Optimization of RNA-based transgene expression by targeting Protein Kinase R

Dissertation

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Summary

The aim of this thesis was to reach an expression lasting for several days of mRNA-based transgenes by inhibition of the protein kinase R (PKR) and the cellular interferon response. The expression of recombinant proteins by *in vitro* transcribed mRNA (IVT-RNA) has progressed substantially in the last few years. There is hope for an improvement in cancer therapy by using IVT-RNA for vaccination or as a T-cell therapy. The great advantage of an RNA-based technique is that integration into the patients' genome can be excluded and it is therefore seen as clinically safe, in contrast to viral based approaches.

The main drawbacks of using IVT-RNA for long lasting protein expression are a fast cellular derived degradation of the mRNA and the induction of the cellular defense. The cellular defense is mediated mainly by the interferon response and is accompanied by a translational shutdown. Another consequence of the antiviral state is the induction of apoptosis. When starting this work no method for long-term, continuous protein expression by IVT-RNA had been described. This was needed for providing IVT-RNA encoded proteins for several days or weeks; either for therapeutic purposes or for generating so called induced pluripotent stem cells (iPS).

Due to the short half-life of IVT-RNA, frequent transfections in short intervals are essential in order to reach a continuous protein expression. The cells are able to discriminate between "self" and "non-self" RNA and once the IVT-RNA is transfected, the cellular defense is initiated. This reaction is counterproductive for IVT-RNA-based protein expression, where the viability of the targeted cells should be maintained. Initially, the transfected IVT-RNA induced, in our hands, an interferon response and a shutdown of translation. The reason for the upregulated interferon response and the stalled translation was confirmed to be the PKR and thus the PKR was identified as a main target for intervention. To achieve an improved transgene expression, we aimed to inhibit PKR activation and the interferon response. Therefore the following four strategies were tested:

- small molecule PKR inhibitor

- siRNA mediated PKR knockdown

- overexpression of a kinase inactive PKR mutant

- co-transfection of viral inhibitors of PKR and interferon response

To evaluate, which strategy would be most effective in hampering the interferon response, the effect on the induction of transcripts representing the interferon response and the translation

of reporter genes were analyzed subsequent to IVT-RNA-based transfection. The use of small molecule PKR inhibitor caused an enhanced protein expression and hampered the induction of interferon-transcripts, but the inhibitor had to be excluded due to cytotoxicity. Both the siRNA mediated PKR knockdown and the overexpression of a kinase inactive PKR mutant elevated the protein expression, but the down-regulation of the interferon response remained insufficient for continuous IVT-RNA based protein expression. In contrast to the previously tested strategies, the co-transfer of viral inhibitors of PKR and interferon response was successful. Not only was an optimized protein expression achieved but also the interferon response was reduced to background level. For this strategy three Vaccinia virus proteins were chosen: E3, K3 and B18R. E3 acts as ds RNA binding protein and K3 as PKR pseudo substrate. Thereby both proteins prohibit its activation. B18R is an extracellular decoy-receptor that binds type I interferon. Thus, the interferon cannot bind to its original receptor in the plasma membrane, resulting in an interrupted interferon signaling cascade. The co-transfection of E3, K3 and B18R enabled repeated IVT-RNA-based transfection of human fibroblasts. Thereby the suppression of the interferon response was sufficient to perform frequent transfections with IVT-RNA coding for stem cell specific transcription factors, until an induction of their target genes was observed.

Taken together, this thesis describes a method which provides a basis for continuous expression of foreign proteins by IVT-RNA. The therapeutic protein expression could profit from this work and advance IVT-RNA based vaccination or T-cell therapy. The developed protocol allows a continuous IVT-RNA encoded protein expression of stem cell specific transcription factors, which could be the basis for the generation of the induced pluripotent stem cells (iPS). iPS cells derived from IVT-RNA-based transfection could be of high value for several therapeutic applications in regenerative medicine or in drug research.

Zusammenfassung

Das Ziel dieser Arbeit war eine Verbesserung der mehrtägigen Expression mRNA-basierter Transgene durch eine Inhibition der Protein Kinase R (PKR) sowie der zellulären Interferonantwort. Die Expression von rekombinanten Proteinen mit Hilfe von *in vitro* transkribierter mRNA (IVT-RNA) hat in den letzten Jahren große Fortschritte erzielt. Nun erhofft man sich durch deren Einsatz bei der Vakzinierung oder T-Zell Therapie verbesserte Behandlungsmöglichkeiten von Krebs. Ein großer Vorteil des IVT-RNA basierten Gentransfers ist, dass deren Integration in das Genom von Patienten ausgeschlossen ist. Daher gilt diese Form der Gentherapie, im Gegensatz zur Gentherapie mit retroviralen Vektoren, als sichere klinische Anwendung.

Die Nachteile von IVT-RNA, im Zuge einer langfristig ausgelegten Proteinexpression, sind neben einer schnellen zellvermittelten Degradation der mRNA die Induktion zellulärer Abwehrmechanismen. Diese werden hauptsächlich durch die Interferonantwort vermittelt und sind von einer allgemeinen Herunterregulation der Translation begleitet. Als letztmögliche Verteidigungsmaßnahme der Zellen wird Apoptose ausgelöst. Zu Beginn dieser Arbeit war keine Methode zur langfristigen, kontinuierlichen Proteinexpression mit IVT-RNA beschrieben. Diese ist allerdings notwendig, um Proteine IVT-RNA-basiert für Tage oder Wochen bereitstellen zu können; sei es für therapeutische Zwecke oder um sogenannte induzierte pluripotente Stammzellen (iPS) zu generieren.

Wegen der kurzen Halbwertszeit von IVT-RNA sind mehrmalige Transfektionen in kurzen Abständen nötig um eine kontinuierliche Expression der Proteine gewährleisten zu können. Da Zellen allerdings in der Lage sind zwischen eigener bzw. fremder RNA zu unterscheiden, löste die Transfektion mit IVT-RNA eine zelluläre Verteidigung aus. Diese ist kontraproduktiv für IVT-RNA-basierte Expression von Proteinen, bei welcher die Viabilität der Zielzellen erhalten werden sollte. Zunächst induzierte die Transfektion mit IVT-RNA auch bei uns eine Induktion der Interferonantwort und eine Herunterregulation der Translation. Es wurde die PKR als Ursache für hochregulierte Interferonantwort sowie die Inhibition der Translation bestätigt und damit als primäre Zielstuktur identifiziert. Um eine verbesserte IVT-RNA-basierte transgene Expression zu erzielen, wurde folglich ein Verhindern der PKR Aktivierung und Interferonantwort angestrebt.

Dabei wurden folgende vier Strategien verfolgt:

- niedermolekularer PKR Inhibitor

- siRNA vermittelte PKR Herunterregulation

- Überexpression einer Kinase inaktiven PKR Mutante

- Kotransfektion viraler Inhibitoren der PKR und Interferonantwort

Zur Überprüfung, welche Strategie das Auslösen einer Interferonantwort am effektivsten verhinderte, wurde im Anschluss an eine IVT-RNA-basierte Transfektion der Effekt auf die Induktion von Transkripten der Interferonantwort sowie die Translation von Reportergenen untersucht. Mit Hilfe eines niedermolekularen PKR Inhibitors konnte zwar die Proteinexpression gesteigert und die Induktion von Interferon-Transkripten verhindert werden, allerdings schlossen zytotoxische Effekte eine weitere Verwendung aus. Sowohl durch die siRNA vermittelte Herunterregulation der PKR, als auch durch die Überexpression einer Kinase inaktiven PKR Mutante, konnte die Proteinexpression verbessert werden. Die Herunterregulation der Interferonantwort war jedoch nicht ausreichend für eine kontinuierliche Proteinexpression durch IVT-RNA. Im Gegensatz dazu war von den getesteten Strategien der Kotransfer von viralen Inhibitoren der PKR und Interferonantwort ein Erfolg, da nicht nur eine optimierte Proteinexpression erzielt wurde, sondern auch die Interferonantwort auf ein Hintergrundlevel reduziert werden konnte. Für diese Strategie wurden drei Proteine des Vaccinia Viruses ausgewählt: E3, K3 und B18R. E3 wirkt als ein ds RNA bindendes Protein und K3 als Pseudosubstrat der PKR. Beide Proteine verhindern auf diese Weise deren Aktivierung. Das Protein B18R, welches ein extrazellulärer Täuschziel-Rezeptor für Interferon ist, unterbindet die Interferon-Signalkaskade indem Typ I Interferon an B18R bindet anstatt an seinen originalen Rezeptor in der Plasmamembran. Die Kotransfektion von E3, K3 und B18R erlaubte repetitive IVT-RNA-basierte Transfektionen in humanen Fibroblasten. Dabei wurde die Interferonantwort soweit unterdrückt, dass auch IVT-RNA kodierte stammzellspezifische Transkriptionsfaktoren durch repetitive Transfektionen so lange exprimiert werden konnten, dass eine Induktion von deren Zielgenen beobachtet wurde.

Die Ergebnisse dieser Arbeit zeigen, dass die entwickelte Methode als Grundlage für eine kontinuierliche Expression fremder Proteine, kodiert durch IVT-RNA, dienen kann. So könnte die therapeutische Proteinexpression von den Erkenntnissen profitieren und möglicherweise die IVT-RNA basierte Vakzinierung oder T-Zell Therapie voranbringen. Eine

besonders vielversprechende Anwendung ist die nun geschaffene Voraussetzung für eine kontinuierliche IVT-RNA kodierte Proteinexpression von stammzellspezifischen Transkriptionsfaktoren, so dass die Grundlage zur Herstellung induzierten pluripotenten Stammzellen (iPS) gelegt worden ist. Die durch IVT-RNA generierten iPS könnten für vielfältige therapeutische Anwendungen im Rahmen der regenerativen Medizin, oder auch zur Wirkstoffsuche genutzt werden.

Abbreviations

*****	p value smaller than 0.05
******	p value smaller than 0.01
*******	p value smaller than 0.001
°C	degree centigrade
Ab	antibody
2-AP	2-Aminopurine
ARCA	anti-reverse cap analog
bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CLR	C-type lectin receptors
CMV	cytomegalovirus
c-Myc	myelocytomatosis viral oncogene homolog
CO_2	carbon dioxide
Ct	cycle threshold
DC	dendritic cell
DCP1,2	decapping enzyme 1,2
dist	distilled
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dN-PKR	dominat negative protein kinase R
dNTP	deoxyribonucleotide triphosphate
DPPA4	developmental pluripotency associated 4
ds	double-stranded
DspS	sarvenger decapping enzyme
DTT	dithiothreitol
F	Farad
ECR	electron-coupling reagent
EDTA	ethylenediaminetetraacetic acid
EF1a	elongation factor-1 alpha
eIF2α	eukaryotic initiation factor-2 alpha
EP	electroporation
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig.	Figure
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDF3	growth differentiation factor 3
GDP	guanosine di-phosphate
GFP	green fluorescent protein
GTP	guanosine tri-phosphate

h	hour(s)
H_20	water
HPRT	hypoxanthine-phosphoribosyl-transferase
IFITM1	interferon-induced transmembrane protein 1
IFN	interferon
Im-13	imidazole derivate
IKK	inhibitor of κB kinase
iPS	induced pluripotent stem cells
IVT	*in vitro* transcribed
JAK	janus kinase
JNK	jun amino-terminal kinases
k	kilo
KLF4	krueppel-like factor 4
l	liter
LB	lysogeny broth
Lin28	Lin28 protein; a micro RNA binding protein
Luc	luciferase
M	molar
m5C	5 methylcytidine
MDA-5	melanoma differentiation-associated gene 5
min	minute(s)
ml	milliliter
MM	master mix
MOPS	3-(N-morpholino) propanesulfonic acid
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response gene (88)
MX	myxovirus resistance
NANOG	protein that is named after a land of enduring youth in the celtic mythology
NEAA	non-essential-amino acids
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B-cells
NLA	normalized luciferase activity
NLR	nucleotide-binding oligomerization domain receptors
nm	nanometer
OAS	oligoadenylate synthetase
OCT4	octamer binding transcription factor 4
OD	optical density
PABP	poly A binding protein
PAMPs	pathogen associated molecular patterns
PAN2-PAN3	PABP-dependent poly A nuclease 2,3
PARN	poly A specific ribonuclease

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	Peridinin chlorophyll protein complex
PGK	phosphoglycerate kinase
PKR	protein kinase R
PRPs	pattern recognition receptors
ΨU	pseudourinide
REX1	reduced expression protein 1 (also known as ZFP42, zink finger protein 42)
RIG-I	retinoic acid inducible gene I
RLR	RIG-I-like receptors
RM	reaction mix
RNA	ribonucleic acid
RNaseL	ribonuclease L
rpm	rounds per minute
RT	room temperature
s	second(s)
siRNA	short interfering RNA
SOX2	sex determining region Y-box 2
ss	single-stranded
STAT	signal transducer and activator of transcription
T	time
T1/2	half-life
TAE	tris-acetate-EDTA
TCR	T-cell receptor
TERT	telomerase reverse transcriptase
Tetrazolium salt XTT	((sodium 3´-[1-(phenylaminocarbonyl)-3,4tetrazolium]-bis (4-methoxy-6-nitro))
TF	transcription factor
TLR	Toll-like receptor
U	unit
UTR	untranslated region
UV	ultraviolet
VACV	vaccinia virus
w/v	weight per volume
WT	wild type
XRN1	exoribonuclease 1

1 Introduction

1.1 RNA

1.1.1 Transgene expression by *in vitro* transcribed messenger RNA

To make transgenes available for mammalian cells is a challenge in life science research. The direct delivery of proteins is possible (Kim et al., 2009), but for providing protein expression of the genes of interest mostly deoxyribonucleic acid (DNA) (Scholz et al., 2013) or ribonucleic acid (RNA) (Tavernier et al., 2011) is brought into target cells, using a number of techniques, which are collectively termed transfections. Eukaryotes use a conserved two-step system for gene expression: The transcription from DNA into messenger RNA (mRNA) is located in the nucleus; subsequently the mRNA is transported to the cytoplasm, where it serves as template for protein translation.

Both nucleic acids, DNA and RNA, can serve as vectors for transgene delivery endowed with specific advantages and drawbacks for each of them. DNA is more stable than RNA and in contrast to RNA the genomic integration of DNA allows long-term transgene expression and the possibility to generate stably transfected cell lines. Thereby the possibility of insertional mutagenesis exists (Nichols et al., 1995; Würtele et al., 2003; Wang et al., 2004). When using RNA as a vector, the risk of genomic integration is abrogated, since the transgene expression of the gene of interest is transient. Technically, for RNA the transfection is easier because only one bi-lipid barrier must be crossed to reach the cytoplasm, whereas the DNA must additionally overcome a second barrier, the nuclear membrane. Therefore RNA transfection is more efficient than DNA transfection (Ponsaerts et al., 2003; Zhao et al., 2006).

1.1.2 Cellular mRNA degradation

The mRNA degradation occurs in all cells, although mRNA half-life differs depending on the organism and the mRNA function. The mRNA half-life can vary from several minutes up to several hours. As an example unstable RNA in yeast has a half-life of less than 7 min whereas the stable ones can be detected for more than 25 up to 90 min (Herrick et al., 1990; Wang et al., 2002). In mammalian cells the half-life of unstable RNA is in the range of 15 min, like c-fos mRNA (Shyu et al., 1989) but stable mRNA can be present for 24 h, like β-globin RNA (Ross and Sullivan, 1985; Peixeiro et al., 2011). The life time of mRNA is limited because it is cleaved into its constituent nucleotides, which are recycled by the organism. Differences in

mRNA stability are regulated by specific interactions between the structural elements of the mRNA and RNA binding proteins. Through mRNA turnover the gene expression in the cells can be influenced. Thereby internal (e.g. cytokines) or external (e.g. temperature) stimuli affect mRNA half-life, as well as deregulation due to diseases (Guhaniyogi and Brewer, 2001). The following section focuses on the mRNA degradation (Fig. 1.1 A + B) while details of the structural elements will be addressed later. The natural mRNA consists of basic structural elements itemized as follows from 5' to 3' direction: A 5'-cap structure, an 5'-untranslated region (UTR), the coding region of the gene of interest and a 3'-UTR afterwards. Finally the last structural element consists of a stretch with repeating adenine nucleotides, termed the poly A tail.

The degradation of mRNA is prevalently catalyzed by three enzymes classes: 5'-exonucleases and 3'-exonucleases attack the ends of mRNA and endonucleases degrade mRNA by internal hydrolysis of the ester linkages (Garneau et al., 2007; Harris and Cassano, 2008). The 5'- and 3'-terminal structures of mRNA, the 5' cap and the 3' poly A tail, protects the ends of the mRNA from immediate degradation and delay complete mRNA hydrolysis (Coller and Parker, 2004; Garneau et al., 2007; Houseley and Tollervey, 2009). Usually mRNA degradation starts by 3' poly A tail deadenylation (Fig. 1.1), but a deadenylation independent decay does also exist (Badis et al., 2004; Garneau et al., 2007). Enzyme complexes (PAN2-PAN3 or PARN) are responsible for starting the deadenylation (Meyer et al., 2004; Balagopal et al., 2012). The degradation from 5' to 3' end is triggered by deadenylation and starts with the cleavage of the protective cap by the decapping enzyme complex DCP1-DCP2. For cleavage, DCP2 prefers a methylated cap that is bound to RNA which consists of more than 25 nucleotides (Liu and Kiledjian, 2006). Then the exoribonuclease 1 (XRN1) continues RNA degradation in a 5' to 3' direction (Chang et al., 2011; Jinek et al., 2011). After the poly A tail has been shortened to fewer than 30 to 60 nucleotides (Shyu et al., 1991; Chen et al., 1994) RNA degradation is continued in a 3' to 5' direction by the exosome, a ribonucleolytic multiprotein complex composed of nine core subunits and two exosome-associated subunits (Rrp44, belonging to the core exosome and Rrp6) for the exonuclease activity (Houseley et al., 2006; Lorentzen et al., 2008).

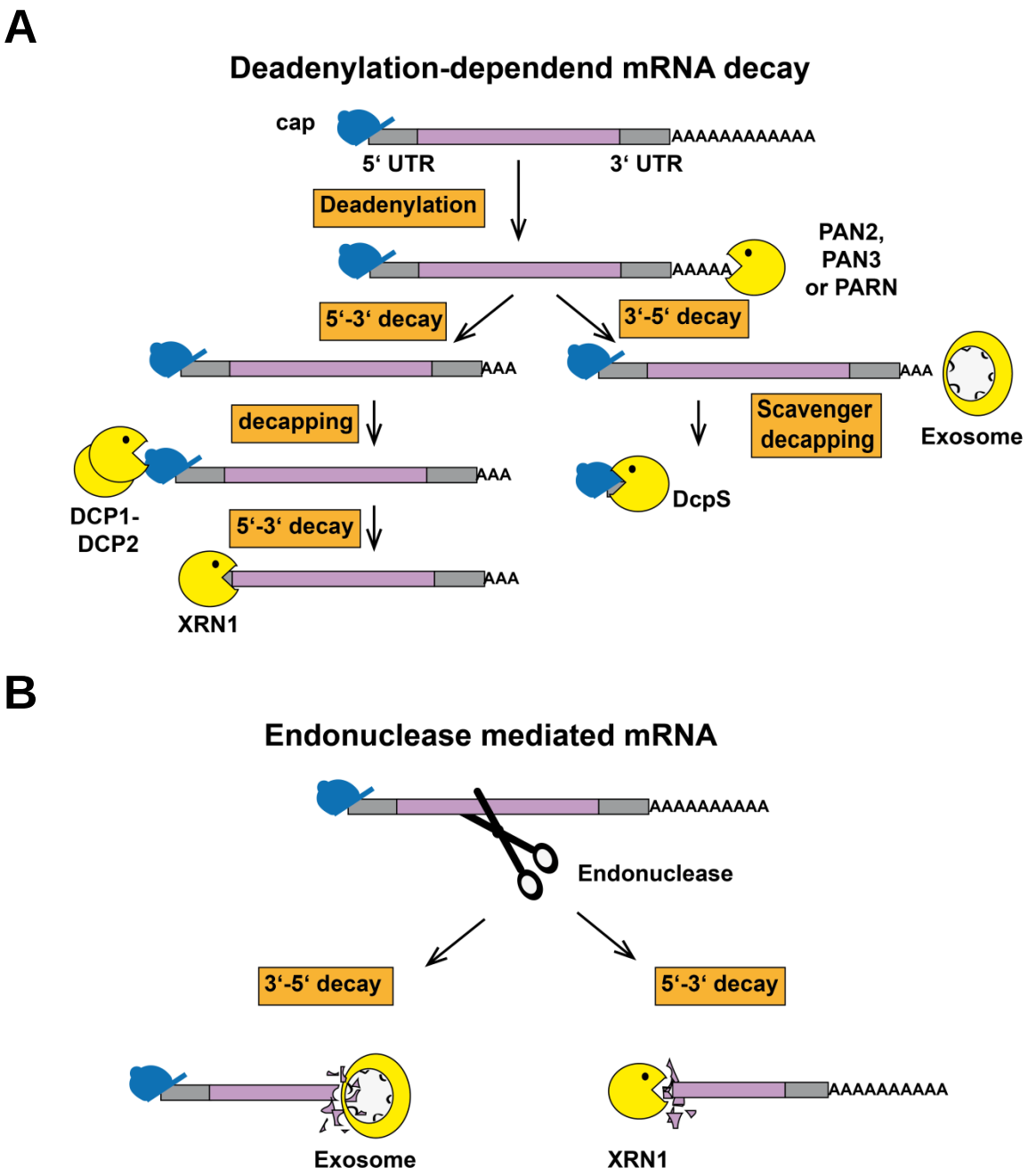

Figure 1.1 Mechanisms of mRNA degradation The degradation of mRNA, consisting of a protective cap, a 5’ untranslated region (UTR), the coding sequence, a 3’UTR and a poly A tail, occurs mainly in a deadenylation dependent manner: Deadenylase shortens the poly A tail of the mRNA (**A**). Afterwards the degradation proceeds either in 5’-3’ or in 3’-5’ direction. In case of 5’-3’ decay the decapping enzyme complex removes the cap and the exoribonuclease 1 continues mRNA degradation. In the second mechanism the exosome degrades the mRNA in 3’-5’ direction which is finished by hydrolyzing the cap by the scavenger decapping enzyme. In the endonuclease mediated decay (**B**) the mRNA is cut into two parts with unprotected ends, which were susceptible for the exosomal mediated 3’-5’ decay and 5’-3’ degradation by XRN1. poly A binding protein dependent poly A nuclease (PAN), poly A specific ribonuclease (PARN), decapping enzyme complex (DCP1-DCP2), exoribonuclease 1 (XRN1), scavenger decapping enzyme (DspS). Figure adapted and modified from (Garneau et al., 2007).

After the exosomal degradation, the remaining capped oligonucleotide is then hydrolyzed by DcpS, a scavenger decapping enzyme (Liu et al., 2004; Liu et al., 2008) (Fig. 1.1).
Degradation, however, is not limited to the exonucleolytic decay, thus efficient degradation of mRNA can occur by endoribonucleolytic decay. A prominent example is the cleavage of mRNA by RNA interference (Wilusz, 2009; Li et al., 2010; Tomecki and Dziembowski, 2010). After the internal cutting of endonucleases the fragments are susceptible for further degradation by the exosome and XRN1 (Garneau et al., 2007) (Fig. 1.1). To protect mRNA, in particular from exonucleolytic degradation, the *in vitro* transcribed RNAs (IVT-RNAs) can be modified on their 5' and their 3' end in order to enhance the stability.

1.1.3 Structure of mRNA and implications for IVT-RNA design

Typically, IVT-RNA mimics natural mRNA and consists of the same structural elements that were mentioned before: 5'cap and 3'poly A, as well as 5'- and 3' UTRs and a central coding region. For many research applications a long availability of the transfected IVT-RNA is required. With modifications in each part of the IVT-RNA structure, beginning with the protective cap structures at the 5' end and ranging to the poly A tail at the 3' end, improved stability and translational efficiency can be achieved (Gallie, 1991). In eukaryotic cells, the 5' end of the mRNA is post-transcriptionally modified, resulting in the enzymatic addition of a 7-methylguanosine (m7G) cap by a triphosphate connexion (ppp) to the first transcribed nucleotide. The capping procedure involves three enzymatic reactions, starting with a dephosphorylation of the terminal phosphate group by a triphosphatase, the guanylyltransferase adds the nucleotide guanosine and lastly the methylation of guanosine at the position N7 is performed by the methyltransferase (Shuman, 2001). This m7GpppG modification is important for mRNA splicing, for mRNA transport to the cytoplasm, for translation initiation where it helps to recruit the ribosomes by the eukaryotic translation factor eIF4E and for mRNA stabilization (Sonenberg et al., 1978; Gingras et al., 1999; Kapp and Lorsch, 2004).
The benefit of the 5' cap on natural mRNA stability was proven by comparative studies with uncapped RNAs generated by chemical removal of the cap (Furuichi et al., 1977). The terminal structure of the 5' cap is important, not only for stability, but also for the translational efficiency. The cap of the IVT-RNA can either be added enzymatically (Martin et al., 1975; Shuman et al., 1980; Venkatesan et al., 1980) or co-transcriptionally by addition of a dinucleotide (m7GpppG) to the reaction mix, which acts as structural homolog to the

natural cap. As a consequence dinucleotides and the GTP compete for initiating the transcription. The dinucleotide can be incorporated into the IVT-RNA in both directions (Gpppm7GpN as well as m7GpppGpN) (Pasquinelli et al., 1995). Translation cannot initiate with the wrongly incorporated m7GpppGpN, this reverse orientation is the reason for reduced translation efficiency compared to mRNAs with caps that were invariably incorporated in the correct orientation. The reverse orientation is avoided when a modified dinucleotide is used: $m_2^{7,2'O}$GpppG and $m_2^{7,3'O}$GpppG termed anti-reverse cap analogs (ARCA), ARCA improve translation since they can only be incorporated in the correct orientation due to methylation of the 2'- or 3' oxygen (Stepinski et al., 2001; Peng et al., 2002; Jemielity et al., 2003). The ARCA cap is the current gold standard for IVT-RNA capping (Kirkegaard et al., 2004; Medzhitov, 2007; Kuhn et al., 2010). By an additional modification, the so called phosphothioate cap analog, an oxygen atom is substituted by a sulfur atom within the triphosphate bridge. This substitution is more resistant to DcpS decapping resulting in less mRNA degradation and thus an increase in translation in mammalian cells (Fig. 1.2 B) (Grudzien-Nogalska et al., 2007; Kowalska et al., 2008).

In eukaryotic mRNA the 5'- and 3' UTR plays a role in both, translational efficiency and stability of the RNA (Pesole et al., 2001; Liu et al., 2012): the 5'UTR is important for the regulation of translation (Araujo et al., 2012), and the 3'UTR enhances the stability (Jiang et al., 2006). Examples of naturally stable mRNAs are the alpha- and beta-globin mRNAs where a conserved pyrimidine-rich element in both globin 3' regions was identified for RNA stabilization. The conserved element is recognized by RNA-binding proteins, the alpha-CP/hnRNP-E family members, which then are responsible for recruiting trans-acting factors that are important for mRNA stability (Yu and Russell, 2001; Waggoner and Liebhaber, 2003). The enhanced stability of beta-globin is related to a nucleolin-binding element within 3' UTR (Jiang et al., 2006). Sequence comparison of different stable eukaryotic mRNA revealed closely related pyrimidine-rich sequences (Holcik and Liebhaber, 1997).

For messenger IVT-RNA translation optimization, the 5'- and 3' untranslated region (UTR) can be addressed for modifications. Hereby the protein translation depends to some extend on the translation efficiency governed by suitable UTRs. In the first studies that analyzed IVT-RNA-based transgene expression the globin UTRs sequence of the model organism *Xenopus leavis* were used (Malone et al., 1989). Due to a possible later use for clinical applications, our group switched to human beta-globin UTRs for gene expression in dendritic cells. The tandem repeat of human beta-globin 3' UTR, which was fused head to tail, resulted in significantly enhanced protein expression in DCs (Holtkamp et al., 2006).

Furthermore the gene of interest itself can have an impact on the stability and translation efficiency. Some codons are translated more efficiently and with fewer mistakes than others (Bossi and Ruth, 1980; Sørensen et al., 1989) and certain codons are used more frequently than others, with differences between the organisms (Grantham et al., 1980; Grantham et al., 1981). For an efficient protein expression it is necessary that the codon usage reflects the available transfer RNAs (tRNAs) in the cell (Ikemura, 1985) and a balanced codon usage increases the efficiency (Qian et al., 2012). Because of the degenerated genetic code it is feasible to alter the coding region to some extent without changing the amino acid sequence. This can be done to modify the coding region of IVT-RNA. With the knowledge of the preferred codons the sequence of interest can be optimized in order to enhance either the accuracy or the amount of translation (Gingold and Pilpel, 2011).

At the 3' end of the RNA a poly A tail is found in most mature mRNAs, but with differences in length ranging from 25 to 250 adenine nucleotides (Brawerman, 1981; Wahle and Keller, 1996). A prominent exception in eukaryotes is the mRNA of histones; they lack a poly A tail (Marzluff et al., 2002). Besides its role in translation initiation, the pivotal function of the poly A tail is to protect the RNA from degradation. In mammalian cells the complete turnover of mRNA is initiated only after a shortening of the poly A tail to less than 30 to 60 nucleotides (Shyu et al., 1991; Chen et al., 1994). The protective function of the poly A tail is provided by proteins, such as the poly(A)-binding proteins (PABP), that cover and protects the poly adenine stretch from deadenylation (Bernstein et al., 1989; Bernstein and Ross, 1989; Mangus et al., 2003).

Similar to natural mRNA the IVT-RNA is poly adenylated as well. The poly A tail can be added either enzymatically to the 3' end of the IVT-RNA by recombinant poly A polymerase or the adenine stretch can be encoded by a poly T stretch on the vector template used for *in vitro* transcription. The disadvantage of the enzymatic poly adenylation is that the poly A tails differ in length and a high batch to batch variability is observed (Holtkamp et al., 2006). Both drawbacks can be circumvented when the IVT-RNA is produced from a vector template encoded poly A tail. With this technique a robust and defined adenine stretch is added to the *in vitro* transcribed IVT-RNA. Our group observed in dendritic cells a robust reporter gene expression encoded by IVT-RNA with 120 adenines at the 3' end (Holtkamp et al., 2006).

Synthetic mRNA is commonly produced by *in vitro* transcription (Geiduschek et al., 1961). Therefore a linear DNA-template is required that can be produced either by digestion of a plasmid or by PCR. For the IVT-RNA production the following components are necessary: a suitable promoter, the sequence information encoded in the linear DNA-template, nucleotides

and an appropriate transcription enzyme such as the bacteriophage derived RNA polymerase (T7, T3 or SP6) (Nielsen and Shapiro, 1986; Leary et al., 1991; Moll et al., 2004). Finally the purified IVT-RNA can be stored ready to use at – 80 °C.

The IVT-RNAs used in this study were synthesized from vectors established in accordance to Holtkamp et al. (Holtkamp et al., 2006). The IVT-RNA consists of a protective cap followed by a 5'-UTR, the codon-optimized coding sequence of a corresponding gene and the 3' 2-beta-globin UTR. Lastly, the IVT-RNA is completed at the 3' end by 120 adenines (poly A tail) (Fig. 1.2 A). Beside the natural RNA caps, several modified caps have been synthesized and, as previously mentioned, the ARCA cap is seen as standard in IVT-RNA capping (Mockey et al., 2006; Bontkes et al., 2007; Kore et al., 2009; Kuhn et al., 2010). Compared to the natural cap, the 3'-OH group is substituted by a $–OCH_3$ group in the ARCA cap. For dendritic cells (DCs) Kuhn and coworkers showed (Kuhn et al., 2010) that an ARCA derivate cap, termed D1 cap, exists which is superior to both the natural and ARCA caps in prolonging the transgene expression (Fig. 1.2 B).

In summary the expression duration and translational efficiency of IVT-RNA encoded proteins can be improved by optimization of each structural element.

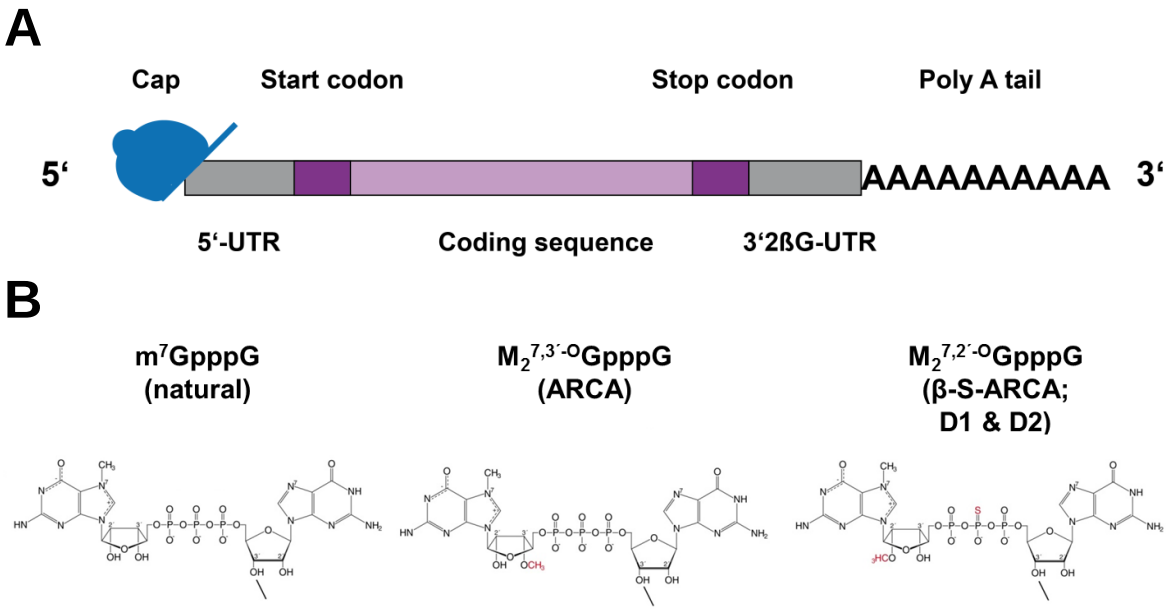

Figure 1.2 IVT-RNA structure and different RNA caps (**A**) A typical IVT-RNA structure includes in 5’ to 3’ direction a protective cap, a 5’ untranslated region (UTR), the codon optimized gene of interest and the 3’UTR which consists of two serial head-to-tail fused human β-globins. Lastly the IVT-RNA has a poly A tail consisting of 120 adenines (AAAAA). Chemical structures of the IVT-RNA caps (**B**). Depictured are the naturally occurring m7GpppG (m7G = 7-methylguanosine), the ARCA (anti-reverse cap analogs) 5‘cap-structure of mRNA and the sulfide modified versions of the ARCA 5‘-cap (ß-S-ARCA = D1/D2), which were shown to stabilize mRNA.

1.1.4 Distinguishing between self and non self RNA in cells

The discrimination of self and non-self enables cells to defend themselves against invading pathogens. Thereby the pattern recognition receptors (PRPs) are specialized for sensing conserved structures of microorganisms termed *pathogen associated molecular patterns* (PAMPs). The four families of PRPs include: the transmembranal *C-type lectin receptors* (CLRs), the *toll-like receptors* (TLR) the *NOD-like receptors* (NLR) and the *(RIG)-I-like receptors* (RLRs), which are located in the cytoplasm (Takeuchi and Akira, 2010). This defense system has been optimized during evolution and is now able to sense the PAMPs. In the following part, I will focus on receptors that are able to sense RNA. For invading viruses either single stranded (ss) or double stranded (ds) RNA is characteristic. After an infection the hosts’ machinery is used for biosynthesis. Thus, prior to initiating the cellular defense, the cell has to distinguish between self and non-self mRNA. For sensing foreign mRNA, which also includes the artificial produced messenger IVT-RNA, several membrane-bound and

cytoplasmic sensors exist. The toll-like receptors (TLR) can sense beside fungal, bacterial components also viral mRNA (Medzhitov, 2007). Hereby the endosomal TLR3 senses ds RNA that is usually absent in mammalian and therefore a trigger for anti-viral defense. Furthermore, the endosomal TLR7 and TLR8 sense ss RNA (Diebold et al., 2004; Heil et al., 2004; Diebold, 2010). In the cytoplasm of mammalian cells until now, the following constitutively expressed ds RNA recognition receptors are known: The *retinoic acid inducible gene* I (RIG-I) (Yoneyama et al., 2004), the *melanoma differentiation-associated gene* five (MDA-5) (Kang et al., 2002) and the RNA dependent *protein kinase R* that is termed PKR (Clemens et al., 1993; Clemens, 1997) (Fig. 1.3 A and B). The signaling pathways initiated by RIG-I and MDA-5 have the *interferon regulated factor* 3 and 7 (IRF3, IRF7) as targets which dimerizes upon activation, enter the nucleus and result in the upregulation of proinflamatory cytokines such as the interferon's (Barral et al., 2009). It is assumed that MDA-5 is the receptor for longer mRNAs whereas for RIG-1 activation shorter mRNAs are required (Barral et al., 2009; Pichlmair et al., 2009). A further cytoplasmatic enzyme which is able to sense ds RNA is the *oligoadenylate synthetase* (OAS). It is constitutively expressed at basal levels and accumulates in the cytoplasm of the cells as an inactive monomer. In the presence of ds RNA OAS is activated resulting in the activation of *Ribonuclease L* (RNAseL) which triggers the cleavage of all intracellular RNA. This is a cellular defense mechanism especially for avoiding viral replication (Fig. 1.3 A) (Samuel, 2001; Sadler and Williams, 2008; Kristiansen et al., 2011).

