Functional studies of GR and MR function by RNA interference

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(Hee-Young Lim)

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I. Introduction

1.1 Steroid hormones; Glucocorticoids

The term "steroids" refers to a group of small lipophilic compounds derived from a common percursor, cholesterol. There are four major steroid hormone types: progestins, androgens, estrogens, and corticoids. They differ in the number of carbon atoms, the binding receptor and their biological activities. Corticoids are divided into two groups:
a) mineralocorticoids such as aldosterone, which regulate sodium / potassium transport and thus fluid and electrolyte for homeostasis and b) glucocorticoids, which have many activities including the response to stress, regulation of intermediary metabolism, cell development and immunosuppressive effects (Ashwell et al., 2000).
Steroid hormones such as mineraol- and glucocorticoid are secreted and synthesized by the adrenal glands and controlled by a neuroendocrine cascade called the hypothalamus-pituitary-adrenal (HPA) axis (Palkovits, 1987; Reichardt and Schutz, 1996). Stimulation by stress or inflammation leads to the release of corticotropin-releasing hormone (CRH) in the hypothalamus. CRH is transported to the anterior pituitary where it induces synthesis and secretion of adrenocorticotrophic hormone (ACTH). Finally ACTH causes an instant increase in the secretion of glucocorticoids as well as an increase in the production of steroid biosynthetic enzymes in the adrenal cortex (Reichardt, 2004; Simpson and Waterman, 1988). Glucocorticoid levels are controlled by inhibition of their own production and secretion by binding to the GR via a negative feedback-loop, thus protecting the organism from chronically elevated systemic steroid levels (Bradbury et al., 1994). An additional feedback-loop is established between the immune system and the hypothalamus (Ashwell et al., 2000; Wilckens and De Rijk, 1997).

Under normal conditions secretion of glucocorticoids (GCs) occurs in a circadian rhythm. In humans GC levels are high in the morning, prior to waking, and reach low GC levels in the evening, whereas this pattern is reversed in rodents (Arriza et al., 1988; Bradbury et al., 1994; Vinson GP, 1992).

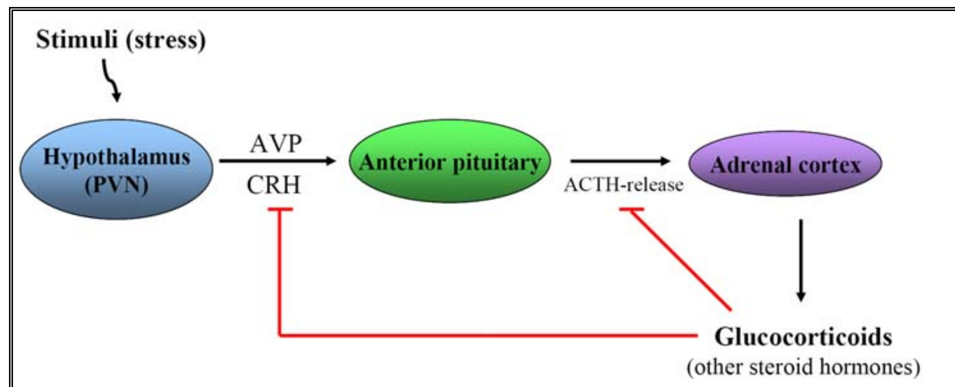


Figure 1.1 Regulation of GC levels by the HPA axis via negative feedback loops

1.2 Steroid hormone receptors

1.2.1 Glucocorticoid receptor (GR)

1.2.1.1 Structure of the glucocorticoid receptor

The glucocortiocid receptor (GR), a phosphorlyated 85 ~ 92kDa protein, belongs to the nuclear receptor subfamily that includes the receptors for other hormones such as mineralocorticoids, estrogens, thyroid hormones, vitamin D3, retinoic acid and a number of orphan receptors (Ashwell et al., 2000). The GR is expressed in almost all tissues throughout the body (Yamamoto, 1985).

The GR is an evolutionarily highly conserved transcription factor found in several animal species; *Xenopus*, rat and human etc. The gene encoding the GR consists of nine exons, the protein-coding regions are located in exons 2 ~ 9, and three promoters (1A, 1B, and 1C) have been identified in human (Breslin et al., 2001).

As mentioned before, the GR has a conserved modular structure containing three major domains(Beato et al., 1995); a variable N-terminal gene transactivation domain containing the AF-1 transcriptional activation domain required for transcriptional enhancement and association with basal transcription factors (Evans and Hollenberg, 1988; Thompson et al., 1988), a central DNA binding domain which is composed of two highly conserved zinc-finger regions for dimerization and DNA binding and the first of two nuclear localization signals (NSL1), and a C-terminal ligand-binding domain that serves as the hormone binding site and contain a second NLS (NLS2) connected by a hinge region.

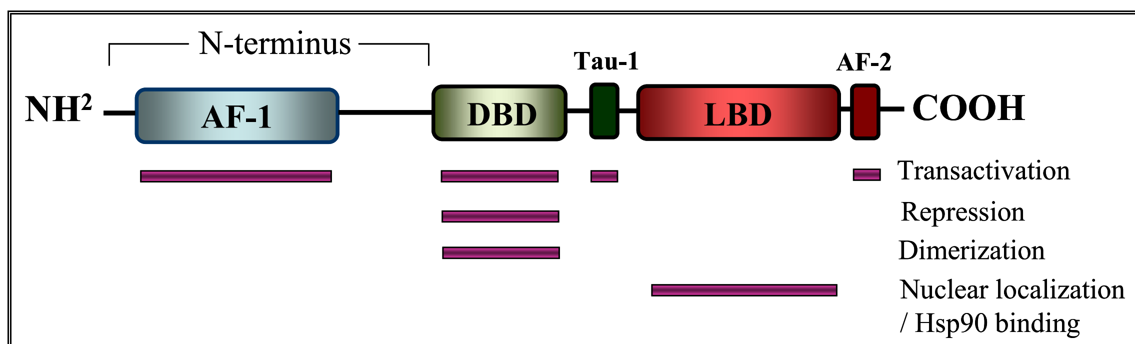


Figure 1.2 A diagram of the molecular structure of the glucocorticoid receptor

1.2.1.2 Mechanisms of glucocorticoid action through the GR

The mechanisms of GC actions via the GR can be divided into genomic and non-genomic effects.

The genomic effects are known to be mediated by transactivation and transrepression.

In the absence of hormone, an inactive GR exists in the cytoplasm within a heterocomplex with the chaperones hsp70, hsp90, hsp40, the co-chaperone p23 and the immunophilins FKBP32 and Cyp-40 (Pratt et al., 1996). The chaperon hsp90 prevents

nuclear location of the unoccupied receptor and formation of insoluble cytosolic GR aggregates (Karin, 1998; Pratt et al., 1996).

After the hormones passively diffuse into the cell, they bind to the soluble GR, which resides in the cytosol in the multiprotein complex, resulting in conformational changes of the receptor protein. The ligand-binding complex induces the release of the chaperones and then translocates into the nucleus. Within the nucleus, the ligand-bound GR homodimer recognizes and binds to palindromic sequences, GRE (glucocorticoid response element), present in the promoter and enhancer regions of several target genes. The bound GR modulates transcription from these response elements.

Figure 1.3 gives a schematic representation of what happen after glucocorticoids bind to receptors in cells. Upon hormone binding the hormone receptor complex rapidly adopts an "activation" or "repression" status depending on the target genes.

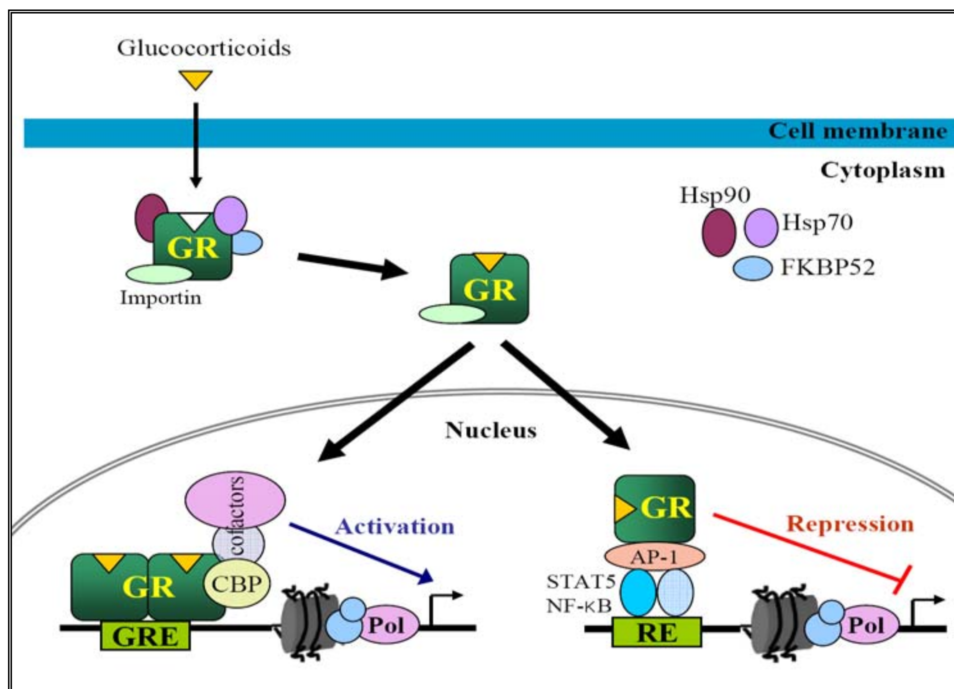


Figure 1.3 Molecular modes of glucocortcioid receptor actions

Traditionally, two GR molecules as a homodimer bind to GREs which leads to an increase in target gene transcription, called transactivation, while interaction with negative GRE sites leads to the suppression of genes, called cis-repression. Inhibition of transcriptional activity of target genes take place either by binding of the activated glucocorticoid receptor to a negative GRE within the 5'flanking region or by cross-coupling e.g. protein-protein interaction of the GR with transcription factors induced under stimulation conditions (Jonat et al., 1990; Scheinman et al., 1995).

The immunesuppressive activity of glucocorticoids is mainly derived from the ability to repress the transcription of numerous pro-inflammatory mediators. It accomplishes this by interfering with transcription factors such as NF-κB, AP-1, NF-AT, CREB and members of the STAT family that are pivotal to the immune response. Intriguingly, the GR is able to transrepress promoters in this way without binding to DNA itself (Heck et al., 1997; Heck et al., 1994). The GR also participates in numerous physiological processes needed for cell homeostasis in diverse cell types that range from hepatocytes and epithelial cells to neurons. The GR is able to regulate many genes that encode enzymes of carbohydrate and amino acid metabolism, hormones, components of antibacterial defense, and serum proteins by three basic mode, (i) binding to GREs in target genes leading to activation of transcription, (ii) inhibition of transcription of genes through direct DNA binding, and (iii) gene regulation by physical interaction with other transcription factors (Necela and Cidlowski, 2004).

The other GR response, the rapid non-genomic GCs effects, is assumed to be mediated by three different mechanisms; (i) physicochemical interactions with cellular membranes known as non-specific non-genomic effects (Buttgereit and Scheffold, 2002), (ii) membrane-bound glucocorticoid receptor (mGR)-mediated non-genomic

effect (Croxtall et al., 2002), and (iii) cytosolic glucocorticoid receptor (cGR)-mediated non-genomic effects (Bartholome et al., 2004).

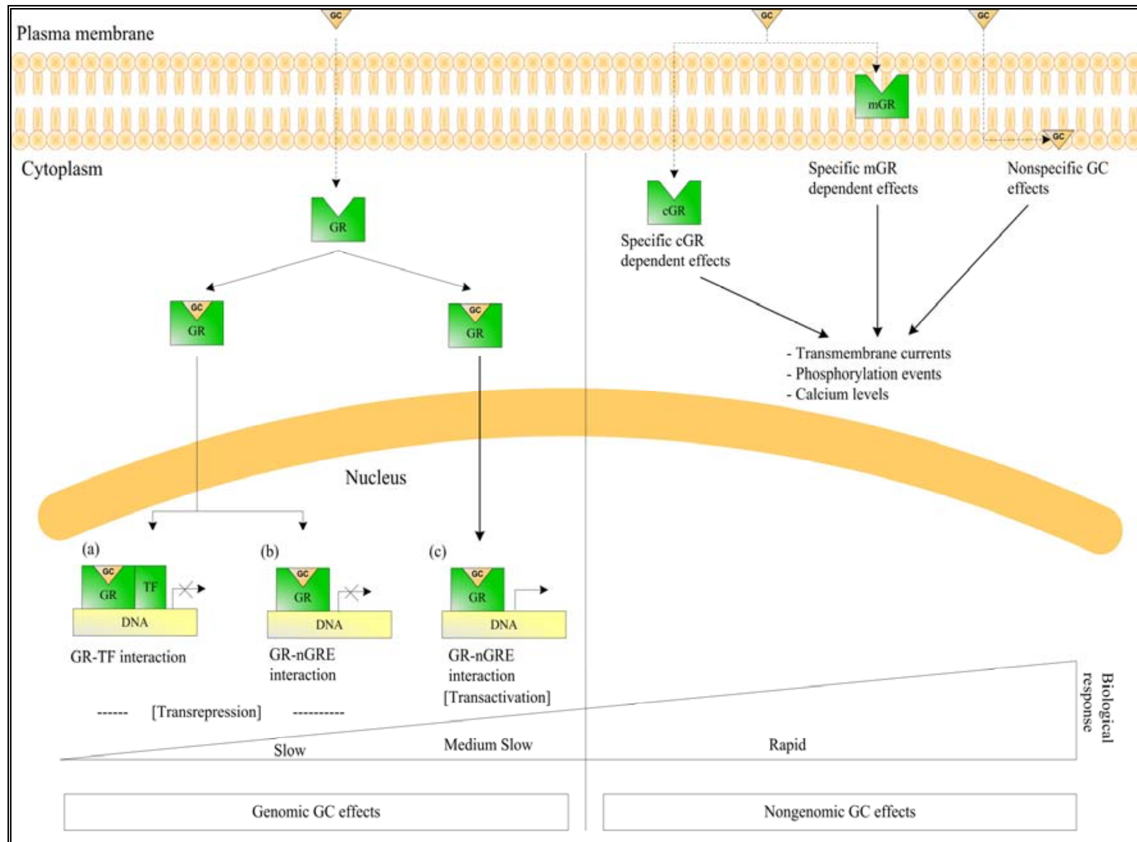


Figure 1.4 Schematic representation of the molecular mechanisms of glucocorticoid action including genomic GC effects and non-genomic GC effects

1.2.2 Mineralocorticoid receptor (MR)

1.2.2.1 Structure of the mineralocorticoid receptor

The mineralocorticoid receptor (MR or NR3C2) is a member of nuclear receptor (NR) superfamliy of ligand-dependent transcription factors and belongs to the steroid receptor (SR) superfamily together with GR (Tsai and O'Malley, 1994). The mineralocorticoid receptor is also referred to as type I receptors for adrenal steroid hormones and has a

higher affinity for naturally occurring adrenal steroids such as cortisol or corticosterone than the glucocorticoid receptor which is referred to as type II steroid receptor. Therefore the MR is occupied and activated at lower concentrations of endogenous hormone (Beaumont and Fanestil, 1983; Reul and de Kloet, 1985; Sutanto and De Kloet, 1987). As all members of the NR family, the mineralocorticoid receptor also consists of three major domains including the N-terminal domain, followed by the central DNA-binding domain, a hinge region, and the C-terminal or ligand-binding domain (Pearce et al., 2003). The region of 66 amino acids in the DNA-binding domain is essentially able to define the subfamliy of NRs. Indeed the N-terminal domain, which contains an activation or repression function bound with co-activators and co-repressors for modulation, is poorly conserved across the NR superfamily and distinguishes the members of the superfamily at the primary structure level (Pearce and Yamamoto, 1993; Rupprecht et al., 1993). The mineraolcorticoid and glucocorticoid receptor proteins share common structural features (Pearce et al., 2003). They are around 60% homologous in the hormone-binding domains, ligand-binding domain and 90% homologous in the DNA binding domain, but only 15% homologous in the N-terminal region, which explains their ability to interact with an overlapping set of hormones and identical DNA binding specificities (Arriza et al., 1987; Martinez et al., 2005). Several members of the SR family mediate gene expression by binding to hormone response elements (HREs) as a homodimer in a ligand-dependent manner. Nevertheless, the GR and MR show different affinities for the same ligand or hormone.

As mentioned above, the mineralocortcioid receptor is able to bind adrenal steroid hormones, glucocorticoids and mineralocorticoids, and has a higher affinity for cortisol and corticosterone (Kp ~ 0.5nM) than the GR (Kp ~ 2.5 - 5nM) and a similar affinity for

the mineralocorticoid aldosterone and the glucocorticoids corticosterone/cortisol (deoxycorticosterone = corticosterone ≥ aldosterone = cortisol) (Krozowski and Funder, 1983; Reul and de Kloet, 1985; Vedder et al., 1993). The MR is mainly expressed in polarized Na+ transporting epithelial cells (e.g. in kidney and colon where it regulates ion homeostasis) and in non-epithelial tissues (e.g. heart and brain where it is especially highly expressed in the hippocampal CA1 and CA2 regions and to a lesser extent in the thalamus, cerebellum, and brainstem, and adipocytes) (Ahima et al., 1991; Arriza et al., 1988; Gomez-Sanchez, 2004; Reul and de Kloet, 1986). Interestingly MR expression on leukocytes has not yet been reported with the exception of human CD34+ hematopoietic progenitor cell (Grafte-Faure et al., 1999) and rat microgial cell (Tanaka et al., 1997), a macrophage-related cell type that resides in the CNS.

1.2.2.2 The role of the mineralocorticoid receptor (MR)

Similar to the GR, the MR also exists like the GR retained in the cytosol in an inactive conformation with the hsp90 complex in the cytoplasm in the absence of hormone (Hong et al., 1997). Due to its high hormone affinity, the MR is essentially always bound by endogenous glucocorticoids or aldosterone. The enzyme 11β- HSD2 prevents occupancy of the MR by cortisol/corticosterone by converting those hormones into an inactive form. The hormone-receptor bound complex results in a conformational change, followed by translocation into the nucleus and binding as a homodimer to GRE-elements. Then various transcriptional modulators are recruited and activate expression of target genes. Those genes regulate sodium reabsorption by a mechanism linking the action of ENaC (Epithelial sodium Channels) at the apical membrane to the Na/K-ATPase pump at the basolateral membrane and thereby mediate electrolyte or fluid

balance (Lingueglia et al., 1994; Rogerson and Fuller, 2000).

The MR is the effector of the cellular response to mineralocorticoids. So far the critical role of the MR for the mineralocorticoid response was demonstrated in several animal models including MR null transgenic mice (Berger et al., 1998). In short, the MR integrates hormonal signaling and activates the expression of aldosterone-target genes, which regulate various physiological processes and play a central role in regulating sodium and potassium homeostasis in the kidney.

In several studies, an important role of the MR was shown not only in the regulation of salt - water homeostasis but also in cardiovascular function, neuronal fate and adipocyte differentiation. Deyregulation of the MR-aldosterone system revealed in various pathologies such as mineralocorticod resistance (Zennaro and Lombes, 2004), disorders of the nervous system (De Kloet et al., 1998), hypertension (Lifton et al., 2001) and cardiac failure (Pitt, 2004). Inactivation or over-expression of the MR mice confirmed the crucial role in renal and cardiac function (Beggah et al., 2002; Berger et al., 1998; Le Menuet et al., 2001).

In epithelial tissues, the MR is coexpressed with the specific enzyme 11β-hydroxysteroid dehydrogenase type 2(11β- HSD2). The physiological role of 11β-HSD2 is to metabolize cortisol to receptor-inactive cortisone in humans or corticosterone to 11-dehydrocorticosterone in rodents (Edwards et al., 1988; Funder et al., 1988). While rats and mice synthesize only corticosterone, humans and many other mammals predominantly synthesize cortisol. This enzyme, converting cortisol to inactive cortisone, serves to “protect” the mineralocorticoid receptor from occupancy and activation by glucocorticoids and thereby allows aldosterone to control its function ((Rogerson and Fuller, 2000). In contrast, non-epithelial tissues such as the

hippocampus do not express 11β-HSD2 that allows GCs to bind to the MR.

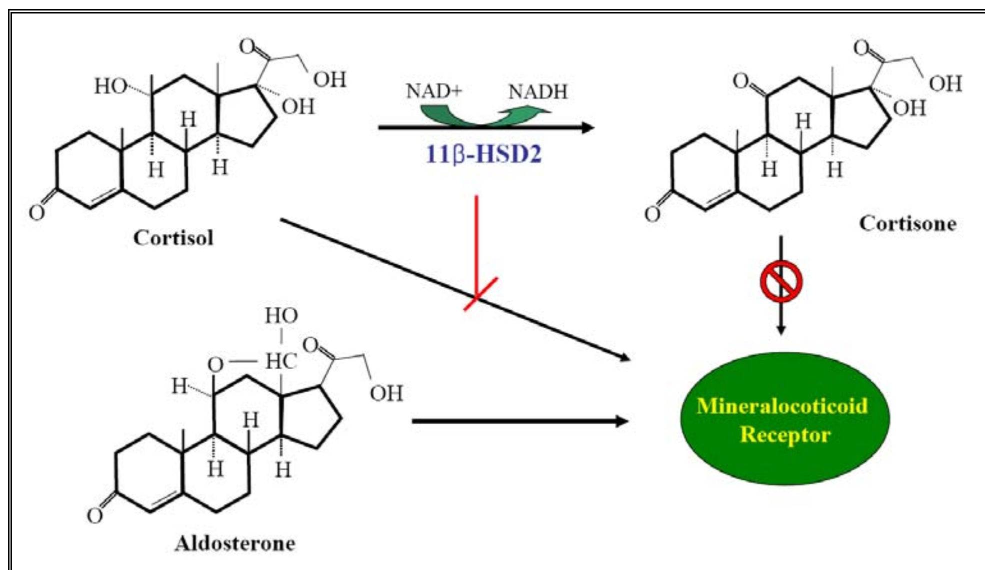


Figure 1.5 Schematic representation of 11β- HSD2 action in epithelial tissues

1.3 Immuno-modulatory actions of glucocorticoids in macrophages

The immune system is divided into two branches: innate immunity, which comprises non-specific defence mechanisms that include physical barriers such as skin and phagocytic cells that attack foreign antigen in the body, and adaptive immunity, which comprises antigen specific immune responses. Innate immunity involves the activation of granulocytes, macrophages, NK cells, and is initiated by the production of inflammatory cytokines. In adaptive immune response, antigen specific lymphocytes such as T and B cells, proliferate in response to specific antigen and differentiate into T effector cells or antibody producing plasma cells that eliminate the invading pathogen.

In animals, defence against infectious agents, in particular rapidly expanding viruses and bacteria, requires an immediate host response in order to limit growth and dissemination, and then stimulation of a prolonged, specific response to prevent re-

infection.

1.3.1 Function of macrophages in innate immunity

Pluripotent stem cells differentiate into two types of stem cells, a common lymphoid progenitor that gives rise to natural killer (NK) cells and T or B lymphocytes, and a common myeloid progenitor that gives rise to different types of granulocyte-macrophage progenitors and megakaryocytes in the bone marrow. The myeloid cells are the monocytes, the myeloid dendritic cells, and neutrophils, eosinophils and basophils which are termed granulocytes. They circulate in the blood and enter tissues only at sites of infection or inflammation. Monocytes, which represent a central part of innate immunity, also enter tissues, where they differentiate into phagocytic macrophages. Monocytes and macrophages are not a homogenous cell population, but rather encompass distinct phenotypes which display a wide-range of pro- and anti-inflammatory activities.

Indeed macrophages are one of the three types of phagocytes in the immune system and distributed widely in tissues throughout the body where they play a key role in innate immunity. Macrophages and neutrophils provide a first line of defense against many microorganisms that is essential for the control of common infections and play a crucial role in the initiation and subsequent direction of adaptive immune response, as well as participating in the removal of pathogens triggered by an adaptive immune response. Phagocytic macrophages conduct the defense against bacteria by means of surface receptors that are able to recognize and bind common constituents of many bacterial surfaces. The same mechanism is applied for viral, fungal, and parasite infections. Almongst them are bacterial endotoxins such as lipopolysaccaride (LPS), which is a

complex glycolipid composed of a hydrophilic polysaccharide moiety and a hydrophobic domain and represents a major component of the outer membrane of Gram-negative bacteria and one of the most potent microbial initiators of inflammation (Cohen, 2002; Raetz, 1990). Cell wall components of bacteria bind to receptors on the surface of macrophages, trigger them to engulf and destroy the invading bacterium and also induce the secretion of biologically active molecules. Activated macrophages in response to pathogen components secrete cytokines, which are proteins that are released by activated immune cells and affect other cells, and chemokines that attract cells with specific chemokine receptors such as monocytes and neutrophils from the bloodstream. Cytokines and chemokines are then able to initiate a process known as inflammation.

Activated macrophages secrete a variety of cytokines, including interleukin (IL) -1, IL-6, IL-8, and TNF-α that mobilize innate immune response, and signal T cells via IL-10, IL-12 and IL-18 to initiate specific response against intracellular and extracellular pathogens. In addition to producing several inflammatory and immunoregulatory cytokines, macrophages and related cells of myeloid origin can differentiate into highly efficient antigen presenting cells (APCs).

1.3.2 Immunomodulatory actions of glucocorticoids

Glucocorticoids (GCs) are important regulators of the immune system (Tuckermann et al., 2005). Synthetic GCs are widely used to treat autoimmune and atopic inflammatory disease including asthma, and inflammatory bowel disease, whereas endogenous steroids protect the organisms from harmful effects of exaggerated immune responses. The release of endogenous GCs is essential for preventing exaggerated immune responses and the management of sepsis (Yeager et al., 2004). Consequently

adrenalectomy or GR antagonist RU486 treatment is shown in high mortality in various models of infection (Bertini et al., 1988; Hawes et al., 1992). In contrast, elevated GR expression is associated with increased resistance to endotoxic shock (Reichardt et al., 2000). GCs modulate the function of leukocytes at different levels including the control of gene expression, enzymatic activity and cellular migration (Tuckermann et al., 2005) and antagonize macrophage differentiation and negatively regulate numerous macrophage functions (Sternberg, 1993).

Adaptive immune responses are primarily modulated via their influence on T cells and dendritic cells whilst granulocytes and macrophages are primary targets in the context of innate immunity. Glucocorticoids act on the immune system by both suppressing and stimulating many pro-inflammatory or anti-inflammatory mediators.

In the case of macrophages, the expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α, chemokines such as CXCL-1, CXCL-10 and IL-8 and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) are repressed by GCs (Tuckermann et al., 2005). Consequently, production of nitric oxide (NO), oxygen radicals and prostaglandins is reduced and the phagocytotic activity impaired. This was shown to antagonize IFN-γ-induced MHC class II (Ia) Ag expression (Fertsch et al., 1987; Snyder and Unanue, 1982) and exhibits both tumoricidal and microbicidal activities (Hogan and Vogel, 1988; Schaffner, 1985).

Indeed, the transcription of numerous anti-inflammatory genes is switched on by glucocorticoids (Figure 6, marked by (a)). The hormone-receptor complex binds to GREs in the promoter region of steroid-sensitive genes and recruits directly or indirectly coactivator molecules such as CBP (cAMP-response-element-binding-protein), pCAF (p300/CBP-associated factor) or SRC-2 (steroid receptor coactivator),

causing acetlyation on histone H4, which then leads to activation of genes encoding anti-inflammatory proteins such as SLP1, MKP-1, IκB-α, and GILZ. Furthermore immunostimulatory functions of GCs lead to the induction of acute phase proteins, the enhancement of delayed-type hypersensitivity responses or the stimulation of T cell mitogenesis (Dhabhar and McEwen, 1999; Hogan and Vogel, 1988; Reichardt, 2004; Wiegers et al., 1993). However, little is known about positive GCs effects on macrophages so far.

In contrast, inflammatory genes activated by such as IL-1β or TNF-α through activation of transcription factor nuclear factor (NF-κB) are suppressed by directly inhibition of HAT activity and recruiting histone deacetylase (HDAC2) through GCs-GRs complex (Figure 6, marked by (b)) .

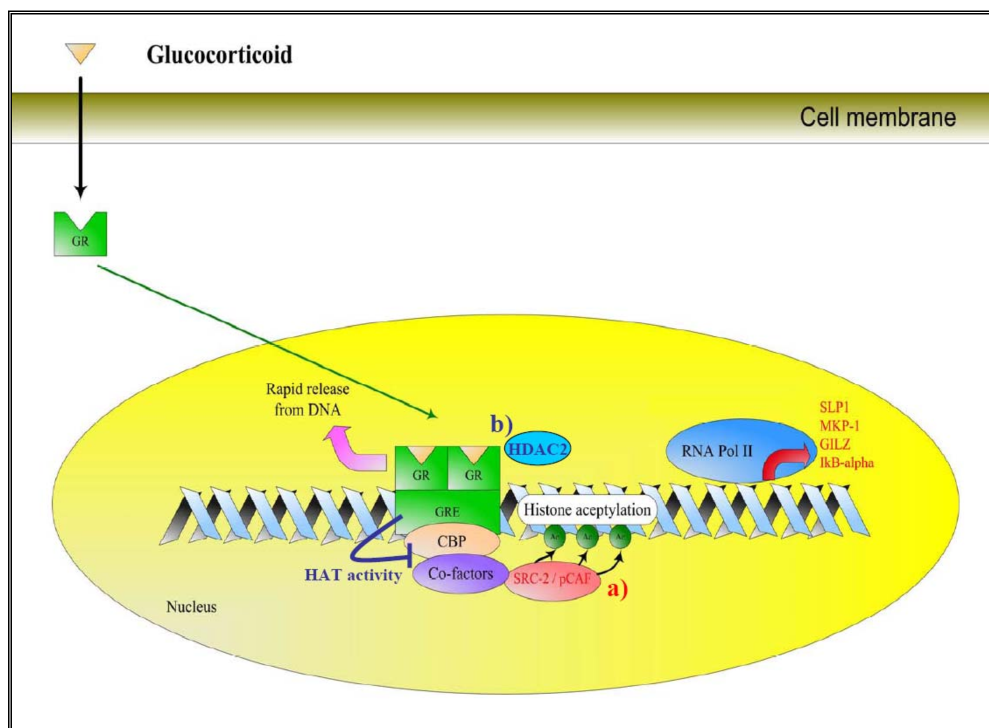


Figure 1.6 Mechanism of GCs actions in inflammatory response

1.4 RNA interference (RNAi)

RNA interference (RNAi) is a well-recognized cellular defense mechanism against viral invasion, transposon expansion, and post-transcriptional regulation of mRNA and represents a unique form of post-transcriptional gene silencing (PTGS). The original observation of RNA interference (RNAi) was made over a decade ago by researchers working in plant and fungal genetics. The term of RNAi was coined after the discovery that injection of double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* leads to specific silencing of target genes that are highly homologous in sequence to the delivered dsRNA (Fire et al., 1998). This evolutionarily conserved phenomenon is observed in almost all eukaryote studied so far (insects, frogs and other animal including mice) (Kennerdell and Carthew, 2000; Svoboda et al., 2000; Wianny and Zernicka-Goetz, 2000). It is closely linked to the PTGS mechanism of cosuppression in plant and fungi (Baulcombe, 2002; Vaucheret et al., 2001).

RNA silencing is also a novel gene regulatory mechanism that limits the transcript level by either suppressing transcription (transcriptional gene silencing) or by activating a sequence-specific RNA degradation process (post-transcriptional gene silencing) or RNA interference (RNAi) (Neema A, 2003). It is a powerful tool for gene function analysis in mammals these days. The natural function of RNAi and its related processes seems to be the protection of the genome against invasion or random integration by mobile genetic elements such as RNA viruses and transposons as well as it orchestrates the developmental programs of eukaryotic organisms.

When a retrovirus or other genetic element enters the cells, the siRNAs bind to the homologous gene sequences of the invading virus. This forms a triplex and initiates a cascade of events resulting in the generation and amplification of siRNAs. The reaction

is catalyzed by several enzymes including a Dicer or RNA-dependent RNA polymerase III that contains a dsRNA binding domain on one or more ribonuclease domains as well as a RNA helicase domain.

MicroRNAs and Small interfering RNAs

RNA silencing is triggered by dsRNA precursors that vary in length and origin. These dsRNAs are rapidly processed into short RNA duplexes of 21 to 28 nucleotides and then trigger the recognition and ultimately the cleavage or translational repression of complementary-single stranded RNAs, such as mRNAs, viral genomic or antigenomic RNAs. According to their origin or function, three types of naturally occurring small RNAs have been described: short (small) interfering RNAs (siRNAs), repeat-associated short interfering RNAs (rasiRNAs) and microRNAs (miRNAs) (Meister and Tuschl, 2004).

Micro RNAs (miRNA) are a family of 21 ~ 25 nucleotide small, non-coding RNAs that have characterized targets and negatively regulate gene expression at the post-transcriptional level in a sequence-specific manner (He and Hannon, 2004). They may also guide mRNA degradation. The small interfering RNAs (siRNAs) are a result of non-coding RNAs, which are part of introns, and other endogeneous retroelements, accumulated throughout evolution. The siRNAs are expressed as dsRNA as part of a large transcript and are broken down to small fragments of ~ 22 base pairs as siRNAs. These siRNAs remain as dsRNA complex: sense(S) and antisense (AS) (Khvorova et al., 2003).

Although the two main classes of short RNA molecules were originally discovered in unrelated studies, siRNAs and miRNAs are fundamentally similar in terms of their

molecular characteristics, biogenesis and effector functions. Both types of small RNA share a common RNase-III processing enzyme, Dicer, and closely related effector complexes, RISCs, for post-transcriptional repression (Hammond et al., 2000).

miRNAs differ from siRNAs in their molecular origins and in their mode of target recognition. miRNAs are produced as a distinct species from a specific precursors that is encoded in the genome. By contrast, siRNAs are sampled more randomly from long dsRNAs that can be introduced exogenously or produced from bi-directionally transcribed endogenous RNAs that anneal to form dsRNA (Hannon and Rossi, 2004). In many cases, miRNAs bind to the target 3' UTRs through imperfect complementarity at multiple sites, and therefore negatively regulate target expression at the translation level (Meister and Tuschl, 2004). But siRNAs often form a perfect duplex with their target at only one site, and therefore direct the cleavage of the target mRNAs at the site of complemetarity (He and Hannon, 2004).

Maturation of miRNA formation in animal occurs via two processing pathways. First, the naturally occurring miRNAs are synthesized by RNA polymerase II (Pol II) in the nucleus in large precursor forms and then the primary miRNA transcripts (pri-miRNA) are processed into ~ 70 nucleotide precursors (pre-miRNAs) by the two RNase-III enzymes, Drosha and Dicer and in second event that follows, this precursor is cleaved to generate 21 ~ 25 nucleotide mature miRNAs (Lee et al., 2002).

In the cytoplasm, Dicer is responsible for cleaving double-stranded molecules, whether derived form endogenously expressed miRNAs or from replicating viruses, into small interfering RNA duplexes (siRNA) of 19 ~ 25 base pairs with 3' dinucleotide overhangs. (Bernstein et al., 2001; Elbashir et al., 2001; Grishok et al., 2001; Hutvagner et al., 2001; Lee et al., 2004).

The process of maturation of small RNAs is similar to the process of miRNAs, where a stepwise process is catalyzed by dsRNA-specific RNase-III type endonuclease, containg catalytic RNaseIII and dsRNA-binding domain (dsRBDs) (Meister and Tuschl, 2004). The siRNA is incorporated into RISC, thus enabling either of the two strands to independently guide target mRNA recognition (Khvorova et al., 2003; Schwarz et al., 2003). The degree of complementarity between the guide strand and the target mRNA is able to determine whether mRNA silencing via site-specific cleavage occurs in the region of the siRNA-mRNA duplex or proceeds through an miRNA-like mechanism of translational repression. For siRNA-mediated gene silencing, the cleavage products are released and degraded, leaving the siRNA-programmed RISC (Schwarz et al., 2003). Figure 1.7 schematic shows the current model of the biogenesis and post-transcriptional suppression of microRNAs and small interfering RNAs (He and Hannon, 2004).

For application of the RNAi technology to analyze the function of certain genes *in vitro*, there are two ways to infect the cells, either through siRNA duplexes or using lentivirus encoding siRNA sequence against the mRNA of interest. To apply siRNA duplexes in vitro and in an *in vivo* model, so far several approaches were developed and two representative methods are generally used, which is direct transfection of siRNA duplex and transduction of lentivirus encoding the shRNA sequence. In this study, we used a lentiviral system for applying siRNA sequences in order to induce down-regulation of target genes. Permanent gene suppression can be achieved by lentiviral vectors transcribing siRNAs from RNA polymerase III promoters (e.g. the mouse U6 promoter). The constitutive lentiviral vector system successfully silences protein expression in cells, other constructs rather permit inducible, Cre-lox-regulated, RNA interference. The

system consists of a lentiviral vector carrying the mouse U6 promoter that is separated from the siRNA sequence by a loxP cassette.

