Investigation of two different aspects of stem cell biology: The role of Stat3 signaling and innate immunity in human pluripotent stem cells

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Sabrina Schoeps
aus Köln

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“Et es wie et es. Et kütt wie et kütt. Et hät noch immer joot jejange. Wat fott es, es fott.”

§1-4, kölsches Grundgesetz

Abbreviations

µg	microgram
µl	microliter
aa	amino acid
ALK	anaplastic lymphoma kinase
APC	adenomatous-polyposis-coli
APS	ammonium persulfate
ATP	adenosine triphopshate
bFGF	basic fibroblast growth factor
bp	base pair
BMP	bone morphogenic protein
BMPR	bone morphogenic protein receptor
BSA	bovine serum albumine
cDNA	complementary DNA
Chi	“Chimera”; IL-6/sIL-6R chimeric fusion protein
CO_2	carbon dioxide
conc	concentration
CTP	cytidine triphosphate
ctrl	control
DMEM	Dulbecco´s modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
EDTA	ethylendiaminetetraacetic acid
EGF	epidermal growth factor
EpiSC	epiblast stem cell
ESC	embryonic stem cell
FB	fibroblast
FCS	fetal calf serum
FGF	fibroblast growth factor
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
goi	gene of interest
GSK3	glycogen synthase kinase 3
GTP	guanosine triphosphate
h	human *or* hour (context dependent)

Abbreviations

HEK	human embryonic kidney
HIV	human immunodeficiency virus
ICM	inner cell mass
IFNβ	interferon beta
IGF	insulin-like growth factor
IL-6	interleukin 6
iPSC	induced pluripotent stem cell
IVT	*in vitro*-transcription
JAK	Janus kinase
kb	kilobase
kDa	kiloDalton
KOSR	knockout serum replacement
l	liter
LIF	leukemia inhibitory factor
Ln	laminin
lt-NES	long-term self-renewing neuroepithelial-like stem
m	murine
M	Mol per liter
MEF	mouse embryonic fibroblast
MEK	mitogen-activated protein kinase kinase
mg	milligram
min	minute
ml	milliliter
mM	millimolar
mod	modified
MOPS	3-(N-morpholino)propanesulfonic acid
ms	millisecond
NEAA	non-essential amino acids
ng	nanogram
NLS	nuclear localization signal
nm	nanometer
NTP	nucleoside triphosphate
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
p	phospho-
PBS	phosphate buffered saline

Abbreviations

PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3 kinase
PM	protein marker
PO	Polyornithine
PSC	pluripotent stem cell
PTD	protein transduction domain
RNA	ribonucleic acid
Rock	Rho-kinase
rpm	Rounds per minute
s	second
SDS	sodium dodecyl sulfate
sIL-6R	soluble interleukin 6 receptor
Smad	small mother against decapentaplegic
SSEA	stage-specific embryonic antigen
Stat3	signal transducer and activator of transcription 3
TAE	Tris, acetic acid, EDTA
TALEN	transcription activator-like effector nuclease
TAT	transactivator of transcription
TBS	Tris buffered saline
TBS-T	Tris buffered saline with Tween
TCF	*Transcription factor* protein
TEMED	tetramethylethylenediamine
TGFβ	transforming growth factor beta
Tris	tris(hydroxymethyl)aminomethane
TTF	tail tip fibroblasts
U	unit
unmod	unmodified
UTP	uridine triphosphate
UTR	untranslated region
UV	ultraviolet
V	volt
XIST	X-inactive specific transcript
ZFN	zinc-finger nuclease

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1 Introduction

1.1 Pluripotent Stem Cells

Since the 1980´s, research of pluripotent stem cells (PSCs) is in focus of life sciences. PSCs have two characteristic features that distinguish them from other cells. First, PSCs are pluripotent, meaning that each single cell can generate all cell lineages of the developing and adult organism, and second they have the ability to self-renewal. Because of that, PSCs hold great promises as a model for development and as a source for cells in regenerative medicine.

1.1.1 Embryonic Stem Cells

Murine embryonic stem cells (mESCs) were derived more than 30 years ago for the first time (Evans & Kaufman, 1981). From the beginning researchers were interested in those cells derived from the preimplantation embryo blastocyst at stage E3.5, as they could differentiate them into cells of all three germ layers; but until 1998 they did not succeed in deriving the human counterpart.

In 1998, Thomson and his colleagues could isolate human embryonic stem cells (hESCs) for the first time from the inner cell mass (ICM) of the blastocyst of a pre-implantation embryo (Thomson et al., 1998). Because an embryo has to be destroyed for the derivation of hESC lines, ethical and depending on the country also legal issues limit the ability to work with those cells.

1.1.2 Induced Pluripotent Stem Cells

In 2006, Takahashi and Yamanaka could reprogram somatic cells into so called induced pluripotent stem cells (iPSCs) by introducing the four key pluripotency factors Oct-3/4, Sox2, Klf4 and c-Myc genetically (Takahashi & Yamanaka, 2006). First, they reprogrammed murine fibroblasts into murine iPSCs (miPSCs), one year later they succeeded to generate human iPSCs (hiPSCs) by transfecting human dermal fibroblasts with the same four factors (Takahashi et al., 2007).

iPSCs resemble ESCs in their characteristics, they are also pluripotent, meaning they have the potential to differentiate into cells of the three germ layers, and can be maintained in culture indefinitely. There are two major advantages of reprogrammed cells; first they do not raise ethical or legal concerns because no embryo has to be

sacrificed. Second in case of human clinical applications patient-specific cells can be differentiated from hiPSCs generated by reprogramming patient cells.

So far, the generation of iPSCs has been improved continuously. Different protocols were established, where factors could be omitted or be replaced by small molecules; different cell types were reprogrammed. The efficiencies increased from initially 0.02% to up to 25% for mouse but only from 0.02% to 0.75% for human cells (Eminli et al., 2009; Sugii et al., 2010; Takahashi & Yamanaka, 2006; Takahashi et al., 2007). In murine reprogramming, principally every diploid cell type can be used as initial cell (Stadtfeld & Hochedlinger, 2010; Takahashi & Yamanaka, 2006). Mostly, mouse embryonic fibroblasts (MEFs) or adult tail tip fibroblasts (TTFs) are used. The requirements for human somatic cells as a starting point are more distinct; it is favorable that the cells are easy to obtain without harming the donor. Until now, different human cell types, for example fibroblasts, keratinocytes and cord blood cells, could be reprogrammed (Aasen et al., 2008; Giorgetti et al., 2009; Takahashi et al., 2007).

1.1.3 Differences between human and murine PSCs

Because of the better accessibility, a lot of research has been performed on murine pluripotent stem cells. But notably, human pluripotent stem cells differ from their murine counterparts in essential characteristics.

The first difference already becomes evident observing the morphology. While mPSCs grow in dome shaped three-dimensional colonies, hPSCs grow in flattened two-dimensional colonies.

Murine and human PSCs also differ in their cultivation behavior. In cell culture, murine PSCs can be grown clonogenic, meaning that the cells can be singularized, and each single cell gives rise to a new colony. Human PSCs need to be splitted in small cell clumps; clonogenicity can only be achieved when the cells are treated with an inhibitor of the Rho kinase, a so-called ROCK inhibitor (ROCKi) (Watanabe et al., 2007). This property makes manipulation of human PSCs hard to achieve, as single manipulated cells can not be grown to a monoclonal population easily (Buecker et al., 2010).

A molecular difference of murine and human PSCs is the occurrence of characteristic cell surface antigens. While SSEA-1 (stage-specific embryonic antigen 1) is a marker for pluripotency in mouse cells, in human cells it is a differentiation marker

(Brambrink et al., 2008; Henderson et al., 2002). Distinct markers for pluripotency in human PSCs are the stage-specific embryonic antigens 3/4 (SSEA-3/4) (Sato et al., 2003).

The most striking and probably most important difference between murine and human PSCs lies in the signaling pathways that need to be activated to maintain their pluripotency. The following chapter will give an insight into the main signaling pathways.

1.2 Signaling in PSCs for pluripotency maintenance

Pluripotent stem cells are cell culture artifacts that do not exist over a longer time *in vivo*. Therefore distinct signaling pathways need to be activated to maintain them in culture. Human and murine PSCs differ in these pathways.

1.2.1 Pluripotency maintenance in human PSCs

Maintenance of human PSCs requires the addition of different extrinsic factors to the cell culture media. In the absence of these factors, hPSCs lose their pluripotency and differentiate spontaneously.

Standard culture of hPSCs depends on a layer of so-called feeder cells, mouse embryonic fibroblasts (MEFs) that are irradiated to prevent an overgrowing of the hPSCs culture (Thomson, 1998). Addition of the extrinsic factors stimulates the fibroblasts to secrete other factors important for maintaining pluripotency. To remove murine cells from the human PSC culture, medium can be conditioned by feeder cells. After filtration, the medium contains no cells. In recent feeder-free cultivation protocols the conditioning of the medium by MEFs is not required. All needed factors, such as bFGF and Activin A, are supplied with the defined cell culture medium. A standard medium commercially available is mTeSR™-1; more recently, a medium with only eight components was published and due to the number named E8 (Chen et al., 2011; Ludwig et al., 2006).

<u>bFGF signaling</u>

Basic fibroblast growth factor (bFGF or FGF2) signaling is activated in hPSC culture by adding the recombinant protein directly to the culture. The way in which bFGF promotes hPSC propagation is still poorly understood. It is known that bFGF

activates the mitogen-activated protein kinase (MAPK) and thus MAPK/ERK signaling (Eiselleova et al., 2009). In general, bFGF promotes proliferation, which is why it is supplemented to a variety of cell culture media for different cell types (Jirmanova et al., 1999; Kotev-Emeth et al., 2002; Luo & Miller, 1997). In 2000, it was reported that bFGF is required for prolonged undifferentiated proliferation of hESCs (Amit et al., 2000). More recently, it was reported that inhibition of FGF/ERK signaling leads to a rapid downregulation of NANOG. Additionally, OCT4 binds to the FGF2 reporter and thus regulates its activation in an autocrine manner (Greber et al., 2010). This indicates a connection of FGF signaling with two core pluripotency-associated transcription factors.

Furthermore it has been shown that, while low levels promote stem cell maintenance, high activity of ERK signaling promotes differentiation. To control the activity of ERK signaling, high concentrations of FGF2 do not only activate MAPK/ERK signaling but also PI3K/AKT signaling. PI3K/AKT signaling itself suppresses MAPK/ERK signaling and thereby prevents too high levels of ERK (Singh et al., 2012).

Activin A signaling

Together with Nodal, Inhibins and BMPs (bone morphogenic protein), Activin A forms the Tgfβ superfamily. In mPSCs, BMP is essential to maintain pluripotency (Ying et al., 2003). When supplemented in human PSC cultures, BMP leads to differentiation into trophectoderm (Xu et al., 2002).

However, another member of the Tgfβ superfamily, Activin A, is an important extrinsic factor for hPSC maintenance culture. Activin A mediates, together with Nodal, pluripotency via Smad-2/3 signaling. In 2008, Xu et al. reported that the pluripotency-related transcription factor Nanog is a direct target of Activin A/Smad-2/3 signaling, highlighting the importance of this pathway (Xu et al., 2008). Furthermore, Activin A is able to induce the expression of other pluripotency regulators such as Oct4, Nodal, Wnt3 and also bFGF (Xiao, Yuan, & Sharkis, 2006). Other members of the Tgfβ family, such as TGFβ itself and Nodal, can substitute for Activin A (Xu et al., 2008).

In co-culture with feeder cells, bFGF stimulates the fibroblasts to secrete Activin A (Beattie et al., 2005; Eiselleova et al., 2008). In feeder-free cultivation systems, Activin A has to be supplemented. Vallier showed in 2005 that the combination of bFGF and Activin A supplementation enabled hPSC culture without feeder cells,

conditioned medium or serum replacement by controlling Nanog expression (Vallier et al., 2009; Vallier, Alexander, & Pedersen, 2005).

Other pathways

The feeder-dependent first-generation hPSC maintenance protocols all include cell culture media containing either fetal calf serum (FCS) or knockout serum replacement (KOSR). These components are not chemically defined and contain factors such as IGF and insulin. The containing factors stimulate a variety of signaling pathways that may also be part of the self-renewal signaling network. For instance, IGF and insulin activate the canonical phosphatidylinositol-3 kinase (PI3K) signaling pathway, which also plays a critical role in murine PSC maintenance (Bendall et al., 2007; Watanabe et al., 2006).

Controversial reports about the role of Wnt signaling in hPSCs have been published. Fibroblasts of the feeder layer secrete several activators of Wnt signaling. In 2004, Sato et al. showed that Wnt signaling prevents differentiation in hESCs (Sato et al., 2004). Conflicting with that, it has been reported that active Wnt signaling promotes differentiation of hPSCs (Bone et al., 2011; Davidson et al., 2012).

1.2.2 Pluripotency maintenance in murine PSCs

As described for hPSCs, mPSCs initially were cultivated on a feeder layer of MEF cells. In 1988, Smith as well as Williams identified factors that made the feeder cells dispensable. One of these factors, the most prominent factor in mPSC maintenance, is the Leukemia Inhibitory Factor (LIF) (Smith et al., 1988; Williams et al., 1988).

LIF signaling

The Leukemia Inhibitor Factor (LIF) is a member of the IL-6 family. It is an essential extrinsic factor in the cultivation of murine PSCs. LIF maintains pluripotency via binding to the LIF receptor (LIFR), which then forms a heterodimer with the signal-transducing transmembrane glycoprotein 130 (gp130). This leads to a Janus kinase (Jak) mediated phosphorylation of the signal transducer and activator 3 (Stat3). Phosphorylated and thereby activated Stat3 (p-Stat3) molecules form homodimers and translocate into the nucleus where they bind to the DNA and activate target gene transcription (Zhong, Wen, & Darnell, 1994). Still, it is not fully understood how LIF/Stat3 signaling maintains mPSC pluripotency. In 2013, Tai and Ying published

that gastrulation brain homeobox 2 (Gbx2) is a target of LIF/Stat3 signaling. Overexpression of Gbx2 maintains mPSC pluripotency in the absence of LIF/Stat3 signaling and improves reprogramming efficiency. Furthermore, when overexpressed, Gbx2 is sufficient to reprogram murine epiblast stem cells (derived from the epiblast; mEpiSCs) into ground state mESCs (Tai & Ying, 2013).

A connection between the extrinsic LIF signaling and the intrinsic transcription factor Nanog was described in 2009 by Niwa et al. LIF activates T-box 3 (Tbx3) via PI3-kinase/Akt-signaling, which itself induces Nanog expression. Furthermore they could show that also the intrinsic transcription factor Klf4 is activated through LIF signaling via Jak/Stat-signaling (Niwa et al., 2009). Correspondent to these findings, for the maintenance of pluripotency in mPSCs in the absence of LIF, either overexpression of Stat3 or Nanog is sufficient (Matsuda et al., 1999; Mitsui et al., 2003). Stat3-knockout-mPSCs can be kept pluripotent when the so-called 2i medium is applied. Double inhibition of GSK3 and MEK with small molecule inhibitors can compensate for lack of LIF signaling (Ying et al., 2008).

BMP signaling

Under serum free conditions, LIF alone fails to maintain pluripotency. mPSCs tend to differentiate into the neuronal lineage upon serum withdrawal (Ying et al., 2003). Addition of Bone Morphogenic Protein (BMP) could, together with LIF, prevent differentiation. BMP binds two forms of cell surface receptors, namely BMP receptor type I (BMPRI) and type II (BMPRII). The binding of BMP leads to heterodimerization of the both receptors. These heterodimers phosphorylate different Smad proteins (1, 5 and 8). The activated Smad proteins form itself heterodimers with co-Smad 4 that translocate into the nucleus where they bind to promoter regions of BMP target genes and thereby activate their expression (Chen, Zhao, & Mundy, 2004; Miyazono, 1999).

Another proposed role for BMP is the inhibition of p38, a tumor suppressor, which inhibits expression of pluripotency-associated genes (Qi et al., 2004).

Wnt signaling

In Wnt signaling, Wnt binds to its receptor Frizzled that then activates Dishevelled. Active Dishevelled inhibits GSK3, Axin and APC to form a complex. The complex phosphorylates β-catenin, which then is degraded. In the presence of a Wnt ligand,

β-catenin is not phosphorylated and therefore can, together with TCF and Lef, induce gene transcription (Barker, Morin, & Clevers, 2000).

It has been reported that in mPSCs TCF represses genes that antagonize stem cell maintenance and therefore would lead to differentiation when expressed (Sokol, 2011).

1.2.3 Naïve versus primed pluripotency

While mPSCs need to be cultured in the presence of leukemia inhibitory factor (LIF), hPSCs do not respond to LIF with the activation of the LIF-Stat3 signaling pathway. In 2004, Daheron et al. reported that LIF/Stat3 signaling cannot maintain hESC pluripotency (Daheron et al., 2004). In contrast, they need the addition of basic fibroblast growth factor (bFGF) and the presence of Activin A and Nodal to remain pluripotent (for summary see Figure 1.1).

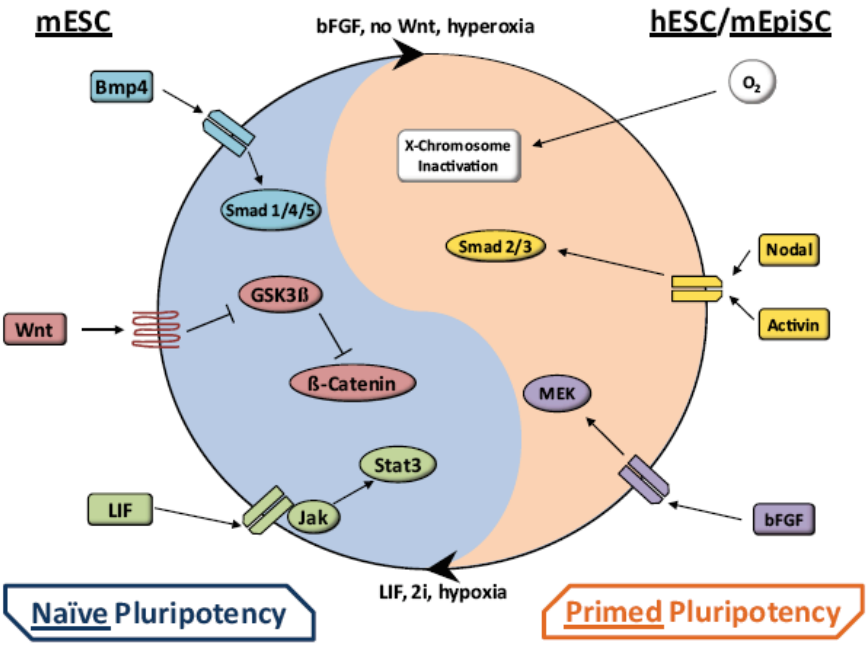

Figure 1.1: Summary of the different pathways that need to be activated in mPSCs and hPSCs/mEpiSCs
(Zhang, Krawetz, & Rancourt, 2013)

Although both cell populations are derived from the inner cell mass of the blastocyst stage, they seem to represent different developmental stages (Najm et al., 2011). Cells that are isolated from the epiblast stage of murine postimplantation embryos at E 5.5 (murine epiblast stem cells, mEpiSCs) resemble hPSCs. In 2007 it was shown

that mEpiSCs show the same morphology as hPSCs as they are growing in two-dimensional colonies. They can not be splitted as single cells, which is a characteristic of mPSCs (Brons et al., 2007; Tesar et al., 2007). Furthermore, mEpiSCs can not contribute to chimeras, which is an important test for pluripotency in mPSCs, but they efficiently form teratomas and embryoid bodies, showing that they have the ability to differentiate into all three germ layers (Guo et al., 2009; Tesar et al., 2007).

Another difference between mEpiSCs/hPSCs and mPSCs is the activation-state of the X chromosomes in the female cells. In mEpiSCs and hPSCs, only one X chromosome is active, the other is inactivated by XIST. Female mPSCs contain two active X chromosomes (Okamoto et al., 2004).

As the chimera-assay cannot be performed with human cells for ethical reasons, other primate ESCs that resemble hPSCs were tested for their potential to contribute to chimeras. Rhesus monkey ESCs can not efficiently home into the inner cell mass of preimplantation blastocysts and thus not generate chimeric monkeys, suggesting that primate PSCs correspond to a rodent primed pluripotent state (Tachibana et al., 2012).

Overall, mEpiSCs seem to represent the same developmental stage as hPSCs, which is obviously later than the mPSCs. Therefore, the state represented by the mPSCs is referred to be the naïve pluripotency, whereas mEpiSCs and hPSCs are in a primed state (Nichols & Smith, 2009). The characteristics of the naïve and the primed pluripotent state are summarized in Table 1.1.

Table 1.1: Characteristics of naïve and primed pluripotency (adapted from Gafni et al., 2013)

	naïve	primed
	mESC	hESC, mEpiSC
morphology	dome shaped	flattened
cultivation	2i/LIF	bFGF
X chromosome	pre-inactivated state	inactivated
Oct4 transcription	distal enhancer driven	proximal enhancer driven
single cell cloning	yes	no
DNA methylation	globally reduced	increased
chimeras	yes	no (mEpiSC)

A lot of effort has been made to find the naïve counterpart of hPSCs. It would be favorable to have human naïve stem cells, because it would allow insights into human development. Furthermore, naïve hPSCs could be plated as single cells; the clonogenicity would permit the translation of gene targeting technologies thus far restricted to murine cells and therefore accelerate dissection of the human genome.

In 2010, attempts to isolate naïve hESCs from human embryos failed. Lengner and his colleagues were able to derive female hESCs with two activated X chromosomes in conventional hESC medium with FGF when the embryos are kept under low-oxygen conditions (5% O_2). Female hESCs that are obtained from embryos under atmospheric oxygen conditions show an upregulation of XIST and an inactivated X chromosome (Lengner et al., 2010). However, it is not reported whether other features associated with naïve ESCs are displayed by the cells with two active X chromosomes. Two other studies reported the attempt to obtain naïve hESCs by applying 2i conditions (MEK and GSK3 inhibition) to human embryos. In both cases naïve hESCs could not be isolated (Kuijk et al., 2012; Roode et al., 2012).

The first successful attempt to directly reprogram human fibroblasts to cells that resemble mESCs was reported in 2009. Lentiviral introduction of the pluripotency factors OCT4, SOX2, NANOG and LIN28 together with the cultivation in 2i conditions and additional inhibition of ALK4/5/7 with the small molecule inhibitor A-83-01 results in cells that can be cultured in presence of LIF/2i for more than 20 passages. However, transgene-independency can not be confirmed, as the transgenes are not silenced properly (Li et al., 2009).

In 2010, Hanna et al. obtained human embryonic stem cells with biological and epigenetic characteristics similar to those of mESCs by switching the media conditions to the so called 2i conditions and ectopically expressing Oct4 and Klf4 or Klf4 and Klf2. The expression system is dox-inducible and after dox withdrawal the cells change back to the primed state (Hanna et al., 2010).

Only few attempts have published the aim to revert primed hPSCs into naïve pluripotency. In 2009, the addition of sodium butyrate, an inhibitor of histone deacetylase (HDAC), was reported to enable hPSC cultivation in the absence of bFGF. Female cells lose their XIST expression. These findings were shown only for one cell line, though. Other characteristics of naïve pluripotency were not reported (Ware et al., 2009). In another study, the application of LIF/2i conditions to hPSCs leads to the generation of primitive neural precursors instead of naïve PSCs (Li et al.,

2011), which led to the speculation that mouse and human cells respond differently to LIF/2i. This could be the reason for the failure to derive naïve hPSC as until then the generation of a stable naïve human PSC line has not been achieved.
Recently, Gafni et al. succeeded in generating stable human naïve pluripotent stem cells by modulating various signaling pathways. The starting cells are primed hPSCs. Addition of LIF, Tgfβ1 and FGF2 and inhibition of ERK1/2, GSK3β, JNK and p38 results in dome-shaped hPSC colonies. Next to the morphology, also the molecular characteristics such as X chromosome activation and the DNA methylation state reveal that their cell population represents naïve hPSCs. Furthermore, applying these conditions to embryos lead to successful generation of naïve hESCs from human blastocysts (Gafni et al., 2013).

1.2.4 IL-6/sIL-6R fusion protein

As mentioned before, hPSCs do not respond with an activation of Stat3 target genes upon LIF addition to standard cultures (Dahéron et al., 2004; Sato et al., 2004). The reasons for that are so far not known. One hypothesis is that LIF is not potent enough to activate Stat3 signaling in hPSCs (Amit et al., 2010). This hypothesis would suggest that a potent LIF agonist could induce Stat3 phosphorylation in hPSCs. One such possible agonist could be a chimeric fusion protein of Interleukin-6 (IL-6) and its soluble receptor (sIL-6R), mainly consisting of the extracellular domain of the membrane bound IL-6R. Interleukin-6 is a cytokine that binds to the membrane-bound IL-6 receptor (IL-6R) and forms a heterodimer. This heterodimer induces to the dimerization of two gp130 and thereby activates Stat3 signaling. IL-6 can also lead to trans-signaling when binding to the soluble form of its receptor. The mechanism of IL-6 transsignaling that occurs *in vivo* is shown in Figure 1.2. In case of the chimeric IL-6/sIL-6R protein, the ligand IL-6 is already bound to its soluble receptor, so that it just has to bind to membrane-bound gp130 to induce Stat3 signaling. The signal-transducing transmembrane glycoprotein 130 is ubiquitously expressed, whereas not every cell type expresses the LIF receptor (LIFR) (Taga & Kishimoto, 1997). Contradictory reports about the expression of LIFR on hPSCs exist in the literature. While Rose-John published in 2002 that hESCs do not express LIFR, other groups showed LIFR expression (Chan et al., 2013; Humphrey et al., 2004; Rose-John, 2002). If hPSCs do not express the LIF receptor or the Interleukin-

6 receptor, the chimeric fusion protein nevertheless could induce Stat3 signaling in hPSCs.

In 2010, Amit et al. showed that hPSCs can be cultivated in suspension when IL-6/sIL-6R is added to the culture medium. Without the chimeric protein, the hPSCs tended to differentiate spontaneously into so called embryoid bodies, whereas in the presence of IL-6/sIL-6R the suspension cultures remained pluripotent (Amit et al., 2010).

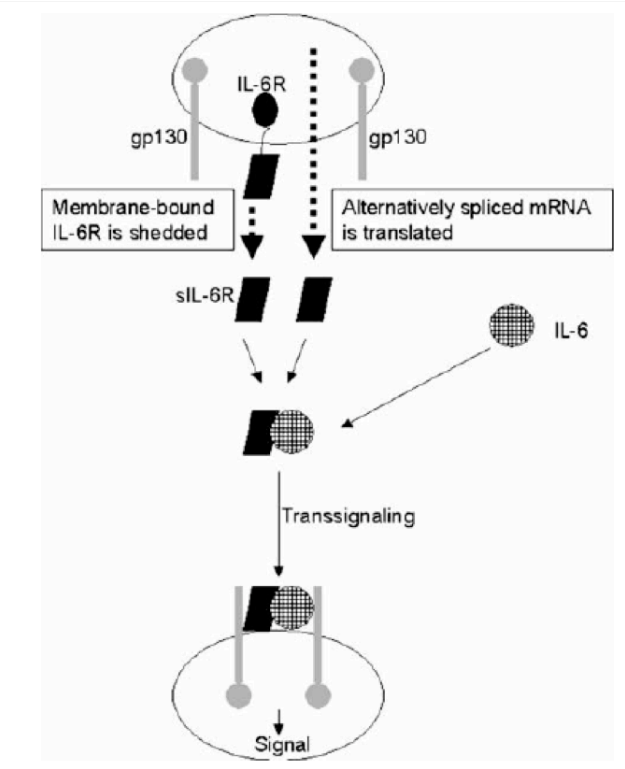

Figure 1.2: Schematic function of IL-6 transsignaling *in vivo*
To get a soluble IL-6 receptor (sIL-6R) either the membrane-bound IL-6R has to be shedded or the IL-6R mRNA has to be spliced alternatively. In the extracellular compartments, sIL-6R can bind to IL-6. The heterodimer can bind to membrane-bound gp130; its dimerization leads to active IL-6 signaling (Knüpfer & Preiss, 2008)

1.3 Manipulation of hPSCs

To gain insight into the molecular mechanisms of cellular processes, it is productive to perform (genetic) manipulation such as gain- or loss-of-function studies. Depending on the desired outcome, cells need to be manipulated either constitutively or transiently.

1.3.1 Transfection of DNA

Nucleic acids can be transfected efficiently into cells via lipofection, electroporation or microinjection. Furthermore, cells can be infected with viral particles. When transfecting DNA, most commonly in form of a plasmid, there always is a risk of integration into the host genome (Wang et al., 2004). Transfected genes can be, depending on their promoter, constitutively active, making a control of transcription impossible. For that purpose, inducible systems have been established, for example the Tet/Dox system. In that case, the gene will only be transcribed when doxycycline is added (Baron, Gossen, & Bujard, 1997). Unfortunately, the Tet/Dox system is leaky, meaning that in some cases a background transcription can be detected (Hofmann, Nolan, & Blau, 1996). Furthermore, integration into the host genome can lead to frameshift mutations and therefore have undesired side effects, for example activation or silencing of genes (Strauss, 1999).

Transfection of DNA into hPSCs is hard to achieve (Zwaka & Thomson, 2003). As the cells grow in tightly packed epithelial-like colonies, the cell surface presented to the environment is relatively small, thus the probability of the lipoplexes or viruses to attach to the cell is rather low (Van Hoof et al., 2008; Watanabe et al., 2007). When plated as single cells in the presence of a ROCK inhibitor, cells are stressed, and the DNA transfection, especially via viral transduction, leads to further stress. This results in a reduced survival of the cells and a poor outcome. The same problems occur when performing electroporation. Dissociation and electroporation lead to a high number of dead cells (Buecker et al., 2010). Thus, the establishment of an efficient transfection protocol of hPSCs is a critical step.

1.3.2 Protein transduction

To circumvent the disadvantages of DNA transfection, recombinant transducible forms of proteins have been developed. These transducible proteins consist of the protein of interest, which is linked to a protein transduction domain (PTD) and optionally to a nuclear localization signal (NLS) (Bosnali & Edenhofer, 2008; Dietz & Bähr, 2004; Peitz et al., 2002). The PTD is important to enable the cellular uptake of the protein. The PTD can be adapted from viral proteins, for example the commonly used TAT (transactivator of transcription)-domain that originates from the HI-virus (Frankel & Pabo, 1988; Green & Loewenstein, 1988; Nagahara et al., 1998). The NLS has to be fused to the protein of interest only if the protein needs to be

transferred to the nucleus and does not contain an internal NLS. In case of transcription factors, an NLS has not to be added, because they already posses an internal NLS (Pan et al., 2004).

Transducible proteins can be applied to cell culture. The cells take up the protein in endosomes. If biologically active, the proteins will exert their effects until degraded.

One disadvantage of transducible proteins is the detrimental effect to the cells. During purification, the protein has to be dialyzed into a buffer that keeps the protein stable. These buffers often contain substances that can stress the cells, e.g. glycerol. Due to the reduced half-life of proteins, transducible proteins usually have to be supplemented more than one time, resulting in repeated stress to the cells. However, the reduced half-life enables a control over the duration of the activity in the cell.

Only a part of the added protein enters the cells, and only a fraction, around 1%, of the transduced protein, gets spontaneously released into the cytosol instead of remaining in endosomes (Jo et al., 2001; Peitz et al., 2002). Therefore, high concentrations of transducible protein have to be applied, making the purification cost-intensive and, furthermore, the application highly stressful to the cells. As described for DNA, hPSCs need to be singularized also for protein transduction. As in many cases protein has to be applied more than once, the growth of hPSCs in colonies raises a problem, as the tightly packed cells have a reduced surface presented to the protein. Protein transduction into hPSCs therefore is hard to achieve.