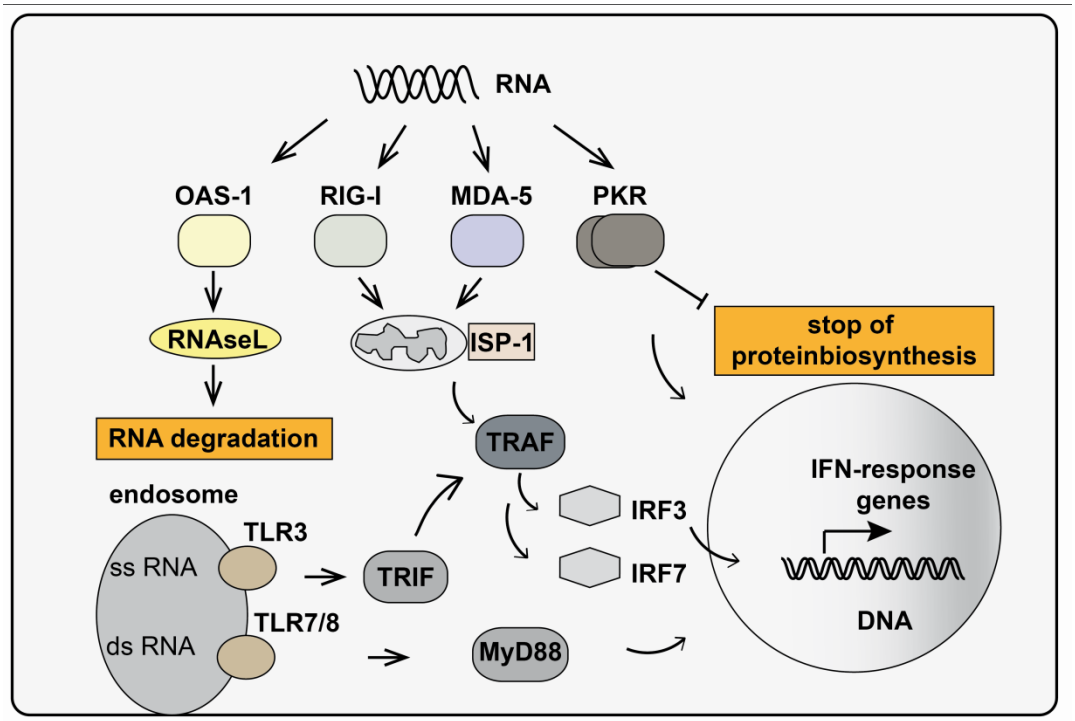

Figure 1.3 (**A**) **Cytoplasmic RNA sensors** Non-self RNA is sensed by the cell by different RNA sensors like endosomal *toll-like receptor* 3, 7/8 (TLR) or the cytosolic receptors *oligoadenylate synthetase* (OAS), *retinoic acid-inducible gene I* (RIG-I), *melanoma differentiation associated gene* 5 (MDA-5) and the double stranded *RNA dependent protein kinase R* (PKR). This simplified schematic illustrates the primary effect of RNA recognition: the IFN response is induced and RNA is degraded by r*ibonuclease L* (RNAseL). When non-self ss RNA is sensed by the TLR3 the signaling cascade is initiated. The adaptor protein *TIR-domain-containing adapter-inducing interferon β* (TRIF) and *TNF receptor-associated factor* (TRAF) are subsequently activated. This is followed by the phosphorylation and activation of the dimeric transducers *interferon regulatory factors* (IRFs) 3 and 7. The IRF complex migrates into the nucleus, where the induction of interferon response genes occurs. When ds RNA is sensed by TLR7/8 the signaling for upregulation of IFN response is inititated by the adaptor protein *Myeloid differentiation primary response gene (88)* (MyD88). In the cytoplasm of the cell the OAS reacts to the foreign RNA by the activation of the RNAseL resulting in a total cleavage of all intracellular RNA. Furthermore RIG-I and MDA-5 activate the mitochondrial associated adaptor *iron sulfur cluster-containing protein*-1 (ISP-1) with activated downstream TRAF and induce the IRF complexes to translocate into the nucleus. There the IFN-response genes are upregulated. Lastly, the PKR can be induced by the presence of RNA, leading to the stop of protein biosynthesis and the induction of IFN response genes (more detailed information in Fig. 1.3 B). (ISP-1 is also known as MAVS - *mitochondrial antiviral signaling protein*)

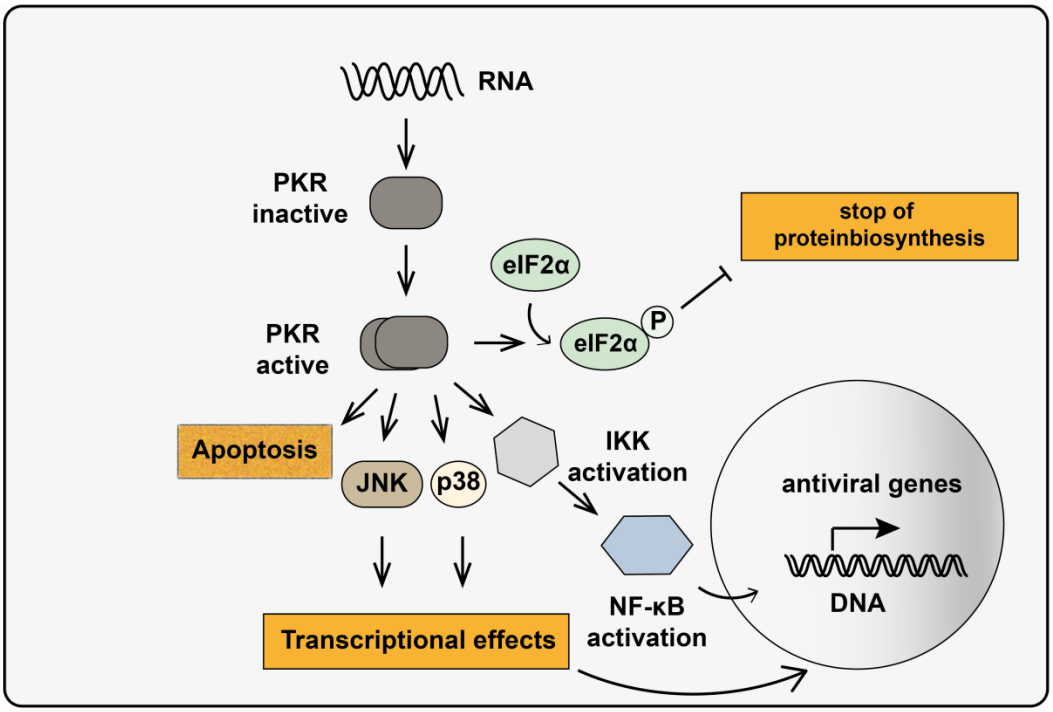

Fig. 1.3 (B) Cellular changes after PKR activation When the RNA dependent *protein kinase R* (PKR) senses ds RNA two inactive monomers assemble into dimers. Subsequently PKR activates several signaling pathways. One substrate is the *eukaryotic translation initiation factor alpha* (eIF2α). The phosphorylation (P) of eIF2α prevents the recycling of *guanosine di-phosphate* (GDP)-eIF2α complex and results into a stop of protein biosynthesis. Furthermore the activated PKR leads to the activation of the *nuclear factor-κB* (NF-κB) which is important for the cellular defense to foreign RNA. The NF-κB activation occurs though the activation of the *inhibitor of κB kinase* (IKK). Activated PKR additionally interferes with the stress activated phospho-kinases p38 and the *c-JUN amino-terminal kinase* (JNK), which results in an altered transcription affecting cellular response to proliferation and cellular survival. Finally PKR activation can lead to the induction of apoptosis as a consequence of sensing foreign RNA in the cell. Figure modified from (Kirkegaard et al., 2004)

The next cytoplasmic RNA sensor that is of special importance for this work is the Protein Kinase R (PKR), which is the subject of the next part.

1.1.5 The RNA sensor Protein Kinase R

The Protein Kinase R was identified in the 1970s. In 1976 Roberts et al. published their work describing an interferon mediated protein kinase and inhibitor of protein synthesis that is dependent on the presence of ds RNA (Roberts et al., 1976), followed by publications of Kerr et al. (Kerr et al., 1977), describing the inhibitor of protein synthesis in a cell free system and Sen et al. (Sen et al., 1978) also describing and partially purifying the kinase. The PKR, also known as DAI (Clemens et al., 1993), is a 68 kDa serine/threonine protein kinase that is induced by interferon's and activated when it senses ds RNA in the cell (Meurs et al., 1990). In humans, the gene EIF2AK2, coding for the PKR, is located on the chromosome 2 (Barber et al., 1993).

The PKR is constitutively expressed in cells at a basal level and is for the most part (Jeffrey et al., 1995) located in the cytoplasm. It consists of two ds RNA binding domains at its N-terminus and a catalytic domain on the C-terminus. An activation of the inactive monomers to the active dimeric PKR is arranged when RNA, consisting usually of at least 30 bp (Lemaire et al., 2008), attaches to the RNA binding domains. Then an allosteric conformation change takes place and the catalytic domain is no longer covered by its N-terminal part. An autophosphorylation of PKR monomers at threonine 446 and 451 (Romano et al., 1998; Zhang et al., 2001) occurs and finally two PKR monomers build up an active dimeric enzyme. The active PKR starts the signaling cascade, which results in the regulation of IFN responsive elements (Fig. 1.3 B) (Sadler and Williams, 2008). An activated PKR has multiple effects, like the phosphorylation of the PKR substrate eIF2α at residue Ser51 (Lu et al., 1999), which shuts down protein biosynthesis. The factor eIF2α is part of the so called ternary complex and is associated with the small subunit of the ribosome (Kapp and Lorsch, 2004). In this constellation a *guanosine tri-phosphate* (GTP) is bound to the ϒ-subunit of eIF2α. When translation is initiated, the GTP is hydrolyzed to *guanosine di-phosphate* (GDP) and the latter is still bound to eIF2α. For maintenance of translation it is essential for the cell to regenerate the active eIF2α. For eIF2α regeneration the GDP to GTP exchange is catalyzed by eIF2B. Due to the phosphorylation step of eIF2α kinases, e.g. by the PKR, the recycling is blocked (Safer, 1983). Furthermore IFN response genes are upregulated as well as apoptotic pathways being initiated by activated PKR (Roberts et al., 1976; Balachandran et al., 1998; Gil et al., 1999; Gil and Esteban, 2000; Gil et al., 2002; García et al., 2006).

Altogether an activated PKR negatively affects protein synthesis and leads to an antiviral cellular state that is counterproductive for long-term IVT-RNA-based protein expression.

1.2 The interferon's and their pathways

The intracellular signaling pathways that are activated upon detection of non-self RNA ultimately result in interferon response. The interferon response was observed for the first time in 1804 by Edward Jenner. He described that the infection with a virus can prevent cells from a second infection with vaccinia virus (Jenner, 1804). Nevertheless the identification of the antiviral agents that interfere with secondary virus infections was not until 1957. It was found that influenza infected chicken cells secreted a factor which transfers virus-resistance to other cells (Isaacs and Lindenmann, 1957). This secreted factor was called interferon (IFN). IFNs are glycoproteins that belong to the family of cytokines. Cytokines are secreted proteins or peptides that regulate intercellular signaling. IFNs can be divided into three classes: interferon type I, II and III (Sadler and Williams, 2008). Type I IFN comprise IFNα and IFNβ and in humans additionally IFNε, IFNκ, and IFNω. The type II class consists of IFNγ (Bach et al., 1997; Sadler and Williams, 2008) whereas the type III class includes IFNλ-1, -2 and -3 (Pestka et al., 2004). The major function of IFNs is to combat viral infections (Samuel, 2001; Borden et al., 2007; Wang and Fish, 2012) but they are also involved in the regulation of tumor suppression (Sen, 2000; Uddin and Platanias, 2004), immune response (Boehm et al., 1997; Taniguchi and Takaoka, 2001), cellular differentiation and cell cycle arrest (McNurlan and Clemens, 1986; Harada et al., 1998; Wang and Fish, 2012). Furthermore type I IFNs are especially involved in inflammatory and autoimmune diseases, where they have either an impact on disease development e.g. by activating the inflammasome or a negative impact e.g. by inducing autoreactive T cells (González-Navajas et al., 2012). The antiviral pathways induced by IFNs are complex. A conserved pathway of cell to cell communication after IFN response induction is mediated by the *janus kinase* and *signal transducer and activator of transcription* (JAK-STAT) pathway (Aaronson and Horvath, 2002). Depending on the IFN class, genes can be regulated differentially (Der et al., 1998). More than 300 genes increase their expression pattern upon IFN response (de Veer et al., 2001). Among them PKR, OAS and Myxovirus resistence (Mx) pathways are induced as consequence to upregulated IFN genes. Furthermore, a down-regulation of some genes was also reported (Der et al., 1998).

1.2.1 Cell response to IVT-RNA resembles the cell response to viral RNA

When cells are transfected with IVT-RNA they are able to sense it as non-self. The cellular reactions to synthetic RNA are assumed to be equivalent to those to invading viral RNA as described before. An induction of IFN response and PKR activation results in cellular

response inducing the IFN production, a shutdown of protein biosynthesis and, after frequent transfection, the induction of apoptosis. All in all this is contra productive for repetitive IVT-RNA treatment of target cells. When cells are in an antiviral state with upregulated cellular defense mechanisms an IVT-RNA-based transgene expression is hampered.

When transfection of IVT-RNA is performed, one should consider upfront, whether the antiviral response will be helpful or detrimental to achieve the intended aim. For example, immunostimulatory reactions to IFNs can be helpful to boost the development of an immune response to RNA-based vaccines (Conry et al., 1995; Leitner et al., 1999; Kreiter et al., 2010), whereas the PKR-mediated translational shutdown apparently limits protein expression. The induction of apoptosis as a response to IVT-RNA precludes mRNA transfection of cells from any experiment that requires living cells. However, cell death after IVT-RNA transfections could be avoided by siRNA-mediated knock-down of the IFN response genes, which enabled repetitive RNA transfections (Angel and Yanik, 2010). Another promising way to reduce the IFN response to IVT-RNA is make it "look self" by incorporation of modified nucleotides (Karikó et al., 2005; Hornung et al., 2006; Nallagatla and Bevilacqua, 2008). In higher organisms nucleotide modifications are introduced into RNA as an adaption to distinguish between self and foreign RNA. Thus, when simple NTPs are substituted with modified nucleotides during *in vitro* transcription, the resulting modified RNA is no longer recognized as "non-self" by cells. A reduced TLR3, 7 and 8 activation as well as a reduced cytokine production was reported when modified RNAs were used for transfection (Karikó et al., 2005). Even a partial substitution of only 25% of cytidine and uridine by 5-methylcytidine (m5C) and thiouridine reduces significantly the binding to TLR3, 7 and 8 as well as to RIG-I and thereby minimizes the IFN response of modified IVT-RNA (Kormann et al., 2011). Similarly, when uridine is totally substituted by pseudouridine (ΨU) in mRNA, activation of PKR is prevented and thereby translation is increased (Anderson et al., 2010). In line with that, a total replacement of cytidine and uridine by 5 methylcytidine and pseudouridine was effectively blocking the IFN response, together with the anti IFN recombinant protein B18R in the medium. Thus they could use IVT-RNA to reprogram fibroblasts to induced pluripotent stem cells (Warren et al., 2010).

Taken together, cells are supposed to respond to transfected IVT-RNA as they respond to viral infections. Therefore it is interesting to see how viruses have evolved methods to evade the innate immunity and to evaluate whether viral proteins can help to improve IVT-RNA transfections.

1.3 Viral anti-interferon proteins

Viruses have evolved a number of strategies to evade the cellular defense mechanism: they block IFN induction and signaling, inhibit RNA sensors and modulate protein biosynthesis (see examples in Tab. 1.1). By blocking the IFN-signaling cascades viruses have the possibility of using the cellular synthesis machinery for their reproduction. The number of IFN antagonists is impressive: 93 different viruses encode for over 170 proteins that target IFN type I response (Versteeg and García-Sastre, 2010).

Several strategies of viruses for blocking the IFN induction and signaling exist. The viral proteins can for example target multiple TLR adaptors and downstream transcription factors, in order to antagonize the IFN response ((A46R) (Stack et al., 2005)). The cellular signaling of cytokines and chemokines is targeted by intracellular immune modulators that interfere either with the IFN regulation (Sen, 2001) or apoptosis signaling (Harrison et al., 2004; Yuan et al., 2012). The signal transduction in response to IFNs is circumvented by recruitment, binding and deactivation of signal transducers (ICP0) (Melroe et al., 2007) or (VH1) (Najarro et al., 2001). In order to avoid the signaling of the transcription factor NF-kB, viral proteins circumvent NF-kB migration into the nucleus (Ghosh and Karin, 2002) (B14) (Chen et al., 2008) (K1) (Shisler and Jin, 2004).

A second strategy of viruses to evade the cellular defense is the inhibition of cellular sensors for viral RNA. As mentioned previously in part 1.1.5, an important sensor for non-self mRNA is the protein kinase R (PKR). Its activation can be inhibited by viral proteins that bind the ds RNA (K3) (Chang et al., 1992) or the PKR (de Levy and Garcia-Sastre, 2001) or when they act as ds RNA (vIF2α) (Rothenburg et al., 2011) (K3) (Davies et al., 1992). For an effective inhibition of the PKR viruses can even degrade the PKR (Black et al., 1993; Habjan et al., 2009). Beside the PKR, the induction of other viral sensor in the cytoplasm could be hampered, like the OAS1 (NS1) (Hale et al., 2008).

A third strategy of viral immune evasion is the modulation of protein biosynthesis. This is essential since viruses are dependent on the host's translation machinery. To avoid their shutdown, phosphatases, which are responsible for dephosphorylation of eIF2α, are recruited (ICP34.5) (Li et al., 2011) or the translation of viral mRNAs are enhanced (NS1) (de La Luna et al., 1995).

Table 1.1 Selected viral immune evasion proteins

protein	mode of action	virus	reference
	Proteins blocking IFN induction and signaling		
A46R	Targets multiple TLR adaptors and downstream transcription factors	Vaccinia Virus	(Stack et al., 2005)
B14	Inhibition of IkB kinase	Vaccinia Virus	(Chen et al., 2008)
ICP0	Recruitment of activated IRF-3 and CBP/p300	Herpes Simplex Virus	(Melroe et al., 2007)
VH1	Binding and dephosphorylation of STAT1	Vaccinia Virus	(Najarro et al., 2001)
VP35	Inhibiting the activation of IRF-3	Ebola Virus	(Basler et al., 2003)
	Proteins inhibiting viral sensors		
NS1	Inhibition of PKR and OAS/RNase L induction	Influenza Virus	(Hale et al., 2008)
P25 (E3)	Inhibition of PKR activation by ds RNA binding	Vaccinia Virus	(Chang et al., 1992)
Us11	Blocks the activation of OAS/RNase L induction	Herpes Simplex Virus	(Sànchez and Mohr, 2007)
V	Blocks MDA-5 signaling	Paramyxovirus	(Andrejeva et al., 2004)
vIF2α	Inhibition of PKR activation by acting as pseudosubstrate	Ranavirus	(Rothenburg et al., 2011)
	Proteins modulating the protein biosynthesis		
2A protease	Internal ribosome-entry side (IRES) dependent initiation of viral mRNA translation by cleavage of eIF4G	Poliovirus	(Ventoso et al., 1998; Fernández-Miragall et al., 2009)
ICP34.5	Ensuring protein translation by recruiting the protein phosphatase 1 (PP1) for dephosphorylation of eIF2α	Herpes Simplex Virus	(Li et al., 2011)
NS1	Enhancer of translation initiation of viral mRNA	Influenza Virus	(de La Luna et al., 1995)

Most viruses modulate the hosts' immune response by using more than one gene product (Alcamí and Koszinowski, 2000; Gale and Foy, 2005). In this regard, VACV is a representative virus. Several VACV proteins modulate IFN signaling and inhibit the PKR (Perdiguero and Esteban, 2009). The three proteins E3, K3 and B18R were considered to be interesting to analyze upon IVT-RNA-based transfection since they contribute towards avoiding the activation and upregulation of the protein kinase R. E3 is a ds RNA binding protein that competes with PKR for binding to RNA (Chang et al., 1992). Thereby the activation of PKR is prevented. K3 acts as a PKR pseudosubstrate by a mechanism that does not involve direct phosphorylation of K3 (Davies et al., 1992). K3 competitively inhibits

eIF2α phosphorylation and activated PKR cannot block protein translation (Beattie et al., 1991; Davies et al., 1992). B18R finally interferes with the IFN signaling. B18R is a decoy receptor which quenches the IFN signaling outside the cell by inhibiting IFN binding to IFN-receptors and thus avoiding the activation of IFN target genes (Colamonici et al., 1995; Symons et al., 1995, 2000).

In summary, viral immune modulating proteins can counteract cellular defense mechanisms by interfering with ds RNA detection by viral sensors, subsequent signaling cascades or by modulating the protein biosynthesis.

1.4 Current and future usage of transgene protein expression by messenger IVT-RNA

The advantage of IVT-RNA is that it cannot integrate into the host genome and that its transfer into cells is very efficient. Currently, IVT-RNA is used for vaccination and for recombinant T cell receptor (TCR) transfer. Furthermore cell reprogramming and direct differentiation of cells are applications of IVT-RNA-based gene transfer (recently reviewed by Kuhn et al., 2012).

Vaccination with mRNA has its basis in the 1990s, when an intramuscular injection of mRNA resulted in detectable amounts of the encoded reporter genes (Wolff et al., 1990). Thereafter, mRNA transfer was used for many different applications (Kuhn et al., 2012) including anti-cancer treatment (Conry et al., 1995; Weide et al., 2008), the generation of antigen specific T cells for an antigen specific immune response that can be used for individualized gene therapy (Schumacher, 2002; Vera et al., 2009) or the adoptive cell therapy (ACT) which manipulates the patients' immune response (Rosenberg et al., 2008). The latter was also reported to be successful in the treatment of human melanoma (Yee et al., 2002). Generally, prospects for new therapies using mRNA based vaccination were seen (Kreiter et al., 2011).

Another implementation is the use of messenger IVT-RNA is the possibility to reprogram human cells to a pluripotent state (Warren et al., 2010). This process, which implies the reactivation of the pluripotent status of a cell, is termed induced pluripotent stem cell (iPS) reprogramming. The groundbreaking work of Takahashi and Yamanaka was published in 2006 for murine cells (Takahashi and Yamanaka, 2006) and then for human cells in 2007 (Takahashi et al., 2007). For the proof of principle of cellular reprogramming using viral based approaches, Yamanaka was accredited in 2012 with the Nobel Prize in medicine. A

drawback for later use in the clinic is the viral background of the reprogrammed cells which bears the risk of genomic integration. Nevertheless iPS cells and their potential for re-differentiation is a promising perspective for regenerative medicine. A further risk of the new technique is their cell type specific memory (Kim et al., 2010; Bar-Nur et al., 2011; Ohi et al., 2011), so that reprogrammed cells might differentiate back into their progenitor cell type. This would be problematic when cells were used e.g. for regenerative medicine. Furthermore standards for the definition of good quality iPS cells and how to rank differences that are detectable between iPS and ESC are missing (Bilic and Belmonte, 2012; Cahan and Daley, 2013). At the moment the most common way of reprogramming, is the use of viral transduction. This virus based approach was improved e.g. by the substitution of oncogenic components or the use of an integration free Sendai virus (Fusaki et al., 2009). In 2009 the generation of iPS using proteins was achieved (Kim et al., 2009; Zhou et al., 2009). Unfortunately until now reprogramming of somatic cell takes a long time as iPS generation has low efficiencies, high costs and often viral background. This is why the usage of iPS cells in the field of regenerative medicine is problematic. It wasn't until 2010 when the first IVT-RNA-based reprogramming was published. Warren and coworkers reported that an integration free reprogramming efficiency of up 4.4% is possible (Warren et al., 2010). A further IVT-RNA-based transfection for reprogramming (Yakubov et al., 2010) underlines the potential of the messenger IVT-RNA-based method by another group. Additionally Warren et al. improved the reprogramming protocol by a feeder–free generation of human iPS cells (Warren et al., 2012). Although the generation of IVT-RNA dependent iPS was achieved, the possibility was seen to improve the protocol by avoiding the use of recombinant proteins, which had to be added into the media, and to focus on unmodified IVT-RNA. When using unmodified IVT-RNA encoded gene transfer, it has to be taken into account that its use can be limited by inducing the cellular antiviral response.

1.5 Purpose of the thesis

The purpose of this thesis was to find a method which allows repetitive transfection of messenger IVT-RNA consisting of unmodified nucleotides. The translation of the IVT-RNA encoded proteins has to be maintained in order to generate a protocol for the long term transgene expression of IVT-RNA encoded proteins. To this aim, the activated antiviral defense that accompanies the IVT-RNA-based gene transfer must be circumvented since it limits the translation of the transfected messenger IVT-RNA.

In this thesis, different strategies to prevent IFN response to IVT-RNA transfection are used. The option to include modified IVT-RNA in order to reduce the IFN response was not exercised since it was already described. My goal was to develop a method that allows the use of unmodified IVT-RNA for gene transfer, circumventing an IFN response. Thereby, the cytoplasmic receptor protein kinase R (PKR), sensing the transferred messenger IVT-RNA, was identified as mainly responsible for the cellular antiviral state. Therefore, the following four possibilities to circumvent PKR activation and IFN response were tested: treatment with small molecules inhibiting the PKR activation, the knockdown of PKR via siRNA, the overexpression of a kinase inactive PKR mutant and the co-transfection of viral anti IFN response proteins. I investigated the benefits of each strategy by analyzing the IFN response induction and reporter gene translation upon IVT-RNA-based transfection. Finally, the best method was validated by performing frequent messenger IVT-RNA-based transfections.

2 Material and methods

2.1 Material

2.1.1 Instrumentation

Name	Manufacturer
7300 Real time PCR system	Life Technologies, Darmstadt, Germany
96-well table centrifuge: Perfect SpinP	Peqlab Biotechnologie, Erlangen, Germany
Blot module XCell II	Life Technologies, Darmstadt, Germany
Blotstation Power Ease 500	Life Technologies, Darmstadt, Germany
Centrifuge Rotina 420R	Hettich, Tuttlingen, Germany
ECM Electroporation System 830	Harvard Apparatus, Holliston, USA
Elektrophoresis chamber XCell SureLock	Life Technologies, Darmstadt, Germany
FACSCanto	BD Bioscience, Heidelberg, Germany
Gel documentation/UV system	Intas, Göttingen, Germany
Gene-Pulser-II apparatus	Bio-Rad, Munich, Germany
Fluorescence microscope Nikon Eclipse TS100	Nikon Instruments Inc., Melville, USA
Image Quant LAS 4000	GE Healthcare, München, Germany
Cell Incubator	Binder Inc, Bohemia, USA
Infinite M200 luminescence reader	Tecan, Crailsheim, Germany
Lamiar flow golden line	Kojair Tech Oy, Viluppa, Finnland
Mr. Frosty Cryo Freezing Container	Nalgene, Rochester, USA
NanoDop 2000c UV-Vis Spectrophotometer	Peqlab Biotechnologie, Erlangen, Germany
Counting chamber Neubauer	LO - Laboroptik, Bad Homburg, Germany
Nitrogen tank 800 Series-190	Cryo-Tech, Geilnau, Germany
QIACube	Qiagen, Hilden, Germany
Sonificator	IKA Werke, Staufen, Germany
Thermocycler T3	Analytik Jena, Jena, Germany
Table centrifuge Heraus Pico21	Heraeus Instruments, Hanau, Germany
Vortex Mixer	Starlab, Hamburg, Germany

2.1.2 Consumables

Name	Manufacturer
Cell culture dish (100 and 145 mm)	Greiner Bio-One, Frickenhausen, Germany
Cellstar tubes (15 and 50 ml)	Greiner Bio-One, Frickenhausen, Germany
Cryo tubes, 1.8 ml	Nunc, Wiesbaden, Germany
Disposable hypodermic needle (diameter: 0.9x40 mm)	B. Braun, Melsungen, Germany
Electroporation cuvettes (0.2 cm and 0.4 cm)	Bio-Rad, Munich, Germany
FACS tubes, polystyrene, 5 ml	BD Biosciences, Heidelberg, Germany
Filter paper	Whatman, Dassel, Germany
Filter tips, Biosphere 100 µl	SARSTEDT, Nümbrecht, Germany
Filter tips, Biosphere 1250 µl	SARSTEDT, Nümbrecht, Germany
Filter tips, Biosphere 300 µl	SARSTEDT, Nümbrecht, Germany
Filter tips, epTIPS, 0,1-10 µl	Eppendorf, Hamburg, Germany
Injekt-F (single use injection) 1 ml	B. Braun, Melsungen, Germany
Luciferase plates 96-well flat-bottomed white	Nunc, Wiesbaden, Germany
Nupage 4-12% Bis-Tris Gel	Life Technologies, Darmstadt, Germany
Protran Nitrocellulose Transfer Membrane BA 83 300 mm x 3 m Pore size 0.2 µm	Whatman, Dassel, Germany
Rnase free eppis (0.5,1.5 and 2 ml)	Eppendorf, Hamburg, Germany
Serological pipette, sterile (5, 10, 25 and 50 ml)	Greiner Bio-One, Frickenhausen, Germany
Tissue culture plate, 6-well	BD Bioscience, Heidelberg, Germany
Top10 chemically competent *E.coli*	Life Technologies, Darmstadt, Germany
XL10-gold ultracompetent cells	Agilent Technologies Inc., Santa Clara, USA

2.1.3 Reagents and enzymes

Name	Manufacturer
2-Aminopurine (2-AP)	Sigma-Aldrich, St. Louis, USA
Agar	Difco, Detroit, USA
Agarose	Applichem, Darmstadt, Germany
*BamH*I	Fermentas, St. Leon-Rot, Germany
beta-mercaptoethanol	Applichem, Darmstadt, Germany
bFGF	Life Technologies, Darmstadt, Germany
Bovine serum albumin (BSA)	Sigma, St. Louis, USA
Bromphenol blue	Sigma, St. Louis, USA
Calf intestine alkaline phosphatase (CIAP)	MBI Fermentas, Burlington, USA
Cryo vials	Greiner Bio-One, Frickenhausen, Germany
Dimethyl sulfoxide (DMSO)	Applichem, Darmstadt, Germany
dNTPS	Roth, Karlsruhe, Germany
*Eci*I	NewEnglandBiolabs, Frankfurt, Germany
Ethanol absolute 96-100% (v/v) p.a.	Roth, Karlsruhe, Germany
Ethidium bromide	Applichem, Darmstadt, Germany
Ethylendiamintetraacetat (EDTA)	Applichem, Darmstadt, Germany
FACS-Clean	BD Bioscience, Heidelberg, Germany
FACS-Flow	BD Bioscience, Heidelberg, Germany
FACS-Rinse	BD Bioscience, Heidelberg, Germany

FCS	PAA, Cölbe, Germany
Formaldehyd 37%	Merck, Darmstadt, Germany
Formamide	Merck, Darmstadt, Germany
GeneRuler 1 kb DNA Ladder	Fermentas, St. Leon-Rot, Germany
GeneRuler 50 bp DNA Ladder	Fermentas, St. Leon-Rot, Germany
Glacial acetic acid	Merck, Darmstadt, Germany
Glucose	Roth, Karlsruhe, Germany
Glycerol 87%	Applichem, Darmstadt, Germany
Glycin	Roth, Karsruhe, Germany
*Hind*III	Fermentas, St. Leon-Rot, Germany
Im-13	Merck, Darmstadt, Germany
Isopropanol p.a.	Merck, Darmstadt, Germany
Kanamycin (Kan)	Sigma, St. Louis, USA
KH_2PO_4 (Potassium dihydrogen phosphate)	Merck, Darmstadt, Germany
L-Glutamine 200 mM	Life Technologies, Darmstadt, Germany
Lumi-Light Western Blotting Substrate	Roche Diagnostics, Mannheim, Germany
MEM medium	Life Technologies, Darmstadt, Germany
MEM NEAA (100x) (Minimum Essential Medium Non-Essential Amino Acids)	Life Technologies, Darmstadt, Germany
Milkpouder	Roth, Karlsruhe, Germany
MOPS (3-(N-Morpholino)propanesulfonic acid)	Sigma, St. Louis, USA
Na_2HPO_4 (Disodium hydrogen phosphate)	Roth, Karlsruhe, Germany
Neomycin	Sigma, St. Louis, USA
Non target PKR siRNA	Santa Cruz Biotechnology, Santa Cruz,USA
Novex Sharp Protein Standard (3.5-260 kDa)	Life Technologies, Darmstadt, Germany
NuPage LDS sample buffer (4x)	Life Technologies, Darmstadt, Germany
NuPage MOPS SDS Running buffer (20x)	Life Technologies, Darmstadt, Germany
Nutristem XF/FF culture media	Stemgent, Cambridge, USA
Opti-MEM	Life Technologies, Darmstadt, Germany
Orange G	Merck, Darmstadt, Germany
PBS	Life Technologies, Darmstadt, Germany
Ponseau S	Sigma, St. Louis, USA
Penicillin-Streptomycin, Liquid	Life Technologies, Darmstadt, Germany
Phusion DNA Polymerase	New England Biolabs, Frankfurt am Main, Germany
Phenol-Chloroform	Roth, Karlsruhe, Germany
PKR siRNA pool	Santa Cruz Biotechnology, Santa Cruz, USA
Potassium chloride	Roth, Karlsruhe, Germany
Restore Western Blott stripping buffer	Thermo Fisher Scientific, Dreieich, Germany
RNase-Free DNase Set	Qiagen, Hilden, Germany
RNaseZAP	Ambion, Austin, USA
RNAiMAX	Life Technologies, Darmstadt, Germany
Sodium chloride	Roth, Karlsruhe, Germany
Sodiumlaurylsulfate (SDS)	Applichem, Darmstadt, Germany
Sodium-pyruvate 100 mM	Life Technologies, Darmstadt, Germany
Sucrose	Merck, Darmstadt, Germany
Superscript II Reverse Transcriptase	Life Technologies, Darmstadt, Germany
SYBR Green PCR Mix	Qiagen, Hilden, Germany

T4 DNA-Ligase	Fermentas, St. Leon-Rot, Germany
Thiazovivin	Stemgent, Cambridge, USA
TRIS	Roth, Karsruhe, Germany
TRIS-HCl	Applichem, Darmstadt, Germany
Trishydroxymethylaminomethane (TrisBase)	Applichem, Darmstadt, Germany
Trypan blue	Applichem, Darmstadt, Germany
TrypLE Express trypsin replacement enzyme	Life Technologies, Darmstadt, Germany
Tryptone	Difco, Detroit, USA
Tween 20	Applichem, Darmstadt, Germany
X-Vivo	Bio Whittaker Europe, Verviers, Belgium
Xylene cyanole	Applichem, Darmstadt, Germany
Yeast Extract	Difco, Detroit, USA

2.1.4 Kits

Name	**Manufacturer**
Bright-Glow Luciferase Assay System	Promega, Madison, USA
Cell proliferation Kit II (XTT)	Roche Diagnostics, Mannheim, Germany
IFN-response detection Kit	System Bioscience, Mountain View, Canada
mMESSAGE mMACHINETM T7 Ultra	Ambion, Austin, USA
MEGAclear	Ambion, Austin, USA
NucleoSpin Plasmid	Macherey-Nagel, Düren, Germany
Pluripotent Stem Cell Transcription factor Analysis Kit (human)	BD Bioscience, Heidelberg, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAquick PCR Purification Kit	Qiagen, Hilden, Germany
RNA Mini Kit	Qiagen, Hilden, Germany
RNeasy Micro Kit	Qiagen, Hilden, Germany

2.1.5 Antibodies

Primary Antibodies

Name	**Manufacturer**
β-actin antibody	Sigma-Aldrich, St. Louis, USA
c-MYC	Santa Cruz Biotechnology Inc., Santa Cruz, USA
eIF2α antibody	Cell signaling Technology Inc., Danvers, USA
KLF4	Santa Cruz Biotechnology Inc., Santa Cruz, USA
OCT4	Santa Cruz Biotechnology Inc., Santa Cruz, USA
phosphor-eIF2α (Ser51) antibody	Cell signaling Technology Inc., Danvers, USA
PKR antibody	Abcam plc, Cambridge, UK
SOX2	Abcam plc, Cambridge, UK

Secondary Antibodies

Name	**Manufacturer**
goat anti-mouse IgG-HRP	Santa Cruz Biotechnology Inc., Santa Cruz, USA
goat anti-rabbit IgG-HRP	Santa Cruz Biotechnology Inc., Santa Cruz, USA

2.1.6 PCR Primer

2.1.6.1 Primer for marker analysis

Name	Primer pair	Sequence	Annealing temperature (°C)
IFN markers (human)			
IFNα	IFNa-537_s	AAATACAGCCCTTGTGCCTGG	60
	IFNa-686_as	GGTGAGCTGGCATACGAATCA	
IFNβ	IFNb-538_s	AAGGCCAAGGAGTACAGTC	60
	IFNb-643_as	ATCTTCAGTTTCGGAGGTAA	
OAS1	OAS1-276_s	AGGTGGTAAAGGGTGGCTCC	60
	OAS1-606_as	GGGTTAGGTTTATAGCCGCC	
OAS2	OAS2-307_s	GAGTGGCCATAGGTGGCTCC	60
	OAS2-622_as	CGATAGATCCAGGGGCTGGG	
PKR	PKR-1146_s	ACACTCGCTTCTGAATCATC	60
	PKR-1253_as	GAGACCATTCATAAGCAACG	
Endogenous housekeeping markers (human)			
GAPDH	GAPDH 49s	CCAGCCGAGCCACATCGCTCA	64
	GAPDH 236as	CCATGGGTGGAAQTCATATTGG	
HPRT	HPRT1 s	TGACACTGGCAAAACAATGCA	62
	HPRT1 as	GGTCCTTTTCACCAGCAAGCT	
ES markers (human)			
DPPA4	hDPPA4-923_s	TGTGTTCACAGGAACAAGGTC	58
	hDPPA4-1167_as	TGTAAGAGTCTCTATCTCCAC	
GDF3	GDF3-293_s	TCCCAGACCAAGGTTTCTTTC	60
	GDF3-530_as	TTACCTGGCTTAGGGGTGGTC	
LIN28	LIN28-519_s	AGGAGACAGGTGCTACAACTG	64
	LIN28-758_as	CCACCCATTGTGGCTCAATTC	
NANOG	Nanog-619_s	AGCTACAAACAGGTGAAGACC	60
	Nanog-877_as	AGGAGTGGTTGCTCCAGGACT	
OCT4	Oct4-857_s	GGCTCGAGAAGGATGTGGTCC	58
	Oct4-1167_as	CTGTCCCCCATTCCTAGAAGG	
REX1	hREX1-61_s	GGTAACAGGGGTTGGAGTGCA	58
	hREX1-542_as	GCGCTGACAGGTTCTATTTCC	
SOX2	Sox2-2202_s	CGATCCCAACTTTCCATTTTG	60
	tag-Primer (=Sox2_as)	GAGATCTCGAGATCTCGATCGTAC	

The primers were ordered and produced by the commercial provider Eurofins MWG operon.