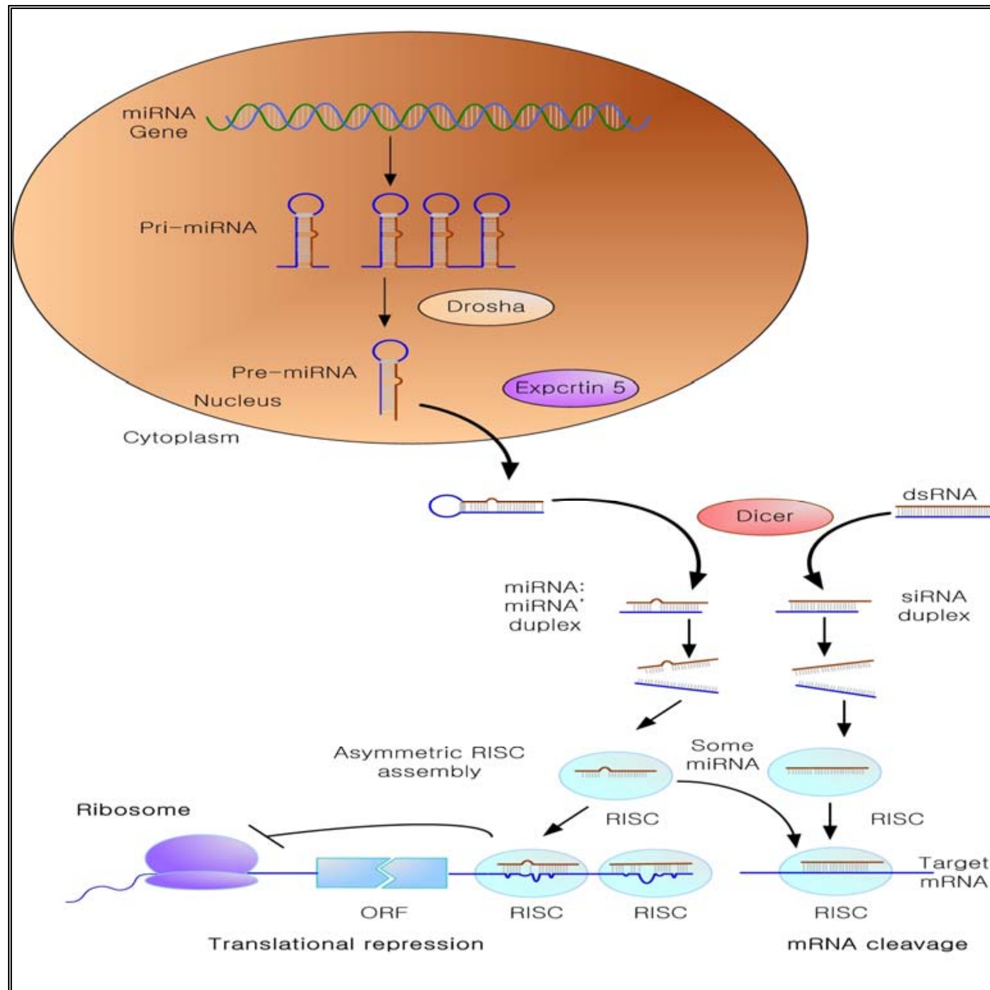


Figure 1.7 The model of the biogenesis and action of miRNAs and siRNAs

Aim of this study :

Steroid hormones are involved in maintaining body homeostasis and modulating the immune system. They act via a class of nuclear receptors dependent on their concentration and pre-receptor mechanisms such as the expression of 11β-HSD2. Amongst them are the glucocorticoids inluding corticosterone and cortisol and the mineralocorticoids such as aldosterone. The mechanism and the physiological role of these hormones was the subject of this work.

Aldosterone primarily binds to the mineralocorticoid receptor (MR) in kidney and colon, which both express 11β-HSD2, and in the limbic system that lacks 11β-HSD2. Thereby aldosterone regulates sodium-potassium transport, fluid homeostasis and neuronal excitability. Previous studies aiming at deleting or overexpressing the MR were performed in mouse models. However, transgenic rats sometimes represent disease models that more closely resemble the situation in human patients. Recently, a new strategy has been reported for the generation of transgenic mice using lentiviral delivery of shRNAs. This allows down-regulation of specific genes in mice and can also be applied to rats. Therefore we generated MR knock-down transgenic rats using a specific lentiviral shRNA to investigate the consequences of MR inactivation and to study whether this model mimics the effects seen in the knock-out mice as well as in human patients. This should allow evaluating this approach for investigating MR function in pathophysiology.

The actions of glucocorticoids (GCs), which are pivotal regulators of the immune system, are mostly mediated via the glucocorticoid receptor (GR). However, an involvement of the mineralocorticioid receptor (MR) in immune cells is also discussed. The MR has a higher affinity for GCs as compared to the GR. Therefore it is assumed

that corticosterone at basal levels acts via the MR in tissues lacking 11β-HSD2 while at elevated concentrations it additionally binds to the GR. Previous studies in microglial cells suggested that the MR mediates immunostimulatoty effects at low corticosterone concentrations while the immunosuppressive activity of GCs at a high concentration is exerted via the GR. Thereby GCs modulate the expression of pro-inflammatory cytokines and other modulators of the immune system. However, the positive and negative GC effects on peripheral macrophages are still unclear. Thus we asked whether i) dose-dependent activities of GCs exist in peritoneal macrophages and whether ii) GCs bind to different receptors, the GR and the MR, dependent on the hormone concentration.

In summary, this work aimed at studying the role of the GR and MR in different physiological situations by inactivating their expression using lentiviral RNA interference in primary cells and transgenic rats.

II. Materials

2.1 Chemicals and general materials

The chemicals were purchased from Merck, Roth, Invitrogen or Sigma Aldrich. Enzymes and fine chemicals were purchased from Biolads, Roche, Gibco/BRL, Promega or Phamacia. Medium and Agar were ordered from Invitrogen and Roth.

Acetone	AppliChem
Acrylamide (Gel 30)	Roth, Karlsruhe
Acrylamide (Gel 40)	Roth, Karlsruhe
Agar powder for bacteriology grade	AppliChem
Agarose	Roth, Karlsruhe
Ammoniumpersulfate	Roth, Karlsruhe
AMP	Sigma
β - Mercaptoethanol	Sigma (Cat.Nr. M7522)
Boric acid	Roth, Karlsruhe
Bovine-Serum Albumin (BSA)	Roth, Karlsruhe
Bromophenol blue	Roth, Karlsruhe
Calcium chloride	Roth, Karlsruhe
Chloroform	AppliChem
Dexamethasone	Sigma Chemical GmbH
Diethanolamin	Merck
Diethylpyrocarbonate (DEPC)	Sigma Chemical GmbH
Dimethylsulfoxide (DMSO)	Roth, Karlsruhe
1,4-Dithiothreitol (DTT)	Invitrogen

dNTPs (dATP, dCTP, dGTP, dTTP)	MBI Fermentas
EDTA	Roth, Karlsruhe
Ethanol	AppliChem
Ethidium Bromide (EtBr)	Sigma Chemie
Fetal Calb serum (FCS)	Gibco/BRL GmbH
Ficoll400	Roth, Karlsruhe
Formaldehyde (37%)	Sigma Chemie GmbH
Glycerol	Ferak, Berlin
Glycogen	Roche(Cat.Nr. 901 393)
Glucose	Ferak, Berlin
HEPES	Sigma Chemie GmbH
Isopropanol	AppliChem
Potassium chloride	Roth, Karlsruhe
LB culture medium	Invitrogen
Magnesium chloride	Roth, Karlsruhe
Milk powder	Roth, Karlsruhe
NP-40	AppliChem
N-(1-Naphthyl)ethylenediamine	Sigma Chemie GmbH
OligodT$_{(15)}$	Roche, Promega
Paraformaldehyde	Sigma Chemie GmbH
Penicillin-Streptomycin solution (50mg/ml)	Gibco/BRL GmbH
Percoll	Amersham
Phenol/chroloform	Roth, Karlsruhe
PMSF	Sigma Chemie GmbH

Polyvynilpyrotidon	Sigma Chemie GmbH
Ponceau S	Sigma Chemie GmbH
Potassium chloride	Roth, Karlsruhe
RPMI 1640 medium	Gibco/BRL GmbH
Sodium acetate	Roth , Karlsruhe
Sodium azide	Roth, Karlsruhe
Sodium chloride	Roth, Karlsruhe
Sodium hydrogen phosphate (Na_2HPO_4)	Ferak, Berlin
Sodium dihydrogen phosphate (NaH_2PO_4)	Ferak, Berlin
Sodium hydroxide	Roth, Karlsruhe
Sodium pyruvate	Gibco BRL
SDS	AppliChem
Sulfanilamide	Sigma Chemie GmbH
TEMED	Roth, Karlsruhe
Tris	AppliChem
Triton X-100	Sigma Chemie GmbH
Trizol Reagent	Invitrogen
Trypanblue	Sigma Chemie GmbH
Tween-20	Roth, Karlsruhe
Urea (Harnstoff)	Roth, Karlsruhe
Xylen cyanol FF	Roth, Karlsruhe

2.2 Enzymes and inhibitors

Alkaline Phosphatase, shrimp	Promega
DNaseI, RNase free	Roche
Iodacetamid	Roche
Klenow Fragment	Roche, MBI Fermentas
Leupeptin	Calbiochem
Pefabloc	Roche
Pfu-DNA ploymerase	Promega
Polynucleotide Kinase	MBI Fermentas
Proteinase K	Roche
Protease inhibitor tablet /complete mini	Roche
Restriction Enzymes	MBI Fermentas, Roche, Promega, NEB
Reverse Transcriptase, SuperscriptII	Invitrogen
RNase A	Qiagen, Hilden
T4-DNA Ligase	Promega, MBI Fermentas
Taq DNA polymerase	Promega, Roth, Genetech

2.3 Reagent kits

Absolute SYBR Fluoroscein Mix	ABgene
BigDye Terminator v3.1 Cycle Sequecing Kit	Applied Biosystems
Calcium Phosphate Transfection Kit	Invitrogen
Complexe Streptavidine-Biotine/PER+	Dako
Gel extraction Kit (QIAEX II)	Qiagen

High Pure PCR product Purification Kit	Roche
iQ™ SYBR Green Supermix	BIO-RAD
QIAquick Nucleotide Removal Kit	Qiagen
QIAfilter Plasmid Midi/Maxi Kit	Qiagen
RNeasy Kit	Qiagen
SuperSignal West Pico Chemiluminescent Substrate	Pierce
Wizard SV Gel and PCR Clean-Up System	Promega

2.4 Radioactive materials

[γ - ^{32}P] ATP (3000Ci/nmol)	GE Healthcare
[α - ^{32}P] CTP (3000Ci/nmol)	GE Healthcare

2.5 Size markers

DNA size marker: I kb Ladder	Invitrogen
DNA size marker: 10bp Ladder	Invitrogen
RNA size marker: Low Range RNA	MBI Fermentas
Protein size marker: Full range Rainbow MW	MBI-Fermentas, PeqLab

2.6 Antibiotics

Ampicilin:	100mg/ml in 70% Et-OH , Sterilize by 0.45㎛ filteration, -20°C store
Kanamycin:	10mg/ml in H_2O, Sterilize by 0.45㎛ filteration, -20°C store
Zeocin:	100mg/ml in H_2O, -20°C store
G418:	350mg/ml in H_2O, Sterilize by 0.45㎛ filteration, -20°C store

2.7 Antibodies

Antibodies for Western blot

Antigen	Source	Dilution	Manufacturer
Glucocorticoid Receptor	Rabbit	1:1000	Santa Cruz Biotech
Mineralocorticoid Receptor	Rabbit	1:200	Santa Curz(H300)
Mineralocorticoid Receptor	Rabbit	1:500	Wolfgaus Sclemid, Heidelberg
Green Fluoroscence Protein	Rabbit	1:1000	Cell Signaling
DsRed polyclonal IgG	Rabbit	1:5000	BD Science
Beta-Tubulin(polyclonal)	Mouse	1:2000	Sigma Aldrich GmbH
Beta- Actin (polyclonal)	Mouse	1:1000	Santa Curz Biotech
Rabbit IgG-HRP	Goat	1:5000	Santa Curz Biotech
Mouse IgG-HRP	Goat	1:5000	Sigma Aldrich GmbH

Antibodies for FACS staining

Antigen	Antibody	Format	Clone	Source
CD11b/c	Rat Intergrinα	Biotin	OX-42	BD Pharmingen
RT1B	Rat MHC classII	Biotin	OX-6	BD Pharmingen
RT1D	Rat MHC classII	PE	OX-17	BD Pharmingen
CD80	Rat B7.1 molecule	Biotin	3H5	BD Pharmingen
CD86	Rat B7.2 molecule	PE	24F	BD Pharmingen

2.8 Consumables

96, 48, 24, 12, 6 well plate Greiner bio-one

96 well plate for ELISA	NUNC
6,10 cm cell culture dish	Greiner
1.5ml Eppendorf centrifuge tube	Sarsted
15 ml, 50 ml centrifuge tube	Greiner bio-one
Absorbant paper	Whatman
Cell strainer	BD Falcon
Cuvette	Bio-Rad, USA
FACS tube	Greiner bio-one
GeneScreeen Plus Nylone membrane (RNA)	PerkinElmer
Hybond-N(Nitrocellulose membrane)	Amersham Buchler GmbH
MACS seperation LS cloumn	Miltenti Biotech
Pipette tips (yellow, blue)	Roth, Karlsruhe
Pipette tips (crystal)	MBP
PVDF membrane	Bio-Rad, USA
Steril filter (0.45 or 0.22㎛)	Millipore, France
Syringe: 2,5ml single-use syringe	B/BRAUN Melsungen
Ultracentrifuge tube	Beckman

2.9 Buffers and solutions

2.9.1. Culture Medium

2.9.1.1. Culture medium for Bacteria

LB-Medium: 20g LB Broth Base / 1L , sterilize by autoclaving

LB-Agar: 1L LB-Medium
15g Agar autoclave

Add the Ampicillin(100㎍/ml) Zeocin (25㎍/ml), Kanamycin (25㎍/ml) and pour in

Petridishes

<u>SOC-Medium:</u>

	Bacto-Peptone	4g
	Yeast-Extract	1g
	NaCl0	1g
	190ml H2O	
	+ 250mM KCl	2ml
	2M MgCl2	1μℓ

Adjust the pH to 7.0 with NaOH
Sterilize by autoclaving and store at 4℃
To 10ml Aliquots add 200μℓ 1M Glucose (steril)

2.9.1.2. Culture medium for eukaryotic cells

<u>DMEM (Dulbeco´s Modified Eagle Medium) of Gibco/BRL</u>

For lentiviral transfection medium:
Glutamax;
25mM HEPES
Pyruvat
50ml FCS (10%)
1ml Pen./Strep (500fold conc., 50kunit/50mg)
Mix and store at 4℃

<u>RPMI 1640 +L-Glutamat (Cat. Nr. 2006-04 of GIBCO)</u>

For peritoneal macrophages:
500ml RPMI1640
5% FCS (25ml)
1ml Pen/Strep
Mix and store at 4℃

<u>MEM (Minimal Essential Medium) institute's production</u>

For Hela (5% FCS) und 293T (10% FCS) cells:
500ml MEM
5% (25ml) or 10% (50ml) FCS
3.5ml Glutamate
1ml Pen/Strep
Mix and store at 4℃

<u>Freezing-medium</u>: Culture medium + 8% DMSO and 20% FCS

2.9.2. Molecular Biological Solution

TE (pH 8.0):	10mM Tris-HCl 8.0	
	1mM EDTA pH 8.0	
	Sterilize by autoclaving, store at RT	
1M Tris (pH 7.0; 7.5; 8.0):	Tris-Base	12.1g
	Adjust to pH 7.0; pH 7.5; pH 8.0 with HCl / 1L	
	Sterilize by autoclaving, store at RT	
0.5M EDTA (pH 8.0):	Ethylendiamintetraacetat -2H2O	186.1g
	Adjust to pH 8.0 with NaOH / 1L	
	Sterilize by autoclaving and store at RT	
3M NaOAc (Sodium Acetate):	NaOAc	40.8g
	Adjust to pH 5.2 with acetic acid / 1L	
	Sterilize by autoclaving and store at RT	

Plasmid Preparation Solutions

Buffer 1(Resuspension buffer)	50mM Tris/Cl pH 8.0
	10mM EDTA
	100μg/ml RNase A, store at 4℃
Buffer 2(Lysis buffer)	1% SDS
	0.2N NaOH, store at RT
Buffer 3(Neutralization buffer)	3M NaOAc, store at 4℃

Tail lysis buffer (TNE S buffer):	10 mM Tris pH 7.5
	100 mM EDTA
	400 mM NaCl
	0.6% SDS

Gel-Loading buffer

for DNA:	0.25%	Bromophenolblue
	0.25%	Xylen cyanol FF
	40%	Saccharose in H_2O

for RNA(Urea-polyacrylamide):	Deionized formamide	90ml
	0.25% Bromophenolblue	
	0.25% Xylen-cyanol FF	
	50% Glycerin	
	1mM EDTA pH8.0	
<u>TBE-Buffer (10x):</u>	Tris	108g
	Boric acid	55g
	0.5M EDTA	40ml
	Adjust to pH 8.0 / 1L	
<u>TAE-Buffer (50x):</u>	Tris	242g
	0.5M EDTA	100ml
	Acetic acid	60ml
	Adjust to 1L	
<u>SSC (20x):</u>	NaCl	175.3g
	Sodium Citrate	88.2g
	Adjust to pH 7.0 with 10N NaOH solution / 1L	
	Sterilize by autoclaving, store at RT	

<u>Hybridization solutions</u>

for southern blot(DNA):	20% SDS	100ml
	1M Na_3PO_4	100ml
	10% BSA	
	0.5M EDTA	400$\mu\ell$
	ad 200ml H_2O	
for northern blot(RNA):	20 X SSC pH7.0	7.5ml
	1M Na_2HPO_4 pH 7.2	600$\mu\ell$
	10% SDS	21ml
	100 X Denhardt's	300$\mu\ell$
	Steriled $2H_2O$	600$\mu\ell$
<u>*Denhardt's solution:</u>	Ficoll	1g
	Polyvynilpyrotion	1g

BSA 1g
Adjust to 50ml H_2O, store at -20℃

DEPC - H2O: 0.1% DEPC in H2O
Incubation at 37℃ for O/N, autoclave and store at RT

2.9.3. Cell biological Solutions

PBS (Phosphate Buffered Saline):

KCl	0.2g
KH2PO4	2g
NaCl	8g
Na_2HPO4	1.15g

Adjust to pH7.4 / 1L

FACS Buffer:
0.1% BSA
0.01%Azid in PBS

Trypanblue dye:
0.4% Trypanblue in basic RPMI 1640

Transfection solutions:

2x HBS:
50mM HEPES pH7,0
10mM KCl
12mM Glucose
280mM NaCl
1.5mM NaHPO4

0.5M CaCl2:
36.5 g $CaCl_2 \cdot 2H_2O$(Sigma) in 500ml H_2O
Filter sterilze through 0.45µM filter
Store at -20℃

2.9.4. Westhern Blot

Cell Lysate buffer:
140mM NaCl
20mM Tris pH7.5
2mM EDTA
1% Nonidet P-40 (NP-40)

RIPA:	0.1% (w/v) SDS	
	150mM NaCl	
	1 % (v/v) NP-40	
	0.5 % (w/v) Deoxycholat	
	5mM EDTA	
	50mM Tris/HCl (pH8.0)	
TGS (5x):	Tris	3.02g
	Glycin	18.7g
	10% SDS	10ml
	Adjust to 1L	
Protein-Loading buffer (5x):	60mM Tris/HCl (pH6.8)	
	25% Glycerin	
	2% SDS	
	0.1% Bromophenol blue	
	72mM β-Mercaptoethanol	
Protein-Staining solution		
Comassin Blue:	Coomassie brilliant blue	0.25g
	Methanol : H2O (1:1)	90ml
	Acetic acid	10ml
Protein destaining solution	30% Methanol;	
	10% Acetic acid in H2O	
TBS-T(0.1%), pH7.6:	Tris	2.42g
	NaCl	8g
	1M HCl	3.8ml
	0.1% Tween 20	
	Adjust to 1L H2O	
Transfer buffer (Semi-dry Blot):	25mM Tris	
	150mM Glycin	
	10%(v/v) Methanol	

<u>Balanced Salt Solution ;</u> BSS I (10X) :

Glucose	10 g
KH_2PO_4	0.6 g
$Na_2HPO_4 \cdot 2H_20$	2.3 g
Phenolred	0.1 g
add 1L H_20	

BSS II(10X) :

$CaCl_2 \cdot 2H_20$	1.86 g
KCl	4 g
NaCl	80 g
$MgCl_2 \cdot 6H_20$	2 g
$MgSO_4 \cdot 7H_20$	2 g
add 1L H_20	

Final solution: 1 vol. BSS I (10X) + 1 vol. BSS II (10X) + 8 vol. H_20

<u>BSS/BSA:</u> BSS with 0.1% Bovine Serum Albumine (BSA)

2.10. Bacteria Hosts

Strain	Company	Genotype
Top10F'	Invitrogen	F´[*lac*Iq Tn*10* (TetR)] *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G
XL-1 Blue	Stratagene	*rec*A1 *end*A1 gyrA96 thi-1 *hsd*R17 supE44 relA1 lac[F´ proAB *lac*IqZΔM15 Tn*10* (Tetr)]
XL-10 Gold	Stratagene	Tet^r *D(mcrA)183 D(mcrCB-hsdSMR-mrr)173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte [F' *proAB* $lacI^q$*ZDM15 Tn10* (Tet^r) Amy Cam^r][a]

2.11. Cell lines

Cell line	Cell type	Morphology
293T	Human embryonal kidney	Adherent fibroblastoid cell
HELA	Human cervix carcinoma	Epithelial-like cell
K562	Human chronic myeloid lekemia in blast crisis	Round large, single cells in suspension

2.12 Vectors

Vector	Object	Company
pBluescript SKII	Molecular cloning	Stratagen
pZero1	Molecular cloning	Invitrogen
pT7 Blue	Molecular cloning	Novagen
pcDNA 3.1	Protein expression vector for mammalian cells	Invitrogen
FUW	Lentiviral vector	Lois *et al* (2003)
FUGW	Lentiviral vector	Lois *et al* (2003)
pLentiLox 3.7	Lentiviral vector	Rubinson *et al* (2004) GFP expression
pLentiLox-DsRed	Lentiviral vector	DsRed protein expression

2.13 Instruments

Agarose gel reader	Mitsubishi
AutoMACs	Miltenyi Biotec
Bacteria Shaking incubator	B.Braun Biotech
CO_2 incubator	Heraeus Instruments

Centrifuge	Eppendorf
	ROTH
	Heraeus Instruments
Electroporator	Bio-Rad
ELISA-Reader ″v-max″	Molecular Devices
FACScalibur	Beckton Dickinson
Gel Dryer	H.Hoelzel GmbH
Heat block	Eppendorf
	Liebisch
Homogenizer	IKA, USA
Hybridization oven	Heraeus
Microscope	Leica
PCR	Eppendorf Mastercycler
	Mastercycler Gradient
pH meter	WTW
Power Supply	Pharmacia
	Consort
Protein Blotter (Semi-Dry)	Hoefer
Spectrophotometer	Pharmacia Biotech
Thermo-Cycler ″Biometra″	Biotron
Ultracentrifuge ″Combi plus″	Sorvall
Vortex (Genie 2)	Scientistic Industrie
Water Bath	LAUDA

2.14 Primer pairs

Oligonucleotides were constructed using Primer3 program (http://www.genome.wi.mit .edu/cgi-bin/primer/primer3_www.cgi) and synthesized by MWG biotech(Ebersberg) or Metabion(Muechen).

Primer name	Direction (5’ → 3’)
rMR1	CCTAGAGACCAATCTTTCCAAC
rMR2	GATGTTCTCCAGGATCATGGC
rMR-rev	CTACTGGATCCTCAAGACCTTCC
rMRF1	TAGTCGGTCTGGGATTTTGCC
rMRF2	CATGGCATCTTCAGTATGCAG
rMRF3	CCTCTGTGTCCAGCCCGGC
rMRF4	GGCTCCAGAAATGCCTTCAAGCTG
rMRF5	TGCAGGCTACGACAATTCC
rMRR1	GGAAGGTCTTGAGGATCCAGTAG
rMRR2	CTGGGAATGCGCTGTCCTCGCA
rMRR3	ACCTTCAGAGCCTGGGATTC
rMR1R	CTCAGGCTTCCTTGTTGGTTC
rGR1	CGGTCAGTGTTTTCTAATGGGT
rGR2	GGATTTTCCGAAGTGTCTTGTG
P/GR1	CAATAGGTCGACCAGCCGTC
P/GR2	CTGACCTCCAAGGACTCTCG
P/GR3	AGAATCCTTAGCTCCCCCTGG
P/GR4	CTGCTGCTTGGAATCTGCCTG
P/GR5	GACGGCTGGTCGACCTATTGA
FLAP-fwd	GGTACAGTGCAGGGGAAAGA
U6.seq	GAAACTCACCCTAACTG
U6-rev	CGAGCTTCTCCCACAAGTCT
31 - 5'	AGGAAACTCACCCTAACTGTAA
32 - 3'	CGGCCGCTTAAGCTTGGAAC
37	GGCTAATTCACTCCCAACGAA
38	GGCGCCACTGCTAGAGATTT

DsRed.fwd	ACCGGACTCAGATCC
DsRed.rev	GGCTGATTATGATCTAGAG
DsRed1.1	CGGCTCCTTCATCTACAAGG
DsRed1.2	GGTGATGTCCAGCTTGGAGT
DsRed1.3	GCTACCGGACTCAGATCCAC
DsRed1.4	GGGTGCTTCACGTACACCTT
FUW1	GACATAATAGCAACAGAC
FUW2	TAGCATGATACAAAGGC
FUW3	GGAGGGATAAGTGAGGCGTC
FUW4	GCTGAAGCTCCGGTTTT
GFP1	CGTGCTGCTTCATGTGGTCG
GFP2	TATATCATGGCCGACAAGCA
GFP3	GAACTCCAGCAGGACCATGT
eGFP-NheI	AAAGCTAGCCATGGTGAGCAAGGGCGAG
IRES1	GCTACAGACGTTGTTTG
pUC/M13 forward	CGCCAGGGTTTTCCCAGTCACGAC
pUC/M13 reverse	TCACACAGGAAACAGCTATGAC
rat 5S	CCCGATCCTGCTTAGCTTCCG
hactin-antisence	CTAGAAGCATTTGCGGTGGACGATGGAGGG
hactin-sence	TGACGGGGTCACCCACACTGTGCCCATCTA
rActin-S	AGCCATGTACGTAGCCATCCAGGCT
rActin-AS	CTCTCAGCTGTGGTGGTGAAGCTGT
rHPRT2a	GTCCCAGCGTCGTGATTA
rHPRT2b	TCAAGGGCATATCCAACAAC
rCXCL1.1	GCGGAGAGATGAGAGTCTGG
rCXCL1.2	TCCAAGGGAAGCTTCAACAC
rCXCL10.1	GCTTATTGAAAGCGGTGAGC
rCXCL10.2	GGGTAAAGGGAGGTGGAGAG
rGM-CSF.1	CGTGCTCTGGAGAACGAAA
rGM-CSF.2	GCCATTGAGTTTGGTGAGGT
rTNF-alpha.1	CTGTGCCTTAGCCTCTTCTC
rTNF-alpha.2	GCAAATCGGCTGACGGTGTG
rIL1b.1	CTGTGACTCGTGGGATGATG
rIL1b.2	GGGATTTTGTCGTTGCTTGT

rIL6.1	AGTTGCCTTCTTGGGACTGA
rIL6.2	ACAGTGCATCATCGCTGTTC
rIL-12.1	ACCCTCACCTGTGACAGTCC
rIL-12.2	CAGAGTCTCGCCTCCTCTGT
riNOS1	AGGGAGTGTTGTTCCAGGTG
riNOS2	TCCTCAACCTGCTCCTCACT
riNOS3	CACCTTGGAGTTCACCCAGT
riNOS4	GCTCCTGGAACCACTCGTAC
rCOX2.1	CTGAGGGGTTACCACTTCCA
rCOX2.2	TGAGCAAGTCCGTGTTCAAG
rCOX2.3	GCAGGAAGTCTTTGGTCTGG
rCOX2.4	CTCGTCATCCCACTCAGGAT
rTLR4.1	TGCTCAGACATGGCAGTTTC
rTLR4.2	TCAAGGCTTTTCCATCCAAC
rCD14.1	TTGTTGCTGTTGCCTTTGAC
rCD14.2	CGTGTCCACACGCTTTAGAA
rScd2.1	CCAGAGCGTACCAGCTTTTC
rScd2.2	TTACCCACTTCGCAAGCTCT
rSHPK1.1	TTCTGGAGGAGGCTGAGGTA
rSHPK1.2	CTCTCCCAGTCAGGTCGTTC
rKcnn4.1	CTGGTTCGTGGCCAAACTAT
rKcnn4.2	CAATGGTCAGGAACGTGATG
rPrss16.1	CGCCTGAGAGAACACATTCA
rPrss16.2	CTTGGCCAGTTCTGTGTTGA
rSgk1.1	ACGCAGCTGAAATAGCCAGT
rSgk1.2	TGTGCTCGATGTTCTCCTTG
rENaC-α.1	CTTCCAACTGTGCAACCAGA
rENaC -α.2	TGAAGCGACAGGTGAAGATG
rENaC -β.1	CCTCATTGAGTTTGGGGAGA
rENaC -β.2	TGTCAGGCTGGAAGACACAG
rENaC -γ.1	ACTGCTGGTGACCTGCTTCT
rENaC -γ.2	ACTTGCAGCCCGTACTCACT

III. Methods

3.1 Methods in Molecular biology

3.1.1 DNA work

For the isolation of plasmid DNA from bacteria or the creation of continuous cultures of these bacteria over night cultures were set up. To this end 1ml culture on 2ml LB medium containing the suitable antibiotics, was inoculated either with a single colony from an agar plate or with 100$\mu\ell$ of a glycerol stockculture. For larger isolation, single colony from an agar plate was picked or 100$\mu\ell$ of a Glycerol stockculture were inoculated into 50ml LB medium. The bacterial culture was placed in 15ml test tubes and/or in 100ml Erlenmeyer flasks and incubated in bacteria shaker at 37°C and180rpm over night.

3.1.1.1. Isolation of plasmid DNA (MINI and QIAplasmid Midi methods)

To isolate plasmid DNA from bacteria culture, a single colony from a streaked plate was picked and inoculated in 2-3ml LB medium containing the appropriate selection antibiotics and incubated at 37°C overnight at 180rpm in a shaking incubator. The bacteria were centrifuged for 1 min at 10000rpm at 4°C. The bacterial pellets were completely resuspended in 300$\mu\ell$ buffer P1 and incubated for 5 min at RT. 300$\mu\ell$ Buffer P2 was added, gently mixed by inverting 5-6 times and incubated for 5 min at RT. And then 300$\mu\ell$ chilled buffer P3 was added, gently mix by inverting the tube a few times and incubated on ice for 5-10 min. The bacterial lysate was centrifuged at 14000rpm for 10min at 4°C. The supernatant was transferred to a fresh tube and 480$\mu\ell$ (0.6 vol.) isopropanol was added, mixed by inverting a few times and incubated at RT

for 10 min. The sample was centrifuged for 10 min at 14000rpm, 4°C, the supernatant discarded and the pellet washed with 70% ethanol. Finally the DNA pellet was air-dried for 5 min at RT and dissolved in 50-100$\mu\ell$ H_2O. The DNA was kept at -20°C until use. For large preparations of plasmid DNA using the QIAfilter isolation kit, a single colony was picked and a starter culture was inocculated in 2ml LB medium containing the selective antibiotics for 8hrs at 37°C with 180rpm shaking. The bacterial culture was diluted 1 : 500 into selective fresh 50ml LB medium and inocculated at the same conditions. The lysis procedure was performed according to the above described protocol. After adding buffer P3, the lysate was gently mixed, poured into the barrel of the QIAfilter cartridge and incubated for 10 min at RT. The supernatant of the lysate was passed through the filter into the equilibrated QIAGEN-tips, washed two times and the DNA eluted. The eluted DNA was precipitated by adding 0.7 vol. isopropanol and washed with 70% ethanol. Finally the plasmid DNA was dissolved in a suitable volume of H_2O. The concentration of DNA was determined by spectrophotometer.

3.1.1.2. Isolation of genomic DNA

For genotyping of transgenic rats genomic DNA was isolated from the tail. Around 3-5mm of rat tail was incubated with TNES tail lysis buffer containing 35$\mu\ell$ protease K (10mg/ml) at 56°C, O/N. The following day 167$\mu\ell$ 6M NaCl was added to the lysate, vigorously mixed for 15 sec and then centrifuged for 10 min at 14000rpm at RT. The supernatant was transferred to a fresh tube and 1 volume cold 95% ethanol was added. The supernatant was incubated for 10min at RT and then centrifuged for 10min at 14000 rpm at 4°C. After spinning down the genomic DNA was collected as white deposits and washed with 70% EtOH. Finally, the genomic DNA was dissolved in 100$\mu\ell$ H_2O,

incubated for 10min at 56°C and stored at 4°C until perform the PCR.

3.1.1.3. Electrophoresis

3.1.1.3.1. Agarose gel electrophoresis

Agarose gels are suitable for separating of DNA fragments with a size of 400 to 20,000 bps. Dependent on the size of DNA fragments 0.7% ~ 2% agarose gels were routinely used with 0.5 X TBE buffer.

Percent of Agarose and expected DNA length

Agarosegel (%)	Separation range (kb)	Xylen Cyanol (kb)	Bromophenol Blue (kb)
0.6%	20kb-2.0	12	0.8
1%	8kb-0.5	2.3	0.375
1.5%	3kb-0.2	1.0	0.15
2.0%	2kb-0.1	0.6	0.06

The gels were made by boiling the agarose powder in 0.5x TBE buffer. After cooling to approximately 60°C, EtBr was added at a final concentration of 100ng/ml and the agarose solution was poured into the gel chamber. The samples were added with 1/6 vol. gel loading buffer (0.25% w/v Xylen cyanol, 0.25% Bromophenol blue, 40% Sucrose) and the gel run. a 1kb-marker (Invitrogen) served as size marker. The running buffer corresponds to the gel buffer (0.5x TBE) in its concentration. The DNA was separated at 5-10V/cm gel length. Ethidium Bromide was interposited into the nucleic acid fragments, which lights up after irradiation with UV light at a visible wavelength.

3.1.1.3.2. TAE-Polyacrylamide gel electrophoresis (non-denaturating gel)

Polyacrylamide gels were used to separate particularly small DNA fragment and to elute

siRNA oligomer duplex. A 10bp ladder by invitrogen served as a marker and the staining materials, bromophenol blue and xylen cyanol, were in the loading buffer.
The running property is dependent on the acrylamide concentration.

Percent of Polyacryamide and expected DNA length

Polyacrylamide (%)	Separation range	Xylen Cyanol	Bromophenol Blue
5	80-500	260	65
8	60-400	160	45
12	40-200	70	20
15	25-150	60	15

The gel apparatus was assembled with 1 ~ 2mm spacers between two cleaned glass plates (10 x 10cm). For a 8% TAE-polyacrylamide gel 2.5ml of 40% acrylamide solution was mixed with 200$\mu\ell$ 50x TAE and filled up with water to total 10ml. After addition of 75$\mu\ell$ 10% ammonium sulfate solution (APS) and 12.75$\mu\ell$ TEMED, the gel mixture was mixed briefly, poured into gel apparatus and allowed to polymerize. Prior to running of the gel, each well in the gel was washed with 1x TAE running buffer. The samples were mixed with 1/6 volume gel loading buffer (0.25% w/v xylencyanol, 0.25% bromophenol blue, 40% Sucrose) and run on the gel at 100V for 30 ~ 40 min. The 10bp size marker of invitrogen served as a size marker. Afterwards the gel was stained in 1μg/ml EtBr solution for 10 min and photographed. The respective DNA fragment was cut out under UV light and eluted.

3.1.1.4. Restriction Reaction

For digestion of 1μg DNA 1 to 10units restriction enzyme were used. The reaction

buffer conditions were dependent on the manufacturer data and enzymes.

The reaction volume and incubation time were generally 20$\mu\ell$ and 1 hour for 1µg DNA. Double digestion was accomplished, if the buffer conditions permitted it, at once. With certain restriction enzymes one had to pay attention that the enzyme volume did not exceed more than 1/10 of the total volume, since high glycerin concentrations from the enzyme storage buffer lead to so-called star activity of enzymes, i.e. to the additional hydrolysis of an alternative interface.

