Aus der Chirurgischen Klinik und Poliklinik – Innenstadt,
Ludwig-Maximilians-Universität München,
Chirurgisches Forschungslabor
Direktor: Prof. Dr. med. Wolf Mutschler

The role of IKK2 in TNF-α-induced migration and proliferation of human mesenchymal stem cells

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Emmanouil Pappou
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Berichterstatter:	Prof. Dr. med. Wolf Mutschler
Mitberichterstatter:	Priv. Doz. Dr. Benedikt Berninger
	Prof. Dr. Christian P. Sommerhoff
Mitbetreuung durch den promovierten Mitarbeiter:	Priv.-Doz. Dr. med. Wolfgang Böcker
Dekan:	Prof. Dr. med. Dr. h.c. M. Reiser, FACR, FRCR
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Table of Contents

Abbreviations

The following is a list of the most commonly used abbreviations. Additional abbreviations used are defined in the text at the appropriate place.

Amino acid A	Alanine
Amino acid K	Lysine
AMV	avian myeloblastosis virus
APS	ammonium persulfate
Att	attachment site
BCA	bicinchoninic acid
Bp	base pairs
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cDNA	complementary DNA
CFU-F	colony forming units-fibroblast
DEPC	diethylpyrocarbonate
D-MEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
Dn	dominant negative
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
dyt medium (2x YT)	double-strength yeast extract/tryptone
E. coli	Escherichia coli
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
EGFP/ eGFP	enhanced green fluorescent protein

ELISA	Enzyme-Linked ImmunoSorbent Assay
EtBr	ethidium bromide
FACS	fluorescent-activated cell sorting
FADD	Fas Associated protein with Death Domain
FBS	fetal bovine serum
FCS	fetal calf serum
GAPDH	glyceraldehyde3-phosphate dehydrogenase
HIV	human immunodeficiency virus
hMSC	human mesenchymal stem cells
HRP	horseradish peroxidase
ICD	intracellular domain
Ig	immunoglobulin
IKK	inhibitor κB Kinase
IKK2/ IKKβ	inhibitor κB Kinase beta
Kb	kilo base pairs
LTR	long terminal repeat
MAPC	multipotent adult progenitor cells
MCS	multiple cloning site
MEM	minimum essential medium
MIAMI	marrow-isolated adult multilineage inducible cells
MSC	mesenchymal stem cells
NEAA	non-essential amino acid
NF-κB	Nuclear factor-κB
NLS	nuclear localizing sequence
P	plasmid
p.A.	pro analysis
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride

RHD	Rel-homology domain
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
RNAse	ribonuclease
RRE	rev-responsive element
RS	recycling stem cells
RSV	respiratory syncytial virus
RT	room temperature
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulfate
SIN	self-inactivating
SODD	silencer of death domain
SV 40	simian virus
TAE	Tris-Acetate-EDTA
TAK1	TGF-β-activated protein 1
Taq	Thermus aquaticus
TE	Tris-EDTA
TEMED	N,N,N',N'-Tetramethylethylenediamine
TNF	tumor necrosis factor
TNF-R	TNF receptor
TRADD	TNF receptor-associated death domain protein
TRAF	TNF receptor-associated factor
Tris	trishydroxymethylaminomethane
UV	ultraviolet light
VCAM	vascular cell adhesion molecule
VSV-G	vesicular stomatitis virus glycoprotein G

System of Units

Symbol	Units
da	Dalton
G	gram(s) / force of gravity
H	hour(s)
L	liter(s)
min	minute(s)
rpm	rounds per minute
S	second(s)
TU	transducing units
U	unit(s)
V	Volt

1 Introduction

1.1 Mesenchymal stem cells

1.1.1 Introduction

About 40 years ago Friedenstein *et al.* first described stromal cells in the bone marrow that were spindle shaped and proliferated to form colonies (Friedenstein, Piatetzky et al. 1966). In that assay system, stromal cells were referred to as colony-forming unit-fibroblasts (CFU-F). These cells attached to plastic and were able to differentiate under defined in vitro conditions into multiple cell types (Friedenstein, Chailakhjan et al. 1970). Later on these cells, obtained from postnatal bone marrow, were called mesenchymal stem cells, or marrow stromal cells and more recently, multipotent mesenchymal stromal cells, all designated by the acronym MSC.

Mesenchymal stem cells (MSCs) have generated a great deal of excitement and promise as a potential source of cells for cell-based therapeutic strategies, primarily owing to their intrinsic ability to self-renew and differentiate into functional cell types that constitute the tissue in which they exist.

Despite diverse and growing information concerning MSCs and their use in cell-based strategies, the mechanisms that govern MSC self-renewal, proliferation and tissue migration are not well understood and remain an active area of investigation. Therefore, research efforts focused on identifying factors that regulate and control MSC cell fate decisions are crucial to promote a greater understanding of the molecular, biological and physiological characteristics of this potentially highly useful stem cell type (Figure 1).

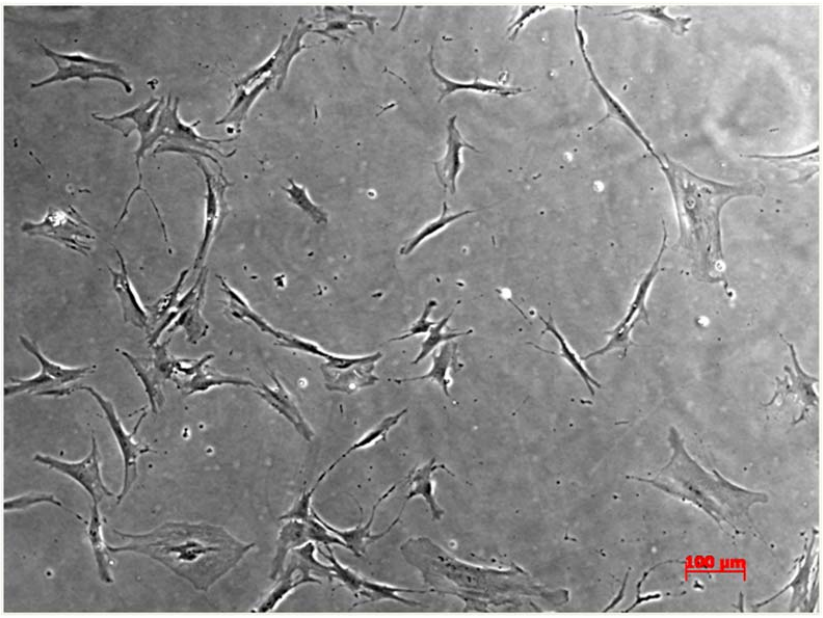

Figure 1. Human mesenchymal stem cells (MSC) are spindle shaped, fibroblast-like cells. Original magnification × 100, light microscopy, scale bar represents 100 µm.

1.1.2 A definition of MSCs

Due to the lack of a single definitive marker and knowledge regarding the anatomical location and distribution of MSCs *in vivo*, their definition has relied primarily on retrospective assays. Since the early work of Castro-Malaspina et al. (Castro-Malaspina, Gay et al. 1980), many researchers have employed different methods to isolate MSCs, in both serum and serum-deprived conditions, and have developed novel approaches to isolate purified populations of MSCs. These advances have furthered our understanding of MSC biology but have also created differences in terminology and read-out measures (i.e., based on morphology, phenotype, gene expression, and combinations thereof) for describing the adherent-capable cells derived from many adult tissue sources displaying fibroblast-like morphology (Caplan 1991; Bianco and Gehron Robey 2000; Shi, Gronthos et al. 2002). There is still a lack of consensus on the hierarchy intrinsic to the MSC compartment (Beyer Nardi and da Silva Meirelles 2006), reflected by the use of terms

such as multipotent adult progenitor cells (MAPCs) (Reyes, Lund et al. 2001), marrow-isolated adult multilineage inducible (MIAMI) (D'Ippolito, Diabira et al. 2004) and recycling stem cells (RS-1, RS-2) (Colter, Class et al. 2000; Colter, Sekiya et al. 2001). Although none of these terms can accurately account for both the developmental origin and differentiation capacity of these cells, the term "mesenchymal stem cell" (MSC) is currently the most often employed broad umbrella designation. However, both this and the other named cell types depend, for their definition, on the adherence of a population of harvested cells to a tissue culture substrate, and therefore none can represent the actual progenitors existant in adult human marrow.

Despite these attendant limitations, the International Society for Cellular Therapy (ISCT) has provided the following minimum criteria for defining MSCs (Dominici, Le Blanc et al. 2006) as follows:

(1) Plastic-adherent under standard culture conditions.

(2) Express CD105, CD73 and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79 or CD19 and HLA-DR.

(3) Must differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*.

1.1.3 The mesenchymal stem cell niche

The exact localization of MSCs *in vivo* remains poorly understood. Mesenchymal stem cells have been conventionally isolated from bone marrow in humans (Digirolamo, Stokes et al. 1999; Pittenger, Mackay et al. 1999). Within the marrow, MSCs comprise 0.001–0.1% of the total population of nucleated cells.

Despite the fact that bone marrow is considered a well-accepted source of MSCs, MSCs have been isolated from other tissue sources, including periosteum (Nakahara, Goldberg et al. 1991; Mason, Grande et al. 1998), trabecular bone (Noth, Osyczka et al. 2002), muscle (Asakura, Komaki et al. 2001), adipose tissue (Zuk, Zhu et al. 2001), synovial tissue (De Bari, Dell'Accio et al. 2001), dermis (Young, Steele et al. 2001), lung (Sabatini, Petecchia et al. 2005), adult peripheral blood (Zvaifler, Marinova-Mutafchieva

et al. 2000), and cord blood (Erices, Conget et al. 2000; Kern, Eichler et al. 2006), suggesting that the MSC niche may not be restricted to just bone marrow. Recent data demonstrate that the MSC compartment is more widely distributed than previously thought and that MSCs are resident in vessel walls (da Silva Meirelles, Chagastelles et al. 2006). These findings reveal that MSCs are diversely distributed *in vivo*, and as a result may occupy a ubiquitous stem cell niche.

1.1.4 Key characteristics of MSCs

1.1.4.1 Surface markers

Considerable progress has been made towards characterizing the cell surface antigenic profile of human bone marrow-derived MSC populations using fluorescence activated cell sorting (FACS) and magnetic bead sorting techniques. To date, however, a single marker that definitively delineates the *in vivo* MSCs has yet to be identified, due to the lack of consensus from diverse documentations of the MSC phenotype (Gronthos, Graves et al. 1994; Pittenger, Mackay et al. 1999; Minguell, Erices et al. 2001; Tocci and Forte 2003). However, analyses using a combination of monoclonal antibodies raised against surface markers of *in vitro*-derived MSCs (e.g., STRO-1, SH2, SH3, SH4) have shown some promise toward immuno-phenotyping these cells (Haynesworth, Baber et al. 1992; Gronthos, Graves et al. 1994). On the other hand, the fact that MSCs share common features with endothelial, epithelial and muscle cells and present a highly variable profile of cell surface antigens (Simmons and Torok-Storb 1991; Jiang, Jahagirdar et al. 2002; Vogel, Grunebach et al. 2003) makes it a daunting task to identify a universal single marker for MSCs. Despite this controversy of what defines a ‘mesenchymal stem cell’, there is general agreement that MSCs lack typical hematopoietic antigens, namely, CD45, CD34 and CD14 (Pittenger, Mackay et al. 1999).

1.1.4.2 Self-renewal potential

One of the defining characteristics of stem cells is their self-renewal potential, the ability to generate identical copies of themselves through mitotic division over extended time periods (even the entire lifetime of an organism). The absolute self-renewal potential of MSCs remains an open question, due in large part to the different methods employed to derive populations of MSCs and the varying approaches used to evaluate their self-renewal capacity. As a population, bone marrow derived MSCs have been demonstrated to have a significant but highly variable self-renewal potential during *in vitro* serial propagation (Bruder, Jaiswal et al. 1997; Colter, Class et al. 2000). Several reports have strongly suggested that MSCs and isolated MSC clones are heterogeneous with respect to their self-renewal capacity (Colter, Class et al. 2000; Bianco, Riminucci et al. 2001).

1.1.4.3 Multilineage differentiation potential

An important feature about MSCs is their multilineage differentiation potential. Under defined inductive conditions, MSCs are able to acquire characteristics of cells derived from embryonic mesoderm. The multilineage differentiation potential of MSC populations has been extensively studied *in vitro*. These studies demonstrate that populations of bone marrow derived MSCs have the capacity to develop into terminally differentiated mesenchymal phenotypes both *in vitro* and *in vivo*, including bone (Aubin, Liu et al. 1995; Bruder, Kurth et al. 1998; Aslan, Zilberman et al. 2006), cartilage (Wakitani, Goto et al. 1994; Kadiyala, Young et al. 1997), tendon (Young, Butler et al. 1998; Awad, Butler et al. 1999), muscle (Galmiche, Koteliansky et al. 1993; Ferrari, Cusella-De Angelis et al. 1998; Kadivar, Khatami et al. 2006), adipose tissue (Rogers, Young et al. 1995), and hematopoietic-supporting stroma (Cheng, Qasba et al. 2000; Koc and Lazarus 2001). Their vast differentiation potential is further expanded, as they have thus far demonstrated the ability to transdifferentiate into neural cells (Kopen, Prockop et al. 1999; Sanchez-Ramos, Song et al. 2000), endothelial cells (Reyes, Lund et al. 2001),

skin cells (Deng, Han et al. 2005), pancreatic islet beta-cells (Chen, Jiang et al. 2004) and hepatocytes (Kang, Zang et al. 2005; Sato, Araki et al. 2005). This capacity to differentiate into mesodermal, ectodermal and endodermal cell lineages characterizes MSCs as pluripotent cells. However, as many studies suggest, MSCs are heterogeneous with respect to their developmental potential (Kuznetsov, Krebsbach et al. 1997; Majumdar, Thiede et al. 1998; Pittenger, Mackay et al. 1999; Muraglia, Cancedda et al. 2000).

1.1.5 Role and Function of Mesenchymal Stem Cells

The complete in vivo role of the MSCs remains to be elucidated; however, various studies suggest that they are likely to play essential roles in supporting hematopoiesis, tissue repair and maintenance within the human body. More recently, MSCs have been recognized as having extensive immunomodulatory properties (Di Nicola, Carlo-Stella et al. 2002; Krampera, Glennie et al. 2003). Given the great potential of these cells and our lack of a complete understanding of that potential, they make excellent subjects of investigation for the current state of tissue repair science.

1.1.6 The potential of MSCs in cell and gene therapy

Over the past two decades, the ability to transfer genes into stem cells has raised hopes towards the feasibility of using gene therapy-based approaches to provide long-term therapeutic impacts (Bianco and Robey 2001; Vollweiler, Zielske et al. 2003). There is mounting evidence that these cells will ultimately be useful as vehicles for cell and gene therapies, especially in the field of tissue engineering. The ultimate goal is to use MSCs in various forms of therapy, as well as tools to understand the mechanisms leading to repair and regeneration of damaged or diseased tissues and organs. This approach has provided a lot of promise in the treatment of bone disorders as well as vascular diseases.

The long lifespan and homing ability of MSCs are attractive assets in the context of gene therapy strategies directed against infectious diseases and metastatic tumours. The use of MSCs in different therapeutic strategies either as immunosuppressive agents or as vehicles to express therapeutic proteins acting against autoimmune processes have been reviewed by Jorgensen *et al*. (Jorgensen, Djouad et al. 2003). There is emerging evidence that MSCs deploy a very powerful array of mechanisms that allow their escape from host allogeneic responses. In *vitro* and *in vivo* observations suggest that MSCs may be potentially used to induce tolerance into allogeneic or xenogeneic hosts, allowing them to facilitate unrelated HSC transplantation, minimise graft-versus-host disease and prevent rejection for organ transplantation.

Genetically manipulated MSCs may have direct applications to impact diseases in a variety of cell types in elaborate microenvironments and in different tissues *in situ*. The ability to genetically modify MSCs provides a means for durable expression of therapeutic genes for the lifetime of the patient for a wide range of diseases. MSCs can be engineered to secrete a variety of different proteins *in vitro* and *in vivo* that could potentially treat a variety of serum protein deficiencies and other genetic or acquired diseases, including bone, cartilage and bone marrow disorders, or even cancer. Improvements in gene delivery into HSCs have provided clues towards crucial improvements required to enhance therapeutic efficacy of MSCs for a variety of different diseases. A better understanding of the molecular mechanisms directing the proliferation, differentiation and tissue migration of MSCs will eventually allow to properly manipulate MSCs both *ex vivo* and *in vivo* to allow the regeneration of complex tissues and organs.

1.2 Lentiviral Vectors for Gene Delivery into Cells

1.2.1 Introduction

Retroviral vectors have been used for delivery of genetic material into cells for over 20 years, with the first reports dating back to the early 1980s (Wei, Gibson et al. 1981). The interest and use of lentiviral vectors came of age in 1996 when it was shown that human

immunodeficiency virus type 1-based vectors were capable of transducing nondividing cells (Naldini, Blomer et al. 1996), thus overcoming one of the important limitations of conventional retroviral vectors (Miller, Adam et al. 1990; Roe, Reynolds et al. 1993). The following properties make them suitable for gene therapy approaches (Trono 2000): (i) Lentiviral vectors have a large insert capacity (8–10 kb); (ii) they efficiently transduce not only dividing but also non-dividing cells; (iii) lentiviruses elicit minimal if any inflammatory response that can compromise the viability of the transduced cells; and (iv) they integrate genes into the chromosome of the target cells, leading to stable long-term expression.

1.2.2 Lentiviruses

The lentivirus family comprises complex retroviruses with the conventional three open reading frames of all retroviruses – *gag*, *pol* and *env* encoding the capsid proteins, the viral enzymes and the envelope glycoproteins, respectively (Lever, Strappe et al. 2004). In addition, they have a varying complement of regulatory and accessory genes, such as tat and rev, with which they control their own gene expression, allowing temporal separation into early and late phases, and with which they manipulate the host cell both for virus production and for virus entry and integration of their genetic material into the cell genome as the provirus (Figure 2). Lentiviruses infect vertebrates, particularly primates and domestic animals. They generally cause a slowly progressive disease (lenti = slow) or, in the case of primate viruses in their natural host, apparently no disease at all. The best studied example for a lentivirus is the human immunodeficiency virus (HIV) (Figure 2b).

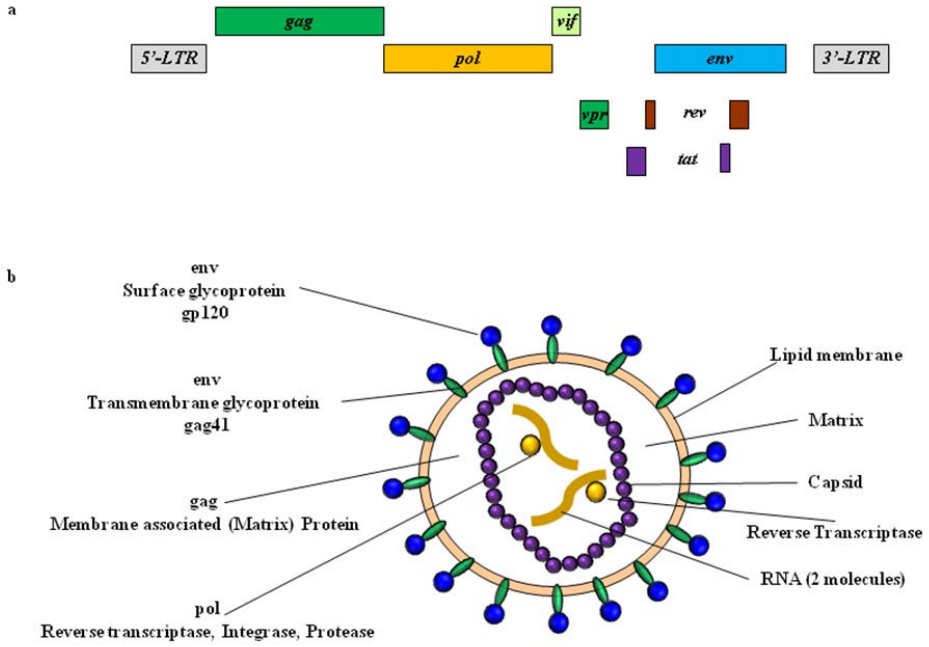

Figure 2. a. Diagram of open reading frame of HIV-1, a typical lentivirus. b. Diagram of mature HIV virion particle with proteins and RNA.

1.2.3 General concept of the design of lentiviral vectors

Lentiviral vectors are derivatives of HIV-1 lentiviruses that have been engineered to carry a foreign gene of interest into a target cell. The vectors are engineered to be replication-defective, being able to complete only a single round of the lentiviral replication cycle. Because of the way replication-defective lentiviral vectors are designed, virus particles containing vector genomes can be produced and can be used to infect target cells. The vector genome then undergoes reverse transcription and integration into the cell's genome, where it can express the foreign gene of interest, but is unable to be replicated

an additional time and spread to other cells; the vectors can undergo only a single round of replication.

Building a replication-defective vector from the parental lentivirus necessitates separating the *cis*- and *trans*-acting sequences of the viral genome. In a practical sense, this entails removal of the *trans*-acting *gag*, *pol*, and *env* genes from the virus (and replacing them with a foreign gene of interest), leaving on the genome only those *cis*-acting regions that are recognized by viral and cellular proteins during the various stages of the viral replication cycle-reverse transcription, integration, transcription, encapsidation.