2.1.6.2 Primer for generating dominant negative PKR

Name	Primer pair	Sequence	Annealing temperature (°C)
PCR 1	PCR-1 tailed with *Hind*III_s	GAGGAAGCTT*CCACC*ATGGCTGGTGATCTT TCAGCAGG	58 and 61 (pool samples afterwards)
	PCR-1 tailed with *BamH*I_as	GGGGGGATCCCAGAAGGGCT*CTA*ACATGTG	58 and 61 (pool samples afterwards)
Mega-primer PCR 1	MegaPCR1 -lys-arg shift_s	AAGACTTACGTTATT**AGG**CGTGTTAAATAT AAT	61
	MegaPCR1 - tailed with *BamH*I _as	GGGGGGATCCCAGAAGGGCT*CTA*ACATGTG	
Mega-primer PCR 2	MegaPCR2 - tailed with *Hind*III _s	GAGGAAGCTT*CCACC*ATGGCTGGTGATCTT TCAGCAGG	61
	MegaPCR2 - tailed with *BamH*I _as	GGGGGGATCCCAGAAGGGCT*CTA*ACATGTG	

AAGCTT = *Hind*III cutting site, GGATCC = *BamH*I cutting site,

CCACC = Kozak consensus sequence, *ATG* = Start-Sequence, *CTA* = Stop-Sequence,

AGG = Switch from lysine to arginine

Primers for control sequencing of the PKR-WT and Mutant

Sequencing primer T7 (sequencing forward from T7 promoter)

Primer1 TGGATAACCGTATTACCGCCATG

Primer2 ACTACCTGTCCTCTGGTTCTTTTG

Primer3 AGAAGAGGCGAGAAACTAGAC

Primer4 AAGGAGTAAGGGAACTTTGCGATAC

Sequencing primer tail (sequencing reverse from end of poly A tail)

Tail reverse ATGAACAGACTGTGAGGACTG

The primers were ordered and produced by the commercial provider MWG operon.

2.1.7 Cell lines

Name	Manufacturer
BJ (human foreskin fibroblast)	ATCC-LGC Standards, Wesel, Germany
CCD-10790Sk fibroblasts	ATCC-LGC Standards, Wesel, Germany
HFF (human foreskin fibroblasts)	System Biosciences, Mountain View, Canada

2.1.8 Culture Media

CCD Medium

500 ml	MEM medium
15%	FCS (heat inactivated)
1%	Non-essential-amino acids (NEAA) (100x)
1%	Sodium pyruvate (100 mM)
1%	L-Glutamine
0.5%	Penicillin (100 U/ml) / Streptomycin (100 µg/ml)

Nutristem XF/FF culture media

500 ml	Nutristem XF/FF culture medium
10 ng/ml	bFGF
0.5 µM	Thiazovivin

- aliquot in 250 ml

Freezing Medium

90%	FCS
10%	DMSO

LB-Medium

1%	Trypton [w/v]
0.5%	Yeast-Extract [w/v]
1%	NaCl [w/v]

- dissolve in H_20_{dist}
- autoclave

LB-Agar

1.5% Agar [w/v]

- dissolve in LB-Medium
- autoclave

2.1.9 Buffers and solutions for molecular biology

DEPC water

1 ml DEPC

- ad 1 L H_20_{dist}
- shake overnight at RT
- autoclave

TAE 50x stock solution

242 g TrisBase

57.1 ml Glacial acetic acid

100 ml 0.5 M EDTA pH 8.0

- ad 1 L H_20_{dist}
- autoclave

TAE gel (1%)

1.5 g Agarose

150 ml 1xTAE

- dissolve agarose by boiling
- cool down to 55 °C
- add 75 µl of ethidium bromide solution (0.05 M)

MOPS 10x stock solution

42.8 g MOPS

6.8 g Sodium acetate

3.7 g EDTA

- adjust pH to 7.0

MOPS gel (1%)

1.5 g Agarose (for 150 ml endvolume)

120 ml DEPC water

- dissolve agarose by boiling
- cool down to 55 °C
- add 15 ml 10x MOPS stock solution
- add 15 ml 37% formaldehyde

RNA sample buffer

120 µl 10x MOPS

500 µl Formamide

350 µl Formaldehyde 37%

150 µl Blue marker

100 µl Ethidium bromide (0.05%)

Blue marker and loading buffer for agarose gel

0.25% Bromphenol blue [v/v]

0.25% Xylene cyanole FF [v/v]

0.25% Orange G [v/v]

1 mM EDTA

40% Sucrose

- prepare in H_2O_{dist}

SOC medium

5 g Yeast extract

20 g Tryptone

0.5g Sodium chloride

2.5 ml Potassium chloride (1M)

- ad 1 L H_20_{dist}

add 20 ml Glucose (1 M, sterile)

2.1.10 Buffers and solutions for western blot

Transfer buffer

0.25 M	Tris
1.92 M	Glycin

PBS 10x stock solution

137 mM	NaCl
2.7 mM	KCl
10 mM	Na_2HPO_4
1.8 mM	KH_2PO_4

PBS 1x

10%	10x PBS stock solution
90%	H_2O_{dist}

PBST

PBS 1x

- add 0.1% Tween20

Blocking buffer

2.5 or 5% milkpowder

0.1% Tween 20

- dissolve in PBS

Lysis Buffer (Laemmli)

0.05 M	Tris-HCl pH 6.8
2%	SDS
10%	Glycerol

2.1.11 Buffer for flow cytometry

FACS-fixation-Buffer

2%	Formaldehyde

- Prepare in PBS

2.1.12 Sequences

The templates for IVT-RNA synthesis were based on the pST1-vector, which was previously described (Holtkamp et al., 2006) and already used as vector backbone before (Kreiter et al., 2008). The pST1-A120 vector is endowed with a T7 promoter, two serial head-to-tail fused β-globin 3'-UTR, a poly A tail consisting of 120 adenines and a neomycin-resistance gene. The generation of the IVT-RNA vector for the reporter gene GFP and luciferase was already done before by members of our working group and were kindly provided for this work. The sequences of both reporter genes are listed in the appendix. The sequences for cloning the vectors with human transcription for producing IVT-RNA were used from PubMed (Tab. 2.1) and the PubMed IDs are listed in the table below. Each sequence was ordered from Geneart codon optimized for the expression in human cells.

Table 2.1: PubMed ID of transcription factors used for codon optimization by geneart

Transkription factor sequence	PubMed ID
NANOG	NM_024865.2
LIN28	NM_024674.4
SOX2	NM_003106.2
KLF4	NM_004235.3
OCT3/4	NM_002701.4
c-Myc	NM_002467.3

The list of the sequences used for cloning into the pST1-vector is attached in the appendix.

2.2 Methods

2.2.1 Molecular biological methods

2.2.1.1 RNA isolation

The RNA isolation was performed in order to produce cDNA for analyzing differences in mRNA expression levels by qRT-PCR. For total RNA isolation the RNeasy Mini Kit (up to $1x10^7$ cells) or the RNeasy Micro Kit (less than $0.21x10^6$ cells) was used, in combination with the QiaCube, according to the manufactures instruction, including the step of DNase digestion. The high-quality RNA was eluted by the machine in RNase free water and optionally quality controlled by MOPS gel analysis.

For the MOPS gel 1 µl of the total RNA was transferred into 20 µl of RNA sample loading buffer and denatured for 10 min at 70 °C. The samples were centrifuged, applied on a MOPS gel and exposed to an electric gradient. The RNA passed the quality control when no degradation of the mammalian ribosomal subunits 28S and 18S was detectable.

2.2.1.2 Measurement of nucleic acid concentration

The concentration of nucleic acids can be determined by spectroscopy using the Nano Drop. Thereby the main absorbance for nucleic acids is at 260 nm (OD_{260}), whereas the maximal absorbance of proteins is at 280 nm (OD_{280}). By measuring the extinction for nucleotides and proteins it is possible to calculate the concentration according to Lambert-Beers law. The ratio of absorbance OD_{260} / OD_{280} is a control for purity. The nucleic acids were acceptable pure with OD_{260} / OD_{280} ratio of 1.8 to 2.0 (DNA) or 1.8 to 2.2 (RNA).

2.2.1.3 Reverse transcription of RNA

After the determination of the RNA concentration of the total RNA isolation the first strand cDNA transcription was performed with SuperScript Reverse Transcriptase II. Therefore a 10 µl master mix (MM) was prepared consisting of the following components per one reaction:

4 µl reaction buffer 5x

2 µl DTT 0.1 M

3 µl H_2O_{DEPC}

1 µl Reverse Transcriptase (200 U)

Subsequently the MM was stored on ice and a second reaction mix (RM) was prepared with

1 µl dT18 (50 µM)

1 µl dNTP (10 mM)

1-8 µl RNA (standard 1 µg)

fill up to 10 µl with H_2O_{DEPC}

Next the samples of the RM were mixed, centrifuged and denatured for 3 min at 70 °C, chilled on ice for 2 min and centrifuged again. To each sample 10 µl of the MM was transferred, carefully mixed and incubated for 1 h at 42 °C. Subsequently the enzyme was denatured at 70 °C for 15 min. Finally the cDNA was diluted 1:10 with H_2O and stored at -20 °C until further analysis.

2.2.1.4 qPCR RT-PCR analyses

The real time quantitative analysis of the gene of interest was performed with ABI 7500 Prism Real-time PCR System including the detection and analyzation software. The total volume of each PCR sample was 30 µl, consisting of 25 µl master mix (MM) and 5 µl of 1:10 diluted (RNAse free H_2O) first strand cDNA. The MM was prepared of the following components:

15 µl SYBRGreen Mix

8 µl H_2O_{DEPC}

1µl Sense-Primer (10 µM)

1µl Antisense-Primer (10 µM)

Each PCR was generally measured in triplicates in a 96-well plate. As negative control a water containing sample was included. For each gene product primer pairs and specific PCR conditions were established (listed in 2.1.6 PCR Primer). The steps of the PCR were performed as indicated below:

step	temperature (°C)	time	number of cycles
1	95	15 min	
2	95	30 s	30
3	Annealing 58-64	30 s	
4	72	30 s	
5	95	15 s	
6	60	1 min	
7	95	15 s	

To prove the specificity of each PCR the melting curves were determined subsequent to the amplification steps. The fluorescence is captured after each amplification cycle since the

fluorescence dye intercalates with the PCR product. The increase of fluorescence is proportional to the increase of the PCR product. We next determined the cycle number in which the fluorescence of a sample exceeded a certain threshold (C_t). The relative changes in gene expression was determined by the $2^{-\Delta\Delta Ct}$ (delta-delta C_t method) (Livak and Schmittgen, 2001; Schmittgen, 2001). As internal reference for normalization the housekeeping gene HPRT was used. The calculation was performed as indicated below:

C_t target gene - C_t constant endogenous HPRT control = ΔC_t

ΔC_t sample - ΔC_t reference = $\Delta\Delta C_t$

Finally, the relative gene expression level of the gene of interest was determined by insertion of the double normalized value into: $2^{-\Delta\Delta Ct}$

This value reflects relative changes in the gene expression level, based on the assumption that the amplification efficiency of target and internal control are equal and double per cycle.

2.2.1.5 Linearization of plasmid DNA and phenol/chloroform extraction

For the linearization of plasmid DNA, 50 µg plasmid vector was incubated for 4 h at 37 °C with the enzyme *Eci*I (1U enzyme per 1 µg plasmid-DNA, final volume: 250 µl). A restriction with this enzyme has the advantage that it generated a cutting side with adenosine at its end with only minimal shortening of the poly A tail. The maintenance of a long poly A tail is essential for the generation of IVT-RNA with maximal stability. The enzyme *Eci*I was inactivated at 65 °C for 30 min.

To remove denatured enzyme and buffer the linear DNA was purified by a phenol/chloroform extraction. First a 1:1 mixture of phenol and chloroform was prepared and was centrifuged for 2 min at room temperature (RT) at maximal speed for separation of the remaining water from the phenol/chloroform mixture. Next an equal volume of 1:1 mixture of phenol/chloroform and DNA was transferred into a tube and centrifuged for 2 min at maximal speed. DNA accumulates in the upper aqueous phase which was carefully transferred into a new tube. For removing residual phenol from the DNA, 400 µl of chloroform was added to the aqueous phase and centrifuged again. The aqueous phase containing the DNA was again transferred into a new eppendorf tube, 40 µl of sodium acetate (3 M) and 1 ml of pure ethanol were added, mixed gently and for precipitation of the DNA incubated at -20 °C for minimal 4 h. Afterwards the DNA was pelleted by centrifugation (21910 g, 30 min, pre-cooled 4 °C) the pellet was washed with 1.5 ml of 70% ethanol and the DNA was pelleted again (21910 g, 30 min, pre-cooled 4 °C). After centrifugation the pellet was air-dried for 5 min and resuspended

in 25 µl of pre-warmed DEPC-PBS. The complete digestion was checked by agarose gel electrophoresis and the concentration of the linearized DNA was determined.

2.2.1.6 IVT-RNA half-life determination

The IVT-RNA half-life (T1/2) indicates the duration that passes by until half of the IVT-RNA is degraded in the cell after transfection. Due to the assumption, that the maximum expression level will be reached after 24 h, cells were harvested after 24, 72, 120 and 168 h. The analyzation was done, using RT-qPCR for the fold induction of the markers compared to the untransfected control. The decay was analyzed by a regression line which was interpolated through the data points which represent the RNA amount of the transcription factor in the cell. The x-coordinate values of each data point therefore get determined by: $\ln [A_t / A_0]$, with t meaning the time being elapsed since the assumed maximum expression level.

Formula for calculating a data point after a time (t) post electroporation: $\ln [A_t / A_0]$
A_t= consecutive measurement points after electroporation, 24 — 168 h
A_0= point of maximal expression, measured 24 h after electroporation

The gradient (λ) was determined from the regression line formula. Finally, the half-life (T1/2) was calculated by the formula: $T1/2 = [\ln 2/ \lambda]$.

2.2.1.7 *In vitro* transcription

The *in vitro* transcription is a method for the synthetically generation of mRNA. For the reaction the mMassage mMachine T7 ultra Kit from Ambion including a T7 polymerase of phage origin is used (Moll et al., 2004). The polymerase binds thereby to the T7 promoter on the pST1 vector backbone and starts the RNA synthesis. As 5'-cap the improved D1 modified anti-reverse cap analog (ARCA) was used (Kowalska et al., 2008). The following reaction mix was prepared at RT:

5 µg	linear template DNA
50 µl	T7 2x NTP/D1 (cap-analogue)
50 µl	10x T7 reaction buffer
10 µl	T7 polymerase
ad 100 µl	RNase-free water

Subsequently the reaction mix was carefully mixed and incubated for 2 h at 37 °C. To get rid of the remaining template DNA 5 µl of Turbo DNase was added to the reaction mix afterwards for 30 min at 37 °C.

Synthesized IVT-RNA was purified using the MegaClear Kit according to the manufactures instructions. Differing from the manual the RNA was eluded with 50 µl buffer after five minutes of incubation. Finally the purified IVT-RNA concentration was measured using the Nano Drop and the material was stored at -80 °C.

2.2.1.8 Plasmid vector construction

The codon optimized coding sequence of a certain gene of interest, flanked with appropriate restriction sites fitting to the linearized pST1 vector (2x *BamH*I for human transcription factors and *Hind*III at the 5' end and *BamH*I at the 3' end for all other genes) was synthesized by a commercial provider (Geneart). In order to introduce the gene of interest into the pST1-vector backbone, the circular plasmid containing the gene of interest and the target vector were linearized by the corresponding restriction enzymes in an end volume of 20 µl (1 U enzyme per 1 µg DNA). To avoid self – ligation of the target vector, the free phosphate ends were removed by the addition of 1 µl calf intestine alkaline phosphatase (CIAP) for 30 min. Next, the fragment and vector backbone must be ligated.

2.2.1.9 Ligation of the vector backbone and the DNA fragments

Before ligating the vector backbone and the DNA fragment, which includes the gene of interest, both components must be purified from buffers and enzymes of the former reactions by agarose gel. For extracting the DNA from the agarose the QIAquick Kit was used. Then the insert and the vector backbone were mixed at a molecular ratio of 3:1 in a reaction volume of 10 µl which includes 1 µl T4 ligase enzyme and 1 µl of the appropriate reaction buffer. The ligation was either performed overnight at 14 °C or for 2 h at 22 °C. Until transformation is performed the vector can be stored at -20 °C.

2.2.1.10 Transformation

The transformation method was used to amplify a plasmid vector of interest in competent bacteria. Chemically competent bacteria (XL-10 or Top10) were thawed on ice. When XL-10 bacteria were used, 2 µl ß-mercaptoethanol was added to 50 µl cell suspension. The cells were carefully mixed every 2 min during their 10 min incubation time on ice. This pretreatment

was not necessary for Top10, here the transformation was started directly after celled were thawed on ice. Generally 0.1 to 50 ng of ligated vector was transferred into the cell suspension; the volume of the plasmid did not exceed 10 % of the complete reaction volume and cells were incubated for 30 min on ice. Than a heat shock was performed for 30 s at 42 °C followed by 2 minutes incubation on ice. Next SOC medium without antibiotics was added and cells were allowed to recover on the shaker for one hour at 37 °C. Finally the cell suspension was plated onto LB-agar plates containing the appropriate antibiotic (Kanamycin 25 µg/ml) and the plates were incubated over night at 37 °C. Positive clones which were able to grow on selective agar plates were picked for DNA purification.

2.2.1.11 Purification of plasmid DNA from bacteria culture

Bacterial colonies chosen for plasmid purification were inoculated in 3 ml LB medium containing the appropriate antibiotic for selection (Kanamycin 25 µg/ml). An overnight incubation step was performed onto an orbital shaker at 37 °C. For purification of plasmid DNA from the bacteria culture the NucleoSpin Plasmid DNA Purification Kit was used as recommended by the provider. The purified the plasmid DNA was eluded in 30 to 50 µl H_20 and stored at -20 °C.

2.2.1.12 Sequencing

The purified DNA was send to a commercial provider (MWG Eurofins, Ebersberg, Germany) for sequencing to verify successful cloning, thereby appropriate sequencing primer pairs were used.

2.2.1.13 Gel extraction of DNA after electrophoresis

This protocol was used to extract and purify DNA fragments of 70 bp to 10 kb from an agarose gel. The isolation of the DNA was performed with the QIAquick Gel Extraction Kit according to the manufacture's instruction.

2.2.1.14 DNA purification

After a treatment of DNA with enzymes or after amplification steps via PCR the DNA must be purified from the inactivated enzymes or the used buffers. Therefore the QIAquick PCR Purification Kit was performed according to manufacturer's instruction.

2.2.1.15 Preparation of cell lysates

For analyzing the proteins by western blot they must be isolated from the cells of interest. Therefore the cells were harvested, if necessary counted and pelleted in a 1.5 ml eppendorf-tube. Subsequently the cells were washed with PBS, pelleted again and stored at -20 or -80 °C. Cells were lysed with 50 µl lysis buffer per $1x10^6$ cells (minimal volume 100 µl) and heated at 95 °C for denaturation reaction for 5 min. If some cellular clusters remain the samples were vortexed. For disrupting the DNA we used a supersonic needle (sonificator, 3 pulses for 5 s) or small needles for injection (up-down pipetting for 20 to 30 times). Finally the protein concentration of each sample was measured using a Nano Drop. Afterwards samples were stored at -20 °C or used for western blot analysis.

2.2.1.16 SDS-PAGE and Western blot

In a next step the samples were prepared for loading the separation gel. Therefore an equal amount of proteins e.g. 30 to 100 µg were required. The lysates were combined with 4x NuPAGE LDS sample buffer adjusted with water to 20 µl, mixed, heated for 10 min at 70 °C and loaded onto a 4 to 12% Bis-Tris-Gel in a electrophoresis chamber (both from Life Technologies) including a reference from 3.5 to 260 kDa (Novex sharp pre-stained protein standard, Life Technologies). The proteins were separated for approximately 50 min at 200 V and 120 mA using the MOPS SDS running buffer.

After the separation by PAGE, the proteins were transferred to a nitrocellulose membrane by western blot using the XCell II blot module according to the manufacturer's instructions. The transfer was performed overnight at 12 V and 100 mA in transfer buffer. Transfer efficiency and equal loading was controlled by penceau staining after the transfer. When the blot passed the quality control the membrane was washed with PBST for three times and blocked with non-fat dry milk for one hour. The protocol proceeded with another three washing steps using PBST, followed by the incubation of the membrane for 1 h or overnight with the primary antibody detecting the targeted protein. The membrane was washed again (3x with PBST) and the secondary antibody, which targets the primary antibody is coupled with a horseradish peroxidase, was next incubated for 1 h with the membrane. The established conditions for the used antibodies are listed below (Tab. 2.2). At the end the membrane was gain washed (3x with PBST) to reduce unspecific signals.

The detection was performed with the Lumi-Light western blot substrate according to the manual. This technique allows an analyzation of the protein lysates by detecting the emitting light at 425 nm using the ImageQuant LAS4000.

Table 2.2 Blotting conditions and antibody dilution for western blot analysis

Blocking condition	1. Ab	Dilution	2. AB	Dilution	Signal (kDa)
5% milk in PBST (1h)	β-actin	1:50.000 (5% milk in PBST 1h)	goat anti-mouse	1:5000 (5% milk in PBST 1h)	42
5% milk in PBST (1h)	c-MYC	1:200 (1% milk in PBST overnight)	goat anti-rabbit	1:2000 (in PBST 1h)	67
2,5% milk in PBST (1h)	eIF2α	1:1000 (2,5% milk in PBST overnight)	goat anti-rabbit	1:2000 (in PBST 1h)	38
5% BSA in PBST (1h)	KLF4	1:200 (0.5% BSA in PBST overnight)	goat anti-rabbit	1:2000 (in PBST 1h)	53
5% BSA in PBST (1h)	OCT4	1:1000 (5% BSA in PBST 1h)	goat anti-mouse	1:2000 (in PBST 1h)	45/33 (isoform A/B)
2,5% milk in PBST (1h)	P~eIF2α	1:1000 (2,5% milk in PBST overnight)	goat anti-rabbit	1:2000 (in PBST 1h)	38
5% milk in PBST (1h)	PKR	1:1000 (5% milk in PBST overnight)	goat anti-rabbit	1:2000 (in PBST 1h)	62
5% milk in PBST (1h)	SOX2	1:500 (0.5% milk in PBST overnight)	goat anti-rabbit	1:2000 (in PBST 1h)	34

Ab = antibody

After the detection the membrane was restored by incubating the membrane for 7 min at RT with western-blot restore buffer followed by three washing steps with PBS for one minute and two for 5 minutes using PBST.

2.2.1.17 Construction of a dominant negative PKR K296R mutant

The overexpression of a dominant negative PKR mutant K296R (dN-PKR) was seen as a method to abrogate the IVT-RNA induced PKR activation. For the generation of the dN-PKR at first the PKR-WT has to be cloned. Therefore c-DNA template of CCD-10790Sk fibroblasts was used. Thereby specific primers, which were introducing a *Hind*III and *BamH*I restriction site at the beginning and the end of the PCR product, were used (Fig. 2.1). Both primers were used at final concentration of 0.5 µM. The 5' primer was thereby tailed with *Hind*III restriction site and was designed corresponding to the Kozak-sequence and the start codon of human PKR (Primer: PCR-1 tailed with *Hind*III_s). The 3' primer tailed with a *BamH*I restriction side (PCR-1 tailed with *BamH*I_as) corresponds to the stop codon of human PKR. For the PCR the Phusion high-fidelity DNA polymerase was used. The PCR steps are shown below:

step	temperature (°C)	time	number of cycles
1	98	3 min	
2	98	1 min	30
3	Annealing 58 and 61	1 min	
4	72	2 min	
5	72	10 min	

The PCR products with the different annealing temperatures were pooled and after restriction the PKR fragment was cloned into the pST1-Vector. For the transformation XL10-bacteria were used. The clone which passed the quality control was controlled to have a tail consisting of more than 100 adenines, the PKR insert and was sequenced as a last validation step.

The introduction of the mutation resulting in a kinase negative mutant was done by a two-step PCR strategy. In the first PCR the mutation on the 5' strand was introduced and the 3' strand primer was tailed with a *BamH*I restriction side. In the second PCR which must include the product from the former PCR as template, the fragment of the dN-PKR with the K296R mutation is completed and amplified. In this PCR the 5' primer targets the PKR start sequence and was tailed with a *Hind*III restriction side (Fig. 2.1). The 3' primer tailed with *BamH*I targets PKR stop codon. In the next PCRs the primers MegaPCR1-lys-arg shift_s and MegaPCR1- tailed with *BamH*I _as) were used at final concentration of 0.5 µM. To avoid undesired mutations the Phusion DNA polymerase was used. The PCR was performed with 3% DMSO in a total volume of 20 µl as listed below.

step	temperature (°C)	time	number of cycles
1	98	3 min	
2	98	1 min	35
3	Annealing 61	1 min	
4	72	2 min	
5	72	10 min	

In the second Megaprimer PCR refilling completes the 5' and 3' strand strands (Fig. 2.1). That's why 5 µl from the former PCR must serve as template after the presence of the 773 bp fragment was verified by and analytical gel. Now the 5' primer MegaPCR2- tailed with *Hind*III _s) and the 3' primer MegaPCR2- tailed with *BamH*I _as were used for the PCR at a final concentration of 0.5 µM. To avoid undesired mutations the Phusion DNA polymerase was used. The PCR was performed with 3% DMSO in a total volume of 20 µl as listed below.

step	temperature (°C)	time	number of cycles
1	98	3 min	
2	98	1 min	27
3	Annealing 61	1 min	
4	72	2 min	
5	72	10 min	

Subsequent to the last PCR the PKR K296R mutant fragment of 1656 bp was separated from the other PCR components and purified from the agarose with the QIAquick gel extraction kit. Then the restricted dN-PKR fragment was cloned into the pST1-Vector and transformed in Top10 bacteria. The clones were controlled for poly A tail length and PKR insert before finally the PKR K296R mutant was verified by sequence analysis.

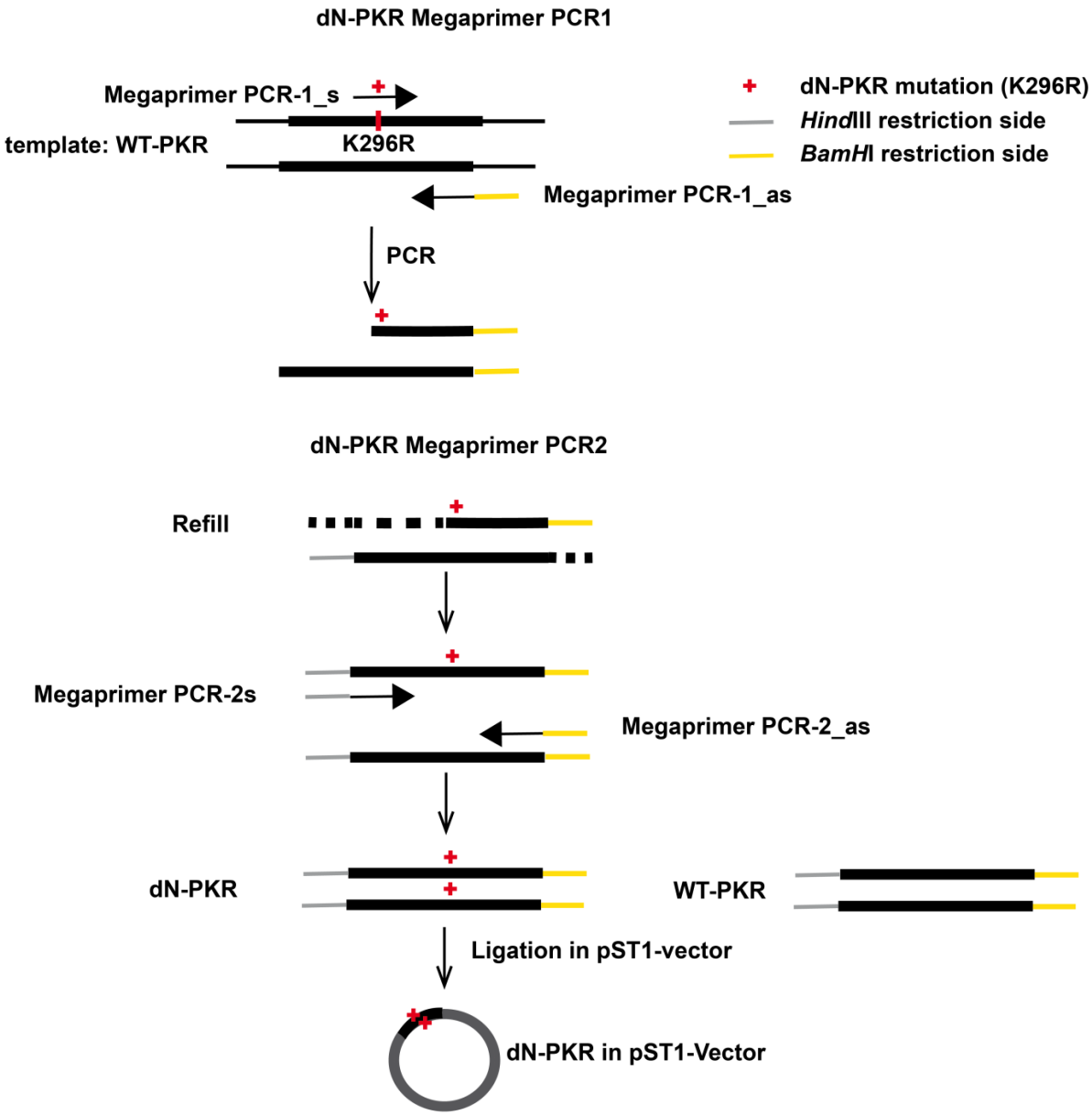

Figure 2.1 Generation of dN-PKR Schema of the dN-PKR pST1-Vector construction starting from a WT-PKR construct is shown. With the first Megaprimer PCR the point mutation for the dominant negative PKR was introduced in the first strand, using a WT-PKR template. After refilling the first strand a second Megaprimer PCR completes the point mutation. Finally the dN-PKR fragment was ligated into the pST1-vector and the mutation was verified by sequencing. Now the template can be used for the production of dN-PKR IVT-RNA.

2.2.2 Cell culture methods

2.2.2.1 Freezing and thawing of cells

Cryostorage is an important tool for preserving cells. Since freezing and thawing can damage cells a cryoprotective reagent (e.g. DMSO) is added to the freezing medium. To prepare cells for the freezing procedure they were harvested and washed twice with chilled PBS (4 °C). Than the cell number was determined and cells were centrifuged in a precooled centrifuge (311 g, 4 °C, 10 min). The pellet was resuspended in adequate volume (e.g. $1x10^6$ cells/ml) of cold freezing medium (4 °C) and transferred into a 1.8 ml chilled cryotube. Next the cryotubes were put overnight at -80 °C in a special container (e.g. Mr. Frosty) which allow a slow cooling rate (1 °C/min). When the cells were used for long term storage the cryotubes were transferred to liquid nitrogen tanks (-196 °C).

In contrast to the freezing procedure, thawing of cells should be rapid which prevents damage of cells by reduced formation of ice during rehydration. After thawing cells in a water bath (37 °C), they were transferred into 9 ml of cold PBS. Cells were centrifuged (311 g, 4 °C, 5 min), washed with PBS and plate as appropriate.

2.2.2.2 Cultivation of cells

Cells were cultivated under aseptic cell culture conditions in appropriate medium (Phelan, 2007) at 37 °C and a CO_2 content of 5%. For avoiding senescence human fibroblasts were used maximal until passage 20 to 25. Adherent cells were cultured subconfluent by passaging twice a week. For splitting the culture media was removed and cells were washed twice with DPBS. Cells were detached from the cell culture dishes by adding trypsin and incubating at 37 °C for a few minutes. The enzymatic reaction was stopped by adding FCS containing medium and the cells were plated again as appropriate.

2.2.2.3 Determination of cell density by Neubauer chamber

The cell number of a cell suspension was determined by Neubauer chamber with the vital dye Trypan Blue. Due to the polyanionic azo dye, which can only enter dead cells, it was possible to distinguish between unstained, living cells on the one hand and death cells, stained in blue on the other. An aliquot of cells was diluted with a Trypan blue solution (e.g. 1:1), brought into the Neubauer chamber and the number of living cells was determined. The cell density was calculated by multiplicating the arithmetic average from the counted cells of one quadrant

with the dilution (2x) and chamber factor (10000) and taking the total volume of cell suspension into account.

Cell density = cell number of one quadrant x dilution factor x 10^4/ml
Total cell number = cell density x total volume of cell suspension (ml)

2.2.2.4 IVT-RNA transfer

In this study two methods were used to transfer IVT-RNA into the target cells.

2.2.2.4.1 Electroporation of RNA

Electroporation is an elegant and commonly used way to introduce charged material like DNA (Wang et al., 2005) and RNA (Saebøe-Larssen et al., 2002; Piggott et al., 2009) into cells. The electric pulse results in an external electric field and pore formation in the cellular membrane occurs (Gehl, 2003). The negatively charged nucleotides can subsequently migrate along the electric field lines and enter the target cells. Since pore formation can lead to cell death, it is necessary to optimize electroporation parameters for efficient transfection with acceptance survival of the cells. For electroporation of RNA it should be taken into account that the transfection has to be performed under RNases free conditions. For inhibiting RNAses on the surface of consumables the reagent RNaseZAP was used.

For the electroporation adherent cells were detached from culture plates, harvested and washed once with PBS-2 mM EDTA. A second washing step with pre-cooled serum-free X-vivo was performed to minimize the source of potential RNase contamination.

Cell number was adjusted to the needed density and transferred to an appropriate cuvette (4 mm gab: $1x10^7$ to $2x10^6$ cells, 2 mm gab: < $2x10^6$ cells). The cell suspension was then electroporated with the optimized electroporation parameters as listed below.

ECM electroporation system

Cells	Parameters for 4 mm gab cuvette/250 µl
CCD	220 V, 3 pulses, 12 ms puls length, 400 ms interval
HFF	250 V, 1 puls, 24 ms puls length, 400 ms interval
BJ	220 V, 3 pulses, 12 ms puls length, 400 ms interval

For 2 mm gab cuvettes the voltage for the electroporation was bisected.

Gene-Pulser-II appararus

Cells	Parameters for 4 mm gab cuvette/250 µl
CCD	250 V, capacity: 300 µF; 1 puls

After electroporation cells were carefully resuspended in a total volume of 1 ml of their appropriate growth medium and plated as desired.

2.2.2.4.2 Lipofection of RNA

The lipid-based nucleotide transfer is an alternative method of transfection (Felgner et al., 1987). Here the RNA or DNA is encapsulated in a lipid-based vehicle which fuses with the target cells. The fusion of the liposome with the cell membrane resulted in the transfer of the nucleic acids into the cytoplasm of the cell. For the lipofection human fibroblasts were harvested, plated $1x10^5$ cells per 6-well in CCD medium and incubated at 37 °C and 5% CO_2. Lipofections were performed 24 h thereafter. The cells don't have to be separated and can remain in their adherent growing state which is an advantage of this method. All lipofections were performed with RNAiMAX as described by the manufacturer. 1.2 µg RNA per lipofection was incubated for 15 min with 6 µl RNAiMAX in 500 µl OptiMem. Afterwards the mixture was given drop vise to the cells for transfection and not removed. When repetitive lipofections were necessary the transfection process was repeated the next day. The lipofection was performed in Nutristem medium or in CCD medium without Pen/Strep.

2.2.2.5 Luciferase assay

The luciferase assay is a method for analyzing the expression of the reporter gene luciferase in cells (Alam and Cook, 1990). The luciferase gene used in this study was derived from the firefly (*Photinus pyralis*). When the 61 kDa monomer is expressed in cells it can catalyze the reaction from luciferin to oxyluciferin as depictured below (de Wet et al., 1987) (Fig. 2.2). The light with a wavelength of 550 to 570 nm is produced through this process and can be detected by a luminometer.