The components were usually used as follows:

10x Reactions Buffer	2 $\mu\ell$
DNA	x $\mu\ell$ (1-2 μg)
Restrictions enzyme	0.5 $\mu\ell$ (10U/$\mu\ell$)
ad 20 $\mu\ell$ with H_2O	

The restriction mixture was incubated for 1-2 hrs at 37°C.

3.1.1.5. Elution of DNA

DNA fragments were isolated from a prepared agarose or naive polyacrylamide gel and cut out using a UV hand lamp (λ=366) with a scalpel. The gel slices were transferred to a new tube and the weight determined on a balance. The 1mg gel was added into 100$\mu\ell$ distilled water and the DNA eluted with the Qiagen gel extraction kit, Ultraclean™15 DNA purification kit according to the weight of gel.

3.1.1.5.1. Gel extraction Kit (QIAEX II and Ultraclean™15 DNA purification Kits)

The elution of DNA from agarose was performed with the QIAEx II (Qiagen) and Ultraclean™15 DNA purification Kits (MO BIO) according to the manufacture's protocol. Finally the DNA was dissolved in 10-20$\mu\ell$ of H_2O / TE.

3.1.1.5.2. Elution from polyacrylamide gels

The polyacrylamide gel was used for elution of small DNA. After electrophoresis the gel band (under 200bp) was cut out using a UV hand lamp (λ =366). The fragment of gel slice was cut as small as possible. The gel slice was transferred to a new tube and 1.5 ~ 2 vol. of the diffusion buffer (0.5M NH_4OAC, 10mM MgOAC, 1mM EDTA, 0.1% SDS) were added, the reaction was incubated at 37°C for fragment below 80bp or 56°C for over 80bp for 30 min or O/N with occasional shaking. Finally it was centrifuged at maximum speed for 1 min. The supernatant was transferred to a new tube and the DNA fragment purified by extraction with phenol: chloroform and ethanol precipitation with glycogen. Finally the DNA was dissolved in 10 ~ 20$\mu\ell$ H_2O.

3.1.1.6. Recombinant DNA manipulation

3.1.1.6.1. Vector and Insert DNA preparation

Each cloning-strategy was accomplished using a certain endonuclease which recognize either 3' - or 5'- overhangs (sticky ends) or sites without overhangs (blunt ends) within a multiple cloning site (MCS).

2-5μg vector DNA was digested with a suitable restriction enzyme in accordance with the manufacturer data. After digestion of the plasmid DNA an alkaline phosphatase or klenow fragment was added and incubated at 37°C for 20 min for making blund ends or DNA dephosphorylation to prevent self-ligation of the vector plasmid DNA. The digested plasmid DNA was run through prepared agarose gel and the DNA fragment eluted from the gel.

In almost of experiments cleaned PCR products were inserted into the prepared plasmid vector. Suitable interfaces for the sticky end of PCR products were added by special

primers including two suitable restriction enzyme sites at the 5'- and/or 3'- end.

These nucleotides facilitate in the following digest the connection of the restriction enzyme to the PCR product and thus the complete modification of the ends. The digested PCR products were cleaned up again from the agarose gel, the concentration of the DNA measured by a spectrophotometer and used for ligation.

3.1.1.6.2. Ligation

For ligation reactions vector DNA and insert were set up a molecular ratio as follows, in 1:10 with blund end cloning, 2 different ratio of 3:1 or 1:3 with sticky ends, and used in total 100 ~ 200ng of DNA per ligation. The ligation mixture contained 0.5$\mu\ell$ T4 DNA Ligase (3U/$\mu\ell$, Promega), 2$\mu\ell$ 10x Ligationbuffer, and H_2O in a final volume of 20$\mu\ell$ and incubated for 2-16hrs at 13-16°C. It was stored at 4°C until transformation into competent cells.

3.1.1.6.3. Transformation to competent cells

3.1.1.6.3.1. Heat-shock transformation

The competent cells for heat shock transformation were treated with ice-cold $CaCl_2$ solution and then briefly heated to transfect with foreign DNA. Aliquots of each 50$\mu\ell$ of the competent bacteria cells were incubated for 10 min on ice before use and 2-5$\mu\ell$ of the ligation mixture added to each tube. The content of the tubes was mixed by swirling gently and incubated for 20 min on ice. Subsequently the tubes including the competent cells and the ligation mix were incubated for 45 sec in 42°C in a water bath, immediately transferred and chilled for 2 min on ice. 250$\mu\ell$ preheated SOC medium was added and incubated for 30 min at 37°C in a bacteria shaker (180rpm). Afterwards

the appropriate volume (up to 200$\mu\ell$) of transformed cells was transferred onto LB agar plate with appropriate antibiotic and gently spread the cells over the surface of the plate. The plate was incubated for 16-18h at 37°C. Single colonies were cultured in LB medium and digested with suitable restriction enzymes, positive recombinant DNA was identified by colony PCR with insert-specific primers and analyzed by gel electrophoresis for the presence of the desired inserts.

3.1.1.6.3.2. Electroporation

Electroporation is an alternative method to transfect foreign DNA into bacteria. This method has a better efficiency than heat-shock transformation. The preparation of cells for electroporation is considerably easier than preparing competent cells. The cells for this method are treated with $CaCl_2$, Glycerin and sometimes also β-Mercaptoethanol dependent on the cell's property. Bacteria were grown up to a O.D. (optical density) of 0.4-0.8 (λ=600nm), chilled, centrifuged and washed with low-salt buffer. The cells were resuspended in 10% glycerol at a concentration of the 2 x 10^{10} cells/ml, frozen in dry ice and stored at -80°C. Overall electroporation was carried out at low temperature. Routinely, the plasmid DNA or ligation mixture was mixed with cold cell, transferred to a chilled cuvette and subjected to an electrical pulse with set-up higher voltage.

3.1.1.6.4. Screening of recombinant DNA

3.1.1.6.4.1. Colony PCR for selection of positive recombinant DNA

After incubation of the plate for over night single colonies were picked, transferred into a PCR tube and filled up with a mixed PCR solution (dNTPs, 1x buffer, specific primers of insert and Taq polymerase) in total volume 50$\mu\ell$. Subsequently, the PCR reaction was

performed for 30-35 cycles using a suitable program and the PCR product analyzed in an agarose gel.

3.1.1.6.4.2. Sequencing reaction

In general the dideoxynucleotide method of Sanger is used for the sequencing of DNA. This sequencing reaction uses fluorescence-marked dideoxynucleotide at its 3'-end which can be identified nucleotide with four colors on each position and/or fragment as 4-color diagram during PCR. Under optimal conditions approximately 500 bases could be read per reaction.

The sequencing reaction consists of the following:

Components:	
DNA in H_2O (e.g. 500ng DNA of ca. 3kb plasmid DNA)	x $\mu\ell$
Primer (5pmol)	1 $\mu\ell$
Big-Dye (included Taq polymerase, dNTPs, ddNTPs, und buffer)	1 $\mu\ell$
5x Reactions buffer	1 $\mu\ell$

Fill up to 10$\mu\ell$ with distilled aqua. The reaction was performed for 25 cycles with 10sec 96°C, 15sec 50°C and 4min 60°C in the PCR. The reaction mixture was transferred to a new 1.5ml eppendorf tube and filled with 90$\mu\ell$ H_2O and added 0.1 vol. 3M sodium acetate(pH 5.2) and 2.5 vol. cold 100% ethanol. The mixture was centrifuged at 14000rpm for 20 min at RT. The precipitate was washed, dried twice with 70% ethanol and dissolved in 25$\mu\ell$ dye form amide buffer (Cat. NR. 4311320 by Applied Biosystems). The sample was denatured at 94 °C for 2 min and cooled down on

ice to analyze in the sequencing gel.

3.1.1.7. Ethanol precipitation

To concentrate DNA or change buffer after enzymatic treatment without DNA loss, the DNA was precipitated with 3M sodium acetate (pH 5.2) and 100% ethanol. This experiment was performed as described above (sequencing reaction cleanup).

3.1.1.8. Southern Blot

Determining the localization and amount of a particular sequence within genomic DNA is usually accomplished by southern blotting.

DNA preparation and separation:

Reagents

0.25N HCl	21ml 37% HCl in 1000ml H2O
Denaturation solution	1.5M NaCl
	0.5M NaOH
Neutralization solution	1.5M NaCl
	0.5M Tris/HCl pH 7.0

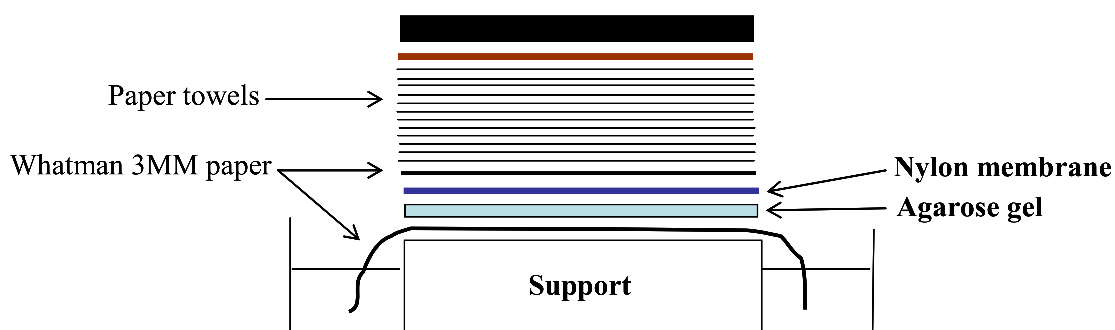
Genomic DNA was isolated from the tail of transgenic rats as described above. 10µg genomic DNA was digested with an appropriate restriction enzyme, 3unit / µg in 100$\mu\ell$ total volume at 37°C for overnight, precipitated with 3M sodium acetate at -80°C and resuspended in 15$\mu\ell$ $2H_2O$. The digested DNA was separated according to its size by electrophoresis through an 0.7% agarose gel containing EtBr in TAE buffer at 80 volts for 6 ~ 8 hrs. After electrophoresis, the agarose gel was washed once in $2H_2O$ and

soaked in at least two gel volumes of 0.25N HCl for 30 min. The DNA on the agarose gel was denaturated by soaking twice for 20 min in denaturation buffer with constant, gentle agitation and then soaked twice in neutralizing solution for 20 min. The gel was washed H_2O between the steps. The nylon membrane was floated thoroughly in distilled water and left in 20 X SSC until use.

Transfer of DNA to membrane:

To transfer fragments of DNA from the agarose gel to the nylon membrane, the capillary transfer method was used in this study. In the capillary transfer method, DNA fragments are carried from the gel in a flow of liquid and deposited on the surface of the membrane.

The apparatus for transfer blotter was built up in larger electrophoresis tray without any air-bubbles using a glass pipet in each layer, sealed the end of tray with parafilm to prevent evaporation of the transfer buffer during transfer and transferred the denaturated DNA from the gel in transfer buffer (20 x SSC; 350g NaCl, 176g Na-Citrate pH 7.0) over night by capillary action as described below:



On the next day, the membrane was soaked in 0.4N NaOH for 1 min and then in 1 x

SSC for 1 min for neutralization. The transferred DNA was immobilized on the wet membrane first by UV-crosslinking and then baked for 1 ~ 2 hrs at 80°C in a conventional oven. The dry membrane was wrapped loosely in a aluminum foil and stored at RT till use for hybridization.

Probe labeling with radioactive material :

For preparing of probe, the vector DNA was digested with restriction enzyme, separated on an agarose gel, the appropriate band extracted by elution kit and dissolved in TE buffer at final concentration 100ng/$\mu\ell$. For the labeling of the probe was used the following protocol:

Components:	Probe (ca. 100ng/$\mu\ell$)	1$\mu\ell$
	H_2O	13$\mu\ell$
	Incubation for 5 min at 95°C	
	Olb-C buffer	5$\mu\ell$
	[α-^{32}P] CTP	5$\mu\ell$
	Klenow	1$\mu\ell$

The double-stranded probe was denaturated by heating for 5 min at 95°C and rapidly chilled on ice. The remaining components were gently mixed and incubated for 2 hours at 37°C, then 75$\mu\ell$ TE buffer was added and the reaction was terminated by adding 1$\mu\ell$ 0.5M EDTA. The radiolabeled-probe was analyzed on chromatography paper in 0.75M Na_2HPO_4 and developed by X-ray film.

Hybridization and Detection

Reagents :

Hybridization solution(C&G hybsolution)	1M NaPi	100ml
	20% SDS	70ml
	10% BSA	20ml
	0.5M EDTA	0.4ml
	ad H_2O to 200ml	
C&G Wash solution(1 liter)	0.5M EDTA	2ml
	1M NaPi	40ml
	20% SDS	50ml

The membrane was prehybridized for 3 hours in hybridization buffer at 55°C, added 50 $\mu\ell$ of the radiolabeled probe and hybridized for over night at 55°C in rotating hybridization oven. Next day, the membrane was soaked once for 10 min in C&G wash solution at 55°C in rotating hybridization oven rotating at very low speed and finally soaked three times for every 15min in C&G wash solution at 68°C.

After washing, the membrane was blotted with filter paper (Whatman 3MM) to remove most of the excess moisture, wrapped in plastic wrap prior to autoradiography and exposed to X-ray film with an intensifying screen at -80°C for 1 to 6 days.

3.1.2 RNA work

3.1.2.1 RNA isolation

At least 1x10^6 primary or cultured cells were re-suspended in 1 ml TRIZOL reagent and stored at -20°C until extraction of RNAs or processed immediately. The samples were thawed, 200$\mu\ell$ of chloroform were added and the samples were vortexed for 15 sec. After 5 min incubation at RT, the sample was centrifuged at 3500 rpm for 15 min and the upper phase was transferred to a fresh tube. RNA was precipitated by addition of 500$\mu\ell$ isopropanol and the mixture was incubated at RT for 10 min. After centrifugation, the RNA was washed with 80% EtOH and dissolved in 89$\mu\ell$ DEPC-treated water.

For removing genomic DNA contamination the extracted RNA was treated with DNaseI. 1$\mu\ell$ DNaseI and 10$\mu\ell$ buffer was added to dissolve RNA and incubated at 37°C for 30 min.

3.1.2.2. RNeasy cleanup of extracted RNA

After the DNaseI digest the sample with RNeasy mini kit by Qiagen in accordance with the manufacture's protocol of RNeasy mini kit. Finally the RNA was eluted with 30 ~ 40 $\mu\ell$ RNase free water. The concentration of extracted RNA was determined in an UV spectrophotometer. RNA was diluted 1:100 and measured at 260 nm wavelength. The absorption of 1 corresponds to a concentration of 40μg/ml RNA.

3.1.2.3 RNA gel electrophoresis

The integrity of the isolated RNA was analyzed in formaldehyde gels. 1μg RNA was added 5$\mu\ell$ formaldehyde loading buffer and incubated at 70°C for 20 min to denature

the RNA. After adding 1㎕ RNA-gel loading dye, samples were loaded on RNA gels.

3.1.2.4 cDNA synthesis

To synthesize cDNA, 1㎍ RNA was mixed with 1㎕ OligodT_{15} primer and the volume was adjusted to 12㎕ with H_20. The mixture was incubated for 10 min at 70°C and then chilled on ice. Afterwards, 4 ㎕ 5x transcription buffer, 2㎕ 100mM DTT and 1㎕ 100mM dNTPs were added and incubated for 2 min at 42°C. 1㎕ reverse transcriptase (Superscript II) was added and incubated for 10 min at 25°C. Thereafter the reaction mix was incubated for 50 min at 42°C and the reaction was terminated by incubation at 70°C for 15 min.

The cDNA synthesis reaction included the following components:

RNA (1µg RNA in 10㎕ DEPC-H_2O)	10㎕
5mM dNTPs	2㎕
Oligo dT Primer (500ng)	1㎕
Incubation for 5 min at 65℃, chill on ice and centrifugation	
First strand Buffer	4㎕
DTT	2㎕
Incubation for 2 min at 42℃	
Superscript ™ II	1㎕

After addition of all components the mixture was incubated for 1 ~ 1.5hrs at 42℃ and incubated for 10 min at 70℃. The cDNA was stored at -20℃ and 0.1-2㎕ cDNA was used for PCR as template.

3.1.2.5 PCR (Polymerase chain reaction)

3.1.2.5.1. Standard PCR

The DNA fragments were amplified from isolated genomic DNA or cDNA by reverse transcription using specific primer pairs. The PCR cocktail contained the following Components:

Component	Volume
10X Reaction buffer containing 15mM $MgCl_2$	5 μℓ
dNTPs mix (each 10mM)	0.4 μℓ
Reverse, Forward primer (100pmol/μℓ)	1μℓ
DNA template	1μℓ
Taq polymerase (5 U/μℓ)	0.1μℓ
ddH2O	up to 50 μℓ

The PCR reaction was performed for 40 cycles using the following protocol:

Pre-denaturation at 95°C for 2 min

40 cycles of: denaturation at 95°C for 1 min

annealing at 55.5°C for 1 min

elongation at 72°C for 1 min

Post-elongation at 72°C for 5 min

The PCR conditions and number of cycles depended on the size of the PCR products and base pair composition of the primers. The sequences of the primers used and their optimal annealing temperatures are indicated below.

3.1.2.5.2. Real-time PCR (Quatitative RT-PCR (qRT-PCR))

To confirm accuracy of the DNA, it could be made by different methods, e.g. digestion with restriction enzymes, sequencing, southern blot and in particular PCR. One of these methods, real-time reverse-transcriptase (RT) PCR quantitates the initial amount of the DNA specifically, sensitively and is more desirable alternative method than other quantitative PCR to detect the amount of the final amplified product at the end-point. The real-time PCR system is based on the detection and quantitation of a fluorescent dye.

The fluorescent signal increases in direct proportion to the amount of the PCR product in a reaction and is possible to monitor the PCR reaction where the first significant increase in the amount of PCR product correlates to the initial amount of target gene by recording the amount of fluorescence emission at each cycle during the reaction. The accumulation of PCR products is determined by an increase in fluorescence of the reporter dye. The SYBR green I, which is a cheap alternative fluorescence dye and bound only to double-strand DNA, permits quantitative real-time analysis of PCR products by measuring fluorescence signals at PCR amplicon including non-specific amplification and primer-dimer complex in the real-time PCR technology. During reaction the amplified dsDNA increases after each cycle and consequently more SYBR green can bind to these dsDNA so that the signal increases according to amount of the amplified dsDNA. The parameter Ct (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold. The quantification of gene can result in absolute copy numbers by comparing with a standard gene of well-known copy numbers. Alternative T values of target gene can be determined quantification N-fold high or down-regulation of the interesting gene by comparing with house keeping gene.

Quantitative PCR (qPCR) was performed on an iCycler instrument (Bio-Rad, Germany) using set of probes and primers specific for certain genes and rat beta-actin or HPRT as internal/endogenous control. The internal standard DNA was diluted from 10^6 to 10^2 molecules per $\mu\ell$ in DEPC-treated water. All samples were prepared as duplicate in one reaction to prevent the error rate in each sample. Each reaction contained the following ingredients:

cDNA (final 5ng ~ 50ng total RNA)	0.1 $\mu\ell$ ~ 1 $\mu\ell$
2x SYBR Green Supermix	12.5 $\mu\ell$
Primer mix (each 10pmol)	0.5 $\mu\ell$

* 2x SYBR Green Supermix: 100mM KCl, 40mM Tris-HCl, pH 8.4, 0.4 mM, dNTP, 50U/ml iTaq DNA polymerase, 6mM $MgCl_2$, SYBR Green I, 20nM fluorescein, stabilizers

The volume was filled up with distilled water to 25$\mu\ell$.

The reaction of amplification was performed in following condition:

Pre-denaturation at 95°C for 3 min with BioRad SYBR mix

(Pre-denaturation at 95°C for 15 min with ABgene SYBR mix)

40 ~ 50 cycles of : denaturation at 95°C for 30 sec

annealing at 62 or 64°C for 30 sec

extension at 72°C for 1 min

Re-denaturation of amplicon at 95°C

Re-annealing for measuring melting point at 55°C for 10 sec

(The temperature rises every 1 °C up to 100°C, annealed for 10 sec)

The final step created the melting curve that shows the purity of the PCR products. The relative amount of amplified DNA molecules of a certain gene were caucluated by normalization with house keeping gene such as HPRT and β-actin.

3.1.2.6 Northern Blot

In general, northern blot is a protocol to determine the size and amount of mRNA molecules by hybridization with radiolabeled DNA or RNA probes followed by autoradiography.

In this study, northern blot was used to detect and confirm the synthesis of specific siRNAs in samples from transgenic animals (in vivo) and siRNA virus-infected cell lines (in vitro). All procedure of northern blot was performed according to the following protocol:

Denaturing Gel Electrophoresis

To separate the mRNA on denaturing gel, there are several ways; Glyoxal / DMSO (Dimethyl sulfoxide) method (McMaster and Carmichael, 1977) and formaldehyde method (Rave et al., 1979). The 15% Urea-polyacrylamide gel was made up at midi-gel thickness on small plates (10cm x 10cm x 2mm) with 15 well combs, allowed polymerization for 30 min and washed out the lanes.

The components of gel were followed below:

40% Acrylamide(38% acryl : 2% bisacryl)	9.5ml
5X TBE buffer	4ml
8.5M Urea	13.5ml
10% APS	250㎕
TEMED	40㎕

The 15μg total RNA samples in a volume of 15$\mu\ell$ or less were mixed with 6 X loading buffer containing BPB, XC, EDTA) by repeated pipetting and then carefully filled into the slot of the gel. Electrophoresis was carried out at 35mA for 40 min until the bromephenolblue dye migrated about 7 cm before the end of the run, in 1 X TBE as running buffer. The gel was stained with 1 μg/ml EtBr in 1 X TBE for 5 min, and analyzed for the tRNA and 5S rRNA bands (78 and 120nt) on the UV-monitor.

RNA transfer (Semi-dry Blot)

The RNA is separated according to size by electrophoresis through a denaturing gel and is then transferred to activated nitrocellulose or nylon membrane. The attachment of denaturated RNA to nitrocellulose is presumed to be nonconvalent but is essentially irreversible. Therefore it is possible to hybridize sequentially RNA immobilized on membrane to a series of radioactive probes without significant loss of the bound nucleic acid. Unfortunately nitrocellulose filters are not usually durable enough to withstand more than two rounds of hybridiziation and washing. This difficulty can be solved by transferring the RNA to positively charged nylon membranes, which stand up well to many rounds of hybridization without loss of hybridization signal. However, the background hybridization is considerably higher than nitrocellulose filters.

Nylon membrane (Genescreen plus) and 6 sheets of 3M Whatman filter paper were prepared to the size slightly larger than gel and presoaked in 1 X TBE. The gel was marked on the left edge after removed from the electrophoresis container and soaked in 1 X TBE. Transfer was set up as such from bottom to top as like southern blot; whatman paper – nylon membrane – gel – whatman paper and transferred at 75mA for 90 min.

Afterwards the RNA was immobilized on the moisted membrane by UV-crosslinking

for 1min at exposure to 1200 Jule (Stratagene Auto-Crosslinker) and baked for 1 hour at 80°C in a conventional oven and stored at 4°C until use for next step.

Oligo labeling with radioactive materials

: For labeling of oligonucleotide the following protocol was used:

Components:	Oligonucleotide (10μM)	2μℓ
	PNK buffer	2μℓ
	Polynucleotide Kinase (10U/μℓ)	1μℓ
	[γ-^{32}P] ATP	7μℓ
	ad sterilized ddH_2O to 20μℓ	

All components were gently mixed and incubated for 30min at 37°C. The reaction was terminated by adding 1μℓ 0.5M EDTA. To remove unlabeled oligonucleotides and radioactive materials the following protocol was used QIAquick nucleotide removal kit as described by the manufacture.

Hybridization with oligonucleotide probes

Reagents:

Hybridization solution	20X SSC (pH 7.0)	7.5 ml
	10% SDS	21 ml
	100X Denhardt's	300μℓ
	1M Na_2HPO_4 (pH 7.2)	600μℓ
	ddH_2O	600μℓ

* 100 X Denhardt's	Ficoll400	1 g
	Polyvynilpyrotidon	1 g
	BSA	1 g
	ad 50ml with ddH_2O , store at -20°C	

Washing solution	10% SDS	500 ml
	20 X SSC (pH 7.0)	150 ml
	ddH_2O	350 ml

The membrane was prehybridized for 1 hour in hybridization buffer at 42°C, added 100 $\mu\ell$ of the radiolabeled probe and hybridized for over night at 42°C in rotating hybridization oven. Next day, the membrane was soaked once for 10 min in wash solution at 42°C in rotating hybridization oven at very low speed and finally soaked twice for every 15min in wash solution into glass container at 42°C in shaking water bath. After washing, the membrane was blotted with filter paper (Whatman 3MM) to remove the excess moisture, wrapped in plastic wrap prior to autoradiography and exposed to X-ray film with an intensifying screen at -80°C for 1 to 6 days.

3.2 Protein work

3.2.1 Preparation of protein extracts

At least 1 X 10^6 cells were washed with PBS and lysed in 25 $\mu\ell$ RIPA buffer containing a cocktail of protease inhibitors. The lysates were incubated for 30 min on ice and centrifuged at 14000 rpm for 10 min at 4°C. The supernatant was transfered to a fresh tube, 6 X lammli protein buffer was added and after denaturing at 100°C for 5 min

proteins were stored at -20°C. The concentration of protein extracts were determined using Bradford assay from Biorad by measurement at absorbance 595mM in spectrophotometer.

3.2.2 SDS-polyacrylamide gel electrophoresis

Protein extracts were separated on 8-12% denaturing SDS polyacrylamide gels. The separating gel mixture was prepared, poured into the gel apparatus and overlaid with water. After the separating gel was polymerized, the overlay was decanted and the prepared stacking gel was poured. Protein samples were mixed with lammli buffer containing 5% 2-mercaptoethanol, loaded and electrophoresed in 1 X TGS running buffer.

Concentration of SDS-Polyacrylamide-Gel:

Acrylamide Cocentration (%)	Expected size of protein (kDa)
15	12-43
10	16-68
8	36-94

	Separating gel			Stacking gel
	8%	10%	12%	3%
ddH_20	4.85 ml	4 ml	3.35 ml	3.7 ml
30% acrylamide	2.5 ml	3.33 ml	4 ml	0.78 ml
1.5 M Tris pH 8.8	2.5 ml	2.5 ml	2.5 ml	-
1.0 M Tris pH 6.8	-	-	-	0.75 ml
10% SDS	100 µl	100 µl	100 µl	60 µl
10% APS	50 µl	50 µl	50 µl	30 µl
TEMED	1 . µl	15 µl	15 µl	7.5 µl
total	10 ml	10 ml	10 ml	6 ml

3.2.3 Western Blot

Reagents:

Transfer buffer:	25mM Tris
	150mM Glycin
	10% (v/v) Methanol
Blocking buffer:	5% milk powder in PBS-0.1% Tween20
Washing buffer:	0.1% Tween20 in PBS

Proteins from the SDS-PAGE gel were electroblotted to a PVDF nitrocellulose membrane at 50mA for 90 min using the semi-dry method. The membrane was stained with Ponceau S to control the transfer of the protein molecular weight marker and proteins. The membrane was then washed several times with H_2O and washing buffer to remove Ponceau S and incubated with blocking solution (3% milk powder in 0.1 % PBST) for 1 hour at RT. The nitrocellulose membrane was incubated with the monoclonal or polyclonal primary antibody solution diluted 1: 200 to 1:1000 in 0.1% PBST for O/N at 4°C or for 1hour at RT. The next day the membrane was washed with washing buffer three times for 10 min each and incubated with a horseradish-peroxidase-conjugated secondary Ab for 1 hour at RT. After incubation, the membrane was washed, treated with enhanced chemiluminescent substrate (ECL; Amersham) for detection and exposed to X-ray film.

3.3 Cellular methods

3.3.1 Isolation of primary peritoneal macrophages

To isolate take peritoneal macrophages, CD:Crl rats purchased from Charles River (Sulzfeld, Germany) were injected i.p. with 5ml of 4% sterile thioglycollate medium. After 4 days the cells were harvested by peritoneal lavage with 20ml balanced salt solution (BSS). The peritoneal lavage was centrifuged at 1550 rpm for 5 min at 4°C and washed twice with cold PBS. The sample was suspended in 1 ~ 2ml BSS containing 1% BSA and subsequently biotinylated Ox42 monoclonal antibody recognizing $CD11b/c^+$ cells was added at 1:5000, mixed well by vortexing and incubated at 4°C for 20 min. The sample was washed twice with BSS/BSA and resuspended in 500$\mu\ell$, adding 1:10 streptavidine microbeads and incubated at 4°C for 20 min. After washing twice with BSS/BSA the cell pellet was resuspended in 1ml BSS/BSA and the $CD11b/c^+$ cells were magnetically purified by MACS. After washing with BSS/BSA, the pellet was resuspended in RPMI-1640 medium complemented with 10% FCS and penicillin/sterptomycin. The cell number was determined using 0.04% trypan blue in PBS diluted 1:50.

3.3.2 Cell culture

3.3.2.1. Thawing and Freezing of cell

Cell lines were kept in liquid nitrogen. In order to avoid the formation of cell destructive ice crystals when freezing, at least 4 X 10^6 cells were harvested in the exponential growth phase and resuspended in freezing medium (RPMI and/or DMEM, 20% FCS, 8% DMSO), freezed slowly in cryotubes at -80°C and then transferred into liquid nitrogen. To thaw the frozen cells in culture, the frozen cells were thawed with

37°C in the water bath, immediately transferred, mixed with culture medium and centrifuged for 5 min at 1550rpm. The cell pellet was resuspended in the appropriate medium and cultured at 37°C in a 5% CO2 incubator with 100% humidity to grow to around 80 ~ 90 % confluence in a culture container.

3.3.2.2. Culture of cells

All cells were cultured in suitable medium at 37°C, 5% CO2 in a H_2O-saturated atmosphere in usual plastic cell culture containers.

To expand suspension cells they were resuspended and transferred to new culture container with a suitable volume of fresh medium at a final 1 X 10^5 cells concentration. Adherent cells such as 293T-cell were treated with small volume of ATV solution and waited for 2 ~ 3min for transition to suspension, stopped the ATV reaction by adding with culture medium. The cells were centrifuged for 5 min at 1600rpm, resuspended in appropriate medium and cultured continuously. Subsequently, the amounts of living cell were determined in counting chamber with 0.4% trypan blue solution.

3.3.3 FACS (Immunfluoresence and flow cytometry)

Flow cytometry is based on the expression of antigens on cellular surface quantitatively and their detection by fluorescently labeled antibodies. Three different wave lengths of light recognize the size of the cells over the so-called "forward Scatter", which forward diversion of the laser beam and the grained cells over the "Side Scatter", which measure lateral dispersion of the laser beam. Usually 10000~50000 cells were measured and analyzed through the virtual window ("gate"). The dates are analyzed in CellQuest software program and represented as linear histograms or two dimensional DOT plots.

$4 \sim 6$ X 10^5 cells were transferred to a FACS tube and washed with FACS buffer (PBS/0.1% BSA/0.01% Azide) for staining. Cells were incubated with PE- or biotin-conjugated antibodies for 20 min at 4°C under dark condition and then washed twice with FACS buffer. In the case of biotinylated Abs cells were incubated with streptavidin-cychrome in a second staining step. Stained cells were analyzed on a FACSCalibur using CellQuest software (Becton Dickinson).

3.3.3.1. Accumulation by fluorescence-activated cell assortment (FACSort)

For the accumulation of certain cell populations preparative cell sorting was used.

The cells were transducted with lentiviruses expressing green fluorescence protein, harvested and passed through 0.45μM filter-cap FACS tube. The prepared cells were sorted through fluorescence-activated cell assortment machine. The sorted cells were washed twice with culture medium and immediately cultured in suitable culture medium.

3.3.4. Stimulation and Cort or Dex treatment

To induce an inflammatory response in primary macrophages the purified peritoneal macrophages were prepared either in tissue-cultured 96well plate at a concentration of 1x 10^5 cells in each well for the analysis of nitric oxide production or 12well plate at concentration of 1x 10^6 cells for the analysis of mRNA expression. The macrophages were incubated for 4 hours at 37°C, 5% CO2 to allow for the adhesion of cells, washed three times to remove the non-adherent cells and cultured continuously in fresh medium as described 3.3.1. Usually the macrophages were stimulated with 100ng/ml LPS (Sigma) and 100 units/ml recombinant rat IFN-γ (R&D system, Germany) for 48 hours to determine nitric oxide production or for 6 hours to analyze changes in mRNA

expression. Corticosterone (Sigma) and Dexamethasone (Dex) (Sigma) were added at various concentrations; 1μM and 0.1nM, from 1000X stock in 70% ethanol.

3.3.5. Nitric Oxide (NO) release assay

The cell-free macrophage culture supernatants were individually collected from the 96 well plates and used for the nitric oxide release assay. 50$\mu\ell$ of supernatant was mixed with 50$\mu\ell$ of each of the two Griess reagent, 1% sulfanilamide and 0.1% N-Naphthyl -ethylene-diamine-dihydrochloride, both diluted in 2.5% phophoric acid, and incubated for 5 min at RT until the color developed. The concentration of NO^{-2} ions was measured by spectrophotometer at 560nM and compared to a $NaNO_2$ standard curve ranging from 1μM to 125μM.

3.4. RNAi technology

3.4.1. shRNA construct with lentiviral vector

The shRNA (small hairpin RNA) sequence was designed as 21 ~ 23 nucleotides based on the targeting sequence following by van parijs' lab's protocol , which are found the appropriate mRNA sequence AAG(N18 ~ 22)TT of target gene. The G base is required to recreate the +1 site of the U6 promotor in lentiviral vector, thereby making it functional for the transcription of shRNAs. In order to use the mRNA sequence for shRNA for effectively gene sliencing, the sequences have to check that lower than 50% GC content, no terminator sequence of = or >4 A or T base and homology to other genes. Designed shRNA sequences consisted of one T base for starting signal of U6 promotor, the restriction sites for insertion into supposed lentiviral vector on 3' frame , the loop sequence , reverse complement of shRNA sequence and ternimator sequence of

the 6 T base, described as below.

e.g.) T G (N18 ~ 22) TTCAAGAGA (N18 ~ 22) C TTTTTT C
A C (N18 ~ 22) AAGTTCTCT (N18 ~ 22) G AAAAAA GAGCT

For annealing of oligomers oligomers were resuspended in water at 60pmol/㎕.

The annealing mixture contained the following components.

Component:	oligomer pairs	1㎕ (each 60pmol/㎕)
	annealing buffer	48㎕
* Annealing buffer:		100Mm KCl
		30Mm Hepes-KOH pH 7.4
		2Mm $MgCl_2$

The oligomer reaction was performed as follow two conditions:

One is faster protocol to anneal the oligonucleotide which is provided by vanparijs lab; oligo-mix was incubated at 95°C for 4 min, 70°C for 10 min. Then it was incubated on decrease temperature to 4°C slowly at 1°C per minute and final incubated at 4°C for 10 min. Alternative protocol was produced by Holger’s lab protocol; oligo-mixture was incubated at 95°C for 2 min, 65°C for 10 min, 37°C for 10 min, 20°C for 20 min and final incubated at 4°C for 10 min. Each step was run one time.

3.4.2. Lentivirus production

3.4.2.1. Transfection with Calcium-phosphate method

The calcium phosphate transfection method for introduction of DNA into mammalian cells is based on the formation of a calcium phosphate-DNA precipitate by slowly mixing with HEPES-buffer saline solution and routinely used to transfect a wide varity of cell types for either transient expression or for the production of stable transformants. An shRNA encoding lentivirus particles were produced following published protocol by transient co-transfection of four- plasmid; the two packing plasmids and a plasmid coding G protein, pMD2G-VSVG, into human embryonic kidney 293T cells.

The 293T cells were cultured in MEM (institute production) supplemented with 10% FCS at 37 ºC, 5% CO_2 incubator. For transfection, 1.8 X 10^6 or 4.5 x 10^6 293T cells were spilted either in 6 cm or 10cm tissue culture dishes on the day before transfection and changed the fresh transfection medium(DMEM medium complemented with 10% FCS and penicillin/streptomycin) 2 ~ 4 hours prior to precipitation.

The following DNAs made with Endo-free Qiagen kits were mixed as at the following amounts:

Name of DNAs	6 cm	10 cm
Lentiviral recombinant DNA	10 μg	20 μg
pMDLg/RRE	5 μg	10 μg
RSV-Rev	5 μg	7 ~ 10 μg
pMD2G-VSVG	3 μg	5 μg

Above table is case for 4 plasmid system to pLentiLox lentiviral vector. For 3 plasmid

system for FUW lentivirial vector, 10μg of pCMV-delta R8.91 DNA is used instead of pMDLg/RRE and RSV-Rev DNAs as packing plasmid. The DNA mixture was filled up with ddH_2O to 250$\mu\ell$, added 250$\mu\ell$ 0.5M $CaCl_2$ with mixing by pipetting and mixed 500 $\mu\ell$ of 2 X HBS buffer dropwise with bubbling for 12 ~ 15 times by pipette.