1.2.4 Production of lentiviral vectors

Lentivectors are generated using a "split-component" production system, the overall objective being to make each component less and less complete in function, to the point where infectious viral particles can only be produced in the packaging cell and not from the final vector preparation. Typically, producer cell lines are transfected with (i) the transfer vector plasmid, containing the gene of interest, lentiviral LTRs for host cell integration and the Rev-responsive element (RRE) for most efficient vector production; (ii) a plasmid encoding the gag and pol viral structural genes, in order to supply reverse transcriptase and integration functions for the therapeutic vector particles; and (iii) "packaging" plasmids encoding envelope proteins for the viral particles and Rev protein. None of the starter plasmids are, by themselves, capable of functioning as autonomous lentiviruses. In addition, most accessory genes of lentiviruses (env, vif, vpr, vpu, and nef) have been removed during the process without any negative effect on vector recovery yield (Zufferey, Nagy et al. 1997; Dull, Zufferey et al. 1998). Only the transfer vector contains the packaging signal and thus, in theory, infectious particles should contain only the envisaged gene of interest.

The ViraPower Lentiviral Expression System of Invitrogen was used to create the replication-incompetent HIV-1-based lentiviruses that were used in this thesis (Figure 3).

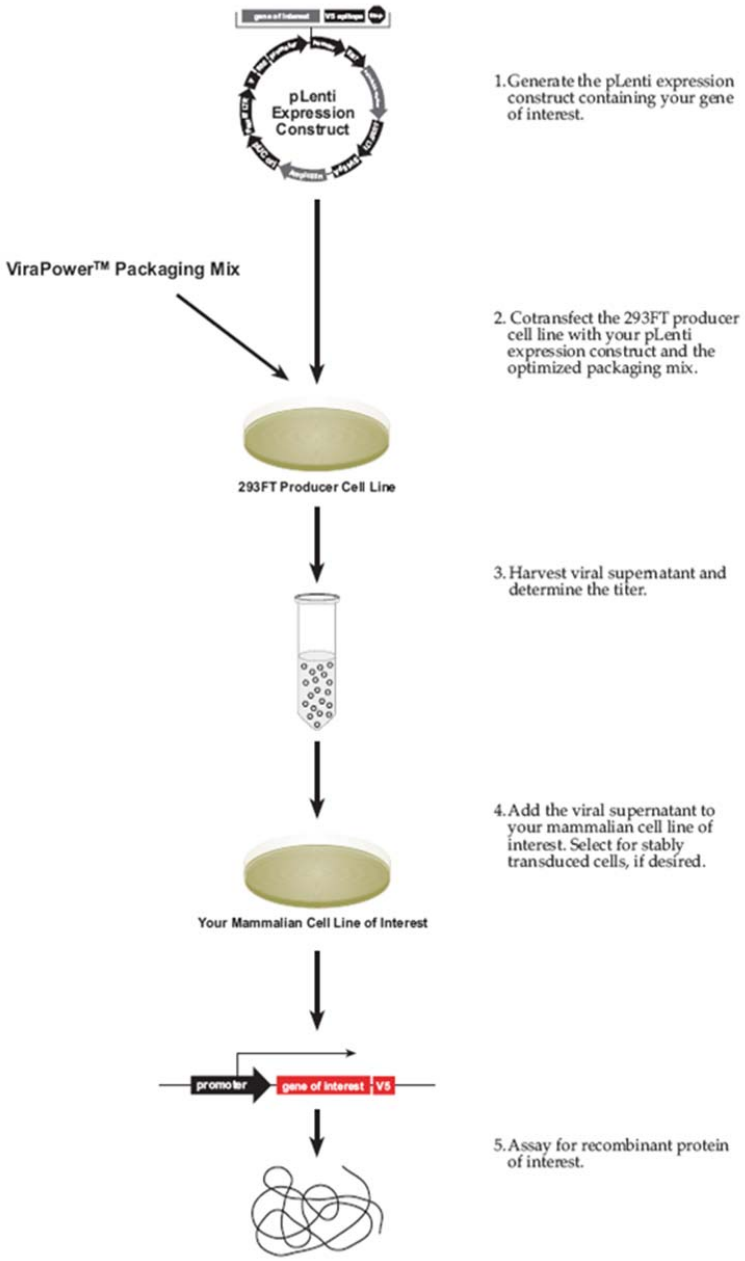

Figure 3. The diagram above describes the general steps required to express the gene of interest using the ViraPower Lentiviral Expression System of Invitrogen. This system was used to create the replication-incompetent HIV-1-based lentiviruses that were used in this thesis. (Source: Invitrogen Manual)

1.2.5 The components of the lentivector production system

1.2.5.1 Transfer Vector

The vector itself is the only genetic material transferred to the target cells. Transfer vector genomes contain all the *cis*-active sequences needed for packaging (ψ), reverse transcription (primer binding site, LTRs), integration (attL and attR integration sites) and transcription (5'-LTR with internal heterologous promoter), as well as the transgene of interest.

The U3 region of the 5'-LTR, which drives the expression of the primary transcripts in producer cells, has been replaced by other heterologous promoting sequences in order to drive a *tat*-independent primary transcription. Specifically, a constitutive promoter (RSV promoter) has been placed upstream of the 5′ LTR in the pLenti expression vector to offset the requirement for tat in the efficient production of viral RNA (Dull, Zufferey et al. 1998).

In addition, the U3 region of the 3'-LTR has also been deleted. The U3 region contains the viral enhancer and promoter, and the 3' copy is the template used to generate both LTR's of the integrated provirus. Thus, transduction of vector deleted in the 3' U3 results in the transcriptional inactivation of both LTR's. Vectors that carry an almost complete deletion in the U3 region of the HIV 3' LTR are called self-inactivating vectors (Miyoshi, Blomer et al. 1998; Zufferey, Dull et al. 1998). Self-inactivating (known as SIN) lentivectors can productively infect and integrate into target cell populations, but generation of proviral transcripts is blocked. Hence, SIN vectors are a sensible precaution. A self-inactivating vector diminishes the concern for oncogenesis by promoter insertion, and it alleviates significantly the risk of vector mobilization and recombination with the wild-type virus, minimizing thus the risk of emergence of replication competent recombinants (RCR). Furthermore, the use of SIN vectors might also avoid gene silencing, because it was postulated that active viral promoter sequence might attract the host silencing machinery to the integrated provirus (Pfeifer 2004).

A special feature of lentiviral vectors is the incorporation of a Rev-responsive element (RRE) that enhances the production of unspliced vector RNA in the packaging cells.

Thus, even complex transgenes containing introns and splicing signals can be incorporated into lentiviral vectors (Dull, Zufferey et al. 1998).

The pLenti6/V5-DEST vector (Figure 4) of Invitrogen is the destination vector used in this thesis for high-level expression of the genes of interest in MSCs using Invitrogen's ViraPower Lentiviral Expression System.

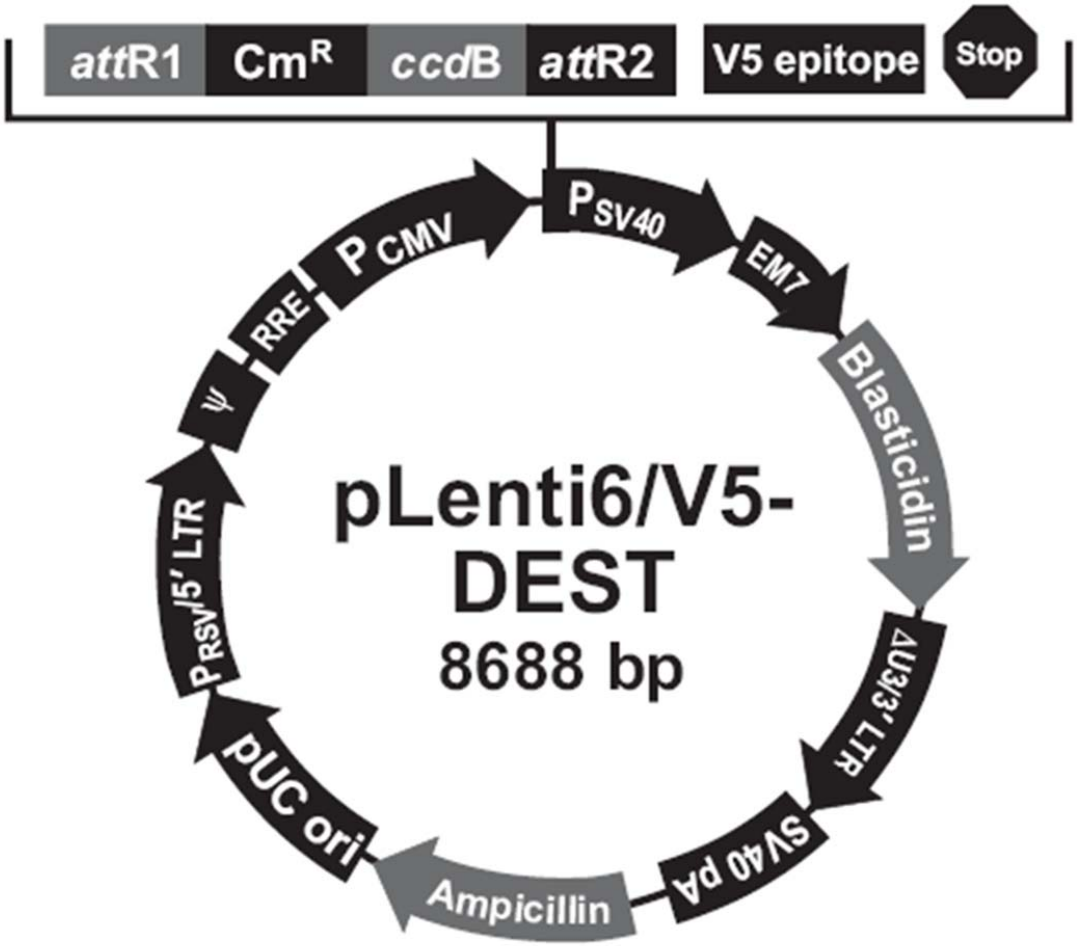

Figure 4. The map above shows the elements of the pLenti6/V5-DEST vector. The Rous Sarcoma Virus (RSV) enhancer/promoter allows Tat-independent production of viral mRNA. The HIV-1 truncated 5' LTR permits viral packaging and reverse transcription of the viral mRNA. The 5' splice donor and 3' acceptors enhance the biosafety of the vector by facilitating removal of the ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev dependent. The HIV-1 psi (ψ) packaging signal allows viral packaging. The HIV-1 Rev response element (RRE) permits Rev-dependent nuclear export of unspliced viral mRNA. The CMV promoter

permits high-level, constitutive expression of the gene of interest. The attR1 and attR2 sites are bacteriophage-derived DNA recombination sequences that permit recombinational cloning of the gene of interest from a Gateway entry clone. The Chloramphenicol resistance gene (CmR) allows counterscreening of the plasmid. The ccdB gene permits negative selection of the plasmid. The V5 epitope allows detection of the recombinant fusion protein by Anti-V5 antibodies. The SV40 early promoter and origin allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen. The EM7 promoter is a synthetic prokaryotic promoter for expression of the selection marker in E. coli. The blasticidin resistance gene permits selection of stably transduced mammalian cell lines. The ΔU3/HIV-1 truncated 3′ LTR allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes. The element also contains a polyadenylation signal for transcription termination and polyadenylation of mRNA in transduced cells. The SV40 polyadenylation signal allows transcription termination and polyadenylation of mRNA. The bla promoter allows expression of the ampicillin resistance gene. The ampicillin resistance gene (β-lactamase) allows selection of the plasmid in E. coli. Finally, the pUC origin permits high-copy replication and maintenance in E. coli. (Source: Invitrogen Manual).

1.2.5.2 The lentiviral packaging system

In order to express viral proteins for *trans*-complementation of a replication-defective vector, cells are transiently cotransfected with the vector DNA construct and plasmids expressing vector and protein-coding constructs (but not containing *cis*-acting sequences necessary for propagation), also termed as "packaging plasmids".

The latest ("third") generation packaging system comprises only three of the nine genes of HIV-1: *gag*, coding for the virion main structural proteins; *pol*, responsible for the retrovirus-specific enzymes; and *rev*, which encodes a post-transcriptional regulator necessary for efficient *gag* and *pol* expression (Dull, Zufferey et al. 1998). The parental virus cannot be reconstituted from such an extensively deleted packaging system since some 60% of its genome has been completely eliminated (Zufferey, Nagy et al. 1997; Trono 1998).

Finally, in lentiviral packaging constructs, the lentivirus' native envelope is typically replaced with a helper plasmid expressing heterologous envelope glycoproteins. This process, termed pseudotyping, can greatly modify the cell and host range tropism of the

vector (Sanders 2002). The vesicular stomatitis virus glycoprotein (VSV-G) has been extensively used to this end (McClure, Marsh et al. 1988; Akkina, Walton et al. 1996). The tactic of pseudotyping has been widely adopted and has the additional benefits of producing a particle that was much more stable than the native virus (Burns, Friedmann et al. 1993; Hopkins 1993). These vectors are capable of ultracentrifugal concentration and survive freezing and thawing. Pseudotyping with VSV has also broadened the tropism of the vectors since VSV-G appears to be pantropic. An obvious additional benefit is in reducing the amount of essential lentiviral genetic sequence, thus enhancing safety by reducing the risk of recombinational reconstruction of a wild-type lentivirus (Lever, Strappe et al. 2004).

The ViraPower Packaging Mix of Invitrogen is the packaging system used in this thesis for the creation of lentiviruses. It contains an optimized mixture of the three packaging plasmids, pLP1 (Figure 5), pLP2 (Figure 6), and pLP/VSVG (Figure 7). These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus.

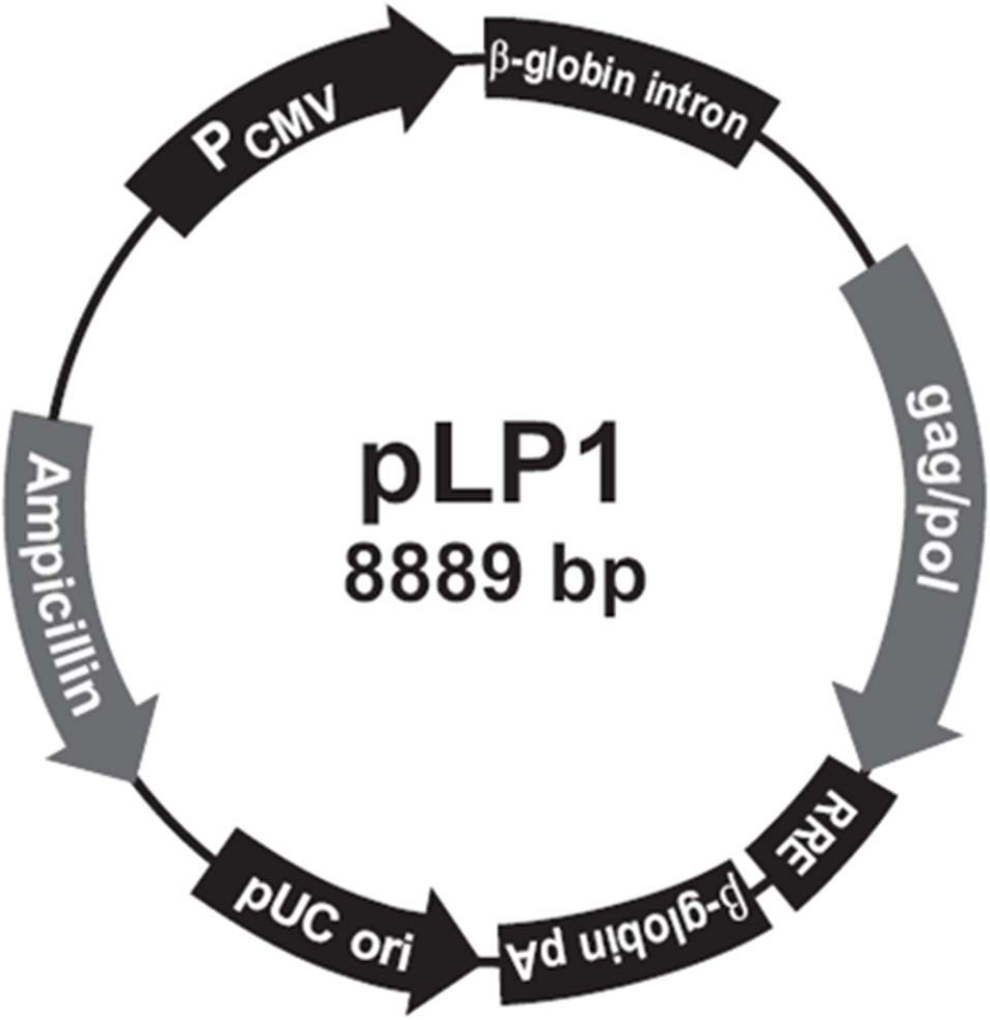

Figure 5. The figure above shows the features of the pLP1 vector. Note that the gag and pol genes are initially expressed as a gag/pol fusion protein, which is then self-cleaved by the viral protease into individual Gag and Pol polyproteins. The human cytomegalovirus (CMV)promoter permits high-level expression of the HIV-1 gag and pol genes in mammalian cells. The human β-globin intron enhances expression of the gag and pol genes in mammalian cells. The HIV-1 gag coding sequence encodes the viral core proteins required for forming the structure of the lentivirus. The HIV-1 pol coding sequence encodes the viral replication enzymes required for replication and integration of the lentivirus. The HIV-1 Rev response element (RRE) permits Rev-dependent expression of the gag and pol genes. The Human β-globin polyadenylation signal Allows efficient transcription termination and polyadenylation of mRNA. The pUC origin of replication (ori) permits high-copy replication and maintenance in E. coli. Finally, the ampicillin resistance gene allows selection of the plasmid in E. coli. (Source: Invitrogen Manual).

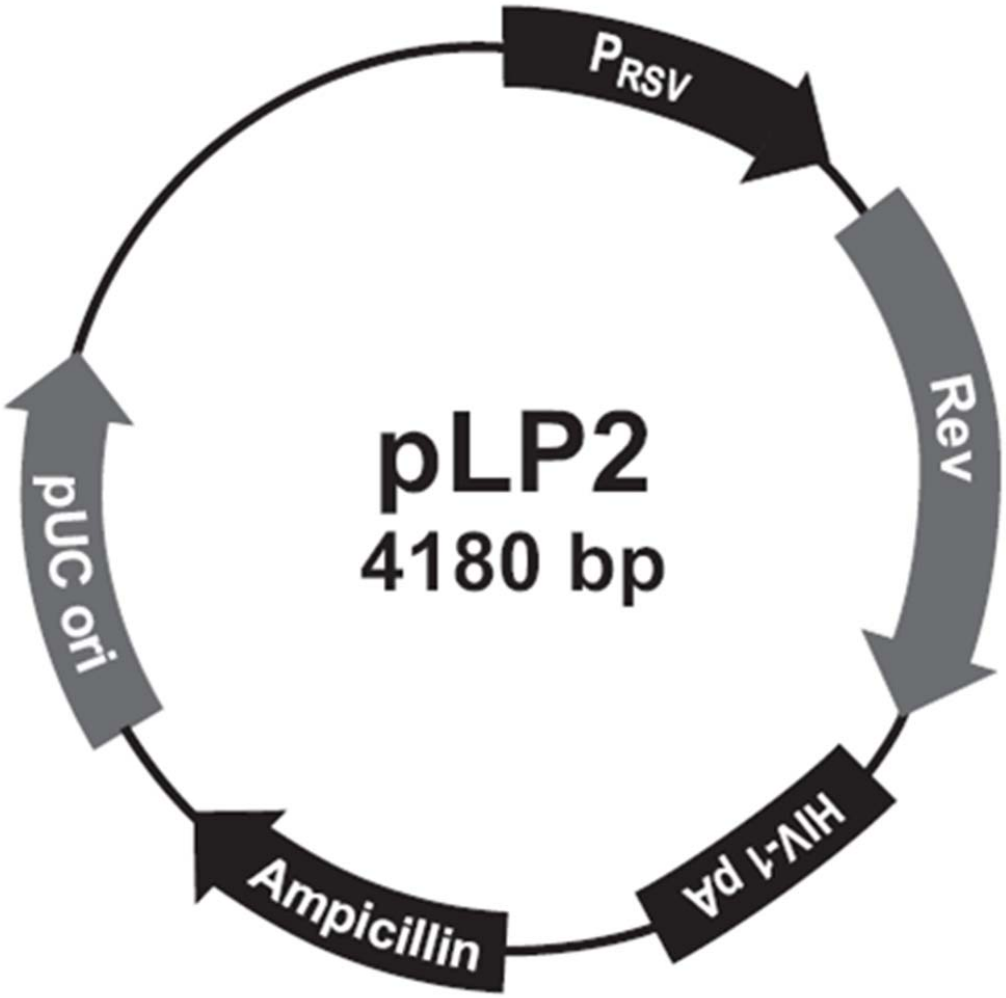

Figure 6. The figure above shows the features of the pLP2 vector. The RSV enhancer/promoter permits high-level expression of the rev gene. The HIV-1 Rev ORF encodes the Rev protein that interacts with the RRE on pLP1 to induce Gag and Pol expression and on the pLenti6/V5 expression vector to promote the nuclear export of the unspliced viral RNA for packaging into viral particles. The HIV-1 LTR polyadenylation signal allows efficient transcription termination and polyadenylation of mRNA. The ampicillin resistance gene allows selection of the plasmid in E. coli. The pUC origin of replication (ori) permits high-copy replication and maintenance in E. coli. (Source: Invitrogen Manual).