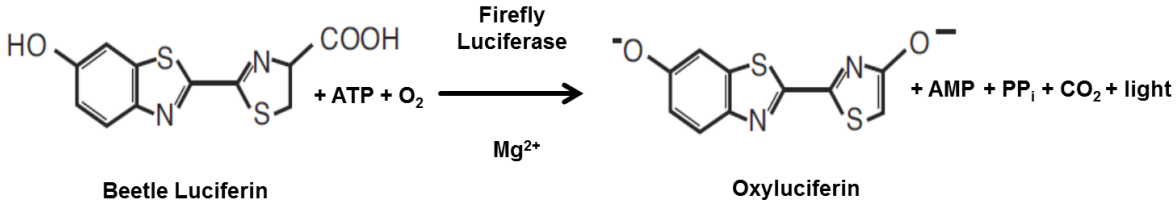

Figure 2.2 The luciferase reaction In the presence of ATP, oxygen and Mg^{2+} the substrate luciferin is catalyzed from luciferase in a mono-oxygenation reaction to oxyluciferin, AMP, PPi, CO2 and light is emitteted at a wavelength of 550 — 570 nm. (ATP, adenosine triphosphate; AMP, adenosine monophosphate; PPi, inorganic phyrophosphate) (Picture adapted from Promega manual)

For the luciferase assay the promega kit was used as recommended by the manufacturer. For analyzing luciferase expression, 10000 cells in each well of a 96-well plate were washed with PBS and 30 µl luciferin were added to the cells. After 2 min of cell lysis the light emission of the luciferase reaction was measured with a luminometer at a constant temperature of 24 °C.

2.2.2.6 Cell proliferation XTT assay

The cell proliferation XTT assay is an elegant non-radioactive technique to characterize cellular viability by a colorimetric assay system. Scudiero et al. (Scudiero et al., 1988) were the first describing the method which was subsequently improved (Roehm et al., 1991). The basis of this assay is a shift in the absorption maximum of the substrate XTT which can only occur in viable cells: The XTT ((sodium 3´-[1-(phenylaminocarbonyl)- 3,4tetrazolium]-bis (4-methoxy-6-nitro)) is turned by mitochondrial dehydrogenase activity from the yellow tetrazolium salt into the orange formazan. Formazan has an absorption maximum of 480 nm and this increase of the absorption can be detected by a photometer.

The assay was performed according to manufacturer's instructions. Per 96-well 50 µl of XTT substrate were mixed with 1 µl of electron-coupling reagent (ECR), added to the well and incubated at 37 °C. Usually differences in the color were measured after 4 h incubation time. For analysis the absorbance was measured with a photometer at 480 nm with a reference at 650 nm.

2.2.2.7 Flow cytometry and intracellular staining

Flow cytometry is a technique to detect, quantify and characterize cells in size, cellular granulation and protein expression up to single cell level. Cells are thereby labeled with a fluorescent dye that is excited by passing through a laser beam in a fine stream of fluid. Beside the absorbance and the scattering of the light, the reemitted fluorescent light can be detected an analyzed. In this work the fluorescent activated cell sorting (FACS) was used for measuring GFP transfected cells or after the intracellular staining of certain stem cell markers. For the intracellular staining cells were harvested and washed twice with PBS, afterwards the cells were fixed for 20 min at RT. Cells were washed again two times with PBS, permeabilisized, washed again twice with 1 ml of 1xPerm/Wash buffer and pellets were resolved in 50 µl 1xPerm/Wash buffer. Now the unstained and the isotype controls were removed from the appropriate sample. Subsequently the samples were incubated for 10 min at RT and the antibodies including the corresponding controls were prediluted 1:10 with 1x Perm/Wash buffer according to the table below (Tab. 2.3).

Table 2.3 Premixes for staining human cells with the pluripotent stem cell transcription factor analysis kit

Component	Volume to add per tube to be labelled	
	Specific stain	Isotype controls
Anti-human-NANOG PE	10 µl	
Anti-OCT4 PerCP	10 µl	
Anti-Sox2-AlexaFluor 647	10 µl	
PE isotype control		10 µl
PerCP isotype control		10 µl
AlexaFluor isotype control		10 µl
1xBD Perm-Wash buffer	70 µl	70 µl

To the unstained control 100 µl H_2O were added and 100 µl of the diluted isotype controls were added to the isotype control samples. 100 µl of the diluted antibodies solution were added to the specific staining samples and afterwards cells were incubated for 30 min at RT in the dark. The cell pellets were washed again two times with 1 ml of Perm/Wash buffer, resuspended in 300 µl PBS with 2% formaldehyde and analyzed by flow cytometry.

2.2.2.8 Translation inhibition

To be able to perform the translation inhibition experiment it was necessary to have human fibroblasts with a stable reporter gene expression. The cells, kindly provided from our working group, have a lentiviral plasmid as expression system. The translation of the reporter

gene was under control of a constitutively expressed and strong promoter. Thereby three promoters from different sources were used to avoid promoter dependent results (Wang et al., 2008). From mammalian origin the human *elongation factor-1 alpha* (EF1a) and the *phosphoglycerate kinase* (PGK) promoter was chosen (Norrman et al., 2010). Furthermore with the *cytomegalovirus* (CMV) promoter a strong promoter usable in a broad range of cell types was selected (Qin et al., 2010). The promoters were cloned into the pST1-Vector. In the experiment human fibroblasts were electroporated with and without 48 ng/µl IVT-RNA and analyzed for the reporter gene expression level by luciferase assay.

2.3 Statistical methods

Statistical analysis of the experiments was performed by GraphPad Prism software (version 5.0) (GraphPad Software Inc., La Jolla, USA) employing unpaired two tailed student's t test. A p-value of < 0.05 was considered as statistically significant.

3 Results

3.1 Characteristic of IVT-RNA-based transgene expression

One of the most important advantages of *in vitro* transcribed RNA (IVT-RNA) based transgene expression is that it circumvents the risk of insertional mutagenesis. One application of IVT-RNA-based gene transfer could be an improvement in the RNA-based reprogramming technology. The reprogramming technique itself was a milestone in stem cell research. The early studies were performed using viral based reprogramming with Oct4, Sox2, Klf4 and c-Myc (OSKM) for murine and human cells (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). This technique harbors the possibility to upregulate pluripotency markers in somatic cells and to dedifferentiate somatic cells to pluripotent cells, termed induced pluripotent stem cells (iPS). A substitution of viral dependent reprogramming by an IVT-RNA-based technique would contribute to a higher safety of the novel reprogramming method. We expect that direct reprogramming with defined factors and the availability of safe and "clean" patient derived pluripotent cells will be a versatile technology which is in line with the German "Embryonenschutzgesetz" (ESchG).

3.1.1 The use of D1 caped IVT-RNA leads to enhanced reporter gene expression in human fibroblasts

Enhanced expression of IVT-RNA encoded transgenes is especially beneficial for RNA-based reprogramming since the height and length of expression from the encoded factors would profit from a prolonged IVT-RNA half-life. The intracellular IVT-RNA concentration at the beginning of an experiment can be adjusted by the amount of IVT-RNA used for the transfection. But once the IVT-RNA is inside the cell it underlies processing and degradation which can be regulated by modification of the structural elements of IVT-RNA to achieve a prolonged half-life and more efficient translation. We intended to use primary fibroblasts for our reprogramming experiments. In order to evaluate, whether the advantage of D1 capped IVT-RNA holds also true for these cells, human fibroblasts were electroporated with the two differently capped IVT-RNAs (ARCA and D1) encoding for the reporter gene luciferase (Luc-RNA). Subsequently the enzymatic activity was analyzed after electroporation as described in material and methods (2.2.2.5) and is depictured in Fig. 3.1. Over the analyzed time we found that D1 capped Luc-RNA resulted in higher and prolonged luciferase activity as compared to the ARCA capped RNA, which indicates that the D1-capped RNA was

translated more efficiently compared to the ARCA capped IVT-RNA. Thus, we concluded to use the D1 cap in all following experiments for an elevated expression of IVT-RNA encoded transgenes.

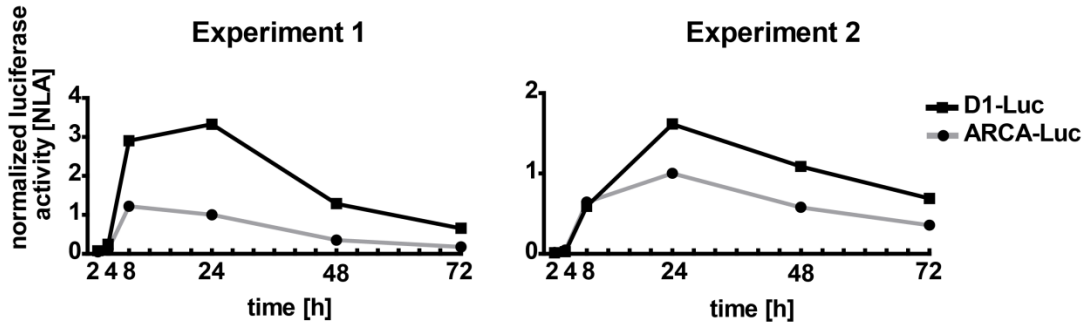

Figure 3.1 Luciferase translation level of ARCA and D1 capped IVT-RNA Comparison of ACRA and D1 capped luciferase (Luc) IVT-RNA measured by luciferase assay. Human fibroblasts (CCDs) were electroporated, using the Gene-Pulser-II apparatus, with 40 ng/µl ARCA and the D1 cap Luc IVT-RNA and the luciferase expression level was monitored from 2 to 72 h.

3.1.2 Duration of the IVT-RNA encoded transcription factor expression

Generally post translational processes modulate the amount of transcripts, thus a difference in IVT-RNA and protein level of transcription factors (TF) can be found (Meyer et al., 2004; Cai et al., 2012). Moreover, the total protein level is dependent on the respective protein stability. In this context, the aim of our next experiments was to investigate the IVT-RNA decay and the presence of protein expression after transfection of the IVT-RNA encoded Yamanaka reprogramming factors. Therefore human fibroblasts were electroporated with D1 capped IVT-RNA coding for OSKM and their corresponding intracellular IVT-RNA level was analyzed for 168 h as indicated in Fig. 3.2 A. We found that IVT-RNA degradation followed an exponential decay which was comparable for all four reprogramming TFs. The calculated half-life (T1/2) for each TF IVT-RNA was comparable to each other, thereby T1/2 being about 10 to 12 h. Thus, 48 h after the transfection approximately four T1/2 had passed by. For monitoring the degradation of the protein expression of OSKM in human fibroblasts protein extracts, we harvested cells and analyzed their protein lysates by western blot at the indicated time points for the presence of the OSKM proteins (Fig. 3.2 B). 24 h after the IVT-RNA-based electroporation we were able to detect OSKM at protein level. After 48 h the signal for

OCT4 is clearly detectable whereas those representing SOX2, KLF4 and c-MYC were nearby or below the detection limit.

In this experiment we found out that the presence of OSKM reprogramming factor IVT-RNA was sufficient to receive a clear presence of the corresponding proteins after 24 hours. After 48 h differences in the availability of the proteins become apparent. Whereas OCT4 was clearly detectable, SOX2, KLF4 and c-MYC were diminished. Thus, we were interested whether the expression of reprogramming factors by IVT-RNA transfer is sufficient to induce reprogramming associated markers after one transfection. This was addressed in the next experiment.

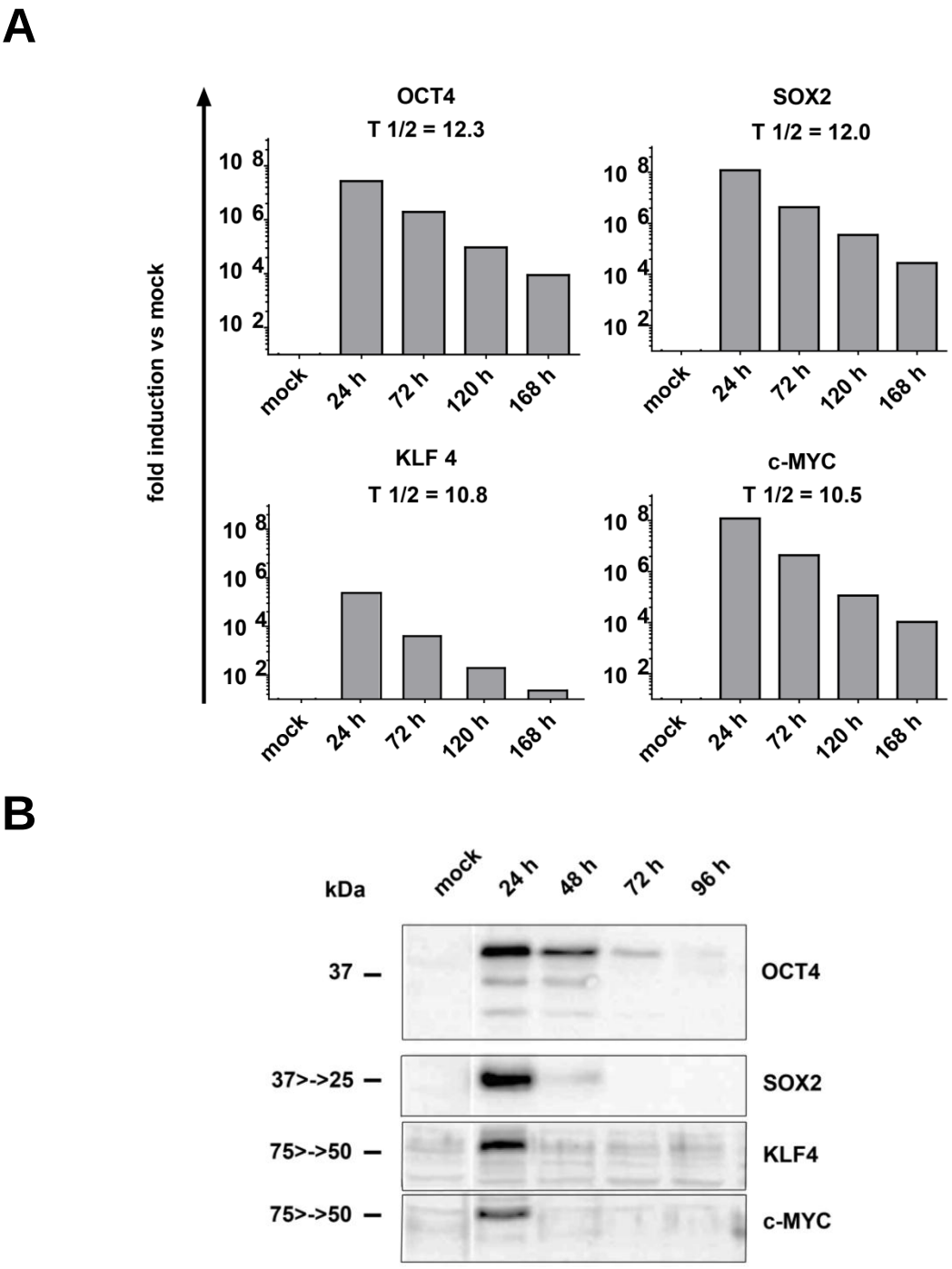

Figure 3.2 Decay of transcription factors on mRNA and protein level Human fibroblasts (CCDs) were electroporated, using the Gene-Pulser-II apparatus, once with a mixture of the Yamanaka reprogramming factors OCT4, SOX2, KLF4 and c-Myc (OSKM). (**A**) For monitoring the intracellular levels of the transcription factors (TFs) cells were analyzed on mRNA level 24 to 168 h after the electroporation with 60 ng/µl OSKM in order to determine the half-life (T ½) of each TF. (**B**) For western blot analysis cells were harvested 24 to 96 h after one electroporation with a concentration of 10 ng/µl of each TF and detected by using specific antibodies.

3.1.3 Single transfection of reprogramming factor IVT-RNA can upregulate pluripotency markers

Until now the retroviral gene transfer is the most widely used technique for cellular reprogramming in which the constant overexpression of OSKM results in the generation of iPS cells. During reprogramming the gene expression changes (Xu et al., 2011). Thereby some genes are down regulated whereas many genes are activated (Brambrink et al., 2008; Jaenisch and Young, 2008; Stadtfeld et al., 2008; Buganim et al., 2012). Finally this reprogramming process results in high expression of pluripotency associated genes like human telomerase reverse transcriptase (hTERT), the growth differentiation factor 3 (GDF3), developmental pluripotency associated 4 (DPPA4) or the endogenous OCT4, NANOG or REX1 (Carey et al., 2011; Sterneckert et al., 2011; Som et al., 2012).
In the following experiment we assessed whether IVT-RNA coding for a reprogramming cocktail, consisting of OSKM, can induce an upregulation of pluripotency markers. Indeed, after one electroporation of human fibroblasts with this reprogramming cocktail we detected an upregulation of selected marker genes, such as TERT, GDF3 or DPPA4, 24 h after the transfection (Fig. 3.3). Unfortunately, the marker induction mostly vanished after 72 h and essential reprogramming markers like OCT4 and NANOG were not upregulated (Fig. 3.3).

It was not possible to generate reproducible data of a constant upregulated pattern of stem cell associated markers. However, we concluded that, as a proof of concept, stem cell markers can be upregulated after transfecting cells with reprogramming factor IVT-RNA. Due to the knowledge of limited RNA based translation it was likely that there was a need for more than one IVT-RNA-based treatment. Therefore we examined the effect of repetitive transfection to achieve the required constant exposure to the reprogramming cocktail. Due to the expression data from Fig. 3.2 B we could conclude that it was necessary to transfect IVT-RNA into cells at least every 48 h to renew a strong expression of the Yamanaka reprogramming factors OSKM. A repetitive transfection every 48 h would result in an availability of IVT-RNA which is represented by a serrated pattern with a huge amount of IVT-RNA short after the transfection that is degraded afterwards. A time gab of 48 h between the transfections guarantees the presence of the most important reprogramming factor OCT4 and minimizes the lack of SOX2, KLF4 and c-MYC on one side and maximizes the recovery time for the cells after each electroporation on the other. Thus, an interval of 48 h between the transfections was used for repetitive transfection in the next experiment.

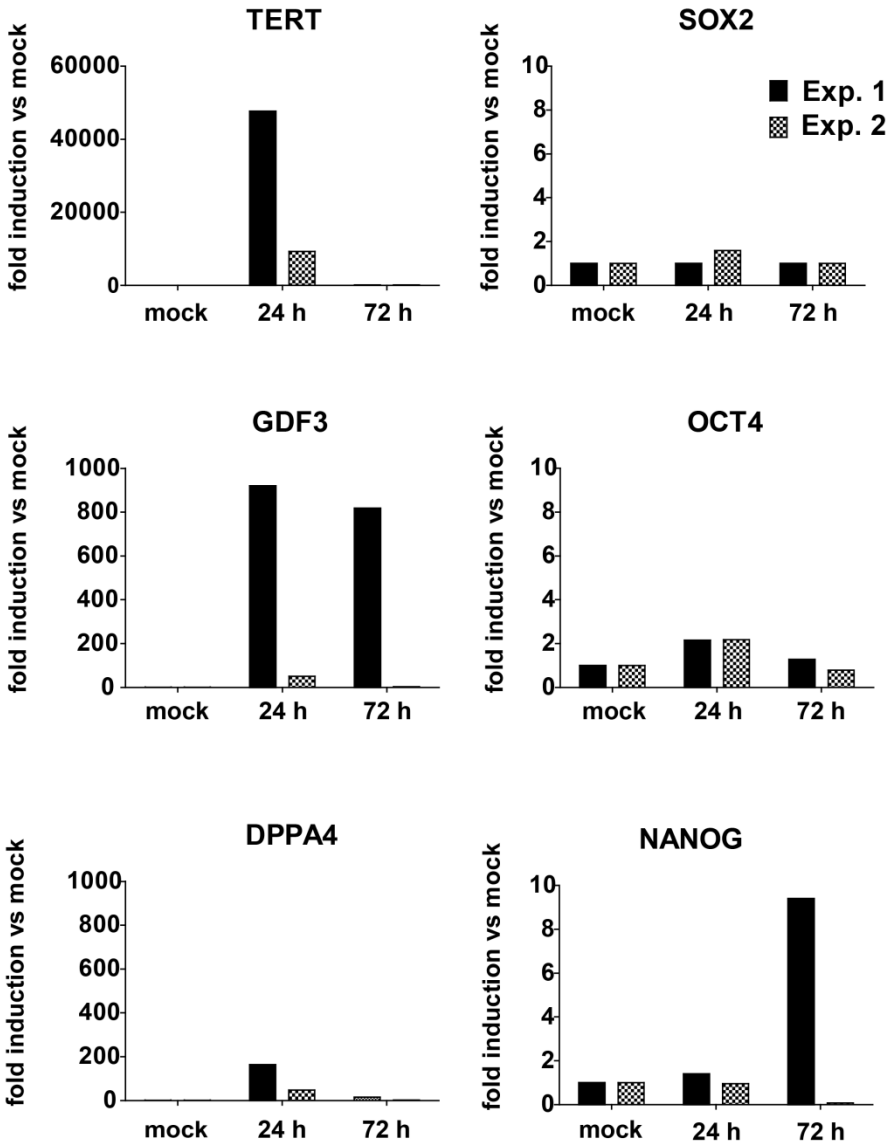

Figure 3.3 Transient upregulation of reprogramming markers 24 and 72 h after transfection Human fibroblasts (CCDs) were electroporated once, using the Gene-Pulser-II apparatus, with 60 ng/µl of OCT4, SOX2, KLF4, c-MYC reprogramming factors and cells were harvested at the indicated time points. As read out for the induction of reprogramming markers the analysis of endogen mRNA levels were performed (telomerase reverse transcriptase (TERT), the growth differentiation factor 3 (GDF3), the developmental pluripotency associated 4 (DPPA4), the sex determining region Y-box 2 (SOX2), the octamer binding transcription factor 4 (OCT4) or the endogenous stem cell marker NANOG). Data from two representative experiments (Exp.1 and 2) are shown.

3.2 Enduring expression of IVT-RNA encoded transgenes by repetitive electroporation

The need for frequent transfections in order to generate IVT-RNA-based iPS cells was manifested by the expression of stem cell associated markers after viral transduction, which leads to enduring reprogramming marker expression (Takahashi and Yamanaka, 2006). Thus, for a stable upregulation of stem cell markers a repetitive transfection seems indispensable.

3.2.1 Repetitive electroporation of IVT-RNA is accompanied by extreme cell death

Due to the aforementioned analysis of IVT-RNA encoded protein expression of reprogramming factors (Fig. 3.2) and for cellular revival we decided to perform the electroporation in a 48 h interval. Human fibroblasts were electroporated with the ECM electroporation system 830. Human fibroblasts were transfected with and without RNA coding for the reprogramming factors OCT4, SOX2, KLF4 and c-MYC and the cell number was counted before each transfection. In Fig. 3.4 an exemplary cell number is depictured: While nearly all cells survived the first EP with or without IVT-RNA we observed massive cell loss upon subsequent electroporation with IVT-RNA but not in the samples without IVT-RNA. Due to the observation of only minor cell death few hours after the first electroporation these result indicated that the used electroporation conditions were principally suitable for human fibroblasts. 24 h and 48 h after the second electroporation a massive loss of cells was detected in the IVT-RNA electroporated sample. The mock electroporated sample was able to maintain and even increase the cell number due to proliferation of the cells. The mock and the IVT-RNA containing samples were detached from the plate and several washing steps which are required to prepare the electroporation, were performed. Therefore the massive cells death in the IVT-RNA containing sample could not be explained by technical loss alone.

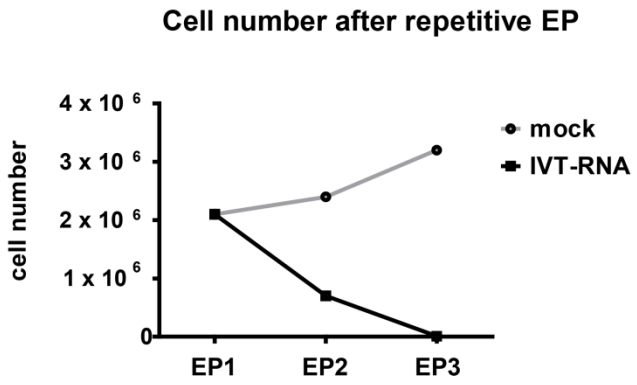

Figure 3.4 Exemplary cell number after repetitive electroporation Human fibroblasts (CCDs) were electroporated (EP), using the ECM electroporation system, with the Yamanaka reprogramming mix of OCT4, SOX2, KLF4 and c-Myc IVT-RNA (60 ng/µl of each TF and 10 ng/µl reporter gene IVT-RNA). The intervals between the transfection were 48 h and prior to each electroporation cells were counted and compared to the starting cell number.

In summary, human fibroblasts survived the first electroporation with IVT-RNA but failed to survive sequential RNA transfer. The main subject to be addressed in the next set of experiments was to find out the reason for the massive cell death upon IVT-RNA-based transfection.

3.2.2 Upregulation of IFN response markers upon IVT-RNA-based transfection

Recognition of non-self RNA is known to be sensed by the cells and they respond with the activation of effective self defense mechanisms (Pichlmair and Reis e Sousa, 2007). Among others, cytoplasmic sensors, e.g. the PKR, are able to activate IFN and NF-kB-dependent pathways which result in an anti-viral cellular state (Diebold et al., 2004; Pichlmair et al., 2006; Kawai and Akira, 2007; Uematsu and Akira, 2007; Sadler and Williams, 2008). Since we were not able to perform repetitive RNA-based transfections we hypothesized that cytoplasmatic RNA sensors and subsequent IFN response might be the major reason for the observed cell death in our experiments. Thus, human fibroblasts were electroporated with IVT-RNA and after an incubation time of 24 h cells were harvested and tested for the upregulation of anti-viral marker genes induced by IFNs. We could demonstrate by RT-PCR that the IFN response genes OAS1 and OAS2, MX1, IFITM1 and IRF9 were upregulated in cells electroporated with IVT-RNA (Fig. 3.5). These results show that 24 h after the

transfection with IVT-RNA cellular pathways, representing an IFN response, were induced. Cells which were not exposed to IVT-RNA during the electroporation had no induced IFN response genes. Therefore we could exclude that the application of an electric field or the overall handling procedures were the reason for the elevated levels of IFN response marker.

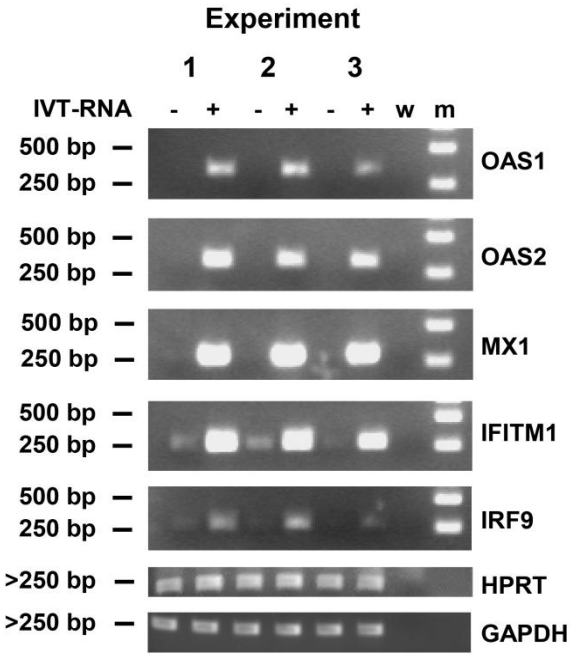

Figure 3.5 Upregulated interferon responses after electroporation with IVT-RNA Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, with a total of 133.6 ng/µl of IVT-RNA and cells were harvested 24 h later. By PCR the endogen levels of IFN response markers were analyzed with the IFN-response detection kit. The housekeeping genes HPRT and GAPDH were analyzed with our own control primers. The transfected IVT-RNA mix consisted of 118 ng/µl of reprogramming factors OCT4, SOX2, KLF4, LIN28, c-Myc and NANOG (OSKLMN), 5 ng/µl GFP reporter gene IVT-RNA and the rest was filled up with IVT-RNA encoding for E6 and SV40 to stimulate proliferation and survival. (OAS, oligoadenylate synthetase; MX, myxovirus resistance; IFITM1, interferon-induced transmembrane protein 1; IRF9, interferon regulating factor 9; HPRT, hypoxanthine-guanine phosophoribosyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; w, water control; m, marker). Data from three experiments are shown.

These experiments indicated that an upregulated IFN response, induced by transfected IVT-RNA is most likely the obstacle for cell survival. Therefore the aim was to identify the major component in IVT-RNA mediated upregulation of the IFN response in order to evaluate way to circumvent its induction. When we were able to combat the IFN response induction than frequent IVT-RNA-based transfections shout be feasible.

3.2.3 Translation inhibition upon IVT-RNA transfer

Several RNA sensors like RIG-I, MDA-5, PKR or TLRs are optional target molecules for inhibiting an IVT-RNA induced upregulation of IFN response genes. From these receptors PKR is of special interest due to its key position in activating the IFN response as part of the innate immunity and mediation of complex signaling cascades resulting in prevention of protein biosynthesis and facilitating the induction of apoptosis (Gil and Esteban, 2000; García et al., 2006). To underline the hypothesis that PKR is indeed the main player of IFN-response against IVT-RNA-based gene transfer we looked more closely to the effects of IVT-RNA transfer on protein biosynthesis, representing an indirect read out for PKR activation. To this aim human fibroblasts were stably transduced with a lentiviral vector which expresses firefly luciferase. IVT-RNA coding for renilla luciferase was then transfected in these cells by electroporation. We expected an IVT-RNA induced activation of PKR which would result in reduced firefly luciferase expression and therefore analyzed firefly reporter gene expression levels after the electroporation of IVT-RNA. To generate comparable read out conditions for the reporter gene expression level, identical numbers of cells were plated after electroporation with and without IVT-RNA. The firefly luciferase was thereby under the control of three different constitutive promoters, with one promoter in each of the three fibroblast cell lines. With this setting we planned to exclude promoter dependency in our experiments. We used the human *elongation factor-1 alpha*- (EF1α), the human promoter *phosphoglycerate kinase*- (PGK) and the viral *cytomegalovirus*- (CMV) promoter. In all cell lines we observed that the electroporation of IVT-RNA reduced the expression level of the firefly luciferase independently from the promoter which drove the expression (Fig. 3.6).

Since one hallmark of activated PKR is the downregulation of protein biosynthesis, the result of the reduced plasmid encoded reporter gene strengthens the hypothesis that the PKR is mainly involved in response to IVT-RNA.

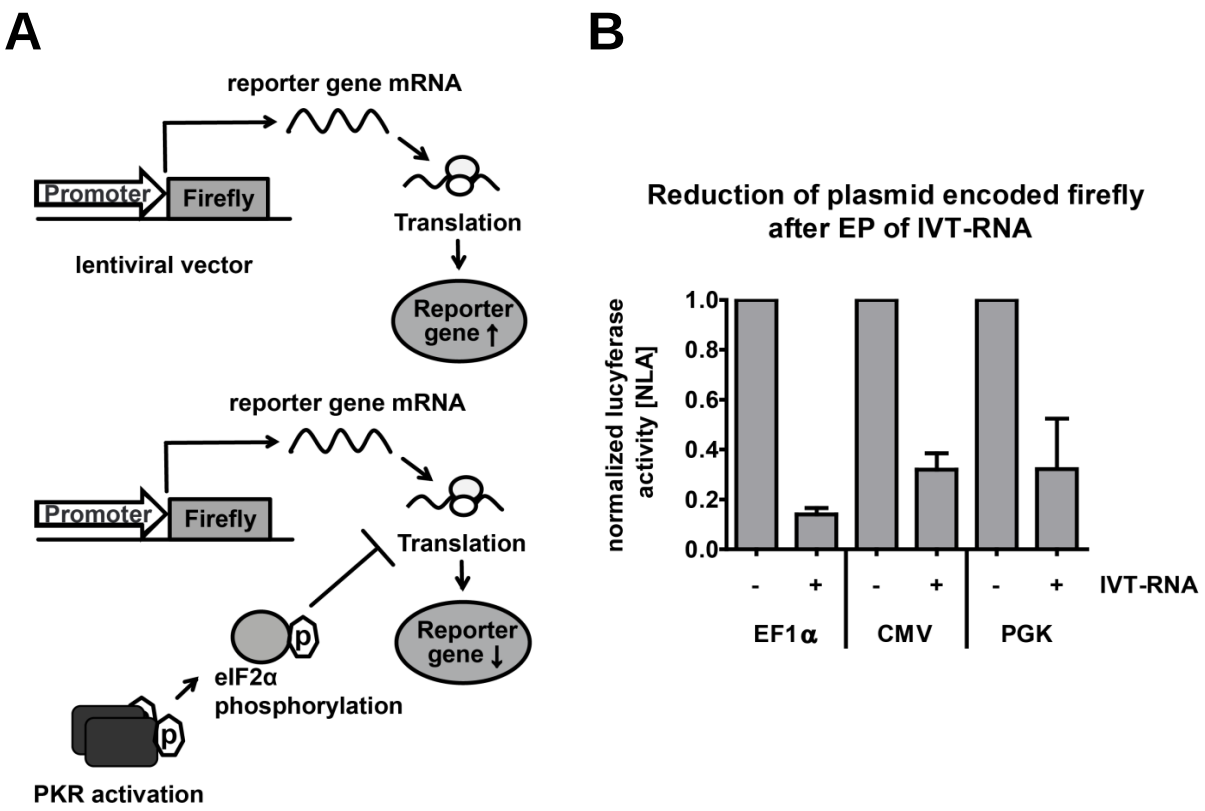

Figure 3.6 Translation inhibition after IVT-RNA transfer (**A**) Schematic presentation of the translation inhibition experiment. The stable transfected reporter gene firefly luciferase is constitutively expressed under the control of different promoters. When the target cells were electroporated with IVT-RNA an activation of PKR results in reduced translation of the firefly reporter gene. (**B**) Human fibroblasts (HFFs) containing stable transfected firefly luciferase as reporter gene were used in the experiment. Firefly was under the control of three different promoters in a lentiviral vector. Cells which were constitutively expressing the reporter gene were electroporated, using the ECM electroporation system, with 48 ng/µl IVT-RNA and after 24 h the reporter gene expression level was measured by luciferase assay. For *elongation factor-1 alpha* (EF1α) and *phosphoglycerate kinase* (PGK) promoter data are represented as mean + sd from three experiments, for *cytomegalovirus* (CMV) data from two experiments are shown.

3.2.4 IVT-RNA transfection leads to the phosphorylation of the PKR substrate eIF2α

For further evidence that PKR is activated by IVT-RNA, we performed a set of experiments which analyzed the phosphorylation status of the *eukaryotic initiation factor alpha* (eIF2α). Upon activation of PKR the substrate eIF2α gets phosphorylated which results in a halt of translation (Gebauer and Hentze, 2004; Sadler and Williams, 2008). For this aim human fibroblasts were electroporated with reporter gene IVT-RNA encoding for luciferase and

GFP. As positive control for a strong eIF2α phosphorylation cells were additionally transfected with IVT-RNA encoding for PKR wild type (WT-PKR). 24 h after transfection the protein extracts were analyzed by western blot using specific antibodies detecting the PKR substrate eIF2α in its unphosphorylated and phosphorylated form. We observed that the phosphorylation status of eIF2α increased from cells electroporated without IVT-RNA to cells exposed to 48 and 96 ng/µl IVT-RNA (Fig. 3.7). The strongest phosphorylation signal of the PKR substrate eIF2α was detected as expected in the WT-PKR overexpressing sample. These results verified the importance of PKR activation after IVT-RNA-based gene transfer.

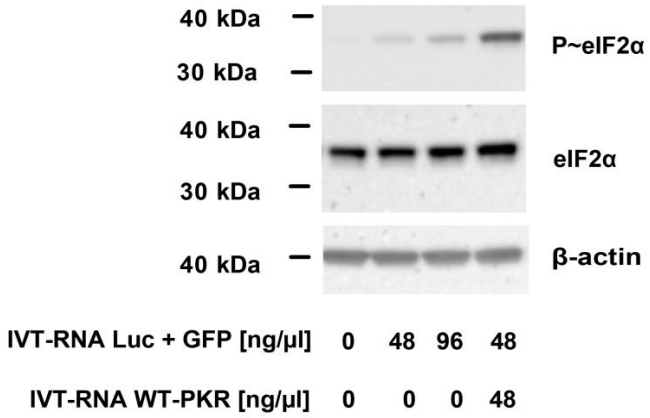

Figure 3.7 Phosphorylation of the PKR substrate eIF2α after electroporation with IVT-RNA Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, with 48 and 96 ng/µl IVT-RNA (luciferase (Luc) and GFP) and 48 ng/µl IVT-RNA (Luc and GFP) combined with 48 ng/µl WT-PKR to generate a positive control for eIF2α phosphorylation by PKR overexpression. 24 h after the transfection, cells were harvested and analyzed by western blot using specific antibodies. The antibodies recognize either the *eukaryotic initiation factor alpha* (eIF2α), its phosphorylated form (eIF2α~P) or *beta-actin* (β-actin) for loading control. A representative western blot image is shown.

So far we found that upon IVT-RNA transfer the cellular response is upregulated mediated by the IFNs whereas the protein biosynthesis is diminished. Moreover upon IVT-RNA transfection an elevated phosphorylation of eIF2α was observed. This substrate phosphorylation is thereby most likely mediated by the PKR. All results point therefore to the PKR as essential molecule mediating the major aspects of the cellular IFN response after IVT-RNA-based transfection. Thus, the inhibition of the PKR could pave the way for frequent transfections with IVT-RNA and since the PKR remains inactivated, improvements in the transgene expression might be achieved.

3.3 Inhibiting the PKR activation

Upon IVT-RNA transfection we observed diminished protein translation, upregulated IFN response genes and cell death upon frequent transfections. By inhibiting RNA dependent activation of the key protein PKR we planned to improve both, cell survival as well as IVT-RNA-based expression. Thus in the following experiments we investigated whether small molecules, inhibiting the PKR activation, can minimize the upregulation of IFN response genes after IVT-RNA transfection.