The DNA-calcium phosphate precipitate was incubated for 30 min at RT, added drop-wise all over the plate and gently swirled from front to back and incubated O/N at 37 ºC, 5% CO_2. The following day fresh MEM5 supplemented with 10% FCS was exchanged and returned to the incubator. The supernatant medium containing lentivirus particles was harvested at 24 and 48 hours after changing the fresh medium, filtrated through 0.45µm filters, and concentrated by ultracentrifugation at 25000rpm for 90 min at 4°C in Sorvall AH629 rotor. After spinning, the virus pellet was resuspended in 20-40$\mu\ell$ /tube of cold PBS and incubated for O/N at 4°C with horizontal shaking. Next day the suspension solution was centrifuged for 5 min at 2500rpm at 4°C, transferred to new tube and stored at -80°C. The titer of the virus was determined by measuring a green fluorescent protein (GFP) with serial dilutions in transducing HeLa cells by using flow cytometry.

3.4.2.2. Stable transfection with pcDNA construct in HeLa cells

Generation of stable long-term clones in cells is necessary to plasmid DNA has integrated into the chromosome. Plasmid DNA for transfection into mammalian cells must be clean and free from phenol and sodium chloride. The mammalian expression vector (pcDNA 3.1) contains the neomycin resistance gene for selection of stable ell lines using geneticin. The transfection of mammalian express vector was accomplished in HeLa cells. 5 X 10^5 HeLa cells were plated in 5ml medium on 6 cm culture dish and

on the next day the medium was replaced by warmed fresh medium. Prior to transfection, the pcDNA 3.1 construct was linearized with *Bgl*II restriction enzyme which located in upstream of CMV promoter on vector for 3 ~ 4 hours at 37°C, precipitated by sodium acetate and 100% ethanol and dissolved in 20 ㎕ distilled water. The construct was transfected into mammalian cell line using calcium phosphate method as desired protocol.

The precipitated DNA construct was mixed with 25㎕ 2M $CaCl_2$, added 250㎕ 2 X HBS with bubbling for a few seconds and incubated for 30 min at RT. After 24 hours of transfection, the cells were washed and added fresh culture medium. 48 hours after transfection, the confluent cells were split 1 : 10 or 1 : 15 as such less than 25% confluent into new fresh medium containing geneticin (G418 ; Gibco BRL) at pre-determined concentration 350 µg/ml and sed with selective medium every 3 ~ 4 days until geneticin-resistant single colony can be identified. After 10 to 12 days the single colonies was picked, transferred and expended in fresh medium containing 175 µg/ml concentration of geneticin on 96 or 48 well plates.

3.4.3. Lentivirus titration

The titer of virus particles and efficiency of the viral infection were determined on HaLa cells 3 or 4 dyes after transduction of viruses by FACS analysis.

At the same day as harvesting of virus particles, 1 X 10^4 HeLa cells were plated in 12well plate and incubated for 4 hours at 37°C, 5% CO2. The viruses were serially diluted in culture medium and added dropwise to cells. 3 ~ 4 days after transduction of the virus to cells the medium was sucked off, treated with 0.5ml ATV solution and washed with FACS buffer. Finally the cell pellet was resuspended in 200 ~ 500㎕

FACS buffer and analyzed on a FACSCalibur using CellQuest software (Becton Dickinson).

3.5. Immunohistochemistry

Immunohistochemistry protocol is widely used and applied to the detection of disease progression and differences between antigens and their recognition by antibody.

Preparation of paraffin embedding samples

The tissues were dissected and fixed in 4% paraformaldehyde solution for 2 hours or O/N depending on the size of the tissue. The protocol of paraffin embedding was followed generally published protocol and cut section of the tissues at 5-10 micrometers thick and smoothed out using water bath with distilled cold / warm water, allow the sections to dry upright in order to facilitate adhesion between the section and the charged glass surface and baked the slides for O/N at 37°C.

3.5.1. Hematoxylin-Eosin staining

The preparing paraffin section was deparaffinized as follows. The slides were incubated twice for 10 min each in xylene, followed by twice rinsed for 2 min each in 99% ethanol, hydrated twice by placing in 96%, 70% ethanol for 2 min each and washed in distilled water. The slides was stained for 10 min in hematoxylin and washed in flowed water for 10 min and stained for 7 min in 1% eosin. Afterwards the samples were washed in distilled water and dehydrated in reverse order of protocol of hydration; dehydrated twice by placing in 70%, 96%, 99% ethanol for 2 min each, incubated twice in xylene for 10 min each. One drop of mounting media was applied to tissue on slide

and placed coverslip over slide.

3.5.2. Immuno-staining for the Rat Mineralocorticoid receptor

Reagents:

Citrate Buffer	0.1M Citric acid	18 ml
	0.1M Sodium citrate	82 ml
	ad to 1000ml with H_2O pH 6.0	
POD Block	Methanol	8 ml
	2N NaN_3	1.2 ml
	3% H_2O_2	2 ml
AB-Complex (POD)	1 X PBS	1 ml
	Solution A	10 μl
	Solution B	10 μl
	Pre-incubation for 15 ~ 20 min	
DAB solution	DAB	1 tablet
	1 X PBS	10 ml
	3% H_2O_2	75 μl
	0.2µM filtration	

The paraffin section was deparaffinized by following as described above.

After hydration in ethanol the samples were washed for 5 min in fresh distilled water,

The slides in glass container were immersed and boiled for 30 min in citrate buffer (pH 6.0) using a microwave oven, rinsed four times in distilled water and PBS and blocked for 30 min in 10% BSA-PBS at RT. The slides were incubated O/N with primary antibody diluted 1 : 200 in blocking buffer at 4°C, on the next day excess lipid was removed from slides and treated with POD-Block for 13 min at RT. The slides were washed three times in PBS, treated with anti-rabbit IgG biotin as secondary antibody diluted 1 : 200 in 1% BSA/PBS for 45 min at RT, washed three time in PBS. The slides were immersed for 35 min in AB-complex diluted 1: 100 in PBS as for 35 min at RT, washed three times in PBS again. As last step, the slides were react with DAB solution at RT till visualize a certain brown color, immediately washed in distilled water for stop of the reaction. Depends on used primary antibody the slides were counterstained with hematoxylin for 30 sec to 3 min and washed in flowing water for 10 min. Finally the slides were dehydrated by followed protocol as described above and mounting media was applied one drop to tissue on slide and placed coverslip over slide.

IV. Results

Part I. Generation and Analysis of mineralocorticoid receptor knock-down rats

4.1.1 Expression of the mineralocorticoid receptor is efficiently down-regulated by RNA interference *in vitro*

4.1.1.1 Generation of lentiviral constructs encoding a specific sequence targeting the MR

To inactivate mineralocorticoid receptor expression in the rat, we identified a suitable siRNA (small interfering RNA) sequence based on the rat MR mRNA sequence (Accession Nr. M36074). Following the rule, which is a AA-(G N18)-TT sequence that is characterized by a low G/C content of ≈ 50% and no terminator sequence of ≥ 4 A / T, we found five possible candidate sequences for an siRNA against the MR. We designed shRNA-MR oligonucleotides by adding a signal sequence (T) at the first position for expression under the U6 promoter, a sense strand of 19 base pairs, a loop sequence followed by the reverse complementary sequence of the sense strand, the serial six T tail as a RNA polymerase III terminator, and a suitable restriction enzyme sequence, *Xho*I, on the 3' flank region (Table 4.1).

+1		Loop		Terminator	
T	GACAATAGTCGGTCTGGGA	TTCAAGAGA	TCCCAGACCGACTATTGTC	TTTTTT	CTCGAG
A	CGATGTTGATGATGTACTG	AAGTTCTCT	CAGTACATCATCAACATCG	AAAAAA	GAGCTC

Table 4.1 Sequence of the shRNA-MR stem loop

The designed shRNA sequence against the MR was annealed by the standard protocol established in the Reichardt laboratory, eluted from a TAE-polyacrylamide gel, and cloned into the lentiviral vector, pLentiLox–DsRed, which encodes the red fluorescent protein DsRed under the control of the early promoter of CMV (cytomegalovirus, P_{CMV}) and the mouse RNA polymerase III promoter U6 used to drive expression of the shRNA (Figure 4.1).

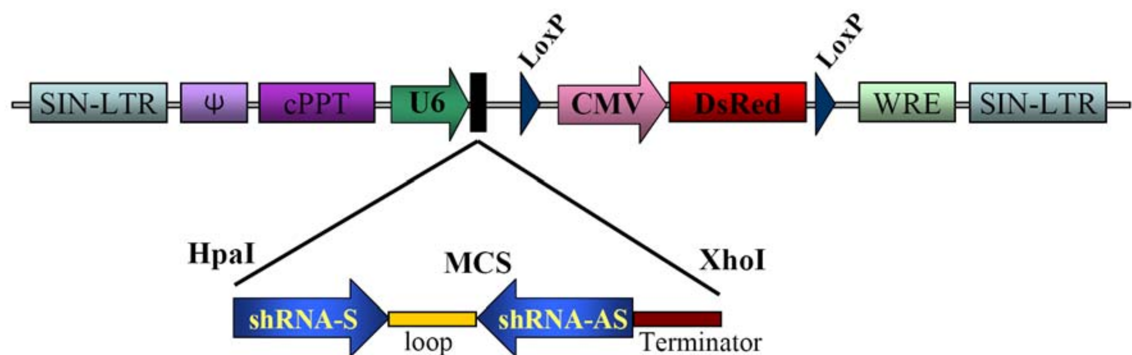


Figure 4.1 Schematic representation of the shRNA-MR expressing pLentiLox-DsRed lentiviral vector. We modified the pLentiLox 3.7 vector (Rubinson et al., 2003) to express the CMV-DsRed cassette by replacing eGFP (enhanced green fluorescent protein) with the red fluorescent protein DsRed excised from the pCMV-DsRed expression vector (BD bioscience, USA) using the *Nhe*I / *Nco*I restriction sites. pLL-DsRed is carrying two loxP sites, a CMV promoter, the mouse U6 promoter and downstream restriction sites to allow the efficient introduction of suitable shRNAs. An shRNA against the rat MR was inserted into the pLentiLox-DsRed vector using *Hpa*I as blund end and *Xho*I as 5'-sticky overhang restriction sites.

Figure 4.2 shows the annealed shRNA against the MR on a TAE-polyacrylamide gel. The annealed shRNA oligonucleotides run between 50 and 60 bp.

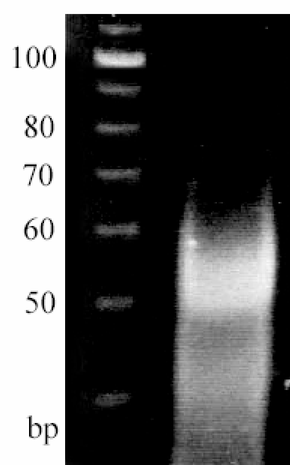


Figure 4.2 Annealing of the shRNA against the MR. The shRNA-MR oligonucleotides were annealed by the established protocol, separated through a 8% TAE-polyacrylamide gel, and the ≈ 60 base pairs oligonucleotides eluted. The 10bp DNA molecular marker was used to determine the size of the oligonucleotides.

To make the lentiviral shRNA-MR construct, the eluted shRNA-MR oligonucleotides were cloned into the pLL-DsRed lentiviral vector (pDR) using the restriction enzymes, *Hpa*I / *Xho*I. The recombinant shRNA-MR in the pDR construct was analyzed by suitable restriction enzymes (Figure 4.3). At first, we selected potentially positive clones by restriction analysis of the colony PCR (Figure 4.3 (A)). The amplified PCR product including the shRNA-MR sequence comprised two fragments of 1.4kb and 110bp whereas the control pDR product comprised two fragments of 1.4kb and 70bp. The positive constructs were analyzed by *Xba*I / *Xho*I double digestion. As in (A), the construct including the shRNA-MR sequence has an around 50bp bigger fragment than the control vector (Figure 4.3 (B)).

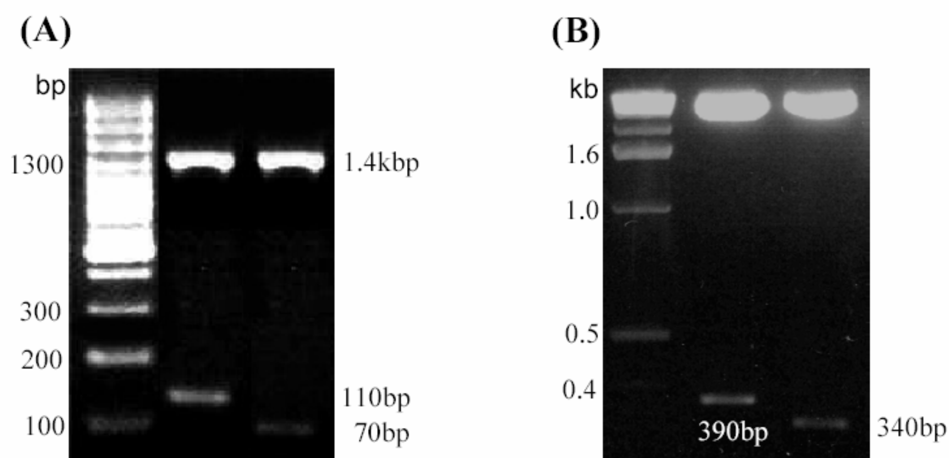


Figure 4.3 Restriction analysis of the shRNA-MR construct in pLL-DsRed. The recombinant shRNA-MR in the pLL-DsRed construct was analyzed by restriction digest. At first, we selected positive clones by colony PCR using specific primer sets and restriction analysis of PCR products with *Xho*I (A) and then confirmed the selected recombinant DNA by digestion with *Xba*I/*Xho*I enzymes (B).

4.1.1.2 Generation of the stably MR expressing HeLa cells

To accomplish the inactivation of MR expression by our shRNA *in vitro*, we made HeLa cells that stably express the MR by transduction with the FUrMRW lentiviral vector. This construct was cloned by inserting the rat MR full length cDNA into the FUW lentiviral vector (Lois et al., 2002) via *Bgl*II and *Bam*HI sites. This allows expression of the MR protein under the control of the ubiquitin promoter in cells. We performed sequence analysis to check the recombinant FUrMRW construct and analyzed it with several restriction digest (Figure 4.4). The FUrMRW construct was used to generate lentiviral particles by co-transfection of the three plasmids consisting of a packing plasmid, pCMV- Δ8.9, and a plasmid coding G protein, pMD2G-VSVG, by the calcium phosphate method in 293T cells. The lentiviral particles were then used to transduce wild type HeLa cells and analyzed for MR expression by western blot

using a specific MR antibody (Santa Curz, Germany) (Figure 4.5).

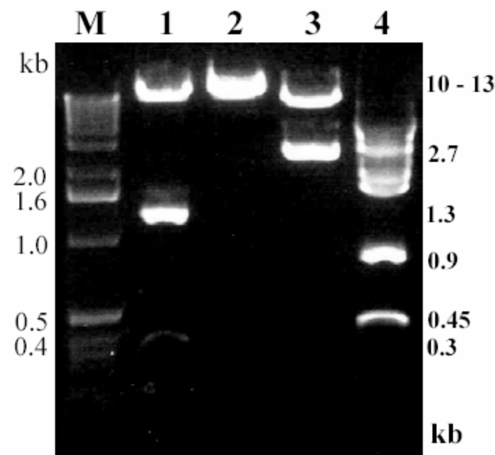


Figure 4.4 Restriction analysis of the FUrMRW construct. The rat MR cDNA was inserted into the FUW lentiviral vector using the *Bgl*II and *Bam*HI sites. The construct FUrMRW was digested with various restriction enzymes, *Eco*RI in lane1, *Bam*HI in lane 2, *Xba*I in lane3, and *Bgl*II in lane 4. The 1kb ladder was used to determine the size of the fragments by digestion.

As shown in Figure 4.5, the FUrMRW-transduced HeLa cells strongly expressed the MR similar to K562 cells. The stably MR expressing HeLa cells were used for further studies in this work.

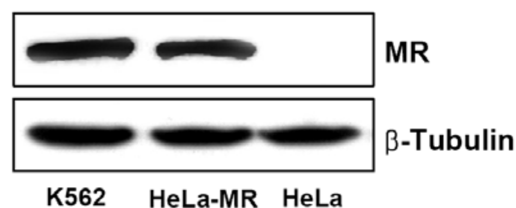


Figure 4.5 Analysis of the stably MR expressing HeLa cells. The lentiviruses encoding the rat MR were used to transduce HeLa cells for 4 days. We performed a western blot to test MR expression in the lentivirus-transduced HeLa cells. As a control we used K562 cells that strongly express the MR protein and native HeLa cells as negative control. Staining with an anti β-Tubulin antibody was used as a loading control.

4.1.1.3. RNAi-mediated silencing of MR expression *in vitro*

The construct containing the shRNA against the MR cloned into pLL-DsRed was called pLL-siMR. This plasmid and the control pLL-DsRed vector were used to produce lentiviral particles by transient co-transfection using the four plasmid system consisting of the packing vectors, pMDLg/pRRE and pRSV-Rev, and the envelope coding G protein gene, pMD2G-VSVG, using the calcium phosphate method and 293T cells. In order to determine the titer of the lentivirus precipitation, the virus particles were used to transduce HeLa cells by serial dilutions. The titer was determined by measuring DsRed expression by flow cytometry.

Next, to assess the efficacy of gene inactivation by the shRNA *in vitro*, the rat MR expressing HeLa cells were transduced either with pLL-siMR or the control virus pLL - DsRed. Flow cytometric analysis for DsRed expression showed that transduction efficiency was greater than 95% (Figure 4.6).

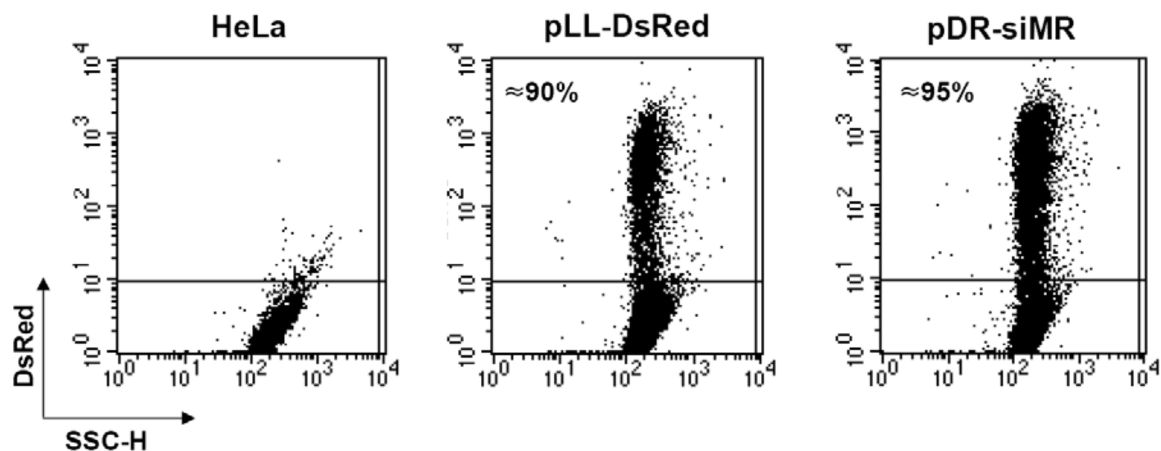


Figure 4.6 Analysis of DsRed expression on lentivirus-infected HeLa-MR cells.

After transducing HeLa-MR cells with pLL-siMR or pLL-DsRed, they were harvested, washed with FACs buffer, and DsRed expression measured by FACS.

The lentivirus-infected HeLa-MR expressing cells were analyzed by quantitative PCR and western blot. Importantly, MR expression was strongly diminished in pLL-siMR transduced cells, both on the mRNA as well as the protein level (Figure 4.7 (A), (B)). Quantitative PCR confirmed that the amounts of mRNA transcript were also reduced by more than 90% (Figure 4.7 (C)).

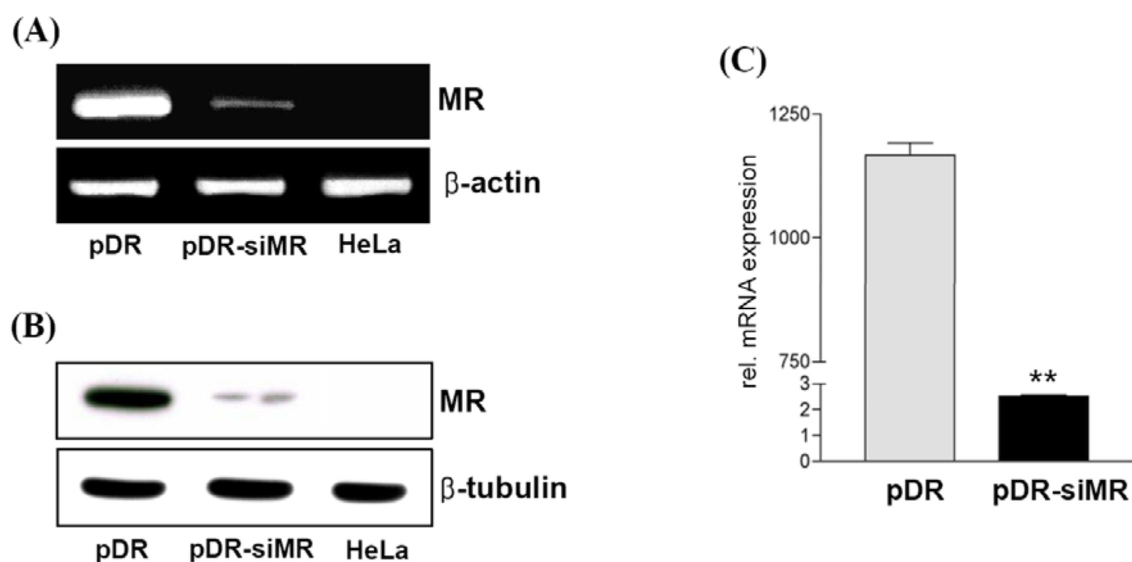


Figure 4.7 mRNA and protein levels of MR in MR-expressing HeLa cells transduced with pDR-siMR or pDR. We examined the MR mRNA expression levels by standard RT-PCR (A) and protein levels by western blot (B) in pLL-DsRed (pDR) and pDR-siMR transduced HeLa-MR cells 5 days after transfection with the viruses. The shRNA-MR leads to reduced mRNA and protein levels of the MR *in vitro*. We also performed a quantitative real-time PCR to compare MR mRNA expression between control and shRNA-MR transduced cells. The result of the q RT-PCR shows that expression of MR mRNA is reduced around 350 fold as compared to control virus-transduced cells.

In addition, northern blot analysis confirmed the synthesis of the specific siRNA in lentivirally transduced HeLa cells (Figure 4.8). In general, the single antisense strand of the siRNA in RISC is bound as a dsRNA complex to the corresponding complementary

specific target sequence and triggers mRNA cleavage (Martinez et al., 2002; Martinez and Tuschl, 2004). So we supposed that the antisense strand of the shRNA duplex was processed and regulated mRNA expression in our lentivirus-transduced cells. To examine processing of the specific siRNA, the annealed shRNA-MR oligonuloetide was cloned into pLentiLox-3.7, see result part II. This construct was used to produce lentiviruses, the lentiviral particles were used to transduce HeLa cells, which then were sorted depending on GFP expression by FACS. Two radioactively-labeled probes, which corresponded to the sense or the anti-sense shRNA sequence, were used for northern blot hybridization.

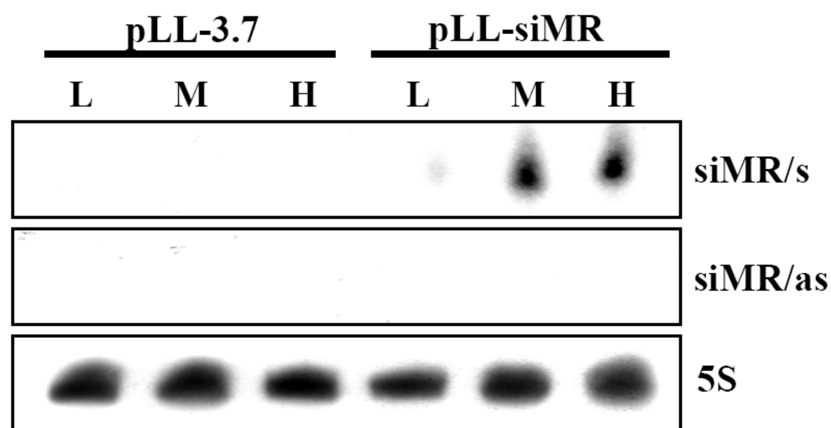


Figure 4.8 Northern blot analysis to detect processing of the shRNA-MR *in vitro*.

15㎍ of total RNA from pLL-3.7 and pLL-siMR lentivirus-transduced HeLa cells were used to analyze the expression of shRNA processing. The lentivirus-transduced cell was sorted into low, middle, and high GFP expression by FACS. The total RNA was separated on an urea-TBE polyacrylamide gel, transferred to a nylon membrane, hybridized with the radioactively-labeled specific MR sequence (sense and antisense primer) and visualized by X-ray film. The 5s RNA probe was used to control for equal RNA loading.

In Figure 4.8, following the supposition, a specific siRNA was detected by the sense probes, but not the antisense probe. This indicated that the specific siRNA is synthesized in lentivirally transduced HeLa cells.

4.1.2 Analysis of siMR knock-down rats

4.1.2.1 Generation of mineralocorticoid receptor knock-down rats

To generate MR knock-down rats, we used a highly-concentrated pDR-shRNA-MR lentivirus preparation and injected it into the perivittelin space of fertilized oocytes from Crl:CD rats. We confirmed that three out of nine pups born had stably integrated the provirus into the genome by PCR and southern blot analysis (Figure 4.9).

These founder rats were designated siMR7, 8 and 9. However, in spite of the integration of the transgene (shRNA-MR construct with the DsRed fluorescence protein), we were not able to demonstrate the presence of DsRed in any of the three animals, neither by flow cytometry of peripheral blood leukocytes nor by western blot analysis.

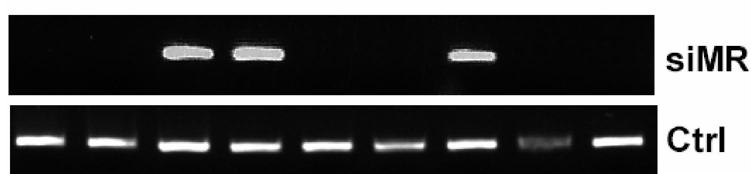


Figure 4.9 Analysis of the phenotype of siMR know-down transgenic rats. The genomic DNA from transgenic rats was confirmed by PCR using specific primer. The amplified PCR product separated through a 1.5% TBE agarose gel. Two primers of specific for Notch were used to control for equal loading.

To confirm that the siRNA was expressed, we performed a northern blot analysis of kidney and hippocampus samples from siMR transgenic rats. This confirmed that the

siRNA was correctly expressed in the transgenic rats (Figure 4.10).

As shown in Figure 4.10, the transgenic rats 1, 2 and 3 expressed different amount of shRNA-MR in kidney and hippocampus (A) and the reduction in MR mRNA expression corresponded to the level of the shRNA (B).

This means that high expression of the shRNA leads to strongly reduced MR mRNA expression and low expression of shRNA leads to weakly reduce mRNA expression.

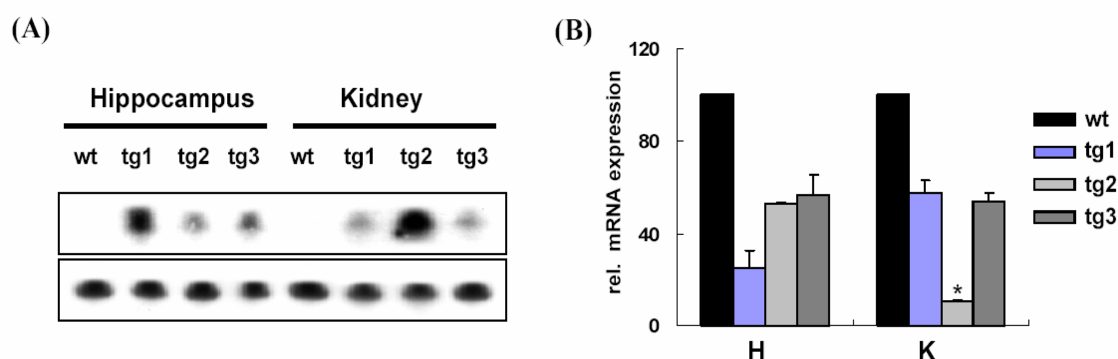


Figure 4.10 Processing of the shRNA-MR in transgenic rats. We confirmed that the siRNAs was expressed in transgenic animal although the DsRed fluorescent proteins was absent by northern blot analysis of RNA samples extracted from kidney and hippocampus of siMR transgenic rats. 15μg of total RNA were used to analyze the processing of the shRNAs. The 5s RNA probe was used to control for equal RNA loading (A). (B) shows the relative MR mRNA expression levels in the corresponding samples analyzed by qRT-PCR.

4.1.2.2 Efficient inactivation of MR expression in shRNA-MR transgenic rats

To assess the efficiency of the MR knock-down we analyzed composite F1 offspring of the founder rats. In particular, we were interested whether different levels of gene inactivation can be achieved by this way. Therefore, we crossed the male founder rat siMR9 with the two female founder rats siMR7 and siMR8. The offspring was devided into wild-type and transgenic animals based on their genotype as determined by PCR. Subsequently, we studied the mRNA levels in the two major areas of MR expression,

namely the kidney and the hippocampus.

Firstly, we investigated MR mRNA expression by quantitative PCR in 2 ~ 3 week old siMR transgenic rats. Expression of the highly related nuclear receptor GR served as an internal control. Analysis of 33 transgenic rats revealed a reduction in MR mRNA levels by 70% on average in kidney and 76% in hipppocampus (Figure 4.11 (A, B)). In contrast, GR mRNA levels were unaltered in both organs.

Furthermore, we observed a great diversity of different MR expression levels ranging from a reduction by less than half to almost complete gene inactivation.

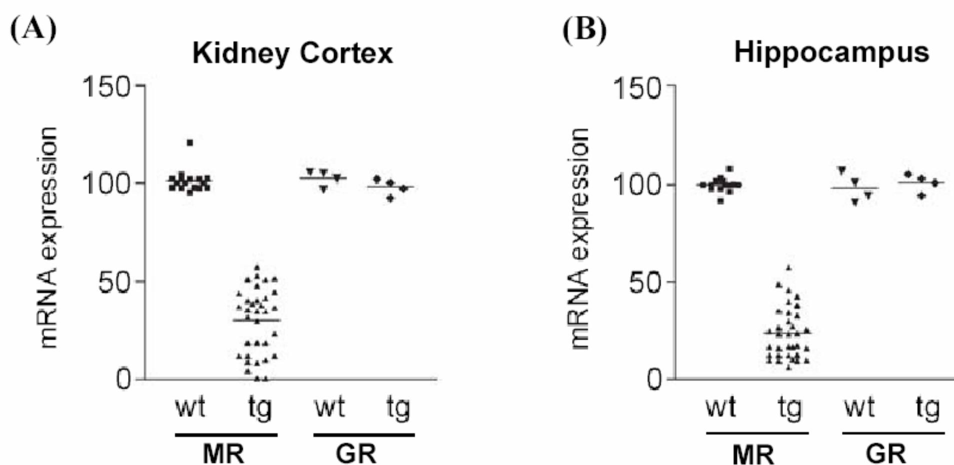


Figure 4.11 Analysis of MR mRNA levels in transgenic rats. We performed quantitative PCR using kidney (A) and hippocampus (B) samples of 2 ~ 3 week old rats to compare MR or GR mRNA expression values between wild type and transgenic rats. To calculate the relative values of MR mRNA expression, HPRT or β-actin, two known house keeping genes, served as an internal control.

To confirm our results on the protein level we performed a western blot analysis of kidney and hippocampus. Similar to our results on mRNA expression above, MR protein levels were reduced in both organs, accompanied by unaltered expression of the

GR (Figure 4.12)

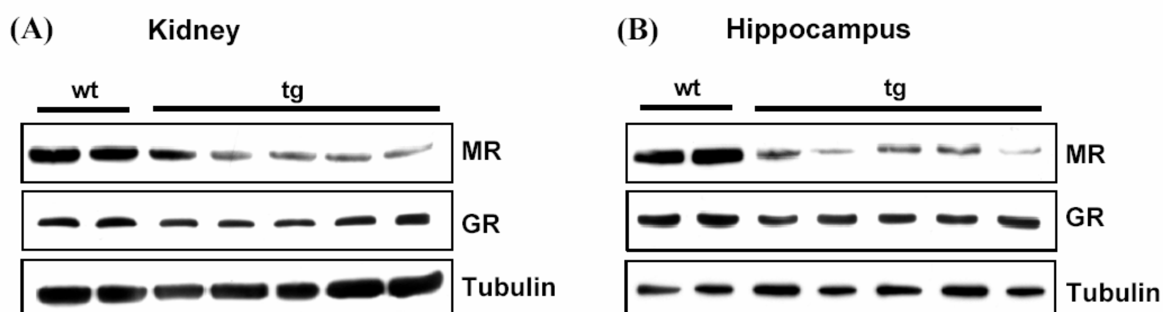


Figure 4.12 Western blot analysis of MR and GR protein levels in siMR transgenic rats. Protein lysates of kidney (A) and hippocampus (B) from wild type (wt) and shRNA-MR transgenic rats (tg) were analyzed by western blot using MR and GR specific polyclonal Abs. This shows that the MR, but not GR protein levels are reduced by the activity of shRNA-MR in the transgenic rats. As a control for equal protein loading a anti β-tubulin Ab was used.

In addition, we performed an immunohistochemical analysis of the brain to determine MR expression. It is known that MR is expressed only in neurons, with highest abundance in the limbic system, particularly within the hippocampus (Kretz et al., 2001). As shown in Figure 4.13, transgenic rats show the lower density in the whole region of hippocampus compared to wild type controls. This also shows that MR protein levels were reduced in hippocampus similar to the western blot result.

In summary, the knock-down rats show a profound downregulation of the MR, confirming that gene inactivation can be achieved in rats using such an approach.

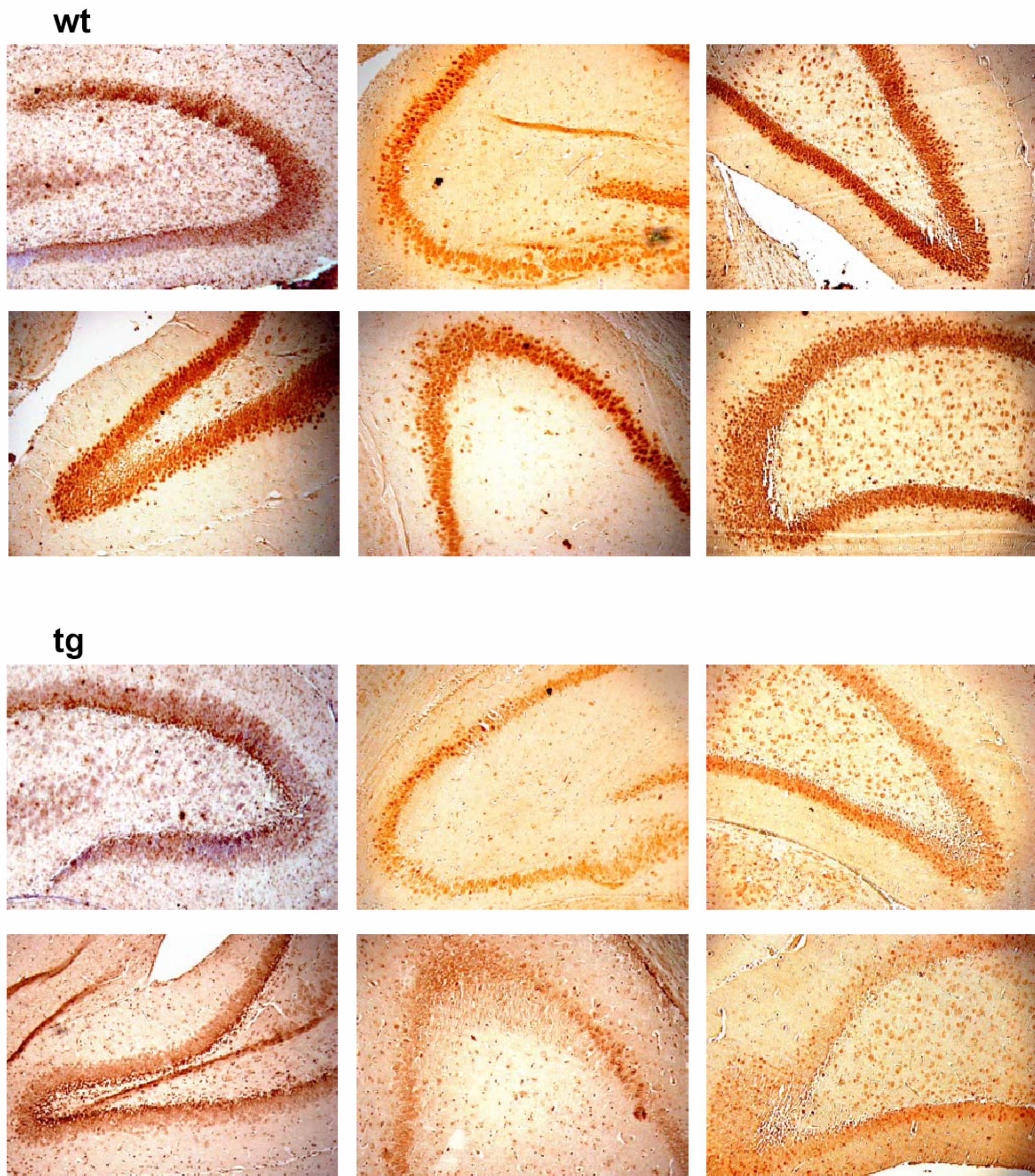


Figure 4.13 Immunochemical analysis of MR expression in brain. We made paraffin section of the brain and analyzed them by immunochemistry using MR-specific polyclonal antibody. The transgenic rats showed a lower density of MR expression in the hippocampus.