1.3.3 Transfection of synthetic mRNA

Recently, the transfection of synthetic mRNAs came to focus. It combines the advantages of DNA-transfection and protein transduction. mRNAs can be transfected into the cells with a high efficiency without the risk of integration into the host genome. Furthermore, mRNAs have a limited half-life making a control over the duration of activity in the cell possible.

For the generation of synthetic mRNA, the open reading frame (ORF) of the gene of interest (goi) has to be fused to so-called untranslated regions (UTR). At the 5´ end, the UTR contains a strong Kozak sequence (Warren et al., 2010). At the 3´ end, an alpha-globin UTR is added which ends with an oligo(dT) sequence resulting in a polyA tail after transcription.

This construct of 5´UTR-ORF-3´UTR can be transcribed *in vitro*. *In vitro*-transcribed mRNA activates the innate immune system via binding to the TLR3 (Karikó et al., 2004). In order to prevent innate immunity stimulation, the mRNA needs to be modified. The activation potential of RNA correlates inversely with the extent of their nucleoside modification (Karikó et al., 2005). Instead of using all four standard ribonucleosides, two modified ribonucleosides are used routinely. Instead of uridine, pseudouridine (psi) is added to the *in vitro* transcription (IVT) reaction, and 5-methyl cytidine (5mC) replaces cytidine. After the IVT, a 5´ Cap analog is added to the synthetic mRNA. The incorporation of 5mC and the addition of a 5´ Cap are typical features of mammalian mRNA, whereas pseudouridine is often found in ribosomal RNA (rRNA), with 80% the major constituent of cellular RNA (Bokar & Rottman, 1998; Bachellerie & Cavaille, 1998). Taken together, these modifications make the synthetic mRNA resemble cellular mRNA more closely, and such modifications distinct intrinsic RNA from viral RNA, thus preventing immune reactions upon mRNA-transfection (Warren et al., 2010).

After the IVT and capping, the synthetic mRNA has to be purified and can be transfected via lipofection. The half-life of synthetic mRNA depends on the goi and is rather short with a maximum of expression 12 to 18 hours after transfection (Warren et al., 2010).

In 2010, it was shown that synthetic mRNAs can be transfected into hPSCs; Warren et al. used synthetic mRNAs to induce differentiation in hiPSCs efficiently (Warren et al., 2010).

1.3.4 Tools for genetic engineering

One possible way to achieve genetic engineering is to use engineered nucleases. These nucleases cleave the DNA either on a random position, or on a specific target site (Sun, Abil, & Zhao, 2012). After cleaving the DNA, the DNA has to be repaired by the cell. This happens either by homology-dependent repair (HDR) or by non-homologous end joining (NHEJ) (Jackson, 2002; Shiloh, 2003).

In case of NHEJ, both sides of the double-strand brake are reconnected even if there is no sequence overlap. This repair mechanism can lead to errors in the genome (Seyffert, 2003). If foreign DNA shall be introduced, both cellular repair mechanisms NHEJ and HDR can be exploited. By introducing a donor DNA fragment with homology to the restricted genome, the foreign DNA can be incorporated into the

host genome via HDR. Donor DNA without homology to the cut host genome can be inserted spontaneously via NHEJ (Pan et al., 2013).

One type of engineered nucleases are the Zinc Finger Nucleases (ZFNs). ZFNs can be engineered in that way that they bind to a specific DNA region and cut the targeted gene, inducing a double-strand break at the desired target to either inactivate the gene or to introduce foreign DNA (Kim, Cha, & Chandrasegaran, 1996; Miller et al., 2007).

A disadvantage of the ZFN technology is that off-target effects can occur when the ZFN is not designed specifically enough. Off-target cleavage could lead to undesired genome editing (Gaj et al., 2012). The synthesis of specific ZFNs depends partly on chance, which makes it impracticable to design it in each lab. Routinely, ZFNs are designed by specialized companies.

In 2009, hPSCs were engineered successfully using ZFN technology (Hockemeyer et al., 2009). Since 2010, a novel engineered nuclease called TALEN has gained impact on genome editing. Its modular structure and simple DNA binding code makes it highly specific and easy to design (Christian et al., 2010; Miller et al., 2011).

1.3.5 TALENs as a novel tool for genetic manipulation of hPSC

Transcription activator-like effector nucleases (TALENs) are proteins that consist of a DNA cleavage domain fused to a DNA binding domain. The DNA binding domain bases on the naturally occurring TALE proteins. TALEs are expressed by the plant pathogen *Xanthomonas* to undermine host genome regulatory networks (Boch & Bonas, 2010) For that, they specifically bind and regulate plant genes (Kay et al., 2007). TALEs consist of a central repeat domain responsible for the DNA binding. Multiple of these so-called TALE repeats are arranged in tandem. The sequence of the 33-35 amino acids of each repeat unit are nearly identical, except for two adjacent amino acids that are highly variable. These two amino acids are called repeat variable di-residue (RVD) and are responsible for the target base recognition. A simple code shows which RVD targets which base (Boch et al., 2009).

For genetic engineering, TALEs are fused to the cleavage domain of the FokI restriction enzyme resulting in a protein with nuclease activity (Cermak et al., 2011; Miller et al., 2011). To cleave the double-stranded DNA, two TALENs have to be designed. Both TALENs bind to two targeted sequences separated by 14 to 20 bases. When both TALENs bind, their FokI catalytic domains dimerize and introduce

a double-strand brake (see Figure 1.3; Sanjana et al., 2012). As described for other engineered nucleases, the double-strand brake is repaired by the cellular repair mechanisms HDR or NHEJ.

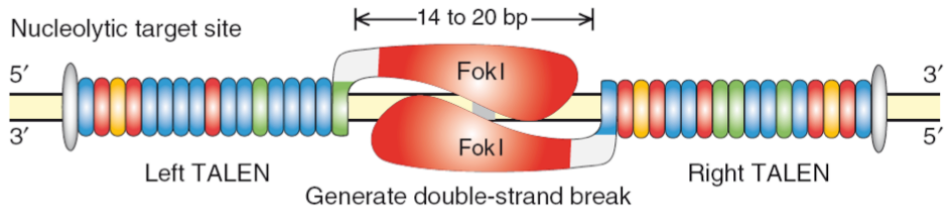

Figure 1.3: Scheme of TALEN binding and cleavage
Schematic illustration of a TALEN pair. In the binding domain, each color represents a RVD that binds a specific base. The binding sites of both TALENs are in a distance of 14 to 20 base pairs, were the catalytic FokI domains dimerize and cut the genome (Sanjana et al., 2012)

In 2011, Hockemeyer et al. transfected human pluripotent cells with plasmids encoding for TALENs. They succeeded in engineering hESCs as well as hiPSCs genetically (Hockemeyer et al., 2011a).

1.4 Innate immunity

The immune system is the system that protects an organism against diseases by recognizing foreign agents and pathogens and distinguishing them from host origin. In vertebrates, the immune system consists of two subtypes, the adaptive or acquired immune system and the innate immune system.

The adaptive immune system creates an immunological memory. After a first contact to a pathogen, it creates a stronger immune response specific to this antigen. Instead of the adaptive immune system, which is only found in vertebrates, an innate immune system can be found also in invertebrates as well as in plants and prokaryotes as it is cell-autonomous and present in each cell. The innate immune system is the first line of defense against pathogens. It is non-specific and responds to pathogens in a generic way; therefore it is distinct to the adaptive immune system.

1.4.1 Innate immunity in somatic mammalian cells

Mammalian cells are exposed to a variety of external threats. For example, an infection with a virus conveys nuclear acids into the cell. The cell then has to be able to distinguish between its own and pathogenic material. Responsible for the detection of and the defense against pathogenic substances is the innate immune system of the cell.

The targets of the innate immune recognition are the so-called pathogen-associated molecular patterns (PAMPs). They are conserved molecular patterns, which are recognized by receptors of the innate immune system that are called pattern-recognition receptors (PRR) (Meylan, Tschopp & Karin, 2006; Takeuchi & Akira, 2010). There are many different PRRs; they can be expressed on the cell surface or in intracellular compartments. Furthermore, they can be secreted and therefore sense for microbial infections in blood or in tissue fluids (Medzhitov, 2001).

PAMPs are constitutive and conserved products of the microbial metabolism, which are essential for the survival of microorganisms and do not occur in the host cells. The matter that they are essential for survival is important, as microorganisms tend to mutate frequently. Existential proteins will not mutate in a high frequency, as mutation could lead to cell death or at least to a reduced adaptive fitness (Medzhitov, 2001).

The major type of PRRs consists of the so-called Toll-like receptors (TLRs). They have two characterizing domains: extracellular it is a leucine-rich repeat (LRR) domain and intracellular a Toll/IL-1 receptor (TIR) domain. The LRR domain is responsible for ligand recognition, whereas the TIR domain is essential for protein-protein interactions (Hashimoto et al., 1988; Medzhitov, Preston-Hurlburt, & Janeway, 1997).

The different members of the TLR family recognize different PAMPs. For example, TLR3 is responsible for recognition of dsRNA. TLR3 is found both on cell surface and endosomes (Matsumoto et al., 2002). After TLR3 recognizes dsRNA, it recruits TRIF to endosomes, which there functions as a docking platform that interacts with several signaling proteins to activate NFκB, IRF3 and IFNβ (Jiang et al., 2004).

Another important player in the response to dsRNA infection is PKR (dsRNA-activated protein kinase) (McAllister & Samuel, 2009). It is directly activated by dsRNA (Lemaire et al., 2008). Upon activation, several substrates, such as IκB, are phosphorylated. This leads to an activation of different target genes, for example

IFNβ (Kumar & Carmichael, 1998). PKR does not only activate the transcription of the genes important for immune response, but also limits viral replication by inhibiting the proliferation of the host cell (García, Meurs, & Esteban, 2007).

1.4.2 Immunity during human development

The developing embryo has basically no immunity. It develops in the sterile environment of the womb and therefore is, when no infections occur, not exposed to pathogens to that it could adapt immunity. In the fetal stage, immunoglobulins of the IgG class are transferred from the mother to the fetus (Saji et al., 1999). This so called passive immunity protects the newborn against pathogens the mother adapted immune response against. However, the IgGs are degraded and therefore protect the newborn only for a limited time (Hinton et al., 2006).

The innate immune system does not require exposure to pathogens. Nonetheless, TLR responses in the embryo develop relatively late and are suppressed in the neonatal period (Levy, 2007). In the first trimester of pregnancy, cells of different tissues of the placenta express TLRs (Holmlund et al., 2002). TLR2 is activated through its ligand Peptidoglycan upon infection with gram-positive bacteria in trophoblasts. But instead of cytokine secretion, it induces apoptosis (Abrahams & Mor, 2004). Accumulated apoptosis in the placenta leads to abnormal pregnancy outcomes as abortions and preterm births (Jerzak & Bischof, 2002).

Taken together, these data suggest that the placenta is responsible for the protection of the embryo against pathogens. The embryo itself seems not to have an innate immune system.

1.4.3 Innate immunity in PSCs

An interesting feature of pluripotent stem cells is their proposed attenuated capacity to react on the infection with viral RNA.

Chen et al. showed in 2010 that in hESCs and hiPSCs transfection with dsRNA did not lead to an activation of IFNβ expression. hPSCs do express the dsRNA-activated protein kinase (PKR) and some of its downstream signaling targets like IκB, NFκB and IRF3, but PKR is not phosphorylated and thus activated. Strikingly, hPSCs do not express TLR3. However, after 4 days of BMP4-induced differentiation into trophoblasts, IFNβ induction was enhanced 10fold (Chen, Yang, & Carmichael, 2010).

These results stand in conflict with results published also in 2010. It was reported that H7 hESCs express TLR3 at a similar level as adult human endothelial cells, whereas TLR1, 4 and 6 are expressed less. TLR8 and 10 are not expressed at all, but TLR5 is expressed at an even higher level. The expression levels of NFκB- and TLR signaling genes are comparable to the adult endothelial cells. Furthermore it was reported that, upon PAMP stimulation, hESCs do not release CXCL8, which act as a biomarker for cell activation in that study. Remarkably, cells that were differentiated for up to 4 months into endothelial cells do not react with CXCL8 release to PAMP stimulation. Only IL-1β, which acts independently of TLRs, induces CXCL8 secretion (Földes et al., 2010).

In another report, prolonged differentiation leads to increased innate immunity. Differentiation of hESC-derived neurons into hESC-derived neural precursor cells (NPCs) increases the expression of the IFN type I signaling pathway components IRF-9 and IFNAR2. Therefore, differentiation leads to an increased protection against viral infections (Farmer et al., 2013).

For murine PSCs, it was also reported that they posses a low immunogenic potential. In 2006, it was published that mESCs as well as cells differentiated from ESCs (endothelial cells (esECs) and smooth muscle cells (esSMCs)) do not react with cytokine release upon LPS treatment. mESCs as well as esECs and esSMCs do not express TLR4 (Zampetaki et al., 2006).

Viruses can infect mPSCs of the D3 line and exert their cytopathic effects, but the cells do not express type I IFNs as a response to that. Furthermore it was shown that mESCs express TLR3 only at a very low level compared to fibroblasts (Wang et al., 2013). Conflicting with these two publications, another study investigating the role of TLRs in mPSCs reported that murine ESCs as well as adult stem cells express functional TLRs. Their activation even leads to an increased proliferation (Lee et al., 2009).

Overall, innate immunity of PSCs still is poorly understood, making it an interesting field of research. In case of a possible future use in regenerative medicine, the immunogenic potential of the cells need to be elucidated. When differentiating transplant cells from hPSCs, it has to be clarified whether the cells acquire an innate immunity; otherwise, transplanted cells would be amenable to viral infections and thus a risk for the host.

1.5 Aim of the Thesis

Aim of this thesis is to analyze two different aspects of the biology of human pluripotent stem cells (hPSCs).

First of all the role of Stat3 signaling in hPSCs shall be analyzed. With a potent LIF agonist, an IL-6/sIL-6R chimeric fusion protein, the signaling shall be activated in hPSCs, cells that usually do not respond to LIF.
Initially, it shall be determined if the chimeric protein is feasible to maintain pluripotency in hPSCs in the absence of feeder cells.
Further it shall be investigated whether it is possible to activate Stat3 in hPSCs. Via immunoblot it shall be shown whether Stat3 is getting phosphorylated. Expression of putative target genes of the Stat3 signaling shall be shown with qRT-PCR.
To further investigate the role of Stat3-signaling, a robust genetic loss-of-function model shall be established. For that TALEN technology shall be used. To gain a high efficiency, an mRNA encoding for TALEN that target Stat3 shall be synthesized.
In order to improve mRNA transfection efficiency, different synthetic mRNAs will be tested. Preliminary data of other groups let assume that hPSCs could be transfected with unmodified synthetic mRNA.
Because of that the immune response of hPSCs is another general aspect of stem cell biology, which will be investigated in this thesis.
It will be examined whether hPSCs can be transfected efficiently with unmodified synthetic mRNA. For that, an mRNA encoding for GFP will be used. Efficiencies will be estimated via flow cytometry. The activation of the innate immune system upon transfection with modified and unmodified synthetic mRNA shall be shown by using qRT-PCR.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used are obtained from Sigma-Aldrich GmbH (Steinheim, Germany), Carl Roth GmbH (Karlsruhe, Germany), Merck (Darmstadt, Germany), Life Technologies (Darmstadt, Germany) and Fluka (Bad Homburg, Germany).

In general, solutions are prepared using water from a Millipore filter system (Millipore, Eschborn, Germany).

2.1.2 Equipment

Table 2.1: Technical equipment

Appliance	Name	Manufacturer
Autoclave	D-150	Systec (Wettenberg, Germany)
Bacterial shaker		New Brunswick Scientific (Nürtingen, Germany)
Block heater	Thermomixer compact	Eppendorf (Hamburg, Germany)
Cell culture centrifuge	Megafuge 1.0R	Heraeus (Hanau, Germany)
Centrifuge	RC5B plus	Thermo (Waltham, USA)
Chemiluminescence detection	Chemidoc XRS	Biometra (Göttingen, Germany)
Counting chamber	Fuchs-Rosenthal	Faust (Halle, Germany)
Digital camera	Canon Power Shot G5	Canon (Krefeld, Germany)
Flow Cytometer	FACS Calibur	BD (Mountainview, USA)
Fluorescence cam	DFC345 FX	Leica (Wetzlar, Germany)
Fluorescence lamp		Leica (Wetzlar, Germany)
Fluorescence microscope	DMIL LED Fluo	Leica (Wetzlar, Germany)
Freezing container	“Mr.Frosty” 5100 Cryo	Nalgene (Roskilde, Denmark)
Gel electrophoresis chamber	Agagel	Biometra (Göttingen, Germany)
Gel Documentation	Geldoc2000	Biorad (Munich, Germany)
Incubator (Cell culture)	HERAcell 150	Heraeus (Hanau, Germany)
Inverse light microscope	Axiovert 40C	Carl Zeiss (Jena, Germany)
Liquid nitrogen store	MVE 611	Chart Industries (Burnsville, USA)
Magnetic stirrer		Stuart Scientific (Staffordshire, UK)
Micropipette	2µl, 10µl, 100µl, 1000µl	Eppendorf (Hamburg, Germany)

Micro-Spectrophotometer	Nanodrop ND-1000	Peqlab (Erlangen, Germany)
pH-meter	CG840	Schott (Mainz, Germany)
Pipetteboy	Accu-Jet II	Brand (Wertheim, Germany)
Powersupply for agarose electrophoresis	Standard Power Pack P25	Biometra (Göttingen, Germany)
Refrigerator 4°C/-20°C	G 2013 Comfort	Liebherr (Lindau, Germany)
Refrigerator -80°C	HERAfreeze	Heraeus (Hanau, Germany)
Scales	LA310S; BL610	Sattorius (Göttingen, Germany)
SDS-PAGE system	ProteanIII MiniGel System	Biorad (Munich, Germany)
Sterile laminar flow hood	HERAsafe	Heraeus (Hanau, Germany)
Table centrifuge	Centrifuge 5415R	Eppendorf (Hamburg, Germany)
Thermocycler	T3000	Biometra (Göttingen, Germany)
UV transilluminator	GelVue GVM20	Syngene (Cambridge, UK)
Vacuum pump	Vacuubrand	Brand (Wertheim, Germany)
Vortexer	Genie 2	Scientific Industries (Bohemia, USA)
Water bath	1008	GFL (Burgwedel, Germany)

2.1.3 Disposables

Table 2.2: Disposables

Disposable	Manufacturer
Adhesive PCR films	Peqlab (Erlangen, Germany)
Centrifuge tubes	Corning (Kaiserslautern, Germany)
Cryovials	Nunc (Wiesbaden, Germany)
“Whatman” filter papers	GE Healthcare Biosciences (Pittsburgh, USA)
Nitrocellulose membrane	Carl Roth GmbH (Karlsruhe, Germany)
Parafilm	Pechinery (Chicago, USA)
PCR plate (96 well)	Peqlab (Erlangen, Germany)
PCR stripe tubes 0.2ml	Peqlab (Erlangen, Germany)
Reaction tubes (1ml, 1.5ml)	Eppendorf (Hamburg, Germany)
Serological pipettes (5ml, 10ml, 25ml)	Corning (Bodenheim, Germany)
Syringe filter 0.2µm	Millipore (Schwalbach, Germany)
Syringes	BD Biosciences (Heidelberg, Germany)
Tissue Culture dishes	BD Biosciences (Heidelberg, Germany) Corning (Kaiserslautern, Germany) Nunc (Wiesbaden, Germany)

Disposables used for cell culture are either delivered as sterile material or autoclaved prior to use.

2.1.4 Enzymes

Table 2.3: Enzymes

Enzyme	Manufacturer
Phusion Taq polymerase	NEB (Frankfurt, Germany
Proteinase K	Sigma-Aldrich (Steinheim, Germany)
Restriction endonucleases	Thermo Scientific (Dreieich, Germany)
T7 endonuclease I	NEB (Frankfurt, Germany)

2.1.5 Antibodies

Table 2.4: Antibodies

Antibody	Dilution	Manufacturer
anti beta-actin	1:800	EMD Millipore (San Diego, USA)
Monoclonal anti-FLAG® M2 antibody produced in mouse	1:400	Sigma (Steinheim, Germany)
anti pStat3 (Tyr 705) rabbit	1:500	Cell Signaling Technology (Boston, USA)
anti Stat3 rabbit	1:500	Cell Signaling Technology (Boston, USA)
anti-Mouse IgG, HRP-linked antibody	1:1000	Cell Signaling Technology (Boston, USA)
anti-Mouse IgG, HRP-linked antibody	1:1000	Cell Signaling Technology (Boston, USA)

2.1.6 Kits

Table 2.5: Kits

Kit	Manufacturer
AmpliScribe™ T7-*Flash*™ Transcription Kit	Epicentre (Madison, USA)
DryEase®	Life Technologies (Karlsruhe, Germany)
GoTaq Flexi DNA polymerase Kit	Promega (Mannheim, Germany)
iScript™ Reverse Transcription Kit	Biorad (Munich, Germany)
Lipofectamine ®LTX Transfection Kit	Life Technologies (Karlsruhe, Germany)
NucleoBond® Xtra Midi/Maxi Kit	Macherey-Nagel (Düren, Germany)

Nucleospin RNA II	Macherey-Nagel (Düren, Germany)
PathScan® Phospho-Stat3 (Tyr705) Sandwich ELISA Kit	Cell Signaling Technology (Boston, USA)
ScriptCap™ m7G Capping System	Cellscript (Madison, USA)
ScriptCap™ 2´-O-Methyltransferase Kit	Cellscript (Madison, USA)
SuperSignal™ West Femto Substrate Kit	Thermo Scientific (Dreieich, Germany)
SuperSignal™ West Pico Substrate Kit	Thermo Scientific (Dreieich, Germany)
TransIT® RNA Transfection Kit	Mirus Bio LLC (Madison, USA)
Wizard® SV Gel and PCR Clean-Up System	Promega (Madison, USA)

2.1.7 Markers

Table 2.6: Markers and ladders

Marker	Manufacturer
1kb DNA ladder	NEB (Frankfurt, Germany)
1000bp DNA ladder	NEB (Frankfurt, Germany)
Prestained Protein Marker	NEB (Frankfurt, Germany)
ssRNA ladder	NEB (Frankfurt, Germany)

2.1.8 Cell lines and bacterial strains

CRL-2097 (ATCC; Manassas, USA)

CRL-2097 cells are human foreskin fibroblasts obtained from a male Caucasian. CRL-2097 are kept in culture until passages around 20, in higher passages they lose their replication potential.

DH5α (Life Technologies; Karlsruhe, Germany)

DH5α bacteria cells are used for amplification of plasmid DNA. They posses a high transformation efficiency.

DravetFB

Dravet fibroblasts are primary fibroblasts. They were obtained from a Dravet patient through a skin biopsy (kindly provided by Johannes Jungverdorben, Institute for Reconstructive Neurobiology, University of Bonn).

E14T

E14T is a murine embryonic stem cell line.

H9.2

H9.2 are wildtypic human embryonic stem cells from Haifa, Israel and were generated in 2000 (Amit et al., 2000).

HEK 293T (Invitrogen, Karlsruhe, Germany)

HEK 293T is an immortalized human cell line extracted from the kidney (Human embryonic kidney cells). They have a modal chromosome number of 64. Usually they are used for the production of lentiviral particles. In this thesis they are used as control cells.

HepG2

HepG2 is a human liver carcinoma cell line that is a suitable model for human hepatocytes. In this thesis they are used to test the functionality of the IL-6/sIL-6R fusion protein. HepG2 cells are known to have a functional Stat3 signaling upon stimulation. HepG2 cells were kindly provided by Ines Raschke (Life and Medical Sciences Institute, University of Bonn).

I3

I3 is a wildtypic human embryonic stem cell line derived from the inner cell mass of a blastocyst (Amit & Itskovitz-Eldor, 2002).

iLB-30-r12

The iPSC line iLB-30-r12 was generated in our lab by Johannes Jungverdorben (unpublished) using the four classical reprogramming factors Oct4, Sox2, Klf4 and c-Myc. They are characterized as *bona fide* iPS cells exhibiting all characteristics of hPSCs.

lt-NES cells

lt-NES cells are human neural stem cells differentiated from hPSCs. The lt-NES cells examined in this thesis were differentiated from the iPSC line iLB-30-r12 by Johannes Jungverdorben (unpublished) using the protocol published by Li et al. (2011).

2.1.9 Cell culture stock solutions

Table 2.7: Cell culture stock solutions

Stock Solution	Manufacturer
β-mercaptoethanol (50mM)	Life Technologies (Karlsruhe, Germany)
B27 Supplement (50x)	Life Technologies (Karlsruhe, Germany)
Basic FGF (bFGF; FGF-2)	Life Technologies (Karlsruhe, Germany)
Defined Trypsin Inhibitor	Life Technologies (Karlsruhe, Germany)
Dimethyl sulfoxide (DMSO)	Life Technologies (Karlsruhe, Germany)
DMEM/F-12	Life Technologies (Karlsruhe, Germany)
DMEM high glucose	Life Technologies (Karlsruhe, Germany)
D-PBS	Life Technologies (Karlsruhe, Germany)
0.5mM EDTA/PBS	Life Technologies (Karlsruhe, Germany)
EGF	Life Technologies (Karlsruhe, Germany)
ESGRO LIF (1000U/µl)	EMD Millipore (San Diego, USA)
Fetal Calf Serum (FCS); heat-inactivated	Life Technologies (Karlsruhe, Germany)
Glucose (4.5g/ml in DMEM/F-12)	Carl Roth (Karlsruhe, Germany)
GlutaMAX (100x)	Life Technologies (Karlsruhe, Germany)
hLIF (10µg/ml)	EMD Millipore (Billerica, USA)
KnockOut DMEM	Life Technologies (Karlsruhe, Germany)
KnockOut Serum Replacement	Life Technologies (Karlsruhe, Germany)
Laminin (natural mouse)	Life Technologies (Karlsruhe, Germany)
Matrigel™ hESC qualified matrix	BD Biosciences (Heidelberg, Germany)
mTeSR™-1	StemCell Technologies (Grenoble, France)
N2 Supplement (100x)	Life Technologies (Karlsruhe, Germany)
Non-Essential Amino Acids (NEAA; 100x)	Life Technologies (Karlsruhe, Germany)
OptiMEM	Life Technologies (Karlsruhe, Germany)
Sodium Pyruvat	Life Technologies (Karlsruhe, Germany)
2.5% Trypsin (10x)	Life Technologies (Karlsruhe, Germany)

2.1.10 Inhibitors and small molecules

Table 2.8: Inhibitors and small molecules

Name	Inhibitor of	Manufacturer
B18R	IFN signaling	eBioscience (San Diego, USA)
CHIR	GSK3	EMD Millipore (San Diego, USA)
HALT protease inhibitor	proteases	Thermo Scientific (Dreieich, Germany)
IL-6/sIL-6R		Merck (Darmstadt, Germany)
Y-27632	ROCK	EMD Millipore (San Diego, USA)

The IL-6/sIL-6R fusion protein is not available commercially.

2.1.11 Oligonucleotide primers

All oligonucleotides are displayed in 5´ to 3´ direction.

Table 2.9: Primers used for qRT-PCR

Primer	Sequence
GAPDH fwd	GAGTCAACGGATTTGGTCGT
GAPDH rev	GACAAGCTTCCCGTTCTCAG
IFNβ fwd	CAGCATCTGCTGGTTGAAGA
IFNβ rev	CATTACCTGAAGGCCAAGGA
Pim1 fwd	GCCCTCCTTTGAAGAAATCC
Pim1 rev	GGACCTGGAGTCTGGAATGA
Socs3 fwd	TCAAGGGACACGCCTTCTGA
Socs3 rev	TGTAAACCTCGAGGTGGAAG

All primers used for qRT-PCR are designed to have an annealing temperature of 60°C.

Table 2.10: Primers used for T7-Assay

Primer	Sequence
Stat3 target fwd	ATGCTCACGGGTAAGTATACAGAGC
Stat3 target rev	CTAGGGCATATGCGGCCAGC

Table 2.11: Primers used for the generation of IVT templates

Primer	Sequence
5´UTR fwd	TTGGACCCTCGTACAGAAGCTAATACG
3´UTR rev	GCGTCGACACTAGTTCTAGACCCTCA
3´TailT rev	T_{120}CTTCCTACTCAGGCTTTATTCAAAGACCA

2.1.12 Software

Table 2.12: Software

Name	Application	Producer
CellQuest Pro	Flow cytometry data acquisition	BD
FlowJo 8.7	Flow cytometry data analysis	Tree Star, Inc.
iCycler analysis software	qRT-PCR analysis	Bio-Rad
ImageJ 10.2	Densiometric analysis	NIH
Microsoft Office 2007	Figure- and text processing	Microsoft
Quantity One 4.6.3	Electrophoresis gel documentation	Bio-Rad
Photoshop CS3	Image processing	Adobe

2.2 DNA

2.2.1 Transformation of E.coli

An aliquot of 100 µl of competent E.coli cells (strain: DH5α) is thawn on ice. Once thawed, 10 ng of the plasmid to be amplified is added to the cells following incubation for 30 minutes on ice. Subsequent to a heat shock at 42°C the cells are incubated on ice for another 2 minutes. 900 µl of SOC medium are added, the solution is incubated for 1 hour at 37°C while shaking in a thermo shaker devise. The transformed E.coli can be used for inoculation of an overnight culture for plasmid preparation.

2.2.2 Preparation of DNA

An overnight culture of 250 ml LB medium containing 250 µl ampicillin (final concentration 0.1 mg/ml) is inoculated with transformed E.coli of the strain DH5α. The beaker containing the solution is incubated overnight at 37°C at 120 rpm in a bacterial shaker. The next day, the cells are pelleted by centrifugation for 10 minutes

at 4000 rpm at 4°C. Plasmid DNA is extracted using the NucleoBond® Xtra Midi / Maxi Kit (Macherey-Nagel) following the manufacturers protocol.
The plasmid DNA is resuspended in 100 µl nuclease-free H_2O.

2.2.3 Sodium Acetate precipitation of DNA

In order to get DNA into another buffer or to concentrate it, it can be precipitated. First, 1/10 volume of 3M sodium acetate (pH 5.2) is added to the aqueous DNA solution followed by 2.5 volumes of 100% ethanol. After mixing by inverting the reaction tube, the solution is incubated for 5 minutes on ice. Subsequently, the precipitate is centrifuged for 30 minutes at 16 000 rpm at 4°C. The resulting pellet is washed twice with 70% ethanol, each followed by a centrifugation for 15 minutes at 16 000 rpm, 4°C. The DNA pellet is dried and recovered in an appropriate volume of the desired buffer or water.

2.2.4 Quantification of DNA

The concentrations of DNA solutions are determined using the NanoDrop system (PeqLab). This system measures the absorption at a wavelength of 260 nm, the wavelength at which nucleic acids posses their maximum absorption. Furthermore it measures the absorption at a wavelength of 280 nm as proteins that naturally contain aromatic amino acids absorb at 280 nm. Therefore the ratio of the optical densities ($OD_{260/280}$) defines the purity of the DNA. A ratio of >1,8 is desirable for further applications.