3.3.1 Inhibition of the PKR by small molecules targeting the PKR leads to reduced induction of IFN response markers

Several small molecules with the ability of inhibiting PKR are described in the literature (Tiwari et al., 1988; Hu and Conway, 1993; Jammi et al., 2003). One of them is $C_{13}H_8N_4OS$, an imidazole derivate, which was discovered by screening a library of 26 different ATP-binding site directed inhibitors (Jammi et al., 2003). To ease reading, I will from now on abbreviate the imidazole derivate $C_{13}H_8N_4OS$ as "Im-13". In the following indicative experiments Im-13 was used to analyze its ability of minimizing the IVT-RNA induced IFN response. Therefore, human fibroblasts were electroporated with IVT-RNA and incubated either with 2 µM Im-13 or not. After 24 h cells were analyzed exemplary for the expression of IFN response genes oligoadenylate synthetase (OAS) 1 and 2 by PCR. Hereby an electroporation with IVT-RNA results in a strong induction of OAS1 and 2 (Fig. 3.8). No IFN response genes induction was detectable in untransfected cells with and without Im-13. We could demonstrate that IVT-RNA dependent upregulation of IFN response genes was remarkably minimized by Im-13 treatment of the target cells.

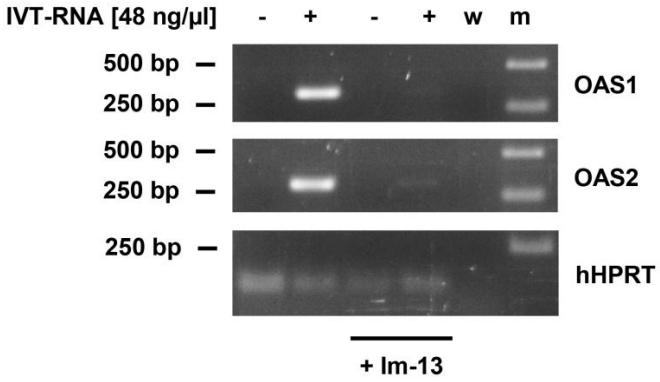

Figure 3.8 Activation of IFN response genes by IVT-RNA transfection and the reduction of the IFN response marker induction by Im-13 Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, with 48 ng/µl IVT-RNA. One part of the transfected cells were additional treated with 2 µM of the PKR inhibitor Im-13. After 24 h cells were harvested and analyzed via PCR for the presence of IFN response genes OAS1 and 2 with and without the PKR inhibitor treatment. (OAS, oligoadenylate synthetase; hHPRT, human hypoxanthine-guanine phosophoribosyltransferase; w, water control; m, marker)

3.3.2 Small molecule inhibitors of the PKR increase the translation of an IVT-RNA encoded reporter gene

After we were able to confirm the reduced IFN response in the presence of Im-13 upon IVT-RNA-based transfection the question was whether small molecules, which target the PKR, are also able to increase protein expression. To this aim human fibroblasts were electroporated with IVT-RNA encoding the reporter gene luciferase and the transfected cells were incubated with increasing concentrations of Im-13 as indicated in Fig. 3.9 A. The luciferase expression was analyzed from 7 to 72 h. Thereby we detected a dose dependency between increasing Im-13 concentration and enhanced reporter gene expression. In particular a treatment with 2 µM Im-13 resulted in a plateau phase of stabilized reporter gene expression after 24 h. The reporter gene expression after the Im-13 incubation with 0.5 to 1 µM leads to a fast decrease of luciferase after peaking at 24 h (Fig. 3.9 A). For Im-13 we observed the best elevated expression when target cells were incubated in medium containing 2 µM of the inhibitor.

Having observed elevated transgene expression with the small molecule Im-13 we tested whether 2-Aminopurine ($C_5H_5N_5$) (2-AP) as an alternative PKR inhibitor is also able to prolong and enhance transgene expression of the reporter gene luciferase. Since 2-AP is a mutagenic base analogue for adenine (Sowers et al., 1986; Tiwari et al., 1988) we decided, after verifying enhanced reporter gene expression upon 2-AP treatment, to use Im-13 in the

further experiments. Again, human fibroblasts were electroporated with IVT-RNA encoding the reporter gene luciferase and subsequently incubated with 0 to 20 mM 2-AP (Fig. 3.9 B). The read out, based on the expression of luciferase, was performed. The maximal reporter gene expression after an incubation of cells with 0 to 10 mM of the PKR inhibitor 2-AP was reached after 24 h. The curve representing the luciferase translation after a treatment with 20 mM 2-AP peaked at 48 h and declined afterwards. The small molecule PKR inhibitor 2-AP showed its best elevated reporter gene expression at a concentration of 20 mM. Similar to Im-13 we can detect a dose dependency between increasing 2-AP concentration and elevated reporter gene expression.

These results show that beside the small molecule Im-13, 2-AP is also able to increase and stabilize reporter gene expression. Nevertheless the usage of a small molecule PKR inhibitor is dependent on reversible inhibition since the PKR is involved in other cellular procedures like differentiation (Alisi et al., 2008; Yoshida et al., 2009), cell cycle (Zamanian-Daryoush et al., 1999) and growth control (Meurs et al., 1993; Lee and Esteban, 1994) beside sensing non-self RNA. Especially for long term experiments and the later use in e.g. clinical trials the reversibility of the PKR inhibition might be of particular importance.

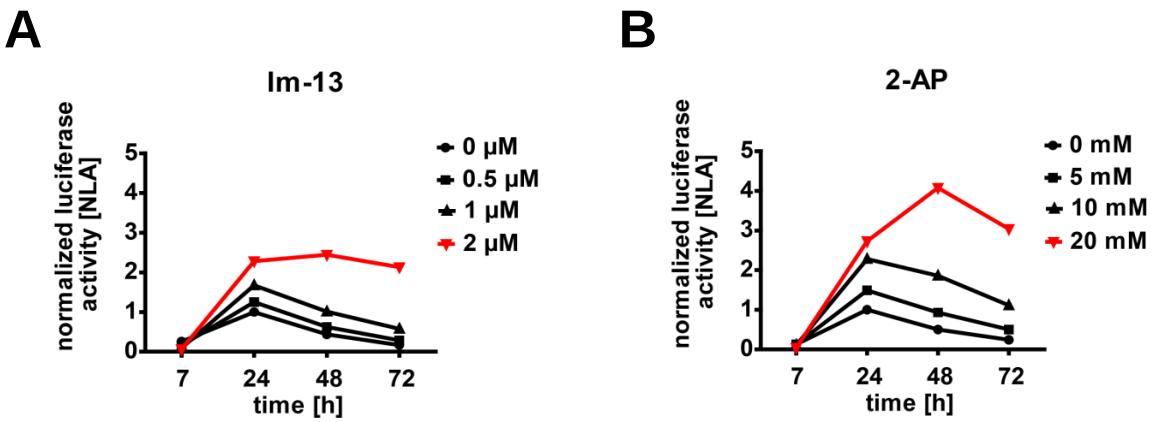

Figure 3.9 Enhanced translation by inhibiting PKR with small molecules Human fibroblasts were electroporated, using the ECM electroporation system, with reporter gene IVT-RNA (A= CCDs, 14 ng/µl; B= HFFs, 48 ng/µl) and incubated with 0 to 2 µM Im-13 (**A**) or 0 to 20 mM 2-AP (**B**). As read out the luciferase activity was measured at the indicated time points from 7 to 72 h. Shown are the normalized data of two concentration rows with two different PKR inhibitors. Data from one experiment is shown.

3.3.3 The process of PKR inhibition by Im-13 is reversible

In our next experiments we assessed whether enhanced reporter gene translation after Im-13 treatment is reversible. To this aim human fibroblasts were electroporated with IVT-RNA encoding for an reporter gene. Afterwards the cells were incubated either continuously or for the indicated time points with 2 µM of the PKR inhibitor Im-13. As read out the reporter gene expression luciferase was measured (Fig. 3.10 A and B). A limiting effect of enhanced protein translation was seen in the conditions were the PKR inhibitor was removed after 8 and 24 h of incubation. But the restricted effect of enhanced protein translation decreased when the cells were incubated with Im-13 for 48 h or longer. Collectively, these data indicate that Im-13 worked as reversible PKR inhibitor.

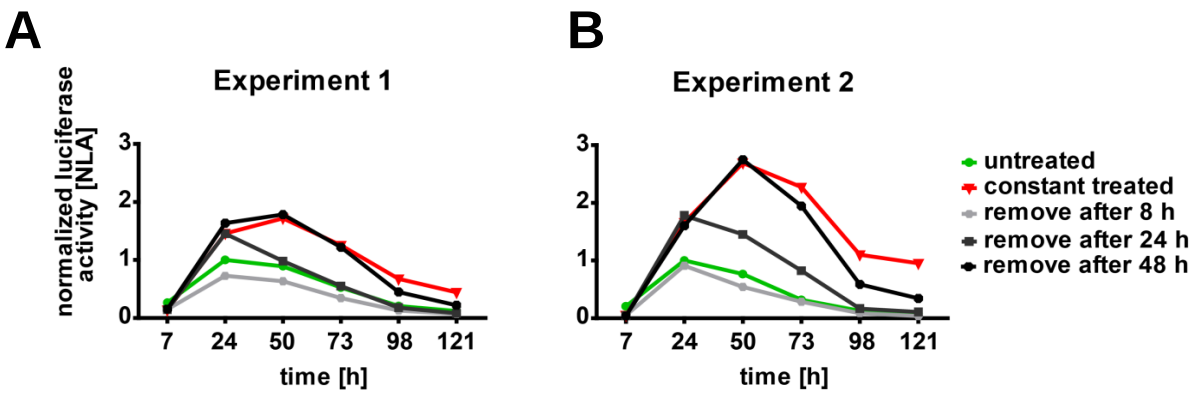

Figure 3.10 The process of PKR inhibition by Im-13 is reversible Human fibroblasts were electroporated, using the ECM electroporation system, with 28 ng/µl (**A** = BJs) or 14 ng/µl (**B** = CCDs) reporter gene IVT-RNA. 8, 24 and 48 h after the electroporation and treatment with the PKR inhibitor, the small molecule Im-13 was removed from the cells. At the indicated time points the reporter gene amount on luciferase level was determined. Additionally, untreated and permanently with Im-13 treated cells were used. For the analysis data were normalized to the 24 h reporter gene control. Data from two representative experiments are shown.

With the former experiments we have answered the question concerning the reversibility of the PKR inhibitor Im-13. Next we investigated whether the non-mutagenic PKR inhibitor Im-13 is suitable for long term experiments at its most effective dose. Since we aimed to achieve long term IVT-RNA-based transgene expression an effective PKR inhibitor must be tolerated by the targeted cells during the experiment.

3.3.4 The PKR inhibitor Im-13 is cytotoxic when incubated at its effective concentration

The suitable PKR inhibitor Im-13 was most effective at a concentration of 2 µM. To allow frequent transfections in long term experiments the cells must survive the exposure to the PKR inhibitor Im-13. To address this, human fibroblasts were electroporated mock and were incubated with 2 µM Im-13 for 96 h. Finally the condition of the fibroblasts was documented by microscopy. Thereby we observed a toxic effect of the PKR inhibitor Im-13 (Fig. 3.11). The cells treated with 2 µM Im-13 alter their appearance from the typical spindle-shaped fibroblast to thin cells with long filamentous protrusions. Consequently the use of Im-13 in long term experiments after repetitive IVT-RNA transfer was excluded and it was not further used in this study.

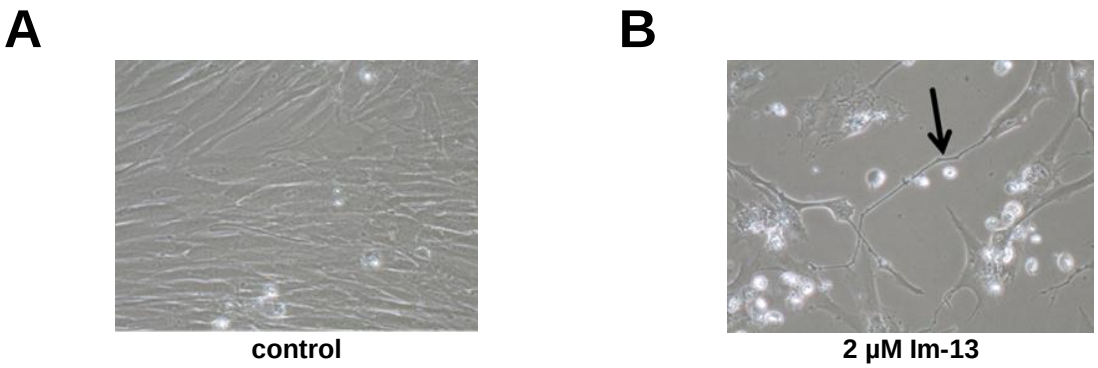

Figure 3.11 Cytotoxicity after 96 h Im-13 treatment Human fibroblasts (CCDs) were treated for 96 h with the PKR inhibitor Im-13 at its best stabilization concentration of 2 µM. Im-13 treated cells alter their appearance from spindle-shaped fibroblasts (**A**) to long filamentous protrusions thin cells indicated with the black arrow (**B**). (Magnification 100X)

To summarize the results with the small molecule Im-13 it should be mentioned that the tested inhibitor was in general effective in inhibiting the activation of the kinase PKR which was supposed to be the major obstacle in IVT-RNA induced upregulation of the IFN response. After Im-13 treatment we observed a reversible and enhanced translation of reporter genes. Furthermore the IFN response after IVT-RNA transfection was reduced in the presence of the small molecule inhibitor. Unfortunately Im-13 was cytotoxic for our proposed target cells at its effective concentration and therefore its usage is limited.

3.4 Inhibition of the PKR with siRNA, a dominant negative PKR mutant or viral anti-IFN IVT-RNA

We had identified the PKR as target structure for the optimization of IVT-RNA-based gene transfer. Due to cytotoxic effects of the small molecule Im-13 we targeted the PKR with alternative strategies in the following set of experiments with three different approaches: First by short interfering RNA (siRNA) for a knockdown of PKR expression, secondly an overexpression of a dominant negative PKR mutant (dN-PKR) and at last a co-expression of viral IFN response inhibitors was evaluated.

3.4.1 PKR knockdown with siRNA increases translation and reduces the IFN response upon IVT-RNA transfection

siRNAs are noncoding RNA elements which are able to interact with cellular mRNA and thereby leading to a specific and robust cleavage of the targeted mRNA. We expected that a knockdown of PKR mRNA would result in the downregulation of the IFN response followed by an elevated translation of reporter genes. Thus, we targeted the constitutively expressed PKR with a PKR siRNA-pool, consisting of three different PKR siRNAs which had the size of 19 to 25 nt. First of all, we had to verify the knockdown of PKR on mRNA transcript and protein level. To this aim human fibroblasts were electroporated with PKR siRNA and incubated for 48 h. Afterwards cells were analyzed on mRNA level for PKR knockdown by qRT-PCR. With 80 nM PKR siRNA we investigated a robust knockdown of PKR on mRNA level for nearly 90% (Fig. 3.12 A). Additionally we collected cells after 48 h for western blot analysis of protein lysates using specific antibodies detecting the PKR and β-actin. We observed that the PKR signal is downregulated 48 h after the electroporation of the PKR siRNA mixture. Moreover we could verify a knockdown of PKR on protein level (Fig. 3.12 B).

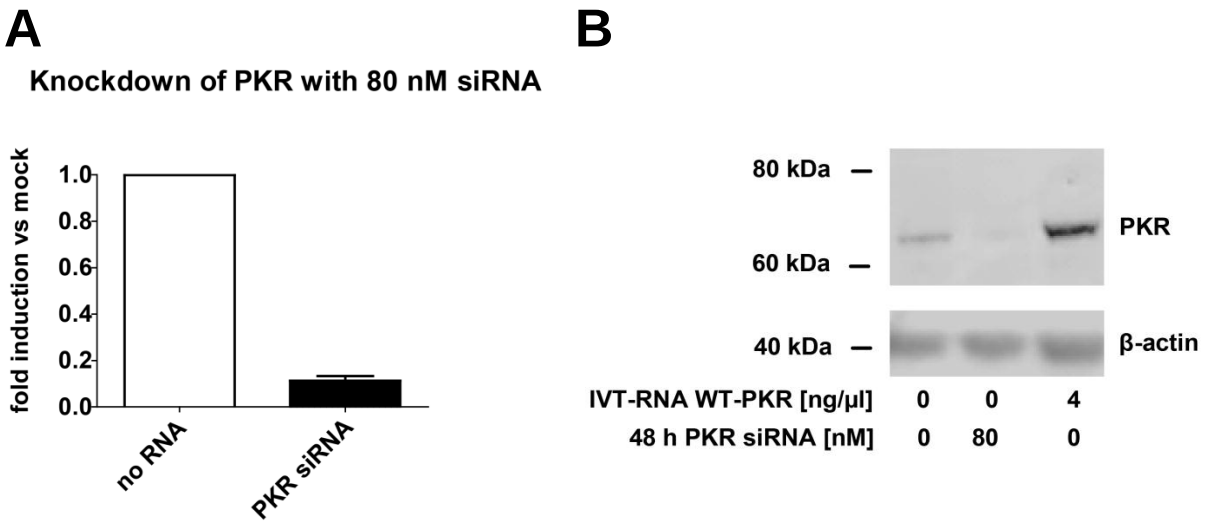

Figure 3.12 Knockdown of PKR on mRNA and protein level after 48 h incubation with PKR siRNA Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, with 80 nM PKR siRNA mixture and (**A**) analyzed 48 h later for PKR mRNA level. Within four experiments a robust reduction of the PKR mRNA level for nearly 90% was detected. (**B**) Western blot analysis for analyzing the PKR protein in human fibroblast was performed and compared to untransfected and PKR overexpressing cells. At protein level a knockdown could also be detected. The antibodies recognize either the *RNA dependent protein kinase* (PKR) or *beta-actin* (β-actin).

Especially for later application we had to rule out the seldom effect of inducing IFN response by the siRNA pool itself (Nanduri et al., 1998; Reynolds et al., 2006). To this aim human fibroblasts were electroporated with PKR siRNA or IVT-RNA as positive control generating a maximal IVT-RNA depended induction of IFN response genes. After 24 h cells were harvested and evaluated by qRT-PCR. We observed no upregulation of IFN response genes for IFNβ and OAS1 in the sample electroporated with PKR siRNA (Fig. 3.13). These results confirmed that the chosen pool of PKR siRNA is not inducing the IFN response and can therefore be used in our next experiments.

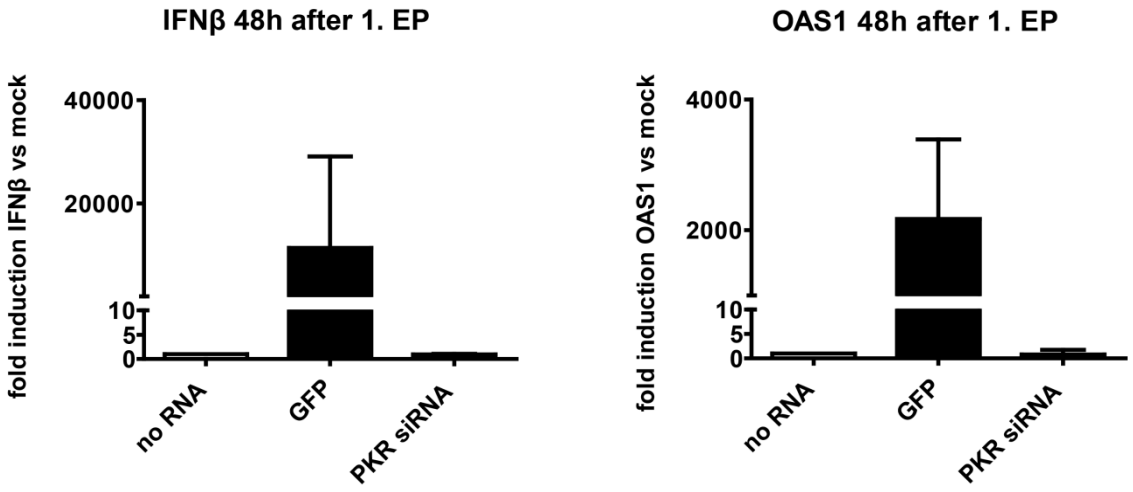

Figure 3.13 PKR siRNA alone has no effect on IFN response induction Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, either with 80 nM PKR siRNA or with GFP reporter gene IVT-RNA as positive control for maximal induction of IFN response genes. After the cultivation of cells for 48 h the induction of IFN response genes IFNβ and OAS1 were assayed. Data are represented as mean + sd of three experiments

We hypothesized that by siRNA mediated knockdown we should increase the translation and reduce IFN response towards electroporated IVT-RNA. Therefore, human fibroblasts were electroporated mock, with PKR siRNA or a control siRNA mix (non-target siRNA). After an interval of 48 h, the cells were additional electroporated with and without reporter gene IVT-RNA encoding for luciferase and GFP alone or either without siRNA co-transfer or in combination with PKR target or non-target siRNA. 24 h later cells were harvested and analyzed for electroporation efficiency and expression value. Furthermore the translation of the reporter gene luciferase was measured in a time course of 7 to 120 h after electroporation (Fig. 3.14 B). In Fig. 3.14 A we illustrated a comparable electroporation efficiency of the different samples. We observed no changes in the GFP expression intensity between the samples containing reporter gene IVT-RNA with and without non target siRNA whereas the addition of PKR siRNA resulted in 2-fold enhanced reporter gene expression (Fig. 3.14 B). The same situation was observed under the influence of PKR siRNA in combination with the reporter gene luciferase. We measured no differences between reporter gene IVT-RNA with and without non-target siRNA (Fig. 3.14 B). In the PKR siRNA containing sample we measured for the maximal translation efficiency of the reporter gene luciferase a more than 2-fold induction (Fig. 3.14 B).

For evaluating the reduction of IFN response genes 24 h after the last electroporation the cells were harvested and analyzed for the expression level of the IFN response gene IFNβ and OAS1 by qRT-PCR. For the PCR analysis the normalization was based on the maximal induction of expression after electroporation of IVT-RNA encoding for reporter genes (Luc and GFP). Thereby we observed after an siRNA mediated PKR knockdown a clear downregulation of IFNβ. However, this downregulation was not reached with the IFN dependent marker OAS1, where we could not detect a clear reduction within five experiments (Fig. 3.14 C).

To sum up the results of the last experiments it should be mentioned that we were able to enhance the translation of the reporter gene 2-fold with a treatment of PKR siRNA. Also on IFNβ marker level a clear reduction could be reached but only a minor reduction in expression of the IFN response gene OAS1 was achieved by PKR siRNA knockdown.
We concluded that PKR siRNA in principal worked robustly. Moreover, an elevated translation in IVT-RNA encoded reporter gene expression was achieved by PKR siRNA mediated knockdown. Nevertheless the data sets for IFN response induction are ambivalent. A clear reduction of IFN response gene markers was seen on IFNβ level whereas, to our disappointment, the IFN dependent target and IFN marker OAS1 was not reliable minimized. We therefore decided to target PKR in another way.

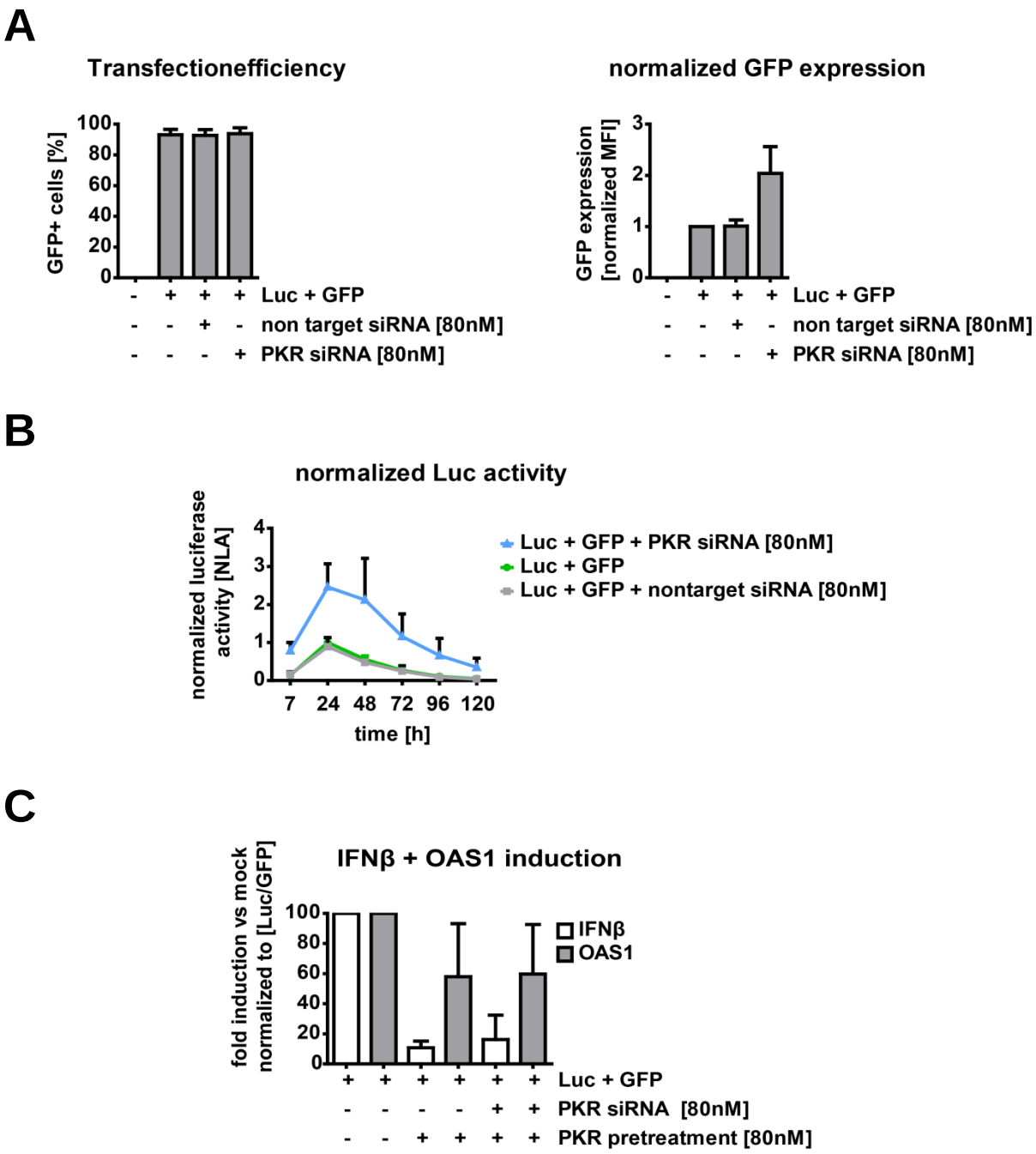

Figure 3.14 Targeting PKR with PKR siRNA Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, mock, with PKR siRNA (80 nM) or a control siRNA mix (80 nM) (non-target siRNA). After 48 h cells were additional electroporated with and without 4 ng/µl luciferase (Luc) IVT-RNA and 10 ng/µl GPF IVT-RNA reporter gene alone, or in combination with PKR target or non-target siRNA. (**A**) 24 h later cells were analyzed for electroporation efficiency and expression value. (**B**) The translation of the reporter gene luciferase was measured 7 to 120 h after electroporation. Data from two experiments are shown (A and B). (**C**) Human fibroblasts were electroporated mock or with 80 nM PKR siRNA mix. After 48 h cells were additional electroporated mock or including the reporter gene mixture consisting of 4 ng/µl luciferase IVT-RNA and 10 ng/µl GPF IVT-RNA alone or in combination with PKR siRNA. 24 h later cells were analyzed for the expression level of the IFN response gene IFNβ and OAS1 by qRT-PCR analysis. Data from five experiments

+ sd are shown.

3.4.2 The overexpression of a dominant negative PKR increases translation but fails to inhibit the IFN response

For a next approach of targeting PKR we decided to test the overexpression of a dominant negative PKR (dN-PKR) mutant. We hypothesized that an overrepresented catalytically inactive PKR (Katze et al., 1991) will reduce the IFN response after IVT-RNA electroporation because it's signaling is interrupted (Taylor et al., 1996). A dN-PKR was generated by directed mutagenesis from a PKR wild type clone as described in material and methods. The mutation in the kinase domain disturbs its kinase activity, since the targeted ATP binding domain is necessary for phosphorylation and activation steps leading to antiviral response (Meurs et al., 1990; Taylor et al., 1996). Therefore we analyzed whether the overexpression of a dN-PKR can enhance the translation of the reporter gene luciferase and reduce the IFN response to IVT-RNA. In this set of experiments we made the assumption that an increased reporter gene translation is an indirect read out for a reduced phosphorylation of the PKR substrate eIF2α as we had done before (Fig. 3.7). To address this possibility, human fibroblasts were electroporated mock or with IVT-RNA either encoding for the reporter gene luciferase and GFP alone or in combination with dN-PKR IVT-RNA. We evaluated the reporter gene translation from 7 to 120 h. The curves representing the reporter gene translation peaked for all samples 24 h after electroporation. The strongest expression of reporter gene translation was thereby observed for the dN-PKR overexpressing sample, followed by the only reporter gene containing sample. After 24 h the translation of the reporter gene luciferase was 1.5 fold stronger when the dN-PKR was co-expressed. In addition the weakest signal was detected as expected for the PKR-WT overexpression control (Fig. 3.15 A). Beside the enhancement of translation we investigated the expression of IFNβ and the IFN target gene OAS1 by qRT-PCR 24 h after the electroporation. In the mock electroporated sample no induction of IFN response genes was observed, whereas in the reporter gene containing sample induced levels for IFNβ and OAS1 were measured. Due to the excess of the kinase inactive dN-PKR we hypothesized that after IVT-RNA transfection we might detect a minimized IFN response in the dN-PKR containing samples compared to the samples which encodes only for the reporter genes. However, we detected induced levels of the IFN response markers IFNβ and OAS1 in the dN-PKR overexpression sample (Fig. 3.15 B).

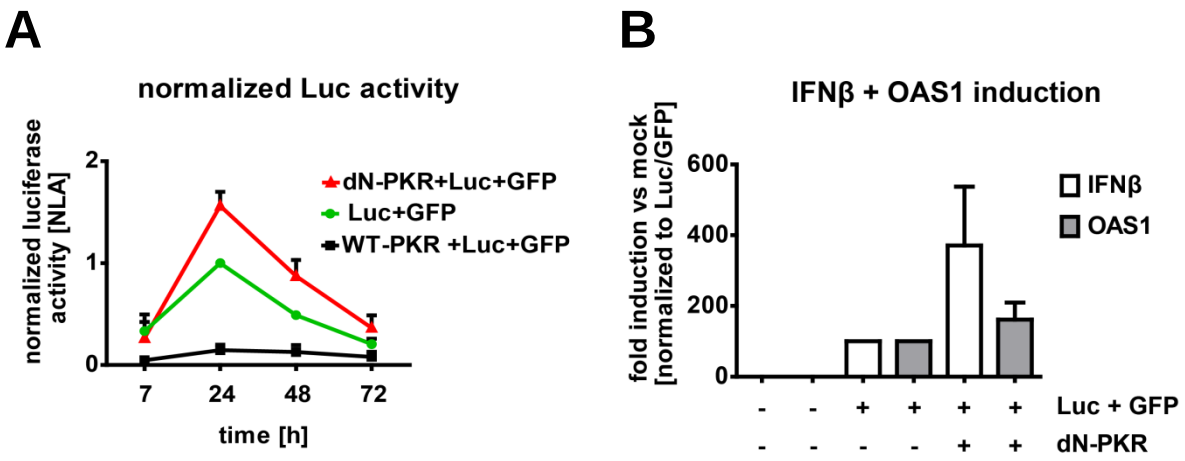

Figure 3.15 Reporter gene expression and induction of IFN markers after dN-PKR overexpression in human fibroblasts Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, mock or with 4 ng/µl luciferase (Luc) and 10 ng/µl GFP reporter gene IVT-RNA alone or in combination with 24 ng/µl dominant negative PKR (dN-PKR) or 24 ng/µl wild type PKR (WT-PKR) as control. (**A**) After electroporation the reporter gene translation was measured from 7 to 120 h. For analysis data were normalized to the reporter gene expression of Luc and GFP after 24 h. Data from three experiments + sd are shown. (**B**) After 24 h cells were harvested for analyzing the expression of IFNβ and the IFN target gene OAS1 by qRT-PCR. Data from three experiments + sd are shown.

Taken together, these results show that the overexpression of a dN-PKR resulted in enhanced reporter gene expression although the presence of the kinase inactive PKR failed to reduce the IFN response induction. On the basis of these observations we decided to reject dN-PKR overexpression for the purpose of enhanced reporter gene translation. Although it would be interesting to explore the background why it is possible to generate an improved translation in the presence of increased IFN response genes, it is not productive for our approach. Therefore we decided to focus on targeting the PKR with viral IFN response inhibitors.

3.4.3 The co-expression of viral inhibitors of the IFN response increase the translation, decrease the IFN induction and reduce the PKR activation

3.4.3.1 Viral inhibitors of the IFN response

We considered viral inhibitors of the IFN response to be an approved strategy for minimizing the IFN dependent response to IVT-RNA since virus and host cells have been exposed to a long co-evolution. This resulted in specialized virus recognition by the host on the one hand and in optimized viral immune evasion strategies to circumvent the cellular defense effectively on the other. Therefore, we assessed whether it is possible to reduce the activation of PKR by the co-expression of IVT-RNA encoding for viral proteins which were inhibiting the IFN response.

VACV proteins which directly inhibit either the PKR activation or the IFN response were of preferred interest and we decided to produce IVT-RNA from the proteins E3, K3 and B18R (EKB). E3 is able to inhibit the PKR by blocking its activation whereas K3 acts as pseudosubstrate blocking the PKR. The third protein B18R is described as IFN-decoy-receptor in the extracellular compartment acting as IFN receptor analogue and quenching the IFN response (Symons et al., 1995). The effectiveness of the B18R is supported by the successfully RNA-based reprogramming by Warren et al. (Warren et al., 2010; Warren et al., 2012) where he used B18R as a recombinant protein to supplement the reprogramming media.

3.4.3.2 The EKB co-expression increases the expression of IVT-RNA encoded reporter genes

The capacity of viral anti IFN response treatment was assessed by the electroporation of cells either with or without the co-expression of viral proteins. Thereby we compared whether the IVT-RNA encoded viral proteins were able to elevate the expression of the reporter gene luciferase or not. Therefore human fibroblasts were electroporated mock and with reporter gene IVT-RNA coding for luciferase (Luc) and GFP alone or in combination with E3 and K3, B18R or E3, K3 and B18R together (EKB). After electroporation the cells were cultured and the reporter gene expression was monitored at four time points from 7 to 72 h. Additionally the incubation with the small molecule PKR inhibitor Im-13 was used as positive control for enhanced transgene reporter gene expression. In Fig. 3.16 A we observed that all curves peaked 24 h after electroporation. Nearly no difference in reporter gene translation can be detected between the sample which contained the reporter genes IVT-RNA and the sample

that was additional treated with B18R IVT-RNA. The sample representing E3 and K3 co-transfection showed a 2-fold increased reporter gene expression level after 24 h, but was rapidly diminishing thereafter. A remarkably high expression level was measured after the co-expression of all three anti IFN components (EKB) in the target cells. Here the peak induction was 3-fold higher than in the control sample and comparable to the sample incubated with the small molecule Im-13. From 24 to 72 h the reporter gene expression of the EKB treated sample remains at a high level. However, the luciferase expression is lower than observed after the incubation with 2 µM of the PKR inhibitor Im-13. In Fig. 3.16 B we observed the GFP expression intensity of samples either containing EKB co-expressed together with the reporter genes or not. We illustrated that the expression of the reporter gene GFP 24 h after transfection in combination with EK or EKB was twice compared to the reporter gene expressing sample. No differences in the expression intensity of GPF were observed between the reporter gene control and the sample which was additionally transfected with B18R. A treatment with 2 µM of the PKR inhibitor Im-13 resulted in an elevated GFP expression that was higher compare to the reporter gene containing sample without Im-13 treatment but lower than the EKB co-transfected sample.

Having analyzed the expression of two different IVT-RNA encoded reporter genes after the transfection either with or without the viral proteins EKB we achieve twice enhanced reporter gene expression. These results implied that the combinatory treatment of viral anti IFN IVT-RNA encoding for EKB is a promising tool for enhancing IVT-RNA-based transgene expression.

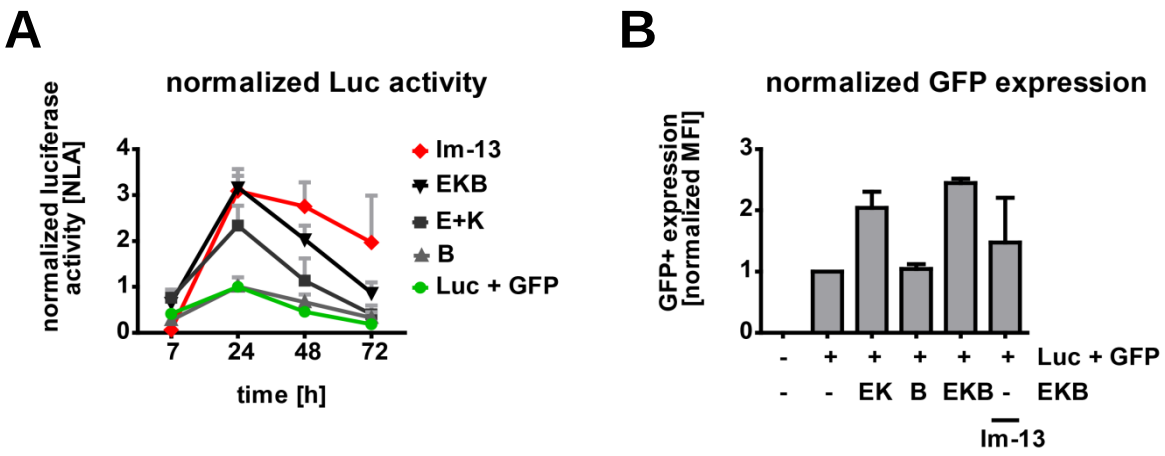

Figure 3.16 Reporter gene expression after co-expression with VACV anti IFN IVT-RNA Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, mock and with 8 ng/µl luciferase (Luc) and 40 ng/µl GFP reporter gene IVT-RNA alone or in combination with E3 and K3, B18R or E3, K3 and B18R together (EKB). The concentration of each viral IVT-RNA was 24 ng/µl. (**A**) The expression of reporter gene Luc was monitored from 7 to 72 h by luciferase assay. (**B**) 24 h after the electroporation the cells were analyzed for the expression value of the reporter gene GFP. Data are represented as mean + sd of three experiments. Data for Im-13 are shown from two experiments.