4.1.2.3 The siMR knock-down transgenic rats show signs of pseudohypoaldosteronism

The mineralocorticoid receptor binds the adrenal steroid hormone aldosterone and thereby mediates sodium and potassium ion transport, maintains the salt/water homeostasis by regulation of electrogenic sodium reabsorption in kidney (Canessa et al., 1994). In previous reports, it was shown that MR knock–out mice die in the second week of age due to a severe disturbance of the salt-water balance. The phenotype includes growth retardation and disturbed levels of aldosterone and renin, two hormones involved in the regulation of sodium reabsorption in the kidney (Berger et al., 1998).

To investigate whether siMR rats show similar physiological alterations, we analyzed them at 14-28 days of age. Strikingly, a small number of newborn pups were always lost by around two weeks of age. Similar to MR knock-out mice, we suspect that the down-regulation of the MR in these rats was too efficient to be compatible with life. However, most of the transgenic offspring survived and could be analyzed.

Firstly, the MR knock-down transgenic rats showed a significantly lower body weight as compared to wild-type littermates, i.e. the transgenic rats were 25% lighter on average compared to wild types (Figure 4.14 (A)). Since the offspring showed different levels of gene inactivation as shown in Figure 4. 10, we also observed a considerable degree of heterogenicity amongst the offspring. However, there was a significant correlation between the reduced body weight and the expression of the transgene (Figure 4.14 (C)). This suggests that the phenotype of the MR knock-down rats is a direct consequence of gene inactivation by RNAi.

Pseudohypoaldosteronism is caused by a loss of MR function in human patients and is mimicked by the phenotype of MR knock-out mice (Armanini et al., 1985; Cheek and Perry, 1958; Hanukoglu, 1991). Thus, we measured the concentration of two hormones,

which are involved in the renin-angiotension-aldosterone system (RAAS), in the serum of the shRNA-MR transgenic rats (Figure 4.14(B)).

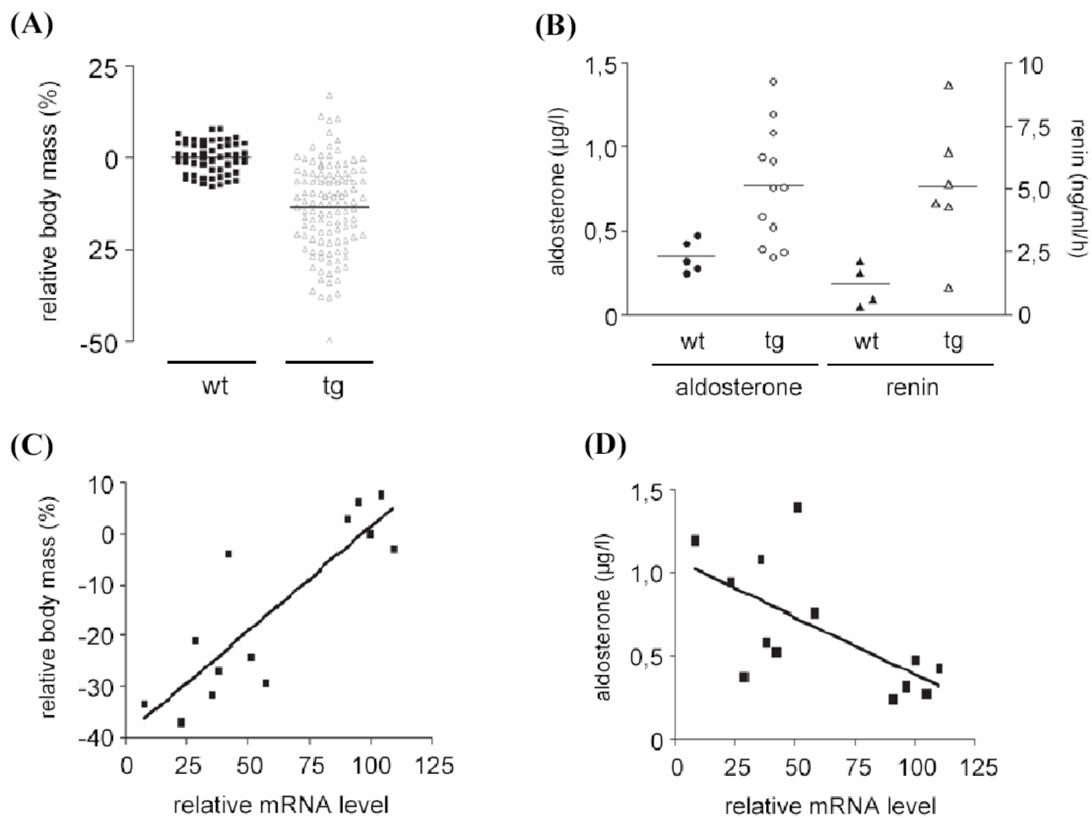


Figure 4.14 The pathophysiological consequences in transgenic rats expressing the shRNA-MR correlate with the degree of gene inactivation. In the offspring of the transgenic rats the weight (A) and the renin and aldosterone concentration in the serum of blood samples was measured at an age of 14 ~ 28 days (B). We analyzed the correlation between the loss of body weight and MR mRNA levels of the transgenic rats (C) and between MR mRNA level and aldosterone (D).

Figure 4.14 (B) shows that, similarly to $MR^{-/-}$ mice, the levels of aldosterone and renin in the serum of siMR rats were increased by a factor of three to four (Figure 4.14 (B)). Again we tested whether there was a correlation between the degree of gene inactivation

and the phenotype of the rats. To this end, we blotted the aldosterone level against the relative decrease in MR mRNA expression in the kidney. As shown in Figure 4.14 (D), there was a significant inverse correlation between both parameters, suggesting that the salt-water balance directly depends on the MR dosage. The hormone measurements were kindly performed by Dr. Martin Fassnacht, Department of Endocrinology, University of Würzburg.

We also supposed that inactivation of MR protein by shRNA-MR could lead to morphological alteration of kidney. In a previous study of MR function using the knock-out system (Berger et al., 1998), it was shown that conspicuous morphological changes occurred at the glomerular vascular pole in kidney, i.e. enlargement of the macula densa segment of the distal tubule and prominent hyperplasis in the extragolmerular mesangium. To address this question, we performed a histological analysis of kidney. However we observed no striking morphological changes in kidney by Hematoxlin-Eosin staining (Figure 4.15)

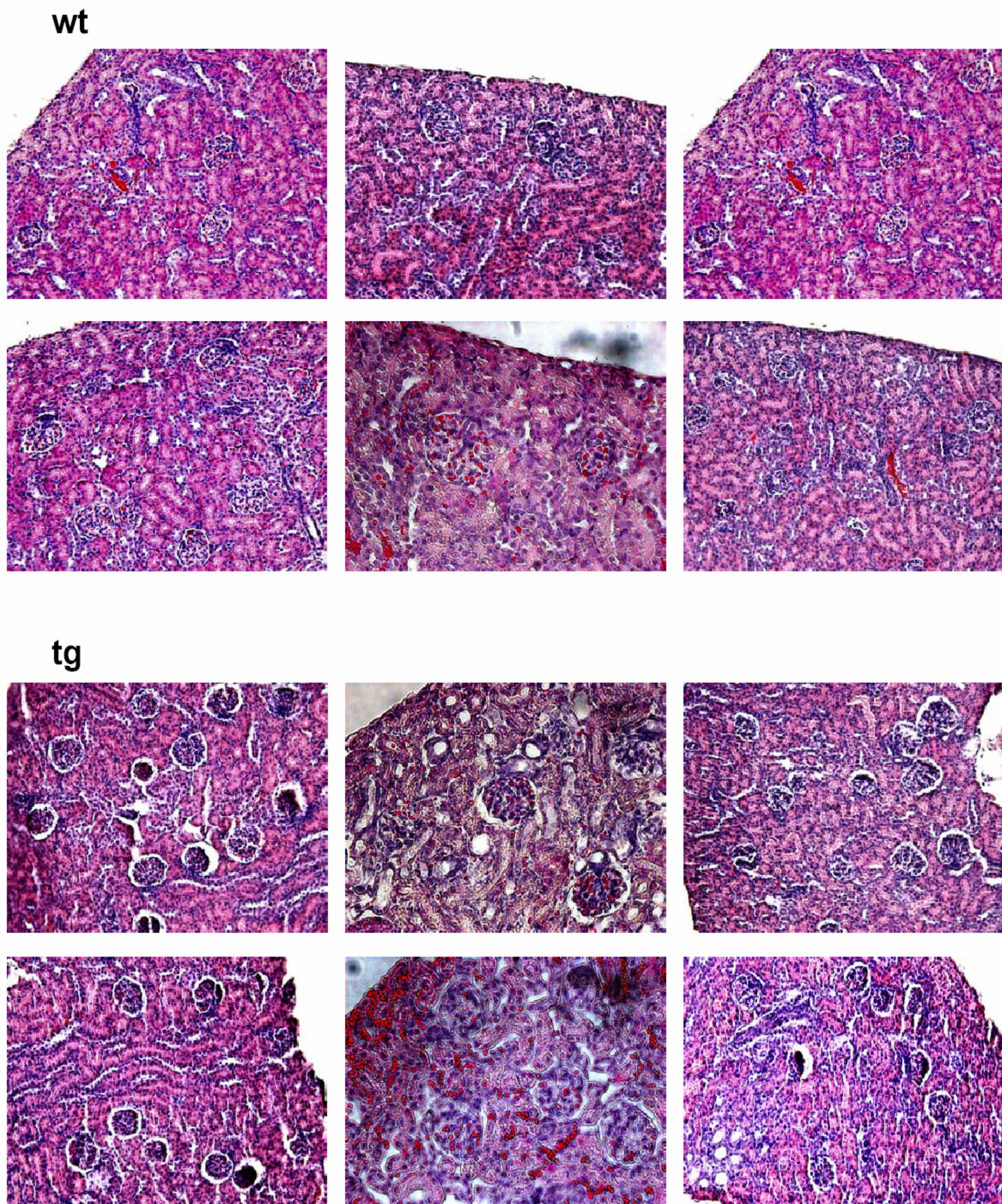


Figure 4.15 Histological analysis of the kidney. We made paraffin section of the kidney, which express MR protein, and analyzed them by histology. We could not show any morphological changes in kidney in shRNA-MR knock-down rat using HE staining

We also investigated the influence of the MR knock-down on the expression of genes in kidney and hippocampus. Based on the function of the MR for homeostasis in kidney and brain, the mRNA expression of IK1 (Ca^{2+} activated intermediate potassium channel, Ac. Nr. NM023021) and SCD2 (stearoyl-CoA desaturase 2, Ac.Nr. AB032243), which are involved in ion transport and cellular synthesis of fatty acid, was performed by quantitative PCR.

Transgenic rats revealed a reduction in IK1 mRNA levels by 50% on average in kidney, 55% in hipppocampus and SCD2 mRNA levels by 60% on average in kidney, 50% in hipppocampus (Figure 4.16). Similar to MR expression levels in Figure 4.11, we observed a big diversity of different expression levels of the two genes ranging from a reduction by less than half to almost complete gene inactivation.

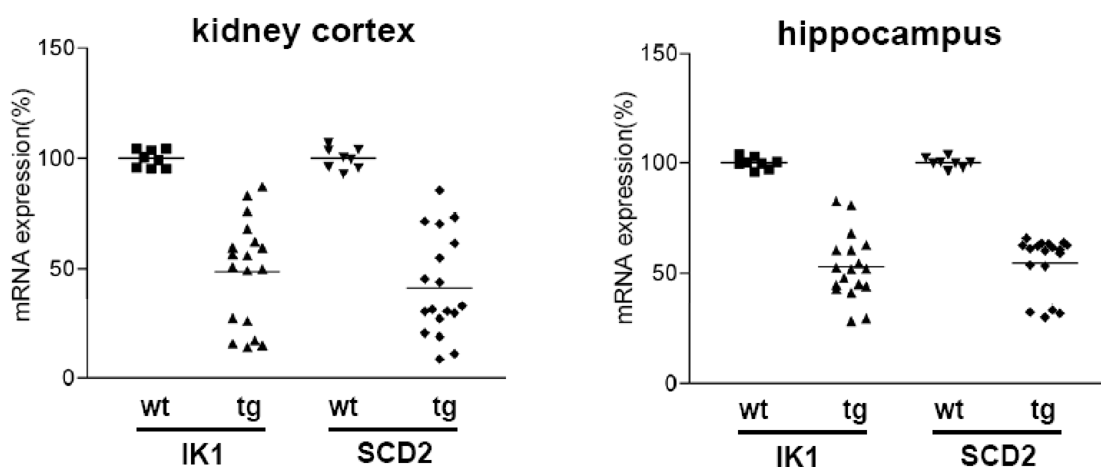


Figure 4.16 mRNA expression levels of IK1 and SCD2 in transgenic rats. We performed quantitative real-time PCR to measure mRNA expression of the IK1 and SCD2 genes in kidney and brain from wt and transgenic rats. β- actin expression served as an internal control.

In addition, we analyzed the mRNA expression of the epithelial aldosterone-MR target genes, SGK1 (serum- and glucocorticoid-induced kinase1, Ac. Nr. NM019232) and

sodium channel (ENaC) subunits (Ac. Nr. of ENaC- α: NM031548, - β: NM012648, - γ: NM017046) by quantitative PCR. In a previous report on MR-knock out mice, it was shown that the abundance of the mRNAs encoding the ENaC and ($Na^{+}K^{+}$)-ATPase subunits is not changed, but that amiloroid-sensitive Na^{2+} reabsorption was altered (Berger et al., 1998). The Sgk1 and ENaC genes are activated by the MR in epithelial and non-epithelial cells and induced by aldosterone (Bhargava et al., 2001; Brennan and Fuller, 2000). The expression of the three subunits (α, β, and γ) of ENaC is also regulated by corticosteroid in tissues-specific manner (Rogerson and Fuller, 2000). The expression of the Sgk1 phosphorylates ENaC regulatory protein, subsequently regulates the expression of the ENaC, and result in increased ENaC-dependent Na^{2+} reabsorption (Boyd and Naray-Fejes-Toth, 2005; Naray-Fejes-Toth et al., 2004).

We chose a group of transgenic rats that shows strongest reduction of the MR expression in both organs based on results of Figure 4.16 and performed qRT-PCR using specific primers. Interestingly, Sgk1 and the three subunits of ENaC, especially the α- and γ- subunits, mRNA levels were increased by a factor of 3 ~ 20 folds in kidney and 2 ~ 10 folds in hipppocampus (Figure 4.17). Similar to MR expression levels in Figure 4.11, we observed a big diversity of different expression levels of two genes. This indicates that Sgk1 and ENaC maybe regulate in an MR-dependent manner in our system.

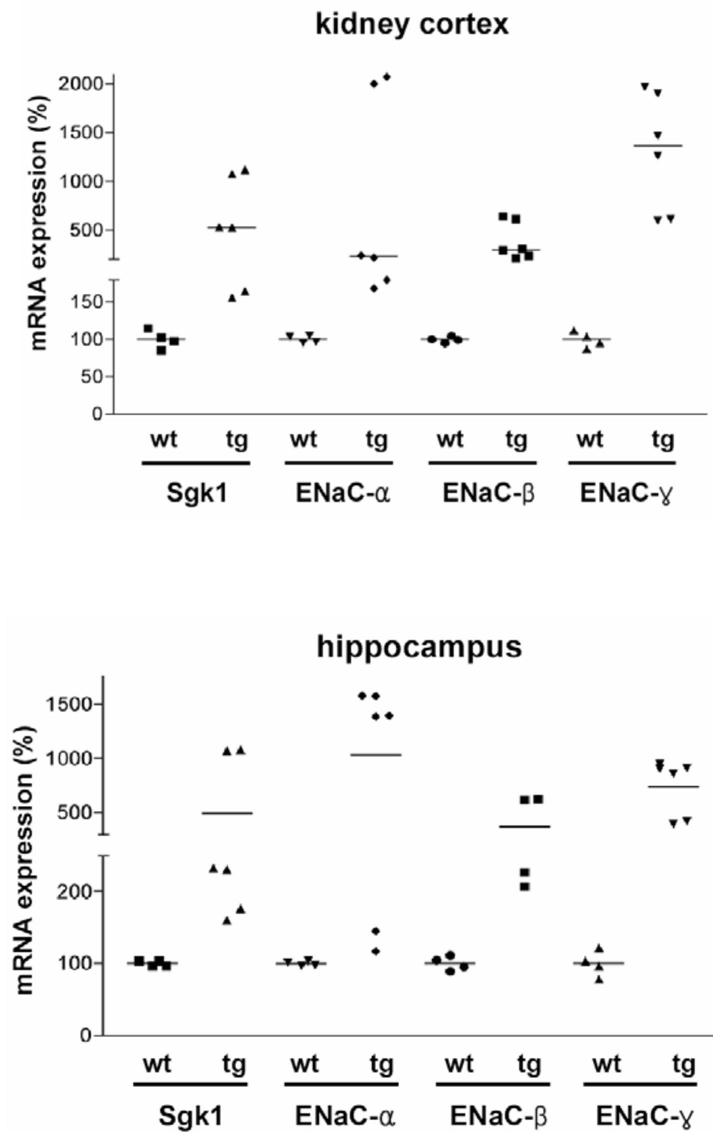


Figure 4.17 mRNA expression levels of Sgk1 and ENaC subunits in transgenic rats. At first, we chose the transgenic rats based on mRNA expression levels of IK1 and SCD2 in Figure 4.16 and performed quantitative RT-PCR to measure mRNA expression values of the Sgk1 and three subunits (-α, -β, and –γ) of ENaC genes in kidney and hippocampus. β-actin was used for the normalization as an internal control.

Part II. Analysis of immunomodulatory effects of glucocorticoids via the GR or MR on macrophages by RNA interference

4.2.1 Lentiviruses expressing an shRNA-GR or MR efficiently down-regulate the expression of the GR or MR *in vitro*

4.2.1.1 Generation of lentiviral shRNA-GR and MR constructs

To inactivate glucocorticoid receptor or mineralocortcioid receptor expression *in vitro*, we cloned two recombinant lentiviral vectors, pLL-siGR or F6UGW-siGR and pLL-siMR. Appropriate specific siRNA sequences of the GR (Table 4.2) based on the rat GR mRNA sequence (Accession Nr. M14053) were identified and the hairpin stem loop structure designed according to the rules described in 4.1.1.1. In addition we used an identical MR siRNA sequence as for the knock-down transgenic rats in Part I.

The annealed oligonucleotides encoding shRNA sequences against the GR or MR were cloned into the lentiviral vector pLentiLox 3.7, which expresses an shRNAs driven by the mouse U6 promoter and contains the enhanced green fluorescent protein under the control of CMV promoter, and a multiple cloning sites (Rubinson DA *et al*; Nat Genet 2003). In addition, F6UGW was used in which the green fluorescent protein is expressed under the control of the ubiquitin promoter instead of the CMV promoter. The siRNA-GR1 is 19 nucleotides in length and positioned in the N-terminal domain, the siRNA-GR2 is 22 nucleotides in length and positioned in the DBD (DNA binding domain).

	Sequence of Small interfering RNAs
siRNA-GR1	GGATACTGGAGATACAATC
siRNA-GR2	GAG CAG TGG AAG GAC AGC ACA A

Table 4.2 siRNA sequences targeting the GR

The shRNA-GR1 or 2 oligonucleotides were annealed by the established protocol (Figure 4.18), and inserted into the lentiviral pLentiLox 3.7 or F6UGW vector using the *Hpa*I / *Xho*I sites.

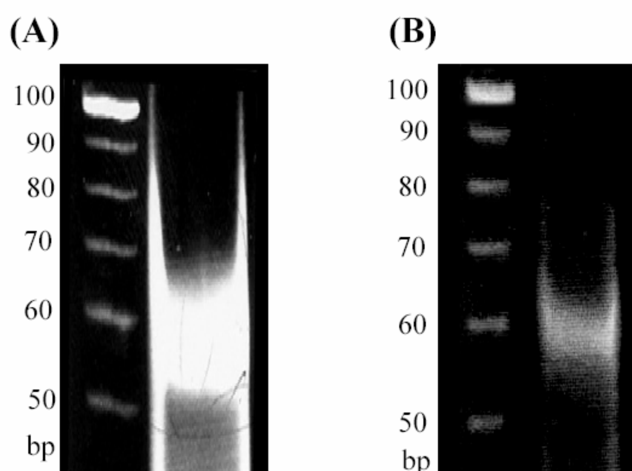


Figure 4.18 Separation of the annealed shRNAs against GR. Two shRNA oligonucleotides were annealed, separated on a 8% TAE-polyacrylamide gel, and eluted. The 10bp DNA molecular marker was used to determine the size of the oligonucleotides. The annealed shRNA-GR was 60bp. (A): shRNA-GR1 oligonucleotides , (B) : shRNA-GR2 oligonucleotides

At first we performed colony PCR using specific primer sets for the pLL-3.7 vector and analyzed the PCR products by restriction digest with *Xho*I. The amplified PCR products including an shRNA-GR sequence corresponded to 1.4kb and 110bp fragments whereas the control pLL vector product corresponded to 1.4kb and 70bp (Figure 4.19(A)).

The potentially positive shRNA-GR constructs in the F6UGW lentiviral vector were analyzed with several restriction enzymes. Figure 4.19 (B) shows the restriction analysis of the shRNA-GR2 construct cloned in the F6UGW lentiviral vector. The F6UGW-siGR2 construct contained a 50bp bigger fragment as compared to the control vector (Figure 4.19(B)). The shRNA-GR sequence in the construct was confirmed by sequencing analysis.

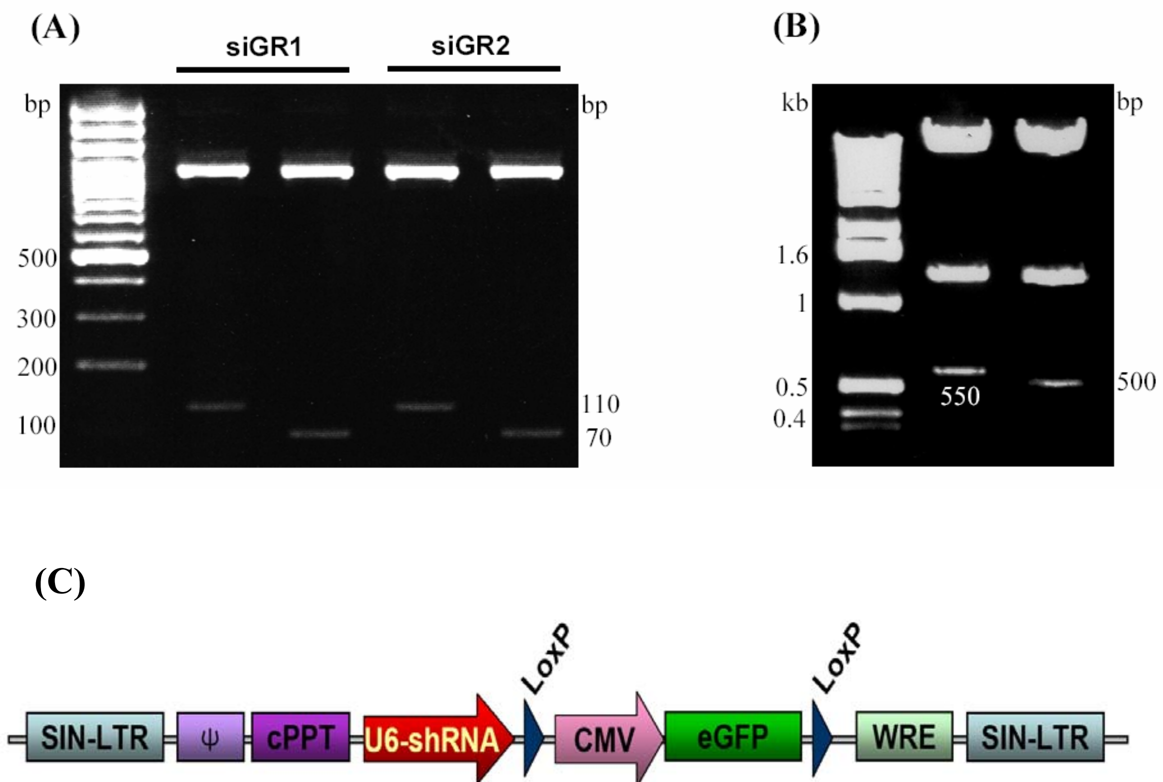


Figure 4.19 Restriction analysis of the pLL-siGR and F6UGW-siGR constructs. The recombinant shRNA-GR in the lentiviral vectors was analyzed by restriction enzymes. At first we selected positive clones by colony PCR using specific primer sets and then analyzed them by digestion with *Xho*I (A). The selected recombinant F6UGW-siGR DNA confirmed by digestion with *Pst*I and *Xho*I enzymes, which are positioned in the vector (B). (C) is a schematic representation of the shRNA-GR construct in pLentiLox 3.7 (Rubinson et al., 2003). In the F6UGW-shRNA construct eGFP is expressed under the control of the ubiquitin promoter.

4.2.1.2. Effects of GR knock-down in stably transduced cells by siRNA-GR

To test the efficiency of the siGR constructs, HeLa cells stably expressing the GR were transduced with the F6UGW-siGR or pLL-siGR lentiviruses.

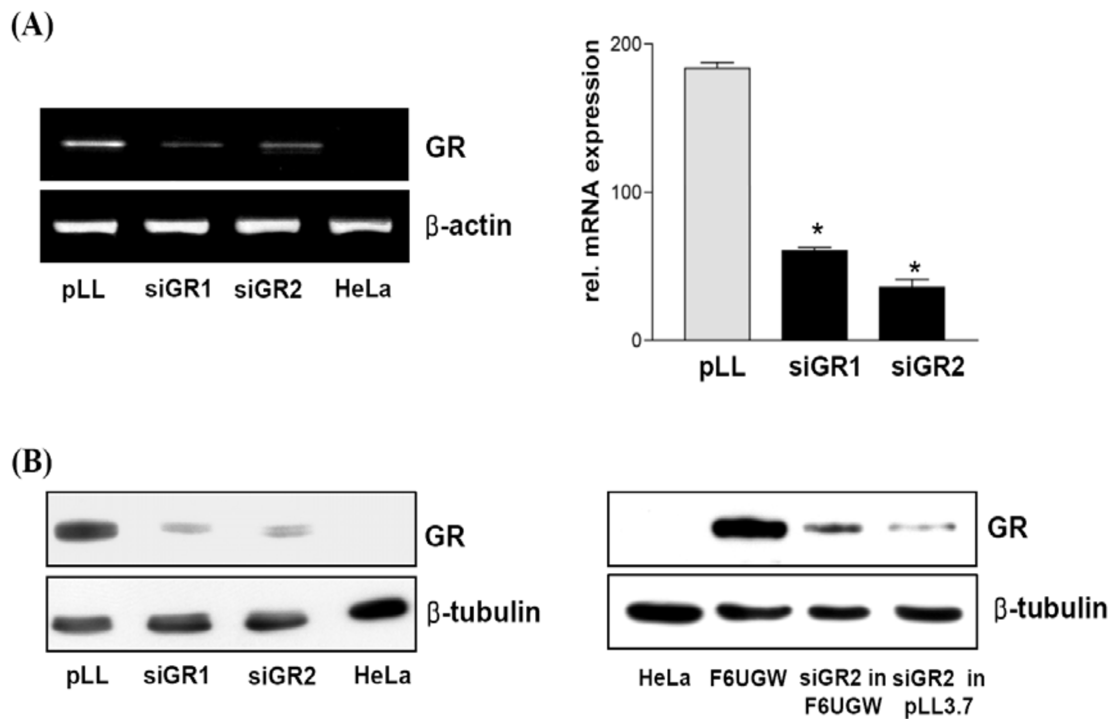


Figure 4.20 The two shRNA-GR lentiviruses reduce GR expression in HeLa cells.

We analyzed GR expression on the mRNA and the protein level in shRNA-GR virus-infected cells after 3 days. We performed standard RT-PCR and quantitative PCR using specific primer sets to determine the GR mRNA levels (A) and western blot for protein levels (B). Beta-actin expression served as a loading control for both PCRs, β-tubulin as a control for western blot.

The lentiviral particles were generated following published protocols, and GFP expression was measured to determine the titer of the lentiviruses by flow cytometry. The lentiviral particles were used at a 1:1 ratio to transduce HeLa cells, which ubiquitously expressed GR and generated by transduction with the FUGW lentivirus (Lois et al., 2002). After 3 ~ 4 days, cell lysates form the shRNA virus-transduced cells were analyzed for

mRNA and protein levels of the GR by RT-PCR and western blot. As shown in Figure 4.20, the shRNA-GR1 and 2 lentiviruses effectively reduced GR expression by around 80% on the mRNA and the protein level in GR expressing HeLa cells.

In comparison, the shRNA-GR1 and shRNA-GR2 lentiviruses efficiently reduce GR expression in stable cells, although shRNA-GR2 seems to blocke it more strongly. Then, we used the shRNA-GR2 construct in further experiments.

4.2.2. Analysis of glucocorticoid effects via GR / MR on macrophage function

4.2.2.1 Glucocorticoid and mineralocorticoid receptors are expressed in peritoneal macrophages

It is known that the MR is expressed only in selected cell types, whereas the GR is expressed ubiquitously. In previous reports, it was shown that the MR is expressed in microgial cells (Tanaka et al., 1997). Because these cells are functionally related to macrophages, we asked whether peritoneal macrophage may also express the MR. To this end, we studied mRNA expression of the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) in thymocytes, mature lymphocytes, peritoneal macrophages, kidney and hippocampus (Figure. 4.21 (A)). While the GR was present in all cell-types, the MR was only expressed in kidney, hippocampus and macrophages (Funder, 2005; Tanaka et al., 1997). Lack of 11β-hydroxysteroid dehydrogenase type II (11β-HSD2) expression in macrophages further suggests that both, the GR and the MR in these cells can be regulated by corticosterone (Figure. 4.21 (A)). To confirm MR expression on the protein level we analyzed peritoneal macrophages by immunofluoresence (perfromed by Dr. Nora Müller, Universitry of Würzburg). Using

confocal microscopy, we demonstrated the presence of both receptors in the cytosol as well as the nucleus (Figure. 4.21 (B) (Lim et al., 2007).

In summary, these data suggest that the MR is expressed in thioglycollate-elicited peritoneal macrophages and is able to respond to corticosterone.

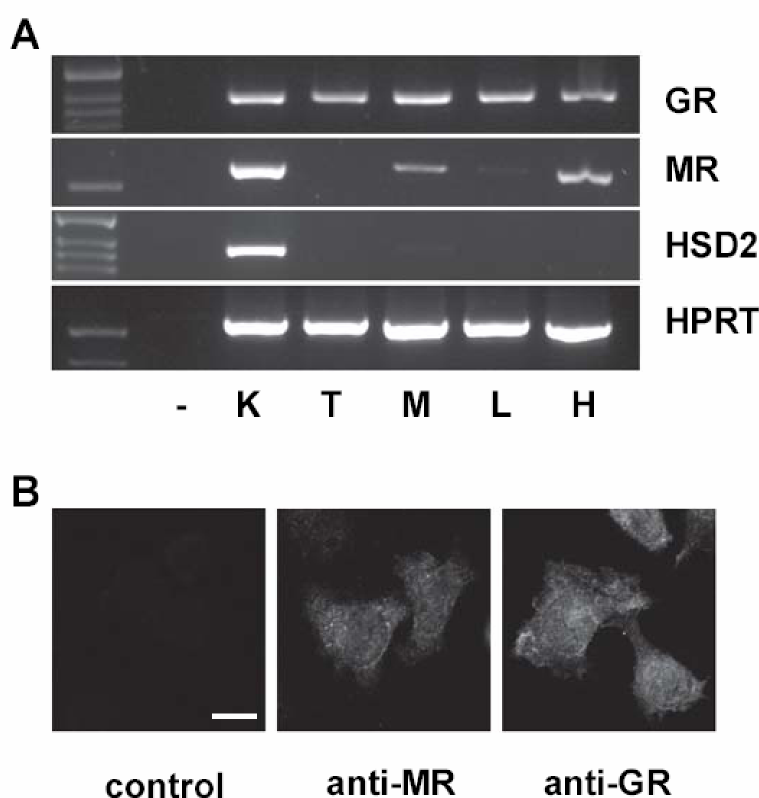


Figure 4.21 Expression of the GR and the MR in several cell types and tissues.

We analyzed whether GR or MR is expressed in various cell types by PCR and immuofluorescence. (A) Analysis of GR, MR and 11β-HSD2 mRNA expression in various cell types and tissues. We performed PCR using specific primer, HPRT served as a loading control. K = kidney, T = thymocytes, M = peritoneal macrophages, L = lymph node cells, H = hippocampus. (B) Immunofluorescence analysis of peritoneal macrophages using GR and MR specific antibodies. Staining with the secondary antibody anti-rabbit Alexa488 but lacking primary antibody is shown for control. The result is kindly provided by Dr. Nora Müller.

4.2.2.2 Lentiviruses expressing siRNAs against the GR and the MR allow efficient gene inactivation in peritoneal macrophages

In order to selectively inactivate the GR and the MR in peritoneal macrophages we generated shRNA-MR or GR lentiviruses using the designated lentiviral constructs.

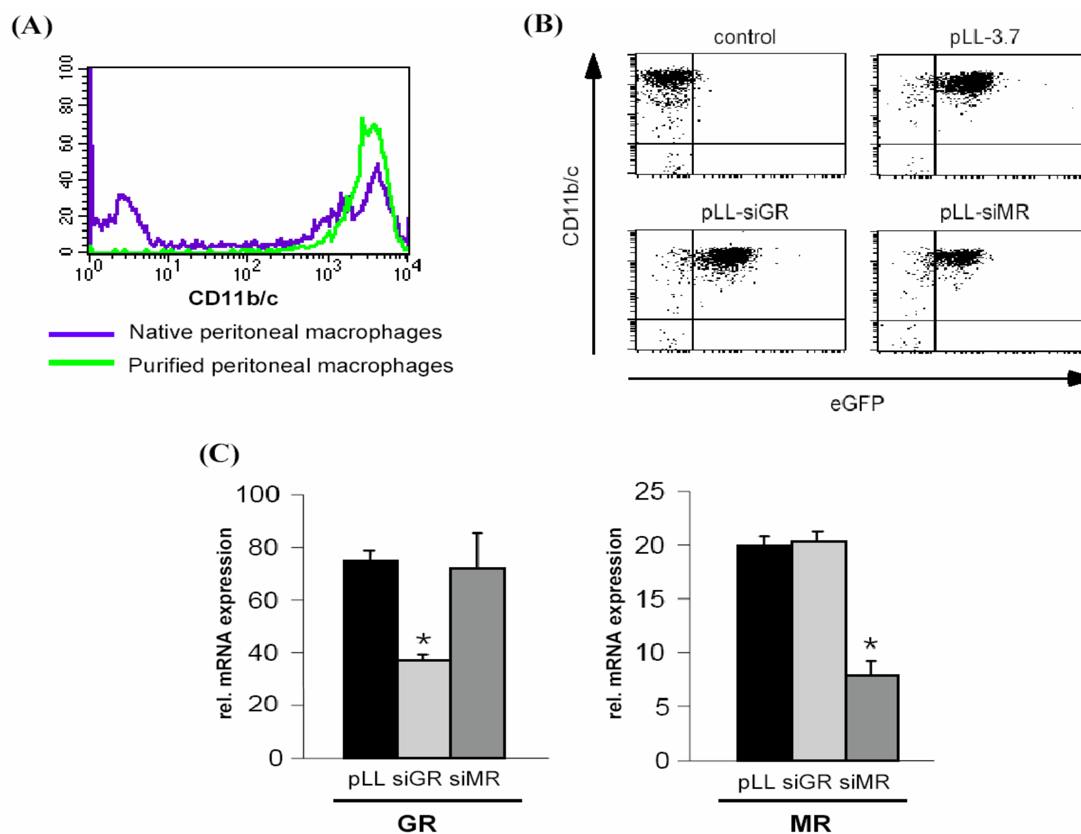


Figure 4.22 Analysis of the shRNAs against GR or MR lentiviruses in infected peritoneal macrophages. (A) Purification of CD11b/c+ peritoneal macrophages. To elicit peritoneal macrophages, CD:Crl rats were injected i.p. with 4% sterile thioglycollate medium (Sigma, Taufkirchen, Germany) and the peritoneal lavage was harvested with balanced salt solution (BSS). The peritoneal macrophages were magnetically purified using a CD11b/c+ antibody and their purity analyzed by FACS staining. (B) Flow cytometric analyses of peritoneal macrophages transduced with the lentiviruses pLL-3.7, pLL-siGR and pLL-siMR for expression of CD11b/c and eGFP. Untransduced control cells are shown for comparison. (C) GR and MR mRNA expression was determined by quantitative PCR in macrophages after lentiviral gene inactivation as in panel A. Statistical analysis was performed by students' t-test. The asterisk indicates $p<0.05$.