2.2.5 Restriction Hydrolysis for TALEN-mRNA synthesis

In order to prepare synthetic TALEN-mRNA, it is necessary to have templates in the form of 5´UTR-TALEN-3´UTR. The TALEN plasmids are designed in that way that a simple restriction hydrolysis with 2 enzymes, namely Bsp120I and HindIII, results in a fragment containing the desired structures. For further procedures, a high number of templates are needed; so 10 µg of plasmid DNA was digested.
Therefore, a double digest is performed using the following protocol:

10 µg	DNA
5 µl	Tango Buffer
1 µl	Bsp120I
1 µl	HindIII
x µl	H_2O (adjust to 50µl)

Digestion is performed for 2 hours at 37°C. Afterwards, the enzymes are heat-inactivated for 20 minutes at 80°C.
The obtained fragments can be separated using agarose gel electrophoresis; the fragment to be worked with can be cut out and purified as described in 2.2.7.

2.2.6 Agarose gel electrophoresis

DNA fragments can be separated according to their size by using gel electrophoresis. For that, an electric field is applied to a chamber that holds an agarose gel loaded with DNA samples. Due to the negative charge of the phosphate backbone of the DNA, the fragments move towards the anode with a velocity that is inverse proportional to the logarithm of the fragments molecular weight.
To visualize the DNA fragments, a fluorescing dye is added to the gel. This dye, ethidium bromide, intercalates to nucleic acids. It can be visualized with short-wave UV light.
For preparation, agarose is diluted in TAE Buffer at a concentration of 1-2% (w/v) and shortly boiled in the microwave until the agarose powder is dissolved. After cooling down, ethidium bromide is added 1:10 000, resulting in a concentration of 1 µg/ml; the gel solution immediately is poured into a gel chamber with a slot comb. Once solidified, the gel is transferred into a gel electrophoresis chamber and loaded with the samples. To picture its running front, the samples are mixed 1:10 with 10x Agarose Gel Loading Buffer. To estimate the size of the DNA fragments, at least one slot is loaded with a DNA ladder.
After loading the samples to the gel, an electric field with a voltage of 60-120 Volts is applied for 30 to 60 minutes, depending on the size of the fragments. Visualization and documentation can be performed in the GelDoc system (Biorad, Munich, Germany).

TAE Buffer:	40mM Tris 20mM Acetic acid 1mM EDTA pH of 50xTAE has to be adjusted to 8.4
10x Agarose Gel Loading Buffer:	30% Glycerin spatula tip of bromphenol blue spatula tip of xylenxyanol

2.2.7 Purification of DNA from agarose gels

DNA can be purified from agarose gels or PCR reactions using the Wizard® SV Gel and PCR Clean Up System (Promega).

When planning to extract DNA from an agarose gel, low melting agarose is used for electrophoresis. After running the gel as described before, DNA can be visualized with UV light and the desired band can be cut out with a scalpel. Extraction is performed according to manufacturers protocol.

2.2.8 Quantitative reverse transcriptase polymerase chain reaction

Quantitative reverse transcriptase PCR (qRT-PCR) is a method to determine the concentration of a given cDNA molecule. As a first step, RNA is isolated from the cells of interest as described in 2.3.1. Subsequently, the RNA is treated with DNase to remove any genomic DNA and then reverse transcribed into cDNA using the iScript Kit (Biorad) according to manufacturers instructions.

2 µg of RNA are used for the reverse transcriptase reaction. The obtained cDNA is diluted 1:5 before using it for qRT-PCR.

Reaction Mix:

1 µl	cDNA (diluted 1:5)
1 µl	Primer Mix
10.375 µl	H_2O
12.5 µl	qRT-PCR Supermix
0.125 µl	GoTaq Flexi DNA polymerase

qRT-PCR Supermix: 1511.5 µl H_2O
2000 µl 5x GoTaq Flexi Buffer
1000 µl $MgCl_2$
80 µl 100mM dNTP (20µl each)
400 µl DMSO
1 µl 100µM Fluorescin
7.5 µl 1000x SYBR green

The qRT-PCR reaction is performed in the iCycler with the following cycle parameters:

1. 95°C 5 min
2. 95°C 60 sec
3. 60°C 60 sec
4. 72°C 40 sec
5. 72°C 5 min

Steps 2-4 were repeated 40 times.

qRT-PCRs are done in triplicates. Expression levels get normalized to GAPDH expression.

2.2.9 T7-Assay

To determine the functionality of TALEN pairs, a T7-assay can be performed. This assay makes use of the T7 endonuclease I, an endonuclease derived from the T7 bacteriophage. The T7 endonuclease I recognizes and cleaves non-perfectly matched DNA. After genomic engineering with TALENs, the restricted DNA is ligated via nonhomologous end-joining (NHEJ) or homology-dependent repair (HDR). To test whether a TALEN pair has engineered the genome, cells are lysed two days after transfection in lysis buffer. The reactions are incubated at 65°C for 10 minutes and subsequently at 95°C for 15 minutes. 1µl of the lysate is used for a 25 µl PCR reaction using Phusion Taq-Polymerase according to manufacturers protocol. Primers were chosen to amplify a 300-400bp-sized fragment containing the target side of the TALEN. In case of the Stat3 TALEN pair used in this thesis, the primers *Stat3 target fwd* and *Stat3 target rev* are used.

PCR program:
1. 98°C 30 sec
2. 98°C 30 sec
3. 64°C 30 sec
4. 72°C 15 sec
5. 72°C 10 min
6. 4°C Hold

Steps 2-4 were repeated 32 times.

5µl of the reaction are mixed with 1.1 µl 10x NEB buffer 2 and 4.4 µl H_2O. After heating to 95°C, the reaction is cooled down at a ramp rate of 0.7°C per minute. After reaching room temperature, 0.5 µl of T7 endonuclease I are added, the reaction is incubated at 37°C for 20 minutes. Subsequently, reaction can be, mixed with 10x Agarose Gel Loading Buffer, separated on a 2% agarose gel at 100V. Densiometric quantification of DNA bands are performed using ImageJ. Mutation frequencies can be calculated using the formula:

$$\textit{fractional modification} = 1 - (1 - (\textit{fraction cleaved}))^{0.5}$$ (Miller et al., 2007)

Lysis Buffer:
0.2 µg/ml proteinase K
1mM $CaCl_2$
3mM $MgCl_2$
1mM EDTA
1% Triton X100
10mM Tris pH 7.5

2.3 RNA

2.3.1 Purification of RNA

In order to get RNA for qRT-PCR, cells get detached and centrifuged. The pellet is washed once with PBS. Subsequently, RNA is isolated using the Nucleospin RNA II kit (Macherey-Nagel).

To get purified RNA after *in vitro* transcriptions, the same kit can be used, following the “PCR clean up”-protocol.

Both procedures are carried out according to manufacturers instructions.

2.3.2 Quantification of RNA

The concentrations of RNA solutions can be determined using the NanoDrop system (PeqLab). This system measures the absorption at a wavelength of 260 nm, the wavelength at which nucleic acids posses their maximum absorption. Furthermore it measures the absorption at a wavelength of 280 nm as proteins that naturally contain aromatic amino acids absorb at 280 nm. Therefore the ratio of the optical densities ($OD_{260/280}$) defines the purity of the RNA. A ratio of >1,8 is desirable for further applications.

2.3.3 Generation of templates for synthetic mRNA

To generate synthetic mRNA, a template is needed which consists of the open reading frame (ORF) of the gene of interest, flanked by the so-called untranslated regions (UTR) important for the following *in vitro* transcription (IVT). So-called splint oligonucleotides, short primers that bind to the ends of the ORF and to the UTR, mediate the ligation of these UTRs to the ORF. Both GFP (Dominic Seiferling, unpublished) and TALEN (kindly provided by Tobias Schmidt) constructs were cloned in our lab and by the Hornung Lab in a plasmid in that way that they were already flanked by UTR. So UTR ligation can be skipped. As an overview for template generation, a scheme is depicted in Figure 2.1.

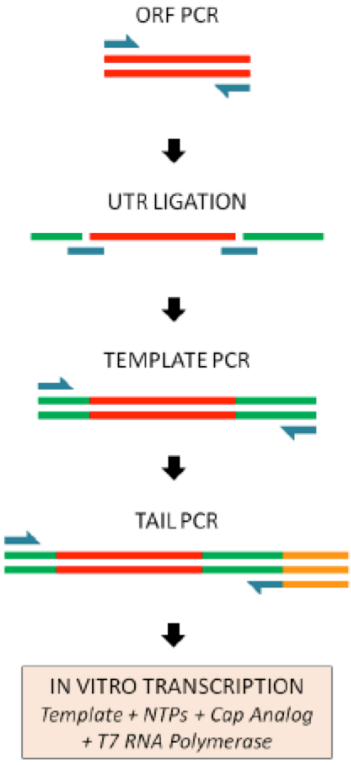

Figure 2.1: Schematic overview of template generation (modified after Warren et al., 2010)
The steps "ORF PCR" and "UTR ligation" could be skipped. Red:open reading frame of the gene of interest; green: UTR, yellow: polyA tail

Template PCR

As a first step, the template needs to be amplified using special primers that bind to the UTR, meaning that for every template the same primers could be used.

Reaction Mix:

10 µl	5x HF Buffer
1 µl	dNTP
1 µl	*5´UTR fwd*
1 µl	*3´UTR rev*
10 µl	DNA
0.2 µl	Phusion Taq-Polymerase
36.8 µl	H_2O

In case of the TALEN constructs, 1.5 µl of H_2O are replaced with 1.5 µl of DMSO in order to improve the outcome of the PCR, as the DNA fragment to be amplified is comparatively long.

PCR program:
1. 98°C 30 sec
2. 98°C 30 sec
3. 58°C 30 sec
4. 72°C 1 min (GFP)/1:45 min (TALEN)
5. 72°C 10 min
6. 4°C Hold

Steps 2 – 4 were repeated 35 times.

Whether the reaction results in a template of the expected size is tested using gel electrophoresis. Afterwards, DNA is extracted from the gel as described before.

Tail PCR

In order to get synthetic mRNA with a polyA tail, as a next step a polyA tail have to be added to the template. For that, a special primer (*3´TailT rev)* is used with a 120 nucleotide long T-stretch. The primers used for the tail PCR bind at the UTR, so that again for each template the same primers can be used.

Reaction Mix:

40 µl	5x HF Buffer
6 µl	DMSO
2 µl	dNTP
2 µl	*5´UTR fwd*
2 µl	*3´TailT rev*
4 µl	DNA
4 µl	Polymerase
144 µl	H_2O

PCR program:
1. 98°C 30 sec
2. 98°C 30 sec
3. 58°C 30 sec
4. 72°C 1 min (GFP)/ 1:45 min (TALEN)
5. 72°C 10 min
6. 4°C Hold

Steps 2 – 4 were repeated 35 times.

Afterwards, the tailed template gets precipitated with Sodium Acetate as described in 2.2.3. The pellet is recovered in 5 µl H_2O, resulting in a high DNA-concentration of >1µg/µl, which is important for the following *In Vitro* Transcription.

2.3.4 Generation of synthetic mRNA

In Vitro Transcription

For the *In Vitro* Transcription (IVT) reactions the AmpliScribe™T7-Flash™ Transcription Kit (Epicentre) is used. The IVT reaction is driven by a T7 promotor within the 5´UTR.

Reaction mix:

1 µg	Template DNA
2 µl	AmpliScribe T7-Flash 10x Reaction Buffer
1.8 µl	100mM ATP
1.8 µl	100mM CTP or Methyl-CTP
1.8 µl	100mM GTP
1.8 µl	100mM UTP or Pseudo-UTP
2 µl	100mM DTT
0.5 µl	RiboGuard RNase Inhibitor
2 µl	AmpliScribe T7-Flash Enzyme Solution
X µl	H_2O (adjust to 20 µl)

The IVT reaction mix gets incubated for 45 minutes at 37°C (GFP mRNA) or 60 minutes at 42°C (TALEN mRNA) respectively. According to the manufacturers protocol, the enzymes have an activity range for temperatures between 37-42°C. Increasing incubation temperature as well as duration leads to improved outcome for the long TALEN mRNA (data not shown).

After incubation, 1 µl RNase-free DNase is added to the reaction and incubated for further 15 minutes at 37°C. Subsequently, the RNA is cleaned up using the Nucleospin RNA II kit as described above.

For generation of synthetic mRNA as published by Warren (Warren et al., 2010) the modified ribonucleotides methyl-CTP and pseudo-UTP were used. These modifications are supposed to reduce innate immunogenic reactions caused by ssRNA (Warren et al., 2010). In order to investigate these effects, next to the

modified version, a modified as well as an unmodified version of GFP-mRNA was synthesized using the standard ribonucleotides CTP and UTP.

Capping of synthetic mRNA

Following the IVT reaction, the obtained synthetic mRNA needs to be capped, as the 5´Cap also decreases the reactions of the innate immune system and leads to increased stability. For this purpose, two kits are used for simultaneous capping: ScriptCap™ m7G Capping System and ScriptCap™ 2´-O.Methyltransferase Kit (Cellscript).

For that, 60 µg IVT product is adjusted with H_2O to a volume of 67 µl. This RNA solution gets heat-denatured for 10 minutes at 65°C and subsequently cooled on ice.

Reaction mix:

10 µl	10x ScriptCap Capping Buffer
10 µl	10mM GTP
2.5 µl	20mM SAM
2.5 µl	20mM ScriptGuard RNase Inhibitor
4 µl	ScriptCap 2´-O-Methyltransferase (100 U/µl)
4 µl	ScriptCap Capping Emzyme (10 U/µl)
67 µl	Heat-denatured RNA

The capping mix was incubated for 30 minutes at 37°C.

Afterwards the mRNA is cleaned up using the Nucleospin RNA II kit (Macherey-Nagel) resulting in a RNA solution with a concentration between 500 and 1500 ng/µl. The RNA can be washed of the column with sterile nuclease-free water; this procedure was appropriate to use the synthetic mRNA for cell culture applications.

2.3.5 Agarose gel electrophoresis

In order to estimate the size and quality of RNA molecules, agarose gel electrophoresis is used. Two different kinds of gel electrophoresis can be performed.

Native gel electrophoresis

The advantage of the native gel electrophoresis for RNA is that a good visualization is possible and that it can be performed at the normal ethidium bromide working bench without any further precautions. The disadvantage is that RNA is easily degraded in native conditions.

The gel gets prepared as described above for DNA electrophoresis (2.2.6). TAE Buffer is prepared freshly to avoid RNase contaminations.
As soon as the gel is solidified, probes are loaded to the slots. Prior to loading, the samples (1µg of RNA each) and 2 µg of the ssRNA ladder (NEB) are prepared using 2x RNA loading buffer (supplied with the ladder) and boiled for 10 minutes at 70°C, subsequently chilled on ice.
Electrophoresis is performed in TAE Buffer. A voltage of 5 V/cm of gel is applied for 30 minutes to one hour. RNA fragments can be visualized using a UV transilluminator.

Denaturing gel electrophoresis

To prevent degradation during electrophoresis, denaturing gel electrophoresis can be performed. A disadvantage of this method is that the visibility of the bands is not as good as in the native gel electrophoresis. Furthermore, the use of strong denaturing substances as formaldehyde requires special security precautions. Contact with skin and inhalation of the vapors need to be avoided.
For preparation, agarose (1% w/v) is boiled in water and the respective amounts of 10x MOPS Buffer and formaldehyde (final concentration 7% v/v) are added afterwards. Samples and ladder are prepared as described above, unless before loading ethidium bromide is added at a final concentration of 10 µg/ml to them. Electrophoresis is performed in 1x MOPS buffer with an applied voltage of 5 V/cm for 30 minutes to one hour. Visualization is performed as described before.

10x MOPS Buffer: 400mM MOPS, pH 7.0
0.1M sodium acetate
0.01M EDTA

2.4 Proteins

2.4.1 Isolation of total protein

To analyze total protein of cell lysates, cells are lysed using RIPA Buffer. Prior to use, the RIPA Buffer is prepared freshly. For that, 200 µl of each 5x stock solution are mixed resulting in the wanted 1x concentration of each component; 10 µl of HALT protease inhibitor are added to prevent degradation of the proteins.

Cells are lysed directly on the cell culture dishes. For that, medium gets aspirated and the cells are washed once with PBS. Subsequently, the dishes are put on ice, cold RIPA Buffer is added (60 µl per well of 6well dish). Following 20 minutes of incubation, cells get detached using a cell scraper. The lysate is transferred to a reaction tube and centrifuged for 20 minutes at a speed of 16 000 rpm at 4°C. The supernatant is collected in a new reaction tube and stored at -20°C.

RIPA Buffer Stock Solutions:

5x Buffer	250mM Tris-HCl, pH 7.5
	5mM EDTA
5x NaCl	750mM NaCl
5x DOC	Deoxycholic acid, sodium salt
5x SDS	0.5% Lauryl sulfate
5x Igepal	5% Igepal

2.4.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Polyacrylamide gel

Proteins are separated using a discontinuous polyacrylamide gel consisting of a stacking gel and a separation gel (Laemmli, 1970). Due to a lower concentration of acrylamide, the pores in the stacking gel are bigger; therefore all the proteins accumulate in the stacking gel and enter the separation gel simultaneously. The higher amount of acrylamide in the separation gel leads to a smaller pore size leading to a separation of the proteins according to their molecular masses.

At preparation, first the separation gel is poured into a tightly assembled gel chamber and subsequently covered with isopropanol to ensure a plane surface. After polymerization, isopropanol gets removed using Whatman Paper and the stacking gel is poured into the gel chamber. A comb is inserted to the stacking gel. Polymerization is accomplished after approximately 30 minutes.

Stacking gel:	3.05 ml H_2O
	0.65 ml 30% Bis-/Acrylamide
	25 µl 10% APS
	5 µl TEMED
	1.25 ml TrisCl/SDS pH 6.8

Separation gel: 3.15 ml H_2O
2.5 ml 30% Bis-/Acrylamide
30 µl 10% APS
8 µl TEMED
1.9 ml Tris-Cl/SDS pH 8.8

Sample preparation for SDS-PAGE

Before loading the protein sample onto the polyacrylamide gel, it has to be diluted in 2x sodium dodecyl sulfate (SDS) buffer. SDS is a highly anionic detergent and has two beneficial effects for protein separation; first it charges the proteins negatively according to their size, and second it denatures the secondary and tertiary structures of the proteins. β-mercaptoethanol, another component of the 2x SDS buffer, reduces the disulfide bonds.

For preparation, the protein lysate is diluted 1:1 with 2x SDS buffer. After heating it to 95°C for 5 minutes, the samples are cooled down on ice and centrifuged subsequently.

2x SDS Buffer: 250mM Tris-Cl, pH 6,8
6% (w/v) SDS
20% (w/v) glycerol
0.01% (w/v) bromphenol blue
6% (w/v) β-mercaptoethanol

Gel electrophoresis

After preparation, the samples are either frozen down for later use or directly loaded onto the gel. 5 to 10 µl are loaded in each slot. Additionally, the Prestained Protein Marker is loaded in at least one slot to determine the size of the proteins.

After loading, the gel gets covered with SDS Running Buffer. Electrophoresis is carried out at 200V. The duration depends on the expected size of the proteins to be separated. After the gel run, the gel can be stained or used for an immunoblot.

SDS Running Buffer: 25mM Tris
192mM Glycin
0.1% SDS, pH 8.3

Staining of gels

After completion of the gel run, the gel is transferred into a glass dish, washed with ddH_2O and then covered with Imperial Protein Stain Solution (Perbio). After an incubation of at least 1 hour the staining solution gets removed, ddH_2O and a tissue are added. On the next day, the residual staining was washed of the gel; only the proteins in the gel lanes remain stained. Gels are dried using the DryEase® System (Life Technologies). Shortly, two membranes supplied with the system and the gel are incubated with Gel Drying Buffer for 2 minutes and then fixed into a frame. After 24 hours, the gel is dried and can be scanned for documentation.

Gel Drying Buffer:	20% methanol
	2% glycerol

2.4.3 Immunoblot

To determine whether a protein is present in the whole protein lysates of cells and to estimate the running size of this protein, an immunoblot can be used. Briefly, the proteins separated using SDS-PAGE first need to be blotted onto a membrane. After that, an antibody against the protein to be detected gets incubated with the membrane. A secondary antibody coupled to an enzyme enables detection.

Blotting

The proteins are blotted onto the membrane using the so-called “wet” technique, meaning the whole blotting complex is covered with Blot Transfer Buffer all the time. First, the gel, a piece of blotting membrane in the size of the gel, and 6 Whatman papers in the same size as well as two fiber pads get pre-wetted in Blot Transfer Buffer. To assemble the blot, one fiber pad and 3 Whatman Paper are put onto the black side of the cassette followed by the gel and the membrane. On top, another 3 Whatman papers and the second fiber pad complete the assembly. The cassette is closed and put into a Mini Trans-Blot buffer tank together with a cooling unit and covered with Blot Transfer Buffer. An electric current of 100V is applied for 1 hour.

Blot Transfer Buffer (wet):	25mM Tris
	192mM Glycin
	20% methanol

Blocking and antibody incubation

After the blotting, the membrane is put into a 50 ml centrifugation tube. Depending on the antibody to be used, the blocking solution differs; Stat-3 and pStat-3 are detected using blocking solution and antibody solution containing BSA. After one hour of incubation at room temperature in 5 ml of the appropriate blocking solution, the first antibody is diluted in 3 ml of 2.5% BSA/TBS-T or 2.5% milk powder/TBS-T respectively. Incubation is either performed overnight at 4°C or for one hour at room temperature. Subsequently, the membrane gets washed 3 times for each 5 minutes with TBS-T, followed by the incubation of the secondary antibody, which is HRP-coupled and diluted 1:1000, as described before overnight at 4°C or 1 hour at room temperature. After three times of washing with TBS-T the membrane is ready for detection. All incubation and washing steps are performed in the centrifugation tube while rotating it overhead to prevent drying out of the membrane and to ensure a full coverage of the membrane.

Blocking solution 1:	5% milk powder
	TBS-T

Blocking solution 2:	5% bovine serum albumine (BSA)
	TBS-T

TBS-T:	50mM Tris-HCl, pH 7.4
	150mM NaCl
	0.05% Tween 20 (v/v)

Detection

After antibody incubations, the immunoblot is ready to be detected using either the SuperSignal West Pico Substrate or the SuperSignal West Femto Substrate (Thermo Scientific). The detection itself is carried out using the ChemiDoc XRS system (Biorad), which harbors a CCD camera able to detect the emerging chemiluminescence.

Immunoblot stripping

In some cases it is interesting to check whether a second protein can also be detected on the membrane. For that, the antibodies used before can be stripped of the membrane. To accomplish this, the membrane is incubated at 50°C in Stripping Buffer containing strong detergents. After 30 minutes of incubation, the blot gets washed with PBS (Life Technologies) 2 times for 30 minutes and another 5 times for 10 minutes. After that, the blot is ready to be used again, starting with blocking.

Stripping Buffer:	2% SDS
	100mM β-mercaptoethanol
	50mM Tris-Cl, pH 6.8

2.5 Cell Culture

All cells are cultivated at 37°C in a humidified atmosphere with 5% CO_2.
All cell culture procedures are performed under a sterile working bench with laminar flow using sterile media, glass and plastic ware.

2.5.1 Coating of cell culture dishes

For cell culture, only cell culture treated plastic ware was used. Nevertheless, for some cells, these dishes had to be coated to assure appropriate culture conditions.

Matrigel™ coating

hPSCs used in this thesis are cultivated on 6well-dishes coated with BD Matrigel™ hESC-qualified matrix (BD Biosciences). Matrigel™ is aliquoted and frozen following manufacturer's instructions; the volume of the aliquots is batch-dependent. For use, one aliquot of Matrigel™ gets thawed on ice, once thawed it is immediately transferred into a centrifugation tube containing 25 ml of ice-cold DMEM/F-12. Into each well of a 6well-dish, 1 ml of the Matrigel™-dilution is pipetted. The dishes are sealed with Parafilm and stored at 4°C for up to 2 weeks. Before use, the dishes are put to room temperature for 30 minutes. Before plating cells on the dishes, the Matrigel™-dilution has to be aspirated, no washing is necessary.

PO/Ln coating

The cultivation of lt-NES cells requires dishes coated with Polyornithine (PO) and Laminin (Ln). First, 1 ml of PO (1.5 mg/ml in PBS) is pipetted into each well of a 6well-dish and incubated for at least 2 hours at 37°C. After PO incubation, the dishes are washed twice with PBS and subsequently Laminin is added at a concentration of 1 µg/ml in PBS. After 2 hours of incubation at 37°C, the dishes are used immediately or sealed with Parafilm and stored for up to one week at 4°C.

Gelatin coating

When needed for experiments as mRNA transfection, human fibroblasts and 293T cells are seeded on gelatin-coated dishes. For that, cell culture plastic ware is incubated with 0.1% (v/v) Gelatin in H_2O for 20 minutes at 37°C.

2.5.2 Cultivation of human pluripotent stem cells (hPSCs)

hPSCs routinely are cultivated in 6well-dishes coated with BD Matrigel™ hESC-qualified matrix (BD Biosciences) in mTeSR™1 medium (STEMCELL Technologies). Cell culture medium is changed daily and the cells are passaged at a confluence of around 70-80%. For passaging, culture medium gets aspirated and replaced by 1 ml of fresh mTeSR™1, then the colonies are scraped of using a cell scraper. The cell suspension is gently pipetted up and down three times with a 1000 µl-tip to dissociate the colonies into smaller clumps. The cells are transferred into a 15 ml centrifuge tube; the volume gets adjusted to obtain the desired dilution. An appropriate amount of cell solution is then transferred on a new Matrigel™-coated 6well dish, e.g. for a splitting ratio of 1:6, one well of cells are detached, the volume is adjusted to 3 ml, and 500 µl of the solution is transferred to each well of the new dish already containing 1 ml of mTeSR™1, resulting in a total volume of 1.5 ml/well of culture medium.

In order to test the influence of the IL-6/sIL-6R fusion protein, mTeSR™1 is replaced with hPSC Medium containing the fusion protein. hPSCs require a layer of radiated mouse fibroblasts, so-called Feeder Cells, when not cultivated in special systems as the mTeSR™1 system. It gets tested whether the fusion protein can compensate for the lack of Feeder Cells.

hPSC Medium:
- Knockout DMEM
- 20% Serum Replacement
- 1:100 NEAA
- 1:100 GlutaMax
- 1:500 β-Mercaptoethanol
- 10 ng/ml bFGF
- 200 pg/ml IL-6/sIL-6R

As a control, hPSC Medium is supplemented with human LIF at a concentration of 10 ng/ml instead of fusion protein.

2.5.2.1 Single cell suspension of hPSCs

For some experiments it is necessary to plate hPSCs as single cells instead of colonies. For that, one hour before splitting, the cells are incubated with 10µM Rock-

inhibitor at 37°C, 5% CO_2. After 1 hour, the culture medium is aspirated and the cells are washed once with PBS. PBS is replaced with 500 µl of 0.5mM EDTA in PBS. After an incubation of 10-20 minutes at 37°C, 5% CO_2, cells are detached. Adding 500 µl of culture medium stops the enzymatic reaction. The cells are dissociated by roughly pipetting the cells up and down using a 1000 µl-pipette. The cell solution is transferred into a 15 ml centrifuge tube and the well is washed once with 2 ml of culture medium which then also gets transferred into the tube. The single cells are centrifuged at 1000 rpm for 5 minutes. Following, the supernatant is aspirated, and the cell pellet is resuspended in an appropriate volume. The single cell suspension were transferred onto a new Matrigel™-coated 6well dish in mTeSR™1 supplemented with 10µM of Rock-inhibitor.

2.5.2.2 Freezing and thawing of hPSCs

To freeze hPSCs, a single cell suspension is done following the protocol described in 2.5.2.1. After centrifugation, the supernatant is aspirated. The cell pellet gets resuspended in an appropriate volume of hPSC Freezing Medium (1 ml freezing medium per well of a 6well plate). The cell suspension immediately is pipetted into cryovials that then are put into a Mr. Frosty™ freezing container (Nalgene) filled with isopropanol. After 1 day at -80°C, cells ideally are transferred in a liquid nitrogen container at -196°C for longer storage.

To thaw hPSCs, a cryovial with cell suspension is thawed in a 37°C water bath. As soon as only a small clump of medium was left frozen, the content of the vial is transferred in a 15 ml centrifugation tube already containing 9 ml of hPSC medium.
After a centrifugation at 1000 rpm for 4 minutes, supernatant is aspirated and the cell pellet are resuspended in hPSC medium. The cells of a confluent well of a 6well plate, corresponding to about 2 x 10^6 cells, are usually seeded onto 3 wells of a Matrigel™-coated 6well plate. It is essential to supplement the hPSC medium with 10µM ROCK inhibitor for at least 24 hours.

hPSC Freezing Medium:	10% DMSO
	90% Serum Replacement

2.5.3 Cultivation of human lt-NES cells

lt-NES cells routinely are cultivated in 6well-dishes coated with PO/Ln in lt-NES cell medium. Medium is changed every day and cells are splitted when reaching 100% confluence. For passaging, the cell culture medium is aspirated and the cells are washed once with PBS (Life Technologies). 1 ml of trypsin is added per well and the cells are incubated for 5 to 10 minutes at 37°C, the detachment of the cells is observed with the microscope. As soon as the cells are detached, 1 ml/well of trypsin inhibitor is added to stop the enzymatic reaction. The cell suspension is transferred into a 15 ml centrifuge tube; the well is washed once with 2 ml of lt-NES cell medium. After 4 minutes of centrifugation at 1000rpm/4°C, the supernatant gets aspirated and the pellet is resuspended in an appropriate volume of lt-NES cell medium. The splitting ratio is between 1:3 and 1:6.

lt-NES cell medium: DMEM/F-12
1% N2
0.1% B27
1:100 Glucose Stock Solution
1:1000 bFGF
1:1000 EGF
0.8µM CHIR

2.5.4 Cultivation of human fibroblasts, HEK 293T cells and HepG2 cells

Human fibroblasts, HEK 293T cells and HepG2 cells are cultivated in Fibroblast Medium on uncoated cell culture dishes. Cells are splitted after reaching 90-95% confluence. For that, the cell culture medium is removed and the cells are washed once with PBS. An appropriate volume of trypsin is added to the cells and incubated for 3-5 minutes at 37°C. After detachment, addition of an equal volume of fibroblast medium stops the enzymatic reaction. The serum in the medium is sufficient to inhibit trypsin; no addition of trypsin inhibitor is needed. The cell suspension is transferred into a 15 ml centrifuge tube; the dish is washed once with fibroblast medium that is also transferred to the tube. After 4 minutes centrifugation at 1200rpm, the supernatant is discarded and the cell pellet is resuspended in an appropriate volume.

The splitting ratio lies between 1:4 and 1:10 for human fibroblasts and HepG2 cells and between 1:10 and 1:30 for HEK 293T cells.

Fibroblast Medium: DMEM (high glucose)
10% FCS
1:100 NEAA
1:100 Sodium Pyruvate
1:100 GlutaMAX

2.5.5 Cultivation of murine pluripotent stem cells (mPSCs)

mPSCs are cultivated on gelatin coated cell culture dishes in mPSC Medium. Medium is changed on a daily base. The cells are splitted every 3 to 4 days, depending on the confluence and the size of the colonies. To split mPSCs, cell culture medium gets aspirated and the cells are washed once with PBS. An appropriate amount of Trypsin is added to the cells following an incubation of 4 to 6 minutes at 37°C. After incubation, the enzymatic reaction is stopped by adding the same amount of mPSC Medium. The cell suspension is pipetted up and down to dissociate the colonies and subsequently transferred into a 15 ml centrifuge tube for a centrifugation at 1000rpm for 4 minutes. The supernatant is aspirated and the cells get resuspended in fresh mPSC medium. The splitting ratio is between 1:20 and 1:50.