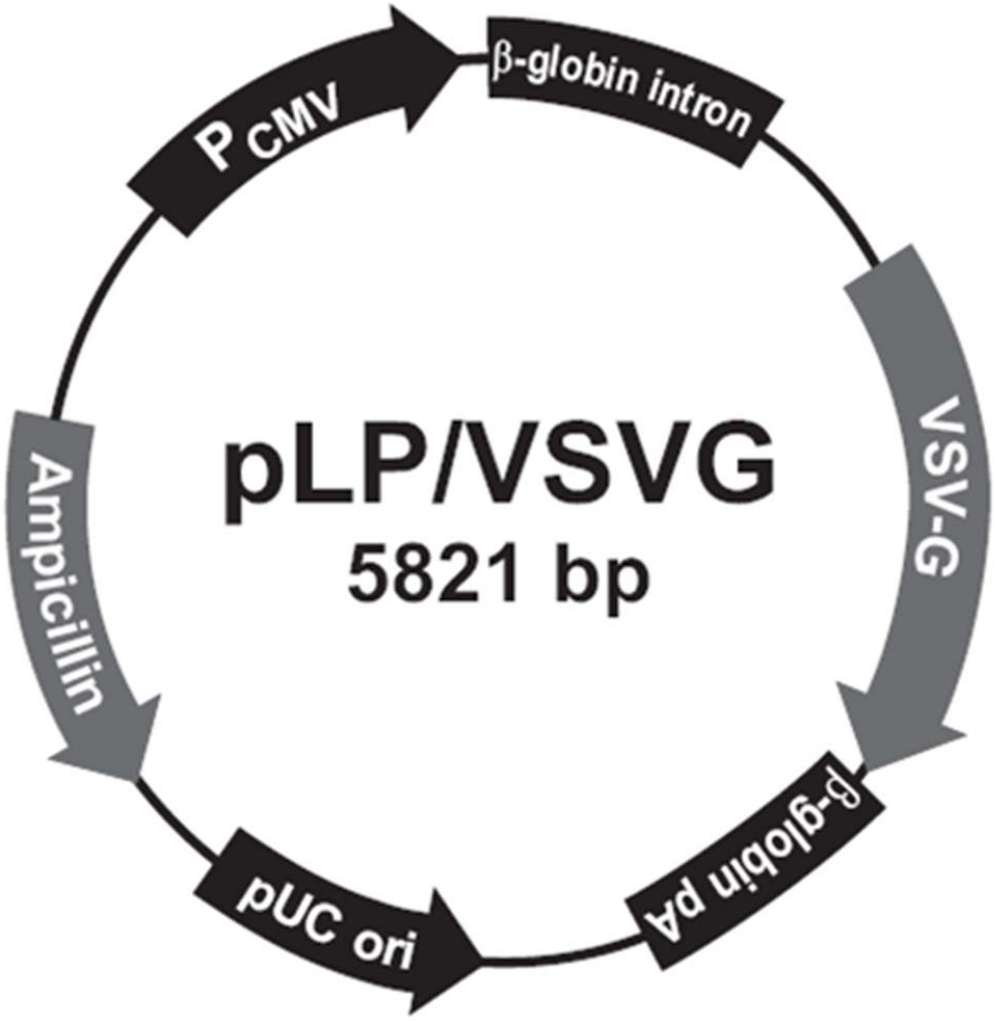

Figure 7. The figure above shows the features of the pLP/VSVG vector. The human CMV promoter Permits high-level expression of the VSV-G gene in mammalian cells. The human β-globin intron enhances expression of the VSV-G gene in mammalian cells. The VSV G glycoprotein (VSV-G) encodes the envelope G glycoprotein from Vesicular Stomatitis Virus to allow production of a pseudotyped retrovirus with a broad host range. The human β-globin polyadenylation signal allows efficient transcription termination and polyadenylation of mRNA. The pUC origin of replication (ori) permits high-copy replication and maintenance in E. coli. Finally, the ampicillin resistance gene allows selection of the plasmid in E. coli. (Source: Invitrogen Manual).

1.2.5.3 Packaging cells

The vast majority of the lentiviral vectors are currently made by transient transfection of the vector and packaging plasmids into human embryonic kidney (HEK 293FT) cells (Pfeifer 2004). Studies have demonstrated maximal virus production in human 293 cells expressing SV40 large T antigen, making the 293FT Cell Line a particularly suitable host for generating lentiviral constructs (Naldini, Blomer et al. 1996).

1.2.6 Transgene delivery into mesenchymal stem cells using lentivirus-based vectors

Recent results from several labs have indicated that HIV-1-based vectors are very efficient at delivering and expressing transgenes into MSCs (Totsugawa, Kobayashi et al. 2002; Zhang, La Russa et al. 2002; Davis, Humeau et al. 2004; Lee, Kohn et al. 2004; Zhang, La Russa et al. 2004). A single round of transduction using unconcentrated HIV-1-based lentiviral vectors can lead to the efficient transduction of human MSCs and sustained transgene expression for up to at least 5 months (Zhang, La Russa et al. 2002). An advantage of lentiviral vectors over vectors based on oncogenic retroviruses is that they are capable of transducing non-dividing cells (Reiser, Harmison et al. 1996; Mochizuki, Schwartz et al. 1998). This is important given the fact that a relatively large subset (20%) of MSCs has been described to be quiescent (Conget and Minguell 1999). Studies have shown that lentivirus-transduced MSCs retain their *in vitro* ability to differentiate into adipocytes, osteocytes and chondrocytes as well as into myocyte- and astrocyte-like cells, suggesting that HIV-1-derived lentiviral vectors can efficiently transduce MSCs without inhibiting their differentiation potential (Zhang, La Russa et al. 2002; Anjos-Afonso, Siapati et al. 2004).

All in all, lentiviral vectors are proving to be versatile and effective agents for the delivery and sustained expression of a transgene into MSCs, so far without detectable immunological or pathological consequences attributed to the vector.

1.3 The Nuclear Factor-κB

1.3.1 Introduction

NF-κB is a transcription factor (DNA-binding protein) that plays a key role in a wide variety of cellular processes such as innate and adaptive immunity, cellular proliferation, apoptosis and development. It stands out as an exceptionally important factor due to its pleiotropic effects, the inducible and expression patterns, its unique regulatory mechanisms, large number of activating signaling pathways and number of genes that it controls and is therefore a topic of intense investigation in numerous laboratories. NF-κB was first identified as a factor regulating the expression of κ light chains in mouse B lymphocytes in 1986 (Sen and Baltimore 1986), but has subsequently been identified in most cell types and is found in species as far back as insects.

1.3.2 NF-κB proteins

NF-κB is not a single protein but a family of dimeric transcription factors composed of members of the Rel family with five closely related DNA binding proteins: RelA (p65), RelB, c-Rel, NF-κB1/p50 and NF-κB2/p52 (Figure 8). All five NF-kB members share a highly conserved 300-amino-acid-long N-terminal Rel homology domain (RHD). The RHD contains a nuclear localization sequence (NLS) that determines dimerization, nuclear localization and binding to kB elements in the promoter and enhancer regions of target genes. NF-κB/Rel proteins can exist as homo- or heterodimers. By far the commonest member of the NF-κB family is p50:p65 heterodimer that is often used synonymously for NF-κB, but various combinations are possible.

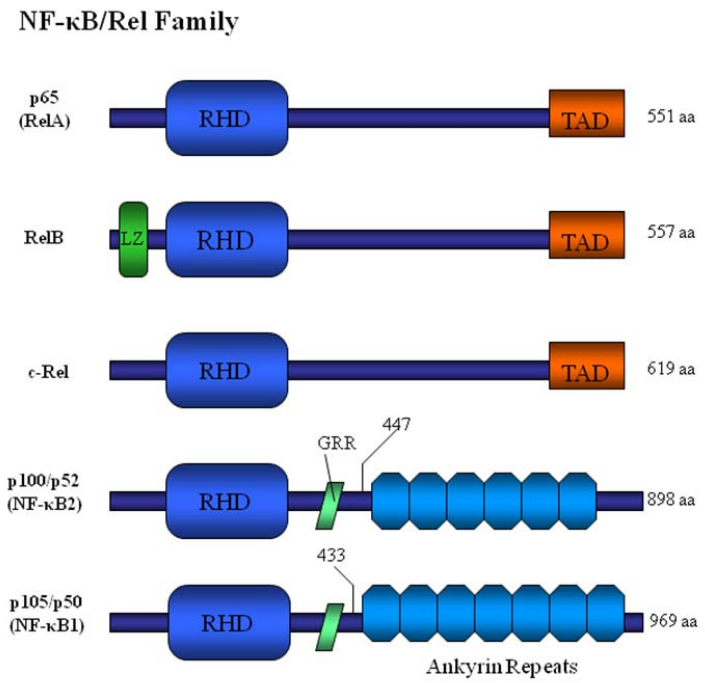

Figure 8. Schematic representation of the NF-κB family of proteins. The number of amino acids in each protein is indicated on the right. There are five nuclear factor (NF)-κB family members, RelA (p65), RelB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). p50 and p52 are derived from the longer precursor proteins p105 and p100, respectively. Presumed sites of cleavage for p100 (amino acid 447) and p105 (amino acid 433) are shown. Phosphorylation and ubiquitination sites on p100 and p105 proteins are indicated. All NF-κB family members contain an N-terminal Rel-homology domain (RHD), that mediates DNA binding and dimerization and contains the nuclear-localization domain. The Rel subfamily, RelA, RelB and c-Rel, contain unrelated C-terminal transcriptional activation domains (TADs). TA1 and TA2 are subdomains of the RelA transactivation domain. (RHD) Rel homology domain; (TAD) transactivation domain; (LZ) leucine zipper domain; (GRR) glycine-rich region.

NF-κB is normally held in the cytoplasm in an inactive form bound to an inhibitory protein, IκB, of which several types are recognized. The IκB family contains seven known mammalian members, IκBα, IκBβ, IκBγ, IκBε, Bcl-3 and the precursor Rel proteins p100 and p105 (Figure 9) (Ghosh, May et al. 1998; Jacobs and Harrison 1998;

Cramer and Muller 1999; Croy, Bergqvist et al. 2004). IκB molecules are characterized by the presence of multiple ankyrin repeats, which are protein-protein interaction domains that interact with NF-κB via the RHD (Cramer, Larson et al. 1997; Chen, Huang et al. 1998; Malek, Huang et al. 2003). The IκB proteins bind to the RHD region of NF-κB protein and interfere with the NLS function to prevent NF-κB translocation into nucleus, thereby maintaining NF-κB in an inactive state (Jacobs and Harrison 1998; Johnson, Van Antwerp et al. 1999; Prigent, Barlat et al. 2000; Croy, Bergqvist et al. 2004).

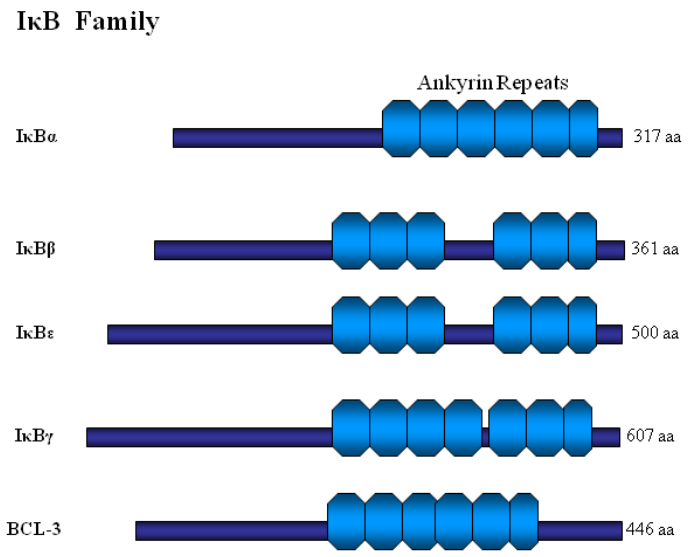

Figure 9. Schematic representation of the inhibitor of NF-κB (IκB) family. The number of amino acids in each protein is indicated on the right. The IκB family consists of IκBα, IκBβ, IκBε, IκBγ and BCL-3. Like p105 and p100, the IκB proteins contain ankyrin-repeat motifs in their C termini.

1.3.3 Overview of the NF-κB pathway

There are several distinct NF-κB-activation pathways. The most frequently observed is the canonical, or classical, pathway , which is induced in response to a large variety of stimuli including the pro-inflammatory cytokines tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1), mitogens, microbial components and DNA damage (Figure 10) (Pahl 1999). These stimuli lead to activation of a specific IκB kinase (IKK), a multiprotein complex with a high molecular weight of approximately 700–900 kDa. Once activated, IKK phosphorylates specific serines within the IκB proteins, triggering their ubiquitination by an ubiquitin ligase complex containing the β-transducin repeat-containing protein (β-TrCP) (Karin and Ben-Neriah 2000).The IκB is then degraded by the 26S proteasome, thus allowing the release, modification and translocation of NF-κB dimers into the nucleus to induce gene expression. The resulting free NF-κB interacts with a specific DNA motif (5′-GGGPuNNPyPyCC-3′, where Pu is purine, Py is pyrimidine and N is any nucleotide) and activates or represses a large collection of target genes. In the alternative pathway (or non-canonical), IKKα, one catalytic subunit of IKK is activated and phosphorylates p100, leading to its processing by the proteasome (Bonizzi and Karin 2004). Thus, IKK is required for activation of both canonical and non-canonical NF-κB signaling pathways.

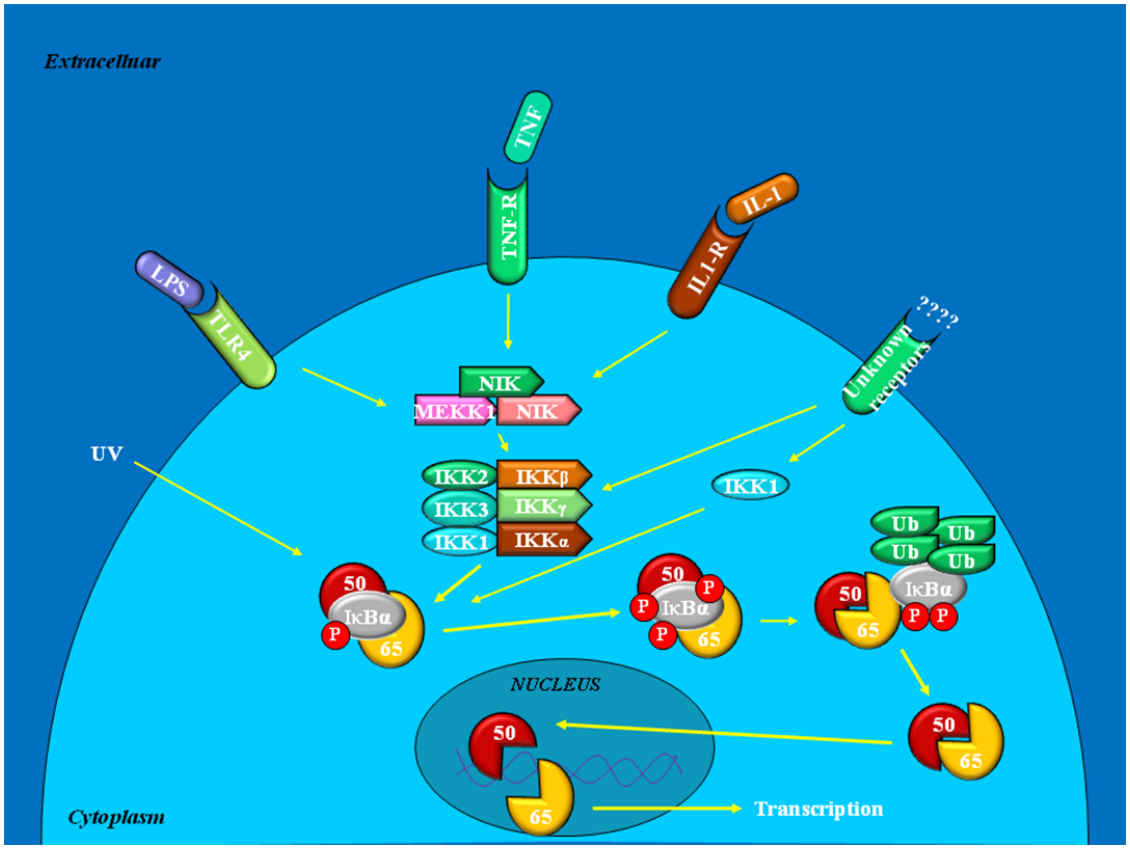

Figure 10. Overview of the canonical NF-κB pathway. In its inactive form, NF-κB is sequestered in the cytoplasm, bound by members of the IκB family of inhibitor proteins. The various stimuli that activate NF-κB cause phosphorylation of IκB, which is followed by its ubiquitination and subsequent degradation. This results in the exposure of the nuclear localization signals (NLS) on NF-κB subunits and the subsequent translocation of the molecule to the nucleus. In the nucleus, NF-κB binds with a consensus sequence of various genes and thus activates their transcription. Tumor necrosis factor (TNF) is the best-studied activator of NF-κB.

1.3.4 Structure and function of the IKK complex

As described above, the common feature of all pathways leading to the activation of NF-κB is the activation of one of the IκB kinases. Consequently, this is the most important regulatory step in determining the NF-κB response to a given stimulus.

The IKK complex consists of three core subunits, the catalytic subunits IKKα and IKKβ (also known as IKK1 and IKK2) and several copies of a regulatory subunit called the NF-κB essential modifier (NEMO, also known as IKKγ) (Figure 11) (Karin and Ben-Neriah 2000). IKKα and IKKβ are serine/threonine kinases that are characterized by the presence of an N-terminal kinase domain, a C-terminal helix–loop–helix (HLH) domain, and a leucine zipper domain. They are capable of phosphorylating multiple members of the IκB family at multiple sites. NEMO is a 48-kDa protein that is not related to IKKα and IKKβ and contains a C-terminal zinc finger-like domain, a leucine zipper, and N-terminal and C-terminal coiled-coil domains (Rothwarf and Karin 1999).

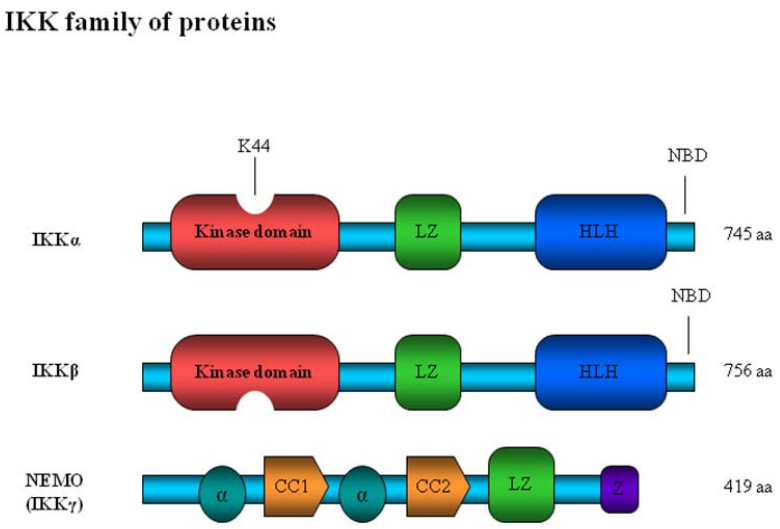

Figure 11. Schematic representation of the IKK family of proteins. The number of amino acids in each protein is indicated on the right. The three core subunits of the IκB kinase (IKK) complex are shown: the catalytic subunits IKKα (also known as IKK1) and IKKβ (also known as IKK2) and the regulatory subunit called the NF-κB essential modifier (NEMO, also known as IKKγ). The principal structural motifs of each protein are shown, together with amino-acid numbers corresponding to the human proteins. (TAD) transactivation domain; (LZ) leucine zipper domain on IKKα/β; (GRR)

glycine-rich region; (HLH) helix–loop–helix domain; (Z) zinc finger domain; (CC1/2)coiled-coil domains; (NBD) NEMO-binding domain; (α) α -helical domain.

IKKα and IKKβ share 52% overall sequence identity and 65% identity in their catalytic domains (Mercurio, Zhu et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al. 1997). They can form homo- and heterodimers via their leucine zipper motifs. Once IKKα and IKKβ dimerize, they can associate with IKKγ through a short interaction motif located at the very C terminus of either catalytic subunit (May, D'Acquisto et al. 2000; Hu, Baud et al. 2001). The association of IKKα and IKKβ dimers (usually IKKα and IKKβ heterodimers) with IKKγ results in formation of a large complex, composed of IKKα:IKKβ heterodimers held together via dimeric interactions between two IKKγ molecules (Miller and Zandi 2001). Activation of this large IKK holocomplex by all proinflammatory and innate immune stimuli depends on phosphorylation of either the IKKα or IKKβ catalytic subunits at two conserved serines located within their activation loops (Ling, Cao et al. 1998; Delhase, Hayakawa et al. 1999).
Although both IKKα and IKKβ are capable of phosphorylating IκBα at Ser 32 and Ser 36, and IκBβ at Ser 19 and Ser 23, IKKα is less efficient and cannot replace IKKβ in knockout studies (DiDonato, Hayakawa et al. 1997; Mercurio, Zhu et al. 1997; Regnier, Song et al. 1997). Genetic experiments have shown that IKKβ is the predominant IκB kinase in the canonical pathway (Bonizzi and Karin 2004; Pasparakis, Luedde et al. 2006).

1.3.5 TNF-α: a model NF-κB stimulus

Among the major signaling pathways targeting IKK is the one used by a major pro-inflammatory cytokine, known as tumor necrosis factor (TNF-α). Its anticancer activity was first described more than a century ago. However, it wasn't until 1984 that human TNF-α was purified and its encoding cDNA was cloned and expressed. The subsequent availability of recombinant TNF led to a rapid cataloging of TNF-α's pleiotropic

activities. In addition to triggering apoptosis of certain tumor cells, TNF mediates the inflammatory response and regulates immune function.