3.4.3.3 The EKB co-expression reduces the IFN response after IVT-RNA-based transfection

Since the co-expression of EKB IVT-RNA was beneficial for enhancing the reporter gene expression. These data raised the possibility that the IFN response, which we assessed in the next set of experiments, could be reduced in the presence of viral anti IFN proteins EKB. To this aim human fibroblasts were electroporated mock and with reporter gene IVT-RNA coding for luciferase and GFP alone or in combination with E3 and K3, B18R or all three proteins (EKB) together. Afterwards cells were cultured for 48 h followed by harvesting and subsequently analyzing the expression of IFNβ and the IFN target gene OAS1 by qRT-PCR. For data analysis, the sample of reporter gene transfected cells was set as reference for maximal induction of IFN response. As depictured in Fig. 3.17 one electroporation without RNA generates no induction of IFN response genes, whereas the reporter gene IVT-RNA samples represent the maximal induction of the IFN response genes IFNβ and OAS1. For the following samples the induction pattern was similar for both markers. We observed that E3 and K3 IVT-RNA alone were not able to reduce the IFN response. Interestingly, B18R IVT-RNA alone was able to reduce IFN response strongly. For OAS1 it was possible to suppress

the induction to background level. Nevertheless the best reduction of both markers was seen when all three IVT-RNAs were combined (Fig. 3.17).

In summary, these results confirm the hypothesis, that IVT-RNA from VACV IFN evasion proteins can reduce the IFN response of IVT-RNA transfected human cells.

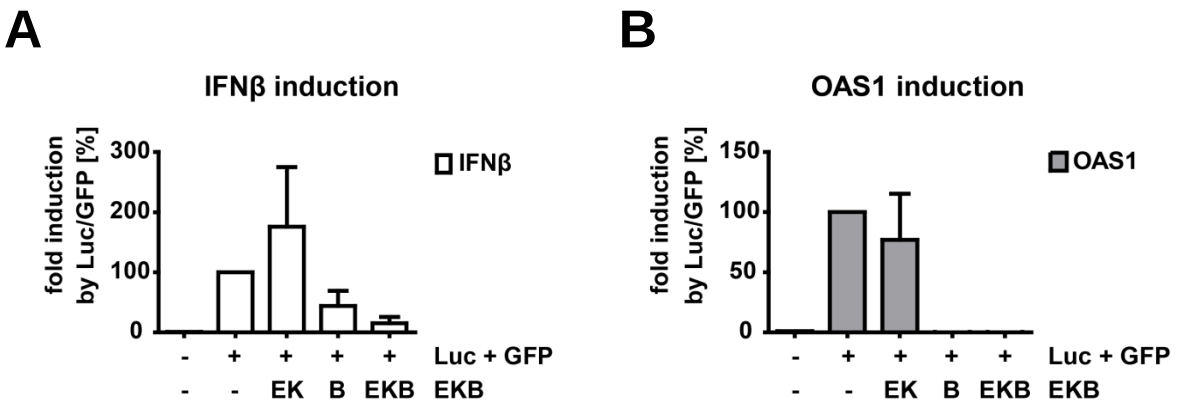

Figure 3.17 Reduction of IFN response with VACV anti IFN IVT-RNA after electroporation Human fibroblasts (CCDs) were electroporated mock and with 8 ng/µl luciferase (Luc) and 40 ng/µl GFP reporter gene IVT-RNA alone or in combination with E3 and K3, B18R or E3, K3 and B18R (EKB) together. The concentration of each viral IVT-RNA was 24 ng/µl. The expression of IFNβ (**A**) and the IFN target gene OAS1 (**B**) was performed after 48 h by qRT-PCR. For analyzing the data, the sample of reporter gene transfected cells were set as maximal induction of IFN response gene activation. Data are represented as mean + sd of three experiments.

In the aforementioned set of experiments it became apparent that viral proteins had a great potential for targeting PKR and subsequent signaling with high impact on protein expression. Next the PKR substrate phosphorylation status was analyzed.

3.4.3.4 Reduction of PKR substrate phosphorylation in the presence of EKB

Unfortunately, by direct antibody based detection of the PKR substrate phosphorylation we observed a variable background of eIF2α phosphorylation level. Due to mostly very weak signal intensity of the PKR substrate phosphorylation it was not possible to show the reduction of the eIF2α phosphorylation in the presence of the anti IFN proteins EKB on western blot. Therefore we had to establish an indirect method to judge PKR substrate phosphorylation level. Initially, we induced a huge eIF2α phosphorylation signal by including IVT-RNA encoding for WT-PKR. The overexpression of the WT-PKR in combination with reporter gene IVT-RNA ensures the artificially high eIF2α phosphorylation level. Next, we investigated whether the elevated PKR substrate phosphorylation level can be reduced by the co-expression of the viral anti IFN proteins EKB. Therefore human fibroblasts were electroporated mock, two controls without WT-PKR overexpression containing IVT-RNA coding for the reporter gene luciferase and luciferase combined with GFP respectively (lane 1 and 2). The third control contained IVT-RNA encoding the reporter genes luciferase and GFP in combination with IVT-RNA for WT-PKR (lane 3). In lane 4 reporter gene IVT-RNA and WT-PKR were co-electroporated and in lane 5 to 7 reporter gene IVT-RNA and WT-PKR were co-electroporated without E3 and K3, B18R and the combination of EKB as indicated. The cells were harvested 24 h after the electroporation and their protein lysates were analyzed for eIF2α phosphorylation status by western blot (Fig. 3.18). The equal loading of the gel was verified by western blot using specific antibodies for the detection of unphosphorylated eIF2α and β-actin which revealed comparable expression levels. We observed a weak phosphorylation of eIF2α after mock electroporation of the cells (lane 1). Furthermore the samples containing equal amounts of reporter gene IVT-RNA resulted in comparable induced eIF2α phosphorylation status as expected (lane 2 compared to lane 3). The IVT-RNA encoding for the reporter genes combined with WT-PKR overexpression showed a strong induction of PKR substrate phosphorylation and represented the control for maximal phosphorylation (lane 4). In the pictured experiment we observed that E3 and K3 alone can reduce eIF2α phosphorylation (lane 5) whereas B18R did not (lane 6). It should be mentioned that these results for the eIF2α phosphorylation status in lane 5 and 6 were not reproducible in all repeated experiments. Nevertheless within all three experiments we reproducibly observed a clear reduced eIF2α phosphorylation when E3 and K3 together with B18R were co-electroporated with the IVT-RNA encoding for WT-PKR and the reporter genes, which is marked in lane 7 by the black arrow (Fig. 3.18).

Altogether these experiments underline the huge potential using VACV anti IFN proteins EKB, since they are able to reduce the PKR substrate phosphorylation even after WT-PKR overexpression, resulting in an enhanced PKR level that is much higher than usually.

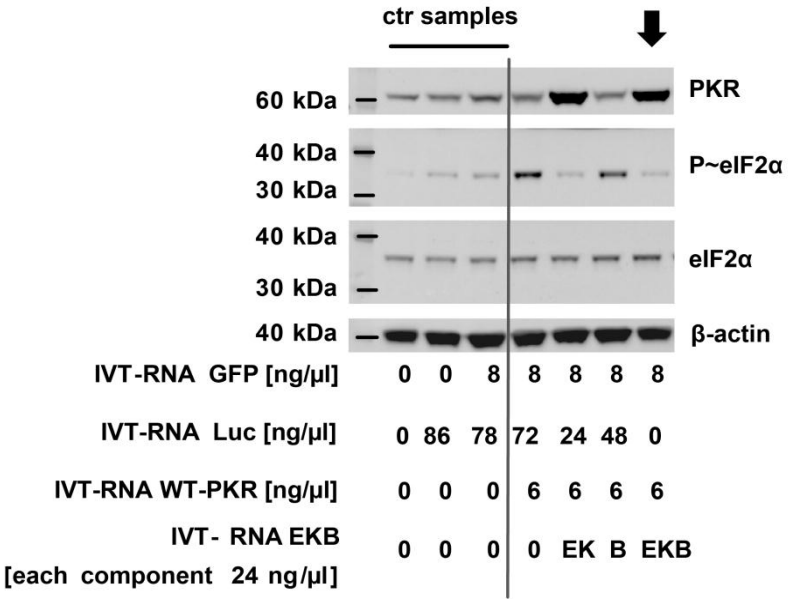

Figure 3.18 Reduction of eIF2α phosphorylation after WT-PKR overexpression Human fibroblasts (CCDs) were electroporated, using the ECM electroporation system, mock, two controls without WT-PKR overexpression containing luciferase (Luc) reporter gene and Luc and GFP reporter gene IVT-RNA and one control with Luc and GFP IVT-RNA overexpression in combination with 6 ng/µl wild type PKR (WT-PKR) overexpression. Luciferase was used to assimilate the IVT-RNA concentrations. For the other samples, reporter gene IVT-RNA and WT-PKR was co-electroporated with E3 and K3, B18R and the combination of EKB. Each compound was used at 24 ng/µl concentration. 24 h after electroporation cells were harvested and analyzed by western blot using specific antibodies. The antibodies recognize either the *RNA dependent protein kinase* (PKR), the *eukaryotic initiation factor alpha* (eIF2α), its phosphorylated form (eIF2α~P) or *beta-actin* (β-actin). One out of three blot data are shown.

In the last part, we had characterized, beside the usage of small molecule inhibitors of the PKR, three more strategies. Initially, we characterized PKR siRNA, secondly the overexpression of a dN-PKR was analyzed and at last, we investigated the possibilities of reducing IFN response by co-expression of viral anti IFN IVT-RNAs EKB. A summary of the results can be found in Tab. 3.1. The siRNA approach was excluded since the siRNA was able to decrease IFNβ expression but not the induction of OAS1. The overexpression of the dN-PKR was also ruled out since it leads to an upregulated IFN response upon IVT-RNA transfer. Only the combinatorial co-expression with EKB was successfully in both, reducing

the IFN response on the one hand and in enhancing protein expression on the other (Tab. 3.1). Notably the EKB combination minimized strongly the phosphorylation of the PKR substrate eIF2α. Thus, the co-expression of IVT-RNA encoding for EKB was the selected method for the next experiments to achieve repetitive IVT-RNA-based transgene expression.

Table 3.1 Summary of targeting PKR

inhibition of PKR activation by	transkription	IFN response measured by qRT-PCR (IFNβ/OAS1)	comment
small molecule (Im-13)	↑	n.a.	cytotoxic
PKR siRNA	↑	↓/→	
dN-PKR	↑	↑/↑	
viral proteins EKB	↑	↓/↓	

n.a. = not analyzed in the depictured experiments

3.5 Repetitive IVT-RNA transfer

In the next set of experiments we aimed to test our approach of EKB co-expression as possibility to achieve repetitive transfer of IVT-RNA.

3.5.1 The EKB co-transfer permits repetitive lipofection with unmodified IVT-RNA

For establishing an own protocol with permits repetitive transfections with unmodified IVT-RNA we decided to use lipofection instead of electroporation. Our decision was affected according to the highly efficient reprogramming protocol published by Warren and coworkers that was based on lipofection (Warren et al., 2010). Moreover, we aimed to circumvent the daily trypsinisation, which is accompanied by enzymatically and mechanically disruptions, to prevent further cell damage.

To assign the usage of EKB to lipofections we validated whether in presence of the anti IFN proteins EKB frequent IVT-RNA-based transfections can be performed. To this aim human fibroblasts were daily lipofected in antibiotic free CCD medium for four times with unmodified reporter gene IVT-RNA in the presence of EKB. In this set of experiments, GFP was used as transfection control and the reporter gene Luc was used to adjust the IVT-RNA concentration in each sample to 0.47 ng/ µl IVT-RNA. Using RNAiMax cells were lipofected mock or with reporter gene IVT-RNA combined with IVT-RNA coding for E3 and K3 or B18R or all three of them. All samples depictured in line 2 to 5 consist of unmodified IVT-RNAs. An additional sample consists of pseudouridine and 5 methylcytidine (ΨUm5C) modified IVT-RNA coding for luciferase and GFP. Those modifications were used in the successful reprogramming approach with IVT-RNA of Warren and coworkers in 2010 and 2012 (Warren et al., 2010; Warren et al., 2012). The experiment was analyzed 24 h after the last lipofection by measuring cellular viability by XTT assay (Fig. 3.19). As hypothesized we observed the strongest loss in viability in the sample with unmodified IVT-RNA alone (bar 2). The sample containing E3 and K3 (bar 3) showed approximately 50% of the viability compared to the mock lipofected control (bar 1). By contrast, the co-transfer of the IVT-RNA encoding only for B18R (bar 4) the cellular viability was low and comparable to the reporter gene sample (bar 2). Nevertheless the best results in maintaining the cellular viability after frequent transfection were achieved with the co-expression of EKB IVT-RNA (bar 5) which resulted in an 80% survival rate compared to the mock cells (bar 1). Moreover we observed a twice better viability of EKB treated cells (bar 5) compared to the sample which was

transfected with ΨUm5C modified reporter gene IVT-RNA (bar 6) (Fig. 3.19). Thus, there is increasing evidence that the co-expression of unmodified EKB IVT-RNA is most likely superior to the usage of modified IVT-RNA in maintaining cellular viability upon RNA-based treatment.

Furthermore cells were harvested 24 h after the fourth lipofection and prepared for IFN response analysis by qRT-PCR. Given that the IFN-markers were successfully downregulated after single transfection, we wondered whether the IFN-markers can be maintained at low expression levels even after frequent daily lipofections. Thereby we looked at the marker gene induction of IFNβ and OAS1 after co-transfection with and without EKB Fig. 3.20 (A and B). An additional sample consists of pseudouridine and 5 methylcytidine (ΨUm5C) modified IVT-RNA coding for luciferase and GFP. In the first lane of each picture the mock lipofected sample is shown in which no IFN response induction was observed. For IFNβ and OAS1 the strongest induction was detected in the samples where the unmodified IVT-RNA encodes for the reporter genes (lane 2). By contrast, the IFN response was significantly reduced in the presence of EKB for both markers (lane 3). Furthermore, no significant difference was detectable for both markers when the sample, transfected with unmodified IVT-RNA EKB (lane 3), was compared to the modified only the reporter genes containing sample (lane 4).

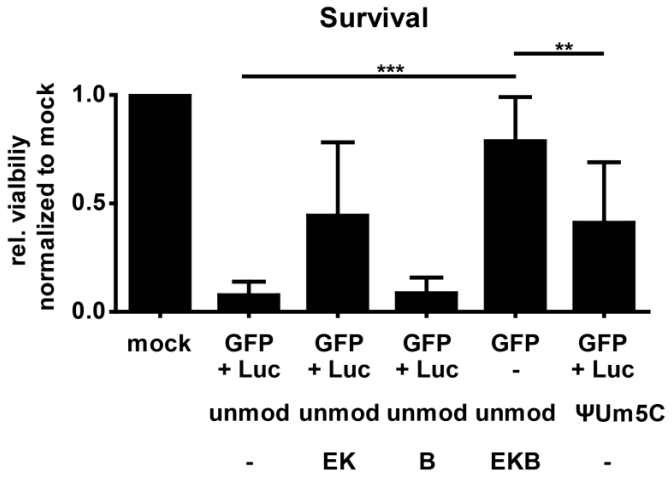

Figure 3.19 Survival after four lipofections Human fibroblasts (CCDs) were daily transfected for four times in antibiotic free CCD medium with unmodified (unmod) reporter gene IVT-RNA and viral anti IFN response IVT-RNA of E3, K3 and B18R (EKB) (bar 2 to 5). To compare the survival of cells transfected with unmodified or *pseudouridine* and *5 methylcytidine* modified (ΨUm5C) IVT-RNA bar 6 represents the control sample for modified reporter gene IVT-RNA. In total each IVT-RNA containing sample consits of 0.47 ng/µl IVT-RNA. The reporter gene GFP was used at a concentration of 0.27 ng/µl in all samples and luciferase (Luc) was used to fill up the amount of IVT-RNA with a maximal concentration of 0.2 ng/µl. The Luc concentration was reduced when anti IFN IVT-RNA encoding for EKB was added. E, K and B was used at a concentration of 0.07 ng/µl for each component. After four daily lipofections the viability was measured by XTT assay. Data were normalized to the maximal survival of mock lipofected fibroblasts. Data are represented as mean + sd of eight experiments; Significance niveau t-test: $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***

In summary, we observed that frequent lipofections of human fibroblasts with the co-expression of EKB, leads to a reduced induction of the IFN response markers IFNβ and OAS1 compared to a transfection without EKB. Furthermore the lipofection of unmodified EKB and reporter gene IVT-RNA results in comparable minimized IFN response induction as the use of ΨUm5C modified reporter gene IVT-RNA in the absence of EKB.

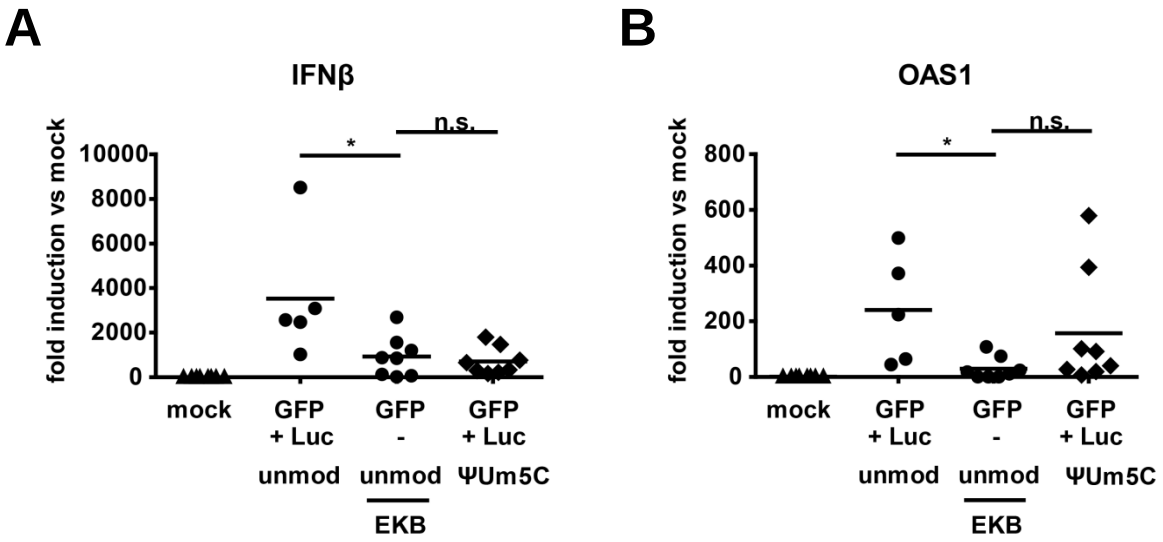

Figure 3.20 IFN response of modified and unmodified Luc and GFP IVT-RNA compared to unmodified reprogramming mix with EKB after four lipofections Human fibroblasts (CCDs) were daily transfected with unmodified (unmod) reporter gene IVT-RNA and viral anti IFN response IVT-RNA of E3, K3 and B18R (EKB) in antibiotic free CCD medium. As control sample *pseudouridine* and *5 methylcytidine* modified (ΨUm5C) IVT-RNA encoding for the reporter genes luciferase (Luc) and GFP without EKB was as well daily lipofected. In total each IVT-RNA containing sample consits of 0.47 ng/µl IVT-RNA. The reporter gene GFP was used at a concentration of 0.27 ng/µl in all samples and Luc was used to fill up the amount of IVT-RNA with a maximal concentration of 0.2 ng/µl. The Luc concentration was substituted completely when anti IFN IVT-RNA encoding for EKB was added. E, K and B was used at a concentration of 0.07 ng/µl for each component. After four daily lipofections cells were harvested and the IFN response was measured by qRT-PCR assay either for IFNβ (**A**) or for OAS1 (**B**). Data showed results of eight experiments, Luc and GFP samples were from five experiments (due to cell death) Significance niveau t-test: $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***, n.s. = not significant

3.5.2 Fibroblasts survive repetitive lipofection of IVT-RNA encoded reprogramming factors when EKB is co-transfected

Frequent IVT-RNA-based lipofections are feasible when EKB is co-transferred as shown in the experiment before. In the context of reprogramming we next focused on the question whether repeated lipofections of human fibroblasts using unmodified Yamanaka factors in the presence of viral IFN inhibiting proteins EKB can be performed. To this aim human fibroblasts were mock lipofected on four consecutive days, with unmodified reporter gene IVT-RNA and with modified or unmodified IVT-RNA coding for OSKMLN with and without EKB as indicated in Fig. 3.21. All probes were adjusted with Luc IVT-RNA to an end concentration of 0.47 ng/µl. After four lipofections cellular viability was analyzed by XTT

assay. The results of this set of experiments are pictured in Fig. 3.21 were we observed a 96% reduction of viability in the samples with unmodified IVT-RNA encoding for the reporter genes (bar 2) or for the reprogramming factors (lane 4). When the IVT-RNA coding for the reprogramming factors was pseudouridine and 5 methylcytidine modificated in average a 43% reduction in viability after four lipofections was measured (bar 3). The last two samples, in which the IVT-RNA mix was combined with IVT-RNA coding for anti IFN proteins EKB show impressively the considerable effect of EKB on cellular viability. The viability of the lipofected cells was only reduced for about 9% or 11% for modified or unmodified IVT-RNA respectively (bar 5 and 6) in the presence of EKB compared to the mock transfected sample. As depictured in Fig. 3.20 the breakthrough in enhancing the viability after four lipofections was reached by the co-transfection of EBK in combination with the reprogramming cocktail. Since then, we were able to elevate the cellular viability after transfection with unmodified IVT-RNA to 89% (lane 6) compared to the mock transfected sample (lane 1). Furthermore when we compared the viability of repetitive transfection with modified transcription factors (lane 3) with the sample lipofected with unmodified reprogramming factor and EKB IVT-RNA we saw improved viability.

The viability of the cells was consistent with microscopic picture of the samples captured after four lipofections (Fig. 3.21 B exemplary photos). Only few cells were left in the samples where unmodified IVT-RNA coding for the reporter genes or the six reprogramming factor was transfected. Furthermore the cells seem to alter their formation to a more compact packaging in the sample were the cells were transfected four times with unmodified reprogramming factors and EKB IVT-RNA. This altering was not seen in the mock lipofected cells or in the modified transcription factor transfected sample with EKB, where as well a high confluence was reached. This might indicate that the presence of unmodified TF in combination with EKB may lead to expression changes in the transfected cells.

Thus, we could show within this set of experiments that, in our hands, it was possible to overcome the IVT-RNA dependent cell death within frequent IVT-RNA-based lipofections. Through the usage of IVT-RNA encoding for EKB daily transfections of the reprogramming cocktail consisting of IVT-RNAs coding for OSKMLN were feasible.

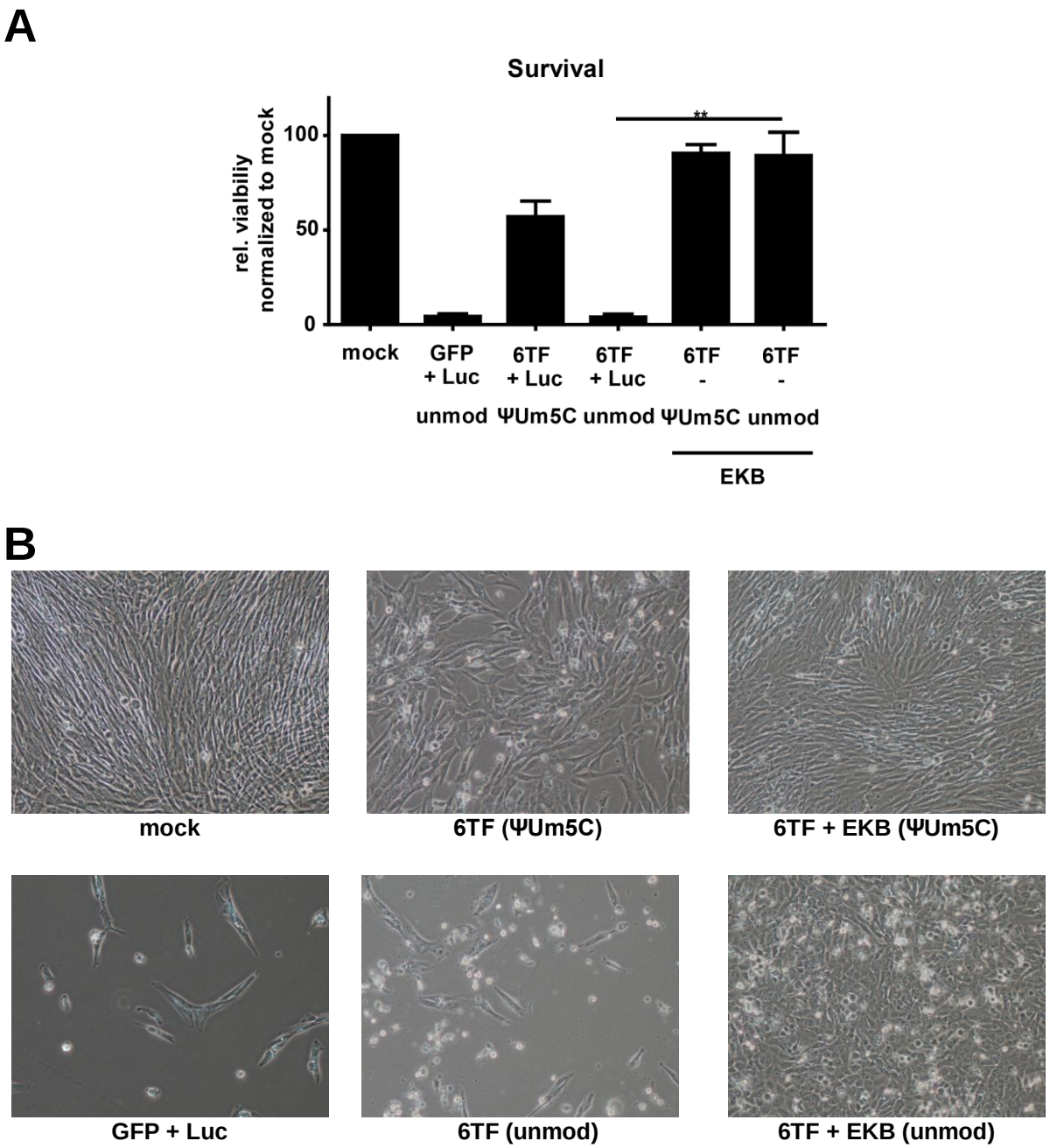

Figure 3.21 Viability after four repetitive lipofections Human fibroblasts (HFFs) were daily transfected in Nutristem medium with 0.47 ng/µl IVT-RNA consisting of unmodified (unmod) or *pseudouridine* and *5 methylcytidine* modified (ΨUm5C) IVT-RNA coding for the six reprogramming associates transcription factors OCT4 (O), SOX2 (S), KLF4 (K), c-MYC (M), LIN28 (L) and NANOG (N) (6TF) with and without the viral anti IFN response IVT-RNA of E3, K3 and B18R (EKB). The reporter gene control was lipofected with 0.27 ng/µl GFP and 0.2 ng/µl luciferase (Luc). In samples with the transcription factor cocktail 0.27 ng/µl OSKMNL IVT-RNA was used instead of the GFP. The luciferase either modified or not was used to reach an IVT-RNA endconcentration of 0.47 ng/µl. In samples 5 and 6 the luciferase IVT-RNA was substituted by 0.2 ng/µl of the anti IFN IVT-RNA EKB. (A) After four lipofections the viability was measured by XTT assay. Data were normalized to the maximal survival of mock lipofected fibroblasts. (B) Additionally the cell shape

was documented via microscope (Magnification 40X). Data are represented as mean + sd of three experiments. Representative pictures were shown after four repetitive lipofections. Significance niveau t-test: $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***

3.5.3 Minimized IFN response correlates with the induction of stem cell marker after four lipofections of IVT-RNA encoding for reprogramming factors and anti IFN proteins EKB

Since we have observed that we could transfect unmodified IVT-RNA of reprogramming factors frequently when co-transfered with viral proteins to combat the IFN response, we aimed to use this approach for reprogramming of human cells. In a subsequent step we therefore tested whether an induction of stem cell markers after the lipofection with IVT-RNA coding for reprogramming factors and anti IFN proteins EKB can be achieved.

To this aim human fibroblasts were mock lipofected on four consecutive days, with modified IVT-RNA coding for OSKMLN and with modified and unmodified IVT-RNA coding for OSKMLN in the presence of EKB as indicated in Fig. 3.22. All probes were adjusted with Luc IVT-RNA to an end concentration of 0.47 ng/µl. One day after the last transfection cells were harvested and analyzed by qRT-PCR for the induction of the IFN response markers IFNβ and OAS1 (Fig. 3.22 A and B). The cells were mock lipofected (lane 1), with all six transcription factors (OSKMLN) either with (lane 3, 4) or without EKB (lane 2). As indicated the IVT-RNA in each sample consisted of unmodified (lane 4) or pseudouridine and 5 methylcytidine (ΨUm5C) modified (lane 2, 3) IVT-RNA. We saw that the co-transfection of EKB in combination with OSKMLN (lane 3, 4) results in a reduced IFN response compared to the sample without EKB (lane 2). With EKB nearly no differences were detectable for the downregulated IFN response in the modified (lane 3) or unmodified (lane 4) samples. The usage of pseudouridine and 5 methylcytidine modified IVT-RNAs coding for the reprogramming factors (lane 2) still resulted in IFNβ and OAS1 induction and by the co-expression of unmodified EKB in combination with the transcription factors (lane 4) it was possible to maintain the minimized IFN response induction, indicating that the use of modified IVT-RNA is dispensable in the presence of EKB.

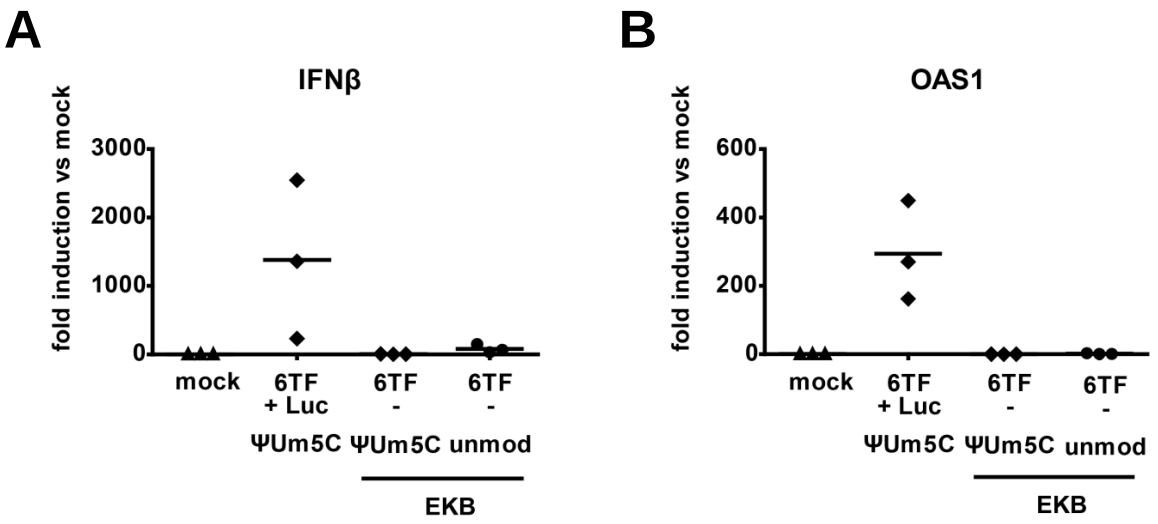

Figure 3.22 Interferon marker inductions after four lipofections Human fibroblasts (HFFs) were harvested after four daily transfections with 0.47 ng/µl IVT-RNA in Nutristem medium. Thereby unmodified (unmod) or *pseudouridine* and *5 methylcytidine* modified (ΨUm5C) IVT-RNA encodes for the six reprogramming factors OCT4, SOX2, KLF4, c-MYC, LIN28 and NANOG (6TF) with or without the modi- or unmodified viral anti IFN response IVT-RNA coding for E3, K3 and B18R (EKB). The sample without EKB was filled up with 2 ng/µl luciferase (Luc) whereas in EKB containing probes Luc was substituted by 2 ng/µl EKB IVT-RNA. All samples were analyzed one day after the last transfection for the induction of IFN response genes IFNβ (**A**) and OAS1 (**B**) by qRT-PCR. Data were normalized to mock lipofected sample. Data from three experiments are shown.

These data showed that the effect of EKB in minimizing the IFN response is at least equal to the usage of modified IVT-RNA. Therefore we turned, as aforementioned, to the analysis of the stem cell marker induction due to transfection of human fibroblasts with reprogramming factors and EKB. The cells for analyzing the induction of stem cell associated markers by qRT-PCR were as well harvested one day after the last transfection with the afore mentioned IVT-RNA end concentration of 0.47 ng/µl. Thereby we analyzed mock lipofected samples and samples transfected with modified IVT-RNA coding for the six transcription factors (6TF) OSKMLN with and without EKB (lane 3, 2). Lane 4 represents the marker induction of the transfection with unmodified IVT-RNA coding for OSKMLN and EKB (Fig. 3.23). We observed that some pluripotency associated markers were induced. Interestingly, it has to be pointed out that a marker induction of TERT, DPPA4 and GDF3 was only detected in samples transfected with unmodified IVT-RNA the reprogramming cocktail OSKMLN and the viral anti IFN proteins EKB. Thereby we determined that the induction was significant for

TERT and DPPA4. However, the markers LIN28 and REX1 were not significantly upregulated at the analyzed time point.

In summary, we observed a stronger induction of stem cell markers in samples lipofected with unmodified reprogramming IVT-RNA compared to pseudouridine and 5 methylcytidine modified IVT-RNA when co-transfected with EKB. Therefore we hypothesized that the expression of unmodified RNA might be more efficient compared to modified RNA in presence of the viral proteins. Furthermore the IFN response remained in the presence of EKB downregulated.

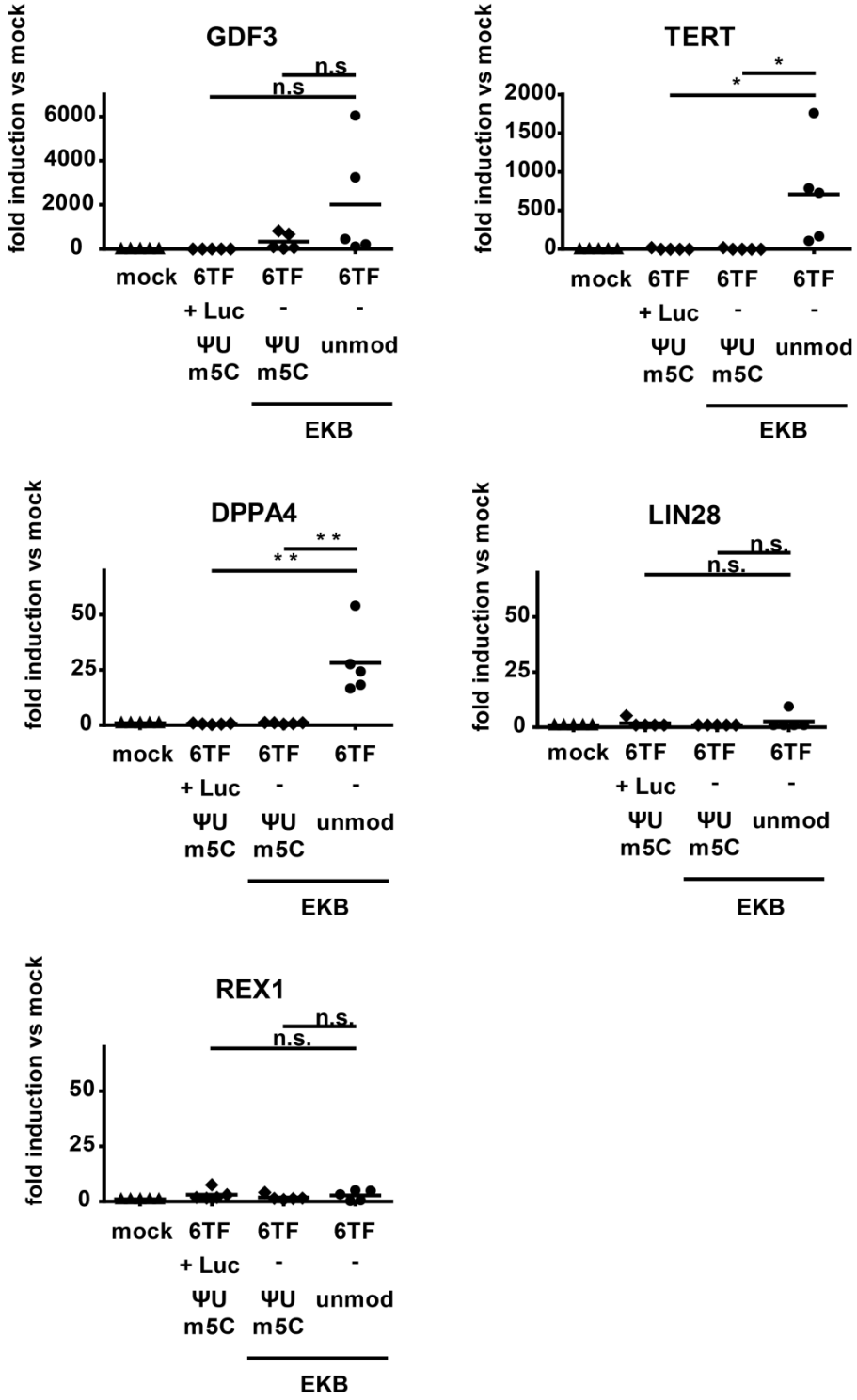

Figure 3.23 Stem cell marker inductions after four lipofections Human fibroblasts (HFF) were harvested one day after four daily transfections in Nutristem medium with 0.47 ng/µl IVT-RNA and analyzed by qRT-PCR for the induction of pluripotency associated markers. As indicated the samples consists of either unmodified (unmod) or *pseudouridine* and *5 methylcytidine* modified (ΨUm5C) IVT-RNA. The IVT-RNA encodes for the *six transcription factors* (6TF) OCT4, SOX2, KLF4, c-MYC, LIN28 and NANOG and was lipofected at a concentration of 0.27 ng/µl with and without 0.2 ng/µl IVT-RNA coding for the anti IFN proteins E3, K3 and B18R (EKB). Samples without EKB were filled up with IVT-RNA encoding for luciferase. Data were

normalized to mock lipofected sample. Data from five experiments are presented. Significance niveau t-test: $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***; n.s. = not significant

3.5.4 Enhanced expression level of transcription factors in the presence of EKB after repetitive lipofections using unmodified IVT-RNA

We aimed to generate experimental evidence to underline, that unmodified IVT-RNA coding for reprogramming factors is translated with higher effectiveness than modified IVT-RNA in the presence of the anti IFN proteins EKB. Therefore human fibroblasts were transfected for three times, using either modified or unmodified IVT-RNA, encoding for reprogramming transcription factors (OSN), the reporter gene GFP and EKB. In all samples an equal IVT-RNA end concentration of 0.47 ng/µl was used. We analyzed the expression of the transcription factors by specific intracellular FACS staining as described in material and methods. The GFP expression was used as additional transfection control. Data depictured in Fig. 3.24 showed exemplary the results of the intracellular FACS staining. No signals were detected in the mock transfected samples depictured on the left. In the middle and on the right the signals of NANOG, OCT4 or SOX2 expression after the transfection with unmodified or modified IVT-RNA is shown. Thereby we observed the hypothesized enhanced protein expression after unmodified IVT-RNA transfection. The highest protein expression was recorded for OCT4 were the signal was 25-fold increased compared to the modified IVT-RNA transfected sample. For NANOG a 10-fold difference was detected and the SOX2 induction was five times stronger than in the samples transfected with modified IVT-RNA. Together with EKB co-transfection an elevated protein expression was provided upon frequent unmodified IVT-RNA transfer.