For a dose response test, the mPSC Medium gets modified, instead of LIF different concentrations of the IL-6/sIL-6R fusion protein are added.

mPSC Medium: DMEM (high glucose)
15% FCS
1:100 NEAA
1:100 Sodium Pyruvate
1:500 β-Mercaptoethanol
1:10 000 LIF (10ng/ml)
or IL-6/sIL-6R in different concentrations

2.5.6 Counting of cells

For some experiments it is necessary to determine the exact cell number, e.g. to plate cells for mRNA transfection. For that, cells are detached as described before. 20 µl of the cell suspension are transferred into a reaction tube containing 20 µl of trypan blue. After mixing, 20 µl of the cell suspension are pipetted in a Neubauer counting chamber. The mean cell number per main squares is determined by counting four main squares.

In case of dense cell suspensions, a dilution with PBS can be useful.

With the following formula, the cell number can be calculated:

$$cells/ml = (mean\ cell\ number/main\ square) \times dilution\ factor \times 10^4$$

2.5.7 mRNA transfection

Cells are plated 24 hours before transfection as single cells on the required coating. The cell number is determined as described before. Due to toxicity, the different cell lines can not all be transfected with the same transfection reagent, so Lipofectamine® LTX (Life Technologies) and TransIT® mRNA transfection reagent (MirusBio) have to be used. In table 2.13 the cell number plated per well of a 6well plate and the transfection reagent is shown.

Table 2.13: plated cell number for mRNA transfection

Cells	Number/well	Transfection reagent
hPSCs	1 000 000	Lipofectamine® LTX
lt-NES	200 000	Lipofectamine® LTX
hFibroblasts	150 000	TransIT®
HEK 293T	300 000	TransIT®

2.5.7.1 Transfection with Lipofectamine® LTX

Per well to be transfected, an appropriate amount of mRNA (usually between 500 ng and 1 µg) is diluted in 100 µl OptiMEM in a reaction tube. 1.5 µl of PlusReagent were added. After an incubation of 5 minutes at room temperature, 4.5 µl Lipofectamine® LTX are added, the solution is incubated for 20 minutes at room temperature. During the last incubation, the medium on the cells is replaced with a 1:1 mixture of the normal cell culture medium and OptiMEM. This is necessary because some components of the medium as serum replacement can interfere with the lipoparticles. After incubation, the transfection mix is added dropwisely to the cells.

2.5.7.2 Transfection with TransIT®

Per well to be transfected, an appropriate amount of mRNA (usually between 500 ng and 1 µg) is diluted in 100 µl OptiMEM in a reaction tube. Subsequently, Boost reagent is added at an amount of 1 µl per 1 µg RNA. After mixing, TransIT® reagent is added (1 µl per 1 µg RNA). The solution is mixed again and incubated at room temperature. During incubation, the medium on the cells is exchanged as described before in the protocol for transfection with Lipofectamine®LTX. After 2 to 5 minutes of incubation, the solution is added dropwisely to the cells.

After addition of the transfection mixture the cells subsequently are put in a 37°C incubator and incubated over night. 16 hours after mRNA transfection, the medium is exchanged with fresh medium.

2.5.7.3 Treatment with B18R

To examine whether the TLR3 activity affects mRNA transfection efficiencies, the TLR3 inhibitor B18R is added in some experiments. For that, B18R is added at a concentration of 200 ng/ml to the cells one hour prior mRNA transfection. Before that, the medium on the cells is replaced with a 1:1 mixture of the normal cell culture medium and OptiMEM. The transfection protocol is followed as described above, except the medium change during incubation.

2.6 Flow Cytometry

Flow cytometry is a method to quantify fluorescent events. For example it is possible to stain cells for surface markers and use a secondary antibody that is coupled to a fluorescent dye. In this thesis, flow cytometry was used to quantify efficiencies of

GFP-mRNA transfection. For analysis, the transfected cells are detached as described above (see 2.5). In case of hPSCs, the cells need to be singularized. After centrifugation, the pellets are resuspended in a small volume of cold PBS (200 – 500 µl). Subsequently, the cell solutions are transferred into flow cytometry tubes, put on ice and immediately measured.
Data acquisition is performed using CellQuest Acquisition software; obtained data can be analyzed using FlowJo software.

2.7 Enzyme-linked immunosorbent assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) is an assay that enables to quantify the amount of a substance in a sample, using antibodies against this antigen. In this thesis, the amount of phosphorylated Stat3 (pStat3) is determined with the PathScan® Phospho-Stat3 (Tyr705) Sandwich ELISA Kit from CellSignaling Technology.
The assay is performed according to manufacturers protocol.

3 Results

3.1 The role of Stat3 in hPSC

The role of Stat3 signaling in human pluripotent stem cells is discussed controversially.

In murine PSCs, Stat3 signaling needs to be activated via LIF addition to maintain pluripotency. So far, the widespread opinion is that hPSCs are in the primed state of pluripotency and do not respond to extrinsic factors with Stat3 signaling. Only the naïve counterparts react with Stat3 signaling upon stimulation and are even dependent on Stat3 activation (Gafni et al., 2013).

In 2010, it was published that hPSCs could be maintained pluripotent in suspension cultures by adding the chimeric fusion protein IL-6/sIL-6R, which is a LIF agonist and was claimed to be more potent than LIF (Amit et al., 2010). If the reason for the absence of Stat3 signaling in hPSCs is the missing of LIF receptors on its cell surface, the fusion protein could circumvent this lack; only the gp130 receptor needs to be presented on the cell surface to initiate Stat3 signaling.

In suspension cultures, cells do not have a matrix to bind to, they can only attach to themselves. The standard spontaneous differentiation protocol for hPSCs requires as a first step to detach the colonies and bring them into suspension. The medium used for that is hPSC medium without supplementation of bFGF. In the published protocol, Amit and colleagues use the same medium, only supplemented with bFGF and a IL-6/sIL-6R fusion protein, for maintenance in suspension (Amit et al., 2011). They did not observe a lot of spontaneous differentiation. This let suggest that the IL-6/sIL-6R fusion protein could also enable hPSCs to be maintained pluripotent without a feeder layer. Cultivation of hPSCs without a feeder cell layer of irradiated mouse embryonic fibroblasts normally requires special coatings and cell culture mediums, as the mTeSR™-1/Matrigel™ system. To evaluate this, the company Merck kindly provided the IL-6/sIL-6R fusion protein in order to repeat the suspension culture experiments (performed in another group) and to transfer the results to adherent conditions.

As a first step, the IL-6/sIL-6R fusion protein was examined on quality aspects. An SDS gel stained with Imperial Protein Stain Solution showed that the delivered protein was pure, only a single band could be detected (Figure 3.1). The marker revealed that the size of the protein in the solution was slightly less than 80kDa,

which is consistent with the size of the IL-6/sIL-6R fusion protein given by the manufacturer.

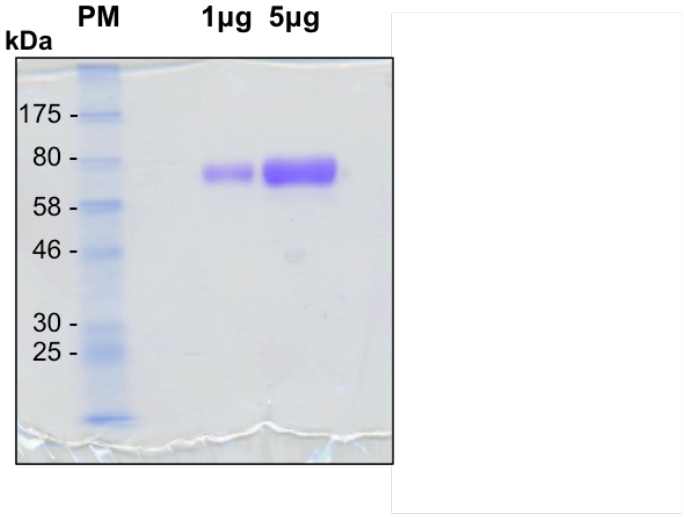

Figure 3.1: SDS-PAGE analysis to estimate size and purity of the IL-6/sIL-6R fusion protein
SDS-PAGE reveals a single protein with a size of around 80kDa; no contamination could be seen in the lanes. 1 µg or 5 µg of the fusion protein were loaded in two slots of the SDS gel; PM= proteinmarker

Furthermore, the concentration of the IL-6/sIL-6R fusion protein was estimated using a Bradford assay. The concentration given by the manufacturer was 680 µg/ml. The measured concentration differed from this; the Bradford assay resulted in a concentration of 318 µg/ml. All further dilutions of the IL-6/sIL-6R fusion protein were calculated using the measured concentration.

3.1.1 hPSCs express Stat3

After confirming the quality of the IL-6/sIL-6R fusion protein, it was checked whether hPSCs that served as cellular models in this thesis in fact express Stat3. If Stat3 is not expressed in hPSCs, no activation of the Stat3 signaling is expected to occur. Whole cell protein lysates of I3 hPSCs were loaded on a gel and an SDS-PAGE was performed. The cells were either cultivated in full hPSC medium without additional supplements (Figure 3.2, lane 1) or in presence of the IL-6/sIL-6R fusion protein (lane 2) or human LIF (lane 3) for 1 day, respectively. The IL-6/sIL-6R fusion protein was supplied at a concentration of 200 pg/ml, which was in the range of the published concentration feasible to maintain pluripotency in suspension cultures (100 pg/ml; Amit et al., 2010). hLIF was added with a final concentration of 10 ng/ml.

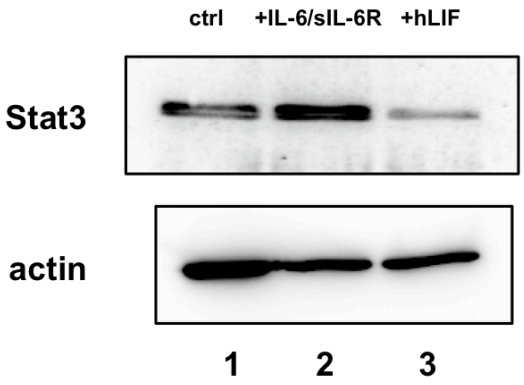

Figure 3.2: Immunoblot confirms Stat3 expression in hPSCs
This immunoblot exemplarily shows a strong Stat3 signal for I3 hESCs cultivated in hPSC medium only and supplemented with IL-6/sIL-6R fusion protein and hLIF, respectively. The signal for cells treated with LIF appears to be weaker. 1= control (hPSC medium), 2= cells treated with IL-6/sIL-6R fusion protein, 3= cells treated with hLIF
Development: Stat3, actin= SuperSignal West Pico Substrate

The immunoblot depicted in figure 3.2 shows that hPSCs express Stat3. It appeared that, when compared to the actin control, cells treated with the IL-6/sIL-6R fusion protein express more Stat3. This could not be observed in this impact in iLB-30-r12 hiPSCs.

3.1.2 Stat3 cannot be activated in hPSCs by IL-6/sIL-6R fusion protein

Next, experiments to check whether the IL-6/sIL-6R fusion protein can activate Stat3 signaling in hPSCs were performed. Medium conditions were changed on iLB-30-r12 hiPSCs that were plated on Matrigel™ hESC qualified matrix. Instead of mTeSR™-1, hPSC medium supplemented with either 200 pg/ml IL-6/sIL-6R fusion protein or 10 ng/ml hLIF was added to the cells plated as small clumps one day in advance. One day after the medium change (figure 3.3, A-D), IL-6/sIL-6R fusion protein as well as hLIF changes the hPSC morphology. In the absence of these factors, cells spontaneously lost the compactness of the colonies indicating differentiation (Figure 3.3, B). The colonies lost its sharp borders, and in the inside of the colonies single cells could be distinguished, which is normally a first sign of differentiation. In contrast to that, the presence of both, IL-6/sIL-6R fusion protein (Figure 3.3, C) and hLIF (Figure 3.3, D), kept the colonies in a compact shape, comparable to the morphology of the positive control, cells cultured in mTeSR™-1 (Figure 3.3, A).

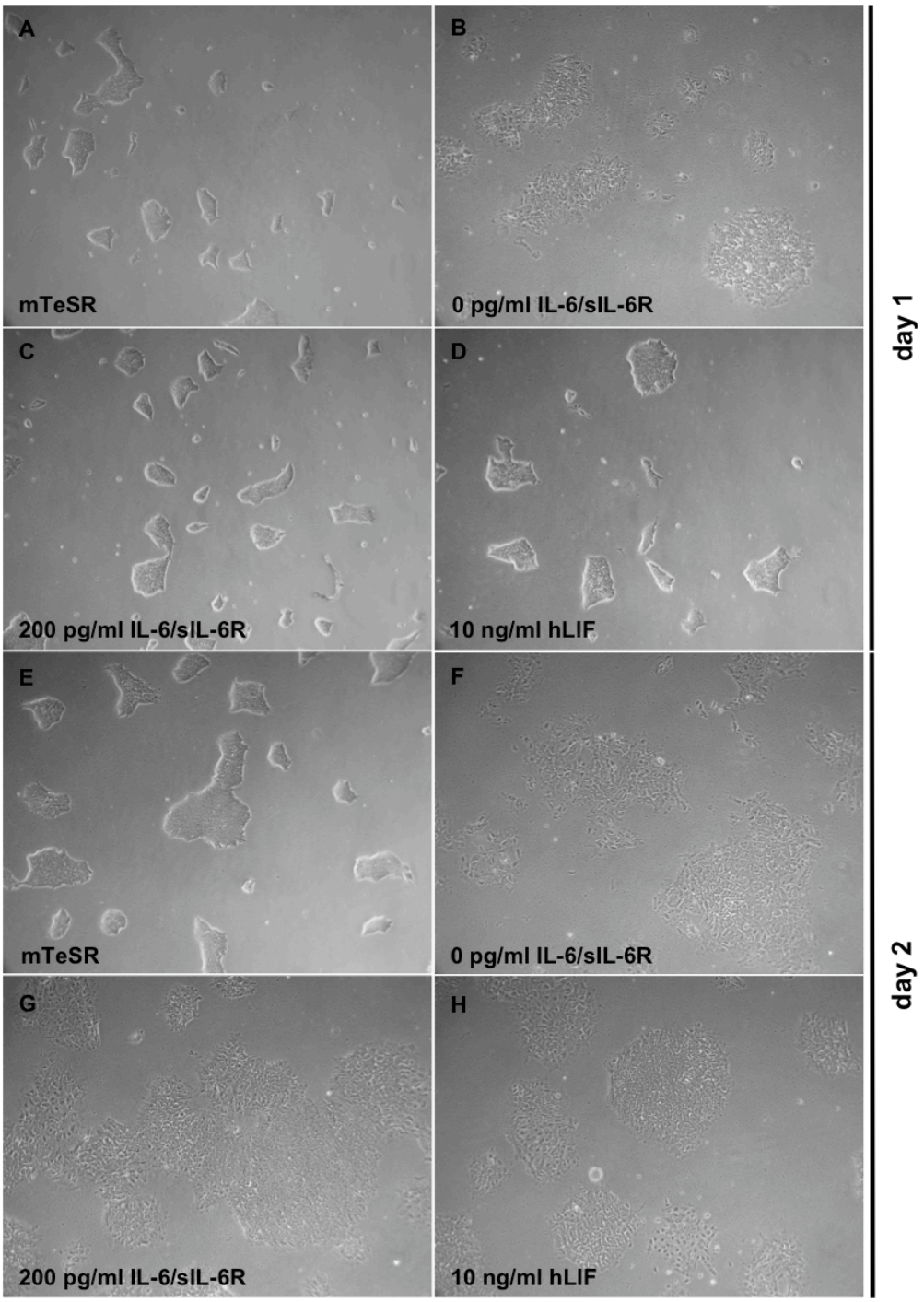

Figure 3.3: Morphological changes after one and 2 days of application show disability of the IL-6/sIL-6R fusion protein to maintain pluripotency-associated morphology
hPSCs (iLB-30-r12) were treated with IL-6/sIL-6R fusion protein or hLIF as indicated.
(A-D): Examination of the morphology 24 hours after changing the medium conditions indicate that spontaneous differentiation can be circumvented by the presence of the fusion protein (C) or hLIF (D) in hPSC medium. The positive control in mTeSR medium (A) exhibits an undifferentiated morphology, whereas cells in the negative control in hPSC medium without fusion protein or hLIF starts to differentiate.
(E-H): 48 hours after the change of the medium conditions, also the cells treated with the fusion protein (G) and hLIF (H) begin to differentiate and exhibit the same morphology as the negative control with hPSC medium only(F). The positive control in mTeSR medium retains its morphology (E).
Magnification 40x

Two days after the change of the medium conditions, this effect could not be observed anymore. While the cells under control conditions still exhibited the morphology of undifferentiated hPSC (Figure 3.3, E), the cells treated either with IL-6/sIL-6R fusion protein (Figure 3.3, G) or hLIF (Figure 3.3, H) showed a changed morphology. The colonies lost their compactness. Therefore, after two days they resembled the negative control (hPSC medium without supplements; Figure 3.3, F) and not the positive mTeSR™-1 control (Figure 3.3, E). This indicates that the spontaneous differentiation was just delayed by the addition of LIF or fusion protein.

Notably, there was no apparent difference between the cells treated with LIF or fusion protein. This suggests that the reported failure of activation of Stat3 signaling is not necessarily due to lacking LIF receptors, as hLIF has an effect on the culture.

The morphological changes showed in Figure 3.3 do only give a hint that hPSCs differentiate in the presence of IL-6/sIL-6R fusion protein or LIF. Further analysis of the differentiation state of the treated cells could not be performed, because an elongated culture under these conditions led to the loss of the cells. It is a commonly known feature of Matrigel™ matrix that spontaneously differentiated cells detach from the matrix and consequently get lost.

The reported change in morphology did not give any indication whether the IL-6/sIL-6R fusion protein and LIF did fail in activating Stat3 signaling. Possibly Stat3 was phosphorylated, but this was not sufficient to maintain pluripotency in adherent cultures. Therefore, the phosphorylation of Stat3 should be investigated by performing an immunoblot with an antibody against the phosphorylated and thus activated form of Stat3 (pStat3). Whole cell protein lysates were obtained from I3 hESCs cultivated for 24 hours either in hPSC medium without supplement (Figure 3.4, lane 1) or IL-6/sIL-6R fusion protein (200 pg/ml; Figure 3.4, lane 2) or hLIF (10 ng/ml; Figure 3.4, lane 3).

No phosphorylated Stat3 could be detected in protein lysates from control cells. Surprisingly, Stat3 was found phosphorylated in hPSCs, but not by IL-6/sIL-6R fusion protein but by hLIF. Nevertheless, the signal of pStat3 was very weak, indicating that Stat3 activation is a rare event in hPSCs. For detection, the membrane had to be incubated for several seconds with the SuperSignal West Femto Substrate, which is only necessary for low protein concentrations. These findings were made in 4 individual experiments.

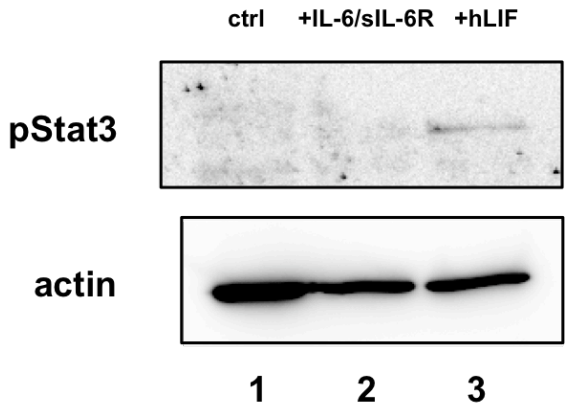

Figure 3.4: IL-6/sIL-6R fusion protein fails to phosphorylate Stat3 in hPSCs
I3 hESCs were cultivated for 24 hours in the presence of 200 pg/ml IL-6/sIL-6R fusion protein (lane 2) or 10 ng/ml hLIF (lane 3). As a control, cells were cultured in hPSC medium without addition of an activator of Stat3 signaling (lane 1). In this exemplary immunoblot, no phosphorylated Stat3 can be seen for the fusion protein (lane 2).
Development: pStat3= SuperSignal West Femto Substrate; actin= SuperSignal West Pico Substrate

Further validation of Stat3 signaling upon treatment with the IL-6/sIL-6R fusion protein was performed by investigating the expression of Stat3 target genes using quantitative RT-PCR (qRT-PCR).

For that, I3 hESCs were cultivated in hPSC medium with different concentrations of IL-6/sIL-6R fusion protein. As a control, hPSC medium without supplementation of Stat3-stimulators was added to the cells. As another control, RNA was prepared from cells that were cultivated under standard conditions, meaning in mTeSR™-1 medium. Before starting the treatment of the cells, cells were starved meaning cultivated in hPSC medium without supplements for 16 hours. Following this, cells were kept in the corresponding media for 6 hours, then RNA was isolated and reverse transcribed. qRT-PCR was performed for two candidate target genes Pim1 and Socs3, which are known to be expressed after activation of Stat3 signaling. The results were normalized against the housekeeping gene GAPDH. In Figure 3.5, the expression rates relative to the expression in mTeSR™-1 cultivated cells (“mTeSR”) can be seen.

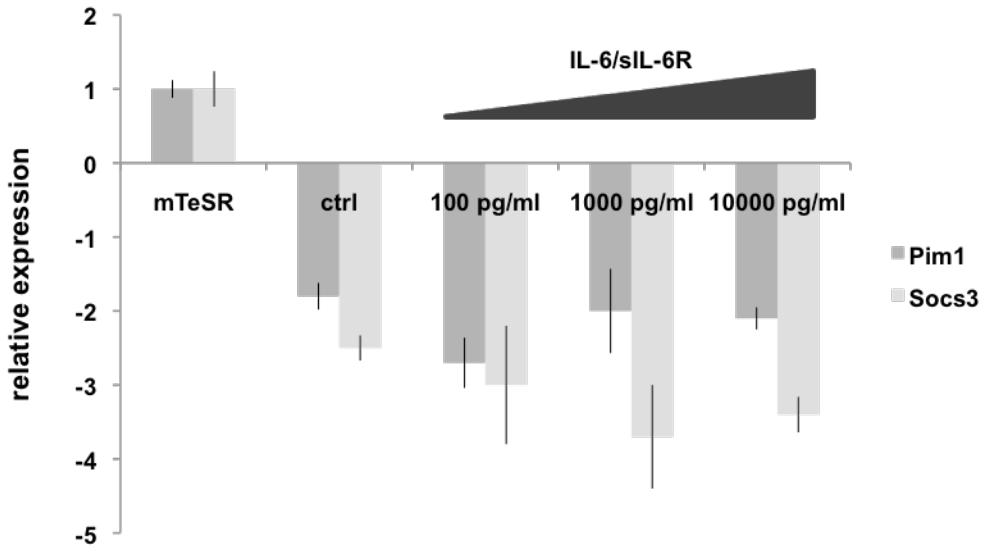

Figure 3.5: Increasing concentrations of IL-6/sIL-6R fusion protein cannot activate the expression of Stat3 target genes
I3 hESCs were treated for 6 hours with different concentrations of IL-6/s-IL6R fusion protein; RNA was isolated subsequently. As a control, hESCs were cultured in hPSC medium lacking any Stat3 stimulator ("ctrl"). qRT-PCR was performed to check for expression of the two Stat3 target genes Pim1 (dark grey bars) and Socs3 (light grey bars). Bars show expression relative to cells cultured under standard conditions ("mTeSR").
Results were normalized against GAPDH. All RT-PCRs were performed in triplicates; error bars show standard deviation.

Pim1 expression (Figure 3.5, dark grey bars) decreased when medium was switched from mTeSR™-1 to hPSC medium without supplements ("ctrl"), which could indicate that mTeSR™-1 contains substances inducing Pim1 expression, whereas the hPSC medium lacks a stimulator. Contradictory, the addition of different concentrations of the IL-6/sIL-6R fusion protein did not lead to an increase of Pim1 expression. No dose dependent effect could be observed. This indicates that the IL-6/sIL-6R fusion protein is not feasible to activate Pim1 expression at all and that not only the concentrations needed to be adjusted.

No positive effect of IL-6/sIL-6R fusion protein could also be observed for another Stat3 target gene, namely Socs3 (Figure 3.5, light grey bars). The highest relative expression could be detected for mTeSR™-1 cultivated hPSCs. The expression of Socs3 even decreased with increasing IL-6/sIL-6R fusion protein concentrations.

Notably, the concentration of the IL-6/sIL-6R fusion protein used in the published suspension culture protocol (Amit et al., 2010) was 100 pg/ml and therefore as low as the lowest concentration used in the presented experiments. Even with the double amount of fusion protein (200 pg/ml) no Stat3 phosphorylation could be observed on protein level with immunoblot (Figure 3.4). The qRT-PCR covers a higher range of

concentrations, but did not reveal any activation of Stat3 signaling. Even a thousand-fold increase of the concentration did not lead to detectable increase of target gene expression. The only observable effect of the IL-6/sIL-6R fusion protein is the delay of differentiation in feeder-free cultivation of hPSCs.

However, the fact that LIF has the same effects as the IL-6/sIL-6R fusion protein suggests that the fusion protein is not more potent than LIF.

3.1.3 In mPSCs, Stat3 can be activated by IL-6/sIL-6R fusion protein

In order to investigate whether the IL-6/sIL-6R fusion protein can be more potent than LIF, cells known to react on Stat3 activation should be tested. Murine PSCs (mPSCs) are known to depend on LIF presence in their cell culture medium.

As a first test, mPSCs of the E14T line were cultured with mPSC medium either supplemented with 10 ng/ml LIF (ESGRO) or 200 pg/ml IL-6/sIL-6R fusion protein. Whole cell protein was prepared 2 days after start of treatment.

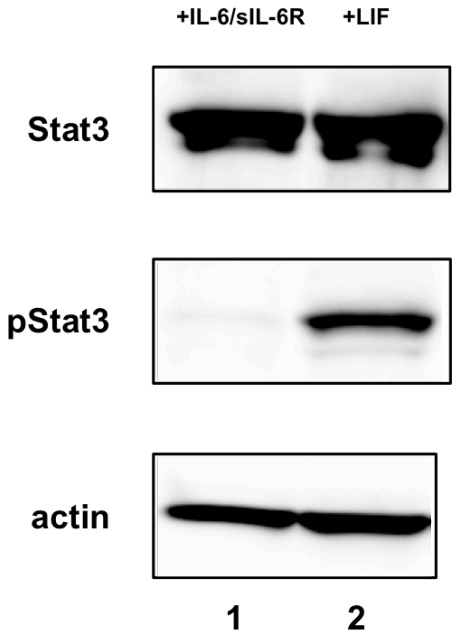

Figure 3.6: Stat3 gets phosphorylated in mPSCs weakly upon activation with fusion protein

E14T mPSCs were cultivated for 48 hours in the presence of either 200 pg/ml of IL-6/sIL-6R fusion protein (1) or 10 ng/ml of LIF (2). Immunoblot reveals that Stat3 is expressed in cells cultivated with fusion protein; only a very weak signal could be detected for pStat3 (lane 1). A strong signal is detectable for pStat3 in LIF dependent culture (lane 2).

Development: pStat3= SuperSignal West Femto Substrate; Stat3, actin= SuperSignal West Pico Substrate

LIF induced a strong Stat3 phosphorylation when compared to the fusion protein, which only led to a weak phosphorylation of Stat3 that was hardly detected on the membrane. Based on this observation it was assumed that 200 pg/ml of IL-6/sIL-6R fusion protein may not be sufficient to activate Stat3 signaling in mPSCs. To investigate that, a dose-response test should be performed.

In a dose-response test, mPSCs of the E14T line were cultivated in media containing different IL-6/sIL-6R fusion protein concentrations from 100 pg/ml up to 10 ng/ml. As a positive control, mPSCs were cultivated in their standard medium supplemented with 10 ng/ml ESGRO (murine LIF). As a negative control, mPSC medium without addition of LIF or fusion protein was used.

After 4 days of cultivation in the different media, morphology was checked and pictures were taken. The positive control (Figure 3.7, A) showed a typical morphology with small, compact colonies that are dome-shaped. The borders of the colonies were sharp and refracted light. After removal of LIF, the colonies lost their compact form, at the outer parts of the colonies cells were growing out. The borders of the colonies were not bright anymore, indicating that they lost their domed shape (Figure 3.7, B). mPSCs cultivated in the presence of 100 pg/ml IL-6/sIL-6R fusion protein could not be distinguished from cells without Stat3 stimulation (Figure 3.7, C). A lot of differentiated cells could be seen around the residuals of the colonies. The cells surrounding the colonies almost formed a monolayer. The same was true for cells cultivated with 200 pg/ml of the IL-6/sIL-6R fusion protein (Figure 3.7, D). The addition of 1 ng/ml of the IL-6/sIL-6R fusion protein to the mPSC medium led to a slight improvement: the colonies that were left appeared to have a three-dimensional morphology, and fewer cells were growing outside the colonies (Figure 3.7, E). Still, some cells grew in between indicating that 1 ng/ml of the fusion protein is not sufficient to replace 10ng/ml of LIF. Only the equivalent concentration of 10 ng/ml of the fusion protein restored the morphology of the positive control. The cells were growing in compact colonies, only few cells seemed to be differentiated (Figure 3.7, F).

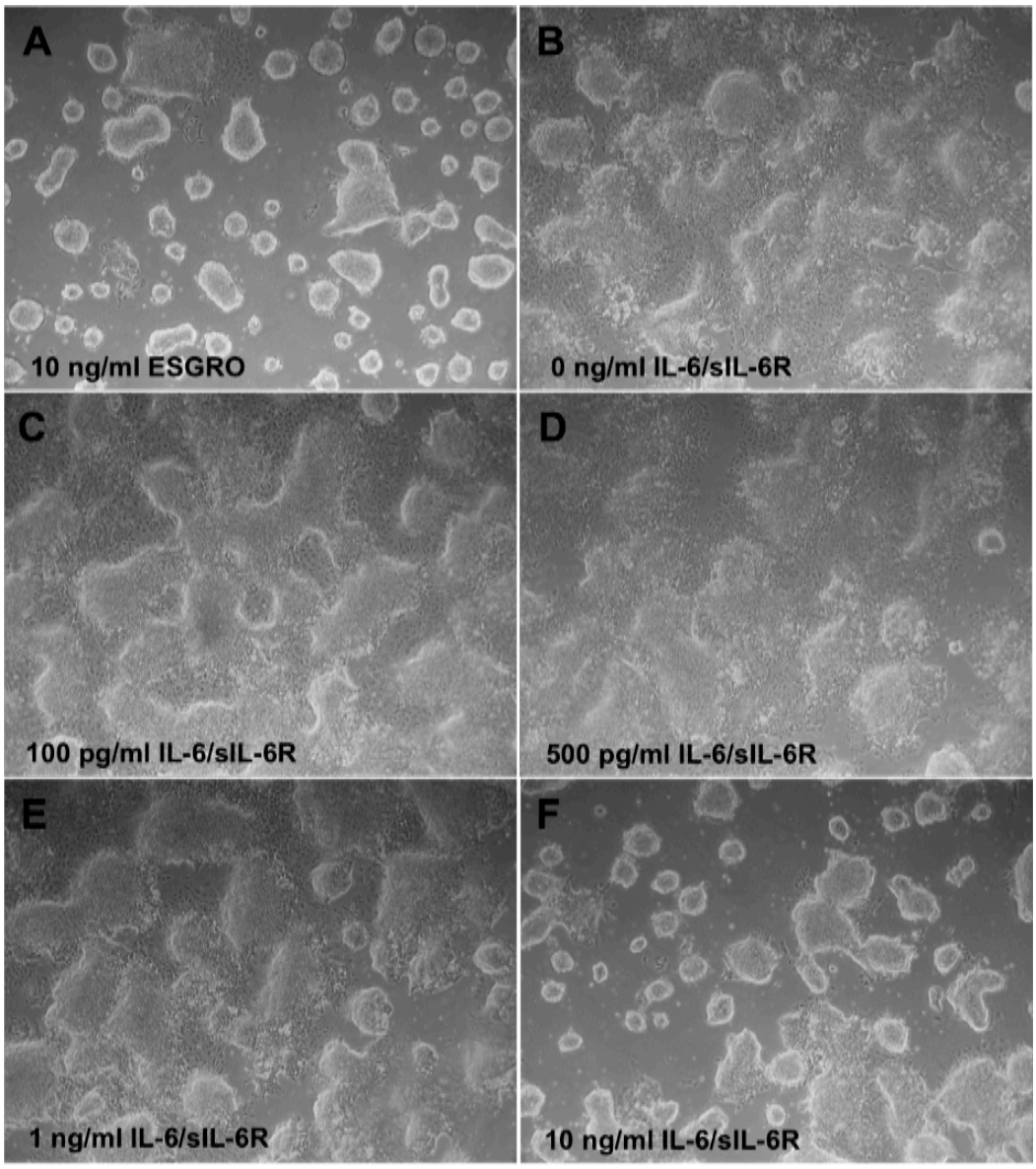

Figure 3.7: mPSCs maintain a pluripotency-associated morphology after 4 days of culture with IL-6/sIL-6R fusion protein
mPSCs tend to differentiate spontaneously when cultivated with (C) 100 pg/ml or (D) 500 pg/ml of fusion protein.1ng/ml of fusion protein (E) leads to a tighter colony formation and sharper boarders, but only cells cultivated with 10 ng/ml of fusion protein (F) can mimic the morphology of the positive control (A)
Magnification 40x

Now that it has been shown that low concentrations of the IL-6/sIL-6R fusion protein were not sufficient to activate Stat3, higher concentrations of the fusion protein should be tested. mPSCs of the E14T line were cultivated following the standard protocol. These cells were used as a positive control (Figure 3.8, lane 2). To exclude that pStat3 detection is due to residual protein phosphorylated in LIF presence, cells were starved overnight (16 hours) cultivating them in mPSC medium without LIF. After starvation, mPSC medium either with 10ng/ml LIF (Figure 3.8, lane 3) or different concentrations of the IL-6/sIL-6R fusion protein (Figure 3.8, lanes 4-6) was added. As a negative control, one batch of cells remained in mPSC medium without supplementation. After 30 minutes, whole cell protein lysates were prepared, an SDS-PAGE and an immunoblot were performed.