TNF-α is a homotrimer of 157 amino acid subunits primarily produced by activated macrophages. TNF signals through two distinct cell surface receptors, TNF-R1 and TNF-R2. Multiple experimental approaches have revealed that TNF-R1 initiates the majority of TNF-α's biological activities. The binding of TNF-α to TNF-R1 triggers a series of intracellular events that ultimately result in the activation of NF-κB.

The initial step in TNF-α signaling involves the binding of the TNF-α trimer to the extracellular domain of TNF-R1 and the release of the inhibitory protein silencer of death domains (SODD) from TNF-R1's intracellular domain (ICD) (Figure 12). The resulting aggregated TNF-R1 ICD is recognized by the adaptor protein TNF-α receptor–associated death domain (TRADD), which recruits additional adaptor proteins receptor-interacting protein (known as RIP), TNF-R–associated factor 2 (TRAF2), and Fas-associated death domain (FADD). Each of them plays a specific role in IKK activation, RIP being able to directly interact with NEMO and to recruit IKK, whereas TRAF2 activates the TAK1 complex that in turn induces IKKα/IKKβ phosphorylation (Hsu, Shu et al. 1996; Devin, Cook et al. 2000; Zhang, Kovalenko et al. 2000).

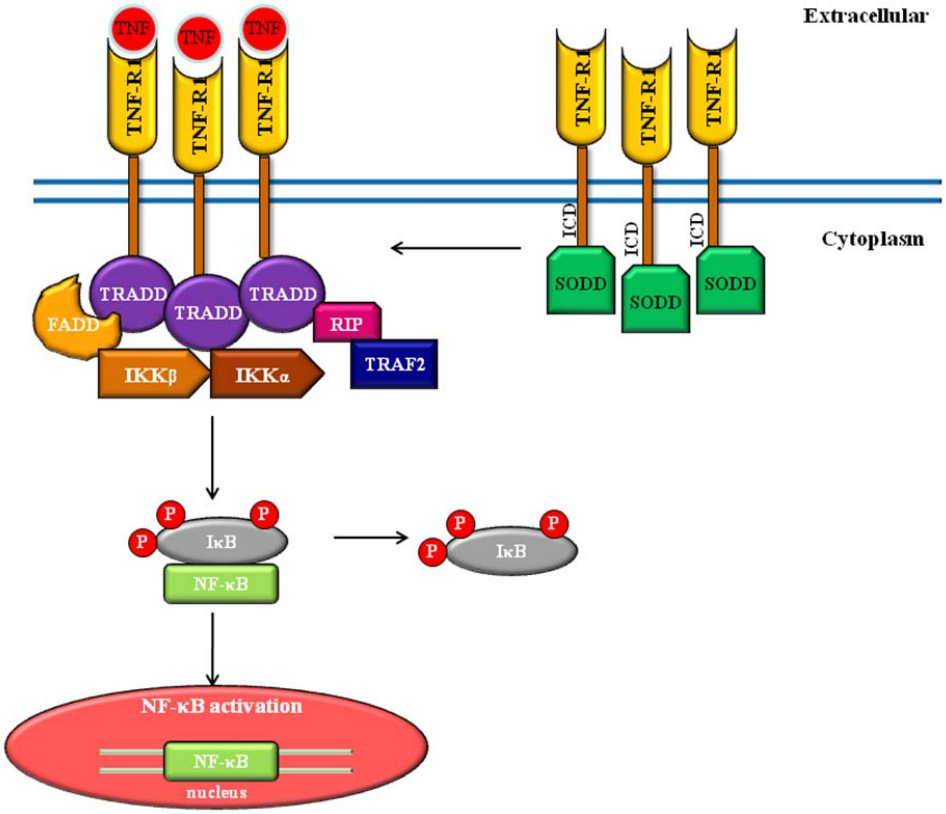

Figure 12. TNF signal transduction pathway. Engagement of TNF with its cognate receptor TNF-R1 results in the release of SODD and formation of a receptor-proximal complex containing the important adaptor proteins TRADD, TRAF2, RIP, and FADD. These adaptor proteins in turn recruit additional key pathway-specific enzymes (for example IKKβ) to the TNF-R1 complex, where they become activated and initiate downstream events leading to NF-κB activation.

Several studies have established that IKKβ is essential for NF-κB activation by TNF-α, while IKKα plays only a minor role in TNF-α-induced activation of NF-κB (Karin and Ben-Neriah 2000; Chen, Chou et al. 2001; Baxter, Came et al. 2006).

1.3.6 Inhibition of NF-κB activation using dnIKKβ

Since phosphorylation of IκB proteins by the IKK complex is a key step in NF-κB activation, IKK has been a prime target for the development of NF-κB signaling

inhibitors. IKK activation can be efficiently blocked by gene-based inhibitors. Specifically, a dominant-negative form of IKKβ (dnIKKβ or dnIKK2), which is capable of blocking activation of NF-κB, has been created through substitution of a single lysine (K) codon present at position 44 with an alanine (A) codon within the predicted ATP-binding site (Figure 13) (Mercurio, Zhu et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al. 1997). The resulting mutant is defective in binding ATP and therefore catalytically inactive. Since IKKβ (IKK2) is the predominant IκB kinase in the canonical pathway as already described above, stable overexpression of dnIKKβ can selectively block activation of NF-κB induced by TNF-α.

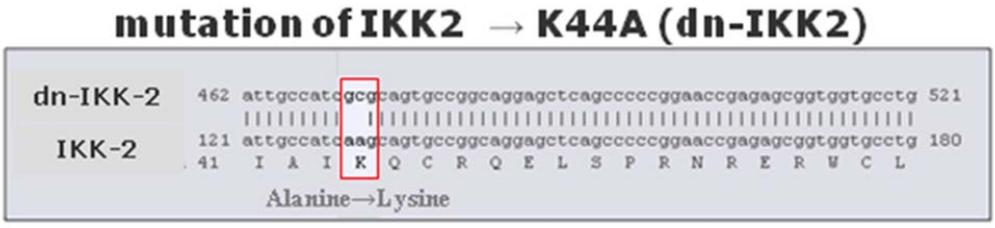

Figure 13. Sequence of the wild-type IKK-2 gene, compared to the sequence of the highly specific and effective dominant negative IKK-2 mutant, bearing a replacement of Lysine in position 44 with Alanine (K44A).

DnIKKβ and dnIKK2 are both valid terms and describe essentially the same protein, but to avoid confusion we will use the term dnIKK2 throughout the rest of this thesis. The cDNA of the dominant-negative mutant (K44A) of IKK2 (dnIKK2) used in this thesis was generously provided by Tularik Inc. (San Francisco, USA).

1.3.7 The biological function of NF-κB

Activated NF-κB controls the expression of many genes, including those encoding inflammatory cytokines, chemokines, immune receptors, and cell surface adhesion molecules and is required for many fundamental cellular activities (Karin and Ben-Neriah

2000; Ghosh and Karin 2002). NF-κB proteins play an important role in innate immunity (Senftleben, Li et al. 2001), stress responses (Pahl 1999), cell proliferation (Rayet and Gelinas 1999), survival (Wu, Lee et al. 1996; Sonenshein 1997), developement and differentiation (Franzoso, Carlson et al. 1997; Gerondakis, Grossmann et al. 1999; Silverman and Maniatis 2001). Moreover, there is growing evidence implicating roles for NF-κB in inflammatory diseases and human cancer (Karin, Lawrence et al. 2006).

The exact role of the NF-κB transcription factor in MSCs is largely unknown though. Further biochemical and genetic studies are required to identify its biological function in this field. The further understanding of the NF-κB-dependent processes in MSCs might also provide an opportunity for the development of treatments for controlling inflammation and cancers.

2 Aims of the thesis

The main focus of this thesis was to investigate the role of NF-κB in TNF-α-mediated migration and proliferation of human MSCs *in vitro*. The project consisted of the following parts:

1. Cloning the dnIKK2 gene that inhibits TNF-α-mediated NF-κB activation into an appropriate entry vector.
2. Cloning a gene (EGFP) that encodes a green fluorescent protein into an appropriate entry vector, that will serve as a control in order to demonstrate an effective lentiviral transduction of cells and to investigate whether the transduction by itself has any effects on hMSCs.
3. Creating two different types of lentiviruses that contain each one of these vectors.
4. Tranduction of hMSC cells with the created lentiviruses.
5. Documenting the overexpression of dnIKK2 at RNA and protein level in the cells transduced with the dnIKK2 lentivector.
6. Documenting the effective inhibition of function of NF-κB in the cells transduced with the dnIKK2 lentivector through immunohistochemistry.
7. Observing and comparing the migration and proliferation potential of hMSCs transduced with dnIKK2 and those transduced with EGFP after stimulation with TNF-α, using invasion and proliferation assays in vitro.
8. Searching for differences in expression of downstream targets of NF-κB in cells transduced with dnIKK2 and EGFP with the help of RT-PCR, which could potentially explain the effect of dnIKK2 on the migration and proliferation of hMSCs.

3 Materials

3.1 Laboratory Equipment

- 80°C freezer, type 6485	GFL, Burgwedel
Analytical balance, type 770	Kern, Balingen
Analytical balance, type A 120 S	Sartotius, Göttingen
Autoclave, type Varioklav 300	H+P Labortechnik, Oberschleißheim
Cell culture centrifuge, type Universal 16R	Hettich-Zentrifugen, Tuttlingen
Cell culture incubator, type 1G150	Jouan, Unterhaching
Cell culture microscope, type Diavert	Leitz, Wetzlar
Cooling centrifuge, type BR4 with fixed angle rotor AB 2.14	Jouan, Unterhaching
Cooling centrifuge, type Megafuge 1.0R	Heraeus, Hanau
Electrophoresis chambers, horizontal type	Owl Scientific, MA, USA
Electrophoresis chambers, vertical type	Bio-Rad, Mannheim
Electrophoresis power supply, type E 802	Consort, Turnhout, Belgium
Electrophoresis power supply, type EV 232	Consort, Turnhout, Belgium
ELISA reader, type MRX	Dynex, Denkendorf
Flow cytometer, type FACSCalibur	BD Biosciences, San Jose, CA, USA
Gel photo imager	Pharmacia, Freiburg
Incubator, type BE 40	Memmert, Schwabach
Magnetic stirrer, type RCT	IKA, Staufen
Microscope, type Axiovert S100	Carl Zeiss, Berlin
Microwave oven, type HZ 86 B 000	Siemens, Munich
Multilabel microtiter plate reader	CBM, Copenhagen, DN
Neubauer cell counting chamber	Paul Marienfeld, Lauda-Königshofen
Nitrogen tank, type Locator JR	Linde, Wiesbaden

PCR Thermal Cycler, type PTC-200	MJ Research, MA, USA
pH-meter, type inoLab Level 1	WTW, Weilheim
Pipette, type multichannel	Eppendorf, Hamburg
Pipette, type Pipetus-akku	Hirschmann Laborgeräte, Eberstadt
Pipettes 10, 20, 200, 1000 µl	Gilson, WI, USA
Schott Duran glassware	Schott Duran, Mainz
Shaker Rotamax 120	Heidolph Instruments, Schwabach
Shaking incubator	Infors, Bottmingen, CH
Sonicator, type Branson sonifier	Branson Ultrasonics, Danbury, CT, USA
Spectrophotometer, type Biophotometer	Eppendorf, Hamburg
Sterile hood, type HeraSafe	Heraeus Instruments, Hanau
Tabletop centrifuge, type 5415D	Eppendorf, Hamburg
Tabletop centrifuge, type Biofuge Pico	Heraeus, Hanau
Tabletop centrifuge, type GMC-360	BMG Labtech, Offenburg
Thermo block, type HLC BT 130-2	Scientific Plastics, KS, USA
UV transilluminator, 302 nm	Vilber Lourmat, Marne-la-Vallee, France
Vacuum concentrator, type Jouan RC1010	Jouan, Unterhaching
Vacuum pump	IBS Integra Biosciences
Vortexer, type Genie-2	Scientific Industries, NY, USA
Vortexer, type REAX 1R	Heidolph, Schwabach
Water bath, type 1012	Gesellschaft für Labortechnik, Burgwedel
X-ray film cassette	Amersham, Little Chalfont, UK

3.2 Consumables

Cell culture flasks 25 cm^2, 75 cm^2, 225 cm^2,	Nunc, Wiesbaden
Cell culture plates 6-, 24-, 96-well	Nunc, Wiesbaden
Cell scraper	Sarstedt, Nümbrecht

Falcon tubes 15 ml, 50 ml	Sarstedt, Nümbrecht
Freezing vials, 1.8 ml	Nunc, Wiesbaden
Laboratory film, type Parafilm M	American Can Company, CO, USA
PCR tubes, 0.2 ml	Biozym, Oddendorf
Photometer cuvettes, type Uvette Original	Eppendorf, Hamburg
Pipette tips 10, 20, 200, 300 µl	Gilson, WI, USA
Pipette tips 1000µl	Sarstedt, Nümbrecht
Plastic syringes, type BD Discardit II	Becton Dickins, Basel, CH
Polycarbonate membrane 8 µm pore filters	Costar, Pleasanta, CA, USA
Polystyrene Petri dishes, d = 94 mm	Greiner, Solingen
PVDF-membrane	Roche, Oldenburg
Reaction tubes, 1.5 ml, 2 ml	Sarstedt, Nümbrecht
Scalpels, No. 11	Feather Safety Razor, Osaka, Japan
Serological pipettes 2, 5, 10, 25 ml	Sarstedt, Nümbrecht
Syringe Filters, type Millex GP 0.22, 0.45 µm	Millipore, Eschborn
Transwell invasion chambers, 24-well	Costar, Pleasanta, CA, USA

3.3 Chemicals

Chemicals used in this thesis were ordered at "A.C.S" or "p.A." purity grade. All standard solutions, buffers, and media were prepared according to Sambrook *et al*. (Sambrook J. 2001).

Media for cultivating bacteria were sterilized by autoclaving for 20 minutes at 15 psi (1.05 kg/cm^2), all other solutions were sterile filtered. Thermo labile components such as antibiotics were sterile filtered and added to the media after autoclaving and cooling to 50°C.

Acrylamide, 40 %	Appligene, Heidelberg

Agarose, Seakem LE	Cambrex, NJ, USA
Ammonium peroxydisulfate (APS)	Serva, Heidelberg
Ampicillin sodium salt	Sigma, Deisenhofen
AMV First-Strand cDNA Synthesis Kit	Invitrogen, Karlsruhe
Aqua ad iniectabilia	Braun, Melsungen
Bacto Agar	Becton Dickins, Basel, CH
Bacto-Tryptone	Becton Dickins, Basel, CH
Bacto-Yeast extract	Becton Dickins, Basel, CH
BCA protein assay Kit	Pierce Biotechnology, Rockford, IL, USA
BigDye terminator RR mix	Applied Biosystems, Weiterstadt
Blasticidin	Invitrogen, Karlsruhe
Bovine serum albumin (BSA)	Sigma, Deisenhofen
BrdU-Kit, colorimetric type	Roche, Mannheim
Bromophenol Blue	Sigma, Deisenhofen
Chloramphenicol	Sigma, Deisenhofen
Coomassie Brilliant Blue R-250	Pierce Biotechnology, Rockford, IL, USA
Crystal violet	Sigma, Deisenhofen
Diff Quik Stain Kit	Dade Diagnostika, Munich
Dimethyl sulfoxide (DMSO)	Merck, Darmstadt
Dithiothreitol (DTT)	Merck, Darmstadt
DNA Ladder 1 kb GeneRuler	Fermentas, St. Leon-Rot
DNA Ladder 100 bp	Invitrogen, Karlsruhe
DNA Ladders No. V, VII, VIII	Roche, Mannheim
dNTP Mix	Roche, Mannheim
ECL Western Blotting Kit	Amersham, Little Chalfont, UK
EDTA	Merck, Darmstadt
Ethanol, absolute	Merck, Darmstadt
Ethidium bromide	Merck, Darmstadt
Extracellular Matrix (ECM)	Becton Dickins, Basel, CH
Fetal Bovine Serum (FBS)	Invitrogen, Karlsruhe
Fetal Calf Serum (FCS)	Gibco, Karlsruhe

Gateway LR Clonase Enzyme Mix	Invitrogen, Karlsruhe
Gel Loading Buffer, type 6x loading Dye	Fermentas, St. Leon-Rot
Gel Loading Buffer, type blue juice 10x	Invitrogen, Karlsruhe
Geneticin	Invitrogen, Karlsruhe
Glycerol	Merck, Darmstadt
Glycine	Sigma, Deisenhofen
Glycine sodium salt	Sigma, Deisenhofen
Isopropanol	Merck, Darmstadt
Kanamycin sulfate	Sigma, Deisenhofen
L-Glutamine	Invitrogen, Karlsruhe
Lipofectamin 2000	Invitrogen, Karlsruhe
Medium, type Alpha-Mem	Gibco, Karlsruhe
Medium, type DMEM	Gibco, Karlsruhe
Medium, type MSCGM BulletKit	Cambrex, NJ, USA
Medium, type OptiMEM I	Gibco, Karlsruhe
Medium, type SOC	Invitrogen, Karlsruhe
Methanol	Merck, Darmstadt
Penicillin/Streptomycin solution	Gibco, Karlsruhe
Phosphate buffered saline (PBS)	PAA Laboratories, Linz, Austria
Protease Inhibitor tablet Complete, Mini	Roche, Mannheim
Protein Standard SeeBlue Plus2 Pre-stained	Invitrogen, Karlsruhe
QIAEX II Gel Extraction Kit	Qiagen, Hilden
Qiagen Plasmid Maxi Kit	Qiagen, Hilden
Qiagen Plasmid Midi Kit	Qiagen, Hilden
Qiagen Plasmid Mini Kit	Qiagen, Hilden
Qiagen RNeasy Mini Kit	Qiagen, Hilden
QIAquick Gel Extraction Kit	Qiagen, Hilden
Quick Ligation Kit	New England Biolabs, Frankfurt
Restriction endonuclease buffers 1,2,3,4	New England Biolabs
Restriction endonucleases type II	New England Biolabs
SDS Ultrapure	Roth, Karlsruhe

Sodium acetate	Sigma, Deisenhofen
Sodium chloride	Merck, Darmstadt
Sodium deoxycholate salt	Sigma, Deisenhofen
Sodium fluoride	Sigma, Deisenhofen
Sulfuric acid	Roth, Karlsruhe
TEMED	Sigma, Deisenhofen
TNF-α	Biomol, Hamburg
Tris-Base	Sigma, Deisenhofen
Tris-HCl	Sigma, Deisenhofen
Triton X-100	Sigma, Deisenhofen
Trypan Blue Stain	Invitrogen, Karlsruhe
Trypsin-EDTA	Gibco, Karlsruhe
Tween 20	Merck, Darmstadt
ViraPower Lentiviral Expression System	Invitrogen, Karlsruhe
β-Mercaptoethanol	Sigma, Deisenhofen

3.4 Stock solutions, media and frequently used buffers

Culture medium of E. coli strains

dyt (2x YT)	Per liter: 16 g Tryptone 10 g Yeast extract 5 g Sodium Chloride

DNA Gel electrophoresis

TAE (1x)	Tris acetate EDTA 40 mM Tris-acetate 1mM EDTA

Protein Extraction

RIPA-Buffer	RadioImmuno Precipitation Assay Buffer 0.1 % (w/v) SDS 1 % (w/v) Na-DOC sodium deoxycholate 1 % (w/v) Triton X-100 50 mM Tris-HCl (pH 8.0) 150 mM NaCl 10 mM EDTA (pH 8.0) 20 mM NaF ¼ tablet of Protease Inhibitor per 2.5 ml RIPA-Buffer

SDS-PAGE

Acrylamide stock solution 30 %	75 % (v/v) Acrylamide 40% in H_20
APS 10%	10 % (w/v) Ammonium persulfate in H_20
Laemmli Buffer	225 mM Tris-HCl pH 6.8 20 % (w/v) SDS 0.05 % (w/v) Bromphenol blue 50 % (v/v) Glycerol 250 mM Dithiothreitol (DTT)
SDS running electrophoresis buffer (10x)	250 mM Tris-Base pH 8.3

	1.92 M Glycine
	1 % (w/v) SDS
Tris Buffer (1x) for SDS Separating Buffer	1 M Tris-Base, pH 8.8
Tris Buffer (1x) for SDS Stacking Buffer	1 M Tris-Base, pH 6.8

Western Blot

Blotting buffer (10x)	250 mM Tris-Base pH 8.3
	1.92 M Glycine
Blotting buffer (1x)	10 % (v/v) 10x Blotting buffer
	20% (v/v) Methanol

Immunodetection

Blocking solution	5% skim milk powder in Wash Buffer
Tris HCl pH 7.4	1 M Tris-Base, pH 7.4
Wash buffer (TBS-Tween 20)	1% (v/v) 1 M Tris HCl pH 7.4
	150 mM NaCl
	0,05 % (v/v) Tween 20

Stripping

Stripping buffer	62.5 mM Tris-HCl pH 6.7
	100 mM 2-Mercaptoethanol
	2 % (w/v) SDS
Wash buffer (TBS-Tween 20)	1% (v/v) 1 M Tris HCl pH 7.4
	150 mM NaCl
	0,05 % (v/v) Tween 20

Coomassie Staining

Isopropanol fixing solution	10 % (v/v) Acetic acid 25 % (v/v) Isopropanol
Coomassie Blue staining	7 % (v/v) Acetic acid 40 % (v/v) Methanol 0.025 % (w/v) Coomassie brilliant blue R-250
Destaining solution	7 % (v/v) Acetic acid 40 % (v/v) Methanol

3.5 Antibodies

3.5.1 Primary antibodies

Antibody	**Source**	**Supplier**
Anti-NF-κB (p65)	Rabbit	Santa Cruz Biotechnology, CA, USA
Anti-IKKα/β (H-470)	Rabbit	Santa Cruz Biotechnology, CA, USA
Anti-GAPDH	Mouse	Biotrend, Cologne

3.5.2 Secondary antibodies

Antibody	Source	Supplier
Anti-rabbit IgG, HRP conjugated	Goat	Acris Antibodies, Hiddenhausen
Anti-mouse IgM, HRP conjugated	Goat	Santa Cruz Biotechnology, CA, USA
Anti-rabbit IgG, rhodamine red fluorochrome conjugated	Goat	Santa Cruz Biotechnology, CA, USA

3.6 Escherichia coli strains

Competent cells have been prepared by a patented modification of the procedure of Hanahan (Hanahan 1983). The E. coli strains used in this study are presented below.