Virus particles were concentrated by ultracentrifugation and used to transduce magnetically purified CD11b/c+ macrophages from the peritoneal lavage (Figure 4. 22(A)). Transduction efficiency was confirmed by flow cytometric analysis of enhanced green fluorescent protein (eGFP) expression two days after infection (Figure. 4.22(B)). The level of gene inactivation was determined by quantitative PCR, which confirmed that the expression of the GR and the MR were both specifically reduced by 50-70% (Figure. 4.22(C)).

Thus, our approach should allow assessing the role of these receptors for the modulation of macrophage function by GCs.

4.2.2.3 Corticosterone modulates nitric oxide production by activated macrophages in a dose-dependent manner via the glucocorticoid receptor

To analyze whether corticosterone exerts different effects on macrophage function dependent on its concentration, we first analyzed the production of nitric oxide (NO), an important anti-bacterial mediator. Thioglycollate-elicited peritoneal macrophages were activated by lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) and concomitantly treated with increasing concentrations of corticosterone and dexamethasone, a synthetic glucocorticoid, ranging from 10^{-10} M to 10^{-6} M. 48 hours later, we measured NO production in the supernatant using a photometric assay. As hypothesized, low concentrations of corticosterone potentiated the ability of macrophages to produce NO while high levels significantly repressed NO production (Figure 4.23). In contrast to the corticosterone treatment, low and high concentrations of dexamethasone repressed NO production in LPS/IFN-γ activated macrophages.

It was suggested that GR and MR would mediate the opposite activities in macrophages

dependent on the concentration and the kind of hormones. In other words, the GR could mediate immunosuppressive actions by glucocorticoid and the MR would allow the permissive actions on basal levels of hormones.

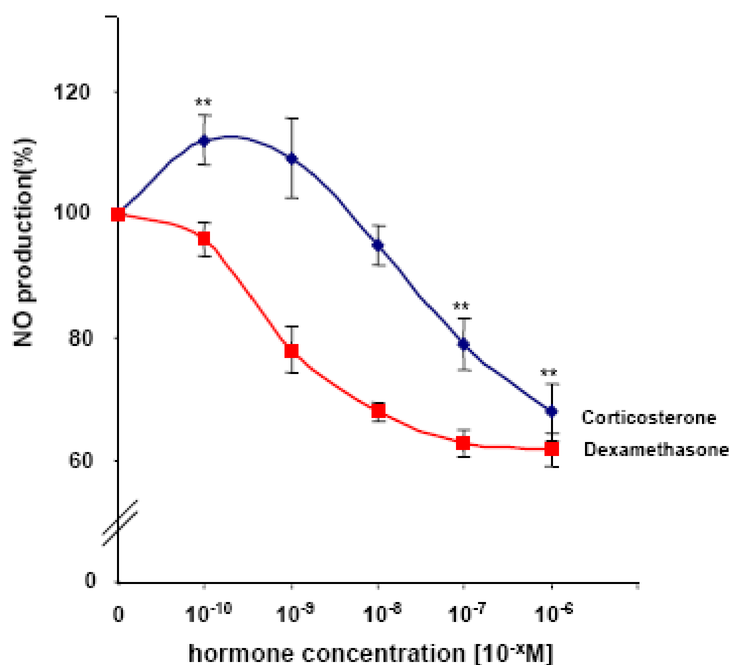


Figure 4.23 Dose-dependent effect curves of the corticosterone and dexamethasone. The purified peritoneal macrophages were activated by LPS and IFN-γ in the absence or presence of increasing amounts of corticosterone or dexamethasone, which are ranging from 10^{-10} M to 10^{-6} M. After 48 hours nitric oxide (NO) production was determined in the culture supernatant. NO production in the absence of corticosterone or dexamethasone was set 100% and the values calculated in the presence of corticosterone or dexamethasone.

Importantly, GC treatment did not induce apoptosis of cultured macrophages (Figure 4.24). Whilst 89.5 ± 2.3% of the cells in control cultures were still alive after two days based on AnnexinV-binding, this was the case for 88.2 ± 4.4% after treatment with 10^{-6} M corticosterone (n=3). We conclude that GCs exert opposing effects on macrophage function dependent on their concentration and that this effect is independent of

apoptosis induction.

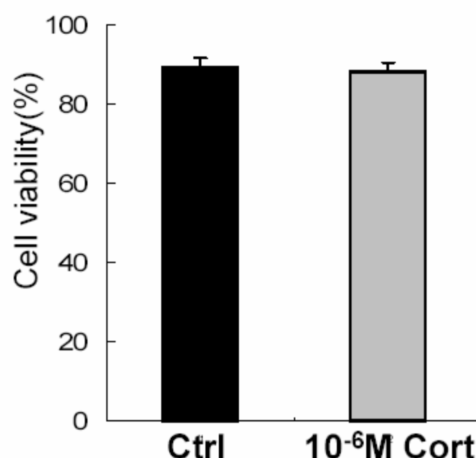


Figure 4.24 Percentage of cell viability under corticosterone treatment. Peritoneal macrophages were treated with a high concentration of corticosterone (10^{-6}M). The macrophages were stained and the percentage of living cells was measured based on AnnexinV-binding by FACS; the black bar means untreated cells and the grey bar means high-dose corticosterone treated macrophages.

Next, we investigated the role that the GR and the MR play in the immunostimulatory and immunosuppressive activities of corticosterone and dexamethasone. To address this question, we transduced macrophages with the lentiviruses pLL-3.7 as a control, pLL-siGR or pLL-siMR for two days, activated them with 100ng/ml LPS, 100units/ml rat IFN-γ and determined NO production as a measure for macrophage function. In addition, corticosterone or dexamethasone were added either at a low (10^{-10}M) or a high (10^{-6}M) concentration.

As shown in Figure 4. 25, similar to the result of non-transduced macrophages in Figure 4.23, pLL-3.7-transduced macrophages produced high amounts of nitric oxide following activation, which could be further potentiated by low doses of corticosterone and repressed by high doses of corticosterone. Dexamethasone repressed NO production at both concentrations.

Importantly, in cells transduced with pLL-siGR, a low concentration of corticosterone did no longer increase NO production and a high dose only inefficiently reduced it (Figure 4.25(A)). A high concentration of Dex did also strongly repress NO production and a low dose of Dex had no effects on NO production (Figure 4.25(B)).

In contrast, inactivation of the MR had not influence on the ability of corticosterone or dexamethasone to modulate NO production, neither at a low nor at a high concentration. Thus, the immunostimulatory and the immunosuppressive activities of GCs are both mediated via the GR and not the MR.

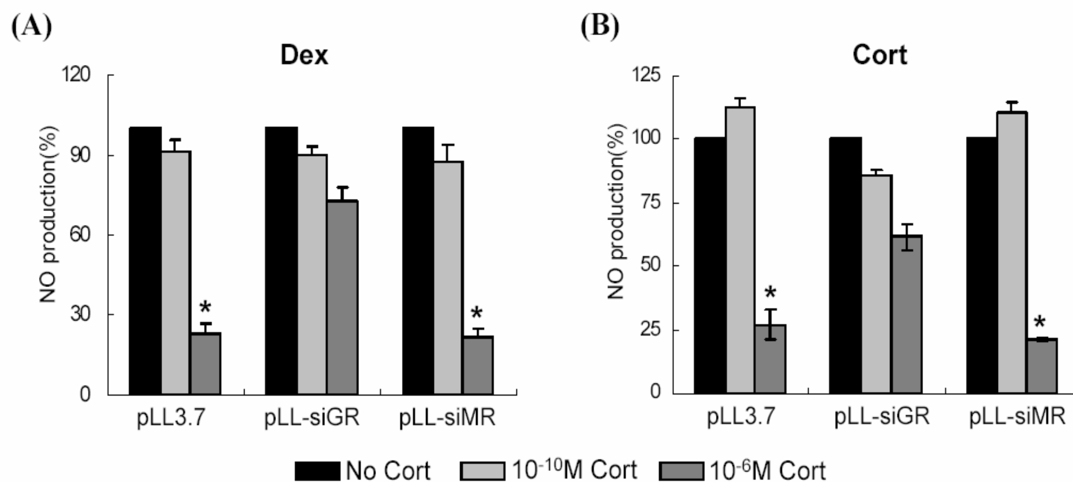


Figure 4.25 Impact of GR and MR inactivation by shRNAs on the ability of corticosterone to modulate NO production. Purified peritoneal macrophages were transduced with the lentiviruses pLL-3.7, pLL-siGR2 or pLL-siMR. Afterwards macrophages were activated with 100ng/ml LPS and 100units/ml IFN-γ in the absence or presence of low dose or high dose corticosterone or dexamethasone for 48 hrs. At day 4, we measured NO production under each condition using the cell supernatants and Griess reagent.

4.2.2.4. Dose-dependent modulation of mRNA expression in activated peritoneal macrophages by corticosterone

To extend our findings to other macrophage functions involved in the control of inflammation we studied the mRNA expression of a variety of genes.

As in the previous experiment, thioglycollate-elicited peritoneal macrophages were plated at a density of 10^6 cells per well and transduced with the three kinds of lentiviruses pLL-3.7, pLL-siGR2 or pLL-siMR. Subsequently, the cells were activated with LPS / rat recombinant IFN-γ in the absence or presence of a low or a high concentration of corticosterone or dexamethasone for 6 hrs. Then, we analyzed mRNA expression of cytokines (TNF-α, IL-1β, IL-6, IL-12), chemokines (CXCL-1, CXCL-10), enzymes necessary for the production of mediators (iNOS, COX-2), membrane receptors (CD14, TLR4) that are activated in response to LPS and other related genes involved in macrophage activity (GM-CSF, MIF) by quantitative PCR.

Figure 4.26 shows that both, a high and a low dose of dexamethasone reduced mRNA expression of all genes in pLL-3.7 transduced macrophages. This pattern of mRNA expression was similar to the effect on NO production in Figure 4.25. As shown in Figure 4.27, a low dose of corticosterone increased mRNA expression of all genes analyzed, whereas a high corticosterone concentration reduced mRNA gene expression in control, pLL-3.7 transduced macrophages.

In summary, dexamethasone and corticosterone show different effects depending on their concentration. In addition, corticosterone and dexamethasone modulate macrophage function in a concentration-dependent manner by activating the GR but not the MR.

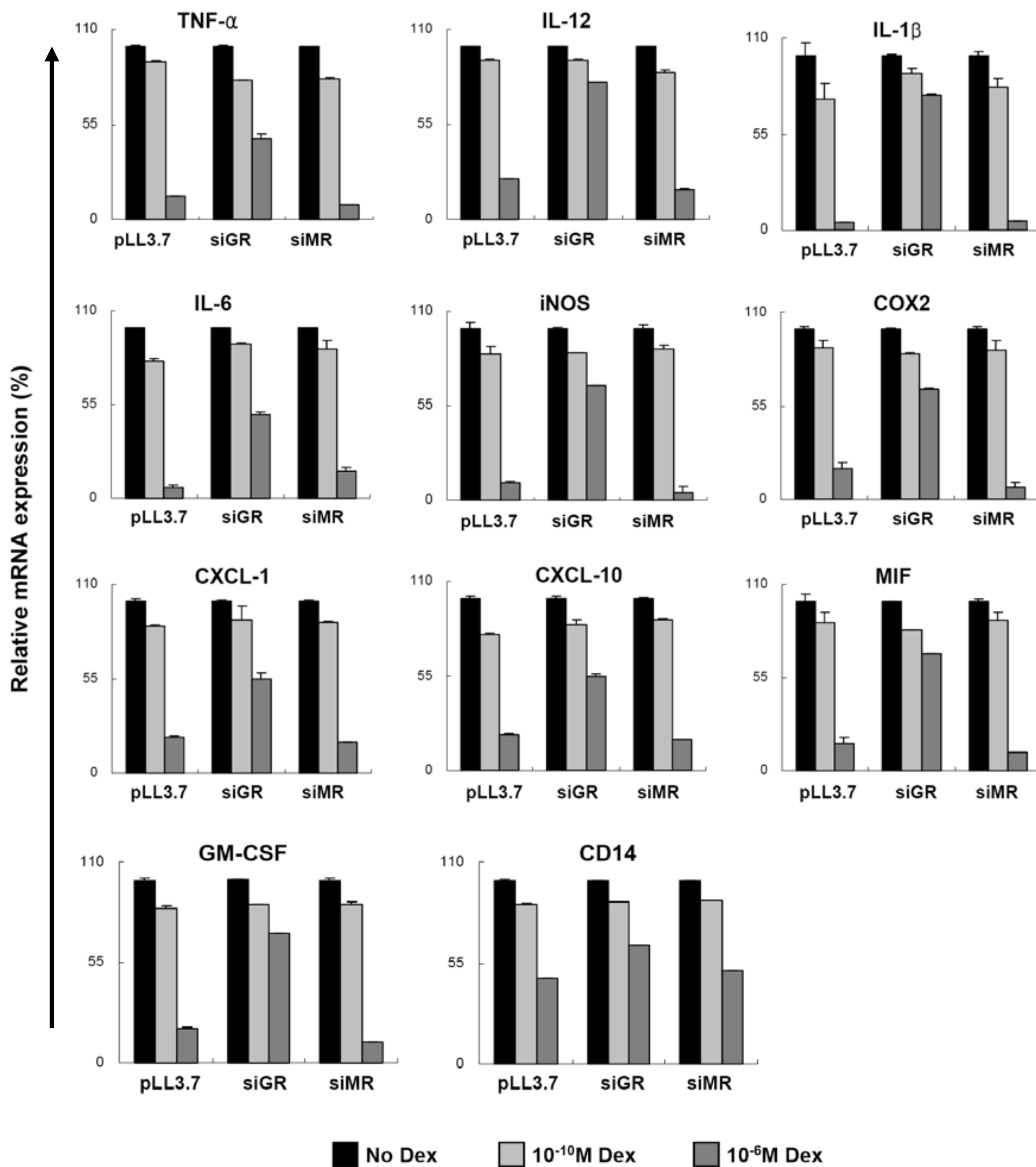


Figure 4.26 Modulation of mRNA expression of various inflammatory mediators by dexamethasone. Thioglycollate-elicited peritoneal macrophages were transduced with pLL-3.7, pLL-siGR2 or pLL-siMR for 2 days and afterwards activated with LPS / IFN-γ in the absence or presence of low or high dose dexamethasone for 6 hrs. mRNA expressions of various inflammation related genes was determined by quantitative PCR. The mRNA levels in the absence of dexamethasone treatment were set at 100%.

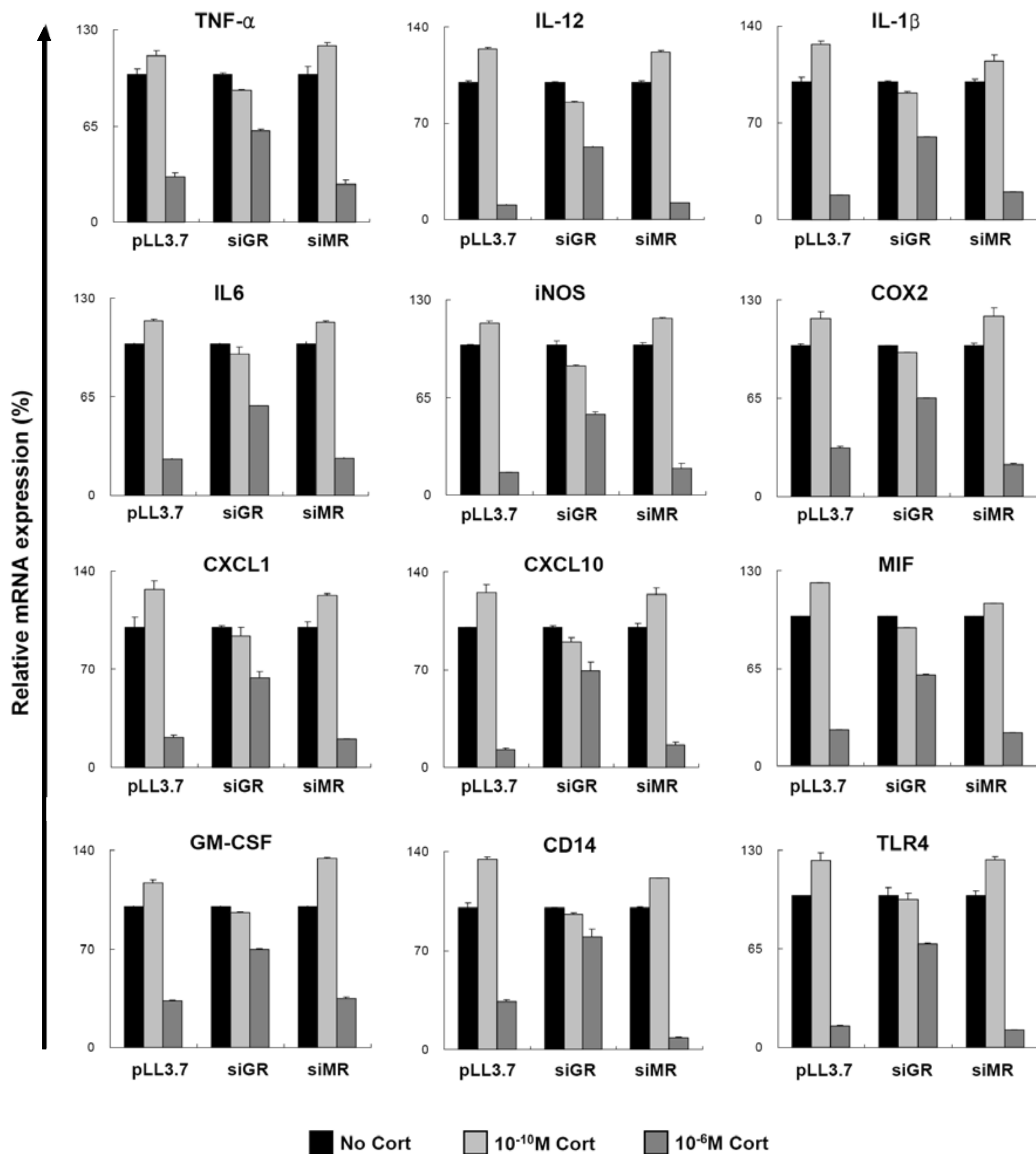


Figure 4.27 Modulation of mRNA expression of various inflammatory mediators by corticosterone. Thioglycollate-elicited peritoneal macrophages were transduced with pLL-3.7, pLL-siGR2 or pLL-siMR for 2 days and afterwards activated with LPS / IFN-γ in the absence or presence of low or high dose corticosterone for 6 hrs. mRNA expression of various proinflammatory genes was determined by quantitative PCR. The mRNA levels in the absence of corticosterone treatment were set at 100%.

4.2 3. The impact of glucocorticoids on innate immunity

4.2.3.1 Adrenalectomy induces macrophage priming

To investigate how the removal of endogenous GCs impacts on innate immunity, we isolated thioglycollate-elicited peritoneal macrophages from adrenalectomized (ADX) and sham-operated rats and took them in culture. As a control, macrophages from both types of animals were activated by LPS and IFṆ-γ for 48 hrs. In addition, corticosterone was either added at a low or a high concentration. Most importantly, macrophages from ADX rats produced elevated levels of NO even in the absence of LPS/IFN-γ induction, which could only slightly be enhanced by LPS/IFṆ-γ treatment (Figure. 4. 28(A)). Nevertheless, NO production by peritoneal macrophages from ADX rats could still be positively and negatively modulated by corticosterone like in macrophages from sham-operated rats.

Similarly, macrophages from ADX rats also expressed elevated mRNA levels of pro-inflammatory mediators (iNOS, TNFα, CXCL10, SPHK1) and other potentially important genes (Ca^{2+} activated intermediate potassium channel (IK1) and , stearoyl-CoA desaturase 2(SCD2)), indicating that these cells were generally pre-activated and must have been primed by the lack of endogenous GCs (Figure. 4.28(B)). We conclude that the removal and the addition of increasing concentrations of corticosterone impact on macrophage function in a complex manner.

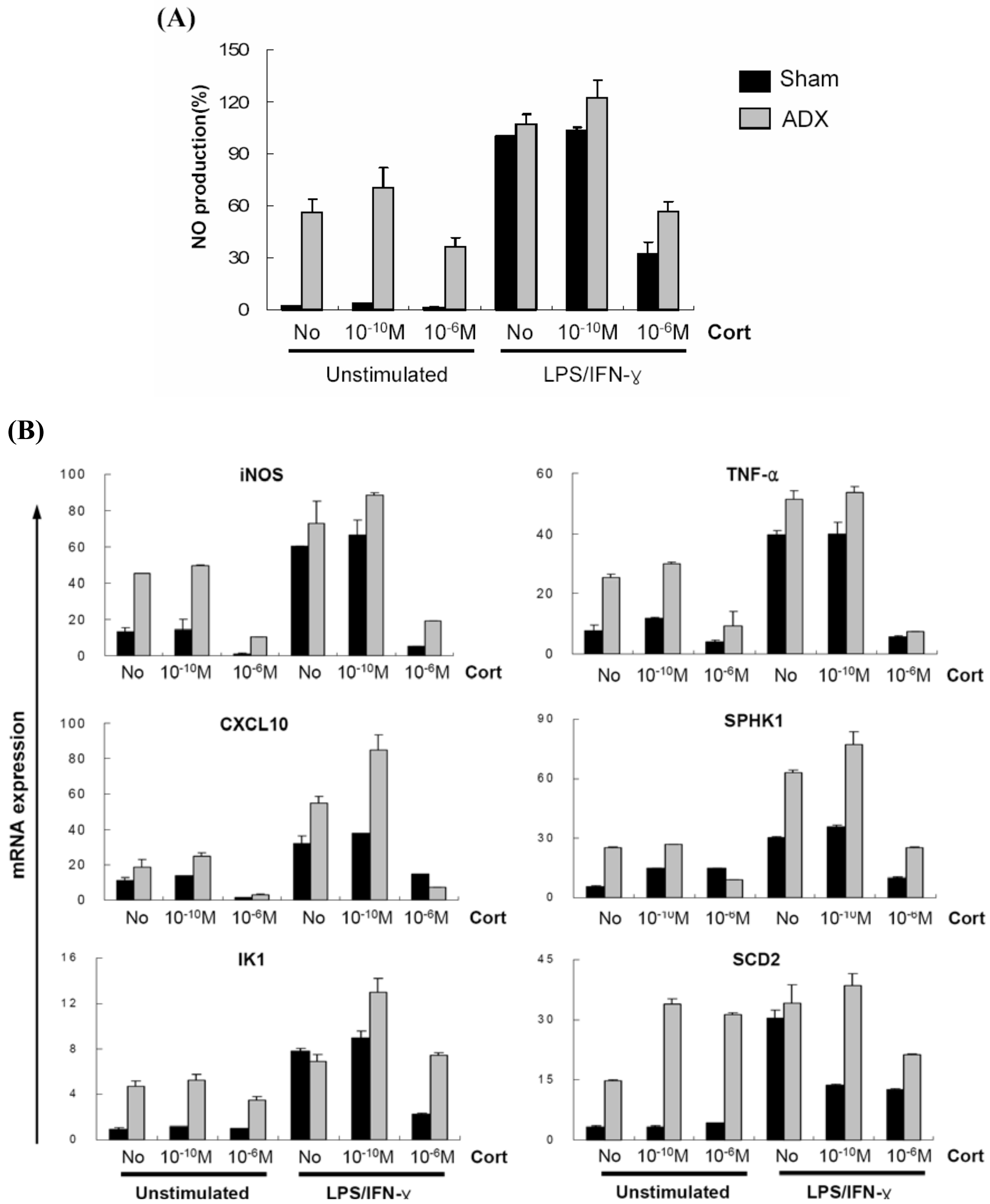


Figure 4.28 Peritoneal macrophages from adrenalectomized rats produce inflammatory mediators. Adrenalectomized or sham-operated rats were obtained by operating CD:Crl rats and removing the adrenal glands to get rid of endogenous GCs or the same procedure were performed without removing the adrenals. After the rats had recovered, peritoneal macrophages were isolated from adrenalectomized (ADX) and sham-operated rats, left unstimulated or activated with LPS / IFN-γ in the absence or presence of low or high

corticosterone. (A) We determined NO production in the supernatant of cells from both rats after 48 hrs by photometric assay. (B) mRNA expression of the inflammatory mediators was measured by quantitative PCR 6 hrs after activation.

4.2.3.2. The role of the GR for GC actions after adrenalectomy

In the previous experiments, we confirmed that dexamethasone and corticosterone modulate inflammatory signaling in response to LPS / IFN-γ stimulation via the GR, not the MR. Next, to further study of the function of the GR and the MR in ADX macrophages, we transduced macrophages from ADX and sham-operated rats with pLL-3.7, pLL-siGR or siMR lentiviruses and added a low or a high concentration of corticosterone. After 48 hours, we determined NO production using the cell supernatant.

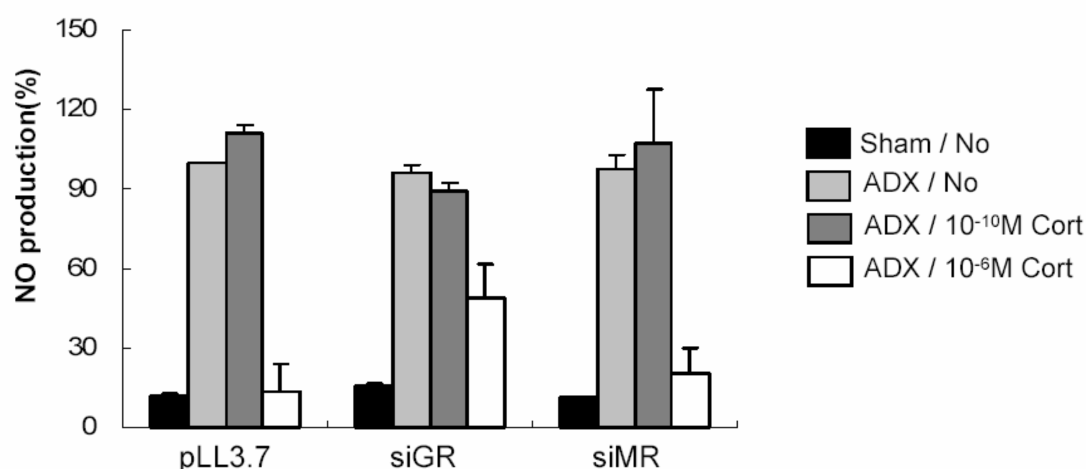


Figure 4.29 Impact of GR and MR inactivation on the ability of corticosterone to modulate NO production in ADX rats. Purified peritoneal macrophages from sham-operated and adrenalectomized (ADX) rats were transduced with the lentiviruses pLL-3.7, pLL-siGR2 or pLL-siMR. Afterwards macrophages were incubated in the absence or presence of low dose or high dose corticosterone for 48 hrs. We measured NO production under each condition in the cell supernatant using Griess reagent.

Figure 4.29 shows a similar pattern as the previous results in control, siGR or siMR lentiviruses-transduced LPS-activated macrophages in the absence or presence of a low or a high dose of corticosterone. Thus, the shRNA-GR prevents the reduction of NO production by high concentrations of corticosterone in LPS-stimulated and ADX-macrophages and the shRNA-MR has no effect on this modulation.

Thus, in ADX macrophages the immunomodulatory activity of GCs is also mediated via the GR.

Next, we investigated the role that the GR play in the immunomodulatory activitiy of corticosterone in ADX macrophages. To address this, we transduced ADX and sham-operated macrophages with the lentiviruses pLL-3.7 and pLL-siGR for two days, and activated only sham-operated macrophages with LPS/IFN-γ. In addition, corticosterone was added at a low (10^{-10}M) or a high (10^{-6}M) concentration. After 6 hours, we analyzed mRNA expression of various inflammatory mediators by quantitative PCR. Both pLL-3.7-transduced macrophages show a similar pattern of mRNA expression as in the previous results, i.e. increased mRNA expression at low doses and repression of inflammatory mediators at high doses. Interestingly, inactivation of the GR leads to a more increased mRNA expression of various inflammatory mediators as compared to control, pLL-3.7 transduced macrophages in the absence of corticosterone (Figure 4.30).

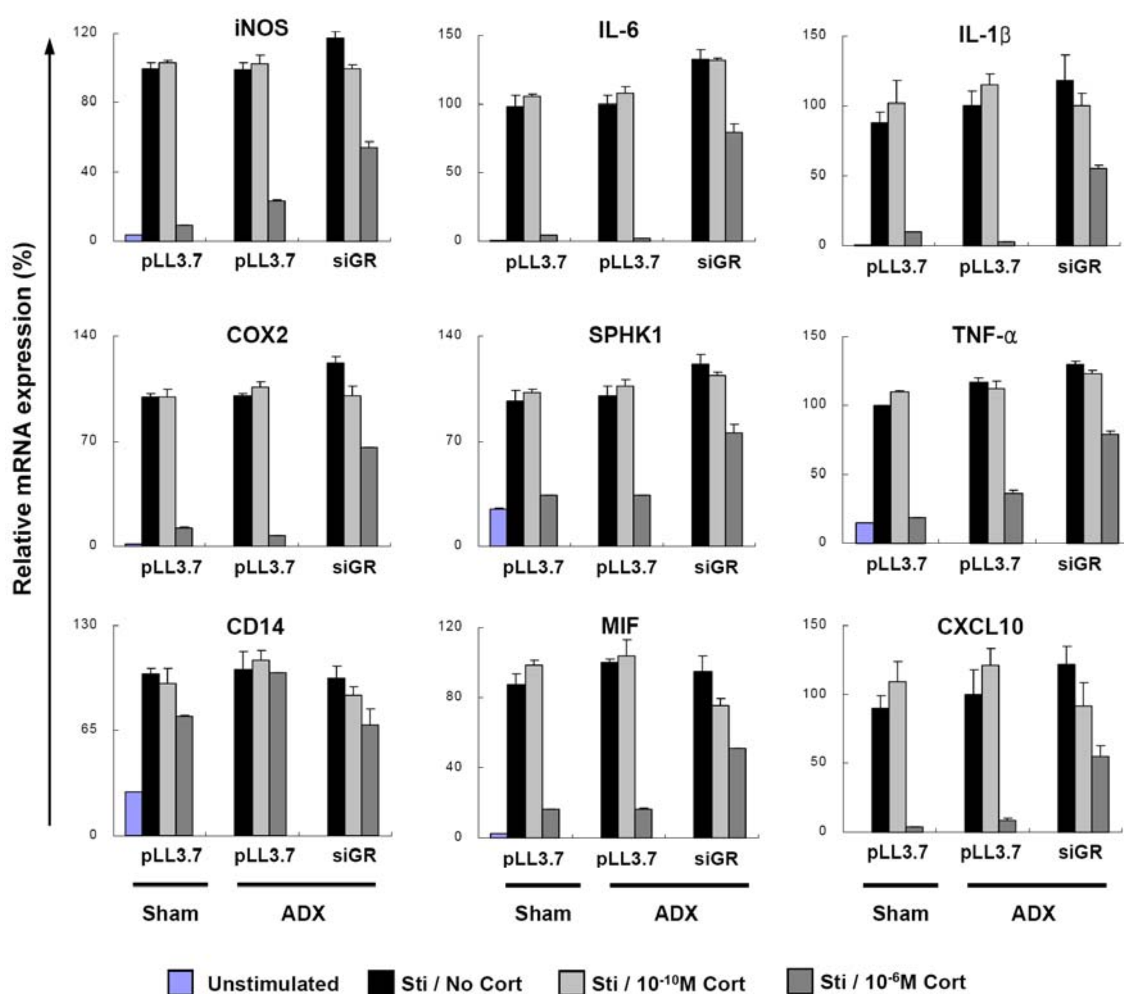


Figure 4.30 Analysis of mRNA expression of inflammatory mediators in ADX macrophages. Purified peritoneal macrophages from sham-operated and adrenalectomized (ADX) rats were transduced with the lentiviruses pLL-3.7 or pLL-siGR2. Afterwards macrophages were incubated in the absence or presence of low dose or high dose corticosterone for 6 hrs. We performed quantitative PCR to compare mRNA expression of various inflammatory mediators in pLL-3.7 or siGR lentivirus-transduced ADX macrophages. β- actin was used for normalization of mRNA values.

4.2.3.3. Expression of immune molecules on macrophages in response to stimulation

In general, macrophages are activated by a variety of stimuli, such as LPS, in the course of an immune response. Activated macrophages are more effective in eliminating potential pathogens by exhibition phagocytic activity, increased secretion of

inflammatory mediators, and an increased ability to activate T cells. In addition, activated macrophages also express higher levels of MHC class II molecules and the co-stimulatory B7 family of membrane molecules, allowing them to function more effectively as antigen-presenting cells (Zhu FG, 2001 Immuno.

We asked whether corticosterone is involved in the regulation of class II MHC molecules and the co-stimulatory B7 family on macrophages via GR or MR.

To this end, we transduced peritoneal macrophages with the lentiviruses pLL3.7, pLL-siGR or pLL-siMR for two days, activated them with 100ng/ml LPS, 100units/ml rat IFN-γ and analyzed the expression of several immune molecules, which are class II MHC molecules (RT1B and D) and the co-stimulatory B7 molecules (CD80 and CD86), by FACS staining. In addition, corticosterone or dexamethasone was added either at a low (10^{-10}M) or a high (10^{-6}M) concentration.

We first analyzed transduction efficiency by flow cytometric analysis of enhanced green fluorescent protein (eGFP) expression two days after infection (Figure. 4.31(A)).

Next, we asked whether lentiviral infection impacts on MHC class II and B7 expression on macrophage under unstimulated condition. Table 4.3 shows that all four molecules weakly increase compared to wild type cells.

Thus, lentivirus infection probably induces the expression of B7 molecules on macrophages.

	WT	Con	siGR	siMR
RT1B	1.00	**1.20**	1.13	1.03
RT1D	1.00	**0.86**	0.92	1.28
CD80	1.00	**1.27**	1.85	1.50
CD86	1.00	**1.60**	1.28	1.25

Table 4.3 Relative expression values of immune molecules on unstimulated peritoneal macrophages. Purified peritoneal macrophages were transduced with the lentiviruses pLL-3.7, pLL-siGR2 or pLL-siMR for 2 days. Afterwards macrophages were analyzed for the expression of MHC class II and B7 molecules by FACS staining. The expression values of each molecules in wild type was set to 100% (≈ 1.00).

As shown in Figure 4.31 (B, C), in wild type macrophages, RT1B and RT1D were strongly induced around 5 ~ 6 fold by LPS-stimulation as compared to the unstimulated condition and were not altered by glucocorticoids treatment. Either lentivirus-transduced macrophage showed a similarly pattern MHC class II expression (Figure 4.31(B)).

The expression of the B7 molecules, CD80 and CD86, was induced to a lower degree than the expression of MHC class II molecules on wild type and lentiviruses-transduced macrophages by LPS stimulation and was not altered by glucocorticoid treatment.

Moreover both inactivation of the GR and the MR by shRNA had no effects on the expression of MHC class II and B7 molecules.

This indicates that the expression of these immune molecules could be induced by LPS / IFN-ɣ stimulation, but is not modulated by GCs.