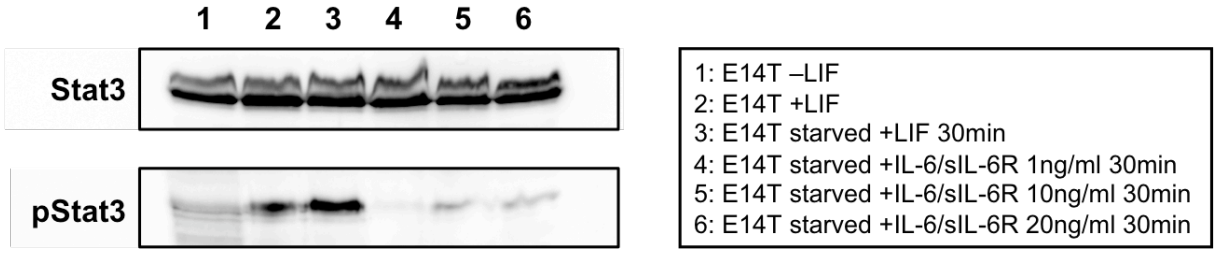

Figure 3.8: Phosphorylation of Stat3 by fusion protein is weaker than by LIF
By immunoblot it could be observed that the IL-6/sIL-6R fusion protein is able to phosphorylate Stat3 in mPSCs (lanes 5,6). The signal, though, is much weaker than for the cells that were cultivated in the presence of LIF (lanes 2, 3).
Development: Stat3= SuperSignal West Pico Substrate; pStat3= SuperSignal West Femto Substrate

As expected, mPSCs cultivated in the presence of LIF showed a strong signal for phosphorylated Stat3, either in the standard cultivation or after starvation. In Figure 3.8 lane 1 a weak signal at the expected height for pStat3 could be observed in the cells growing in the absence of LIF, which likely is due to a high background. The addition of 1ng/ml of the IL-6/sIL-6R fusion protein to the mPSC medium did not lead to a notable phosphorylation of Stat3 (Figure 3.8, lane 4), but concentrations of 10 ng/ml and 20 ng/ml showed a detectable band (Figure 3.8, lanes 5 and 6). Notably, there seemed to be no dose dependent effect, the pStat3 signal for 10ng/ml fusion protein is as strong as for 20 ng/ml. Therefore it was assumed that the IL-6/sIL-6R fusion protein may not be able to activate Stat3 beyond a certain extent.

Taken together, the results obtained by cultivating mPSCs with the IL-6/sIL-6R fusion protein showed that the fusion protein seems to be a functional LIF agonist, but not as potent as expected. In mPSCs it was possible to gain Stat3 phosphorylation and maintain pluripotency, unless the published concentration of 100 pg/ml was not sufficient. A possible reason for that might be that the fusion protein was designed to replace human LIF in human cells. It may be that mPSCs are not an appropriate model to test for IL-6/sIL-6R fusion protein activity.

3.1.4 IL-6/sIL-6R fusion protein is not more potent than hLIF and IL-6

To check whether the IL-6/sIL-6R fusion protein is more potent in human cells, HepG2 cells were taken into culture. HepG2 is a human cell line that was generated from a liver carcinoma and can be used as a model for polarized hepatocytes. It is known that Stat3 signaling can be activated in HepG2 cells (Lutticken et al., 1994). Therefore, they can be used to test the functionality and activity of the IL-6/sIL-6R fusion protein in human cells without interfering with the pluripotency status. In standard culture, HepG2 cells were cultured without addition of LIF. Only after plating cells for sample preparation for an ELISA, cells were treated for 6 hours with different concentrations of either hLIF, IL-6/sIL-6R fusion protein or the cytokine IL-6, which is not bound to its soluble receptor. LIF and IL-6 treated cells were used as a reference to estimate the activity of the fusion protein.

Notably, at a concentration of 100 pg/ml (0.1 ng/ml; the concentration published to maintain hPSC pluripotency in suspension culture) the IL-6/sIL-6R fusion protein (Figure 3.9, blue line) seemed to have hardly any effect as nearly no phosphorylated Stat3 was detected. The relative amount of pStat3, indicated by OD 450nm Reading, at this concentration was already higher in hLIF treated cells (Figure 3.9, red line). Even in cells cultivated in the presence of IL-6 alone (Figure 3.9, green line), Stat3 got phosphorylated at lower concentrations.

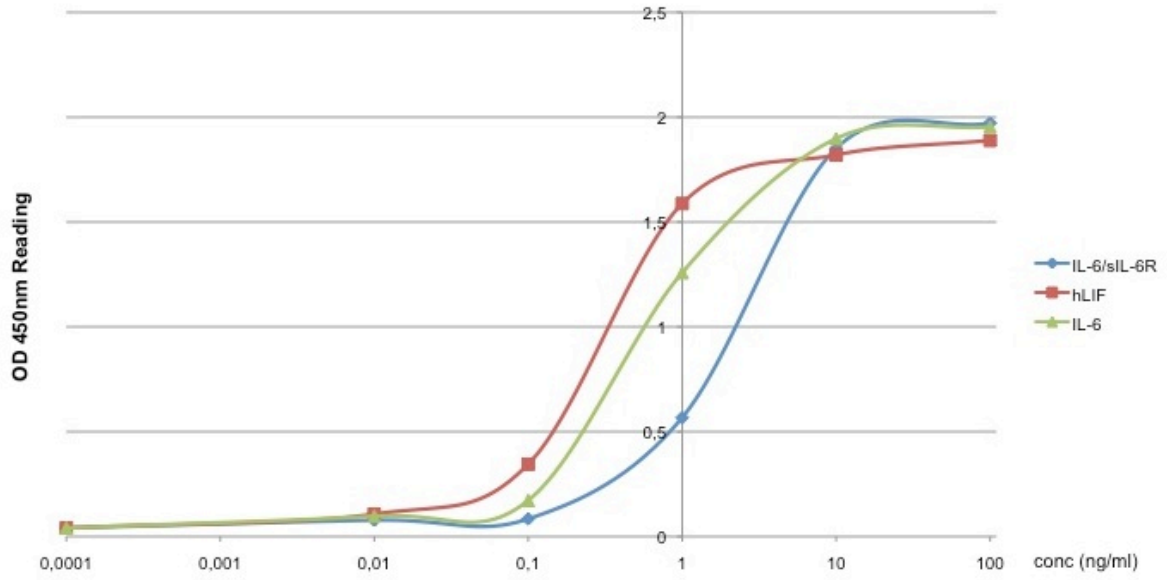

Figure 3.9: IL-6/sIL-6R fusion protein is less potent than hLIF or IL-6 alone
HepG2 cells were treated with IL-6/sIL-6R fusion protein, hLIF and IL-6 for 6 hours; subsequently whole protein lysates were prepared. An ELISA for pStat3 shows that the fusion protein (blue) needs to be applied in higher concentrations to obtain the same level of pStat3 than IL-6 and hLIF

When treated with 10 ng/ml of each molecule, the cell lysates contained approximately the same relative amount of pStat3, at 100 ng/ml a maximum was reached for all three Stat3 stimulators. This led to the conclusion that the IL-6/sIL-6R fusion protein is as potent in Stat3 activation as hLIF and as IL-6 alone.

In total, two conclusions can be drawn from the experiments performed. First, the IL-6/sIL-6R fusion protein is an agonist for LIF feasible to replace it. It seems not to be stronger, though, as the concentrations needed for Stat3 activation are even higher as the LIF concentrations. Second, the role of Stat3 downstream signaling still remains unclear. It was published that the IL-6/s-IL6R fusion protein can maintain pluripotency in hPSC suspension cultures. In the experiments described in this chapter, the fusion protein could not activate Stat3 downstream genes.

In order to further investigate the role of the Stat3 signaling pathway in hPSC biology, it was decided to establish a loss-of-function model.

3.2 Loss-of-function of Stat3 signaling via genome editing

Several techniques are known to edit the genome of cells. Since the TALEN technology was reported to be applicable for genetic engineering in hPSCs, this technology should be used to establish a targeted gene inactivation of Stat3 in hPSCs.

3.2.1 Establishment of a TALEN pair against Stat3

TALEN pairs can be designed to bind and cut at specific loci. Using a modular system, an assembly of a desired TALEN pair can be achieved by just using few special plasmids. The working group of Professor Hornung (Bonn University) established a system to clone TALEN constructs by using a pentamer library of the TALEN repeat units (Schmid-Burgk, 2013).

The Hornung Lab kindly provided a TALEN pair against Stat3, namely 3L and 3R, which was shown to functionally edit the genome in the here not relevant cell system HEK 293T when transfected as plasmids. Functionality in hPSCs should be shown in this part of the thesis. All TALEN constructs hold a flag-tag enabling a read out of transfection and translation.

As nucleofection via electroporation and lipofection did not lead to successful transfection of hPSCs with TALEN plasmids (for electroporation see Table 3.1), an mRNA synthesis was started. The target cells for the TALEN were supposed to be hPSCs; initial experiments had shown that hPSCs could be transfected efficiently with synthetic mRNA.

Table 3.1: Overview of electroporation attempts

Cell line	Buffer	DNA	Volume	Cuvette	Volts	µF	attached	succesful
iLB-30-r12	PBS	4µg 3L+3R	400µl	4mm	250	500	no	no
iLB-30-r12	PBS/OptiMEM 1:1	4µg 3L+3R	300µl	4mm	250	500	no	no
iLB-30-r12	OptiMEM	4µg 3L+3R	300µl	4mm	200	500	no	no
iLB-30-r12	BioRad	4µg 3L+3R	300µl	4mm	200	400	yes	no
iLB-30-r12	BioRad	8µg GFP	100µl	2mm	250	500	no	no
iLB-30-r12	BioRad	8µg GFP	800µl	4mm	200	450	yes	no
iLB-30-r12	BioRad	16µg GFP	800µl	4mm	250	450	yes	no
I3	PBS/OptiMEM 1:1	4µg 3L+3R	300µl	4mm	250	500	no	no
I3	BioRad	8µg GFP	100µl	2mm	200	400	yes	no

Read-out for TALEN transfection was a T7-Assay, for GFP the presence of a fluorescence signal. Single cells ($1x10^7$) were electroporated in the indicated buffer and afterwards plated on Matrigel™ coated 6 well-dishes

3.2.2 Generation of TALEN mRNA

The TALEN plasmids constructed by the Hornung Lab are designed in that way that a double digestion with two restriction enzymes leads to a construct in the form of 5´UTR-TALEN-3`UTR. The construct has a size of approximately 3.4kb. After digestion, the fragments were separated by electrophoresis. The band at the height of 3.4kb was cut out and purified. To amplify this template, a template PCR was performed using a primer pair that binds in the 5´ and 3´ UTR (*5´UTR fwd* and *3´UTR rev*). The efficiency of the PCR was checked on an agarose gel (see Figure 3.10). In case of the 3L construct, the template PCR was successful, as a band at the expected height could be seen. However, several additional bands could be seen that represent fragments of a smaller size. The PCR on the 3R construct led to similar results, as several unspecific bands occurred. The band on the expected size was much weaker, though. PCR conditions were adapted; because of the length of the TALEN constructs, the extension time was increased from 1 minute to 1:45 minutes. This and the addition of DMSO led to a slight improvement of the PCR outcome. Nevertheless, the contamination of unspecific bands and the concentration of the desired PCR product were still not satisfying (Figure 3.10, A).

The PCR product was used anyway as a template for the following tail PCR. The primers used in this PCR bind to the same sites of the DNA as the template PCR primer pair. The 5´UTR primer is exactly the same (*5´UTR fwd*), whereas the 3´UTR primer has a 120 base long T-tail (*3´TailT rev*). The fact that the primers bind to the same spots means that the tail PCR should amplify only the correct fragment of the template. The outcome again was poor (Figure 3.10, B). To get more tailed templates for the *in vitro* transcription (IVT), it was attempted to run the tail PCR directly on the fragment cut out from the plasmid. The tail PCR resulted in one prominent band at the height of the expected PCR product at around 3.4kb (Figure 3.10, C). This was unexpected, as the primers bind to the same region of the DNA. PCR conditions were the same as mentioned above, with elongated extension time and the addition of DMSO.

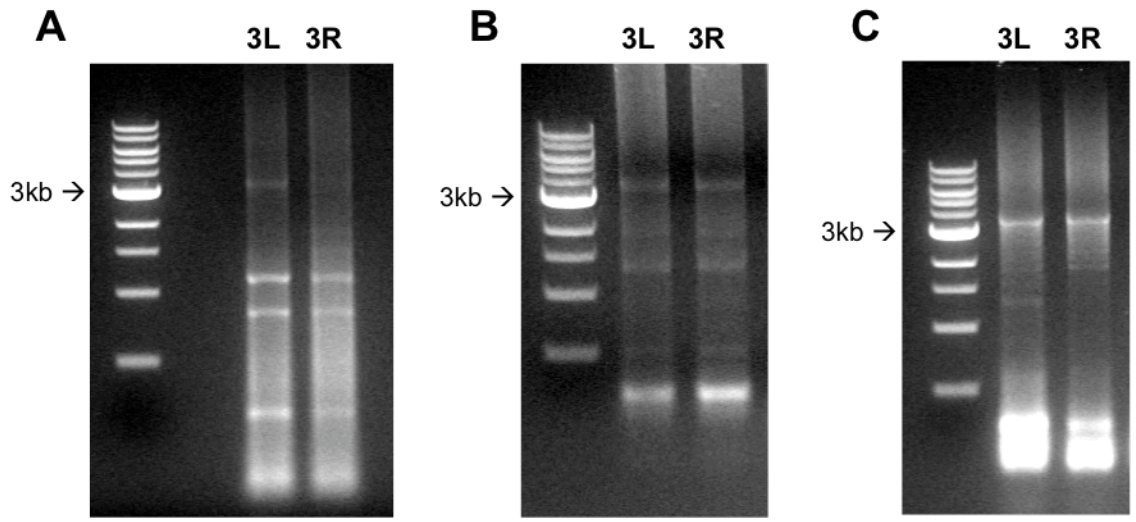

Figure 3.10: Amplification of two TALEN constructs (3L and 3R)
Template PCR with the *5´UTR fwd* and *3´UTR rev* primer pair (A) results in a lot of additional bands, the PCR product with a size of 3.4kb can hardly be detected. Tail PCR does not succeed when the PCR product from (A) is used as a template (B). Tail PCR of the fragment of the digested plasmids using *5´UTR fwd* and *3´TailT rev* has the best outcome (C).

As a conclusion, tailed templates for the IVT were generated by digesting the TALEN plasmids; the 5´UTR-TALEN-3´UTR fragment was purified and used as a template for the following tail PCR. Due to the large size of the construct, it was necessary to lengthen the extension phase and to add DMSO.
In vitro transcription and capping was performed as described in 2.3.4. Afterwards, RNA gel electrophoresis should be performed to estimate the quality and purity of the synthetic mRNA. mRNA preparations showing a single sharp band at the correct height (>3kb) were considered to have a high quality. Only high quality mRNAs were used for transfections. Because denaturing gel electrophoresis had a very weak quality (data not shown), native gel electrophoresis was performed.

Both synthetic 3L and 3R TALEN mRNA could be generated. IVT resulted in a single mRNA with a size of >3kb. The band on the gel was sharp, only a slight smear could be seen below the band, indicating that some smaller RNA fragments were synthesized or that mRNA molecules got degraded (Figure 3.11, A).

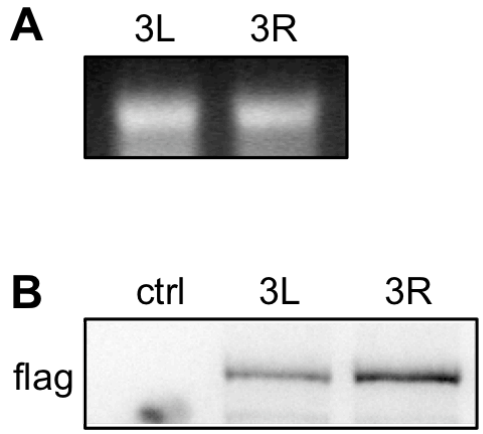

Figure 3.11: Stat3-TALEN mRNA were synthesized and translated by HEK 293T cells
(A) Native gel electrophoresis shows an mRNA with a size of >3kb
(B) Immunoblot: HEK293T cells were transfected with synthetic TALEN mRNA of both 3L and 3R construct; the TALEN mRNA that also encodes for a flag-tag was translated, indicated by the flag signal in the immunoblot. Development: SuperSignal West Femto Substrate

In order to analyze whether the mRNA could be translated to proteins, Stat3-TALEN mRNA was transfected into HEK 293T cells. Per well of a 6 well plate, 1 µg of each TALEN mRNA was transfected. After 16 hours of incubation, medium was changed to culture medium. The cells were set to 37°C for 6 hours in order to recover, then transfection procedure was repeated. After a second incubation of 16 hours, whole cell protein lysates were obtained and separated with SDS-PAGE. In the immunoblot, a strong signal could be detected using a flag antibody (Figure 3.11, B). This indicates that TALEN mRNA could be transfected into HEK 293T cells. It gives also a hint about the quality of the mRNA, as the mRNA can be translated into protein *in vivo*.

3.2.3 Functional TALEN mRNA can be transfected into different cell lines

Even though the flag-immunoblot gave a hint about the quality of the TALEN mRNA, functionality still was not proven. To assess this, a T7 Assay was performed. For this assay, HEK 293T cells were transfected with both 3L and 3R TALEN mRNA and with one single TALEN mRNA (3L) alone as a control. As described before, transfection was performed twice, both times 1 µg of each mRNA was transfected.
In case of successful transfection of both functional TALEN mRNA, the DNA should be cut at the target site. The DNA strand is repaired via NHEJ. In the T7 Assay, the T7 endonuclease recognizes non-perfectly matched DNA and cleaves it. Therefore,

in case of successful editing, the T7 Assay results in 2 bands, as the amplified target region is cut.

HEK 293T cells were chosen as a first test cell line because it has been seen that they can be transfected very efficiently with synthetic mRNA.

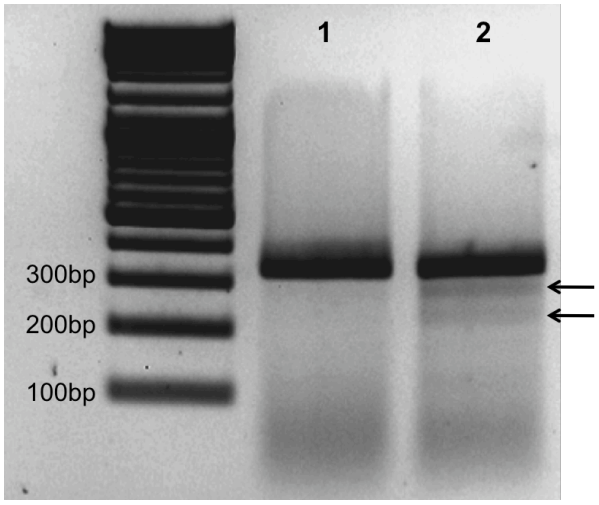

Figure 3.12: T7 Assay shows that Stat3 TALEN mRNA transfection leads to genome editing in HEK 293T cells
Lane 1: Transfection of only one TALEN mRNA (3L) does not lead to editing of the Stat3 gene as only a single band at the height of approximately 320bp can be detected.
Lane 2: Transfection of both 3L and 3R TALEN mRNA results in restriction of the Stat3 gene at the target side, as indicated by the smaller fragments (arrows); densitometric quantification reveals an editing efficiency of 5%.

As shown in Figure 3.12, the transfection of only one TALEN mRNA did not result in any cutting of the target side (lane 1). When both mRNAs of the TALEN pair were transfected, though, both TALEN could bind to the DNA and lead to an editing of the target side in the Stat3 gene, as indicated by the two smaller bands (arrows, lane 2). Quantification with ImageJ resulted in an efficiency of approximately 5%.

As TALEN mRNA shall be used to manipulate hPSCs, it is necessary to show functionality of synthetic TALEN mRNA in hESCs or hiPSCs. First of all iLB-30-r12 cells were transfected with 3L or 3R TALEN mRNA. Total cell protein lysates were analyzed for flag-tagged protein via immunoblot (Figure 3.13, A). This analysis demonstrated that 3L as well as 3R TALEN mRNA could be transfected into iLB-30-r12 and were translated into TALEN proteins. To assess the functionality, hiPSCs of the line iLB-30-r12 were transfected with the 3L/3R TALEN pair and with the 3L alone.

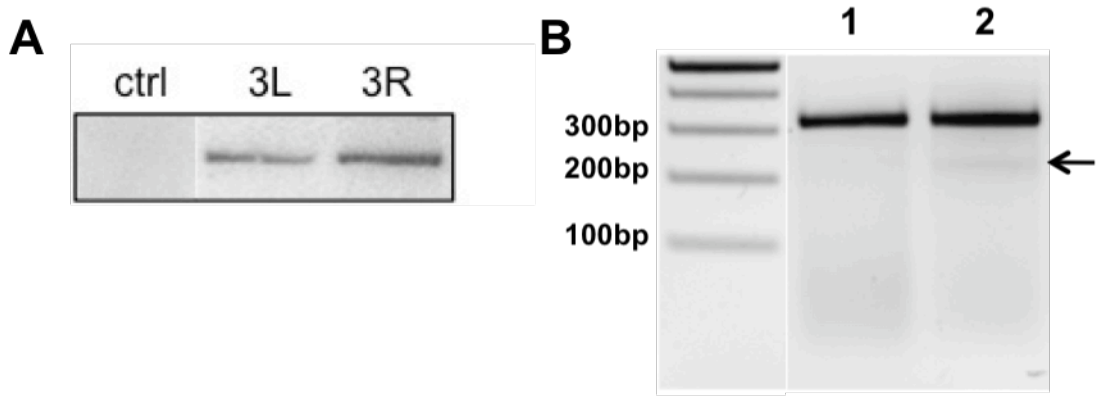

Figure 3.13: Immunoblot and T7-Assay after transfection of TALEN mRNA into hiPSCs
(A) Immunoblot against flag confirms transfection and translation of TALEN mRNA holding a flag-tag into hiPSCs; Development: SuperSignal West Femto Substrate
(B) T7-Assay shows that, when transfected into hiPSCs of the iLB-30-r12 line, Stat3 TALEN mRNA led to editing at the target site (lane 2) indicated by a shortened band, whereas the control (3L only; lane 1) shows no effect. The efficiency of the editing is 5.1%.

When transfected into the human iPSC line iLB-30-r12, the synthetic Stat3 TALEN mRNA led to a comparable result as in HEK 293T cells. They edited the target site with an efficiency of 5.1% (Figure 3.13, B, lane 2). Transfection of 3L TALEN mRNA alone did not result in restriction of the target side (Figure 3.13, B, lane 1).
With the positive results of the T7 Assay, functionality of the synthetic TALEN mRNA has been proven. To further improve the outcome, transfection efficiencies of synthetic mRNA should be increased. To accomplish that, the effect of the modifications of synthetic mRNAs shall be tested.

3.3 Innate immunity in hPSCs and their derivatives

Standard protocols of mRNA synthesis require the use of at least two modified ribonucleotides, namely 5´methyl-CTP and pseudo-UTP (Warren et al., 2010). These modifications are claimed to reduce the response of the cell autonomous innate immunity upon mRNA transfection. When transfected with mRNA, cells normally react with the activation of the innate immune system, as mRNA resembles viral RNA. Due to the modifications of the synthetic mRNA, the cell cannot distinguish between synthetic mRNA and mRNA produced by the cell, resulting in higher viability of the cell upon transfection and an increased mRNA half-life. Initial results (data not shown) unexpectedly revealed that hPSCs could be transfected efficiently with modified as well as unmodified mRNA. To elucidate whether hPSCs do not require modified mRNA, two different synthetic GFP mRNA have been produced. First, the

standard protocol (Warren et al., 2010) was followed and the modified ribonucleotides 5´methyl-CTP and pseudo-UTP replaced CTP and UTP; this synthetic mRNA will be referred as “modified” (short: “mod”). Second, the unmodified ribonucleotides CTP and UTP were used; this synthetic mRNA will be referred as “unmodified” (short “unmod”). The capping protocol was followed for both synthetic mRNA types.

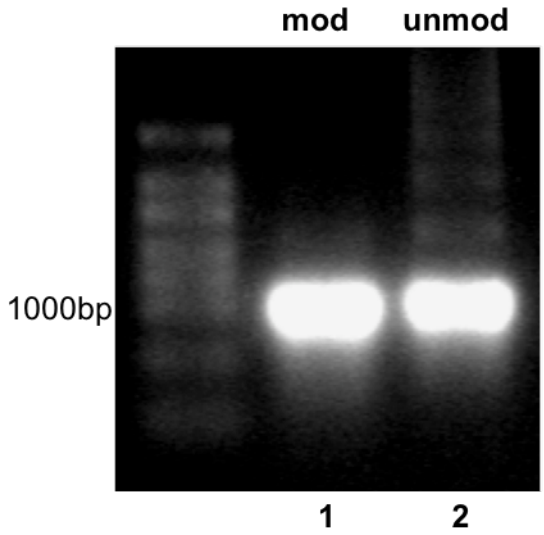

Figure 3.14: Generation of two different synthetic GFP mRNA
Lane 1: synthesis of modified GFP mRNA results in a single pure mRNA with the expected size of around 1000bp. Lane 2: unmodified GFP mRNA synthesis also give rise to a prominent mRNA of the expected size; several bands can be found in the lane indicating contaminating RNA fragments

Modified as well as unmodified synthetic GFP mRNA could be generated (Figure 3.14). On a native agarose gel, the modified GFP mRNA showed a single band at the expected height of 1000bp (Figure 3.14, lane 1). Unmodified GFP mRNA synthesis also resulted in a 1000bp-sized RNA, however some additional bands were detected exhibiting bigger sizes (Figure 3.14, lane 2). For both mRNA syntheses, the same template was used.

3.3.1 hPSCs and lt-NES cells can be transfected with unmodified mRNA

To confirm that hPSCs can be transfected with unmodified mRNA, hiPSCs of the line iLB-30-r12 and hESCs of the H9.2 and I3 lines were plated on 6-well dishes. 24 hours later, they were transfected with 800 ng/well of modified mRNA or 800 ng of unmodified mRNA. As a control, cells were transfected with the same amount of polyA, a polynucleotide. The transfection reagent used was Lipofectamine® LTX. 16

hours after transfection, the transfection mix was exchanged with fresh medium and pictures have been taken to estimate the fluorescence. To facilitate comparison of the fluorescence intensities and efficiency of the transfection, the fluorescence pictures were taken using the same exposure time for each experiment.

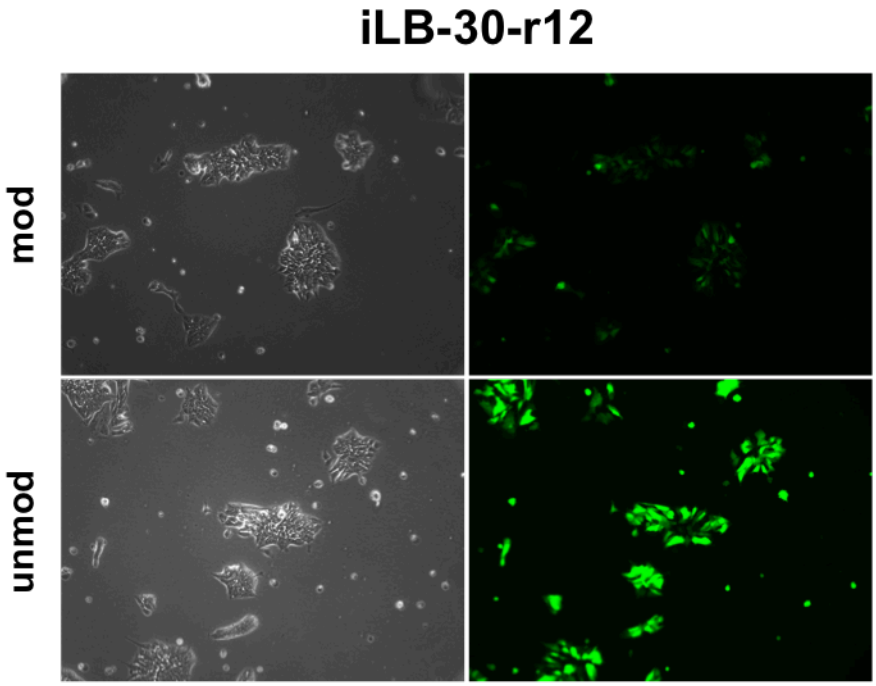

Figure 3.15: Transfection of unmodified GFP mRNA into hiPSCs results in a robust translation of GFP
Transfection of iLB-30-r12 iPSCs with 800 ng of unmodified mRNA leads to a stronger fluorescence signal than with modified mRNA.
Magnification: 40X; exposure time= 125ms

The human iPSC line iLB-30-r12 could be transfected efficiently with modified and unmodified synthetic GFP mRNA. As it can be seen in Figure 3.15, both cells transfected with modified and unmodified mRNA survived mRNA transfection. The colonies have a similar size and a similar amount of debris is present in the supernatant, indicating no additional stress induced by unmodified synthetic mRNA. Transfection with unmodified mRNA seems to lead to stronger fluorescence intensity, which could be due to a better translation. Both fluorescence pictures depicted in Figure 3.15 were exposed for 125ms.

A similar effect could be observed in H9.2 human embryonic stem cells (Figure 3.16); however, the effect was less striking as the difference between the fluorescence intensities of cells transfected with modified and unmodified GFP mRNA was not as strong as observed in iLB-30-r12 hiPSCs. Nonetheless, the transfection efficiency of unmodified GFP mRNA appeared to be higher.