One SHOT TOP10 Chemically Competent E. coli (Invitrogen, Karlsruhe)

Genotype: F- mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu)7697 galU galK rpsL (Str^R) endA1 nupG

Description: TOP10 E. coli cells are ideal for high-efficiency cloning and plasmid propagation and allow stable replication of high-copy number plasmids.

RapidTrans TAM1 Chemically Competent E. coli (Active Motif, Carlsbad CA, USA)

Genotype: mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araΔ139 (ara-leu)7697 galU galK rpsL endA1 nupG

Description: RapidTrans TAM1 cells are high-efficiency competent E. coli that allow stable replication of high-copy number plasmids.

Library Efficiency DH5α Competent E. coli (Invitrogen, Karlsruhe)

Genotype: F- φ80lacZΔM15 Δ(□lacZYA-argF)U169 recA1 endA1 hsdR17(r_k^-, m_k^+) phoA supE44 thi-1 gyrA96 relA1 λ^-

Description: These cells are suitable for efficient transformation of large plasmids.

LIBRARY EFFICIENCY DB3.1 Competent E. coli (Invitrogen, Karlsruhe)

Genotype: F- gyrA462 endA1 Δ (sr1-recA) mcrB mrr hsdS20(r_B-, m_B-) supE44 ara-14 galK2 lacY1 proA2 rpsL20(Sm^R) xyl-5 λ- leu mtl1

Description: These cells contain the gyrA462 allele which renders the strain resistant to the toxic effects of the ccdB gene. They can be used for propagating plasmids containing the *ccd*B gene such as Gateway System vectors of Invitrogen.

One Shot Stbl3 Chemically Competent E. coli (Invitrogen, Karlsruhe)

Genotyp: F^- *mcr*B *mrr hsd*S20(r_B^-, m_B^-) *rec*A13 *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Str^R) *xyl*-5 λ^- *leu mtl*-1

Description: The Stbl3 E. coli strain is designed for cloning direct repeats found in lentiviral expression vectors. Unlike TOP10 E. coli, these cells reduce

the frequency of unwanted homologous recombinations of long terminal repeats (LTRs) found in ViraPower Lentiviral and other retroviral vectors.

3.7 Eukaryotic cell lines

hMSC

Cell type:	Human mesenchymal stem cell line
Obtained from:	Cambrex, NJ, USA
Source:	Harvested and cultured from normal human bone marrow.
Description:	Cells are positive for CD105, CD166, CD29, and CD44. Cells test negative for CD14, CD34 and CD45.

293FT

Cell type:	Genetically modified human embryonal kidney cell line
Obtained from:	Invitrogen, Karlsruhe
Source:	Derived from the 293F primary embryonal human kidney cell line
Description:	The 293FT strain is a particularly suitable host for generating lentiviral constructs. It stably expresses the SV40 large T antigen from the pCMVSPORT6TAg.neo plasmid. Studies have demonstrated maximal virus production in human 293 cells expressing SV40 large T antigen (Naldini, Blomer et al. 1996).

3.8 Plasmid vectors

pRK5 IKK-β C-Flag, Length 4.7 kb (Tularik Inc., South San Francisco, CA, U.S.A)

The pRK5-IKKβ (K44A)-C-FLAG plasmid was constructed by John Woronicz and was kindly provided to us by Dr. David V. Goeddel. It consists of the pRK5-C-FLAG vector containing the IKKβ cDNA encoding amino acids 1–755 with an alanine substitution of the conserved lysine residue at position 44 (K44A). pRK5-IKKβ (K44A)-C-FLAG encodes a dominant-negative mutant of IKKβ kinase (Regnier, Song et al. 1997; Woronicz, Gao et al. 1997; Zandi, Rothwarf et al. 1997).

pBC SK (+) vector, Length 3.4 kb (Stratagene, La Jolla, CA, USA)

The pBC SK (+) vector is a cloning vector designed to simplify commonly used cloning and sequencing procedures. The pBC SK+ phagemid (plasmid with a phage origin) carries a chloramphenicol-resistant gene.
The pBC SK + vector was used as an intermediate vector for the cloning reactions, due to the versatile and convenient multiple cloning site (MCS) it contains.

pEGFP-N3 vector, Length 4.7 kb (Clontech, Heidelberg)

pEGFP-N3 encodes a red-shifted variant of wild-type GFP (Prasher, Eckenrode et al. 1992) which has been optimized for brighter fluorescence and higher expression in mammalian cells. (Excitation maximum = 488 nm; emission maximum = 507 nm.) pEGFP-N3 encodes the GFPmut1 variant (Cormack, Valdivia et al. 1996) which contains the double-amino-acid substitution of Phe-64 to Leu and Ser-65 to Thr. The coding sequence of the EGFP gene contains more than 190 silent base changes which correspond to human codon-usage preferences. Sequences flanking EGFP have been converted to a

Kozak consensus translation initiation site (Kozak 1987) to further increase the translation efficiency in eukaryotic cells.

pENTR 11 vector, Length 2.7 kb (Invitrogen, Karlsruhe)

The pENTR11 vector allows restriction cloning of a gene of interest into a vector for entry into the Gateway System available from Invitrogen.

The vector contains the rrnB T1 and T2 transcription termination sequences to prevent basal expression of the cloned gene in E. coli, thereby reducing possible toxicity (Orosz, Boros et al. 1991) .The attL1 and attL2 sites in the vector allow site-specific recombination of the entry clone with a Gateway destination vector (Landy 1989). A Kozak consensus sequence allows efficient translation initiation in eukaryotic systems (Kozak 1987). The ccdB gene located between the two attL sites makes a negative selection of expression clones possible. A Kanamycin resistance gene also allows selection of the plasmid in E. coli. Finally, the vector contains a pUC origin of replication that allows high-copy number replication and growth in E. coli.

In order to propagate and maintain the pENTR11 vector, DB3.1 Competent Cells have to be used for transformation, since this particular E. coli strain is resistant to ccdB effects and can support the propagation of plasmids containing the ccdB gene. The cloned gene of interest however must replace the ccdB gene located between the two attL sites.

pLenti6/V5-DEST Gateway Vector, Length 8.7 kb (Invitrogen, Karlsruhe)

The pLenti6/V5-DEST vector is a destination vector adapted for use with the Gateway Technology of Invitrogen, and is designed to allow high-level expression of recombinant fusion proteins in mammalian cells using Invitrogen's ViraPower Lentiviral Expression System.

The vector contains the human CMV immediate early promoter to allow high-level, constitutive expression of the gene of interest in mammalian cells (Boshart, Weber et al. 1985; Nelson, Reynolds-Kohler et al. 1987; Andersson, Davis et al. 1989) and two recombination sites, attR1 and attR2, downstream of the CMV promoter for recombinational cloning of the gene of interest from an entry clone. It also contains a Rous Sarcoma Virus (RSV) enhancer/ promoter for Tat-independent production of viral mRNA in the producer cell line (Dull, Zufferey et al. 1998) and a modified HIV-1 5′ and 3′ Long Terminal Repeats (LTR) for viral packaging and reverse transcription of the viral mRNA (Dull, Zufferey et al. 1998) .The U3 region of the 3′ LTR is deleted (ΔU3) and facilitates self-inactivation of the 5′ LTR after transduction to enhance the biosafety of the vector (Dull, Zufferey et al. 1998). The vector contains additionally a HIV-1 psi (Ψ) packaging sequence for viral packaging, a HIV Rev response element (RRE) for Rev-dependent nuclear export of unspliced viral mRNA (Malim, Hauber et al. 1989; Kjems, Brown et al. 1991). A chloramphenicol resistance gene (CmR) located between the two attR sites allows counterscreening. The ccdB gene located between the attR sites allows negative selection, and the C-terminal V5 epitope makes detection of the recombinant protein of interest possible (Southern, Young et al. 1991).

A Blasticidin resistance gene (Takeuchi, Hirayama et al. 1958; Yamaguchi, Yamamoto et al. 1965) makes selection in E. coli and mammalian cells possible. Resistance to Blasticidin is conferred by expression of either one of two Blasticidin S deaminase genes: bsd from Aspergillus terreus (Kimura, Takatsuki et al. 1994) or bsr from Bacillus cereus (Izumi, Miyazawa et al. 1991) .These deaminases convert Blasticidin S to a non-toxic deaminohydroxyderivative (Izumi, Miyazawa et al. 1991).

Finally, the vector contains an Ampicillin resistance gene and a pUC origin for selection and high-copy replication of the plasmid in E. coli respectively.

Stbl3 E. coli is recommended for transformation of this vector, as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats. Transformants containing unwanted recombinants are generally not obtained when this strain is used for transformation.

3.9 Oligonucleotides

3.9.1 RT- PCR primers

For PCR reactions, sense and antisense oligonucleotide primers for GAPDH (Takahashi, Higashino et al. 2003), EGFP (Liu, Dovzhenko et al. 2004), β1 integrin (Lin, Yeh et al. 2005), αV integrin (Lin, Yeh et al. 2005), VCAM-1 (Gregory, Singh et al. 2003) were used as described previously. Primers for IKK2 and CD 44 were designed using Clone Manager 7 (Scientific and Educational Software). Primer sequences are listed below.

Gene name	*Sequence*	*Annealing temperature (° C)*	*Product size (bp)*
GAPDH	Forward: 5'-CAACTACATGGTTTACATGTTC-3 Reverse: 5'-GCCAGTGGACTCCACGAC-3'	50	181
EGFP	Forward: 5'-CAAGCTGACCCTGAAGTTCATCTGC-3' Reverse: 5'-CACGCTGCCGTCCTCGATGTTGTGG-3'	55	409
IKK2	Forward: 5´-AAGTGCGGCAGAAGAGTGAG-3 Reverse: 5´-ACTGAGTGGCAGGCTTATCG-3´	48	407
β1 integrin	Forward: 5´-ATGAATGAAATGAGGAGGATTACTTCG-3´ Reverse: 5´-AAAACACCAGCAGCCGTGTAAC -3	48	322
αV integrin	Forward: 5´-GGAGCACATTTAGTTGAGGTAT -3´ Reverse: 5´-ACTGTTGCTAGGTGGTAAAACT -3´	48	274
VCAM-1	Forward: 5´-AATCCACGTGGAGATCTACT -3´ Reverse: 5´-TTCTCAAAACTCACAGGGCT-3´	50	282
CD 44	Forward: 5`-GCCAAACACCCAAAGAAGAC-3´ Reverse: 5´-TCCACCTGTGACATCATTCC-3´	55	392

All oligonucleotides were purchased from Invitrogen.

3.9.2 Sequencing primers

All sequencing primers used in this thesis were designed with Clone Manager 7.0 and purchased from Invitrogen.

3.10 Data processing

Analysis of sequencing results was performed with the freeware Chromas v 1.45
Clone manager 7.0 (Scientific and Educational Software) was used for various cloning procedures and for designing plasmid vectors.
Axiovision 40 LE 4.4.0.0 (Carl Zeiss Vision) was used for processing of the microscopic images.
FACS data were acquired and analyzed using Cell QuestTM software (Becton-Dickinson Biosciences, San Jose, CA, USA).
Microsoft Excel 2003 was used for statistical analyses and graphics.

4 METHODS

4.1 Microbiological Methods

4.1.1 Cultivation of E. coli

Bacterial strains carrying plasmids or genes with antibiotic selection marker were cultured in liquid or on solid medium containing the selective agent. Stock solutions of antibiotics were sterilised by filtration and aliquots stored at -20°C. Antibiotics were added to freshly autoclaved medium (cooled to below 50°C). Stock and working concentrations of the antibiotics are shown in the following table (Sambrook J. 2001).

Antibiotic	**Stock solution**		**Working concentration**	
	Concentration	Storage		Dilution
Ampicillin	50 mg/ml in water	-20°C	50 µg/ml	1/1000
Chloramphenicol	34 mg/ ml in ethanol	-20°C	170 µg/ml	1/200
Kanamycin	10 mg/ml in water	-20°C	50 µg/ml	1/200

4.1.2 Agar plates

E.coli strains can be streaked and stored on dyt plates containing 1.5% agar and the appropriate antibiotic. With a flamed and cooled wire loop, an inoculum of bacteria was streaked on top of a fresh agar plate and incubated upside down at 37°C for 12-14 h until colonies developed. The plates were stored for up to 1 month at 4°C.

4.1.3 Liquid cultures

Bacterial liquid cultures were grown for plasmid preparations. Cultures were prepared by inoculating a single colony from a freshly streaked selective plate into 4-6 ml (Mini-Prep), or 50 ml (Midi-Prep), or 150 ml (Maxi-Prep) of 2x YT medium containing the appropriate antibiotic. The culture was grown overnight (12-16 h) at 37°C with vigorous shaking (225 rpm). Cells were harvested by centrifugation at 5000 x g for 20 min, and following the cell pellet was treated as indicated in the appropriate plasmid purification protocols.

4.1.4 Glycerol stocks

E. coli strains can be stored for many years at -70°C in 15% glycerol. 0.15 ml glycerol was added to a 2 ml screw-cap freezing vial and sterilised by autoclaving. 0.85 ml of the E. coli culture was added to the vial of pre-sterilized glycerol and vortexed to ensure even mixing of the bacterial culture and the glycerol. Samples were frozen in liquid nitrogen and stored at -80°C.

4.2 Molecular biological methods

4.2.1 Transformation of chemically competent cells

Competent cells were removed from the -80°C freezer and thawn on wet ice. 1 to 5 µl of the DNA (10 pg to 100 ng), or 2 µl of the ligation reaction were added to the 50 µl suspension of competent cells, moving the pipette gently through the cells while dispensing. Cells were incubated for 30 min on ice and heat-shocked for 45 sec in a 42°C water bath afterwards. Then, cells were placed on ice for 2 min, and 0.9 ml of room temperature S.O.C medium was added. The culture was incubated for 1 h at 37°C with shaking (225 rpm). Using a sterile spreader, 10-100 µl of this suspension were plated on pre-warmed 2x YT medium plates containing the appropriate antibiotic. Plates were

allowed to completely absorb any excess media and were subsequently incubated overnight upside-down at 37°C.

4.2.2 Isolation of plasmid DNA

Purification of plasmids from E.coli overnight cultures containing the appropriate antibiotics were routinely performed using the Plasmid Mini Kit for analytical purposes and the Plasmid Midi or Maxi Kit for preparative purposes, according to the manufacturer's protocol.
The plasmid purification protocol is based on a modified alkaline lysis procedure (Birnboim and Doly 1979; Le Gouill, Parent et al. 1994), followed by binding of plasmid DNA to an anion-exchange resin under appropriate low-salt and pH conditions. RNA, proteins, dyes, and low-molecular weight impurities were removed by a medium-salt wash. Plasmid DNA was eluted in a high-salt buffer and then concentrated and desalted by isopropanol precipitation. The precipitated DNA was subsequently washed with 70 % ethanol. Plasmids for transfection of eukaryotic cells were purified under endotoxin free conditions. Plasmid DNA was finally eluted in H_2O or TE buffer and stored at -20°C.

4.2.3 Ethanol precipitation of DNA

Extraction and a cleaning of DNA after enzymatic reactions was achieved with ethanol precipitation. DNA from dilute, aqueous solutions can be precipitated by the addition of monovalent cations and ethanol. 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 100% ethanol were added to the nucleic acid solution, mixed, and incubated at -20°C at least for 30 minutes. Following centrifugation for 30 min at 12,000x g, 4°C, the supernatant was carefully discarded. The pellet was washed once with 70% ethanol to remove remaining salt and centrifuged for additional 10 min at

12,000 x g, 4°C. The DNA or RNA precipitate was briefly air dried and dissolved in sterile H_2O (RNase-free) or TE buffer.

4.2.4 Quantification of nucleic acids

Determination of DNA or RNA concentration was done by spectrophotometry at 260 nm. Nucleic acids absorb UV light of 260 to 280 nm wavelength, with a maximum at 260 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The absorbance of 1 unit at 260 nm (A260) corresponds approximately to 50 µg/ml for double-stranded DNA and 40 µg/ml for RNA. The ratio between the readings at 260 nm and 280 nm provides an estimate for the purity of the nucleic acid. Pure preparations of DNA and RNA have ratios of 1.8 and 2.0, respectively (Manchester 1995; Wilfinger, Mackey et al. 1997). All DNA and RNA solutions used in the experiments of this thesis had a ratio between 1.8 and 2.0.

4.2.5 Gelelectrophoretic analysis of nucleic acids

Analysis of RT-PCR products, restriction enzyme digest, minipreparations of plasmid DNA, or enzymatic modifications of DNA was done with the use of agarose gel electrophoresis.

Gel electrophoresis allows separation and identification of nucleic acids based on charge migration. Migration of nucleic acid molecules in an electric field is determined by size and conformation, allowing nucleic fragments of different sizes to be separated. Agarose gel analysis is the most commonly used method for analyzing DNA fragments between 0.1 and 25 kb. The concentration of agarose (usually 0.7-1.5%) used for the gel depends primarily on the size of the DNA fragments to be analyzed.

Ethidium bromide was included in the gel matrix to enable fluorescent visualisation of the DNA fragments under UV light. Agarose gels were submerged in electrophoresis buffer in a horizontal electrophoresis apparatus.

4.2.6 Separation of DNA on agarose gels

The amount of agarose depending on the concentration required was added to the appropriate volume of 1 x TAE buffer in a bottle and heated in a microwave until the agarose dissolved. Reduced volume of liquid was made up to the original volume with distilled water to ensure correct agarose and buffer concentrations. The cooled agarose (55-60°C) was mixed with ethidium bromide to a final concentration of 0.5 μg/ml and poured onto the horizontal gel tray to a thickness of 3-5 mm. A comb was inserted into the gel immediately after pouring the gel, and the gel was left to set for 20 min. Following, the comb was removed and the tank containing the gel was filled with 1 x TAE buffer. For DNA sample preparation, 1 volume of 6 x DNA loading buffer was mixed with 5 volumes of DNA solution, the samples were applied to the wells of the gel and a molecular-weight marker was included to enable analysis of DNA fragment sizes in the samples. Electrodes were connected so that the DNA would migrate towards the anode. The gel was run at 5-7 V/cm until the dyes have migrated an appropriate distance. An appropriate molecular DNA marker was applied on a separate lane in the same gel, in order to determine weight and size of the DNA sample.
Ethidium bromide–DNA complexes display increased fluorescence compared to the dye in solution. Illumination of a stained gel under UV light (302 nm) allowed bands of DNA to be visualised against a background of unbound dye. The visualised DNA fragments were subsequently documented photographically.

4.2.7 Purification of DNA fragments from agarose gel

DNA fragments were purified after agarose gel electrophoresis using the QIAquick Gel Extraction Kit, according to manufacturer's instructions. This procedure allows DNA fragments separated electrophoretically to be purified from salts or unincorporated nucleotides prior to cloning or other procedures. Purification of DNA fragments with the QIAquick Gel Extraction Kit is based on adsorption of DNA to a silica-gel membrane in the presence of high salt, while contaminants pass through the column. The adsorption of nucleic acids to silica-gel surfaces occurs only in the presence of a high concentration of chaotropic salts, which modify the structure of water (Vogelstein and Gillespie 1979). Impurities are efficiently washed away, and the pure DNA is eluted with Tris buffer or water.

Following electrophoresis, the DNA bands to be isolated were visualised under UV light, excised from the agarose gel with a sterile, sharp scalpel and extracted according to the manufacturer's instructions.

4.3 Analysis and modification of DNA

4.3.1 Fragmentation of DNA with restriction enzymes

Restriction enzymes were discovered about 30 years ago during investigations into the phenomenon of host-specific restriction and modification of bacterial viruses (Arber 1965; Smith and Wilcox 1970; Danna and Nathans 1971). These enzymes were found to cleave DNA at specific sites, generating discrete, gene-size fragments. It is generally accepted that their role in nature is to protect bacteria from infections by viruses. Restriction endonucleases recognize short, specific, and most commonly palindromic base sequences in DNA, which are characteristic for each enzyme. Once a restriction enzyme recognizes its specific DNA sequence, the phosphodiester bond is hydrolyzed whereby the DNA fragment is cleaved at this particular position. Depending on the

restriction endonuclease, this enzymatic reaction generates blunt end or cohesive end termini. The enzymatic activity is indicated in units (u), whereupon 1 u corresponds to the amount of enzyme needed for the digestion of 1 microgram DNA in one hour. It is recommended that the volumetric content of the enzyme does not exceed 10% of the final volume, since glycerin is a major component of the enzyme solution and inhibits the enzymatic activity if its concentration is higher than 5%. Moreover, if restriction endonucleases are used under extreme non-standard conditions (such as high enzyme/DNA ratio, long incubation time, incorrect buffer condition, presence of contaminants), they might be capable of cleaving sequences, which are similar but not identical to their defined recognition sequence, a phenomenon termed "star activity". The restriction reactions in this thesis were conducted under the conditions specified for each enzyme. After the appropriate incubation time, completeness of the enzymatic restrictions was confirmed with agarose gel electrophoresis.