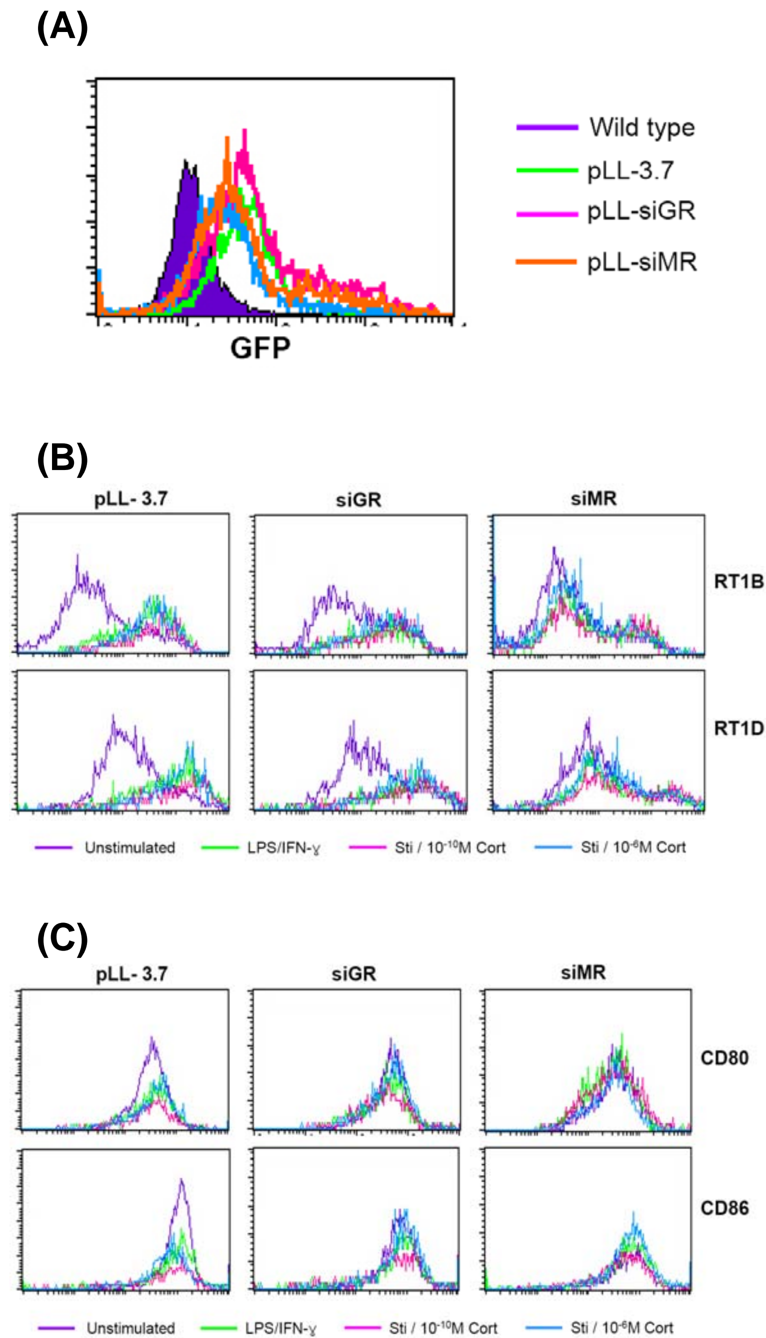


Figure 4.31 Expression of immune molecules on peritoneal macrophages under different conditions. Purified peritoneal macrophages were transduced with the lentiviruses pLL-3.7, pLL-siGR2 or pLL-siMR for 2 days. Afterwards the expression levels of MHC class II and B7 molecules was analyzed on macrophages by FACS staining. The transduction efficiency of the lentiviruses was confirmed by GFP expression through FACS (A) and the expression of the immune molecules on macrophages was demonstrated by histogram plot.

V. Discussion

Part I. Analysis of knock-down mineralocorticoid receptor transgenic rats

5.1.1 Efficient inactivation of MR expression in shRNA-MR transgenic rats

To maintain physiological homeostasis in rodents and humans, numerous steroid hormones are synthesized and released from the adrenal gland under the control of the hypothalamic/pituitary adrenal axis and the RAAS. And then the actions of hormone are mediated via hormone receptors according to their concentration and specificity.

One of the hormone receptors, the mineralocorticoid receptor (MR or NR3C2) is a member of the nuclear receptor superfamily and has a high affinity for endogenous corticosterone and mineralocorticoids. The MR is expressed in restricted organs, which are epithelial tissues including kidney and colon, the limbic system of the brain and heart. In several studies including deficient or over-expression MR mice, it was shown that the function of the MR is involved in controlling the salt-water balance, neuronal excitability and heart function (Berger et al., 1998; De Kloet et al., 1998; Pitt, 2004). In kidney and colon, the adrenocortical hormone aldosterone induces sodium-water reabsorption (Berger et al., 1998), while dysregulation of MR function in the heart has been implicated in pathophysiological conditions (Beggah et al., 2002; Le Menuet et al., 2001). In concert with the highly related glucocorticoid receptor (GR), the MR controls neural excitability in the hippocampus and thereby contributes to behavioral response (Berger et al., 2006). Thus, the MR fullfills diverse functions in mammals.

Therefore, we decided to inactivate the MR in transgenic rats by lentiviral expression of a specific shRNA. To achieve this aim, we generated knock-down mineralocorticoid receptor transgenic rats by lentiviral expression of a specific shRNA and analyzed

which effects inactivation of the MR had in the transgenic rats.

At first we confirmed that the lentiviral specific shRNA sequence against the MR leads to strongly reduced MR expression on the mRNA and protein level by western blot and quantitative PCR in MR expressing stable cells. Afterwards we used high titer concentrated lentivirus particles to generate knock-down transgenic rats.

To examine the efficiency of the MR knock-down we analyzed composite F1 offspring of the founder rats by cross combination between the male founder rat siMR9 and the two female founder rats siMR7 and siMR8. The offspring of the transgenic rats which are determined by PCR showed a reduction in MR mRNA levels by 70% ~ 80% on average in kidney and in hippocampus and strongly reduced protein levels while GR mRNA and protein levels were unaltered in both organs. Moreover, we observed a great diversity of MR expression levels ranging from a reduction by less than half to almost complete gene inactivation. Despite the integration of the shRNA transgene, we were not able to demonstrate the presence of the DsRed protein in any of the transgenic rats. Apparently, the CMV promoter present in the lentiviral construct does not support gene expression in transgenic animals at sufficient levels. In summary, siMR transgenic rats express the siRNA from the integrated provirus although the DsRed reporter gene is silent.

Taken together, these observations suggest that specific and efficient down-regulation of the MR can be achieved in siMR knock-down rats.

5.1.2. Knock-down of the MR by shRNA shows signs of pseudohypoaldosteronism

An important role of the MR is suggested by loss-of-function mutations in humans resulting in pseudohypoaldosteronism (PHA) type I (Armanini et al., 1985; Cheek and

Perry, 1958). The physiological deficits seen in these patients are mimicked by MR knock-out mice and characterized by increased levels of aldosterone and renin and an altered Na^+/K^+ balance. This results in growth retardation and early lethality of the mutants, precluding the analysis of physiological processes in adult MR knock-out mice. To investigate whether siMR rats show similar physiological alterations, we analyzed transgenic rats at 14-28 days of age.

Firstly, we observed a significantly lower body weight by 25% lighter on average in 14 ~ 28 days of age of transgenic rats compared to wild-type littermates and a small number of newborn pups. Strikingly some of them always die by around two weeks of age, but most of the transgenic offspring survived and could be analyzed. In a previous report, all MR homozygous knock out mice (MR -/- mice) did not thrive, lost weight and died at day 8 ~ 13 after birth due to a severe disturbance of the salt-water balance, not caused by growth inhibition (Berger et al., 1998). In addition, we examined mRNA expression of stearoyl-CoA desaturase 2 (SCD2), which is required for the biosynthesis of mono-unsaturated fatty acids and potentially involved in the weight loss of the MR knock-down rats. In general, essential fatty acids are required for normal growth and skin function, and a deficiency in these fatty acids is characterized by growth retardation, skin abnormalities, and increased transepidermal water loss(Hansen and Jensen, 1985; Melton et al., 1987; Wertz and Downing, 1990). SCD2 null mice showed a >80% reduction in SCD activity in the brain of newborn and adult mice and rapidly reduced body weights of newborn *Scd2*$^{-/-}$ mice, indicated that SCD2 is important in lipid synthesis during early development, crucial for the formation of a functional skin permeability barrier and is required for survival (Miyazaki et al., 2005). Our siMR transgenic rats showed that the SCD2 mRNA expression is reduced from 20% to 90% in

kidney and around 50% to 80% in hippocampus of old and young transgenic rats. The reduction of SCD2 showed graded mRNA expression related to the age of transgenic rats. This suggested that SCD2 is an additional factor involved in the induction of weight loss and its action could be influenced by MR expression in kidney and hippocampus.

As mentioned before, the mineralocorticoid receptor is the pivotal effector of the cellular response to mineralocorticoids, namely aldosterone. Aldosterone is classically synthesized in the adrenal gland and binds to specific mineralocorticoid receptors (MRs) located in the cytosol of target epithelial cells. In epithelial cells corticosteroid hormone action is controlled at a prereceptor level by the activity of the 11β-HSD2 enzyme that effectively protects the MR from occupation by corticosterone and gives specificity with aldosterone.

The regulation of hormone synthesis and secretion is mediated by angiotension II (Ang II) and the concentration of extracellular potassium and ACTH(Muller, 1987; Quinn and Williams, 1988) and regulated by the renin-angiotensin system (RAAS). Renin, which is synthesized by the juxtaglomerular cells in the kidney, catalyses the conversion of angiotensinogen to angiotensin I (Ang I). Ang I then is converted to Ang II by angiotension-converting enzyme (ACE). It is known that Ang II stimulates aldosterone production by acting on specific G-protein coupled receptors (Spat and Hunyady, 2004). The other key determinant is the concentration of extracellular potassium ions (K^+). Indeed increased potassium ion concentration stimulates aldosterone secretion and production of aldosterone is sensitive to very small changes in extracellular K^+. Both, extracellular [K+] and Ang II act in synergism and potassium ion influence the concentration/effect relationship of Ang II-mediated aldosterone production (Connell

and Davies, 2005).

MR deficient (-/-) mice had shown that the RAAS is strongly activated by renin and Ang II. Aldosterone is extremely increased and the mice show hyperkalemia, both which are caused by enhanced fractional excretion of sodium ions. We also observed similar patterns, namely renin and aldosterone concentration in the serum were increased by a factor of three to four and the mRNA expression of the Ca^{2+} activated potassium channel, IK1 encoding Kcnn4 gene, in young and adult MR knock-down transgenic rats was reduced. Kcnn4 channels are prominently expressed in cells of the hematopoietic system, in T lymphocyte and in organs involved in salt and fluid transport metabolism including colon, lung, and salivary gland (Joiner et al., 1997; Logsdon et al., 1997; Vandorpe et al., 1998). Homeostasis of potassium ions is controlled via the (NA^+ + K^+)-ATPase channel located in the basolateral membrane together with the cellular K^+ conductance which drives excretion of potassium from the cell to lumen. Kcnn4 null mice showed normal appearance and fertility and lost K^+ permeability in blood cells (Begenisich et al., 2004). We confirmed that Kcnn4 exists in kidney and hippocampus by standard RT-PCR. We supposed that a reduction of the Kcnn4 channel could lead to partially similar effects in kidney like the loss of K^+ permeability and hyperkalemia symptoms. In summary the down regulation of SCD2 and Kcnn4 genes may contribute to growth retardation in our MR knock-down transgenic rats.

The classical genomic action of aldosterone is mediated by the MR, regulates specific "aldosterone-induced' gene transcription in target epithelial cells where it modulates electrolyte, fluid balance, and subsequent blood-pressure homeostasis. In recent studies, it was shown that aldosterone induced proteins through early (1 ~ 6hr) and late (> 6hrs)

phases after binding the hormone-receptor complex to hormone response element in the nucleus. The early phase is mediated by changes in gene expression that activate ion channels and signaling proteins which induce electrolyte-transport proteins. The later phase results from both primary and secondary effects on gene transcription (Connell and Davies, 2005). The representative target genes by the aldosteron-MR complex are sgk1 and ENaC. Sgk1 (serine-theronine glucocorticoid induced kinase 1) is one of the early aldosterone-induced proteins (Naray-Fejes-Toth et al., 2004; Stockand, 2002) and enhances distal nephron Na+ transport, in part by preventing the internalization of ENaC from the plasma membrane (Boyd and Naray-Fejes-Toth, 2005). The other target gene, ENaC (epithelial sodium channel) consists of three homologous subunits, α, β and γ (Canessa et al., 1994), is involved in sodium reabsorption in kidney and their activity, trafficking and gene transcription is regulated by sgk1 at a later phase. Aldosterone increases extracellular fluid volume and blood pressure by activating ENaC. Sgk1 regulates ENaC-mediated Na+ transport at multiple levels in the renal collecting duct cells(Pearce and Kleyman, 2007). MR knock-out mice showed that amiloride-sensitive Na+ reabsorption is reduced, although the abundance of the mRNA encoding ENaC subunits was unaltered or showed only a minor decrease of the β- and γ-subunits in kidney and colon. Other investigations showed that aldosterone depletion has no or weak effects on mRNA expression of ENaC subunits in the kidney (Escoubet et al., 1997). This indicates that mRNA abundance of ENaC subunits is MR-independent and MR-mediated Na+ reaborption is not achieved by transcriptional control of mRNA abundance of sodium channels. However our MR knock-down rat show opposite results. The abundance of the mRNA of sgk1 and ENaC subunits, especially the α and γ subunits, are highly increased in kidney, indicating they might be up-regulated via other

hormone receptor e.g. the glucocorticoid receptor (Mick et al., 2001) and by glucocorticoids (Webster et al., 1993) and a number of other hormones and mediators (Lang and Cohen, 2001). We suppose that increased mRNA abundance of sgk1 and ENaC partially compensates for the occurring sodium loss by the knock-down of the MR.

In other tissues in which the MR is expressed but where no protection mechanism by enzymes exist e.g. in the hippocampus, the MR is occupied mainly by endogenous glucocorticoids because of the higher circulating concentration of corticosterone than aldosterone. In these tissues, both MR and GR controls neural activity and behavioral response via different mechanisms (De Kloet et al., 1998; McEwen and Sapolsky, 1995). The limbic MR deficient mice shown an up-regulation of hippocampal GR expression and behavioral changes associated with abnormal hippocampal mossy fiber layout (Berger et al., 2006). We observed that the expressions of MR protein are reduced in hippocampus by immunohistochemistry and western blot, but no alternation of GR protein expression in siMR transgenic rats.

5.1.3 Strategy for the generation of the transgenic rat by delivery of a specific lentiviral shRNA

Until recently, the analysis of gene inactivation *in vivo* was limited to mice and the development of knock-out mouse strains revolutionized research in many fields of molecular biology and medicine. This technique is based on homologous recombination in embryonic stem (ES) cells, which allows to specifically disrupt or modify gene sequences (Bowers et al., 1999; Gordon et al., 1980; Phillips et al., 1999; Sanford et al., 2001). However, many questions, e.g. related to cardiovascular research, autoimmune

diseases or transplantation are best addressed using rats(Jacob and Kwitek, 2002). Surgical procedures are much easier performed and disease models sometimes more closely reflect the situation encountered in humans. In view of these facts, it is important to develop new tools allowing for gene inactivation in rats. Unfortunately, efforts to derivate germline contributing rat embryonic stem cells over the last twenty years have failed. Furthermore, although nuclear transfer may represent an alternative approach and Zhou *et al* (Zhou et al., 2003) demonstrated that transferred nuclei from somatic cells allowed for the successful reprogramming of oocytes, nulear transfer does not yet provide access to the generation of knock-out rats. Therefore this strategy is not an option for the generation of knock-out rats.

Recently, it was shown for the first time that lentiviral delivery of a shRNAs in transgenic mice allowed for the down-regulation of mRNA and protein levels by up to 90% (Carmell et al., 2003; Hawes et al., 1992). This approach takes advantage of RNA interference (RNAi), which involves the processing of small inhibitory RNA (siRNA) molecules from hairpin structures. Most importantly, this technique can also be applied to rats. Consequently, the delivery of specific shRNAs via transgenesis is presently the only promising strategy for gene inactivation in this species. The feasibility of such an approach has previously been demonstrated in mice (Lois et al., 2002; Tiscornia et al., 2003; Wianny and Zernicka-Goetz, 2000).

In our study, we found that most of the offspring was viable although animals display a graded loss of MR mRNA and protein from 50% to more than 90% and the animals showed a phenotype comparable to both, the knock-out mouse model and human PHA I patients.

However, the advantage of this model is that viable adult MR knock-down rats can be

obtained for studying the function of the MR *in vivo*, thus this technique is an attractive option to achieve gene inactivation in the rat and allows to generate animal models with graded effects of specific gene expression Thus, we believe that this strategy will become instrumental in modeling the pathophysiological consequences of human genetic diseases in transgenic rats and should help to better understand human diseases, which are frequently characterized by a partial loss-of-function rather than by full gene inactivation. Thus, under such circumstances the knock-down technology may therefore be superior to classic knock-out mice.

Part II. Analysis of immunomodulatory functions of glucocorticoids by RNA interference

5.2.1 GCs exert immunostimulatory and immunosuppressive activities via the GR dependent on their concentration

Glucocorticoids are important regulators of the immune system (Tuckermann et al., 2005) and modulate a plethora of physiological functions. Also GCs are involved in the modulation of macrophage function and thereby control the host's immune responses to pathogens. While synthetic GCs are in widespread use to treat autoimmune and atopic inflammatory diseases as potent anti-inflammatory and immunosuppressive agents (Boumpas et al., 1993), endogenous GCs exert a wide range of immunomudulatory activities and physiologic actions involved in maintaining homeostasis (Abe et al., 1995). The major GC in rodents is corticosterone and cortisol in human, which are synthesized in the adrenal gland in response to stress or systemic cytokine release. GCs either act via the glucocorticoid receptor (GR) or the mineralocorticoid receptor (MR), which resides in the cytoplasm sequestered in a heat-shock protein complex. While

actions of GCs are mostly mediated by the GR, the MR plays an equally important role in the hippocampus (Beato et al., 1995). In this case it is assumed that the MR mediates GC effects at basal levels while the role of the GR is largely refined to situations of elevated hormone concentrations, e.g. during stress (Joels and de Kloet, 1994).

After ligand-receptor binding they translocate into the nucleus and modulate transcription in a positive or negative manner. However, neither the role of hormone concentration nor the differential contribution of the GR and the MR to these activities are unknown. Interestingly, it has been reported that MR is expressed in human CD34+ hematopoietic progenitor cells(Grafte-Faure et al., 1999) and rat microglial cells (Tanaka et al., 1997), a macrophage-related cell-type that resides in the CNS. It was suggested that the MR mediates immunostimulatory effects at low corticosterone concentrations whilst immunosuppressive GC effects at high doses are conferred through the GR. However, whether a similar phenomenon also exists for other types of immune cells is presently unknown.

Guided by the observation that GCs are not purely immunosuppressive but also enhance certain immune functions, in particular at low levels (Dhabhar and McEwen, 1999; Wiegers et al., 1993), we asked whether (i) concentration-dependent GC effects can also be found in peritoneal macrophages, and (ii) whether they are mediated by the GR or the MR.

Firstly, in agreement with our expectations, immunofluorescence and PCR analysis provided evidence that thioglycollate-elicited peritoneal macrophages express both the GR and the MR. Furthermore, 11β -HSD2 is not present in macrophages, which is in line with previous data (Gilmour et al., 2006). Thus, corticosterone should be able to regulate these cells.

Secondly, to investigate whether the effects of GCs on macrophage activity are dependent on concentration, macrophages were stimulated with LPS / IFN-γ, and additionally treated with a high or a low dose corticosterone. We measured NO production and analyzed the mRNA expression by quantitative PCR. In macrophages, the expression of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 and TNF-α, chemokines such as CXCL-1, IL-8 and CXCL-10 and the enzymes inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) were repressed by GCs (Almawi et al., 1996; Fushimi et al., 1998; Kunicka et al., 1993). Our data show that a low concentration of corticosterone enhances NO production as well as mRNA expression of various pro-inflammatory mediators and a high corticosterone concentration strongly represses macrophage function. This indicates that macrophage function is enhanced at low corticosterone levels while high concentrations are immunosuppressive. A similar observation was previously made with regard to effects of GCs on a delayed-type hypersensitivity response, the stimulation of T cell mitogenesis and NO production by microglial cells (Dhabhar and McEwen, 1999; Tanaka et al., 1997; Wiegers et al., 1993). Thus, opposing effects of GCs in dependence of their concentration appear to be a general characteristic of the immune system.

Third, previous studies had suggested that the enhancing activities of corticosterone are mediated by the MR while the repressive ones require the GR (Tanaka et al., 1997). We investigated which hormone receptor modulates the glucocorticoid-dependent immune response in macrophages by using lentiviruses encoding specific small interfering RNA (siRNA) molecules. We transduced macrophages with the lentiviruses siGR or siMR, activated then with LPS/IFN-γ, and treated with a high or a low concentration of corticosterone. Our analysis shows that inactivation of the GR by lentiviral delivery of

siRNAs abrogated both the immunostimulatory and the immunosuppressive GC actions whereas inactivation of the MR had no effect. It now clearly shows that the MR is dispensable for both types of GC actions despite its expression in macrophages. However, the mechanism by which the opposing effects are achieved remains unknown. The finding that corticosterone exerts dosage-dependent activities has important consequences for our understanding of immunoregulation by GCs. Whilst the repressive effects of high GC doses prevents exaggerated immune responses, we now found that macrophage functions are potentiated by low levels of corticosterone. We hypothesize that the release of limited amounts of GCs serves to alert the organism of potential threats thereby preparing it to respond to pathogens or wounding. One could imagine a model in which macrophage function is enhanced at an early stage of inflammation when GC levels are only mildly elevated, while leukocytes are held in check at a later stage when the massive release of endogenous GCs prevents a potentially deleterious host response. This would be in line with the model suggested by McEwen and colleagues arguing that acute stress situations are likely to prepare the organism for challenges (Dhabhar and McEwen, 1999).

In the hippocampus, the differential ligand affinities of the GR and the MR underlie the opposing effects of low and high corticosterone concentrations. Pharmacological experiments performed on microglial cells suggested that a similar phenomenon may exist in the immune system (Tanaka et al., 1997). This was further supported by our finding that macrophages express MR mRNA and protein. However, selective inactivation of either the GR or the MR by lentiviral delivery of siRNAs to macrophages *ex vivo* revealed that the presence of the MR was dispensable for all the immunomodulatory functions of GCs tested. Thus, it remains unclear how two different

concentrations of a single hormone are able to achieve opposing effects through one receptor. An explanation would be that different conformations of the GR are induced at low versus high GC levels. For example, the GR binds as a homodimer to the enhancer region of the tyrosine-aminotransferase gene (Schmid et al., 1989) while concerted multimers are found in the case of the phenylethanolamine N-methyltransferase (PNMT) gene (Adams et al., 2003). Alternatively, cofactors could be differentially recruited to the GR dependent on receptor occupancy. Besides the possibility that opposing GC activities are mediated through genomic effects of the GR, non-genomic mechanisms also need to be considered (Tuckermann et al., 2005). It is known that the GR is able to interact with cytosolic signaling molecules such as PI3K, and that GCs can act through a putative membrane receptor. Therefore it is tempting to speculate that genomic effects are seen at high GC concentrations while non-genomic mechanisms rather become effective at lower hormone levels. Taken together, it will be interesting in the future to further dissect the molecular basis of the opposing GC effects.

5.2.2 Adrenalectomy induces macrophage priming that is mediated by the GR

As mentioned before, endogenous GCs are essential for protecting the organism by preventing the harmful effects of exaggerated immune responses, the management of sepsis (Yeager et al., 2004) and play a physiological role in the feedback inhibition of immune responses and in maintaining homeostasis (Abe et al., 1995). Consequently, adrenalectomy or treatment with the GR antagonist RU486 results in high mortality in various models of infection (Bertini et al., 1988; Hawes et al., 1992; Zuckerman et al., 1989). In contrast, elevated GR expression is associated with increased resistance to endotoxic shock (Reichardt et al., 2000). In addition, GCs modulate the function of

leukocytes at different levels including the control of gene expression, enzymatic activity, cellular migration, repress myelopoiesis and inhibit expression of MHC class II induced by IFN-γ (Sternberg, 1993; Tuckermann et al., 2005).

We investigated whether removal of endogenous GCs by adrenalectomy *in vivo* could induce a pre-activated state in peritoneal macrophages and be modulated by corticosterone. To address this, we adrenalectomized (ADX) or sham-operated rats. We measured NO production and analyzed the mRNA expression of various genes involved in macrophage function in unstimulated or LPS/IFN-γ stimulated peritoneal macrophages treated with either a high or a low concentration of corticosterone, from ADX and sham-operated rats. Removal of endogenous GCs *in vivo* induced a pre-activated state in macrophages from ADX rats that produced increased levels of NO and expressed higher mRNA levels of genes related to inflammation (iNOS, TNF-α, CXCL10, SPHK1 (sphingosine kinase 1)) and genes involved in homeostasis (IK1(intermediate potassium channe), SCD2(stearoyl-CoA desaturase 2)) in the absence of stimulus. Moreover, the induction of NO and mRNA expression of inflammatory mediators were partially suppressed by GCs in ADX-macrophages.

Previous data showed that SPHK1 plays a key role in TNF-α triggered intracellular Ca^{2+} signals, degranulation, activation of NF-κB, and pro-inflammatory cytokine production by primary human monocytes (Zhi et al., 2006) and that the circulating concentration of CXCL10 increases in autoimmune primary adrenal insufficiency (Rotondi et al., 2005). In addition, our unpublished date had shown that adrenalectomized animals restored cellularity within 3 weeks after surgery. In this experiment, peritoneal macrophages from ADX rats produced higher mRNA expression of SCD2, which is an essential enzyme for the biosynthesis of saturated fatty acids, as

compared to sham-operated rats, either in absence or presence of stimulus and both dose of a low or a high corticosterone. Then, it could contribute to the recovery from ADX after surgery. Another intriguing finding is the pre-activation of macrophages following the removal of endogenous GCs by adrenalectomy, since it suggests that GCs serve to keep the immune system in an inactive stage in the absence of any stimulus and the presence of GCs prevents premature activation of the immune system in the absence of stimulation. As a consequence, immune functions can be immediately modulated once GC levels rise in response to a challenge. Next, we investigated the role of the GR in the immunomodulatory activity of corticosterone in ADX macrophages using lentiviruses expressing shRNA-GR or shRNA-MR sequences. The shRNA-GR abolished the modulatory activities of GCs at either a low or a high dose of corticosterone in ADX-macrophages and prevents the reduction of NO production and mRNA expression of genes by a high dose of corticosterone and repression at low dose of corticosterone, whereas the shRNA-MR has no influence on this modulation. Thus it seems that the immunomodulatory action of GC in ADX-macrophages and becoming pre-activation state by adrenalectomy is mediated by the GR.

In summary, our results revealed three different functions of GCs in controlling inflammatory macrophages that are all mediated through the GR despite concomitant expression of the MR. The presence of basal levels of GCs guarantees that the immune system is inactive in the absence of stimulation. Release of low amounts of corticosterone enhances immune functions and thereby prepares the organism to challenges. Finally, high concentrations of GCs repress macrophage function and thereby prevent exaggerated immune responses that may harm the organism. Thus, GCs exert complex modulatory activities in the control of the innate immune system.

VI. Reference

Abe, R., Shimosegawa, T., Kimura, K., Abe, T., Kashimura, J., Koizumi, M., and Toyota, T. (1995). The role of endogenous glucocorticoids in rat experimental models of acute pancreatitis. Gastroenterology *109*, 933-943.

Adams, M., Meijer, O. C., Wang, J., Bhargava, A., and Pearce, D. (2003). Homodimerization of the glucocorticoid receptor is not essential for response element binding: activation of the phenylethanolamine N-methyltransferase gene by dimerization-defective mutants. Mol Endocrinol *17*, 2583-2592.

Ahima, R., Krozowski, Z., and Harlan, R. (1991). Type I corticosteroid receptor-like immunoreactivity in the rat CNS: distribution and regulation by corticosteroids. J Comp Neurol *313*, 522-538.

Almawi, W. Y., Beyhum, H. N., Rahme, A. A., and Rieder, M. J. (1996). Regulation of cytokine and cytokine receptor expression by glucocorticoids. J Leukoc Biol *60*, 563-572.

Armanini, D., Kuhnle, U., Strasser, T., Dorr, H., Butenandt, I., Weber, P. C., Stockigt, J. R., Pearce, P., and Funder, J. W. (1985). Aldosterone-receptor deficiency in pseudohypoaldosteronism. N Engl J Med *313*, 1178-1181.

Arriza, J. L., Simerly, R. B., Swanson, L. W., and Evans, R. M. (1988). The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron *1*, 887-900.

Arriza, J. L., Weinberger, C., Cerelli, G., Glaser, T. M., Handelin, B. L., Housman, D. E., and Evans, R. M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science *237*, 268-275.

Ashwell, J. D., Lu, F. W., and Vacchio, M. S. (2000). Glucocorticoids in T cell development and function*. Annu Rev Immunol *18*, 309-345.

Bartholome, B., Spies, C. M., Gaber, T., Schuchmann, S., Berki, T., Kunkel, D., Bienert, M., Radbruch, A., Burmester, G. R., Lauster, R., *et al.* (2004). Membrane glucocorticoid

receptors (mGCR) are expressed in normal human peripheral blood mononuclear cells and up-regulated after in vitro stimulation and in patients with rheumatoid arthritis. Faseb J *18*, 70-80.

Baulcombe, D. (2002). RNA silencing. Curr Biol *12*, R82-84.

Beato, M., Herrlich, P., and Schutz, G. (1995). Steroid hormone receptors: many actors in search of a plot. Cell *83*, 851-857.

Beaumont, K., and Fanestil, D. D. (1983). Characterization of rat brain aldosterone receptors reveals high affinity for corticosterone. Endocrinology *113*, 2043-2051.

Begenisich, T., Nakamoto, T., Ovitt, C. E., Nehrke, K., Brugnara, C., Alper, S. L., and Melvin, J. E. (2004). Physiological roles of the intermediate conductance, Ca2+-activated potassium channel Kcnn4. J Biol Chem *279*, 47681-47687.

Beggah, A. T., Escoubet, B., Puttini, S., Cailmail, S., Delage, V., Ouvrard-Pascaud, A., Bocchi, B., Peuchmaur, M., Delcayre, C., Farman, N., and Jaisser, F. (2002). Reversible cardiac fibrosis and heart failure induced by conditional expression of an antisense mRNA of the mineralocorticoid receptor in cardiomyocytes. Proc Natl Acad Sci U S A *99*, 7160-7165.

Berger, S., Bleich, M., Schmid, W., Cole, T. J., Peters, J., Watanabe, H., Kriz, W., Warth, R., Greger, R., and Schutz, G. (1998). Mineralocorticoid receptor knockout mice: pathophysiology of Na+ metabolism. Proc Natl Acad Sci U S A *95*, 9424-9429.

Berger, S., Wolfer, D. P., Selbach, O., Alter, H., Erdmann, G., Reichardt, H. M., Chepkova, A. N., Welzl, H., Haas, H. L., Lipp, H. P., and Schutz, G. (2006). Loss of the limbic mineralocorticoid receptor impairs behavioral plasticity. Proc Natl Acad Sci U S A *103*, 195-200.

Bernstein, E., Caudy, A. A., Hammond, S. M., and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363-366.

Bertini, R., Bianchi, M., and Ghezzi, P. (1988). Adrenalectomy sensitizes mice to the lethal effects of interleukin 1 and tumor necrosis factor. J Exp Med *167*, 1708-1712.

Bhargava, A., Fullerton, M. J., Myles, K., Purdy, T. M., Funder, J. W., Pearce, D., and Cole, T. J. (2001). The serum- and glucocorticoid-induced kinase is a physiological mediator of aldosterone action. Endocrinology *142*, 1587-1594.

Boumpas, D. T., Chrousos, G. P., Wilder, R. L., Cupps, T. R., and Balow, J. E. (1993). Glucocorticoid therapy for immune-mediated diseases: basic and clinical correlates. Ann Intern Med *119*, 1198-1208.

Bowers, B. J., Owen, E. H., Collins, A. C., Abeliovich, A., Tonegawa, S., and Wehner, J. M. (1999). Decreased ethanol sensitivity and tolerance development in gamma-protein kinase C null mutant mice is dependent on genetic background. Alcohol Clin Exp Res *23*, 387-397.

Boyd, C., and Naray-Fejes-Toth, A. (2005). Gene regulation of ENaC subunits by serum- and glucocorticoid-inducible kinase-1. Am J Physiol Renal Physiol *288*, F505-512.

Bradbury, M. J., Akana, S. F., and Dallman, M. F. (1994). Roles of type I and II corticosteroid receptors in regulation of basal activity in the hypothalamo-pituitary-adrenal axis during the diurnal trough and the peak: evidence for a nonadditive effect of combined receptor occupation. Endocrinology *134*, 1286-1296.

Brennan, F. E., and Fuller, P. J. (2000). Rapid upregulation of serum and glucocorticoid-regulated kinase (sgk) gene expression by corticosteroids in vivo. Mol Cell Endocrinol *166*, 129-136.

Breslin, M. B., Geng, C. D., and Vedeckis, W. V. (2001). Multiple promoters exist in the human GR gene, one of which is activated by glucocorticoids. Mol Endocrinol *15*, 1381-1395.

Buttgereit, F., and Scheffold, A. (2002). Rapid glucocorticoid effects on immune cells. Steroids *67*, 529-534.

Canessa, C. M., Schild, L., Buell, G., Thorens, B., Gautschi, I., Horisberger, J. D., and Rossier, B. C. (1994). Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. Nature *367*, 463-467.

Carmell, M. A., Zhang, L., Conklin, D. S., Hannon, G. J., and Rosenquist, T. A. (2003). Germline transmission of RNAi in mice. Nat Struct Biol *10*, 91-92.

Cheek, D. B., and Perry, J. W. (1958). A salt wasting syndrome in infancy. Arch Dis Child *33*, 252-256.

Cohen, J. (2002). The immunopathogenesis of sepsis. Nature *420*, 885-891.

Connell, J. M., and Davies, E. (2005). The new biology of aldosterone. J Endocrinol *186*, 1-20.

Croxtall, J. D., van Hal, P. T., Choudhury, Q., Gilroy, D. W., and Flower, R. J. (2002). Different glucocorticoids vary in their genomic and non-genomic mechanism of action in A549 cells. Br J Pharmacol *135*, 511-519.

De Kloet, E. R., Vreugdenhil, E., Oitzl, M. S., and Joels, M. (1998). Brain corticosteroid receptor balance in health and disease. Endocr Rev *19*, 269-301.

Dhabhar, F. S., and McEwen, B. S. (1999). Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci U S A *96*, 1059-1064.

Edwards, C. R., Stewart, P. M., Burt, D., Brett, L., McIntyre, M. A., Sutanto, W. S., de Kloet, E. R., and Monder, C. (1988). Localisation of 11 beta-hydroxysteroid dehydrogenase--tissue specific protector of the mineralocorticoid receptor. Lancet *2*, 986-989.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature *411*, 494-498.

Escoubet, B., Coureau, C., Bonvalet, J. P., and Farman, N. (1997). Noncoordinate regulation of epithelial Na channel and Na pump subunit mRNAs in kidney and colon by aldosterone. Am J Physiol *272*, C1482-1491.

Evans, R. M., and Hollenberg, S. M. (1988). Zinc fingers: gilt by association. Cell *52*, 1-3.

Fertsch, D., Schoenberg, D. R., Germain, R. N., Tou, J. Y., and Vogel, S. N. (1987). Induction of macrophage Ia antigen expression by rIFN-gamma and down-regulation by IFN-alpha/beta and dexamethasone are mediated by changes in steady-state levels of Ia mRNA. J Immunol *139*, 244-249.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806-811.

Funder, J. W. (2005). Mineralocorticoid receptors: distribution and activation. Heart Fail Rev *10*, 15-22.

Funder, J. W., Pearce, P. T., Smith, R., and Smith, A. I. (1988). Mineralocorticoid action: target tissue specificity is enzyme, not receptor, mediated. Science *242*, 583-585.

Fushimi, T., Shimura, S., Suzuki, S., Saitoh, H., Okayama, H., and Shirato, K. (1998). Suppression of gene expression and production of interleukin 13 by dexamethasone in human peripheral blood mononuclear cells. Tohoku J Exp Med *185*, 157-160.

Gilmour, J. S., Coutinho, A. E., Cailhier, J. F., Man, T. Y., Clay, M., Thomas, G., Harris, H. J., Mullins, J. J., Seckl, J. R., Savill, J. S., and Chapman, K. E. (2006). Local amplification of glucocorticoids by 11 beta-hydroxysteroid dehydrogenase type 1 promotes macrophage phagocytosis of apoptotic leukocytes. J Immunol *176*, 7605-7611.

Gomez-Sanchez, E. P. (2004). Brain mineralocorticoid receptors: orchestrators of hypertension and end-organ disease. Curr Opin Nephrol Hypertens *13*, 191-196.

Gordon, J. W., Scangos, G. A., Plotkin, D. J., Barbosa, J. A., and Ruddle, F. H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. Proc Natl Acad Sci U S A *77*, 7380-7384.