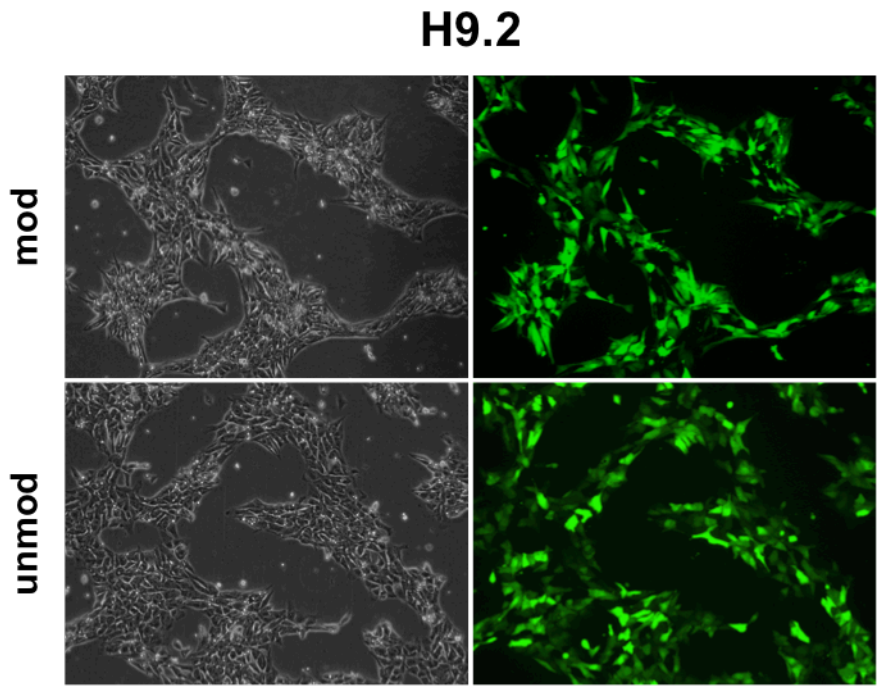

Figure 3.16: Transfection of both modified and unmodified GFP mRNA leads to an intense translation of GFP
The intensities of the fluorescence are on a comparable level for modified and unmodified GFP mRNA transfected into hESCs of the H9.2 line. Both mRNA can be transfected efficiently into the hESCs.
Magnification: 40X; exposure time= 100ms

With I3, another human embryonic stem cell line was tested. Transfection with either modified or unmodified mRNA (Figure 3.17) as well as polyA control (data not shown) resulted in decreased growing or increased dying of the cells as only little cells were attached after 16 hours of incubation with the transfection mix. In spite of this, the surviving cells were transfected with a high efficiency. The synthetic mRNA appeared to be translated very efficiently, as an exposure time of 75ms was sufficient to detect fluorescence in the modified mRNA transfected cells as well as the unmodified, which was even stronger (Figure 3.17).

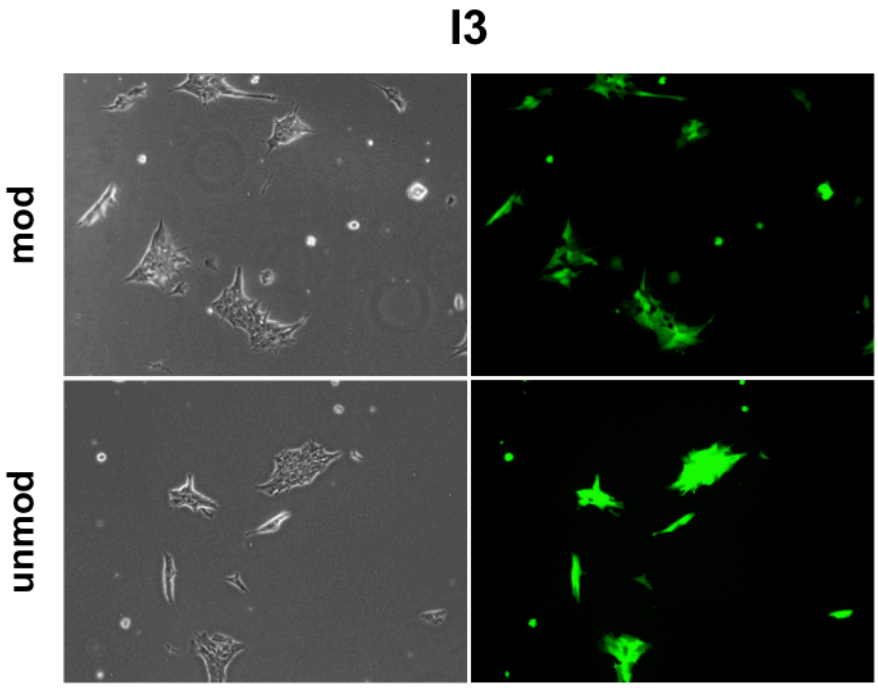

Figure 3.17: hESCs translate both modified and unmodified synthetic GFP mRNA
The hESC line I3 shows a similar transfection capability as iLB-30-r12 hiPSCs. The intensity of the fluorescence is stronger in case of unmodified mRNA transfection. Both mRNAs can be transfected very efficiently into the hESCs.
Magnification: 40X; exposure time= 75ms

In order to quantify the observed effects, flow cytometric analyses were performed. For that, the hiPSC line iLB-30-r12 and the hESC lines H9.2 and I3 were transfected as described before. For flow cytometry, cells had to be singularized.

In Figure 3.18 exemplary histograms of the flow cytometry are shown. iLB-30-r12 cells could be transfected with modified GFP mRNA with an efficiency of 51.9%. When transfected with unmodified mRNA, efficiency increased up to 64.5%. Notably, in the overlay the stronger intensity of the unmodified mRNA (blue line) compared to the modified mRNA (red line) can be seen as the blue curve shifts to the right compared to the red one.

For H9.2, this is not true, which recapitulates the results seen in Figure 3.16. The intensities of both mRNA seem to be comparable, as both lines have the peak at the same point. Nevertheless, the efficiency of transfection with unmodified mRNA was higher (56.2%) than with modified mRNA (40.6%).

The findings found for I3 hESCs via microscopy could be confirmed by flow cytometry as well (Figure 3.18). The blue line has its peak at the far right side of the histogram, indicating a stronger fluorescence than the cells transfected with modified mRNA, as its red line shows a peak more to the left. The efficiencies of transfection

were high, as it was expected after microscopic evaluation (Figure 3.17); transfection with modified mRNA resulted in an efficiency of 67.7%, the use of unmodified mRNA could increase the efficiency up to 78%.

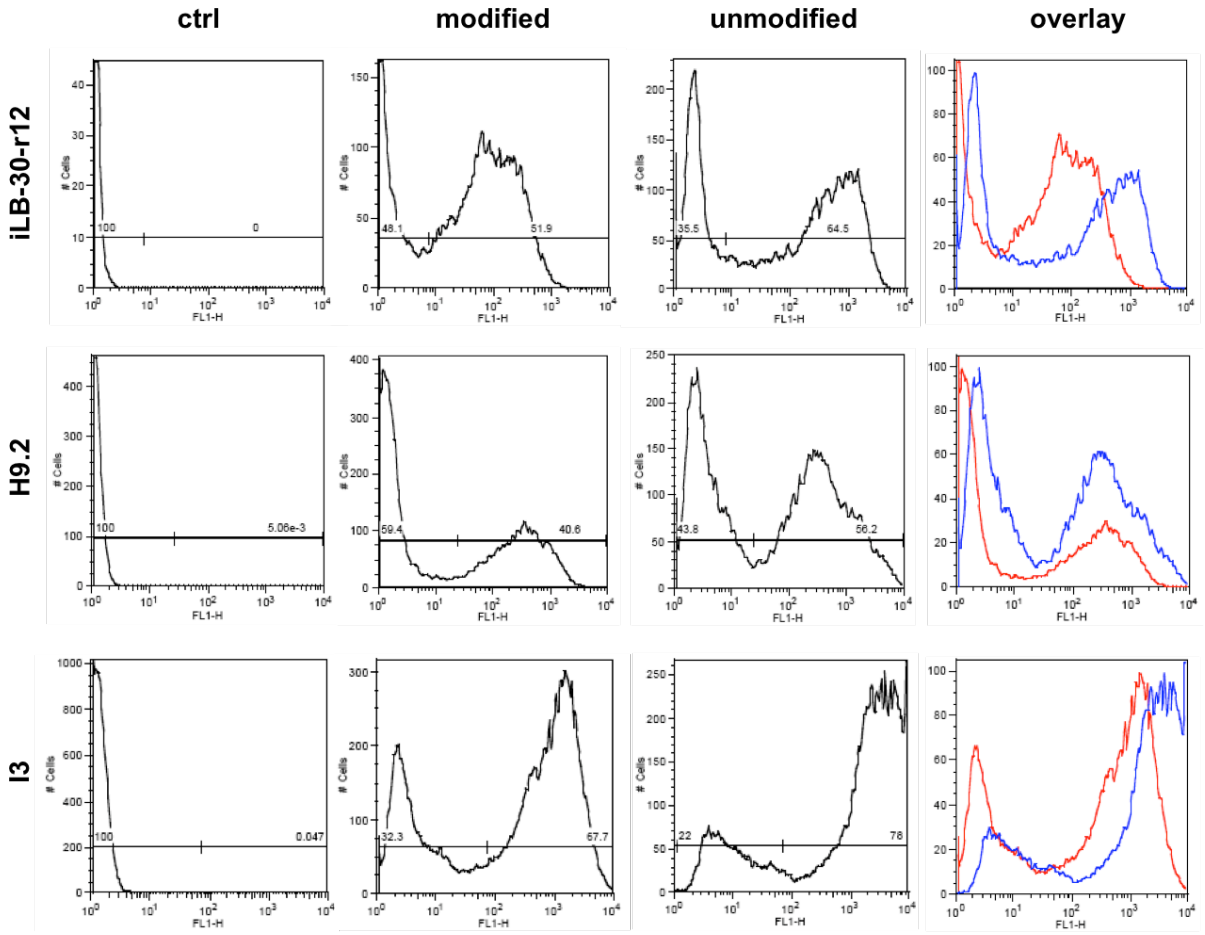

Figure 3.18: Increased efficiency and stronger GFP intensity after transfection with unmodified GFP mRNA in hPSCs
Exemplary histograms show that the transfection efficiency rises from 51.9% to 64.5% (iLB-30-r12), from 40.6% to 56.2% (H9.2) and from 67.7% to 78% (I3) when hPSCs are transfected with unmodified mRNA instead of modified mRNA. In the overlays, the blue line shows that the intensity is stronger in cells transfected with unmodified mRNA compared to modified mRNA (red line)
Blue: unmodified; red: modified

The results shown in Figure 3.18 are just exemplarily, as the histograms are shown only from single experiments. In total, hPSCs were transfected in seven individual experiments with modified and unmodified mRNA.

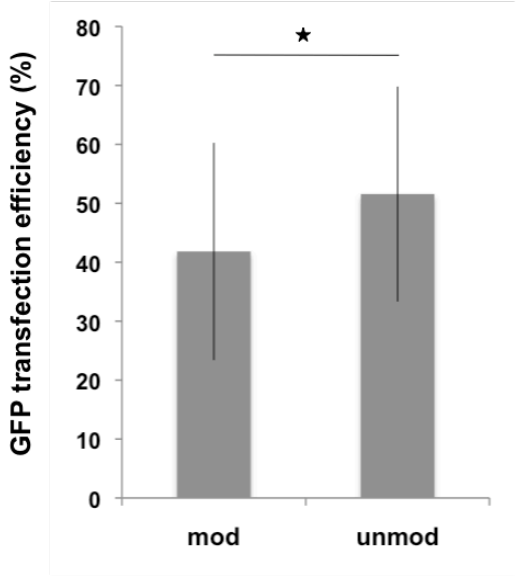

Figure 3.19: Transfection of hPSCs with unmodified mRNA leads to a significantly higher efficiency
Seven individual transfection attempts result in a mean efficiency for transfection with modified mRNA of 41.83% (±18.44). Efficiency is increased up to 51.57% (±18.23%) for unmodified mRNA.
n=7; *: $p<0.05$ according to a paired t-test

These experiments resulted in an average transfection efficiency for modified mRNA of 41.83% (Figure 3.19). Transfection with unmodified GFP mRNA could be achieved with an average efficiency of 51.57%. The standard deviation of 18% in both cases was relatively high, but still a t-test resulted in a p-value lower than 0.05, as a paired t-test was performed. The variety between the efficiencies in the seven experiments was high, but the increase of the efficiencies when unmodified mRNA was transfected did always occur.

Taken together, these results let suggest that hPSCs cannot only be transfected with unmodified synthetic mRNA, but that the efficiency is even increased when unmodified instead of modified mRNA is transfected. If this were due to a reduced innate immune system response, it would be interesting to investigate how derivatives of hPSCs react upon transfection with unmodified synthetic mRNA.

In order to elucidate the immune status of hPSC-derivatives, lt-NES cells, which were differentiated from the iPSC line iLB-30-r12, were transfected with modified and unmodified GFP mRNA. Per well of a 6-well dish, 800 ng of the designated mRNA were transfected into lt-NES with Lipofectamine® LTX. As a control, 800 ng of the polyA polynucleotide were transfected (data not shown).

16 hours after transfection, efficiency and intensity were approximated via fluorescence microscopy.

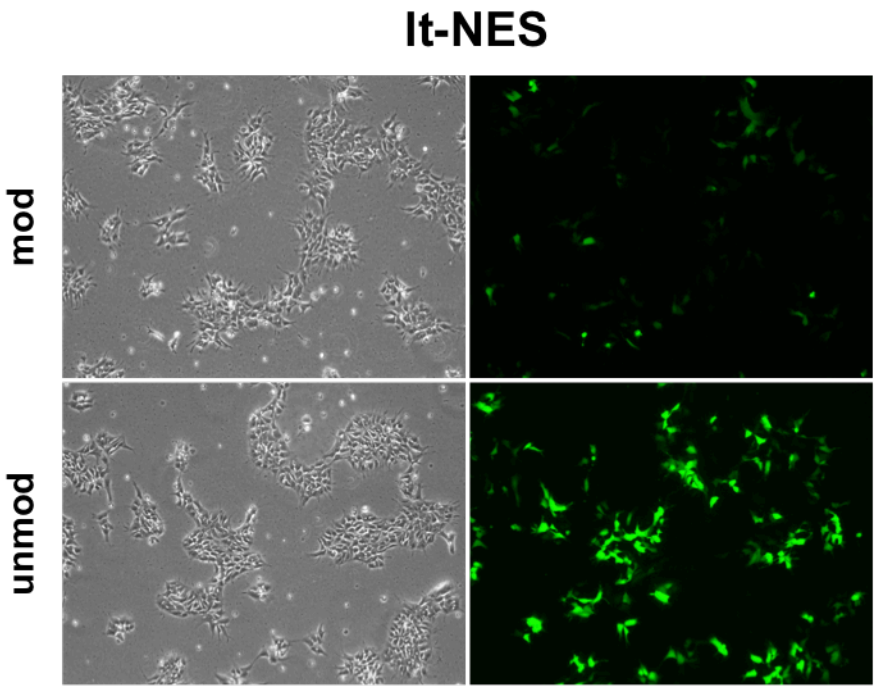

Figure 3.20: Transfection of unmodified GFP mRNA into lt-NES cells results in a robust translation of GFP
Transfection of unmodified GFP mRNA shows higher fluorescence intensity. Efficiency appears to be higher for unmodified mRNA.
Magnification: 40X; exposure time= 75ms

lt-NES cells showed the same reaction as hPSCs upon transfection with unmodified GFP mRNA (Figure 3.20). Apparently, the fluorescence intensity of the cells transfected with unmodified mRNA once more was stronger. Efficiency seemed to be increased as well. The cells kept growing in the same instance, no increased cell death indicated by detached cells in the supernatant could be observed. Quantification with flow cytometry confirmed that the efficiency as well as the intensity increased when unmodified GFP mRNA was transfected (Figure 3.21). Flow cytometry revealed an efficiency of 23.1% for the modified mRNA, whereas the unmodified mRNA could be transfected with an efficiency of 38.3% into lt-NES cells. In the overlay, the blue curve, which represents the histogram for the cells transfected with unmodified mRNA, is shifted to the right, indicating a strong fluorescence intensity.

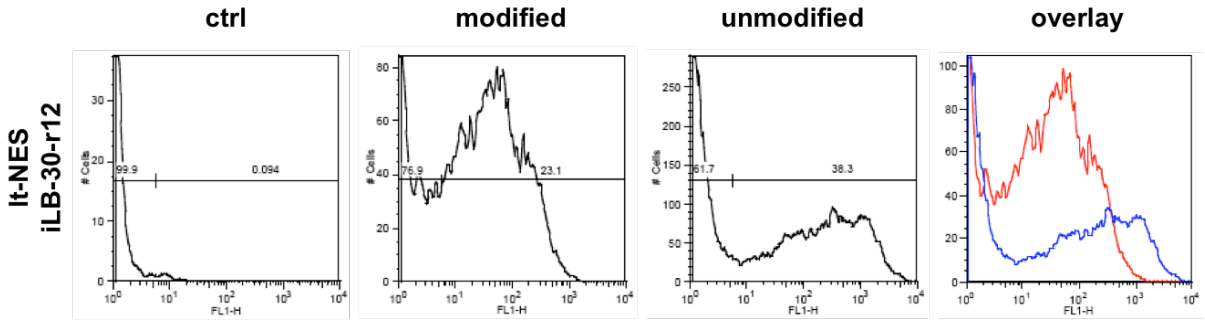

Figure 3.21: Increased efficiency and stronger GFP intensity in lt-NES cells after transfection with unmodified GFP mRNA
lt-NES cells can be transfected more efficiently with unmodified GFP mRNA (38.3%) than with modified GFP mRNA (23.1%). The fluorescence intensity is stronger when lt-NES cells are transfected with unmodified mRNA as in the overlay the peak of the blue histogram (unmodified) is more to the right than the peak of the histogram of modified mRNA-transfected cells (red).

Transfection of lt-NES cells was carried out three times. The variety between the individual experiments was relatively high as indicated by the high standard deviation (Figure 3.22). Nonetheless, in each experiment the efficiency was higher when unmodified mRNA was transfected.

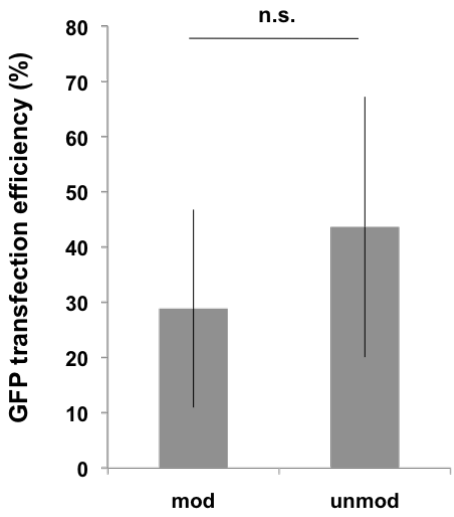

Figure 3.22: Transfection of lt-NES cells with unmodified mRNA leads to an increased efficiency
The mean transfection efficiency increases from 28.87% (±17.91%) for modified mRNA up to 43.63% (±23.57%) for unmodified mRNA.
n=3; error bars show standard deviation; n.s.: not significant; p= 0.06 according to a paired t-test

The average efficiency for modified mRNA was 28.87%. It increased up to 48.63% for unmodified mRNA. Due to a p-value of 0.06, the increase of the efficiency upon transfection with unmodified mRNA can only be considered as a trend, as it is not statistically significant.

3.3.2 hFBs cannot be transfected efficiently with unmodified mRNA

hPSCs and lt-NES cells both could be transfected more efficiently with unmodified mRNA. So far it cannot be excluded that this effect was due to a better quality of the mRNA without modifications. To rule out that option, human fibroblasts should be transfected with both modified and unmodified mRNA. As fibroblasts are fully differentiated somatic cells, the working hypothesis suggests that they have an active innate immune system that distinguishes foreign mRNA from cell-autonomous mRNA. An activated innate immune system could lead to degradation of the potentially harmful foreign mRNA and therefore would lead to a decreased efficiency.
To test whether this occurs in human fibroblasts, different human fibroblasts were transfected with modified as well as with unmodified GFP mRNA. 800 ng of the particular synthetic mRNA were transfected into the cells using TransIT® transfection reagent. 16 hours after transfection, a first evaluation was performed using fluorescence microscopy.
Human CRL-2097 fibroblasts could be transfected efficiently with modified mRNA, whereas the efficiency decreased when the cells were transfected with unmodified mRNA (Figure 3.23). After transfection, the cells kept growing, but seemed to be stressed indicated by morphology. The amount of attached cells appears to be higher in case of transfection with modified mRNA. This effect was not quantified but could give a hint that the transfection with unmodified mRNA led to more frequent cell death.

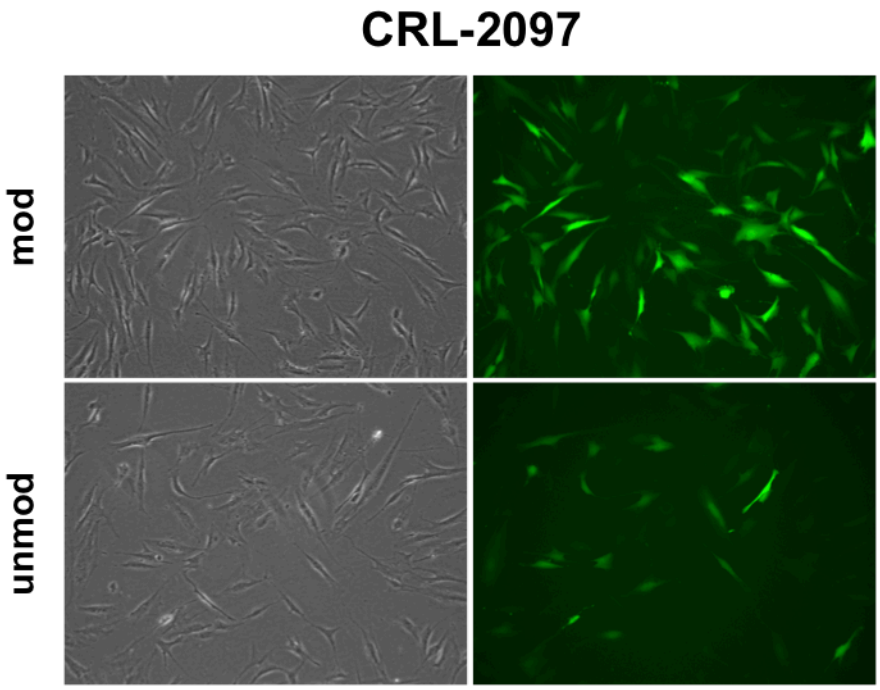

Figure 3.23: CRL-2097 fibroblasts can be transfected more efficiently with modified GFP mRNA
Transfection with modified GFP mRNA results in a higher efficiency and a stronger fluorescence intensity than transfection with unmodified mRNA.
Magnification: 40X; exposure time= 915ms

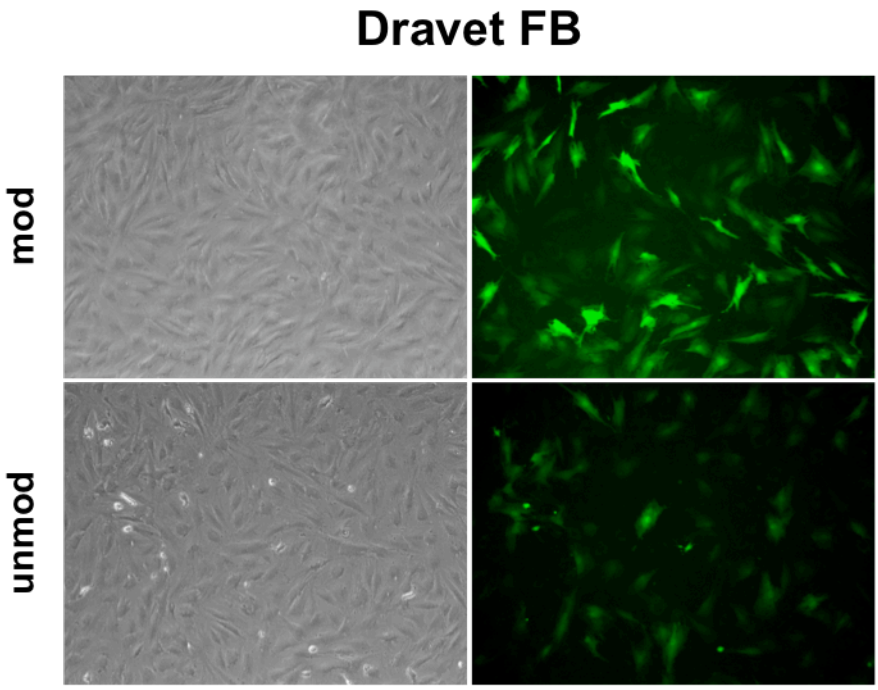

Figure 3.24: Transfection with modified GFP mRNA results in stronger fluorescence intensity
Dravet fibroblasts can be transfected with modified mRNA more efficiently than with unmodified mRNA; the fluorescence intensity appears to be much higher.
Magnification: 40X; exposure time= 2100ms

In case of the Dravet human fibroblast line (Figure 3.24), both modified and unmodified mRNA did not lead to a slowed-down proliferation of the cells as in both experiments as well as in the control (data not shown) cells were confluent 16 hours after transfection. Nonetheless, efficiency and intensity appeared to be higher when Dravet fibroblasts had been transfected with modified GFP mRNA.

When quantified by flow cytometry, both Dravet and CRL-2097 fibroblasts showed similar results (Figure 3.25). Dravet fibroblasts could be transfected with modified GFP mRNA with an efficiency of 43.3%. When transfected with unmodified mRNA, efficiency decreased to 32.1%. Transfection of CRL-2097 fibroblasts with the modified version of the synthetic mRNA resulted in an efficiency of 79.6%, whereas the unmodified version could be transfected with an efficiency of 67%.

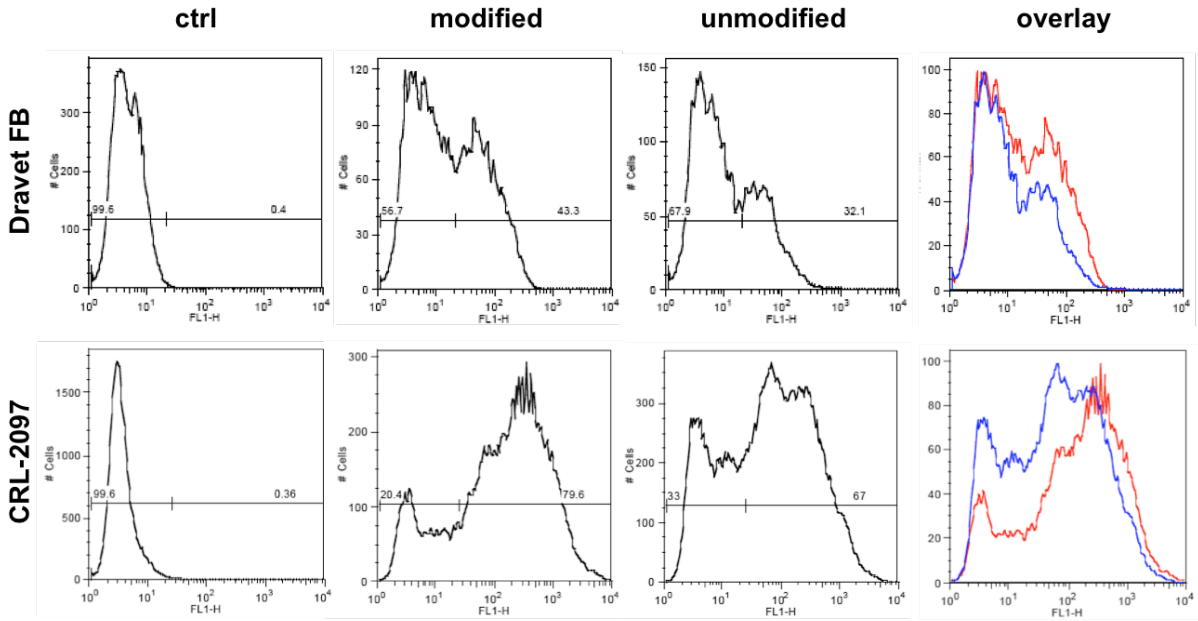

Figure 3.25: Human fibroblasts can be transfected more efficiently with modified mRNA
In this single experiment, transfection of fibroblasts with modified mRNA leads to an efficiency of 43.3% (Dravet FB) or 67% (CRL-2097). Transfecting the unmodified mRNA results in decreased efficiencies (32.1% for Dravet FB and 67% for CRL-2097). The overlay indicates that the fluorescence intensity for modified mRNA-transfected fibroblasts is stronger, as the red histogram (modified) is shifted more to the right than the histogram for the unmodified mRNA-transfected cells (blue histogram).

In total, six independent transfections were performed and validated by flow cytometry. The variation between the experiments was very high, as the best transfection efficiency yielded 92% (CRL-2097 fibroblasts transfected with modified mRNA) and the lowest 23.2% (Dravet fibroblasts transfected with unmodified

mRNA). Despite of this, the experiments that were performed side-by-side always led to a reduced efficiency for unmodified mRNA transfection. Therefore, a paired t-test was performed to estimate the statistical significance.

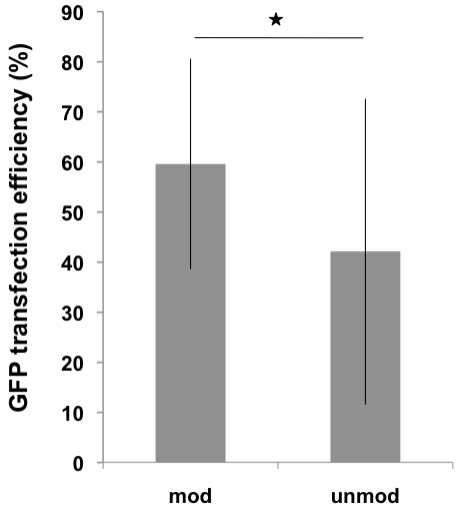

Figure 3.26: Human fibroblasts can be transfected more efficiently with modified GFP mRNA
Transfection of modified mRNA results in an efficiency of 59.57% (±21.03%), unmodified mRNA can be transfected into fibroblasts with an efficiency of 42.08% (±30.51%).
n= 6; error bars show standard deviation; *: $p<0.05$ according to a paired t-test

The six independent experiments led to an average efficiency of 59.57% (±21.03%) for transfection with modified synthetic GFP mRNA. The average efficiency of transfection with unmodified synthetic GFP mRNA was around 17% lower (42.08% ±30.51). Despite of the high standard deviation, a paired student t-test resulted in a p-value of 0.007, indicating that the decrease of efficiency is not a random effect but statistically significant (Figure 3.26).

In Figure 3.27, the quantifications of the flow cytometric analysis are summarized. It can be seen that human pluripotent stem cells could be transfected more efficiently with unmodified than with modified mRNA; efficiency increased about 10% from 41.83% to 51.57%. The difference between the results was statistically significant. Another significant effect could be observed for human fibroblasts; when transfected with unmodified mRNA, efficiency decreased from 59.57% to 42.08% around 17%. As a derivative of hPSCs, lt-NES cells could be transfected successfully with unmodified mRNA (43.63%), whereas the efficiency was 15% lower when modified mRNA was transfected (28.67%). The control cell line HEK 293T cell could be transfected at same efficiencies with modified as well as unmodified synthetic mRNA.