4.3.2 Ligation of DNA fragments

The formation of a phosphodiester bond between juxtaposed 5' phosphate and 3' hydroxyl termini of DNA is catalyzed by DNA ligases. The enzymatic reaction takes place only in the presence of sufficient ATP, and a free 5' phosphate group. Ligation reactions in this study were performed with the Quick Ligation Kit, which enables ligation of cohesive end or blunt end DNA fragments in 5 minutes at room temperature (25°C). For each reaction, 50 ng of vector were combined with a 3-fold molar excess of insert. The volume of the solution was adjusted to 10 µl with sterile water.10 µl of 2x Quick Ligation Buffer were added, and the solution was briefly mixed. Finally, 2 U of recombinant Quick T4 DNA Ligase were added and the sample was incubated at room temperature (25°C) for 5 minutes.

4.3.3 Recombinational cloning

The Gateway LR Clonase Enzyme Mix of Invitrogen was used to catalyze the in vitro recombination between an entry clone (containing a gene of interest flanked by attL sites) and a destination vector (containing attR sites) to generate an expression clone. Recombinational cloning is based on site-specific recombination reactions of the λ bacteriophage in E. coli during lysogenization (Landy 1989). Proteins encoded by λ phage and E. coli mediate the integrative and excisive recombination reactions of λ phage. These recombination reactions, performed in vitro, are the basis of the Gateway cloning technology. The attB (attL) sites are specifically recognized by the recombination proteins that constitute the clonase enzyme mix cocktails. These proteins cut to the left and to the right of the gene in the entry clone and ligate it into the destination vector, creating a new expression clone (Figure 14). There is a counterselection against destination vectors without the gene of interest since they carry the ccdB gene, which is lethal to most E. coli strains (Bernard and Couturier 1992; Miki, Park et al. 1992). Thus, selection for ampicillin resistance chooses for E. coli cells that carry the desired product, which usually comprise 70-90% of the colonies.

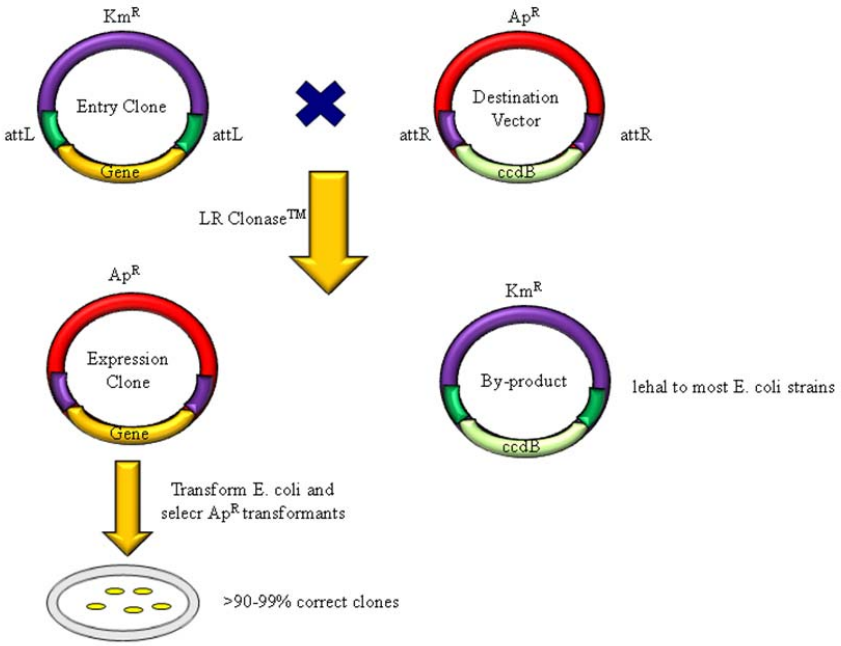

Figure 14. The Gateway LR Cloning Reaction. An entry vector, containing a gene flanked by recombination sites, recombines with a destination vector to yield an expression vector and a by-product plasmid. The result is that a gene sequence in the entry vector is transferred into an expression vector, donated by the destination vector. Only plasmids without the ccdB gene that are also ampicillin (ApR) resistant will yield colonies.

For each reaction, 100-300 ng of the entry clone and 300 ng of the destination vector were mixed with 4 µl 5x LR Clonase Reaction Buffer and then TE buffer, pH 8.0 was added to each sample to a final volume of 16 µl. LR Clonase enzyme mix was thawn on wet ice for about 2 minutes, briefly vortexed and 4 µl of the enzyme mix were added to each reaction. The reactions were incubated at 25°C for 60 minutes. 2 µl of the Proteinase K were afterwards added to each sample to terminate the reaction. Samples were then vortexed briefly and then incubated at 37°C for 10 minutes. Finally, 1 µl of each LR reaction was transformed into 50 µl of Library Efficiency DH5α competent E.coli cells.

4.3.4 Sequencing of DNA

DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. Currently, most DNA sequencing is based on the chain termination method developed by Frederick Sanger (Sanger, Nicklen et al. 1977). This technique uses sequence-specific termination of a DNA synthesis reaction using modified nucleotide substrates. The method takes part in two steps. A labelling-reaction is first performed in order to produce a labelled source sample, followed by a termination-reaction where the synthesis of the fragments produced in the first reaction is ended. Nowadays, labelling of the sequence reaction is done with fluorescent coloring. This is accomplished by labelling each of the dideoxynucleotide chain-terminators with a separate fluorescent dye, which fluoresces at a different wavelength.

Sequencing of plasmid DNA was performed using the BigDye Terminator Cycle Sequencing kit. For the sequencing reaction, 300 ng of PCR-amplified DNA, 3.2 pmol of either the forward or the reverse primer, and 4 µl of BigDye Terminator RR mix were mixed and adjusted to a final volume of 20 µl by adding distilled water. The reaction was run for 30 cycles of 15 s at 94°C, 10 s at 50°C, and 2 min at 60°C. Sequence analysis was carried out with an ABI Prism 3730 automated sequencer by Sequiserve Sequencing Company (Vaterstetten).

4.4 RNA analysis

4.4.1 Isolation of RNA from cultured eukaryotic cells

RNA was purified from cultured eukaryotic cells using the RNeasy Kit of Qiagen. The RNeasy procedure combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly

denaturing guanidine-thiocyanate–containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and the sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water.
Total RNA was isolated from cell cultures according to the manufacturer's suggestions. RNA was dissolved in 60 µl RNase-free H_2O, and the samples were stored at -20°C.

4.4.2 RT-PCR

Sensitive methods for the detection and analysis of rare mRNA transcripts or other RNAs present in low abundance are an important aspect of most cell/ molecular biology studies. RNA cannot serve as a template for PCR, so it must first be reverse transcribed into cDNA (e.g. with reverse transcriptase from avian myeloblastosis virus). RT-PCR is a combined technique in which reverse transcription (RT) is coupled with PCR amplification of the resulting cDNA (D'Alessio and Gerard 1988).

4.4.2.1 Reverse transcription

First strand synthesis was done with the cloned AMV first-strand cDNA Synthesis Kit of Invitrogen using 500 ng RNA and random-hexamer primers according to the manufacturer's instructions. The synthetized cDNA was either used immediately for PCR reactions, or stored at -20°C.

4.4.2.2 PCR amplification

Polymerase chain reaction (PCR) is an in vitro method for enzymatically synthesizing defined sequences of DNA. The reaction uses two oligonucleotide primers that hybridise to opposite strands and flank the target DNA sequence that is to be amplified. The

elongation of the primers is catalyzed by a heat-stable DNA polymerase (such as Taq DNA polymerase). A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by the polymerase results in exponential accumulation of a specific DNA fragment. The ends of the fragment are defined by the 5' ends of the primers. Prerequisites for successful PCR include the design of optimal primer pairs, the use of appropriate primer concentrations, and optimisation of the PCR conditions.

Routinely, the PCR reaction was performed in a total volume of 20 µl, using the Taq DNA Polymerase of Invitrogen. All cDNA samples used for PCR reaction were first diluted 1:10 with DEPC water, to avoid the PCR inhibitory effects of the reverse trascriptase components (Sellner, Coelen et al. 1992; Fehlmann, Krapf et al. 1993; Liss 2002). Transcript levels were normalized using a house keeping gene (GAPDH) as the internal control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes used in comparisons of gene expression data. The assumption was made, that expression of the gene remained constant in the cells under investigation. PCR cycle number was optimized for each reaction to avoid reaching saturation. PCR amplification for GAPDH, EGFP, IKK2, β1 integrin, αV integrin, VCAM-1 and CD44 was performed at 35, 30, 30, 25, 30, 35, and 30 cycles respectively.

4.5 Protein Biochemistry

4.5.1 Protein precipitation

Protein extraction was performed by lysing the cells in radio-immunoprecipitation (RIPA) buffer. The RIPA buffer is a reliable cell lysis buffer that enables efficient cell lysis and protein solubilization while avoiding protein degradation and interference with the proteins' immunoreactivity and biological activity. It is furthermore compatible with the BCA Protein Assay.

Cells were first trypsinized and then collected by low-speed centrifugation at 500 rpm for 5 min at room temperature. The supernatant was carefully removed. The pellet was

washed with PBS and recollected by centrifugation at 500 rpm for 5 min at room temperature. The supernatant was carefully removed and cells were lysed by adding ice-cold RIPA buffer containing a mixture of protease inhibitors (Protease inhibitor tablet Complete, Mini, EDTA-free). After incubation on ice for 30 min cells were disrupted by sonication (2 x 2 sec) and incubated on ice for additional 30 minutes. The insoluble cell debris was removed by centrifugation at 10.000 x g for 10 min at 4°C. The cell lysate was stored at -70°C until assayed.

4.5.2 Determination of protein concentration

For the determination of the protein concentration in samples the Pierce BCA Protein assay kit was used. The assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing BCA. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562 nm that is nearly linear with increasing protein concentrations over a broad working range (20-2,000 µg/ml).
After the assay was set up in a 96-well plate according to the manual of the manufacturer, the assay was automatically measured in a multilabel plate reader at 562 nm wavelength.

4.5.3 SDS-Polyacrylamide gel electrophoresis

In SDS-polyacrylamide gel electrophoresis (SDS-PAGE) proteins are separated largely on the basis of polypeptide length. The solution of proteins to be analyzed is first mixed with SDS, an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures and applies a negative charge to each protein in proportion to its mass. The denatured proteins are subsequently applied to one end of a layer of polyacrylamide gel submerged in a suitable buffer. An electric current is applied across the gel, causing the negatively-charged proteins to migrate across the gel. Depending on their size, each protein will move differently through the gel matrix. After a set amount of time the proteins will have differentially migrated based on their size.

Protein separation was performed using a "discontinuous" buffer system (Laemmli 1970). In this system, two sequential gels are actually used. The top gel, called the stacking gel, is slightly acidic (pH 6.8) and has a lower acrylamide concentration to make a porous gel. Under these conditions the proteins separate poorly, but form thin, sharply defined bands. The lower gel, called the separating, or resolving gel, is more basic (pH 8.8) and has a higher polyacrylamide content, which causes the gel to have narrower channels or pores. As a protein -concentrated into a sharp band by the stacking gel- travels through the separating gel, the narrower pores of the resolving gel have a sieving effect, allowing smaller proteins to travel more easily and hence rapidly than larger proteins.

The protein samples (10 µg) were separated on 10 % SDS-PAGE gel using the BioRad MiniProtean II gel chamber system. Electrophoresis was performed for 2-3 hours under a constant current of 50mA.

Reagents	**Stacking gel**	**10 % Separating gel**
30% Acrylamide mix	415 µl	1.65 ml
10% SDS	25 µl	50 µl
1 M Tris (pH 6.8)	315 µl	-
1 M Tris (pH 8.8)	-	1.875 ml
10% APS	25 µl	50 µl
TEMED	2.5 µl	2 µl
H_20	1.72 ml	1.375 ml

4.5.4 Coomassie blue staining of proteins in polyacrylamide gels

Colloidal Coomassie staining is one of the most sensitive staining protocols. Due to its colloidal properties the dye binds with high specificity to proteins and only minimal to the gel matrix. This allows visualisation of proteins separated by SDS-PAGE with sensitivity as high as 30ng of protein.

Immediately after completion of electrophoresis, gels were incubated in the fixing solution for 15 min with gentle agitation. The fixing solution was then removed and the Coomassie staining solution was added. The dish was covered and boiled for 30 seconds in a microwave oven. After that, it was left at room temperature for 20 minutes with gentle agitation. The Coomassie solution was then removed and destaining solution was added. The dish was covered again, boiled for 30 seconds and then shaken moderately at room temperature for 20 minutes. The destaining solution was removed and the gels were washed with distilled water. After completion of staining, gels can be stored in water at 4°C for a few days.

4.5.5 Western Blot

4.5.5.1 Electroblotting of proteins onto a PVDF membrane

Once a protein of interest has been separated by SDS-PAGE, the pure protein can be transferred to a PVDF membrane for immunodetection (Matsudaira 1987).

Proteins were transferred onto the PVDF membrane using the BioRad Mini Trans-Blot system at a constant voltage of 100V for 1.5 h. The vertical chamber was filled with 1x Blotting Buffer.

4.5.5.2 Immunoblot analysis

Unspecific protein binding sites on the PVDF membrane with the transferred proteins were blocked with blocking solution. After blocking with 5% skim milk solution for 1.5 hours, the blocking solution was discarded and the membrane was washed two times with wash buffer for 5 minutes and directly incubated with 1:1000 diluted primary antibody for IKKα/β (H-470) or 1:5000 diluted primary antibody for GAPDH for 1.5 h at RT, with gentle agitation on a platform shaker. To remove non-specific bound primary antibodies, the membrane was washed 4 times with wash buffer for 7 minutes and then incubated with the horseradish peroxidise-conjugated secondary antibodies for 45 minutes at RT. 1:10000 diluted goat anti-rabbit IgG for detection of IKK or 1:5000 diluted goat anti-mouse IgM for detection of GAPDH were used. To completely remove surplus secondary antibodies, the membrane was washed with wash buffer 4 times for 7 minutes. The signal detection was carried out with the enzymatically catalyzed chemiluminescence (ECL) reaction.

Once the secondary antibody was coupled to horseradisch peroxidase (HRP), the washed membrane was immersed with ECL reaction solution for 5 minutes. Then, the membrane was briefly and gently dried with a paper towel, placed into a sealable clear plastic bag and exposed to an x-ray film in an x-ray cassette. After a suitable incubation time, the film was developed with a Kodak x-ray developer.

4.5.5.3 Stripping of bound antibodies

The complete removal of primary and secondary antibodies from the membrane is possible. The membranes may be stripped of bound antibodies and reprobed several times.

The membrane was incubated in stripping buffer at 50 °C for 30 minutes with occasional agitation and afterwards washed two times with wash buffer for 10 minutes at RT with moderate shaking, using large volumes of wash buffer. Membranes may be incubated

with ECL Plus detection reagents and exposed to film to ensure complete removal of antibodies.

4.6 Methods of cell biology

4.6.1 Cultivation of mammalian cells

All cell lines were incubated at 37 °C in humified cell culture incubators with 5% CO_2. Additionally all used materials, solutions and media were sterilized for cell culture work and the work was performed under sterile cell culture hoods.

4.6.1.1 Human mesenchymal stem cells

Human mesenchymal cells (hMSCs) were purchased from three different donors and were first examined for morphology and adipogenic, chondrogenic and osteogenic differentiation potential. Cells were cultured according to manufactures protocol in complete growth medium (MSCGM hMSC Medium Bullet Kit) and were grown at a confluency of up to 70 %. For all further transduction, proliferation and invasion experiments, cells between 5-9th passage were used.

4.6.1.2 293FT Cells

The 293FT Cell Line is a derivative of the 293F Cell Line, which stably and constitutively expresses the SV40 large T antigen from pCMVSPORT6TAg.neo to facilitate optimal lentivirus production (Naldini, Blomer et al. 1996).
The 293FT Cell line was purchased from Invitrogen and was handled as potentially biohazardous material under Biosafety Level 2.

Cells were cultured according to manufacturer's protocol in D-MEM with 10% fetal bovine serum (FBS), 0.1 mM MEM Non-Essential Amino Acids (NEAA), 6 mM L-glutamine, 1 mM MEM Sodium Pyruvate, 1% Penicillin-Streptomycin to prevent bacterial contamination and Geneticin at a concentration of 500 µg/ml. Cells were not alowed to overgrow before passaging and were 80 % confluent on the day of transfection. Additionaly, 2 h before each transfection, the medium was replaced with 10 ml fresh DMEM

4.6.1.3 Passaging cells

Cells were splitted routinely twice a weak. Cells were harvested by trypsinisation.
For trypsinisation, the medium was removed from the flask and cells were washed carefully with 5 ml PBS to remove residual medium. 2ml of prewarmed Trypsin solution were added to the flask (75 cm^2) and incubated at 37°C until cells had detached (3-5 minutes). Trypsinisation was blocked by adding 2 ml of medium. Following, cells were centrifuged at 500 rcf for 5 min at room temperature and resuspended in an appropriate volume of prewarmed growth medium containing serum.

4.6.1.4 Cell freezing and thawing

The cells were trypsinised first and then resuspended in freezing medium. 1 ml of the cell suspension (approximately 1-2 x 10^6 adherent cells) was transferred into each freezing vial. Freezing vials were placed into a Cryo freezing container (Nalgene) and stored in a -80°C freezer overnight. This allowed freezing of the cells at a rate of 1°C/min. The following day vials were transferred to a liquid nitrogen chamber. For cell thawing, a vial of frozen cells was removed from liquid nitrogen and placed in a 37°C water bath until thawed. To remove DMSO from the freezing medium, the cell suspension was pipetted

into a centrifuge tube containing prewarmed medium, and centrifuged at 500 rcf for 5 min at room temperature. The supernatant was discarded, cells were resuspended in fresh growth medium and transferred to the cell culture flask. Cells were incubated overnight under their usual growth conditions, and the medium was replaced the next day.

4.6.2 Immunofluorescence

HMSCs grown on glass slides were fixed with 100% methanol at -20°C for 8 minutes, washed 3 times with PBS and blocked with 1% BSA for 1 hour at room temperature. The cells were incubated with primary antibody for NF-κB (p65) in a dilution of 1:250 for 45 minutes, which was uncovered with secondary antibody conjugated to rhodamin red fluorochrome in a dilution of 1:100. Photomicrographs were taken with axiocam MR (Zeiss, Jena, Germany) on Axioskope 2 (Zeiss, Jena, Germany).

4.6.3 FACS

Analysis for eGFP transgene expression in hMSCs was performed by flow cytometry. Briefly, after trypsinization hMSCs were collected by centrifugation at 500 x g for 5 minutes at 20°C and resuspended in PBS + 1% FBS. The analyses were performed on a flow cytometer equipped with a 488-nm argon laser. Forward and side scatter was used to establish a collecting gate through which dead cells and debris were excluded. Non specific fluorescence was determined using untransduced hMSCs. Samples were run on the “medium” flow rate setting, until 15000 events had been counted. Data were acquired and analyzed using Cell QuestTM software.

4.6.4 Cell invasion assay

The invasive capacity of hMSCs was analyzed using Costar Transwell invasion chambers (24-well) as previously described (Neth, Ciccarella et al. 2006). Polycarbonate membrane filters with a pore size of 8 μm and a diameter of 6.5 mm were coated with 10 μg of human extracellular matrix (ECM). The filters were dried and prior to the experiment reconstituted with serum-free medium. The lower compartment of the invasion chamber was filled with 600 μl of DMEM. Then the coated filter inserts were placed into the wells forming the upper compartment. HMSCs (5 x 10^3) were suspended in 200 μl of serum-free medium and seeded into the upper compartment of the invasion chamber. Each invasion experiment was performed in triplicate. The invasion chambers were incubated for different time intervals at 37°C in a humidified air atmosphere with 5% CO2. After incubation, cells and ECM on the top surfaces of the filters were wiped off with cotton swabs. Cells that had migrated into the lower compartment and attached to the lower surface of the filter were counted after staining with Diff Quick. Cell viability was assessed with trypan blue staining. The invasion rate was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment.

4.6.5 BrdU proliferation assay

The assay was performed according to the manufacturer's instructions. Briefly, hMSCs (untransduced or transduced with EGFP and dnIKK2) were seeded on 96-well dishes in a density of 3000 cells/well. The cells were grown in complete culture medium which was changed after 12 h with complete media, supplemented with 10 μM BrdU for unstimulated controls or 10 ng/ml of TNF-α and 10 μM BrdU for stimulated cells. BrdU incorporation was measured after 24 h using a multilabel plate reader at 450 nm and a reference wavelength of 690 nm. BrdU uptake was then calculated as percentage of the EGFP unstimulated control hMSCs.

4.6.6 TNF-α stimulation of cells

Treatment of cells with TNF-α was done using concentrations and stimulation times as previously described (Nagasaki, Ishimura et al. 1999; Wolf, Schulz et al. 2002; Sakon, Xue et al. 2003; Cohen, Meisser et al. 2006).
For RNA and protein analysis, cells were pretreated with 50 ng/ml of TNF-α for 4 hours. Treating the cells with 10 ng/ml of TNF-α for 24 hours showed the same results in these experiments.
For cell invasion and proliferation assays, cells were preincubated with 50 ng/ml of TNF-α for 48 hours hours and 10 ng/ml of TNF-α for 24 hours respectively.
For immunohistochemistry, cells were pretreated with 50 ng/ml of TNF-α for 30 minutes.