In summary we finally found an application method for repetitive transfection of unmodified IVT-RNA. This was achieved in the presence of the viral proteins E3, K3 and B18R and is characterized by a good viability of the targeted cells, a minimized IFN response and an enhanced translation of the transgenes. When a reprogramming cocktail is used for frequent transfection we can detect an upregulation of some stem cell related markers, implying that unmodified IVT-RNA is characterized by higher translation efficiency. Furthermore, we were able to detect a stronger expression of selected transcription factors with unmodified than with pseudouridine and 5 methylcytidine modified IVT-RNA. Thus, the elevated translation and stem cell marker induction might correlate. By combating the IFN response using the co-transfection of EKB a major obstacle for unmodified IVT-RNA-based reprogramming will be solved.

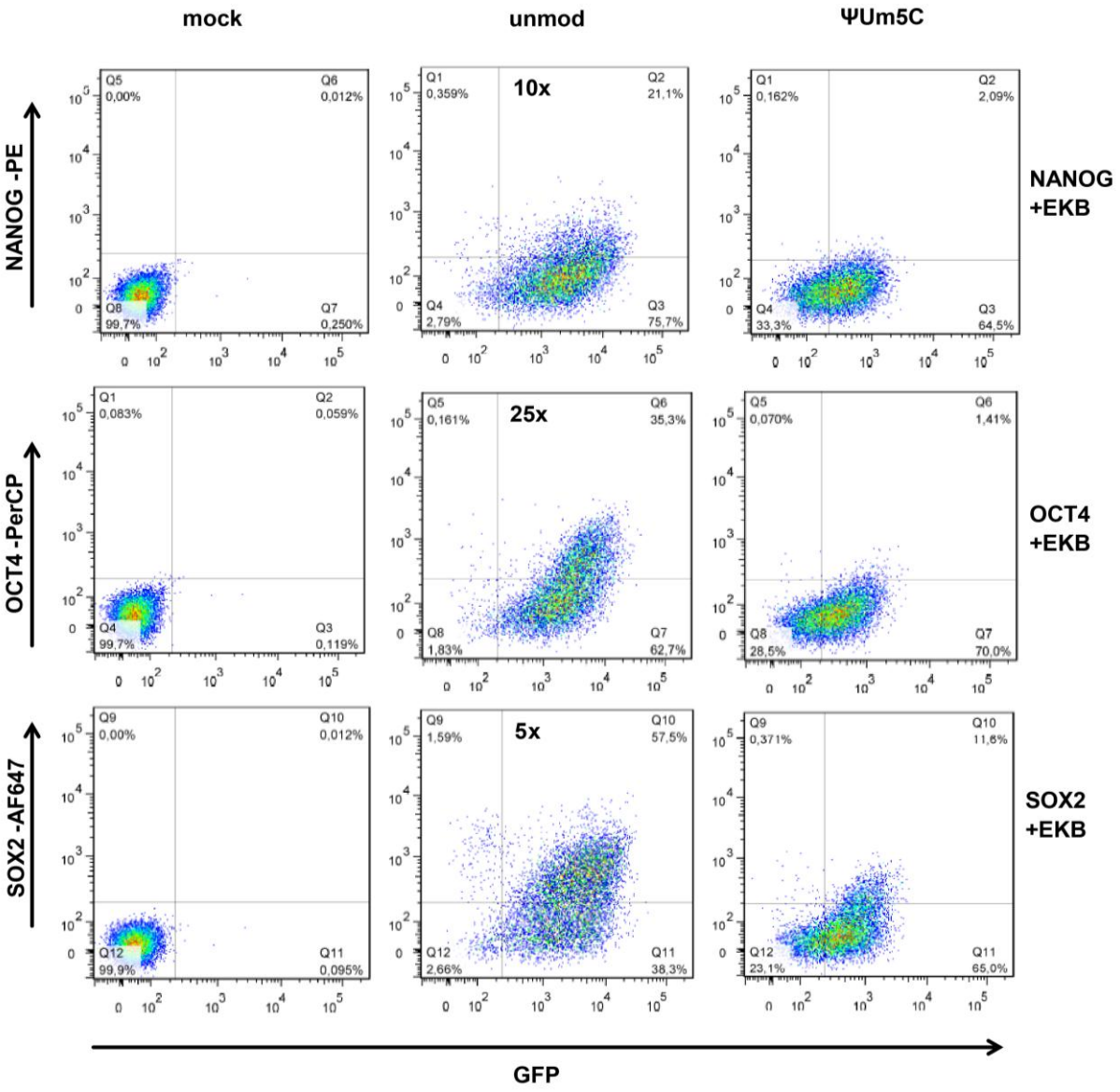

Figure 3.24 Expression levels of transcription factors in the presence of viral proteins after three repetitive lipofections Cells were intracellular stained for OCT4, SOX2 and NANOG (OSN) expression level after three daily transfections with OSN and GFP with an end concentration of 0.47 ng/µl IVT-RNA. Thereby either unmodified (unmod) or *pseudouridine* and *5 methylcytidin* modified (ΨUm5C) IVT-RNA of reprogramming factor mixture OSN and viral anti IFN response inhibitors E3, K3 and B18R (EKB) IVT-RNA were lipofected. In sum the three anti IFN IVT-RNAs were used at a concentration of 0.2 ng/µl as well as the reprogramming factor mixture of OSN. IVT-RNA coding for GFP was used at a concentration of 0.07 ng/µl in each sample. To analyze the presence of the transcription factors each factor was coupled to a specific fluorochrome thus signals can be distinguished due to different filters. NANOG was indicated by PE, OCT4 by PerCP and SOX2 by Alexa Fluor 647. Additionally the signal intensity of the reporter gene GFP is illustrated by its position on the x-axis. Data from one experiment were shown.

4 Discussion

4.1 IVT-RNA-based transfections

4.1.1 IVT-RNA-based transgene expression of reprogramming factors in human fibroblast

The first reprogramming of human fibroblasts was performed by retroviral transduction of the human reprogramming factors OCT4, SOX2, KLF4 and c-MYC (Takahashi et al., 2007). Generally Oct4 is seen as one of the master reprogramming factors and essential for the correct development in the embryo (Pesce and Schöler, 2001). Its absence results in the development of a blastocyst without a pluripotent inner cell mass (ICM) (Nichols et al., 1998). We aimed to achieve the generation of induced pluripotent cells by using messenger IVT-RNA, which encodes for each of the above mentioned reprogramming factors. Since transfected RNA is naturally degraded inside transduced cells, the expression of encoded proteins is limited. We were interested in the presence of the transfected Yamanaka reprogramming factors OCT4, SOX2, KLF4 and c-MYC and analyzed them to find their mRNA half-life. From our experiment the calculated IVT-RNA half-lives of the four transcription factor were similar, ranging from 10 to 12 h. Due to the use of optimized IVT-RNA, including codon optimization, equal poly A tail length and cap structure, we assume a higher stability than the natural transcription factors. The longer mRNA half-life was confirmed compared to the mammalian mRNA half-lives (Oct4: 7.4 h, Sox2: 1.09 h, Klf4: 1.91 h and c-Myc: 0.98 h Sharova et al., 2009). We assume that the use of optimized synthetic mRNA is most likely the reason why the half-lives of the different TFs assimilated.

Next, we analyzed the protein expression of our reprogramming factor coding IVT-RNAs. In our hands the protein expression of the reprogramming factor proteins differs for several hours. Whereas 24 h after transfection all transcription factors were detectable in the protein lysates of the human fibroblasts, the signal intensity of the human protein OCT4 after 48 h was the only one remaining clearly above the detection limit. In the literature the protein half-life of Oct4 was estimated to be 5 to 8 h (Wei et al., 2007; Plachta et al., 2011) or only 1.5 h (Saxe et al., 2009). For KLF4 the protein half-life was determined to be 2 h (Chen et al., 2005) and the protein half-life of MYC was reported be relatively short being around 13 to ~30 min (Hann and Eisenman, 1984; Ramsay et al., 1984; Ramsay et al., 1986; Sears et al., 2000). The only information for the half-life of the Sox2 protein I found was the calculated half-life of murine Sox2 to be 20.5 h (Luo et al., 2011). However, our results indicated that

the SOX2 protein has a shorter half-life. A reason for the different stability is probably due to different cellular systems, or maybe the modeling approach does not correctly represent the kinetics. In our hands it seems that OCT4 and not the SOX2 protein is the most stable one. To provide the cell with reprogramming factors, in order to maintain at least a low level of the master reprogramming factor OCT4, a transfection every two days was reasoned.

Despite a limited expression of reprogramming factors upon one electroporation with IVT-RNA, we found that some pluripotency factors were upregulated to some extent. This early induction of some reprogramming associated markers after one electroporation was expected, because it was shown that within a single transient transfection the gene regulation can be affected (Escudero et al., 2010; Tavernier et al., 2012). The upregulation of TERT, DPPA4 and GDF3 in our experiments shows that an upregulation of pluripotency associated markers is possible when cell were transfected with IVT-RNA encoding for reprogramming factors. For the generation of iPS cells, the induction of TERT is especially helpful since it was shown before in a murine model that the efficiency of iPS generation out of fibroblasts decreases in telomerase-deficient cells (Marion et al., 2009). Furthermore in iPS colonies of human cells it was shown that more than six out of eight embryonic stem cell markers were upregulated in 90% of telomerase unregulated clones (Masaki et al., 2007). Therefore, an upregulation of TERT, as observed in the experiments after a single electroporation of IVT-RNA encoded reprogramming factors, indicates that profound genetic changes can be initiated. Unfortunately the upregulation was neither robust nor uniform between the experiments. Additionally, the highly variable induction of some markers was seen only up until two to three days after transfection. Furthermore, a clear and reproducible upregulation of other reprogramming markers such as endogenous NANOG or OCT4 were absent. In consideration of a published reprogramming time course, where the reprogramming factors were provided constantly by retroviral vectors, the lack of endogenous NANOG or OCT4 after only one transfection was expected. The reactivation of Nanog and Oct4 takes a long time, even in continuous presence of the reprogramming factors, where both markers were weakly detectable after 16 days (Brambrink et al., 2008). Furthermore the IVT-RNA-based reprogramming approach of Warren et al. underlined that important markers like OCT4, SOX2 and NANOG were upregulated in the generated iPS cells after frequent transfections (Warren et al., 2010). The induced markers were also found after retroviral produced iPS cells were endogen murin Oct4 occurs at day 10, Sox2 was upregulated at day 14 and Nanog emerges at day 20 (Xu et al., 2011).

We concluded that frequent transfections were necessary to provide sufficient expression of the reprogramming factors in order to generate iPS cells.

4.1.2 IVT-RNA transfection results in an upregulated IFN response

We performed repetitive IVT-RNA-based transfections in order to obtain long and stable overexpression of reprogramming factors. In experiments with more than one IVT-RNA-based transfection, we observed massive cell death. The number of cells was higher in the mock electroporated control than in the IVT-RNA containing samples, where we detected a reduction of the overall cells. On the one hand the repetitive transfection of IVT-RNA is required for long term IVT-RNA-based protein expression, but on the other, the observed cell death is apparently counterproductive. Technical procedures like harvesting of cells or several washing procedures, which cannot be avoided, are one reason for the observed reduction of the cell numbers. The mock transfected cells were able to balance the cell loss by washing procedures due to proliferation and even increase the cell number. Nevertheless, the high cell death in IVT-RNA containing samples cannot be explained by technical loss alone and we hypothesized that the cellular defense mechanisms were upregulated in the transfected cells. As described in the introduction, cells are able to sense foreign RNA and react with an upregulated IFN-response to transfected messenger IVT-RNA that is similar to the defense of the cell against viruses (Takeuchi and Akira, 2010; Sen et al., 2011). Indeed, I was able to verify that upon transfection with IVT-RNA the IFN response markers were upregulated. The cell death within the first experiments for repetitive RNA transfer underlined the cellular defense. Since the messenger IVT-RNA acts as pathogen associated pattern and induced cellular defense as verified by the set of upregulated IFN response markers we concluded that the IFN response is a major obstacle for frequent IVT-RNA-based transfection which will hamper transgene expression. For our optimized IVT-RNA-based transgene expression, we aimed to circumvent the cellular defense in order to achieve enhanced translation, even after frequent transfections by targeting the PKR. In accordance with the assumption of an upregulated IFN response, we found a block of translation. The stalled reporter gene translation was due to the activation of PKR, which was confirmed by the phosphorylation of the PKR substrate eIF2α. Thus, the question how to combat the IFN response came up.

4.2 Inhibition of the PKR for minimizing the induction of the IFN response after IVT-RNA transfection

The kinase PKR was hypothesized to be one of the key players for sensing the RNA and the initiation of the non-self RNA directed cellular defense. This idea was underlined by the results of the experiments analyzing the induction of the IFN response markers or the translation inhibition. Since we generated experimental evidence for PKR identification as a key kinase in the induction of the IFN response, we targeted the PKR by different approaches such as small molecules, PKR siRNA, dN-PKR overexpression or viral anti IFN proteins. Afterwards we analyzed the impact of the putative PKR inhibitors on the enhanced transgene expression and their effect for avoiding the induction of the IFN response markers upon IVT-RNA-based transfection.

4.2.1 Inhibition of the PKR with small molecules increases the transgene expression but can lead to cell death

In order to reduce the IFN response we inhibited PKR signaling by diverse approaches. First we decided to use small molecules because several of them were known to interact and inhibit PKR (Jammi et al., 2003; Bryk et al., 2011). Two promising inhibitors were Im-13 and 2-AP. We decided to choose Im-13 since it is known that 2-AP is mutagenic when incorporated into the DNA (Sowers et al., 1986). Im-13 was found by a component screening of ATP-binding site addressing inhibitors (Jammi et al., 2003). Indeed, when we incubated cells after IVT-RNA transfection with Im-13 we found a reduced IFN response and an enhanced translation. This was a further indication that an activated PKR is the reason for reduced protein translation after IVT-RNA-based transfections. Unfortunately we noticed in long term experiments that Im-13 was cytotoxic at its best concentration of 2 µM, prohibiting its later use. According to several PKR knockout experiments in mice (Yang et al., 1995; Abraham et al., 1999; Balachandran et al., 2000) leading to viable offsprings, a specific inhibition of the PKR should not be toxic. Therefore the detected cytotoxicity is most probably a side effect of Im-13 and not due to PKR inhibition itself. We found the best stabilization of reporter gene expression generated with 2 µM Im-13. This concentration differs compared to the information given in the data sheet (Merck product information) and the original paper (Jammi et al., 2003). In both references the inhibition of RNA-induced autophosphorylation was declared with IC_{50} of 210 nM and the IC_{50} for inhibiting the block for translation was said to be 100 nM or 1 µM respectively. Thus, our best concentration was 10-fold higher

compared to the published $IC_{50.}$ With lower concentrations the effect of stabilization of the transgene expression was not that distinct. A reason for the differences of the effective dose might be that the original paper analyzed the inhibitory effect in a cell free system (Jammi et al., 2003). Thus, IC_{50} values in a cellular system might differ for the small molecule. Additionally, the effect of Im-13 could be cell line depended meaning that the inhibitor could be more effective in another cellular background. In our experiments we are dependent on cell based systems where the effect of the reduction of the PKR autophosphorylation might be seen later due to amplification of cellular signaling. This means that more than 50% of the PKR autophosphorylation must be avoided in order to achieve a doubling of the translation efficiency, since the effect of PKR autophosphorylation and enhanced translation appears to be not a linear function.

To verify the results of enhanced transgene expression after IVT-RNA transfer when the cells were incubated with Im-13, we additionally tested 2-AP which has previously been described as component targeting the PKR (Tiwari et al., 1988; Hu and Conway, 1993). We observed the highest translation with 20 mM 2-AP, fitting to the *in vitro* and *in vivo* reports (Hu and Conway, 1993). In our hands cellular toxicity in the experiments with 2-AP was not observed. During the first three days Huang and Schneider did not find cytotoxicity but they mentioned cellular degeneration when cells were exposed to 2-AP for more than 3 days (Huang and Schneider, 1990). For further investigations we excluded 2-AP because of its mutagenic abilities (Sowers et al., 1986) and additional known off-target effects such as the upregulation of the mouse hepatic cytochrome P4502A5 (CYP2A5), an enzyme which is associated with liver tumors and liver cell damage (Posti et al., 1999). Furthermore the inhibition of c-fos and c-myc gene expression (Zinn et al., 1988), the inhibition of leptin signal transduction (Hosoi et al., 2006), the inhibition of mRNA splicing of tumor necrosis factor α (Jarrous et al., 1996) and the induction of cell cycle arrest in the G2 phase accompanied by increased gene expression (Maio et al., 1995) are described. One can speculate that the PKR inhibitor Im-13 could react similarly to 2-AP and that the induction of the cell cycle arrest might have an impact of the detected toxicity. Furthermore the PKR is important for several regulatory functions, ranging from growth control (Zamanian-Daryoush et al., 1999; Barber, 2005) to cellular defense (Balachandran et al., 2000; Pichlmair and Reis e Sousa, 2007; Sadler and Williams, 2008). An irreversible inhibition or destruction of PKR might have negative impact on the targeted cells, although viable knockout mice indicate that this is rather unlikely. The observation of Im-13 reversibility was therefore beneficial, but finally due to cytotoxicity, the

usage of Im-13 is not applicable for our aspired frequent transfection, e.g. in reprogramming experiments, where probably more than one week exposure has to be tolerated by the cells.

4.2.2 The knockdown of PKR by PKR siRNA increases the transgene expression and reduces IFNβ induction after IVT-RNA-based transfection

As an alternative to the small molecule Im-13, one further promising way to eliminate the PKR was the use of PKR siRNA because it represents a common and reliable method of knocking down specific targets (Elbashir et al., 2001). Usually siRNA itself is not immunogenic (Kim et al., 2005), but sometimes the activation of IFN response was detected (Sledz et al., 2003; Puthenveetil et al., 2006). Therefore we first confirmed that our chosen siRNA pool actually reduces the PKR level and does not induce IFN response. We verified that, the applications of reporter gene IVT-RNA in combination with PKR siRNA treatment reduced the IFNβ response and increased reporter gene expression. Unfortunately the reduction of the IFN response was different between the monitored markers. Whereas PKR siRNA treatment clearly reduced an IFNβ response, the beneficial effect on the IFN downstream target OAS1 was weaker and more variable. Since the IFN induction was not completely abrogated by PKR siRNA, residual amounts of IFN have most likely still induced the IFN downstream target OAS1 in these experiments. The absence of a reliable IFN response reduction underlined that PKR siRNA is not sufficient for the elimination of the PKR mediated IFN response. Although we achieved a knockdown on PKR mRNA level of approximately 90%, it seems that the remaining PKR molecules achieved an induction of the IFN response. Maybe one could speculate that the IFN upregulation could also be supported by alternative activation pathways induced by the cytoplasmic RNA sensors like the OAS (Sadler and Williams, 2008), RIG-1 or MDA-5 (Barral et al., 2009). Thus, we hypothesized that for long term experiments, the induction of OAS1 indicates that the IFN response is not avoided completely and the remaining cellular defense ends in an anti-viral state of the targeted cells. This would counteract long term transgene expression and we decided to analyze an alternative strategy of inhibiting the key kinase PKR in the IFN response upregulation after IVT-RNA-based transfection.

4.2.3 The overexpression of a dN-PKR for inhibiting the PKR activation increases the transgene expression but also induces the IFN response markers after IVT-RNA-based transfection

An alternative to the PKR knockdown was the overexpression of a dominant negative PKR (dN-PKR). Again, we analyzed the impact on IVT-RNA-based reporter gene expression and the induction of the IFN response markers IFNβ and OAS1. The dN-PKR was generated by a point mutation leading to a catalytically inactive mutant due to a disrupted ATP binding site (Katze et al., 1991; Taylor et al., 1996). We hypothesized that an excess of the inactive mutant will lead to competition for IVT-RNA with the wild type PKR, resulting in a reduced amount of active PKR leading to a minimized IFN response accompanied by an elevated transgene expression (Terenzi et al., 1999). Indeed, the analysis of the IVT-RNA encoded reporter gene expression showed a slightly enhanced reporter gene translation. Unfortunately the IFN response was upregulated compared to samples without the dN-PKR overexpression. Compared to the results described in literature, where a stable PKR deficient cell line was used, we could confirm the elevated reporter gene expression but failed to reproduce the reduced IFN response (Taghavi and Samuel, 2012). As a reason for the upregulated cellular defense we assume that the higher amount of total RNA in the dN-PKR containing sample compared to the reporter gene expression sample only is most likely not the reason, since we would then expect a translational shutdown and not a slightly enhanced reporter gene expression. Perhaps the IFN response marker upregulation might occur faster than the translational shutdown. For the PKR activation, a process termed transphosphorylation might contribute to the phosphorylation by a mostly ATP depended exchange of a phosphate group. Another way of inducing the interferon response describes a model for the dimerization and activation of the PKR, in which WT- and dN-PKR can form heterodimers that were able to phosphorylate the PKR substrate eIF2α (Romano et al., 1995). In the proposed model, based on experiments with yeast, a heterodimeric PKR consisting of WT and dN-PKR is catalytically active, leading to the IFN response induction. If this theory holds true for a successful downregulation of the IFN response the total amount of dN-PKR must be further enhanced. However, we found a hint indicating that further dN-PKR overexpression for successful PKR downregulation is questionable. It was reported that a PKR 296 kinase independent activation of the NF-κB pathways might occur via PKR binding and subsequent activation of the IKK complex (Chu et al., 1999; Bonnet et al., 2000) and this was especially seen after dN-PKR overexpression (Chu et al., 1999).

All in all for frequent transfections we expected an upregulation of the IFN response that will counteract transgene expression. Thus, we decided to skip the analysis of dN-PKR interactions resulting in an enhanced translation but also in induced IFN response markers. We concluded that dN-PKR overexpression is not a feasible approach and began to search for viral immune modulating proteins, which combatted the cellular IFN response.

4.2.4 The co-transfection of IVT-RNA encoding for viral anti IFN modulators increases the transgene expression, decreases the IFN induction and avoids PKR substrate phosphorylation

Neither the small molecule, the PKR siRNA nor the overexpression of a dominant negative PKR was feasible to prevent both the upregulation of IFN response markers on the one hand and to enhance the reporter gene expression after IVT-RNA-based transfection on the other. Therefore we searched for a model that is successful in nature and we decided to include viral inhibitors in our experiments. Viruses have evolved multifaceted mechanisms to escape the host defense (Kotwal et al., 2012) and since the VACV proteins are well characterized, the effect of the proteins E3, K3 and B18R to enhance RNA-based transgene expression and to reduce the IFN response to messenger IVT-RNA was analyzed. The proteins E3 and K3 are known to act intracellular and inhibit the PKR activation. E3 avoids the ds RNA binding to the PKR (Chang et al., 1992) and K3 acts as pseudosubstrate for the kinase (Davies et al., 1992), whereas the B18R protein operates outside the cells as decoy receptor, interrupting the IFNβ signaling pathway by quenching the IFNs (Colamonici et al., 1995; Symons et al., 1995). In my opinion, the inhibition of our major target PKR by an intra- and an extracellular approach was a very good strategy because it based on a successful, two-phase system. Thereby the IFN response induction seems to be blocked effectively, since in a first intracellular step the PKR activation is hampered by two different viral-based immune modulators. In a second step, the remaining IFN response, which might be induced by the remaining activated PKR molecules or other non-self RNA sensors, was quenched outside the cells by the extracellular decoy-receptor protein B18R. This blockade is of special importance because it prohibits the cellular communication by interferon and thereby avoids the induction of IFN responsive elements. This in turn helps to prevent the antiviral state of the cells and counteracts the amplification potential of the IFN response.

To our knowledge it was never addressed before, whether an IVT-RNA encoded co-transfection of the viral anti IFN proteins — together with a reporter gene or the

reprogramming factors — were able to inhibit the PKR mediated induction of the IFN response. We see a benefit in the usage of messenger IVT-RNA alone, since all IVT-RNA can be prepared together, if necessary under GMP compatible conditions, which eases the later application. After transfection the targeted cell will synthesize all needed components on their own, which reduces the costs for e.g. supplemental recombinant proteins in the medium for the reprogramming protocol of Warren et al. (Warren et al., 2010; Warren et al., 2012).

Indeed, the combination of EKB was successful in reducing the IFN response. After one transfection the combination of EKB prevented the upregulation of the IFN response markers while E and K alone were not effective. In contrast to E and K, the extracellular acting protein B was able to minimize the upregulation of the IFN response. This indicates the importance of B18R, which seems to be especially necessary for quenching the IFN response even upon frequent IVT-RNA-based transfections. With regard to the reporter gene translation we could show that in this case B alone was less potent than EK and the EKB co-transfection. These results indicate that E and K are important for elevated translation efficiency. However, both enhanced translation as well as the minimized IFN response induction was best with all three anti IFN modulators. In consideration of the mode of action from E3, K3 and B18R our observation that E3 and K3 are more important for the translation than B18R was expected, since by avoiding the PKR activation the translational shutdown is counteracted as well. For reducing the IFN response B18R was superior to E3 and K3 since it is able to quench IFN outside the cells. Thus our results fit to the known mode of action of the VACV proteins EKB. When frequent messenger IVT-RNA based transfections were performed, we profited from the combination of the intra- and extracellular acting proteins resulting in enhanced translation and reduced IFN response. This result is in line with the knowledge that viruses use several proteins to combat the host defense. Besides the analysis of the phosphorylation status of the PKR substrate eIF2α, we tested and confirmed that the co-transfection of EKB was a reliable method for minimizing phosphorylation of eIF2α. This will enable the targeted cells to continue protein translation, which is also indispensable for successful transgene expression of IVT-RNA encoded proteins.

In summary we recommended the combination of all three proteins since it was the only applicable method that was able to enhance translation and to reduce the IFN response. Additionally, EKB was able to reduce the phosphorylation of the PKR substrate eIF2α. With our experiments we have provided the evidence that EKB co-transfection might enable us to perform repetitive IVT-RNA-based transfections.

4.3 Frequent transfections with IVT-RNA is enabled by the co-transfection of the anti IFN Vaccinia virus proteins E3, K3 and B13R

Initially we had to address the obstacle that our target cells did not survive repetitive transfections of messenger IVT-RNA. We found out that this was dependent on IVT-RNA-based induction of the cellular defense mechanisms mediated by an upregulated IFN response. As seen in the results repetitive transfection of reporter gene IVT-RNA is possible in the presence of EKB even when the nucleotides of the IVT-RNA are not modified. We have thereby achieved cellular viability comparable to the range of mock transfected cells. By Yakubov et al. (Yakubov et al., 2010) frequent messenger IVT-RNA based transfection were only achieved with modified nucleotides and in the presence of the recombinant protein B18R (Warren et al., 2010; Warren et al., 2012). Indeed, one way of reducing the IFN response is the modification of RNA nucleotides (Kormann et al., 2011) In contrast to their approaches, our unique feature is to use exclusively unmodified IVT-RNA encoded for both, for the genes of interest as well as for the viral anti IFN response proteins EKB. The advantage for later applications is that the pharmaceutical consists only of messenger IVT-RNA. The composition containing only one compound most likely eases the procedure for getting a license. Our data suggests that we were successful in maintaining cellular viability during frequent IVT-RNA-based transfections. Moreover, we now had the experimental basis to perform repetitive transfections with reprogramming factors avoiding an induced IFN response as well as the block of protein biosynthesis.

4.4 Frequent IVT-RNA-based transfections of EKB and reprogramming factors leads to the induction of stem cell associated markers and to an enhanced transgene expression level

The co-expression of EKB enabled us to perform frequent transfections with reporter gene IVT-RNA. Now we have a strategy for analyzing cellular viability, the IFN response and the upregulation of stem cell associated markers after repetitive transfection of messenger IVT-RNA encoding for reprogramming factors. Our results verified that we were able to maintain the viability of the human fibroblasts when they were transfected with six unmodified reprogramming factors OCT4, SOX2, KLF4, c-MYC, LIN28 and NANOG in the presence of EKB. Moreover, the IFN induction was abrogated even after four daily lipofections. We

hypothesized that the presence of the reprogramming factors might have an impact on the induction of stem cell associated markers. Indeed with the induction of TERT, GDF3 and DPPA4, we were able to detect an upregulation of stem cell associated markers, although the stem cell dependent markers like REX1 or LIN28 were not reactivated at the analyzed time point. Our results pave the way for successful reprogramming with unmodified messenger IVT-RNA based transfections. By using the strategy of EKB co-expression we might reach a detectable induction of endogenous stem cell markers if the transfections were continued for two or three weeks.

Furthermore, we could show for the lipofected reprogramming factors NANOG, OCT4 and SOX2 that we were able to achieve a better translation of the non-modified reprogramming factors in the presence of EKB compared to the modified control samples. Maybe we can speculate that a more effective translation in the cells might enhance both the speed and efficiency for iPS generation in long term use, or in general we might achieve an improved transgene expression when unmodified IVT-RNA is used as long as the combination with EKB is permitted. Nevertheless an interesting point would be to look not only for viral suppressors of the cellular defense, but also for enhancing the potential of the reprogramming cocktail. In this context further investigations of the adenoviral protein E1A could be interesting, since it can act as a bridging factor activating the transcription of Oct4 (Schöler et al., 1991).

During and after this work other groups were also interested in RNA-based reprogramming (Angel and Yanik, 2010; Plews et al., 2010; Warren et al., 2010; Yakubov et al., 2010; Tavernier et al., 2012; Warren et al., 2012; Mandal and Rossi, 2013). The majority of them generated results that underline and agree with our strategy to combat the IFN response upon frequent messenger IVT-RNA based transfections. When the cells were transfected with RNA a reduction in the cellular viability (Drews et al., 2012) cell density (Angel and Yanik, 2010) or cell growth arrest (Plews et al., 2010) was documented or identified as obstacle after frequent transfections (Warren et al., 2010; Tavernier et al., 2012; Warren et al., 2012; Mandal and Rossi, 2013). Beside Warren et al, a further publication claimed to have achieved RNA based reprogrammed cells in 2010 (Yakubov et al., 2010). The later work has been performed without combating the IFN response. In consideration of the massive cell death we observed at the beginning of our experiments with frequent IVT-RNA transfection, we wondered how to perform successful IVT-RNA transfections without combating the IFN response. It therefore seems questionable to us how iPS colonies can be generated after five repetitive transfections, since in our hands cells wouldn't survive this treatment with

messenger IVT-RNA. In line with this observation is that other researchers combat the IFN response. However, Yakubov et al. were able to show reprogramming associated marker expression on exemplary colonies confirming their success (Yakubov et al., 2010). An alternative approach which was in line with our strategy to combat the IFN response was the use of siRNA against IFNβ, PKR and Stat2 (Angel and Yanik, 2010). Although they were able to perform frequent RNA-based transfection, the generation of iPS cells remained outstanding. For me their results are of importance since I am in line with their approach of hampering the IFN response and a daily RNA treatment. They would probably have been successful in generating iPS cells if the treatment would have been continued for two or three weeks. Alternatively the knockdown of Stat2, a signal transducer being involved in several regulatory processes, might be indispensable also in the signaling for reprogramming. Therefore its knockdown might be problematic and combated reprogramming. Finding the reason why they failed to reprogram cells might help to improve reprogramming. We are convinced that our IVT-RNA-based cocktail coding for the viral proteins EKB and the reprogramming factors might be better since it is focused on minimizing the IFN response upon IVT-RNA treatment, without affecting downstream signaling of factors that might be necessary for altering the cellular expression pattern. However, we still see the possibility to improve IVT-RNA-based translation by searching for alternative or additional viral proteins to support the suppression of IVT-RNA-based cellular defense. Hereby one could think of an alternative for the extracellular acting B18R, because its usage would affect not only the transfected cells, but also the response to inflammation in an organism when the IFN quencher would be exported outside the targeted cell. Moreover besides the chosen EKB proteins several more viral components were able to suppress IFN induction (see Tab. 1.1), and maybe a more complex combination could be more effective as long as signaling pathways, essential for reprogramming, remain unaffected.

Finally we showed the possibility to perform several repetitive messenger IVT-RNA-based transfections also with transcription factors. We generated experimental evidence that the upregulation of stem cell associated markers can be achieved, which paved the way for IVT-RNA based reprogramming or the transgene expression of therapeutic proteins.

4.4.1 The inclusion of the micro RNA mix 302/367 to the IVT-RNA-based reprogramming mix leads to the generation of iPS cells

Subsequent to this work our group managed to generate iPS cells. For this success the use of EKB co-transfection, as was developed in this thesis, led to an essential basis since it ensures frequent lipofection of unmodified IVT-RNA encoding for OSKMLN reprogramming factors. Nevertheless besides the downregulation of the IFN response by EKB it was necessary to add a micro RNA (miRNA) mix 302/367. We decided that the use of miRNA might be helpful to support reprogramming, since it was shown that miRNA based regulation is involved in e.g. cellular renewal and the maintenance of stem cell properties or *mensenchymal to epithelial transition* (MET) (Liao et al., 2011; Berardi et al., 2012; Pfaff et al., 2012) as well as in modulating iPS cells generation (Yang et al., 2011). Furthermore special miRNA clusters, like e.g. the mi302, are upregulated in iPS and ES cells (Wilson et al., 2009) and are reported to be characteristic of the undifferentiated embryonic stem cell state (Suh et al., 2004). Moreover, it was reported previously that the usage of the microRNA cluster 302/367 (Anokye-Danso et al., 2011; Kuo et al., 2012) or miRNA 200c, 302 and 369 (Miyoshi et al., 2011) can reprogram cells into iPS cells. For improving efficiency and duration of iPS generation, the synergistic effects of reprogramming factors and miRNA treatment (Chen et al., 2012) in combination with viral anti IFN proteins is assumed. Indeed, by using miRNA together with IVT-RNA encoded reprogramming factors and anti IFN proteins we observed the generation of iPS colonies. Today our lab is able to robustly reprogram human fibroblast using a protocol which includes IVT-RNA for the reprogramming factors OSKMLN and the viral anti IFN response proteins EKB together with the miRNA mix 302/367.

4.5 Outlook

4.5.1 Viral proteins for targeting the anti IFN after IVT-RNA transfer

For our experiments we decided to use anti IFN proteins from the well characterized VACV virus. The EKB co-transfection was finally shown to be an applicable method for reducing the PKR mediated IFN response after IVT-RNA-based transfections. Although a method was found to perform frequent IVT-RNA-based transfection in human fibroblasts, we are interested in further improvements that could ease messenger IVT-RNA based transfections in the clinic.

In future we could analyze further proteins because although the IFN response is minimized after IVT-RNA transfection the induction of IFN response markers is not avoided completely. So maybe the use of additional IFN response avoiding proteins will be able to further reduce the remaining IFN marker upregulation.

Moreover, the usage of more potent anti IFN response proteins may allow the reduction of the amount of IVT-RNA which is necessary to control cellular defense. This would enable us either to use a more complex anti IFN response IVT-RNA mix or to increase and optimize the amount of the IVT-RNA encoded therapeutic proteins.