It showed the highest efficiencies with 86.7% (±9.9%) for modified and 85.7% (±10.32%) for unmodified mRNA.

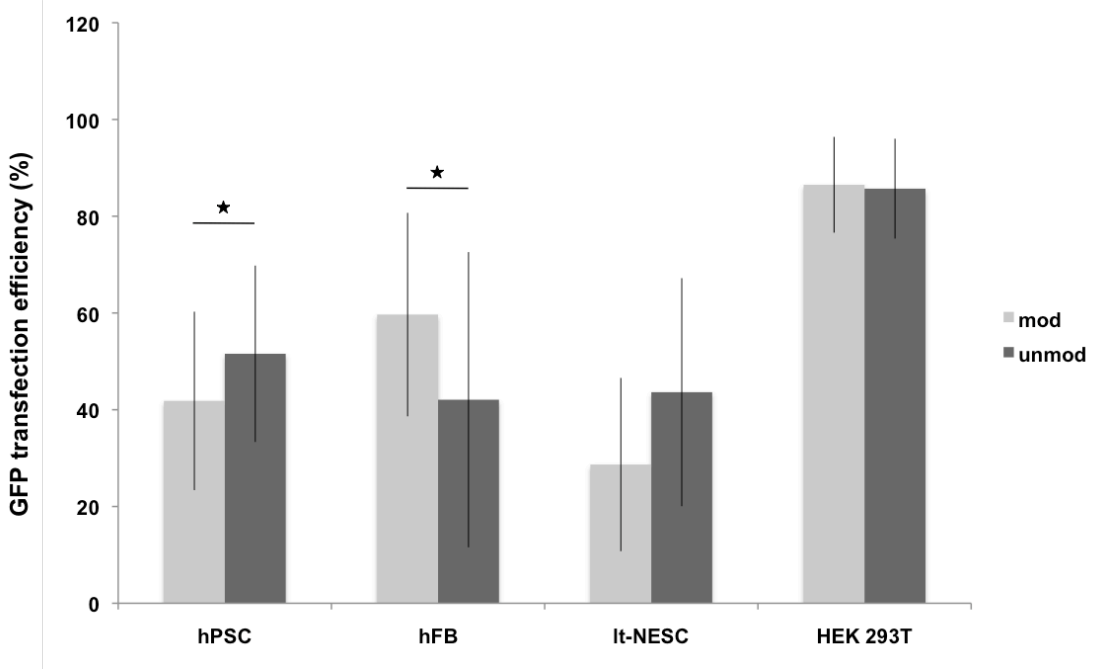

Figure 3.27: Summary of the flow cytometry quantification
hPSCs can be transfected with unmodified mRNA with a significantly higher efficiency than with modified mRNA. Fibroblasts (hFB) show the opposite effect as transfection with unmodified mRNA leads to a significantly lower efficiency. Transfection of lt-NES cells can be increased when using unmodified mRNA; this effect is not significant, though. HEK 293T cells do not show different efficiencies for modified and unmodified mRNA.
Light grey: modified; dark grey: unmodified
n(hPSC)= 7, n(hFB)= 6, n(lt-NES)= 3, n(HEK293T)= 2; error bars show standard deviation; *: $p<0.05$

The results presented in this chapter (summarized in Figure 3.27) let assume that pluripotent cells as well as multipotent precursor cells as lt-NES cells react differently upon transfection with synthetic mRNA without modifications compared to somatic cells. It was hypothesized that this was due to a reduced innate immune system activity.

3.3.2.1 Inhibition of IFNβ leads to increased efficiency

To test whether the non-active innate immune system is responsible for the susceptibility of hPSCs to be transfected with unmodified mRNA, the role of one of the regulators of the innate immune system, the intracellular receptor TLR3, should be investigated. TLR3 activation results in IFNβ signaling. If the innate immune system is responsible for the observed effects, inhibition of one of its activators should enable fibroblasts to be transfected with unmodified mRNA as well.

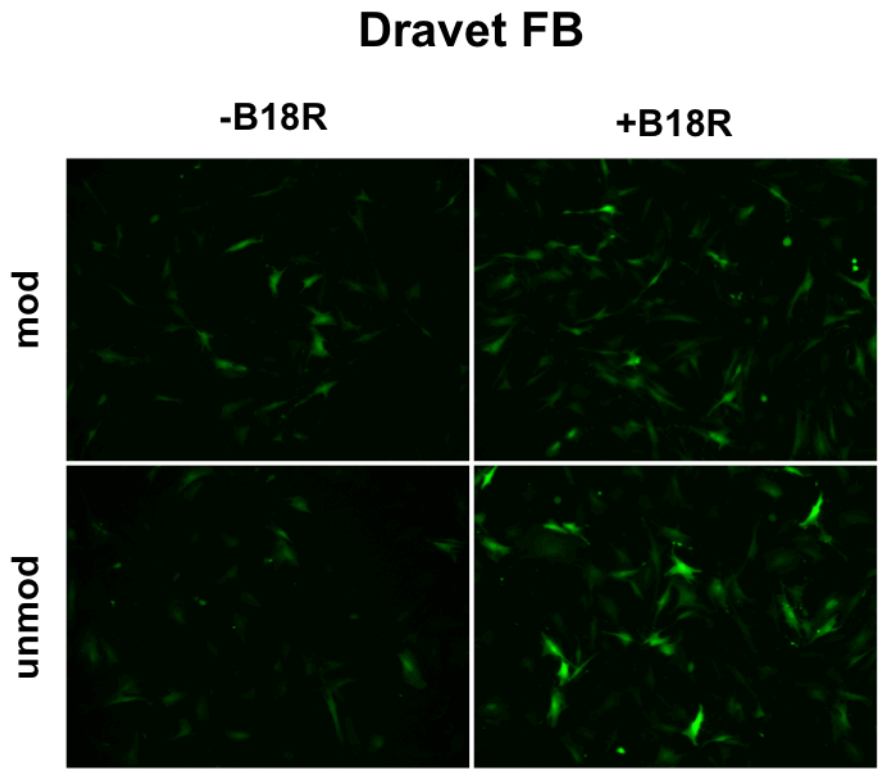

Figure 3.28: Inhibition of IFNβ leads to increased transfection efficiencies in human fibroblasts
Untreated fibroblasts can be transfected with modified mRNA. Transfection with unmodified mRNA results in a low efficiency and weak fluorescence intensity. When treated with the IFNβ inhibitor B18R (200 ng/ml), efficiency and intensity increase strongly for unmodified mRNA.
Magnification: 40X; exposure time= 1600ms

Dravet fibroblasts could be transfected with both modified and unmodified synthetic GFP mRNA (Figure 3.28), whereas the fluorescence intensity were decreased in the unmodified mRNA-transfected cells. When the fibroblasts were treated with the IFNβ inhibitor B18R at a concentration of 200 ng/ml, intensity slightly increased for modified mRNA transfection. For unmodified mRNA transfection, the increase of both parameters was more prominent.

The quantification by flow cytometry showed a transfection efficiency of 53.3% for modified mRNA in CRL-2097. The efficiency reduced to 44.3% for unmodified mRNA. When CRL-2097 fibroblasts were treated with B18R, efficiency of unmodified mRNA transfection rose up to 63% (Figure 3.29). Treatment of B18R also led to an increased efficiency of modified mRNA-transfection for CRL-2097 fibroblasts (data not shown).

For Dravet fibroblasts, a similar behavior could be observed. Modified mRNA was transfected into the cells with an efficiency of 46.4%; unmodified mRNA could only be transfected with 26% efficiency. Treatment with B18R led to an increase of efficiency for unmodified mRNA up to 60.6% (Figure 3.29).

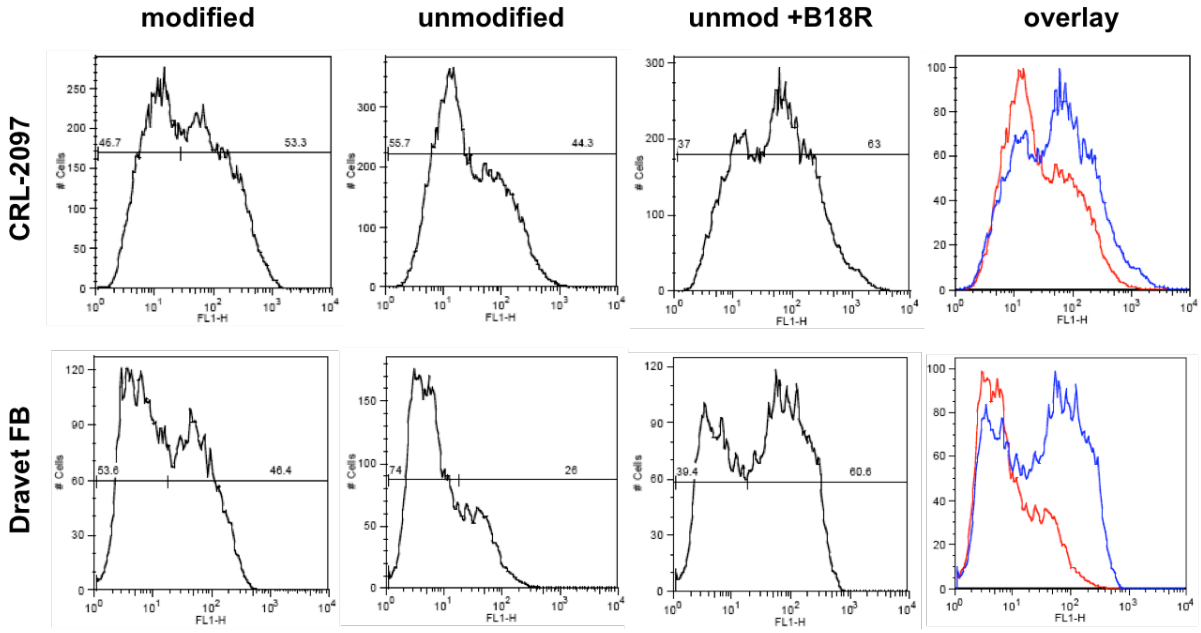

Figure 3.29: IFNβ inhibition enables human fibroblasts to be transfected with unmodified mRNA

CRL-2097 fibroblasts can be transfected with modified mRNA with an efficiency of 53.3% and with unmodified mRNA with an efficiency of 44.3%. Inhibition of IFNβ by B18R treatment (200 ng/ml) leads to an increase of the unmodified mRNA transfection up to 63%.

Dravet fibroblasts show a transfection efficiency with modified mRNA of 46.4%. Unmodified mRNA can be transfected into Dravet FB with an efficiency of 26%. Treatment with B18R improves the transcription efficiency of unmodified mRNA up to 60.6%.

Red histogram: unmodified mRNA; blue histogram: unmodified mRNA with B18R treatment

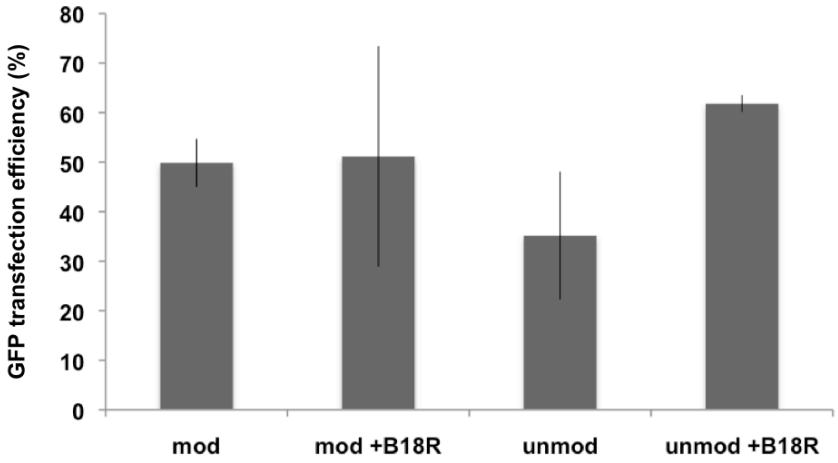

Figure 3.30: Human fibroblasts can be transfected with unmodified synthetic mRNA with a higher efficiency when IFNβ is inhibited
Human fibroblasts can be transfected with modified GFP mRNA with an efficiency of 47.3%. Inhibition of IFNβ with 200 ng/ml B18R does not lead to a notable change. Transfection with unmodified GFP mRNA results in an efficiency of 32.5%; efficiency is increased up to 59.35% when cells were treated with B18R.
n=2; error bars show standard deviation

In summary (Figure 3.30), human fibroblasts could be transfected with modified mRNA with an average efficiency of 49.85% (±4.88%). The inhibition of the TLR3 downstream effector IFNβ led to contradictory results. The average transfection efficiency of modified mRNA with B18R treatment was 51.15%, which would mean that B18R had no effect on transfection, but the high standard deviation of 22.27% indicates the high variability. As described before, transfection of fibroblasts with unmodified mRNA was not as successful as it resulted in an average efficiency of 35.15% (±12.94%) and thereby in a decrease of almost 15% compared to modified mRNA-transfection. Inhibition of IFNβ led to an average efficiency of unmodified mRNA-transfection of 61.8% (±1.70%). Statistical analysis could not be performed as only two individual experiments were performed. Therefore it cannot be said whether the increase of transfection efficiency upon IFNβ inhibition is statistically significant, but it gives a hint that the innate immune system plays a role in mRNA transfection into fibroblasts.

To exclude whether this effect of B18R was not due to the inhibition of IFNβ but a side effect, lt-NES cells and hPSCs were transfected with modified and unmodified mRNA while treated with B18R (200 ng/ml).
As shown in Figure 3.31, no obvious effect could be detected by evaluation with fluorescence microscopy. Both lt-NES cells (Figure 3.31, A) and human iPSCs

(Figure 3.31, B) could be transfected more efficiently with unmodified mRNA and also gave a stronger fluorescence intensity. Treatment with B18R affected neither transfection with modified nor unmodified mRNA.

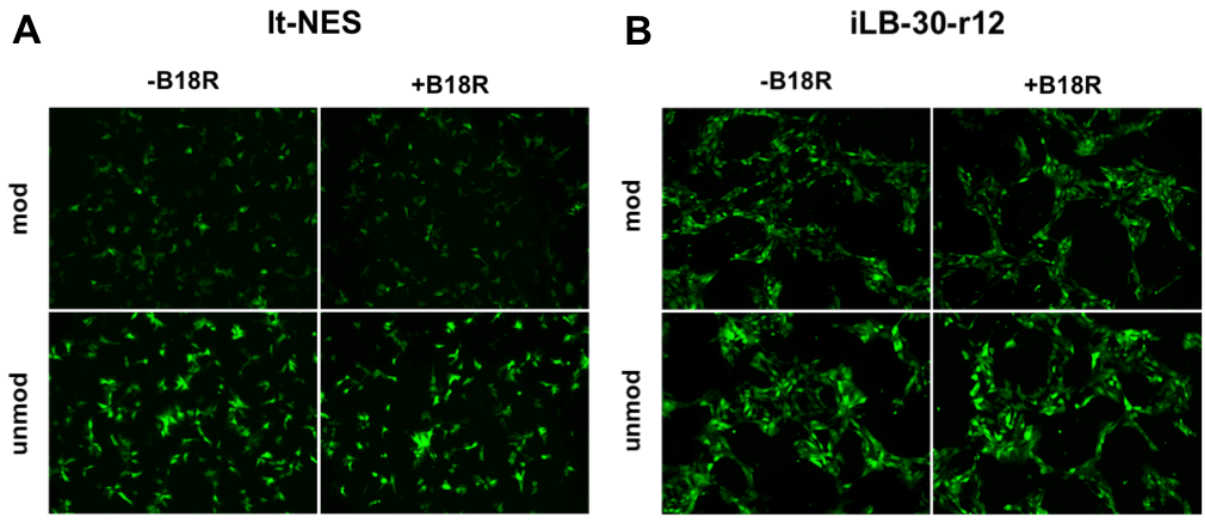

Figure 3.31: IFNβ inhibition has no effect on mRNA transfection into lt-NES cells and hiPSCs
(A) lt-NES cells can be transfected more efficiently with unmodified mRNA than modified mRNA. The treatment with 200 ng/ml of B18R does not lead to an increase of efficiency or intensity. Exposure time= 90ms (B) Transfection of iLB-30-r12 human iPSCs with unmodified mRNA is more efficient than transfection of the modified mRNA. Inhibition of IFNβ does not affect efficiency or fluorescence intensity. Exposure time= 120ms
Magnification: 40X

In summary, the fact that human fibroblasts cannot be transfected efficiently with unmodified mRNA seems to depend on an activated innate immune system. The inhibition of one of its regulator leads to an improved transfection with unmodified and therefore immunogenic mRNA. IFNβ inhibition had no effect on hPSCs and lt-NES cells, cells that are claimed to have no active innate immune system.

3.3.3 hPSCs and lt-NES show less immune response upon transfection with unmodified mRNA than with modified mRNA

To further elucidate the role of the innate immune system of hPSCs, its activation was analyzed via qRT-PCR for Interferon β (IFNβ). IFNβ expression is upregulated upon TLR3 activation. Cells were transfected with 800 ng/well of a 6well dish of either modified or unmodified GFP mRNA. As a control, cells were transfected with 800 ng/well of polyA, a polynucleotide that does not provoke immune reaction. Total RNA was isolated 6 and 24 hours after transfection and subsequently reverse

transcribed into cDNA. As illustrated in Figure 3.32 A and B, fibroblasts of two different lines showed a strong upregulation of IFNβ expression when transfected with unmodified mRNA. Whereas the relative expression was increased 131 fold (CRL-2097) or 1602 fold (BJ) after 6 hours compared to polyA control, 24 hours after transfection the relative IFNβ expression rose up to 5239 fold (CRL-2097) and 5606 fold (BJ), respectively.

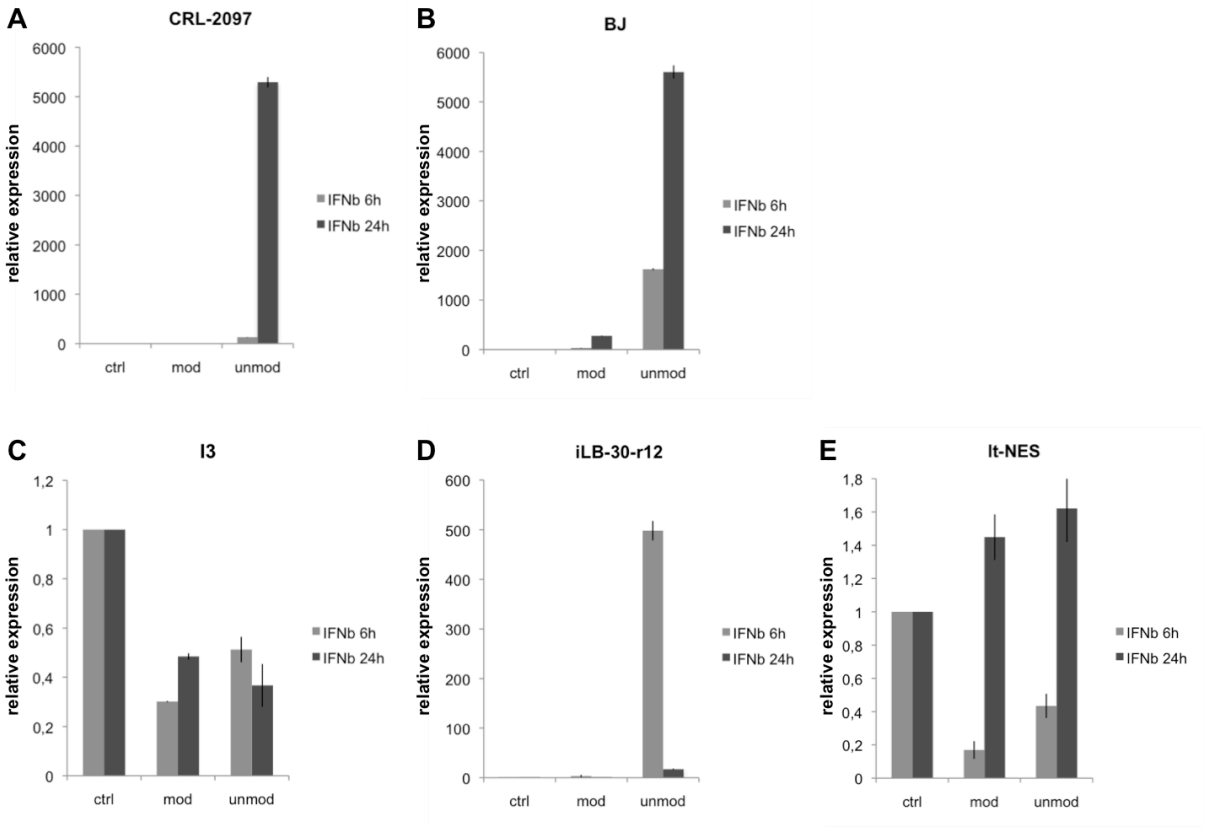

Figure 3.32: IFNβ expression is induced in fibroblasts more strongly than in PSCs and their derivatives
(A)-(B) IFNβ expression is induced strongly in human fibroblasts upon transfection with unmodified mRNA; relative expression increases with time. (C) hESC line I3 does not react with increased IFNβ expression upon mRNA transfection. (D) hiPSC line iLB-30-r12 expresses IFNβ after transfection with unmodified mRNA, but to a less extent than human fibroblasts; after 24 hours, IFNβ expression is strongly reduced. (E) It-NES cells derived from the iLB-30-r12 line do not react to a notable extent to mRNA expression. Results were normalized against GAPDH. All RT-PCRs were performed in triplicates; error bars show standard deviation. Control (transfection with polyA) was set as 1.

Notably, the immune reaction upon transfection with modified mRNA did not lead to a strong upregulation of IFNβ expression. CRL-2097 fibroblasts did not react with IFNβ expression, as its relative expression was only 1.69 after 6 hours and even below 1 (0.87) after 24 hours (Figure 3.32, A). BJ fibroblasts, however, expressed IFNβ after transfection with modified mRNA; with a relative expression of 33.07 after 6 hours

and 275.33 after 24 hours the immune response obviously is weaker than the one induced by unmodified mRNA (Figure 3.32, B).
The hESC line I3 showed no upregulation of IFNβ expression upon mRNA transfection, neither with modified nor with unmodified nucleosides (Figure 3.32, C). All relative expression values were below 1, suggesting no activation of the innate immune system. In contrast, hiPSCs of the iLB-30-r12 line showed an upregulation of IFNβ expression upon transfection with unmodified mRNA (Figure 3.32, D). With a relative value of 498 the upregulation was only a tenth as strong as in fibroblasts. Interestingly, the upregulation could be detected already after 6 hours and was reduced to 17 after 24 hours. In fibroblasts, the IFNβ expression 24 hours after transfection was higher than 6 hours after transfection.

In summary, it can be concluded that the TLR3 mediated immune response, indicated by Interferon β expression, is reduced in hPSCs, as they do not respond strongly upon transfection with synthetic mRNA generated with unmodified nucleosides. lt-NES cells, as a derivative of hPSCs, were also transfected with modified and unmodified mRNA as well as polyA control (Figure 3.32, E). Consistent with the findings in GFP transfection, immune response could better be compared with hPSCs than with somatic fibroblasts. IFNβ expression was only upregulated 1.6 fold upon transfection with unmodified mRNA. These data suggest that lt-NES cells maintain the immunogenic status of pluripotent cells.

4 Discussion

Human pluripotent stem cell biology is a fascinating field of research. The capacity of hPSCs to self-renew indefinitely while being able to differentiate into every cell of the body turned them into the focus of life sciences. The major disadvantage of hPSCs, namely the ethical limitations, could be circumvented by the technique of reprogramming, which was published 2007 for human cells and honored with the Nobel prize in 2012. Reprogramming facilitated the research on hPSCs. Still, a lot of research has to be done to gain an insight into the biology of human pluripotent stem cells. In this thesis, two general aspects of stem cell biology were investigated. First, the role of the Stat3 signaling pathway in human PSCs was analyzed, as it is a key signaling pathway in murine PSCs and understanding its role in human cells could eventually elucidate the differences between PSCs of the two different species. While performing the experiments, another interesting aspect of stem cell biology came up. As human PSCs could be transfected efficiently with synthetic mRNAs lacking modifications, it was hypothesized that the innate immune system of hPSCs differs from somatic cells.

4.1 Human pluripotent stem cells do not depend on Stat3 signaling

In 2004, Dahéron and colleagues published that LIF/STAT3 signaling is not able to maintain hESC pluripotency (Dahéron et al., 2004). Several working groups confirmed this finding (Humphrey et al., 2004; Sato et al., 2004). Before Dahéron and Sato investigated the role of Stat3 signaling more in detail, there were only anecdotal reports about the role of Stat3 signaling in human PSCs. In some publications it was suggested that LIF is not sufficient to maintain pluripotency (Reubinoff et al., 2000; Thomson et al., 1998), whereas another publication claimed that LIF helps to retain hESC pluripotency (Schuldiner et al., 2000). LIF signaling can have a positive influence on human embryos, as its activity has been reported to enhance human blastocyst formation *in vitro* (Dunglison, Barlow, & Sargent, 1996).

The reason why Stat3 signaling was investigated again in this thesis is a publication that claimed that a potent agonist of LIF, the chimeric fusion protein of Interleukin-6 and its soluble receptor (IL-6/sIL-6R), was able to maintain hPSC pluripotency in suspension culture in absence of feeder cells or conditioned medium (Amit et al.,

2010). In this thesis, this should be transferred to and comprehensively investigated in adherent cultures. hPSCs were cultured as described in the published protocol (Amit et al., 2011), but instead of in bacteriological dishes on Matrigel™ coated cell culture dishes to allow attachment of the cells. The medium applied was the same as published, which equals standard culture medium for hPSCs cultivated on a feeder layer, containing serum replacement and bFGF. Due to the lack of feeder cells, Activin A was not present in the medium. The chimeric fusion protein could not prevent differentiation of the cells. While one day after medium change the cells revealed a pluripotency-associated morphology, meaning a compact colony structure with sharp borders, two days after switching the medium all hPSCs showed a morphology typical for spontaneous differentiation (see Figure 3.3). On the one hand, this effect is remarkable. The morphology differed strikingly from the negative control. On the other hand, the effect was just temporary, which makes it hard to explain.

Due to the differentiation, Matrigel™ was not the optimal substrate anymore, therefore the cells detached and could not be kept in long-term cultures. The reason why the differentiation process was delayed in the presence of IL-6/sIL-6R is not known. Notably, not only the chimeric fusion protein, but also hLIF, had the effect of the delayed differentiation. Thus, if the observed effect is Stat3 signaling-dependent, the fusion protein seems not to be more efficient in the applied concentrations to activate the pathway.

The analysis of total protein lysates of I3 hESCs revealed that 200 pg/ml of the fusion protein was not able to phosphorylate Stat3 in hPSCs, whereas hLIF treatment (10 ng/ml) resulted in a weak activation of Stat3 (see Figure 3.4). Two target genes of Stat3 signaling, Socs3 and Pim1, were analyzed via qRT-PCR. The relative expression was lower than in the control cells cultivated under feeder-free conditions, even for a fusion protein concentration of 10 ng/ml. Therefore, even at concentrations as high as the hLIF concentration, the fusion protein was not able to induce Stat3 signaling. In the published protocol, 100 pg/ml of the fusion protein are sufficient to maintain hPSC pluripotency. Unexpectedly, the expression of the target genes did even decrease upon IL-6/sIL-6R treatment. This could imply even an inhibition of Stat3 signaling by the chimeric fusion protein. But as the decrease is only slightly (up to 4 fold) and is not dose-dependent (see Figure 3.5), this is not likely. In order to confirm that Stat3 signaling is not activated upon IL-6/sIL-6R treatment, the expression of several other target genes should be analyzed.

Taken together, IL-6/sIL-6R as well as hLIF was not able to robustly phosphorylate Stat3 in hPSCs. This could be due to missing receptors or signal-transducers. LIF binds to the LIF receptor (LIFR), whereas the fusion protein does not need any receptor, but needs to bind to the signal-transducing transmembrane protein gp130.
After LIF has bound to the LIFR, it dimerizes with gp130 and starts the Stat3 signaling cascade. Therefore, LIF signaling requires the expression of both LIFR and gp130, whereas the induction of Stat3 signaling via IL-6/sIL-6R only needs expression of gp130. The weak, but present, phosphorylation of Stat3 in hPSCs upon hLIF treatment (see Figure 3.4) suggests that gp130 is present on the surface of hPSCs. Several reports support this suggestion. Humphrey et al. published that hESCs do express gp130 (Humphrey et al., 2004). In another study it was shown that hESCs express gp130 as well as LIFR (Dahéron et al., 2004). This is consistent with the findings presented in this thesis, as LIF is able to induce Stat3 activation, even if it is very weak, but contradicted by another publication reporting that LIFR is not expressed on hESCs (Rose-John, 2002).
Chan et al., however, reported that gp130 is only poorly expressed in hPSCs. Interestingly, when hPSCs were cultured in LIF presence while inhibiting MAPK/ERK, Wnt and BMP signaling, the gp130 expression was upregulated. This led to the induction of a pluripotency state more closely to naïve pluripotency, as the cells survived dissociation in absence of ROCK inhibitor and the epigenetic status resembled the status of *in vivo* preimplantation epiblast cells (Chan et al., 2013). This suggests that the extent of gp130 on hPSCs is not sufficient to induce Stat3 signaling activity. For murine PSCs it is known that there is a threshold for the activity of Stat3 to completely maintain their undifferentiated state (Ernst, Oates, & Dunn, 1996; Matsuda et al., 1999; Viswanathan et al., 2002).
Still, it is possible that not the lack of LIFR and/or gp130 is the reason for the deficiency to respond to Stat3 signaling stimulators. Dahéron et al. showed in their report that Stat3 can be phosphorylated in hPSCs; furthermore activated Stat3 dimerizes and translocates to the nucleus (Brierley & Fish, 2005; Dahéron et al., 2004). This would suggest that other mechanisms, for example occupation of the pStat3-dimer binding sites in hPSCs, prevent activation of Stat3 target genes. Supporting this hypothesis, in this thesis it has been shown that Stat3 indeed can be phosphorylated in hPSCs without affecting target gene expression.

In conclusion, the knowledge in the field of Stat3 signaling in hPSCs consists of many ambiguous facts; further elucidation is needed.

The activity of the chimeric fusion protein could not be confirmed to be higher than that of LIF or IL-6 alone. In the published protocol for suspension culture of hPSCs, a concentration of 100pg/ml was sufficient to maintain pluripotency (Amit et al., 2011). In mPSC culture, IL-6/sIL-6R could replace LIF only in high concentrations (10ng/ml; see Figure 3.7 and 3.8). This may be due to the fact that the fusion protein is a human recombinant protein. Therefore, the human cell line HepG2, which can have an active Stat3 signaling, was used as a model as the cells express LIFR as well as IL-6R and gp130 (Lütticken et al., 1994). Even in these cells the fusion protein was not able to phosphorylate Stat3 at lower concentrations (see Figure 3.9). A report from 1996 gives a hint why the chimeric fusion protein is not that active in Stat3 phosphorylation. Ernst et al. published data that suggests that gp130 homodimers, as assembled when the fusion protein binds to gp130, have weaker effects on Stat3 signaling than LIFR/gp130 heterodimers (Ernst et al., 1996). Given that fact, the use of the chimeric fusion protein would only be useful to activate Stat3 signaling in cells that do not express LIF receptor. However, the concentrations need to be high to gain a proper activation.

Taken together, the results presented in this thesis together with the literature lead to two suggestions: first, the IL-6/sIL-6R chimeric fusion protein in the adherent culture is not as potent in pluripotency maintenance as described by Amit et al. for suspension culture, and second, the Stat3 signaling pathway seems to play no role in primed hPSCs. All obtained results need to be confirmed for other primed hPSC lines.