4.7 Production of lentiviral vectors by transient transfection of 293FT cells

4.7.1 Production of lentiviral VSV-G pseudotyped vectors

The ViraPower Lentiviral Expression System of Invitrogen was used to create the replication-incompetent HIV-1-based lentiviruses that were used to deliver and express the dnIKK2 and EGFP genes in human mesenchymal stem cells. The lentiviruses produced with this system were generated at a Biosafety Level 2 (BL-2) laboratory and all published BL-2 guidelines were strictly followed, with proper waste decontamination.
Generally, during this lentiviral production procedure, 293FT cells are cotransfected with the pLenti vector containing the gene of interest and the ViraPower Packaging Mix. The ViraPower Packaging Mix contains an optimized mixture of the three packaging plasmids, pLP1, pLP2, and pLP/VSVG. These plasmids supply the helper functions as well as structural and replication proteins *in trans* required to produce the lentivirus. For the transfection step of 293FT cells, Lipofectamine 2000 is also needed. Lipofectamine 2000 reagent is a proprietary, cationic lipid-based formulation suitable for the

transfection of nucleic acids into eukaryotic cells. After cotransfection the viral supernatant is harvested and the titer is determined.

Lentiviral stocks were harvested according to the manufacturer's instructions: On the day of transfection, 293FT cells were trypsinized and resuspended at a density of 1.2 x 10^6 cells/ ml in Opti-MEM Medium containing 10 % FBS. Antibiotics were not added to this medium. For each transfection sample DNA-Lipofectamine 2000 complexes were prepared as follows: In a sterile 14 ml polystyrol tube, 36 µg of the ViraPower Packaging Mix and 12 µg of pLenti expression plasmid DNA were diluted in 1.5 ml of Opti-MEM® I Medium without serum and gently mixed. In a separate sterile 14 ml tube, 144 µl Lipofectamine 2000 in 6 ml of Opti-MEM I Medium without serum, gently mixed and incubated for 5 minutes at room temperature. After the 5 minute incubation, the diluted DNA was combined with the diluted Lipofectamine 2000, gently mixed and incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine 2000 complexes to form. The DNA-Lipofectamine 2000 complexes were then added dropwise to a T-225 tissue culture flask containing 20 ml Opti-MEM I Medium containing serum. Antibiotics were not included in this medium. Afterwards, 20 ml of the 293 FT cell suspension (24 x 10 6 cells) were also added to the flask containing medium and DNA-Lipofectamine 2000 complexes and mixed gently by rocking the flask back and forth. Cells were incubated at 37°C in a humidified 5% CO2 incubator for 6 hours. After the 6 h incubation period, the medium containing the DNA-Lipofectamine 2000 complexes was removed and replaced with 21 ml of complete growth medium without geneticin. At 48 hours posttransfection the virus-containing supernatants were harvested by removing medium into to a 15 ml sterile, capped, conical tube. Supernatants were centrifuged at 3000 rpm for 15 minutes at 4°C to pellet debris and the supernatad was collected and filtered through a sterile 0.45 µm filter. Viral supernatants were pipetted into cryovials in 1 ml aliquots and stored at -80°C.

4.7.2 Titration of the lentiviral stock in hMSCs

Before proceeding to transduction and expression experiments, the titer of lentiviral stocks was determined. This procedure is necessary in order to control the number of integrated copies of the lentivirus and generate reproducible expression results. These experiments took place in Biosafety Level 2 (BL-2) laboratory and all published BL-2 guidelines were strictly followed, with proper waste decontamination. To determine the titer of the lentiviral stock using hMSCs, the following procedure was performed.

The day before transduction, cells were trypsinized, counted and plated in a 6-well plate such that they would be 30-50% confluent at the time of transduction. Cells were incubated at 37°C overnight in a humidified 5% CO2 incubator. On the day of transduction, the lentiviral stock was thawed and 10-fold serial dilutions ranging from 10^{-2} to 10^{-6} were prepared. For each dilution, the lentiviral stock was diluted into complete culture medium to a final volume of 1 ml. The culture medium was then removed from the cells. Each dilution was mixed gently by inversion and added to one well of cells, the total volume being 1 ml. Polybrene was added to each well to a final concentration of 6 μg/ml. The plate was gently swirled to mix. The cells were subsequently incubated at 37°C overnight in a humidified 5% CO_2 incubator. The following day, the media containing virus were removed and replaced with 2 ml of complete culture medium. The cells were again incubated at 37°C overnight in a humidified 5% CO_2 incubator. The following day, the medium was removed and replaced with complete culture medium containing Blasticidin (10 μg/ml) to select for stably transduced cells. The medium was replaced with fresh medium containing antibiotic every 3-4 days. After 10-12 days of selection, discrete antibiotic-resistant colonies were seen in one or more of the dilution wells. The medium was removed and the cells were washed twice with PBS. 1 ml of crystal violet solution was added and cells were incubated for 10 minutes at room temperature. The crystal violet stain was removed and cells were washed with PBS twice. Finally, the blue-stained colonies were counted and the titer of the lentiviral stock was determined. The viral titer was approximately 1 x 10^5 TU/ml for each lentiviral stock.

4.7.3 Transduction of target cells

Routinely, 5 x 10^5 hMSCs were transduced in a T-75 flask by adding 2.47 x 10^5 TU viral supernatant (2.5 ml) in 5 ml complete growth medium in the presence of 10 µg/ml Polybrene. The cells were incubated at 37°C in a humidified 5% CO_2 incubator overnight with the virus. One day after transduction, the medium was replaced by fresh, non-viral medium. Blasticidine selection (10 µg/ ml) was started on the second day after transduction and it was carried out for additional 7 days.

5 Results

5.1 Construction of lentiviral vectors containing the dnIKK2 and eGFP genes

Two different lentiviral vectors were constructed, the one carrying the dnIKK2 gene and the other, which served as a control, carrying the EGFP gene.

5.1.1 Cloning the dnIKK2 gene into the lentiviral backbone

The cDNA of the dominant-negative mutant (K44A) IKK2 (dnIKK2) encoding for amino acid 1-755 and 100 bp of the 5´ untranslated region was cloned into the SmaI and NotI site of the pBC SK (+) phagemid vector. The dnIKK2 fragment was then subcloned into the entry vector pENTR11 using KpnI and NotI. Subsequently, the insert was transferred into the pLenti6/V5-DEST vector by homologous recombination with Gateway LR Clonase. The pLenti6/V5-DEST-dnIKK2 plasmid clone used for lentiviral production was verified by sequencing (Figure 15a).

5.1.2 Cloning the eGFP gene into the lentiviral backbone

The cDNA encoding enhanced green fluorescent protein (EGFP) was cloned in a similar fashion into an entry pENTR11 vector using KpnI and NotI and thereafter transferred into the pLenti6/V5-DEST vector by homologous recombination with Gateway LR Clonase in order to be used for the production of a control virus (Figure 15b).

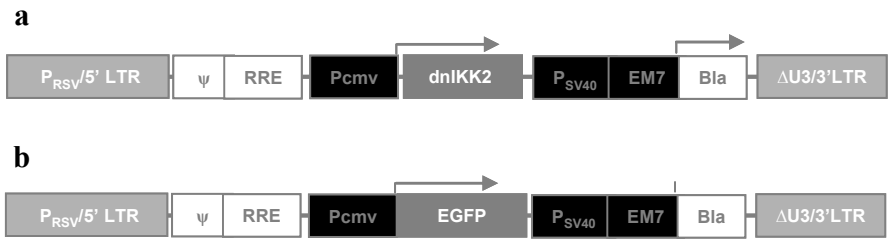

Figure 15. The dnIKK2 and EGFP lentiviral backbones. a. The diagram above shows the dnIKK2 gene cloned into the pLenti6/V5-DEST vector. b. The EGFP gene was cloned into the same sites of the Lenti6/V5-DEST vector. The Rous Sarcoma Virus (RSV) enhancer/promoter allows Tat-independent production of viral mRNA. The HIV-1 truncated 5' LTR permits viral packaging and reverse transcription of the viral mRNA. The 5' splice donor and 3' acceptors enhance the biosafety of the vector by facilitating removal of the ψ packaging sequence and RRE such that expression of the gene of interest in the transduced host cell is no longer Rev dependent. The HIV-1 psi (ψ) packaging signal allows viral packaging. The HIV-1 Rev response element (RRE) permits Rev-dependent nuclear export of unspliced viral mRNA. The CMV promoter permits high-level, constitutive expression of dnIKK2. The SV40 early promoter and origin allows high-level expression of the selection marker and episomal replication in cells expressing the SV40 large T antigen. The EM7 promoter is a synthetic prokaryotic promoter for expression of the selection marker in E. coli. The Blasticidin (Bla) resistance gene permits selection of stably transduced mesenchymal stem cells. The ΔU3 truncated 3′ LTR allows viral packaging but self-inactivates the 5′ LTR for biosafety purposes.

5.2 Generation of lentiviruses expressing eGFP and dnIKK2 and lentiviral transduction of hMSCs

Lentiviruses were generated expressing either dnIKK2 or EGFP as already described and hMSCs were transduced with the lentiviral constructs and selected for blasticidine resistance.

In order to orientate for the efficiency of lentiviral gene transfer in hMSCs, we transduced the cells with EGFP lentivirus. We achieved a transduction efficiency of 77 % in hMSCs, as determined by FACS analysis and when viral titer of 2.47 x 10^5 TU/ml was applied. Furthermore the selection of these cells by 10 µg/ml blasticidine for 7 days

significantly increased the percentage of transgene expressing cells. FACS analysis showed up to 97% EGFP-positive hMSCs after such selection. Therefore, using lentivirus with titer of 2.47 x 10^5 TU/ml followed by blasticidine selection is sufficient to achieve gene expression in almost all hMSCs (Figure 16).

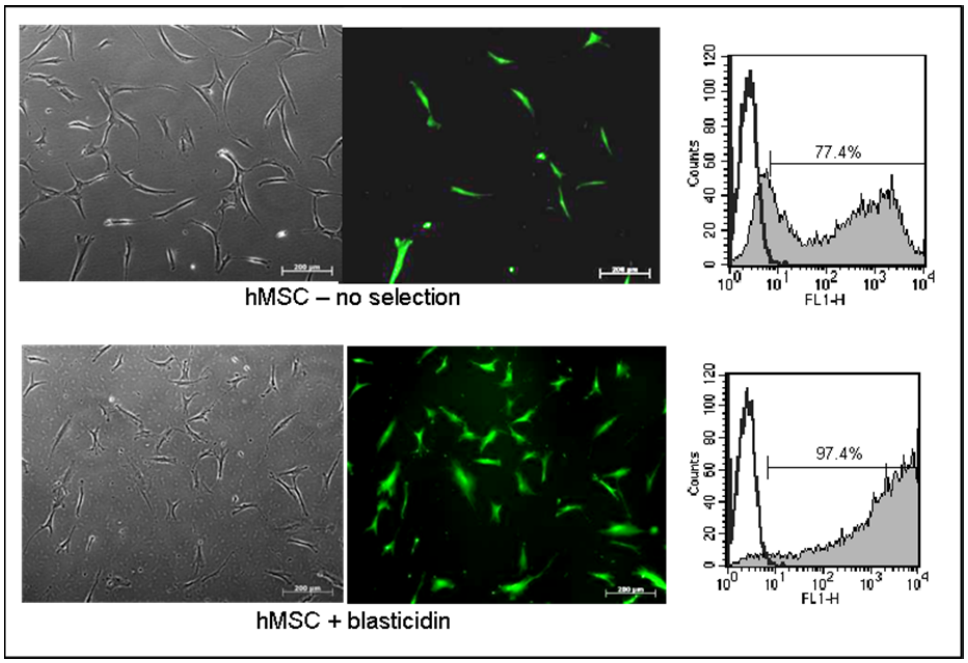

Figure 16. The efficiency of lentiviral transduction with and without blasticidin selection. EGFP-expressing controls showed a strong fluorescent signal in almost all cells, with no morphologic changes after blasticidin selection. A transduction efficiency of 77 % was achieved in hMSCs without blasticidin selection as determined by FACS analysis (standard deviation 6.47 %). FACS analysis showed up to 97 % EGFP-positive hMSCs after such selection (standard deviation 0.20 %).

5.3 DnIKK2 overexpression in mesenchymal stem cells transduced with the dnIKK2 virus

The overexpression of dnIKK2 was verified at RNA and protein level. Semi-quantitative RT-PCR was performed with primers which can not discriminate between IKK2 and its

mutant form (dnIKK2). HMSCs transducted with the dnIKK2 virus showed clearly stronger signal for IKK2 when compared to EGFP-expressing controls and these changes were also confirmed at protein level (Figure 17).

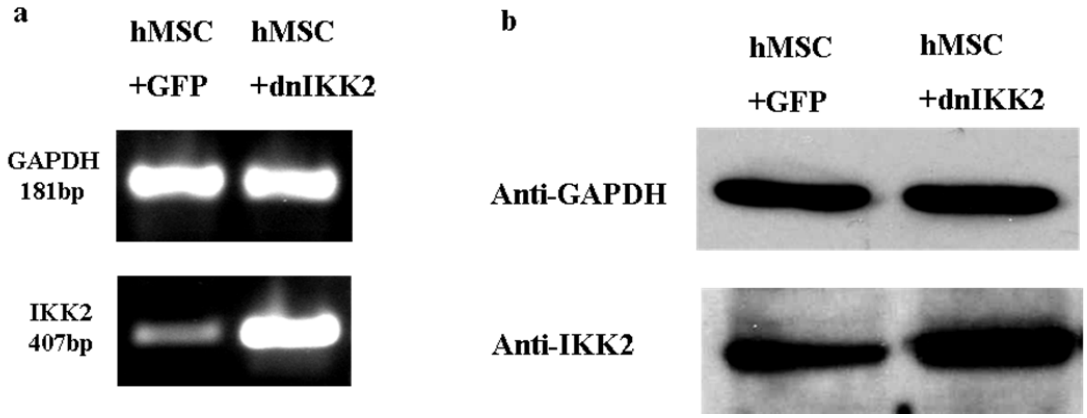

Figure 17. Stable expression of the dnIKK2. Stable expression of the dnIKK2 was verified by PCR (a) and Western blot (b). cDNA and protein samples for RT-PCR and western blot, respectively, were normalized using a housekeeping gene (GAPDH) as the internal control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is one of the most commonly used housekeeping genes used in comparisons of gene expression data.

5.4 Inhibition of function of NF-κB documented through immunohistochemistry

First, we tested for the presence of active NF-κB in hMSCs. We performed immunohistochemistry for p65, a major component of the NF-κB complex and we detected its strong expression in the cytoplasm of hMSCs. Furthermore, NF-κB (p65) translocated into the nucleoplasm of hMSC controls upon stimulation with 50 ng/ml TNF-α for 30 minutes. Finally, we evaluated whether overexpression of the dnIKK2 could block the nuclear translocation of the NF-κB after TNF-α stimulation. As expected, we observed sustained localization of NF-κB (p65) into the cytoplasm of hMSCs

overexpressing dnIKK2, regardless of stimulation with TNF-α, providing further evidence that dnIKK2 effectively blocks translocation of NF-κB into the nucleus (Figure 18).

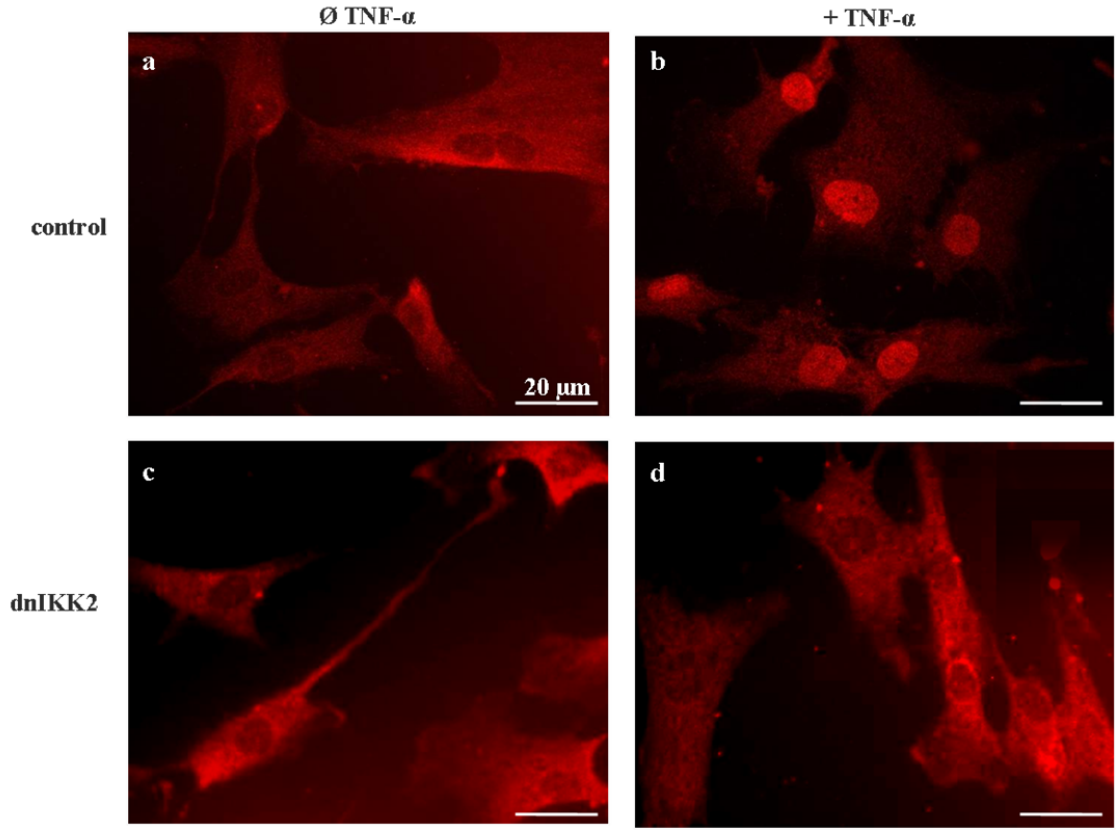

Figure 18. NF-κB (p65) immunohistochemistry on hMSCs. NF-κB (p65) was primarily located within the cytoplasm of unstimulated EGFP- and dnIKK2-transduced hMSCs (a, c). TNF-α stimulation resulted in nuclear translocation of the NF-κB (p65) complex in EGFP-transduced control (b). Nuclear translocation of the NF-κB complex was effectively blocked by overexpression of dnIKK2 (d).

5.5 The effect of dnIKK2 on TNF-α-induced hMSC migration

We performed cell invasion assays with EGFP- and dnIKK2-hMSCs in transwell chambers. Additionally to the complete growth, we used media 50 ng/ml TNF-α as

chemoattractant. Determination of hMSC migration rates after 48 hour-incubation period revealed TNF-α-assosiated-, highly increased- cell trafficking. Stimulation of EGFP-transduced hMSCs with TNF-α led to a 28.9-fold increase in cell invasion ($p<0.00001$), suggesting that TNF-α is an important migratory stimulus for hMSCs. Overexpression of the dnIKK2 caused a significant 43.1% reduction of hMSC invasion ($p<0.0001$), suggesting that the NF-κB signal transduction pathway and IKK2 are involved in hMSCs migration in response to TNF-α (Figure 19).

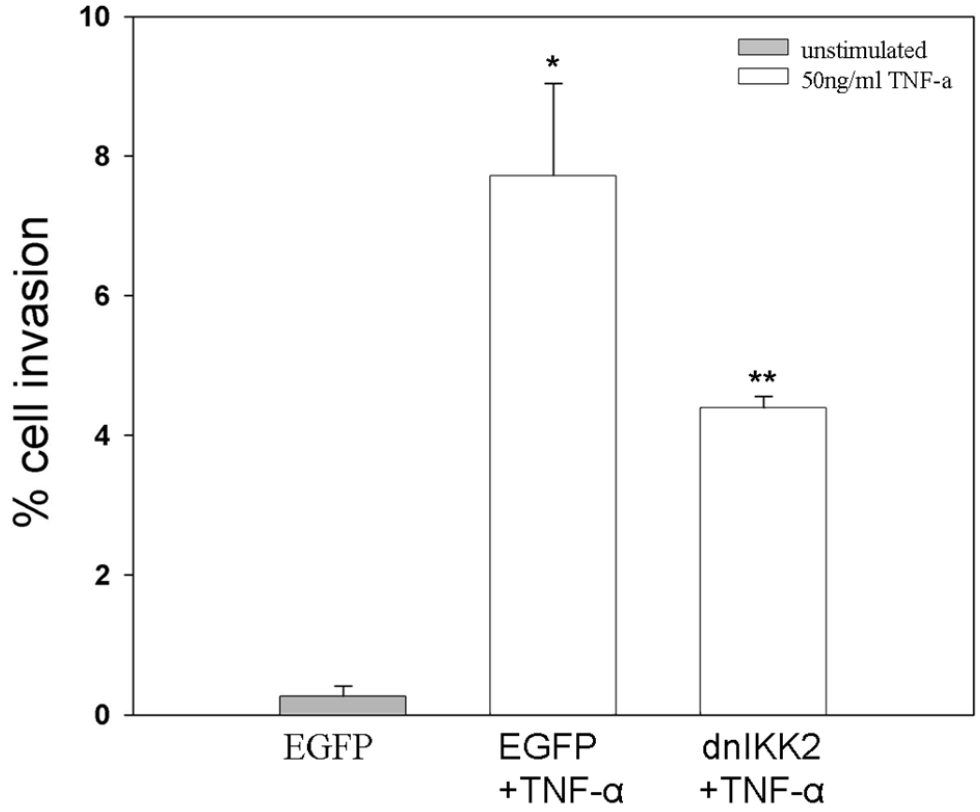

Figure 19. Transwell invasion assay of lentivirally-transduced hMSCs. EGFP-transduced hMSCs show low invasion rate during standard conditions (0.27%). Stimulation with 50 ng/ml TNF-α caused a 28.9-fold increase of EGFP-transduced hMSCs invasion (*p<0.00001), whereas overexpression of the dominant-negative mutant of IKK2 caused a significant 43.1% reduction of hMSC migration (p<0.0001).**

5.6 The effect of dnIKK2 on TNF-α-induced hMSC proliferation

It has been reported that additionally to migration, TNF-α promotes cell proliferation via the NF-κB signal transduction pathway (Widera, Mikenberg et al. 2006). We could show that stimulation of EGFP-transduced hMSCs with 10 ng/ml TNF-α caused a 58 % increase in cell proliferation ($p<0.00005$). Overexpression of dnIKK2 revealed a 28 % reduction of hMSC proliferation ($p<0.0001$) without stimulation, suggesting a role of IKK2 in hMSC proliferation under basal conditions. Most likely, this effect is triggered by the TNF-α contained in the serum supplement of the growth culture medium. Furthermore, when additional TNF-α was added during the culture of dnIKK2-hMSCs no increase in the proliferation was observed. Thus, our results suggest that the positive effect of TNF-α on hMSC proliferation is primarily mediated via the NF-κB signal transduction pathway and IKK2 (Figure 20).

