucose tolerance test

For glucose tolerance test were used A-I (F1) mice and wt mice from the same litters (6 animals per group) at age of 6 moths.

1) Starve the animals over night (16 hours).
2) Next morning measure the weight of the animals.
3) Measure the blood glucose before injection to make sure that levels are low. To take the samples cut a very small piece of the tail and massage it to get a drop of blood.
4) Inject in to the peritoneum 200μl of the glucose solution (20g glucose/100 ml of 0,9 NaCl) per 20 grams of body weight. Measure the blood glucose levels after 15, 30, 60 and 120 minutes.

5. 16. Fluorescence imaging

The pictures from organ sections of RNAi-HNF4γ mice were captured by Zeiss-Axioplan microscope (Zeiss) attached to a Leica DFC 480 Camera (Leica Microsystems) and supplied by a unit consisting of a mercury lamp and EGFP filter. The whole mount photos were taken by binocular Stemi SV6 (Zeiss) connected to the light source HBO 100 (Zeiss).

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