The role of Stat3 signaling in naïve human stem cells will most likely play a different role. Therefore, the investigation of its activation in human naïve pluripotent cells would be interesting. Furthermore, the transition of both states in the aspect of Stat3 signaling would be of interest.

4.2 A TALEN-mediated inactivation of the Stat3 gene

To further elucidate the role of Stat3 signaling, a TALEN pair has been established to generate a double strand brake in the Stat3 gene and therefore induce a gene

inactivation. The TALEN pair was constructed in the Hornung lab, where the plasmids were also tested for their functionality in HEK293T cells. Initial experiments to nucleofect the plasmids into hPSCs failed. It was reported that TALEN plasmids can be introduced into hPSCs via electroporation (Hockemeyer et al., 2011). Neither using the published protocol nor the establishment of an own protocol led to targeting of the Stat3 locus in hPSCs. Furthermore, most of the cells died (data not shown). Establishment of the protocol using a plasmid encoding for a fluorescent protein facilitated the validation of the experiments, but did also not result in functional protocols (data not shown). The reasons for that could not be determined, as also published electroporation protocols for hPSCs were adapted (Costa et al., 2007).

hPSCs are a highly fragile system. A lot of parameters influence the health and survival of the cells. For example, the hPSCs used in successful electroporation attempts were cultivated on a feeder layer (Costa et al., 2005; Hockemeyer et al., 2011b; Zwaka & Thomson, 2003). This could have led to an increased survival after transfection. Electroporation stresses the cells to a high extent (Stacey, Ross, & Hume, 1993). For electroporation, a single cell suspension is needed; hPSCs survive dissociation only when the Rho kinase is inhibited (Watanabe et al., 2007). Even in the presence of the ROCK inhibitor, dissociation leads to additional stress for hPSCs. Furthermore, electroporation requires a large amount of DNA and a lot of cells (Costa et al., 2007; Ding et al., 2013; Hanna et al., 2010).

Therefore, it was decided to assess a different approach to transfect cells with TALENs. As preliminary experiments showed good results for the transfection of hPSCs with synthetic mRNAs, and it was also published that hPSCs can be transfected and manipulated with synthetic mRNAs (Warren et al., 2010), TALEN-encoding mRNAs should be generated. Until then, only few reports have been published that make use of synthetic mRNAs encoding for TALENs. One group generated synthetic TALEN mRNA and injected it into zebrafish embryo (Dahlem et al., 2012). More recently, another group succeeded in inducing a double strand break at the Satb1 locus and introduced sequences of a target construct via homologous recombination by transfecting TALEN mRNAs together with the target construct into the nuclei of mouse pronuclear-stage embryos (Sommer et al., 2013). This data encouraged the hope that also hPSCs can be transfected with synthetic TALEN mRNA.

The protocol for synthetic TALEN mRNA generation followed the standard protocol for synthetic mRNA production. Surprisingly, template PCRs on the digested TALEN plasmid did not result in an amplification of the template (Figure 3.10 A). This was surprising, because the primer pair binds to the untranslated regions (UTRs) and is used for the synthesis for the template of every RNA. Therefore, the primers cannot be the reason for the failed PCR. As the TALEN construct is relatively long (3.4kb) and normally shorter mRNAs are synthesized (approximately 1kb), PCR conditions were adapted; elongation time was increased and DMSO was added. As this did not result in the desired improvement, as an alternative the 5´UTR-TALEN-3´UTR construct obtained via digestion of the TALEN plasmid directly was used as a template for the tail PCR without further amplification. Unexpectedly, tail PCR succeeded, as gel electrophoresis revealed a prominent band at the expected size of >3kb (Figure 3.10 C). This was unexpected since the primers bind to the same sites and the PCR conditions were equal. Even though there were some additional bands visible in gel electrophoresis, the tail PCR product was used as a template for the *in vitro* transcription (IVT). The reason for the overall poor outcome of the PCRs (Figure 3.10 A-C) could be the highly repetitive modular structure of the TALENs. It can be hypothesized that the polymerase has difficulties in reading and polymerizing the repetitive structures. This would suggest that also the T7 polymerase, which is used for IVT, could have problems in reading and transcribing the repetitive template. In the first attempts of TALEN mRNA synthesis, a lot of smaller RNAs could be detected on the agarose gel (data not shown). After adjusting the IVT conditions (increasing the temperature from 37°C to 42°C; extending the incubation time from 45 minutes to 60 minutes), a single prominent band could be detected when analyzed on an agarose gel. A slight signal could be observed around the expected band (see Figure 3.11 A); this could be due to degradation, as instead of denaturing gel electrophoresis a native gel electrophoresis was performed.

Control transfection of the TALEN mRNA was performed into HEK293T cells. As the TALEN constructs also contain a flag tag, immunoblot with an antibody against flag could confirm the efficient transfection. Furthermore, the translation of the mRNA was proven (Figure 3.11 B). Moreover, a T7 Assay revealed functionality in HEK293T cells (Figure 3.12). The mutation frequency achieved in HEK293T cells was 5%. This efficiency is relatively low, if one expects that HEK293T cells can be transfected with mRNA with a high efficiency. Considering that two mRNAs have to be transfected

into the cells, it still is a low efficiency. This could indicate a poor quality in either purity or assembly of the TALEN mRNA. Given the problems during template generation and IVT, the obtained synthetic mRNAs could be partially not functional, for example when not been transcribed from a full-length template. Additionally, the size of the TALEN mRNA with more than 3 kilobases could lead to problems during transfection. Also, the concentrations could need to be adapted, as due to the length of the TALENs, 1µg of RNA equals less mRNA molecules for TALEN mRNA than for example for GFP mRNA. Subsequently it was shown that TALEN mRNA can also be transfected into hPSCs. Human iPSCs from the iLB-30-r12 line were successfully transfected with both mRNAs (see Figure 3.13 A). iLB-30-r12 cells translated the synthetic mRNAs to functional TALENs indicated by an editing efficiency of 5.1% (Figure 3.13 B). As transfection of hPSCs is harder to achieve, transfection protocols still can be optimized to increase the mutation frequency. A possible way to improve mRNA transfection, namely the use of unmodified synthetic mRNAs, was not investigated due to time reasons.

With the results in this thesis, a proof-of-principle was accomplished that the genome of hPSCs can be engineered with synthetic TALEN mRNAs. To examine an effect of the Stat3 inactivation, clonal populations of hPSCs should be generated after TALEN transfection. Subsequently, a T7 assay could reveal the clones positive for Stat3 editing. These cells could be propagated further and used for further analysis.

4.3 Synthetic mRNAs are a robust tool to manipulate hPSCs

As described before, it was not possible to transfect hPSCs with plasmid DNA using electroporation during the experiments for this thesis. As the generation of a corresponding synthetic mRNA as well as the transfection of this mRNA into hPSCs succeeded, it is claimed that synthetic mRNAs are a feasible tool to manipulate hPSCs. The use of synthetic mRNAs to manipulate cells has the advantage that its short half-life and quick translation results in a tight control over application times. No integration into the host genome can occur, as it would be the case for DNA based approaches.

As the targeting efficiency of the TALEN mRNAs (as described in 4.2) was relatively poor, an optimization of mRNA quality could improve the outcome. Warren et al. transfected human cells with synthetic mRNAs in order to reprogram them into

pluripotent stem cells; furthermore, they used synthetic mRNAs to induce directed differentiation of hPSCs (Warren et al., 2010). However, they did not report transfection efficiencies of mRNAs into hPSCs, which might be of interest for further applications. Warren et al. worked with modified mRNAs, as it was reported that the modifications of synthetic mRNA reduce the innate immunity activation (Karikó et al., 2008, 2005; Nallagatla & Bevilacqua, 2008). Posttranscriptional modification of RNA is a process frequently found *in vivo*. Nearly 100 different nucleoside modifications are known (Cantara et al., 2011). Transfer RNA is the RNA subgroup most heavily modified, up to a quarter of the nucleosides are modified. Mitochondrial RNA has fewer modifications, as this organelle is a relic of eubacteria (Margulis & Chapman, 1998).

The lack of modifications leads to a resemblance of synthetic mRNA to viral RNA. Viral RNAs, whether single- or doublestranded, function as PAMPs and get recognized by PRRs. For example, the toll-like receptors TLR3, TLR7 and TLR8 recognize viral mRNA (Karikó et al., 2005, 2004).

Furthermore, RIG-I binds viral RNA and subsequently activates an innate immune response (Uzri & Gehrke, 2009; Yoneyama et al., 2004).

Two groups published successful generation of functional TALEN mRNAs; in the published studies, TALEN mRNAs were generated without employing the modified ribonucleoside bases (Dahlem et al., 2012; Sommer et al., 2013). In both cases, mRNAs were injected directly into embryos. Together with the known attenuated innate immunity of the embryo in the sterile environment of the womb (Levy, 2007), this led to the hypothesis that hPSCs can be transfected with unmodified mRNAs. In order to improve transfection and translation, the efficiencies of unmodified mRNAs should be tested.

4.4 Modified versus unmodified mRNA

The generation of unmodified mRNAs has the advantage that it is less cost intensive, as the modified ribonucleotides are relatively expensive compared to the unmodified standard ribonucleotides. As mRNA *in vivo* also gets modified, the effect of the withdrawal of modifications was subject of this thesis. To have a direct insight into transfection efficiencies, synthetic GFP mRNA was used. First of all, modified as well as unmodified GFP mRNA was generated. Both IVTs were performed using the same batch of template; synthesis was performed side-by-side with the same

aliquots of each reagent to exclude batch-variant differences. After gel electrophoresis, both synthetic GFP mRNAs showed one prominent band with the expected size of 1kb. Interestingly, in the lane of unmodified mRNA (see Figure 3.14, lane 2), additional bands with a smaller size could be detected. The bands were much weaker than the GFP band, but they were from defined lengths. This effect could be observed in three independent *in vitro* translations of unmodified mRNA, but never for modified mRNA. The reasons for that are not known. Vertebrate cells do synthesize modified mRNAs, but the modifications are posttranscriptional (Garcia & Goodenough-Lashua, 1998). The usage of modified ribonucleotides therefore is not likely to be the reason for the improved synthesis of mRNA. The *in vitro* transcription kit used in this thesis depends on a RNA polymerase of the T7 bacteriophage. In T7 phages, no modifications of RNA have been reported. Therefore, an improved mRNA synthesis in the presence of modified ribonucleotides cannot be due to a better performance of the T7 polymerase.
The only different components used for modified mRNA were pseudo-UTP instead of UTP and 5´methyl-CTP instead of CTP. To exclude that a bad quality of this component leads to the different outcome of IVT, two different batches of nucleotides were used leading to no difference. So, the reason for additional bands for unmodified synthetic GFP mRNA could not be detected.

In this thesis, the modified mRNA was synthesized using pseudoUTP and 5-methyl-CTP. These are modifications unique for mammalian RNA, as pseudouridine is a known component of ribosomal RNA (Sumita et al., 2005), and 5-methylcytidine is found in mammalian *in vivo* mRNA (Sibbrit, Patel, & Preiss, 2013). Since these nucleoside modifications play a role in distinguishing host from foreign RNA, the innate immune system may play a role leading to the different results obtained with modified and unmodified mRNA. hPSCs could be transfected more efficiently with unmodified than with unmodified mRNA. Somatic cells, represented by human fibroblasts, could not be transfected efficiently with mRNAs lacking the modifications, suggesting that these cells express the modulators of a functional innate immune system. The control cell line HEK293T could be transfected with modified as well with unmodified mRNA efficiently. HEK293T cells are obtained from kidney tissue. The fact that they have a chromosomal number of 64 and are immortal reveals their mutated genotype. Therefore, even if kidney tissue normally would have an innate

immunity, the abnormality of HEK293T could be responsible for the lack of immune response upon transfection with unmodified mRNA. Interestingly, lt-NES cells, the derivatives of the human iPSC line iLB-30-r12, could be transfected with unmodified mRNA more efficiently than with modified mRNA, and therefore seem to resemble pluripotent stem cells in their immunogenic potential.

Interestingly, the transfection of hPSCs and lt-NES cells with unmodified GFP mRNA resulted in a stronger fluorescence than modified mRNA. As mentioned before, mRNAs are modified *in vivo* as well, partly even with the same modifications used in this thesis. Thus, the modifications of the nucleotides should not lead to decreased translation efficiency. Another possible reason for the stronger fluorescence in case of unmodified mRNA could be a better quality of the unmodified mRNA. This can be excluded, as gel electrophoreses revealed an even slightly worse quality of unmodified mRNA. Moreover, both reasons would also lead to a stronger fluorescence intensity in fibroblasts.

The fact that hPSCs and lt-NES cells can be transfected more efficiently with unmodified GFP mRNA compared to modified GFP mRNA, is also displayed by the fact that the intensity of the fluorescence is higher. This could indicate that the transfected cells translated more mRNA molecules. Nonetheless, it would be puzzling why not more cells were transfected with mRNA, as the transfection efficiencies did not reach more than 78.00% (average transfection efficiency 51.57%). If the transfection mix contains that many mRNA molecules that several molecules enter one cell, it should be enough to transfect more cells. However, this relatively low average efficiency could be due to the accessibility of the cells. During growth, hPSCs form colonies. So it cannot be excluded that cells are in the center of such a forming colony and therefore having a small surface enabling transfection. The same is true for lt-NES cells that form rosettes during growth. A further reason for the efficiency could be the dependency of the cell cycle. It was reported that cells can not be transfected with lipoplexes during G1-phase (Brunner et al., 2000). As hPSCs and lt-NES cells are highly proliferative, a relatively large proportion of cells might be in G1-phase and thus not be accessible for transfection. To confirm this, the transfection could be repeated 6 hours later; the cells then should be accessible, which would result in a higher overall eifficiency.

Nonetheless, the reasons for the better transfection efficiency and stronger intensity of unmodified mRNA in hPSCs are not revealed. In the literature it was described that the use of pseudouridine in mRNA synthesis led to a superior translation in mammalian cells and when injected into mice (Karikó et al., 2008). This is consistent with the findings presented in this thesis for fibroblasts, which are somatic cells. In contrast, hPSCs as well as lt-NES cells showed a stronger translation when transfected with unmodified mRNA, suggesting that the observed effect is specific for stem and precursor cells.

A shift to the right of the histogram in flow cytometric analysis led to the interpretation that the translational efficiency was higher. Remarkably, in case of hPSCs also the negative cells were shifted to the right when transfected with unmodified mRNA (see Figure 3.18). So this effect could also be due to background fluorescence.

4.5 Innate immunity is attenuated in hPSCs

The observation that human pluripotent stem cells can be transfected efficiently with unmodified mRNA whereas somatic fibroblasts could be transfected with modified mRNA more efficiently, resulted in the hypothesis that the innate immune system of hPSCs is attenuated. It is known that the distinction between host and foreign RNA through its modifications is mediated by TLR3, a receptor of the innate immune system (Karikó et al., 2005). Nonetheless, the activation of the innate immune system upon transfection with both forms of mRNA had to be investigated to confirm the contribution of the innate immune system.

As a first experiment, human fibroblasts were treated with B18R during transfection. B18R is an inhibitor of Interferon signaling. Interferon β is stimulated upon TLR3 activation (Oshiumi et al., 2003). If recognition of unmodified mRNA would induce TLR3 mediated Interferon β activation, a treatment with B18R would prevent the transmission of this signal. Indeed, fibroblasts treated with B18R could be transfected more efficiently with unmodified mRNA compared to untreated cells (see Figures 3.28, 3.29 and 3.30). The efficiency was even higher than with modified mRNA. One reason for that could be that also modified mRNA provokes an immune response that however is lower than for unmodified mRNA. This is challenged by the fact that B18R treatment did not lead to an increased efficiency for modified mRNA transfection. B18R was used to enhance cell survival during mRNA mediated reprogramming by

Warren et al. In this study modified mRNAs were transfected, indicating that B18R and thus Interferon signaling can have an effect also on transfection with modified mRNA (Warren et al., 2010).

Expectedly, B18R treatment had no effect on mRNA transfection into hPSCs and lt-NES cells (see Figure 3.31). This supports the hypothesis that these cells do not activate the innate immune system upon mRNA transfection. Therefore, an inhibition of Interferon signaling does not lead to a further increase of transfection efficiency.

It may also be possible that the innate immune system in hPSCs upon RNA transfection is activated through another receptor. Another member of the toll-like receptor family, namely TLR7, also senses for infections with single stranded RNA (Diebold et al., 2004). Upon activation, TLR7 induces Interferon α (IFNα) expression and thus innate immunity activation. As nothing is known about the expression of TLR7 in hPSCs, the role of TLR7 in its attenuated innate immunity has to be investigated.

To confirm the role of Interferon signaling in mRNA transfection, qRT-PCRs were performed to analyze the expression level of Interferon β. An upregulation would suggest an increased innate immune response. As expected, two different human fibroblasts reacted with an upregulated IFNβ expression upon transfection with unmodified mRNA (see Figure 3.32 A, B). The IFNβ expression was increased 131fold or 1602fold respectively after 6 hours, but to a greater extent after 24 hours (more than 5000fold for both cell lines). The transfection with modified mRNA did not result in a strong upregulation of IFNβ expression; in CRL-2097 no effect could be observed, whereas BJ fibroblasts upregulated IFNβ expression 275fold 24 hours after transfection. Compared with the reaction on transfection with unmodified mRNA, the response evoked by modified mRNA is very low. In conclusion, unmodified mRNA activates the innate immune system of fibroblasts, as Interferon β expression is upregulated. Surprisingly, the induction of IFNβ expression could be detected only 24 hours after transfection. Six hours after transfection, no strong effect on IFNβ expression could be detected. A further investigation of the time course of IFNβ upregulation could give insights into the velocity of innate immunity activation and duration of the response.

In case of hPSCs, one hESC line (I3) and one hiPSC line (iLB-30-r12) were tested (see Figure 3.32 C, D). In I3 cells, the IFNβ expression upon mRNA transfection was lower compared to the control. As a control, polyA, a polynucleotide consisting of

only ATPs, was transfected. polyA is supposed to be non-immunogenic (Tobias Schmidt, personal communications). Therefore, transfection of neither modified nor unmodified mRNA seems to have an impact on IFNβ expression in I3 hESCs. However, hiPSCs of the iLB-30-r21 line showed a 500fold increased IFNβ expression upon transfection with unmodified mRNA. This indicates an immune response that only takes place upon transfection with unmodified mRNAs, even though the intensity of this response is low compared to the immune responses of fibroblasts. The fact that this immune response could only be detected in hiPSCs and not in hESCs could suggest that induced PSCs remain an immune status of the cells they were originated from. iLB-30-r12 iPSCs were reprogrammed from human fibroblasts. The weak immune response of the cells upon transfection with unmodified mRNA could be a relic of the fibroblast innate immune system. lt-NES cells, that were differentiated from the iLB-30-r12 iPSC line, did only show a 1.6 fold upregulation of IFNβ expression, though. This contradicts the hypothesis that iLB-30-r12 cells remain the immune response of its origin fibroblasts at least partially; if this was the case, lt-NES cells should react upon transfection with unmodified mRNA more strongly. To further enlighten the immunogenic properties of hPSCs and lt-NES cells, further cell lines should be investigated.

If the observed effect of immune response of iLB-30-r12 hiPSCs is a real biological effect, it has to be noted that the upregulation of IFNβ already occurs six hours after transfection and is decreased strongly 24 hours after transfection. This could suggest that the immune response not only is attenuated in hPSCs, but also accelerated. It may be that the immune response in the I3 hESCs already was finished six hours after mRNA transfection. Therefore, also for hPSCs the time course of innate immune system response needs further examination. In the literature, no studies report an accelerated response of the innate immune system. In the literature it is reported that the innate immune responses in stem cells are attenuated, whereas these studies do not speculate about an acceleration in these processes (Chen et al., 2010; Földes et al., 2010; Hong & Carmichael, 2013). Földes et al. furthermore reported that cells differentiated from hPSCs, in that case endothelial cells, preserve the immune response of the origin cells (Földes et al., 2010). This is contradictory to the findings in this thesis, as lt-NES cells differentiated from hiPSCs, showed not the same immune response as the origin cells. This is also contradictory to the findings

of Chen et al. who showed that BMP4 induced differentiation of hPSCs led to a gain of IFNβ responses (Chen et al., 2010).
Another study from 2013 reported that innate immune responses are enhanced upon neural differentiation of hESCs (Farmer et al., 2013). However, neural precursor cells showed an attenuated immune response, which is consistent with the findings presented in this thesis. Further analysis of differentiated cells could enlighten the innate immunity upon differentiation.

All in all, the knowledge of the innate immunity of human pluripotent stem cells still is poor. But the published data together with this thesis show the same trend. Innate immunity in hPSCs is attenuated; transfection either with PAMPs or with mRNA does not lead to induction of Interferon mediated effects. The reasons for this remain unclear. It is known that during pregnancy, the maternal immune system also protects the embryo; it does not posses either an own innate or adapted immune system. In case of an infection, the placental barrier usually prevents an infection of the embryo. Furthermore, different tissues of the placenta express TLRs, suggesting that the placenta is also responsible for the protection of the embryo against pathogens (Holmlund et al., 2002). Therefore, the innate immunity of trophoblasts would also be an interesting subject of research.
The evolutionary advantage of a lacking innate immune system remains unclear, as a good defense against pathogens should be a benefit for an organism. It can be speculated that the harm of a pathogenic infection would be too high in an early phase of development to put effort in the defense against it. The fact that activation of innate immune response in trophoblast cells lead to apoptosis and therefore to abnormal pregnancy outcomes indicate also a strategy that prevents fight against pathogens (Abrahams & Mor, 2004; Jerzak & Bischof, 2002).
Interestingly, the results reported in this thesis show that human and murine PSCs resemble in their immunogenic potential. As reported several times by a group around Wang, murine PSCs show attenuated innate immunity activation upon viral infection (Wang et al., 2013, 2014). Wang et al. also claim that these unique properties can be exploited for mRNA mediated gene expression strategies (Wang et al., 2014).

Taken together, hPSCs can be transfected efficiently with unmodified mRNA, as the innate immunity is not activated. The reasons for the stronger efficiency and intensity upon transfection with unmodified mRNA still remain unclear. However, in case of manipulation of hPSCs, advantage of the attenuated innate immunity can be taken. But in other fields of research, the missing innate immune system can be a disadvantage. If cells differentiated from hPSCs keeps some of the characteristics of the immune system of the pluripotent cells, transplantation of these cells could hold the risk that these cells can be infected with pathogens. These pathogens could replicate in these cells and then exist in a number high enough to overcome the innate immune system of the other somatic cells.

4.6 Outlook

In this thesis it has been shown that Stat3 signaling seems not to play a role in adherent hPSC culture. Nonetheless, it would be interesting to check whether the signal-transducing transmembrane protein gp130 is expressed on the surface of hPSCs. With that, it could be excluded whether the deficiency of the IL-6/sIL-6R chimeric fusion protein to phosphorylate Stat3 is due to the lack of gp130.

The described data functions as a proof-of-principle. In order to confirm that Stat3 signaling plays no role in hPSCs, several other hiPSC and hESC lines should be included. Moreover, the expression of other Stat3 downstream target genes should be examined via qRT-PCR.

Furthermore, it could be a feature of the chimeric fusion protein to induce Stat3 signaling only in human pluripotent stem cells. To exclude this, naïve human PSCs would be the perfect model. The recently published protocol to gain naïve hPSCs from Gafni et al. could be used to obtain *bona fide* naïve hPSCs (Gafni et al., 2013). Furthermore, the adaption of this protocol to cells in which Stat3 is inactivated by TALEN technology would be interesting.

For that, the TALEN mediated Stat3 editing has to be improved. First of all, clones should be derived, as in the polyclonal cell populations only 5% of the cells show a Stat3 editing. In polyclonal cultures, the not targeted cells could have a growth advantage and therefore overgrow the cultures. In order to facilitate the obtainment of clonal Stat3 edited hPSC lines, the efficiency of the TALEN mRNA transfection should be improved. To reach this, unmodified TALEN mRNAs should be generated, since it was shown that hPSCs can be transfected more efficiently with unmodified synthetic mRNAs. Furthermore, additional transfection rounds could improve the outcome, as the mRNA transfections do not seem to effect cell survival.

It has been shown in this thesis that the replacement of the modified ribonucleotides with their unmodified counterparts leads to an improved transfection of synthetic mRNA into hPSCs. The modifications of ribonucleotides are not the only posttranscriptional modifications of mRNA that occur *in vivo* to prevent the activation of the innate immune system. Furthermore, mRNA gets “capped”, meaning that at the 5´-end of the RNA, a methylated guanine nucleotide is added (Alberts et al., 2002). As the removal of the nucleoside modifications led to increased transfection

efficiency, the effect of further reduction of modifications would be interesting. It could be investigated whether hPSCs can be transfected with completely unmodified mRNA. Additionally, cells could be transfected with mRNAs synthesized with modified ribonucleotides but without the 5´-Cap and thus enlighten the impact of the different modifications to the innate immune activation and to the half-life of the mRNAs. Skipping the capping in the mRNA synthesis would greatly reduce costs of *in vitro* mRNA synthesis, but probably lead to problems in translation.

The innate immune system of hPSCs still is poorly understood; a lot of experiments still can be performed to further elucidate its role in pluripotent cells as well as in multipotent precursors and derivatives of both. First of all, the expression of different receptors of the TLR family could be investigated; of special interest in case of RNA transfection would be the TLR3 receptor. Also the expression of TLR7 should be investigated, as this receptor also recognizes pathogenic RNA. Further, other mediators of RNA-induced immune response, such as RIG-I or the PKR, should be tested for its expression in hPSCs. Additionally it would be interesting to check the expression of the key modulators in trophoblast cells, as it was reported that this extraembryonal tissue seems to be responsible for the protection of the embryo proper.

As the response upon mRNA transfection is mediated by Interferon β, its expression should be investigated more in detail. In this thesis, expression levels were checked only at two time points after transfection. As the IFNβ expression in fibroblasts could be detected only relatively late, further time points between the analyzed 6 and 24 hours would be interesting. The presented data in this thesis gave the hint that immune response in hPSCs could be not only attenuated, but also accelerated; therefore, it would be interesting also to look for IFNβ expression at earlier time points, for example 1,2 and 4 hours after mRNA transfection.

The IFNβ expression of the two analyzed hPSC lines differed; therefore, additional cell lines should be investigated. Of special interest would be the examination of IFNβ expression upon reprogramming and differentiation. To accomplish that, the iLB-30-r12 cells could be used, as from this line fibroblasts, iPSCs and neural precursors already are established. Further differentiation of the iLB-30-r12 lt-NES cells into mature neurons would create fully differentiated somatic cells. Differentiation into different cell types, also from different lineages, could be achieved

without big effort. With that, effects depending on the biological variety can be excluded, as all cells originate from the same background. Not only IFNβ expression, but also TLR3 expression, would be of interest. To confirm that TLR3 is the mediator of the innate immune response upon mRNA transfection, also the expression and activation of TLR7 should be investigated.

Finally, also the activation of the innate immune system upon contact with other PAMPs should be investigated to find out whether also other PRR-mediated immune reactions are attenuated in hPSCs.

4.7 Conclusion

In this thesis it has been shown that the chimeric IL-6/sIL-6R fusion protein cannot maintain pluripotency in hPSCs. It failed to activate Stat3 signaling in hPSCs, and application to mPSCs and HepG2 revealed that this LIF agonist is not as potent as LIF itself. The results still need to be confirmed in different cell lines.

It was shown that functional synthetic TALEN mRNAs against Stat3 can be generated. Transfection into hPSCs resulted in an editing efficiency of the Stat3 gene of 5%.

Synthetic mRNAs represent a robust tool to manipulate hPSCs as they could be transfected efficiently. Furthermore, hPSCs as well as their derivatives lt-NES cells could be transfected more efficiently with unmodified than with modified synthetic mRNA, whereas human fibroblasts showed opposite results. Fibroblasts could be transfected with unmodified synthetic mRNA when IFN signaling was inhibited. This led to the suggestion that the immune response in hPSCs upon synthetic mRNA transfection is attenuated. This assumption was supported by the fact that hPSCs and lt-NES cells show low IFNβ expression upon synthetic mRNA transfection.

This knowledge may be useful to further improve mRNA mediated hPSC manipulation such as genomic engineering with synthetic TALEN mRNAs.

5 Summary

Human pluripotent stem cells (hPSCs) hold great promises as a model for development as well as a source for cells in regenerative medicine. Although hPSC research came into focus in the last decade, many aspects of the biology of hPSCs still remain unclear. Maintenance of pluripotency in murine pluripotent stem cells (mPSCs) depend on LIF-activated Stat3 signaling, whereas human PSCs appear not to require activation of this signaling pathway. In order to investigate the potential role of Stat3 signaling in hPSCs, cells were treated with a chimeric fusion protein ("IL-6/sIL-6R"). The chimeric fusion protein is supposed to be a potent LIF agonist and thus Stat3 signaling activator. While it was reported before that hPSCs can be maintained pluripotent in suspension cultures in the presence of the chimeric protein, here IL-6/sIL-6R failed to maintain pluripotency in adherent cultures. Activation of Stat3 signaling could be observed neither at protein nor at mRNA level. To further analyze the role of Stat3 in hPSCs, a targeted gene inactivation should be established. Classical DNA transfection methods did not lead to an efficient editing. Previously, synthetic mRNA could be transfected into hPSCs with an efficiency up to 78%. Therefore, synthetic Transcription Activator-like Effector Nuclease (TALEN) mRNAs targeting the Stat3 locus were generated. Synthetic TALEN mRNAs edited the hPSC genome with an efficiency of 5%.

In order to improve efficiencies, transfection with several types of synthetic mRNAs should be investigated. Synthetic mRNAs usually contain modified nucleotides in order to prevent innate immunity activation. In order to investigate if innate immunity plays a role in hPSCs, it was tested whether synthetic mRNAs without modified nucleotides ("unmodified mRNA") can be used. As a proof of principle, hPSCs and their derivatives lt-NES cells (multipotent neural precursors) were transfected with unmodified GFP mRNA. Surprisingly, the average efficiency (51.57% for hPSCs; 43.63% for lt-NESCs) was even higher than for transfection with modified GFP mRNA (41.83% for hPSCs; 28.87% for lt-NESCs). Furthermore, the fluorescence of the translated protein appeared to be stronger. Human fibroblasts, representing somatic cells, could be transfected more efficiently (59.57%) with GFP mRNA synthesized with modified nucleotides. Unmodified GFP mRNA transfection resulted in low efficiency (42.08%) and weak fluorescence intensity, most likely due to innate immunity responses.

Summary

In order to test whether attenuated immune response is the reason for accessibility for unmodified mRNAs, the innate immunity associated Interferon signaling was blocked using the inhibitor B18R. Inhibition of Interferon signaling in human fibroblasts resulted in an increase of efficiency from 32.5% up to 59.35% when unmodified mRNA was transfected.

Moreover, fibroblasts expressed higher levels of IFNβ upon transfection with unmodified mRNA compared to hPSCs and lt-NES cells. Together, this let suggest that the IFNβ mediated immune response is attenuated in multi- and pluripotent cells.

These findings may be of interest for further applications of synthetic mRNAs in hPSCs. For example, generation of unmodified synthetic TALEN mRNAs should lead to improved genetic engineering.

6 Declaration

I hereby declare that the work in this thesis is original and has been carried out by myself at the Institute for Reconstructive Neurobiology, Medical Center, University of Bonn. This thesis was prepared under the supervision of Prof. Dr. Frank Edenhofer in fulfillment of the requirements of the doctoral degree of natural sciences of the University of Bonn. I further declare that this work has not been the basis for the awarding of any degree, diploma, fellowship, associateship or similar title at any university or institution.

Bonn, July 2014

Sabrina Schoeps

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