Grafte-Faure, S., Leveque, C., Vasse, M., Soria, C., Norris, V., and Vannier, J. P. (1999). Effects of glucocorticoids and mineralocorticoids on proliferation and maturation of human peripheral blood stem cells. Am J Hematol *62*, 65-73.

Grishok, A., Pasquinelli, A. E., Conte, D., Li, N., Parrish, S., Ha, I., Baillie, D. L., Fire,

A., Ruvkun, G., and Mello, C. C. (2001). Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell *106*, 23-34.

Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293-296.

Hannon, G. J., and Rossi, J. J. (2004). Unlocking the potential of the human genome with RNA interference. Nature *431*, 371-378.

Hansen, H. S., and Jensen, B. (1985). Essential function of linoleic acid esterified in acylglucosylceramide and acylceramide in maintaining the epidermal water permeability barrier. Evidence from feeding studies with oleate, linoleate, arachidonate, columbinate and alpha-linolenate. Biochim Biophys Acta *834*, 357-363.

Hanukoglu, A. (1991). Type I pseudohypoaldosteronism includes two clinically and genetically distinct entities with either renal or multiple target organ defects. J Clin Endocrinol Metab *73*, 936-944.

Hawes, A. S., Rock, C. S., Keogh, C. V., Lowry, S. F., and Calvano, S. E. (1992). In vivo effects of the antiglucocorticoid RU 486 on glucocorticoid and cytokine responses to Escherichia coli endotoxin. Infect Immun *60*, 2641-2647.

He, L., and Hannon, G. J. (2004). MicroRNAs: small RNAs with a big role in gene regulation. Nat Rev Genet *5*, 522-531.

Heck, S., Bender, K., Kullmann, M., Gottlicher, M., Herrlich, P., and Cato, A. C. (1997). I kappaB alpha-independent downregulation of NF-kappaB activity by glucocorticoid receptor. Embo J *16*, 4698-4707.

Heck, S., Kullmann, M., Gast, A., Ponta, H., Rahmsdorf, H. J., Herrlich, P., and Cato, A. C. (1994). A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1. Embo J *13*, 4087-4095.

Hogan, M. M., and Vogel, S. N. (1988). Inhibition of macrophage tumoricidal activity

by glucocorticoids. J Immunol *140*, 513-519.

Hong, H., Kohli, K., Garabedian, M. J., and Stallcup, M. R. (1997). GRIP1, a transcriptional coactivator for the AF-2 transactivation domain of steroid, thyroid, retinoid, and vitamin D receptors. Mol Cell Biol *17*, 2735-2744.

Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T., and Zamore, P. D. (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science *293*, 834-838.

Jacob, H. J., and Kwitek, A. E. (2002). Rat genetics: attaching physiology and pharmacology to the genome. Nat Rev Genet *3*, 33-42.

Joels, M., and de Kloet, E. R. (1994). Mineralocorticoid and glucocorticoid receptors in the brain. Implications for ion permeability and transmitter systems. Prog Neurobiol *43*, 1-36.

Joiner, W. J., Wang, L. Y., Tang, M. D., and Kaczmarek, L. K. (1997). hSK4, a member of a novel subfamily of calcium-activated potassium channels. Proc Natl Acad Sci U S A *94*, 11013-11018.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C., Gebel, S., Ponta, H., and Herrlich, P. (1990). Antitumor promotion and antiinflammation: down-modulation of AP-1 (Fos/Jun) activity by glucocorticoid hormone. Cell *62*, 1189-1204.

Karin, M. (1998). New twists in gene regulation by glucocorticoid receptor: is DNA binding dispensable? Cell *93*, 487-490.

Kennerdell, J. R., and Carthew, R. W. (2000). Heritable gene silencing in Drosophila using double-stranded RNA. Nat Biotechnol *18*, 896-898.

Khvorova, A., Reynolds, A., and Jayasena, S. D. (2003). Functional siRNAs and miRNAs exhibit strand bias. Cell *115*, 209-216.

Kretz, O., Schmid, W., Berger, S., and Gass, P. (2001). The mineralocorticoid receptor expression in the mouse CNS is conserved during development. Neuroreport *12*, 1133-

1137.

Krozowski, Z. S., and Funder, J. W. (1983). Renal mineralocorticoid receptors and hippocampal corticosterone-binding species have identical intrinsic steroid specificity. Proc Natl Acad Sci U S A *80*, 6056-6060.

Kunicka, J. E., Talle, M. A., Denhardt, G. H., Brown, M., Prince, L. A., and Goldstein, G. (1993). Immunosuppression by glucocorticoids: inhibition of production of multiple lymphokines by in vivo administration of dexamethasone. Cell Immunol *149*, 39-49.

Lang, F., and Cohen, P. (2001). Regulation and physiological roles of serum- and glucocorticoid-induced protein kinase isoforms. Sci STKE *2001*, RE17.

Le Menuet, D., Isnard, R., Bichara, M., Viengchareun, S., Muffat-Joly, M., Walker, F., Zennaro, M. C., and Lombes, M. (2001). Alteration of cardiac and renal functions in transgenic mice overexpressing human mineralocorticoid receptor. J Biol Chem *276*, 38911-38920.

Lee, Y., Jeon, K., Lee, J. T., Kim, S., and Kim, V. N. (2002). MicroRNA maturation: stepwise processing and subcellular localization. Embo J *21*, 4663-4670.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J., and Carthew, R. W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell *117*, 69-81.

Lifton, R. P., Gharavi, A. G., and Geller, D. S. (2001). Molecular mechanisms of human hypertension. Cell *104*, 545-556.

Lim, H. Y., Muller, N., Herold, M. J., van den Brandt, J., and Reichardt, H. M. (2007). Glucocorticoids exert opposing effects on macrophage function dependent on their concentration. Immunology.

Lingueglia, E., Renard, S., Waldmann, R., Voilley, N., Champigny, G., Plass, H., Lazdunski, M., and Barbry, P. (1994). Different homologous subunits of the amiloride-sensitive Na+ channel are differently regulated by aldosterone. J Biol Chem *269*, 13736-13739.

Logsdon, N. J., Kang, J., Togo, J. A., Christian, E. P., and Aiyar, J. (1997). A novel gene, hKCa4, encodes the calcium-activated potassium channel in human T lymphocytes. J Biol Chem *272*, 32723-32726.

Lois, C., Hong, E. J., Pease, S., Brown, E. J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. Science *295*, 868-872.

Martinez, E. D., Pattabiraman, N., and Danielsen, M. (2005). Analysis of the hormone-binding domain of steroid receptors using chimeras generated by homologous recombination. Exp Cell Res *308*, 320-333.

Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., and Tuschl, T. (2002). Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell *110*, 563-574.

Martinez, J., and Tuschl, T. (2004). RISC is a 5' phosphomonoester-producing RNA endonuclease. Genes Dev *18*, 975-980.

McEwen, B. S., and Sapolsky, R. M. (1995). Stress and cognitive function. Curr Opin Neurobiol *5*, 205-216.

McMaster, G. K., and Carmichael, G. G. (1977). Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc Natl Acad Sci U S A *74*, 4835-4838.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature *431*, 343-349.

Melton, J. L., Wertz, P. W., Swartzendruber, D. C., and Downing, D. T. (1987). Effects of essential fatty acid deficiency on epidermal O-acylsphingolipids and transepidermal water loss in young pigs. Biochim Biophys Acta *921*, 191-197.

Mick, V. E., Itani, O. A., Loftus, R. W., Husted, R. F., Schmidt, T. J., and Thomas, C. P. (2001). The alpha-subunit of the epithelial sodium channel is an aldosterone-induced transcript in mammalian collecting ducts, and this transcriptional response is mediated

via distinct cis-elements in the 5'-flanking region of the gene. Mol Endocrinol *15*, 575-588.

Miyazaki, M., Dobrzyn, A., Elias, P. M., and Ntambi, J. M. (2005). Stearoyl-CoA desaturase-2 gene expression is required for lipid synthesis during early skin and liver development. Proc Natl Acad Sci U S A *102*, 12501-12506.

Muller, J. (1987). Regulation of aldosterone biosynthesis. Physiological and clinical aspects. Monogr Endocrinol *29*, 1-364.

Naray-Fejes-Toth, A., Helms, M. N., Stokes, J. B., and Fejes-Toth, G. (2004). Regulation of sodium transport in mammalian collecting duct cells by aldosterone-induced kinase, SGK1: structure/function studies. Mol Cell Endocrinol *217*, 197-202.

Necela, B. M., and Cidlowski, J. A. (2004). Mechanisms of glucocorticoid receptor action in noninflammatory and inflammatory cells. Proc Am Thorac Soc *1*, 239-246.

Palkovits, M. (1987). Organization of the stress response at the anatomical level. Prog Brain Res *72*, 47-55.

Pearce, D., Bhargava, A., and Cole, T. J. (2003). Aldosterone: its receptor, target genes, and actions. Vitam Horm *66*, 29-76.

Pearce, D., and Kleyman, T. R. (2007). Salt, sodium channels, and SGK1. J Clin Invest *117*, 592-595.

Pearce, D., and Yamamoto, K. R. (1993). Mineralocorticoid and glucocorticoid receptor activities distinguished by nonreceptor factors at a composite response element. Science *259*, 1161-1165.

Phillips, T. J., Hen, R., and Crabbe, J. C. (1999). Complications associated with genetic background effects in research using knockout mice. Psychopharmacology (Berl) *147*, 5-7.

Pitt, B. (2004). Effect of aldosterone blockade in patients with systolic left ventricular dysfunction: implications of the RALES and EPHESUS studies. Mol Cell Endocrinol

217, 53-58.

Pratt, W. B., Gehring, U., and Toft, D. O. (1996). Molecular chaperoning of steroid hormone receptors. Exs *77*, 79-95.

Quinn, S. J., and Williams, G. H. (1988). Regulation of aldosterone secretion. Annu Rev Physiol *50*, 409-426.

Raetz, C. R. (1990). Biochemistry of endotoxins. Annu Rev Biochem *59*, 129-170.

Rave, N., Crkvenjakov, R., and Boedtker, H. (1979). Identification of procollagen mRNAs transferred to diazobenzyloxymethyl paper from formaldehyde agarose gels. Nucleic Acids Res *6*, 3559-3567.

Reichardt, H. M. (2004). Immunomodulatory activities of glucocorticoids: insights from transgenesis and gene targeting. Curr Pharm Des *10*, 2797-2805.

Reichardt, H. M., and Schutz, G. (1996). Feedback control of glucocorticoid production is established during fetal development. Mol Med *2*, 735-744.

Reichardt, H. M., Umland, T., Bauer, A., Kretz, O., and Schutz, G. (2000). Mice with an increased glucocorticoid receptor gene dosage show enhanced resistance to stress and endotoxic shock. Mol Cell Biol *20*, 9009-9017.

Reul, J. M., and de Kloet, E. R. (1985). Two receptor systems for corticosterone in rat brain: microdistribution and differential occupation. Endocrinology *117*, 2505-2511.

Reul, J. M., and de Kloet, E. R. (1986). Anatomical resolution of two types of corticosterone receptor sites in rat brain with in vitro autoradiography and computerized image analysis. J Steroid Biochem *24*, 269-272.

Rogerson, F. M., and Fuller, P. J. (2000). Mineralocorticoid action. Steroids *65*, 61-73.

Rotondi, M., Falorni, A., De Bellis, A., Laureti, S., Ferruzzi, P., Romagnani, P., Buonamano, A., Lazzeri, E., Crescioli, C., Mannelli, M., *et al.* (2005). Elevated serum interferon-gamma-inducible chemokine-10/CXC chemokine ligand-10 in autoimmune

primary adrenal insufficiency and in vitro expression in human adrenal cells primary cultures after stimulation with proinflammatory cytokines. J Clin Endocrinol Metab *90*, 2357-2363.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., *et al.* (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet *33*, 401-406.

Rupprecht, R., Arriza, J. L., Spengler, D., Reul, J. M., Evans, R. M., Holsboer, F., and Damm, K. (1993). Transactivation and synergistic properties of the mineralocorticoid receptor: relationship to the glucocorticoid receptor. Mol Endocrinol *7*, 597-603.

Sanford, L. P., Kallapur, S., Ormsby, I., and Doetschman, T. (2001). Influence of genetic background on knockout mouse phenotypes. Methods Mol Biol *158*, 217-225.

Schaffner, A. (1985). Therapeutic concentrations of glucocorticoids suppress the antimicrobial activity of human macrophages without impairing their responsiveness to gamma interferon. J Clin Invest *76*, 1755-1764.

Scheinman, R. I., Gualberto, A., Jewell, C. M., Cidlowski, J. A., and Baldwin, A. S., Jr. (1995). Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. Mol Cell Biol *15*, 943-953.

Schmid, W., Strahle, U., Schutz, G., Schmitt, J., and Stunnenberg, H. (1989). Glucocorticoid receptor binds cooperatively to adjacent recognition sites. Embo J *8*, 2257-2263.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N., and Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. Cell *115*, 199-208.

Simpson, E. R., and Waterman, M. R. (1988). Regulation of the synthesis of steroidogenic enzymes in adrenal cortical cells by ACTH. Annu Rev Physiol *50*, 427-440.

Snyder, D. S., and Unanue, E. R. (1982). Corticosteroids inhibit murine macrophage Ia

expression and interleukin 1 production. J Immunol *129*, 1803-1805.
Spat, A., and Hunyady, L. (2004). Control of aldosterone secretion: a model for convergence in cellular signaling pathways. Physiol Rev *84*, 489-539.

Sternberg, E., Wilder, RL (1993). Corticosteroids, Arthritis and Allied Conditions, Philadelphia Lea&Febiger).

Stockand, J. D. (2002). New ideas about aldosterone signaling in epithelia. Am J Physiol Renal Physiol *282*, F559-576.

Sutanto, W., and De Kloet, E. R. (1987). Species-specificity of corticosteroid receptors in hamster and rat brains. Endocrinology *121*, 1405-1411.

Svoboda, P., Stein, P., Hayashi, H., and Schultz, R. M. (2000). Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. Development *127*, 4147-4156.

Tanaka, J., Fujita, H., Matsuda, S., Toku, K., Sakanaka, M., and Maeda, N. (1997). Glucocorticoid- and mineralocorticoid receptors in microglial cells: the two receptors mediate differential effects of corticosteroids. Glia *20*, 23-37.

Thompson, E. B., Yuh, Y. S., Ashraf, J., Gametchu, B., Johnson, B., and Harmon, J. M. (1988). Mechanisms of glucocorticoid function in human leukemic cells: analysis of receptor gene mutants of the activation-labile type using the covalent affinity ligand dexamethasone mesylate. J Steroid Biochem *30*, 63-70.

Tiscornia, G., Singer, O., Ikawa, M., and Verma, I. M. (2003). A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci U S A *100*, 1844-1848.

Tsai, M. J., and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu Rev Biochem *63*, 451-486.

Tuckermann, J. P., Kleiman, A., McPherson, K. G., and Reichardt, H. M. (2005). Molecular mechanisms of glucocorticoids in the control of inflammation and lymphocyte apoptosis. Crit Rev Clin Lab Sci *42*, 71-104.

Vandorpe, D. H., Shmukler, B. E., Jiang, L., Lim, B., Maylie, J., Adelman, J. P., de Franceschi, L., Cappellini, M. D., Brugnara, C., and Alper, S. L. (1998). cDNA cloning and functional characterization of the mouse Ca2+-gated K+ channel, mIK1. Roles in regulatory volume decrease and erythroid differentiation. J Biol Chem *273*, 21542-21553.

Vaucheret, H., Beclin, C., and Fagard, M. (2001). Post-transcriptional gene silencing in plants. J Cell Sci *114*, 3083-3091.

Vedder, H., Weiss, I., Holsboer, F., and Reul, J. M. (1993). Glucocorticoid and mineralocorticoid receptors in rat neocortical and hippocampal brain cells in culture: characterization and regulatory studies. Brain Res *605*, 18-24.

Vinson GP, W. B., Hinson J (1992). The Adrenal Cortex, Englewood Cliffs, NJ : Prentice-Hall).

Webster, M. K., Goya, L., Ge, Y., Maiyar, A. C., and Firestone, G. L. (1993). Characterization of sgk, a novel member of the serine/threonine protein kinase gene family which is transcriptionally induced by glucocorticoids and serum. Mol Cell Biol *13*, 2031-2040.

Wertz, P. W., and Downing, D. T. (1990). Metabolism of linoleic acid in porcine epidermis. J Lipid Res *31*, 1839-1844.

Wianny, F., and Zernicka-Goetz, M. (2000). Specific interference with gene function by double-stranded RNA in early mouse development. Nat Cell Biol *2*, 70-75.

Wiegers, G. J., Croiset, G., Reul, J. M., Holsboer, F., and de Kloet, E. R. (1993). Differential effects of corticosteroids on rat peripheral blood T-lymphocyte mitogenesis in vivo and in vitro. Am J Physiol *265*, E825-830.

Wilckens, T., and De Rijk, R. (1997). Glucocorticoids and immune function: unknown dimensions and new frontiers. Immunol Today *18*, 418-424.

Yamamoto, K. R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet *19*, 209-252.

Yeager, M. P., Guyre, P. M., and Munck, A. U. (2004). Glucocorticoid regulation of the inflammatory response to injury. Acta Anaesthesiol Scand *48*, 799-813.

Zennaro, M. C., and Lombes, M. (2004). Mineralocorticoid resistance. Trends Endocrinol Metab *15*, 264-270.

Zhi, L., Leung, B. P., and Melendez, A. J. (2006). Sphingosine kinase 1 regulates pro-inflammatory responses triggered by TNFalpha in primary human monocytes. J Cell Physiol *208*, 109-115.

Zhou, Q., Renard, J. P., Le Friec, G., Brochard, V., Beaujean, N., Cherifi, Y., Fraichard, A., and Cozzi, J. (2003). Generation of fertile cloned rats by regulating oocyte activation. Science *302*, 1179.

Zuckerman, S. H., Shellhaas, J., and Butler, L. D. (1989). Differential regulation of lipopolysaccharide-induced interleukin 1 and tumor necrosis factor synthesis: effects of endogenous and exogenous glucocorticoids and the role of the pituitary-adrenal axis. Eur J Immunol *19*, 301-305.

Summary

The steroid hormones corticosterone/cortisol and aldosterone are synthesized and secreted by the adrenal gland in response to stress or an altered salt-water balance. This is controlled by a negative feedback mechanism referred to as the HPA axis and the RAAS. Actions of these steroid hormones are mediated by the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR), which reside in the cytoplasm in a complex with heat-shock proteins. Both, the GR and the MR belong to the nuclear receptor superfamily and share a common protein structure consisting of three separate domains. However, they have different affinities for various ligands, their actions depend on hormone concentration, they are modulated by pre-receptor mechanisms such as the 11β-HSD2 and they are differently distributed in several tissues.

Aldosterone acts via the MR in epithelial and in non-epithelial cells and regulates sodium-water homeostasis, cardiovascular function, neuronal excitability and adipocyte differentiation. So far the analysis of gene inactivation *in vivo* was limited to mice, but disease models in rats sometimes more closely reflect the situation encountered in humans. Since embryonic stem cells and thus gene targeting in rats is not available, we generated MR knock-down transgenic rats by lentiviral delivery of a shRNA. The F1 progeny of the founder rats showed a wide range of reduced MR mRNA and protein levels in kidney and hippocampus, the two major sites of MR expression. In contrast, expression of the highly homologous GR was unaltered, indicating specificity of gene inactivation. The two MR target genes, Sgk1 and ENaC, were up-regulated while the mRNA levels of other genes such as IK1 and SCD2 was reduced. Similar to the knock-out mice and human patients, the knock-down rats displayed typical signs of

pseudohypoaldosteronism type I such as increased serum levels of aldosterone and renin as well as growth retardation. Importantly, we found a linear relationship between MR mRNA expression in kidney, serum aldosterone levels and body weight. Thus, our MR knock-down rats are amongst the first examples of RNAi *in vivo* and confirm that this technique allows to accomplish graded levels of gene inactivation that mimick human genetic diseases.

Secondly, we investigated the role of the GR and the MR for the immunomodulatory activities of glucocorticoids (GCs) in peritoneal macrophages. GCs are involved in the modulation of macrophage function and thereby control the host's immune responses to pathogens. Therefore, GCs are widely used for the treatment of inflammation and autoimmune diseases. However, concerning these GC activities neither the role of hormone concentration nor the differential contribution of the GR and the MR are known. At first we confirmed that both receptors but not 11β-HSD2 are expressed in peritoneal macrophages. Next, we showed that low levels of corticosterone enhance NO production as well as mRNA expression of pro-inflammatory cytokines, chemokines and enzymes required for mediator synthesis. In contrast, at high corticosterone concentrations macrophage function was strongly repressed. Importantly, inactivation of the GR by lentiviral delivery of siRNAs abrogated both the immunostimulatory and the immunosuppressive GC actions whereas inactivation of the MR had no effect. Furthermore, removal of endogenous GCs by adrenalectomy *in vivo* induced a pre-activated state in macrophages that could be modulated by corticosterone. We conclude that GCs exert distinct effects on macrophage function dependent on their concentration, and that they act through the GR despite concomitant expression of the MR.

In summary, our results confirm that lentiviral delivery of shRNAs is an efficient means

to down-regulation gene expression in primary cells and transgenic rats and thereby allows to perform functional studies on gene function that were previously limited to mice.

Zusammenfassung

Die Steroidhormone Corticosteron/Cortisol und Aldosteron werden in Folge von Stress oder eines veränderten Salz-Wasser-Haushaltes durch die Nebenniere synthetisiert und sezerniert. Dies wird durch negative Rückkopplungsmechanismen kontrolliert, die als HPA-Achse und RAAS bezeichnet werden. Die Aktivität dieser Steroidhormone wird durch den Glukokortikoid Rezeptor (GR) und den Mineralokortikoid-Rezeptor (MR) vermittelt, die im Zytosol als Komplex mit Hitze-Schock-Proteinen vorliegen. Sowohl der GR als auch der MR gehören zur Kern-Rezeptor Superfamilie und besitzen eine gemeinsame Proteinstruktur die aus drei verschiedenen Domänen besteht. Trotzdem haben sie verschiedene Affinitäten für ihre Liganden, ihre Aktivität hängt von der Hormonkonzentration ab, sie werden durch Prä-Rezeptor-Mechansimen wie der 11b-HSD2 reguliert und ihre Gewebeverteilung ist unterschiedlich.

Aldosteron wirkt in epithelialen und nicht-epithelialen Zellen über den MR und reguliert den Salz-Wasser-Haushalt, die Herzfunktion, die neuronale Erregbarkeit und die Adipozyten-Differenzierung. Bislang war die Analyse der Geninaktivierung *in vivo* auf Mäuse beschränkt, obwohl Krankheitsmodelle in der Ratte die Verhältnisse im Menschen manchmal besser widerspiegeln. Da embryonale Stammzellen und damit die gezielte Genmanipulation in Ratten nicht verfügbar sind, haben wir MR knock-down Ratten mittels lentiviral eingeführten shRNAs hergestellt. Die F1 Nachkommen der Gründer-Ratten zeigten unterschiedlich stark reduzierte MR mRNA und Protein Niveaus in Niere und Hippocampus, den Hauptexpressions-Regionen des MR. Im Gegensatz dazu war die Expression des GR unverändert, was die Spezifität der Geninaktivierung belegt. Die zwei MR Zielgene Sgk1 und ENaC waren hochreguliert

während die mRNA Spiegel anderer Gene wie IK1 und SCD2 erniedrigt waren. Ähnlich wie in den knock-out Mäusen und Patienten zeigten die knock-down Ratten die typischen Merkmale des Pseudohypoaldosteronismus Typ I, so wie erhöhte Serumspiegel von Aldosteron und Renin sowie Wachstumsretardation. Weiterhin fanden wir einen linearen Zusammenhang zwischen der MR Expression in der Niere, den Serum Aldosteron-Werten und dem Körpergewicht. Zusammengefasst sind unsere MR knock-down Ratten unter den ersten Beispielen für RNAi in vivo und belegen, dass diese Technik es erlaubt, abgestufte Ausprägugen der Geninktivierung wie in humanen genetischen Erkrankungen zu erreichen.

Weiterhin haben wir die Rolle des GR und des MR für die immunmodulatorische Aktivität der Glukokortikoide in peritonealen Makrophagen untersucht. GCs sind an der Kontrolle der Makrophagenfunktion beteiligt und regulieren so die Reaktion gegenüber Pathogenen. Aus diesem Grund werden GCs weitverbreitet zur Behandlung von Enzündungen und Autoimmunerkrankungen eingesetzt. Allerdings ist bezüglich dieser GC Aktivitäten weder bekannt welche Kontrolle die Hormonkonzentration spielt noch kennt man den differentiellen Beitrag des GR und des MR. Zuerst bestätigten wir die Expression beider Rezeptoren in peritonealen Makrophagen während die 11b-HSD2 nicht exprimiert war. Anschließend zeigten wir, dass niedrigte Corticosteron-Level die NO Produktion sowie die mRNA Expression von pro-inflammatorischen Zytokinen, Chemokinen und Enzymen die für die Mediator-Synthesee benötigt werden erhöhen. Im Gegensatz dazu war die Makrophagen Funktion bei hohen Corticosteron-Konzentrationen stark reprimiert. Eine wichtige Beobachtung war, dass die Inaktivierung des GR durch lentiviral eingeführte siRNAs sowohl die immunstimulatorischen als auch die immunsuppressiven GR Aktivitäten aufhob

während die Inaktivierung des MR keine Konsequenzen hatte. Weiterhin führte der Verlust endogenener GCs nach Adrenalektomie in vivo zu einem prä-aktivierten Zustand der Makrophagen, welcher durch Corticosteron moduliert werden konnte. Wir schließen hieraus, dass GCs in Abhängigkeit von ihrer Konzentration unterschiedliche Effekte auf die Makrophagen Funktion haben und dass diese durch den GR vermittelt werden, obwohl der MR ebenfalls exprimiert ist.

Zusammengefasst bestätigen unsere Ergebnisse dass die lenivirale Transduktion von shRNAs eine effiziente Methode zur Geninaktivierung in primären Zellen und transgenen Ratten darstellt und es so erlaubt, funktionelle Studien durchzuführen die zuvor auf Mäuse beschränkt waren.

Abbreviations

Ab	antibody
ACTH	adrenocorticotrophic hormone
ADX	adrenalectomized
Ang	angiotension
AP-1	activator protein 1
APS	ammonium persulfate
BSS	balanced salt solution
BSA	bovine serum albumin
CD	cluster of differentiation
COX-2	cyclooxygenease-2
CREB (CBP)	cAMP response element binding protein
Cort	corticosterone
CRH	corticotropin-releasing hormone
Dex	dexamethasone
DTT	dithiothreitol
ECL	enhanced chemo luminescence
ENaC	Epithelial sodium channel
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FSC	forward scatter
GC	glucocorticoid
GCs	glucocorticoids
GILZg	glucocorticoid-induced leucine zipper protein
GR	glucocorticoid receptor
GRE	glucocorticoid responsive elements
HAT	histone acetytransferase
HDAC	histone deacetylase
HPA	hypothalamus-pituitary-adrenal
Hsp	heat shock protein
IFN-γ	interferon gamma
IκB	inhibitor of NF-κB
IK	Intermediate potassium channel

IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccaride
MHC	major histocompatibility complex
MIF	macrophage migration inhibitory factor
miRNA	micro RNA
MKP-1	MAP kinase phosphatase-1
MR	mineralocorticoid receptor
NO	nitric oxide
NF-AT	nuclear fact of activated T cells
NF-κB	nuclear factor κB
NSL	nuclear localization signals
O/N	over-night
PBS	phosphate buffered saline
PNMT	phenylethanolamide *N*-methyltrasferase
PVDF	Polyvinylidene fluoride
RAAS	Renin-angiotension-aldosterone system
RISC	RNA induced gene silencing complex
RT	room temperature
SCD2	stearoyl-CoA desaturase 2
siRNA	small interfering RNA
Sgk1	serum- and glucocorticoid induced kinase 1
shRNA	small hairpin RNA
SLP1	
SPHK1	sphingosine kinase 1
SRC-2	steroid receptor coactivator
TEMED	N,N,N´N´-Tetraethylendiamin
tg	transgenic
TGS	Tris-Glycine-SDS buffer
TLR	toll like receptor
TNF	tumor necrosis factor
wt	wild type
11β-HSD2	11β-hydroxysteroid dehydrogenase type 2

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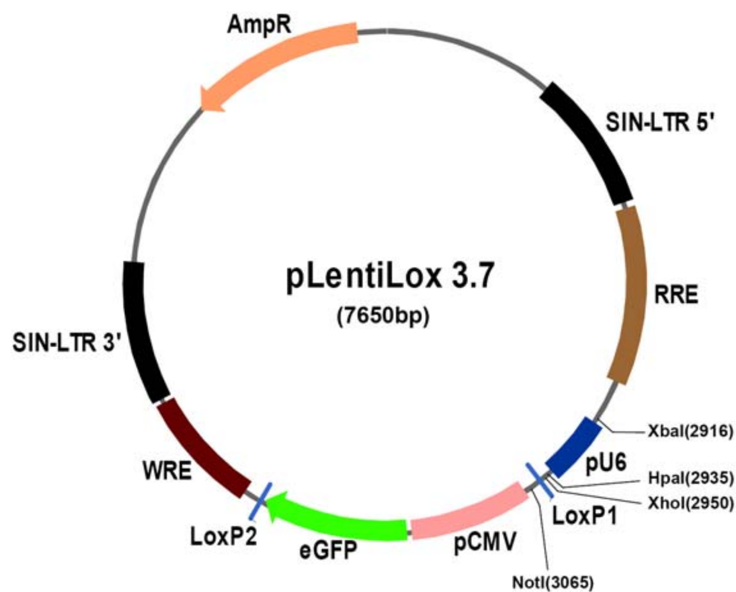
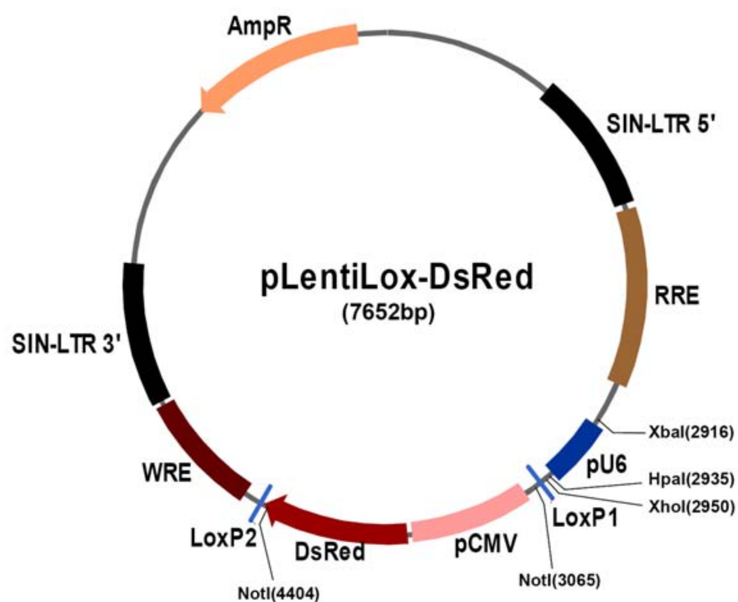
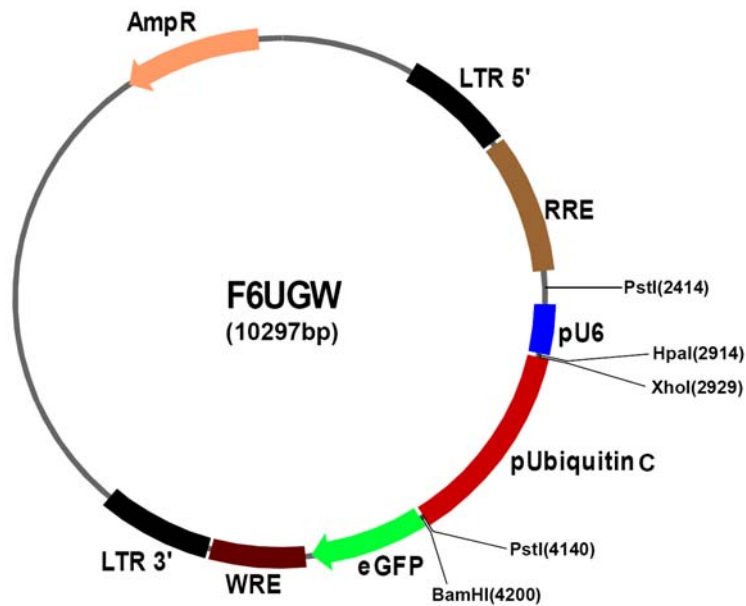
Schematic of lentiviral vectors



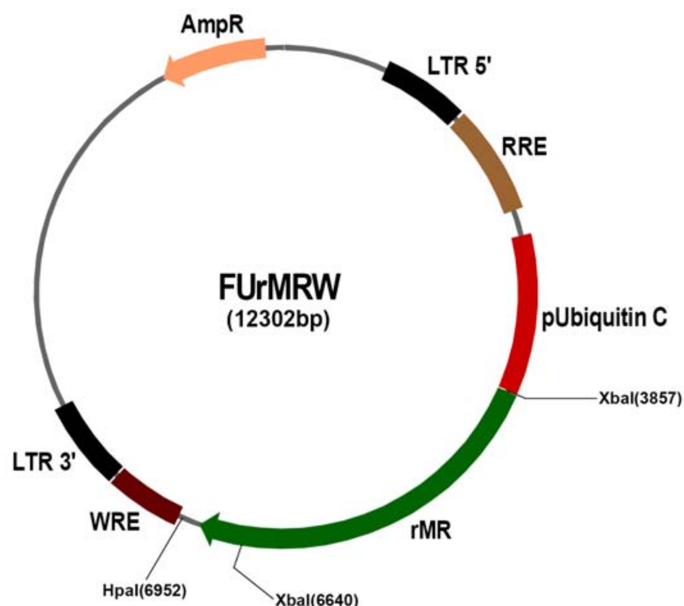
The shRNA expressing Lentiviral vector, pLentiLox 3.7 (Rubinson et al., 2003). An shRNA is cloned into pLL3.7 vector using *Hpa*I / *Xho*I restriction sites. The shRNA expression is driven by U6 promoter and the eGFP is expressed under the control of the CMV promoter.



The shRNA expressing Lentiviral vector, pLentiLox-DsRed. The shRNA is expressed by the U6 promoter and the DsRed is expressed under the control of CMV promoter.



The shRNA expressing Lentiviral vector, F6UGW. We modified the FUGW vector (Lois *et al*, 2002) by adding the U6 promoter for shRNA-expressing. An shRNA is cloned into vector using *Hpa*I / *Xho*I sites and the eGFP is expressed under the control of the uniquitin C promoter.



Construct of rat MR DNA within FUW lentiviral vector. The rat MR full DNA was cloned into the FUW vector (Lois et al, 2002) using *Bgl*II / *Bam*HI restriction sites.
The rat MR is expressed under the control of the ubiquitin C promoter.

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List of Publications

Hee-Young Lim, Nora Mueller, Marco J. Herold, Jens van den Brandt, Holger M. Reichardt. Glucocorticoids exert opposing effects on macrophage function dependent on their concentration. Immunology**,** 2007

Hee-Young Lim, Jens van den Brandt, Martin Fassnacht, BrunoAllolio, Marco J. Herold, Holger M. Reichardt. Generation of mineralocorticoid receptor known-down rats by lentiviral expression of an shRNA. Manuscript in progress

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Shin JA, Song JY, **Lim HY**, Chang MH, Lee HM, Chung KH, Lim YK, Yoon BS. An in vitro vitellogenin bioassay for estrogenic substances in the Rhynchocypris oxycephalus. Journal of Veterinary Science, South Korea. Vol. 46; No.1; pp.35-41, 2006

Lee EJ, **Lim HY**, Kim SH, Kang KS, Park YD, Yun CH, Yoon BS**.** Construction of FMDV VP1 gene using artifical DNA synthesis and Transformation of *Nicotiana tabacum* using *Agrobacterium* vector system. Korean Journal of Plant Biotechology, South Korea. Vol. 31; No.4; pp.285 ~ 293, 2004

Oh YK, **Lim HY**, Kim JS, Yun CH, Kim IG, Yoon BS. Effect of Radiation on mRNA Expression of Ceruloplasmin Gene. Journal of Toxicology and Public Health, South Korea. Vol. 20; No.1; pp. 31~36, 2004.

Kim JS, **Lim HY**, Oh YK, Kim IG, Kang KS, Yoon BS. The mRNA Expression of Radio-Sensitive Genes Exposed to Various Dosage of Ionizing Radiation in U-937 Cell. Journal of Toxicology and Public Health, South Korea. Vol. 20; No.1; pp 21~29, 2004

Lim HY, Oh YK, Kim JS, Lee YS, Lim YK, Yoon BS. Production of Monoclonal Antibody for *Listeria spp*. p60 Protein Based on iap Gene. Jouranl of Food Hygiene and Safety .South Korea, Vol. 18; No.1 pp.25~29, 2003