As listed by Gale, several viruses target the PKR, either by the ds RNA binding motives, by dimerization, by substrate interaction, by influencing PKR expression and protein stability or by regulation of eIF2α (Gale and Katze, 1998). As seen in the western blot of EKB co-transfected cells, we still have a background phosphorylation of eIF2α. Thus it could be that eIF2α dephosphorylation represents a critical step in PKR mediated IFN response upregulation. Therefore the protein ICP34.5 from herpes simplex virus should be interesting to analyze. It acts as mediator of eIF2α dephosphorylation by recruiting the *protein phosphatase* 1 (PP1) to prevent the translational shutdown in the infected cells (Li et al., 2011). An overexpression of ICP34.5 perhaps helps to improve both the translation and the downregulation of the PKR mediated eIF2α phosphorylation.

Besides searching for different ways to inhibit the PKR, it may well be advantageous to look at additional molecules targeting IFN signaling pathways, the attack of TLR signaling components, NF-κB signaling or the Jak-Stat pathway (for examples see Tab. 1.1). The *nonstructural protein* 1 (NS1) encoded by the influenza A virus is an example of a multifunctional protein that is able to attack several steps of cellular defense. It inhibits OAS and PKR activation and improves the viral mRNA translation (de La Luna et al., 1995; Salvatore et al., 2002; Burgui et al., 2003; Hale et al., 2008), additionally NS1 also complexes

with RIG-1 in order to avoids cellular defense induction (Pichlmair et al., 2006). Thus, the NS1 protein is perhaps a powerful IFN response quencher, which might supplement the inhibition of the PKR by avoiding cellular defense initiated by the OAS or RIG-1. The V protein of paramyxovirus A could be interesting to include in future analyses, since the induction of MDA-5, e.g. by 5'-triphosphorylated (5'ppp) or ds RNA, is disrupted due to its avoiding ATP hydrolysis (Andrejeva et al., 2004; Parisien et al., 2009). By the inhibition of the MDA-5 protein a further conserved sensor for non-self RNA is blocked, which might contribute to further reduction of IFN response marker induction.

Another strategy to generally antagonize RNA-based immune response is the inhibition of TLR signaling. Here, for example the protein A46R or A52R from the Vaccinia virus, were reported to block TLR signaling pathways (Bowie et al., 2000; Stack et al., 2005; Lysakova-Devine et al., 2010; Stack and Bowie, 2012). It is possible that a blocked TLR signaling will also contribute to a down-regulated IFN response, as they also play an important role in recognizing pathogen associated patterns and in the upregulation of the cellular defense.

For further applications it might be beneficial to focus on intracellular proteins in order to enhance the transgene expression in the targeted cells. With B18R, one out of three proteins is secreted. Although the combination of EKB was successful in down-regulating the IFN response in cell culture, the secretion of B18R can be seen as drawback for a later clinical usage because it will also quench the IFNs secreted due to pathogen infection. Therefore, it would be beneficial to combat only the IFN response that is released by the messenger IVT-RNA treated cells. However, before B18R can be substituted, we must prove that a suitable protein or protein mix is at least equal in performance to B18R. Perhaps it is difficult to find a replacement for our protocol which consist of a two-step mode of action by intra and extracellular acting viral immune modulator proteins. Maybe an alternative protein target as well IFN receptor signaling, preferably early in the signal cascade. There the VACV protein VH1 could be tested as it binds and dephosphorylates STAT1 resulting into a block of signaling (Najarro et al., 2001). However, interfering with general components of the cellular signaling might be problematic and even hamper reprogramming. Maybe it is more promising to attack the production or secretion of IFNs.

Regarding the composition of the viral proteins that combat the IFN response it could be that due to the introduction of further proteins the amount of EKB could be adapted. Thereby it must be taken into account that, even after frequent transfection the anti IFN effect is sufficient. In future, when our group manages to produce IVT-RNA which precedes the stability of the used D1 capped IVT-RNA, the time between the transfections could be

increased. This, in turn, might provide the opportunity to reduce the amount of RNA required for combating the IFN response.

When cells are infected by a virus they can hamper viral replication by a shutdown of the protein biosynthesis. As mentioned before, viruses have evolved strategies, which counteract the translational shutdown and some viral mRNAs evade the stalled translation or their UTRs lead to enhanced translation (Holden and Harris, 2004). Thus, one could think of improving the IVT-RNA by introducing e.g. viral derived UTRs, which were translated independently of an upregulated IFN response. However, for an IVT-RNA-based reprogramming, the synthesis of just the transfected messenger IVT-RNA encoded reprogramming factors would be insufficient; since the protein biosynthesis of reprogramming factors associated genes is as well indispensable for reprogramming. Thus, when all other proteins, despite of the transcription factors, were affected by the protein synthesis shutdown iPS cell generation is most likely unreachable.

4.5.2 Targeting PKR with a cellular component

In addition to the viral protein approach of targeting PKR, we could also think of using a cellular component to inhibit the PKR activation. For this alternative strategy the cellular protein p58IPK, which avoids the activation of the PKR by a recruitment of the highly conserved 58 kDa heat shock protein 40 (hsp40) (Lee et al., 1994; Melville et al., 1997), might be interesting to analyze. The IVT-RNA encoding for p58IPK has the benefit that it is identical to the highly conserved cellular protein, which probably ensures its effectiveness.

Finally we want to underline that, before using e.g. viral or cellular proteins to support IVT-RNA-based transfection the risk of inhibiting important other components must be calculated. In future its proposed effectiveness has to be verified in cell based experiments. Whereas targeting the PKR for optimized IVT-RNA-based transfection of reprogramming factor is promising, for immune based applications the presence of IFN is necessary to boost the cellular defense. Then a quenching of IFN signaling is counterproductive. With this work it was demonstrated that the expression of IVT-RNA encoded proteins can be optimized. For applications where a safe and non-integrative method is demanded, our findings may pave the way for the IVT-RNA encoded therapeutic protein expressions. Beside the generation of iPS cells, one could think of further applications like the expression of the hormone erythropoietin

(EPO) or antibodies for immune based cancer treatment. Furthermore the use of mRNA for anti-infective applications has been reported (Martinon et al., 1993; Tavernier et al., 2011).

4.6 Conclusion

With the results obtained during this work we could demonstrate, for the first time, that frequent IVT-RNA-based transfection of unmodified IVT-RNA encoding reprogramming factors is possible together with the co-transfection of anti IFN proteins E3, K3 and B18R. Only the presence of the viral anti IFN proteins EKB results in improved cellular survival upon frequent IVT-RNA-based transfection which goes along with reduced induction of IFN response genes. Moreover, prolonged transgene expression could be detected and the activation of the PKR circumvented. This IVT-RNA-based method was implemented for frequent transfections of human cells with transcription factors that are able to convert the targeted cells back into a pluripotent state. Indeed, with our protocol we achieved an induction of pluripotency associated markers. This observation was an important basis for the following frequent transfections performed subsequent to this thesis. Furthermore I'd like to mention that this work profits from the results of the working group, which has improved the IVT-RNA stability in the last few years.

In the future the hurdle of an upregulated IFN response upon frequent IVT-RNA treatment can be solved by inhibiting the activation of the kinase PKR. All applications that are dependent on repetitive IVT-RNA-based transfection will profit from our observations.

5 Appendix

5.1 Nucleotide sequences

The sequences of interest were flanked by restriction enzymes; the restriction enzyme sequence is underlined by a dotted line for *BamHI* and a continuous line for *Hind*III.

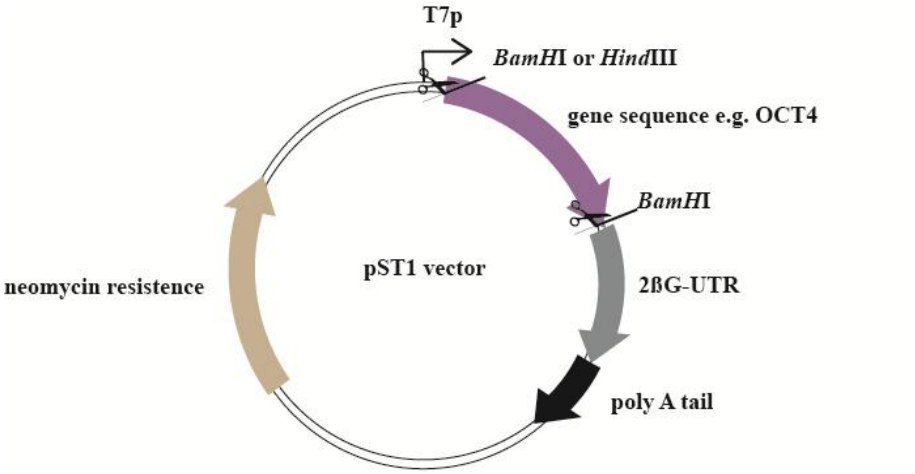

Figure 5.1 Schematic representation of plasmid vectors used for *in vitro* transcription The vectors were based on the pST1 vector and were endowed with a T7 promoter (T7p), two serial human β-globin 3' UTRs (2βG-UTR), a stretch of 120 adenines (poly A tail) and a neomycin resistance gene cassette. The gene of interest (e.g. OCT4) was introduced into the vector by insertion and ligation of the prepared DNA fragments and the vector backbones.

5.1.1 Reprogramming factors

The sequences listed below were synthesized codon optimized.

Sequence for human OCT4:

GGATCCAGCCACCATGGCAGGCCATCTGGCTAGCGACTTCGCCTTCAGCCCACCTCCTGGTG
GAGGAGGGGATGGACCTGGCGGCCCTGAGCCCGGCTGGGTGGACCCTAGAACCTGGCTGTCC
TTCCAGGGCCCTCCAGGTGGCCCAGGCATCGGCCCAGGCGTCGGACCAGGCAGCGAAGTGTG
GGGCATCCCTCCCTGCCCCCCTCCCTACGAGTTCTGCGGCGGCATGGCCTACTGCGGCCCTC
AGGTCGGGGTCGGCCTGGTGCCACAGGGCGGCCTGGAAACAAGCCAGCCCGAGGGCGAAGCC
GGCGTCGGAGTGGAGAGCAACAGCGACGGCGCTAGCCCCGAGCCTTGCACCGTGACCCCTGG
CGCCGTGAAGCTGGAAAAAGAGAAGCTGGAACAGAACCCCGAGGAAAGCCAGGACATCAAGG
CCCTGCAGAAAGAACTGGAACAGTTCGCCAAGCTGCTGAAGCAGAAGCGGATCACCCTGGGC
TACACACAGGCCGATGTGGGCCTCACACTGGGCGTGCTGTTCGGCAAGGTGTTCAGCCAGAC

CACCATCTGCCGGTTCGAAGCCCTGCAGCTGTCCTTCAAGAACATGTGCAAGCTGCGGCCCC
TGCTGCAGAAATGGGTGGAGGAAGCCGACAACAACGAGAACCTGCAGGAAATCTGCAAGGCC
GAGACACTGGTGCAGGCCCGGAAGCGGAAGCGGACCAGCATCGAGAACAGAGTGCGGGGCAA
CCTGGAAAACCTGTTCCTGCAGTGCCCCAAGCCCACCCTGCAGCAGATCAGCCACATTGCTC
AGCAGCTCGGCCTGGAAAAGGACGTGGTGCGCGTGTGGTTCTGCAACCGGCGGCAGAAGGGC
AAACGGTCTAGCAGCGACTACGCCCAGCGGGAGGACTTCGAAGCCGCCGGAAGCCCCTTCAG
CGGTGGACCCGTGAGCTTCCCCCTGGCCCCAGGCCCTCACTTCGGCACCCCCGGCTACGGCA
GCCCCCACTTCACCGCCCTGTACAGCAGCGTGCCCTTCCCTGAGGGCGAGGCCTTCCCCCCC
GTGAGCGTGACCACCCTGGGCAGCCCCATGCACAGCAACTGATGAGGATCC

Sequence for human SOX2:

GGATCCAGCCACCATGTACAACATGATGGAAACCGAGCTGAAGCCCCCTGGCCCCCAGCAGA
CAAGCGGAGGAGGGGGTGGAAATAGCACAGCCGCTGCTGCCGGCGGGAACCAGAAGAACAGC
CCCGACCGGGTGAAGCGGCCCATGAACGCCTTCATGGTCTGGTCCAGGGGCCAGCGGAGGAA
GATGGCCCAGGAAAACCCCAAGATGCACAACAGCGAGATCAGCAAGAGACTGGGCGCAGAGT
GGAAGCTGCTGTCCGAGACAGAGAAGCGGCCCTTCATCGACGAGGCCAAGCGGCTGCGGGCC
CTGCACATGAAAGAGCACCCCGACTACAAGTACCGGCCCAGAAGAAAGACCAAGACCCTGAT
GAAGAAGGACAAGTACACCCTGCCTGGCGGCCTCCTCGCTCCTGGCGGCAACAGCATGGCCA
GCGGCGTGGGCGTGGGAGCCGGCCTGGGAGCCGGGGTGAACCAGCGGATGGACAGCTACGCC
CACATGAACGGCTGGTCCAACGGCAGCTACAGCATGATGCAGGACCAGCTGGGCTACCCTCA
GCACCCCGGCCTGAACGCCCACGGGGCTGCCCAGATGCAGCCCATGCACAGATACGACGTGA
GCGCCCTGCAGTACAACAGCATGACCTCCAGCCAGACCTACATGAATGGCAGCCCCACCTAC
AGCATGTCCTACTCCCAGCAGGGCACCCCCGGCATGGCCCTGGGCTCTATGGGCAGCGTGGT
GAAAAGCGAGGCCAGCAGCAGCCCCCCTGTGGTGACAAGCAGCTCCCACTCTAGGGCCCCTT
GCCAGGCCGGCGACCTGCGGGACATGATCAGCATGTACCTGCCAGGCGCCGAGGTGCCAGAG
CCTGCCGCCCCTAGCCGGCTGCACATGAGCCAGCACTACCAGAGCGGCCCTGTGCCCGGCAC
CGCCATCAACGGCACCCTGCCCCTGAGCCACATGTGATGAGGATCC

Sequence for KLF4:

GGATCCAGCCACCATGGCCGTGAGCGACGCCCTGCTGCCCAGCTTCAGCACCTTCGCCAGCG
GCCCAGCCGGAAGGGAAAAGACCCTGAGACAGGCTGGGGCACCCAACAACCGGTGGCGGGAG
GAACTGAGCCACATGAAGAGACTGCCACCCGTGCTGCCTGGCAGGCCCTACGACCTGGCTGC
CGCCACTGTGGCCACCGACCTGGAAAGCGGAGGAGCTGGGGCTGCCTGTGGCGGCAGCAACC
TGGCCCCCCTGCCCAGGCGGGAGACAGAGGAATTCAACGACCTGCTGGACCTGGACTTCATC

CTGAGCAACAGCCTGACCCACCCCCCCGAGAGCGTGGCCGCCACAGTGAGCAGCAGCGCCAG
CGCATCTAGCAGCTCCTCCCCCAGCAGCTCCGGCCCTGCCAGCGCCCCTAGCACCTGCAGCT
TCACCTACCCCATCAGGGCCGGCAACGACCCTGGCGTGGCCCCTGGCGGCACAGGAGGAGGA
CTGCTGTACGGCCGGGAGAGCGCCCCTCCTCCCACCGCCCCCTTCAACCTGGCCGACATCAA
CGACGTGAGCCCCAGCGGCGGCTTCGTCGCCGAACTCCTGCGGCCCGAGCTGGACCCCGTGT
ACATCCCACCTCAGCAGCCACAGCCTCCTGGAGGAGGGCTCATGGGCAAGTTCGTGCTGAAG
GCCAGCCTGAGCGCCCCTGGCAGCGAGTACGGCAGCCCCTCCGTGATCAGCGTGAGCAAGGG
CAGCCCCGACGGCAGCCACCCTGTGGTGGTGGCCCCCTACAACGGAGGACCCCCCAGGACCT
GCCCCAAGATCAAGCAGGAAGCCGTCAGCAGCTGCACCCACCTGGGAGCCGGCCCACCCCTG
AGCAACGGCCACCGGCCTGCCGCCCACGACTTCCCCCTGGGCCGGCAGCTCCCCAGCAGGAC
CACCCCTACCCTCGGCCTGGAAGAGGTGCTGTCCAGCCGGGACTGCCACCCCGCCCTGCCTC
TGCCCCCTGGCTTCCACCCTCACCCAGGCCCCAACTACCCCAGCTTCCTGCCCGACCAGATG
CAGCCCCAGGTGCCCCCACTGCATTACCAGGAACTGATGCCCCCAGGCAGCTGCATGCCCGA
GGAACCCAAGCCCAAGCGGGGCAGGAGGAGCTGGCCCCGGAAGCGGACCGCCACCCACACCT
GCGACTACGCCGGCTGTGGCAAGACCTACACCAAGAGCAGCCACCTGAAGGCCCACCTGCGG
ACCCACACCGGCGAGAAGCCCTACCACTGCGACTGGGACGGCTGCGGCTGGAAGTTCGCCAG
AAGCGACGAGCTGACCCGGCACTACCGGAAGCACACCGGCCACAGGCCCTTCCAGTGCCAGA
AGTGCGACCGGGCCTTCAGCAGATCTGACCACCTGGCCCTGCACATGAAGAGGCACTTTTGA
TGAGGATCC

Sequence for human c-MYC:

GGATCCAGCCACCATGGATTTCTTCCGGGTGGTGGAGAACCAGCAGCCCCCTGCCACCATGC
CCCTGAACGTGAGCTTCACCAACCGGAACTACGACCTGGACTACGACAGCGTGCAGCCCTAC
TTCTACTGCGACGAGGAAGAGAACTTCTACCAGCAGCAGCAGCAGAGCGAGCTGCAGCCCCC
AGCCCCCTCCGAGGACATCTGGAAGAAGTTCGAGCTGCTGCCCACCCCCCCTCTGAGCCCCA
GCAGGAGGAGCGGCCTGTGCAGCCCCAGCTACGTGGCCGTGACCCCCTTCAGCCTGCGGGGC
GACAACGATGGAGGAGGAGGAAGCTTTAGCACCGCTGACCAGCTGGAAATGGTGACCGAGCT
GCTGGGCGGCGACATGGTGAACCAGAGCTTCATCTGCGACCCCGACGACGAGACATTCATCA
AGAACATCATCATCCAGGACTGCATGTGGAGCGGCTTCTCCGCTGCCGCCAAGCTGGTGTCC
GAGAAGCTGGCCTCTTACCAGGCCGCCAGGAAGGACAGCGGCTCCCCCAACCCCGCCAGGGG
CCACAGCGTGTGCAGCACAAGCTCTCTGTACCTGCAGGACCTGAGCGCCGCAGCCTCCGAGT
GCATCGACCCCAGCGTGGTGTTCCCCTACCCCCTGAACGACAGCTCTAGCCCCAAGAGCTGC
GCCAGCCAGGACAGCAGCGCCTTCAGCCCCTCCAGCGACAGCCTGCTGTCCAGCACCGAGTC
CAGCCCCCAGGGCAGCCCCGAGCCTCTGGTCCTGCACGAGGAAACCCCCCCCACCACCTCCT
CCGACAGCGAGGAAGAACAGGAAGATGAGGAAGAGATCGACGTGGTGTCCGTGGAGAAGCGG

CAGGCCCCTGGCAAGCGGAGCGAGAGCGGCAGCCCCTCTGCCGGAGGACACAGCAAGCCCCC
CCACAGCCCCCTGGTGCTGAAGCGGTGCCACGTGAGCACACACCAGCACAACTATGCCGCCC
CTCCCAGCACCCGGAAGGACTACCCTGCCGCCAAGAGGGTGAAGCTGGACAGCGTGCGGGTG
CTGCGGCAGATCAGCAACAACCGGAAGTGCACCAGCCCCAGAAGCAGCGACACCGAGGAAAA
CGTGAAGCGGAGGACCCACAACGTGCTGGAACGGCAGCGGAGGAACGAGCTGAAGCGGAGCT
TCTTCGCCCTGCGGGACCAGATCCCCGAGCTGGAAAACAACGAGAAGGCCCCCAAGGTGGTG
ATCCTGAAGAAGGCCACCGCCTACATCCTGAGCGTGCAGGCCGAGGAACAGAAGCTGATCAG
CGAAGAGGACCTGCTGCGGAAGCGGCGGGAGCAGCTGAAGCACAAGCTGGAACAGCTGAGGA
ACAGCTGCGCCTGATGAGGATCC

Sequence for human LIN28:

GGATCCAGCCACCATGGGCAGCGTGAGCAACCAGCAGTTTGCCGGAGGATGCGCCAAGGCCG
CCGAGGAAGCCCCCGAAGAGGCCCCCGAGGACGCCGCCAGAGCCGCCGACGAGCCCCAGCTG
CTGCACGGAGCCGGCATCTGCAAGTGGTTCAACGTGCGGATGGGCTTCGGCTTCCTGAGCAT
GACAGCCAGAGCCGGCGTGGCCCTGGACCCCCCCGTGGACGTGTTCGTGCACCAGAGCAAGC
TGCACATGGAAGGCTTCAGAAGCCTGAAAGAGGGCGAGGCCGTCGAGTTCACCTTCAAGAAA
AGCGCCAAGGGCCTGGAAAGCATCCGGGTGACCGGCCCTGGCGGCGTGTTCTGCATCGGCAG
CGAGCGGAGGCCCAAGGGCAAGAGCATGCAGAAGCGGAGGAGCAAGGGCGACCGGTGCTACA
ACTGTGGCGGCCTGGACCACCACGCCAAAGAGTGCAAACTGCCACCCCAGCCCAAGAAGTGC
CACTTCTGCCAGAGCATCAGCCACATGGTGGCCAGCTGCCCCCTGAAGGCCCAGCAGGGCCC
TAGCGCCCAGGGCAAGCCCACCTACTTCCGGGAGGAAGAGGAAGAGATCCACAGCCCCACCC
TGCTGCCCGAGGCCCAGAACTGATGAGGATCC

Sequence for human NANOG:

GGATCCAGCCACCATGAGCGTGGACCCCGCCTGCCCCCAGAGCCTGCCCTGCTTCGAAGCCA
GCGACTGCAAAGAAAGCAGCCCCATGCCCGTGATCTGCGGCCCTGAGGAAAACTACCCCAGC
CTGCAGATGAGCAGCGCCGAGATGCCCCACACCGAGACAGTGAGCCCCCTGCCCTCTAGCAT
GGATCTGCTGATCCAGGACAGCCCCGACAGCAGCACCAGCCCCAAGGGCAAGCAGCCCACCT
CTGCCGAGAAAAGCGTGGCCAAGAAAGAGGACAAGGTCCCCGTCAAGAAGCAGAAAACCCGG
ACCGTGTTCTCTAGCACTCAGCTGTGTGTGCTCAACGACCGGTTCCAGCGGCAGAAGTACCT
GAGCCTGCAGCAGATGCAGGAACTGAGCAACATCCTGAACCTGAGCTACAAGCAGGTCAAAA
CCTGGTTCCAGAACCAGCGGATGAAAAGCAAGCGGTGGCAGAAGAACAACTGGCCCAAGAAC
AGCAACGGCGTGACCCAGAAGGCCAGCGCCCCCACCTACCCCTCCCTGTACAGCAGCTACCA
CCAGGGCTGCCTGGTGAACCCCACCGGCAACCTGCCCATGTGGAGCAACCAGACCTGGAACA
ACAGCACCTGGTCCAACCAGACCCAGAACATCCAGAGCTGGTCCAATCACAGCTGGAATACC

CAGACTTGGTGCACCCAGAGCTGGAATAACCAGGCATGGAATAGCCCCTTCTACAACTGCGG
CGAGGAAAGCCTGCAGAGCTGCATGCAGTTCCAGCCCAACAGCCCCGCCAGCGACCTGGAAG
CTGCCCTCGAAGCCGCCGGAGAGGGCCTGAACGTGATCCAGCAGACCACCCGGTACTTCAGC
ACCCCCCAGACCATGGACCTGTTTCTGAACTACAGCATGAACATGCAGCCCGAGGACGTGTG
ATGAGGATCC

5.1.2 Viral anti IFN modulators

Sequence for B18R:

AAGCTTGCCACCATGACCATGAAGATGATGGTGCACATCTACTTCGTGTCCCTGCTGCTGCT
CCTGTTCCACAGCTACGCCATCGACATCGAGAACGAGATCACCGAGTTCTTCAACAAGATGC
GGGACACCCTGCCCGCCAAGGACAGCAAGTGGCTGAACCCCGCCTGCATGTTCGGCGGCACC
ATGAACGATATCGCCGCCCTGGGCGAGCCCTTCAGCGCCAAGTGCCCCCCCATCGAGGACAG
CCTGCTGAGCCACCGGTACAAGGACTACGTGGTCAAGTGGGAGCGGCTGGAAAAGAACAGAC
GGCGCCAGGTGTCCAACAAGAGAGTGAAGCACGGCGACCTGTGGATCGCCAACTACACCAGC
AAGTTCAGCAACCGGCGCTACCTGTGCACCGTGACCACCAAGAACGGCGACTGCGTGCAGGG
CATCGTGCGGAGCCACATCAGAAAGCCCCCCAGCTGCATCCCCAAGACCTACGAGCTGGGCA
CCCACGATAAGTACGGCATCGACCTGTACTGCGGCATCCTGTACGCCAAGCACTACAACAAC
ATCACCTGGTATAAGGACAACAAAGAGATCAACATCGACGACATCAAGTACAGCCAGACCGG
CAAAGAGCTGATCATCCACAACCCCGAGCTGGAAGATAGCGGCAGATACGACTGCTACGTGC
ACTACGACGACGTGCGGATCAAGAACGACATCGTGGTGTCCCGGTGCAAGATCCTGACCGTG
ATCCCCAGCCAGGACCACCGGTTCAAGCTGATCCTGGACCCCAAGATCAACGTGACCATCGG
CGAGCCCGCCAACATCACATGCACCGCCGTGTCCACCTCCCTGCTGATCGACGATGTGCTGA
TCGAGTGGGAGAACCCCAGCGGCTGGCTGATCGGCTTCGACTTCGACGTGTACAGCGTGCTG
ACCAGCAGAGGCGGCATCACCGAGGCCACCCTGTACTTCGAGAACGTGACCGAAGAGTACAT
CGGCAACACCTACAAGTGCAGAGGCCACAACTACTACTTCGAAAAGACCCTGACCACCACCG
TGGTGCTGGAATGATGAGGATCC

Sequence for E3:

AAGCTTGCCACCATGAGCAAGATCTACATCGACGAGCGGAGCAACGCCGAGATCGTGTGCGA
GGCCATCAAGACCATCGGCATCGAGGGCGCTACAGCTGCCCAGCTGACCCGGCAGCTGAACA
TGGAAAAGCGGGAAGTGAACAAGGCCCTGTACGACCTGCAGCGGAGCGCCATGGTGTACAGC
AGCGACGACATCCCCCCCAGATGGTTCATGACCACCGAGGCCGACAAGCCCGACGCCGATGC
CATGGCCGACGTGATCATCGACGACGTGTCCCGCGAGAAGTCCATGAGAGAGGACCACAAGA
GCTTCGACGATGTGATCCCCGCCAAGAAGATCATCGACTGGAAGGGCGCCAACCCCGTGACC

GTGATCAACGAGTACTGCCAGATCACCAGACGGGACTGGTCCTTCCGGATCGAGAGCGTGGG
CCCCAGCAACAGCCCCACCTTCTACGCCTGCGTGGACATCGACGGCCGGGTGTTCGACAAGG
CCGACGGCAAGAGCAAGCGGGACGCCAAGAACAACGCCGCCAAGCTGGCCGTGGACAAGCTG
CTGGGCTATGTGATCATCCGGTTCTGATGAGGATCC

Sequence for K3:

AAGCTTGCCACCATGCTGGCCTTTTGCTACAGCCTGCCCAACGCCGGCGACGTGATCAAGGG
CCGGGTGTACGAGAAGGACTACGCCCTGTACATCTACCTGTTCGACTACCCCCACTTCGAGG
CCATCCTGGCCGAGAGCGTGAAGATGCACATGGACAGATACGTGGAATACCGGGACAAGCTG
GTCGGAAAGACCGTGAAAGTGAAAGTGATCCGGGTGGACTACACCAAGGGCTACATCGACGT
GAACTACAAGCGGATGTGCAGGCACCAGTGATGAGGATCC

5.1.3 Improving cellular survival and proliferation

Sequence for E6:

AAGCTTGCCACCATGCACCAGAAACGGACCGCCATGTTCCAGGACCCCCAGGAACGGCCCAG
AAAGCTGCCCCAGCTGTGCACCGAGCTGCAGACCACCATCCACGACATCATCCTGGAATGCG
TGTACTGCAAGCAGCAGCTCCTCCGGCGGGAGGTGTACGACTTCGCCTTCCGGGACCTGTGC
ATCGTGTACCGGGACGGCAACCCCTACGCCGTGTGCGACAAGTGCCTGAAGTTCTACAGCAA
GATCAGCGAGTACCGGCACTACTGCTACAGCCTGTACGGCACCACCCTGGAACAGCAGTACA
ACAAGCCCCTGTGCGACCTGCTGATCCGGTGCATCAACTGCCAGAAACCCCTGTGCCCCGAG
GAAAAGCAGCGGCACCTGGACAAGAAGCAGCGGTTCCACAACATCCGGGGCAGATGGACCGG
ACGGTGCATGAGCTGCTGCAGAAGCAGCCGGACCAGACGGGAGACACAGCTGTGATGAGGAT
CC

Sequence for SV40 large T antigen:

AAGCTTGCCACCATGGACAAGGTGCTGAACCGGGAGGAATCCCTGCAGCTGATGGACCTGCT
GGGCCTGGAAAGAAGCGCCTGGGGCAACATCCCCCTGATGCGGAAGGCCTACCTGAAGAAGT
GCAAAGAGTTCCACCCCGACAAGGGCGGCGACGAAGAAAAGATGAAGAAGATGAACACCCTG
TACAAGAAAATGGAAGATGGCGTGAAGTACGCCCATCAGCCCGACTTCGGCGGCTTCTGGGA
CGCCACCGAGATCCCCACCTACGGCACCGACGAGTGGGAGCAGTGGTGGAACGCCTTCAACG
AGGAAAACCTGTTCTGCAGCGAGGAAATGCCCAGCTCTGACGACGAGGCCACCGCCGACAGC
CAGCACAGCACCCCCCCCAAGAAAAAGCGGAAGGTGGAGGACCCCAAGGACTTCCCCAGCGA
GCTGCTGTCCTTCCTGAGCCACGCCGTGTTCAGCAACCGGACCCTGGCCTGCTTCGCCATCT
ACACCACCAAAGAGAAGGCCGCCCTGCTGTACAAAAAGATCATGGAAAAGTACAGCGTGACC

TTCATCAGCCGGCACAACAGCTACAACCACAACATCCTGTTCTTTCTGACCCCCCACCGGCA
CAGAGTGTCTGCCATCAACAACTACGCCCAGAAGCTGTGCACCTTCAGCTTTCTGATCTGCA
AGGGCGTGAACAAAGAATACCTGATGTACAGCGCCCTGACCCGGGACCCCTTCAGCGTGATC
GAGGAAAGCCTGCCTGGCGGCCTGAAAGAGCACGACTTCAACCCCGAGGAAGCCGAGGAAAC
CAAGCAAGTCAGCTGGAAGCTGGTCACCGAGTACGCCATGGAAACCAAGTGCGACGACGTGC
TGCTGCTGCTCGGCATGTACCTCGAATTTCAGTACAGCTTCGAGATGTGCCTGAAGTGCATC
AAGAAAGAGCAGCCCAGCCACTACAAGTACCACGAGAAGCACTACGCCAACGCCGCCATCTT
CGCCGATAGCAAGAACCAGAAAACCATCTGCCAGCAGGCCGTGGACACCGTGCTGGCCAAGA
AACGGGTGGACTCTCTGCAGCTGACCCGCGAGCAGATGCTGACCAACCGGTTCAACGACCTG
CTGGACCGGATGGACATCATGTTCGGCAGCACCGGCAGCGCCGACATCGAGGAATGGATGGC
CGGCGTGGCCTGGCTGCACTGCCTGCTGCCCAAGATGGACAGCGTGGTGTACGACTTCCTGA
AGTGTATGGTGTACAACATCCCCAAGAAGCGGTACTGGCTGTTCAAGGGCCCCATCGACAGC
GGCAAGACCACCCTGGCCGCTGCACTGCTGGAACTGTGCGGCGGCAAGGCCCTGAACGTGAA
CCTGCCCCTGGACCGGCTGAACTTCGAGCTGGGCGTGGCCATCGACCAGTTTCTGGTGGTGT
TCGAGGACGTGAAGGGCACAGGTGGAGAGAGCCGCGACCTGCCTAGCGGCCAGGGCATCAAC
AACCTGGACAACCTGCGGGACTACCTGGACGGCAGCGTGAAAGTGAACCTGGAAAAGAAACA
CCTGAACAAGCGGACCCAGATCTTCCCCCCTGGCATCGTGACCATGAACGAGTACTCCGTGC
CCAAGACCCTGCAGGCCAGATTCGTGAAGCAGATCGACTTCCGGCCCAAGGATTACCTGAAG
CACTGCCTGGAACGCAGCGAGTTCCTGCTGGAAAAGCGGATCATCCAGAGCGGCATTGCCCT
GCTGCTGATGCTGATCTGGTACAGACCCGTGGCCGAGTTCGCCCAGAGCATCCAGAGCCGGA
TCGTGGAGTGGAAAGAGCGGCTGGACAAAGAGTTCAGCCTGAGCGTCTACCAGAAGATGAAG
TTCAACGTCGCCATGGGCATCGGCGTGCTGGACTGGCTGCGGAACTCCGACGACGACGATGA
GGACAGCCAGGAAAACGCCGACAAGAACGAGGACGGCGGCGAGAAAAACATGGAAGATAGCG
GCCACGAGACAGGCATCGACAGCCAGAGCCAGGGCAGCTTCCAGGCCCCTCAGAGCAGCCAG
TCCGTGCACGACCACAACCAGCCCTACCACATCTGCCGGGGCTTCACCTGCTTCAAGAAGCC
CCCCACCCCCCCTCCCGAGCCCGAGACATGATGAGGATCC

5.1.4 Reporter genes

Sequence for GFP:

AAGCTTGGATCCCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCA
TCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG
GGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGT
GCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCG
ACCACATGAAGCAGCACGACTTCTTCAAGTCCGCAATGCCCGAAGGCTACGTCCAGGAGCGC

ACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGA
CACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCTGG
GGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAG
AACGGCATCAAGGTGAACTTCAAGATCCGACACAACATCGAGGACGGCAGCGTGCAGCTCGC
CGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACT
ACCTGAGCACCCAGTCCGCACTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTG
CTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAAGGATCC
C

Sequence for luciferase:

AAGCTTGGCAATCCGGTACTGTTGGTAAAGCCACCATGGAAGATGCCAAAAACATTAAGAAG
GGCCCAGCGCCATTCTACCCACTCGAAGACGGGACCGCCGGCGAGCAGCTGCACAAAGCCAT
GAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCACATATCGAGGTGGACA
TTACCTACGCCGAGTACTTCGAGATGAGCGTTCGGCTGGCAGAAGCTATGAAGCGCTATGGG
CTGAATACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGT
GTTGGGTGCCCTGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCG
AGCTGCTGAACAGCATGGGCATCAGCCAGCCCACCGTCGTATTCGTGAGCAAGAAAGGGCTG
CAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCATACAAAAGATCATCATCATGGATAG
CAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCCATTTGCCACCCG
GCTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGGGACAAAACCATCGCCCTGATC
ATGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCTTG
TGTCCGATTCAGTCATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCCGACACCGCTA
TCCTCAGCGTGGTGCCATTTCACCACGGCTTCGGCATGTTCACCACGCTGGGCTACTTGATC
TGCGGCTTTCGGGTCGTGCTCATGTACCGCTTCGAGGAGGAGCTATTCTTGCGCAGCTTGCA
AGACTATAAGATTCAATCTGCCCTGCTGGTGCCCACACTATTTAGCTTCTTCGCTAAGAGCA
CTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGATCGCCAGCGGCGGGGCGCCGCTC
AGCAAGGAGGTAGGTGAGGCCGTGGCCAAACGCTTCCACCTACCAGGCATCCGACAGGGCTA
CGGCCTGACAGAAACAACCAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAGCCTGGCG
CAGTAGGCAAGGTGGTGCCCTTCTTCGAGGCTAAGGTGGTGGACTTGGACACCGGTAAGACA
CTGGGTGTGAACCAGCGCGGCGAGCTGTGCGTCCGTGGCCCCATGATCATGAGCGGCTACGT
TAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCGGCGACA
TCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAA
TACAAGGGCTACCAGGTAGCCCCAGCCGAACTGGAGAGCATCCTGCTGCAACACCCCAACAT
CTTCGACGCCGGGGTCGCCGGCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGCAGTCG
TCGTGCTGGAACACGGTAAAACCATGACCGAGAAGGAGATCGTGGACTATGTGGCCAGCCAG

GTTACAACCGCCAAGAAGCTGCGCGGTGGTGTTGTGTTCGTGGACGAGGTGCCTAAAGGACT
GACCGGCAAGTTGGACGCCCGCAAGATCCGCGAGATTCTCATTAAGGCCAAGAAGGGCGGCA
AGATCGCCGTGTAATAATTCTAGAGGATCC

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Manuscript of the current work is in preparation

Patent

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Posters

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7 Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig angefertigt und alle benutzten Hilfsmittel angegeben sind. Die aus fremden Quellen (einschließlich elektronischer Quellen) direkt oder indirekt übernommenen Gedanken sind als solche kenntlich gemacht. Weder die komplette noch Teile der Arbeit wurden bei einer anderen Fakultät oder Universität als Prüfungsarbeit bzw. Dissertation eingereicht. Die von mir vorgelegte Dissertation ist von Prof. Dr. Ugur Sahin an der III. Medizinischen Klinik und Poliklinik der Universitätsmedizin der Johannes Gutenberg-Universität Mainz betreut worden.

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8 Curriculum Vitae

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