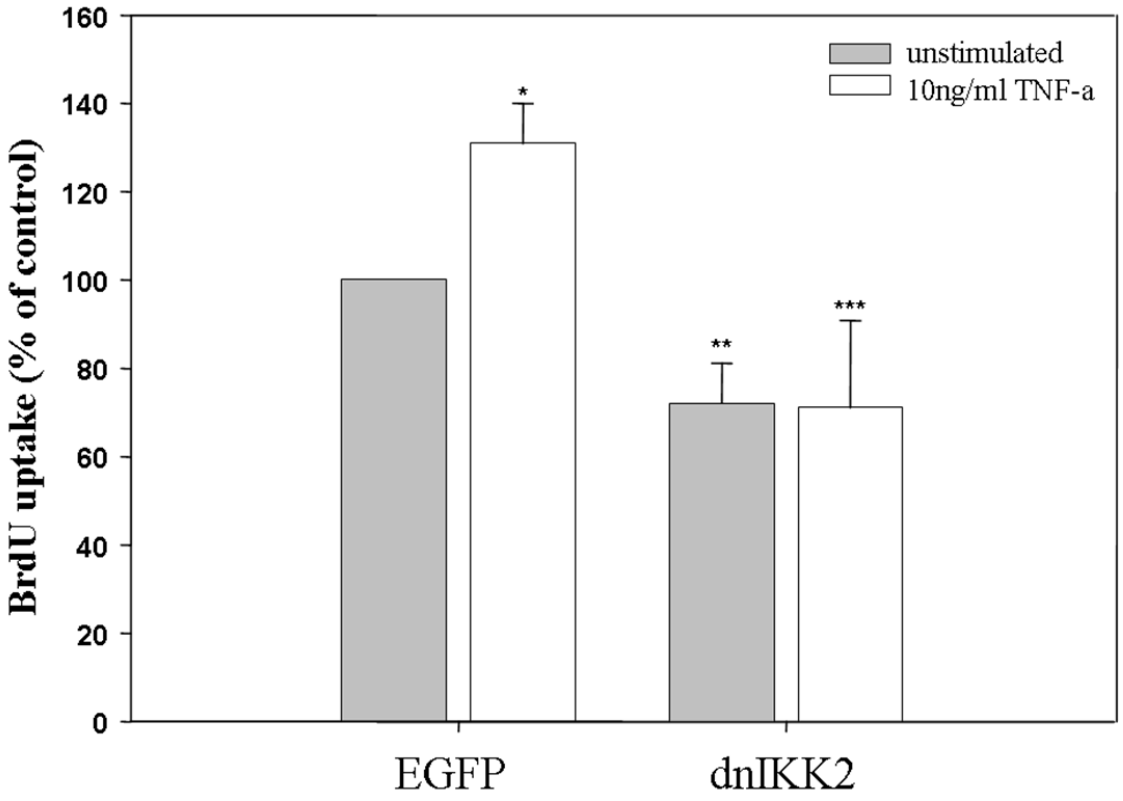

Figure 20. BrdU proliferation assay of lentivirally-transduced hMSCs. Stimulation of EGFP-transduced hMSCs with 10 ng/ml TNF-α caused a highly significant increase in hMSC proliferation in vitro (*p<0.00005 vs. unstimulated control). Overexpression of dn-IKK-2 revealed a significant 28% reduction of proliferation under standard conditions (p<0.0001 vs. unstimulated control) and the stimulating effect of TNF-α was completely blocked in these cells (***p<0.0001).**

5.7 Analysis of plausible downstream target genes of NF-κB

It is known, that once NF-κB localizes in the nucleus, gene transcription is activated. Therefore, we checked if upon TNF-α stimulation the gene expression of known NF-κB target genes, such as VCAM-1 and other hMSC-migration-related genes as β1- and αv-integrins and CD44 are upregulated in hMSCs.

RT-PCR data demonstrated upregulation of VCAM-1 and CD44 in both EGFP- and dnIKK2-transduced hMSCs, whereas the abundant β1- and αv-integrins did not undergo

visible changes when cells were stimulated with TNF-α (Figure 21). Interestingly, dnIKK2 did not have an inhibitory effect in the expression of VCAM-1 and CD44.

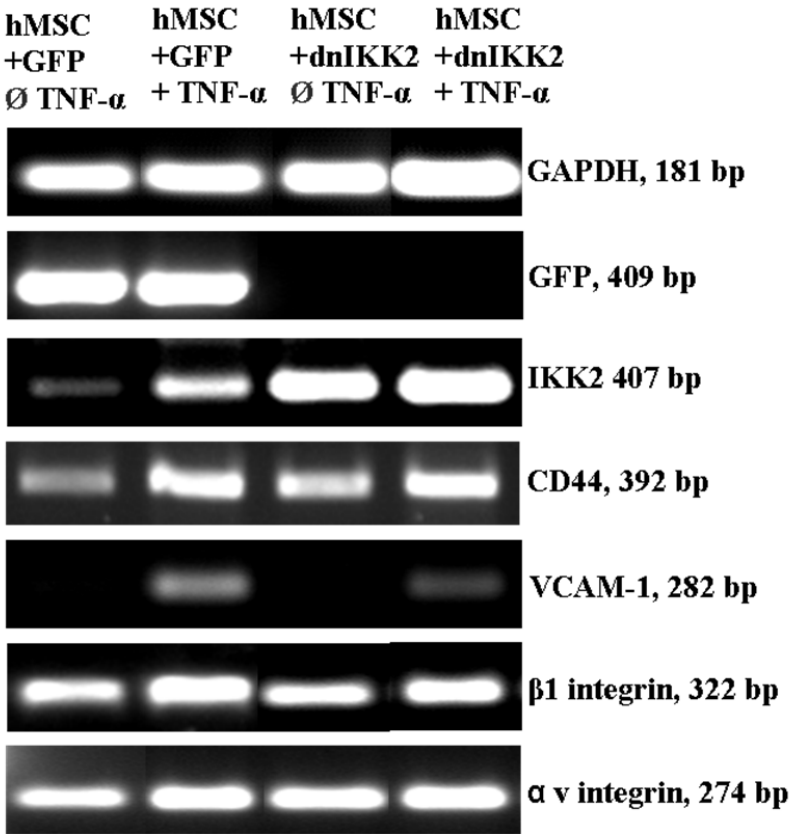

Figure 21. RT-PCR results of plausible downstream targets. Stimulation of EGFP- and dnIKK2-transduced hMSCs with 10 ng/ml TNF-α for 4 hours revealed a strong upregulation of CD44 and VCAM-1, whereas the level of the abundant β1- and αv-integrins remained unchanged.

6 Discussion

Our data give conclusive evidence that bone marrow-derived hMSCs can migrate along a TNF-α gradient and transverse human reconstituted basement membranes. This effect is partly blocked by the overexpression of a dnIKK2. To our knowledge, this is the first report that hMSCs invasion and migration through human extracellular matrix is at least in part mediated through the activation of IKK2, a key regulatory enzyme of the NF-κB signal transduction pathway, after stimulation with TNF-α.

The cytokine TNF-α, which is known to activate the NF-κB pathway, shows increased plasma levels in various human models of tissue injury (Jiang, Tian et al. 1997; Fahim, Halim et al. 2004) and plays an important role in tissue repair (Gerstenfeld, Cho et al. 2003). Cytokines like TNF-α are not only secreted from macrophages and inflammatory cells, but also released from cells of mesenchymal origin (Kon, Cho et al. 2001). TNF-α may recruit hMSCs early in the course of tissue injury. During the initial step of extravasasion TNF-α increases adhesion of hMSCs to endothelial cells (Ruster, Gottig et al. 2006). But currently it is unknown if TNF-α acts systemically to mobilize hMSCs or attract these cells locally within the injured tissue.

So far, the precise molecular mechanisms of hMSC migration and invasion are largely unknown. Neth et al. has recently shown that the Wnt pathway plays a critical role in regulating hMSC migration and invasion (Neth, Ciccarella et al. 2006). C-Jun N-terminal kinase plays a role in PDGF-induced migration of adipose tissue-derived hMSCs (Kang, Jeon et al. 2005). Others have shown that blocking the activation of NF-κB by TNF-α inhibits fibroblast migration and invasion. In immune and cancer cells TNF-α regulates the NF-κB pathway by activation of IKK2 (Karin, Yamamoto et al. 2004). Here, we have shown for the first time that IKK2 is crucial for NF-κB activation in hMSCs after TNF-α stimulation and blocking IKK2 leads to a decreased invasion of hMSC.

In spite of many important proceedings on the field of mesenchymal stem cells migration the target genes which orchestrate homing of MCSs are also still largely unknown. Particularly, it is unknown which NF-κB target genes are responsible for TNF-induced hMSC migration. Ries et al. have shown that TNF-α induces the expression of MMP-9 in

hMSCs (Ries, Egea et al. 2007). In vascular smooth muscle cells MMP-9 expression is regulated by TNF-α via the NF-κB activation. We found that CD44, β1 integrin and VCAM-1 are upregulated in hMSCs after TNF-α stimulation. CD44 has been found to be important for hMSC migration into the extracellular matrix (Zhu, Mitsuhashi et al. 2006). In other cells of mesenchymal origin such as fibroblast β1 integrin and VCAM-1 increase migration. Identification of molecular pathways directing hMSC migration might be crucial for improved therapeutic intervention in several diseases and therefore they have to be further investigated. Additionally to the invasion, our results clearly show that hMSC proliferation is also increased by TNF-α stimulation and can be effectively blocked by overexpression of a dnIKK2. In accordance to our data, in adult neuronal stem cells TNF-α has been shown to strongly increase proliferation by activation of IKK-2 and the canonical NF-κB pathway (Widera, Mikenberg et al. 2006). In intestinal myofibrobasts, TNF-α stimulation resulted in increased proliferation via activation of the TNF receptor 2 (Theiss, Simmons et al. 2005). In a recent study, convincing genetic evidence has been presented for a cell type-restricted requirement for NF-κB in the control of proliferation (Mehrhof, Schmidt-Ullrich et al. 2005). Thus, the role of NF-κB in proliferation has to be investigated separately for each cell type. For example, in some cell types NF-κB controls proliferation through transcriptional regulation of cyclin D1 and delayed progression into G1 cell cycle (Guttridge, Albanese et al. 1999). Moreover, IKK-2 knockout promotes migration and proliferation of mouse embryo fibroblast cells (Chen, Lu et al. 2006). These findings, however, do not necessarily reflect the function of NF-κB in hMSCs. Therefore the precise mechanism by which NF-κB controls hMSC proliferation needs to be still elucidated.

In conclusion, we report here that hMSCs increase invasion and proliferation in response to TNF-α via IKK2 activation in vitro. To our knowledge, this is the first comprehensive report on the involvement of the NF-κB pathway in hMSC migration. Furthermore, this is also the first report showing that TNF has a significant effect on hMSC proliferation in vitro, and that this effect is mediated through IKK-2 and NF-κB pathway activation. Harnessing the migratory and proliferation potential of hMSCs by modulating the NF-κB pathway may be a powerful tool to enhance MSC engraftment after transplantation,

increase their ability to correct inherent disorders of different tissues, or facilitate tissue repair *in vivo*.

7 Summary

Mesenchymal stem cells (MSCs) can contribute to tissue repair by actively migrating to sites of tissue injury. However, the cellular and molecular mechanisms of MSC recruitment are largely unknown. The NF-κB pathway plays a pivotal role in regulating genes that influence cell migration, cell differentiation, inflammation and proliferation. One of the major cytokines released at sites of injury is TNF-α, which is known to be a key regulator of the NF-κB pathway. Therefore, we hypothesized that TNF-α may lead to MSC migration and proliferation by activation of the NF-κB pathway.

Intriguingly, NF-κB (p65) and IκB kinase 2 (IKK2) are expressed in human MSCs (hMSCs), which we documented with immunohistochemistry and PCR respectively. Furthermore, stimulation of hMSCs with TNF-α caused a NF-κB (p65) translocation from cytoplasm to nucleoplasm and upregulation of target genes. Blocking the NF-κB pathway by overexpressing a dominant-negative mutant of IKK2 (dnIKK2) in hMSC, using highly efficient lentiviral gene transfer, effectively inhibited the nuclear translocation of NF-κB (p65). Moreover, TNF-α strongly augmented the migration of hMSCs through human extracellular matrix but its effect was significantly blocked by dn-IKK-2. Additionally, using BrdU assay we showed that inhibition of NF-κB (p65) pathway had a significant effect on the basal proliferation rate of hMSCs. TNF-α stimulated the proliferation of hMSCs, whereas the overexpression of dnIKK2 lead to diminished cell growth.

The precise understanding of the role of NF-κB in hMSC proliferation and migration could have vast clinical applications in a wide spectrum of diseases, including the leading causes of death in the western world, namely cardiovascular disease and cancer (Dai, Moniri et al. 2011; Ding, Shyu et al. 2011). A recent study investigating the potential of hMSC's in cardiac therapeutics demonstrated that activation of NF-κB by pre-treatment of MSC's with TNF-α increased the rate of engraftment of hMSC's into myocardial tissue and improved recovery of cardiac function after myocardial infarction (Kim, Park et al. 2009). Recently, in the field of cancer research, a biologic process that allows

conversion of epithelial-differentiated cells into cells that resemble mesenchymal stem cells has been described (Kalluri and Weinberg 2009; Thiery, Acloque et al. 2009). Studies indicate that this process, termed as epithelial-to-mesenchymal transition, plays an important role in the development and progression of cancer, specifically in the dissemination of cancer cells and formation of metastases, but also in the resistance to apoptosis and chemotherapy (Chaffer and Weinberg 2011). NF-κB has been shown to be a key player in this process in several cancer types (Huber, Azoitei et al. 2004; Huber, Beug et al. 2004; Maier, Schmidt-Strassburger et al. 2010). This finding is of potential therapeutic interest, as shown by a recent study of an orally active pharmacological inhibitor of IKK2, which not only reversed the epithelial-to-mesenchymal transition in murine mammary carcinoma cells, but also suppressed metastatic activity of these cells from orthotopic sites (Huber, Maier et al. 2010).

In conclusion, components of NF-κB pathway (p65, IKK2) are expressed in hMSCs. It is known that NF-κB pathway is regulated by TNF-α through activation of IKK-2. Here, we provide evidence that this signal transduction pathway is also implicated in TNF-α-mediated migration and proliferation of hMSCs. Therefore, our results support the idea that hMSC recruitment to sites of tissue injury may at least in part be regulated by NF-κB signal transduction pathway.

8 Zusammenfassung

Mesenchymale Stammzellen (MSCs) können zur Gewebsreparatur beitragen, indem sie aktiv an Orte mit Gewebsverletzung wandern. Die zellulären und molekularen Mechanismen der MSC Rekrutierung sind jedoch größtenteils unbekannt. Der NF-κB Signalweg spielt eine zentrale Rolle bei der Regulation von Genen die Zellmigration, Zelldifferenzierung, Inflammation und Zellproliferation beeinflussen. Eines der wichtigen Zytokine, das aus verletzten Geweben freigesetzt wird, ist TNF-α. TNF-α ist ein wichtiger Regulator des NF-κB Signaltransduktionsweges. Unsere Hypothese war, dass TNF-α die Invasion und Proliferation von MSCs durch die Aktivierung des NF-κB Signalweges beeinflussen kann. NF-κB und IKK2 werden in humanen MSCs exprimiert, was wir mittels Immunohistochemie und PCR bestätigten. Die TNF-α Stimulation von hMSCs führte zur Translokation von NF-κB vom Zytoplasma in den Kern und zu einer Hochregulierung der Genexpression von NF-κB Zielgenen. Die Blockierung des NF-κB Signalwegs durch eine dominant-negative Mutante von IKK2 (dnIKK2) mittels lentiviraler Transduktion behinderte die Translokation von NF-κB in den Kern der hMSCs. TNF-α löste noch eine verstärkte Invasion von hMSCs durch extrazelluläre humane Matrix aus, dieser Effekt wurde aber signifikant durch dnIKK2 blockiert. Darüber hinaus konnten wir in BrdU Proliferationsversuchen zeigen, dass die Inhibierung des NF-κB Signalwegs einen signifikanten Effekt auf die basale Proliferationsrate von hMSCs hat. TNF-α stimulierte die Proliferation von hMSC Zellen, während die Überexpression von dnIKK2 das Zellwachstum blockierte.

Zusammenfassend kann gesagt werden, dass NF-κB Komponente in hMSCs exprimiert werden. Es ist bekannt, dass der TNF-α-induzierte NF-κB Signalweg hauptsächlich über IKK2 reguliert wird. Wir haben gezeigt, dass dieser Signalweg auch in TNF-α-induzierter hMSC Invasion und Proliferation involviert ist. Unsere Ergebnisse lassen deshalb vermuten, dass die Rekrutierung von hMSC an Ort mit Gewebsverletzung teilweise durch den NF-κB Signalweg reguliert sein könnte.

9 Curriculum Vitae

Emmanouil P. Pappou

Contact information:
Permanent Home Address: Artemidos 5 Str., 85100 Rhodes, Greece
Home Tel. +30 22410 65123
Current Address: 110 W 39th Street, Apt. 1202, Baltimore, MD, USA
Current Tel. +1 443 717 2541
email: manolis.pappou@gmail.com

Date of Birth: February 11, 1982
Citizenship: Greek
Hometown: Rhodes, Greece

UNDERGRADUATE STUDIES

Rhodes, Greece
1991–1994: 4th Junior high school of Rhodes. Absolved with grade 19 $^{13}/_{14}$ (max 20).
1994–1997: 2nd Senior High school of Rhodes. Absolved with grade 20 (max 20).

MEDICAL SCHOOL

Munich, Germany
April 2000 - April 2002: Preclinical part of studies at the medical Faculty of the Ludwig-Maximilians-University.

April 2002- May 2006: Clinical part of studies at the medical Faculty of the Technical University Munich.

Date of degree: 10th May 2006, cumulative grade 1.49 “sehr gut”.
Approbation Certificate acquired on 21st of June 2006.

RESEARCH ACTIVITY

May 2003 - June 2007: Doctoral thesis at the Surgical Research Laboratory of the Surgical Department of the LMU University. (Director: Prof. Dr. Wolf Mutschler).

RESIDENCY

July 1 2007- present: Categorical Resident at the Department of Surgery of the Johns Hopkins Hospital, Baltimore, USA.

UNITED STATES MEDICAL LICENSING EXAMINATION (USMLE)

USMLE Step 1 Exam Date: 14 May 2004, Score 229, 93
USMLE Step 2 CK Exam Date: 23 Oct 2006, Score 234, 95
USMLE Step 2 CS Exam Date: 15 Nov 2006, Passed
USMLE Step 3 Exam Date: 18, 19 June 2007, Score 221, 91
Standard ECFMG Certificate Issue Date: 01 Feb 2007

AMERICAN BOARD OF SURGERY IN-TRAINING EXAMINATION (ABSITE)

Exam Date: January 26, 2008, Score 95th percentile
Exam Date: January 31, 2009, Score 98th percentile

Exam Date: January 30, 2010, Score 90th percentile
Exam Date: January 28, 2011, Score 93rd percentile

AWARDS/HONORS

Johns Hopkins Department of Surgery Core Values in Practice Award, 2009.

Johns Hopkins George D. Zuidema Research Award for Meritorious Laboratory Research, 2010.

Johns Hopkins Hospital House Staff Council Vice-President, 2010-2011.

PUBLICATIONS

1. Böcker, W., D. Docheva, W.C. Prall, V. Egea, **E. Pappou**, O. Rossmann, C. Popov, W. Mutschler, C. Ries, and M. Schieker, *IKK-2 is required for TNF-alpha-induced invasion and proliferation of human mesenchymal stem cells.* J Mol Med, 2008. **86**(10): p. 1183-92.

2. Dhir, M., S. Yachida, L. Van Neste, S.C. Glockner, J. Jeschke, **E.P. Pappou**, E.A. Montgomery, J.G. Herman, S.B. Baylin, C. Iacobuzio-Donahue, and N. Ahuja, *Sessile serrated adenomas and classical adenomas: An epigenetic perspective on premalignant neoplastic lesions of the gastrointestinal tract.* Int J Cancer, 2010.

3. **Pappou, E.P**. and N. Ahuja, *The role of oncogenes in gastrointestinal cancer.* Gastrointest Cancer Res, 2010(Suppl 1): p. S2-S15.

BOOK CHAPTERS

1. **Emmanouil Pappou**, Kent Stevens, *When patients have rib fractures, always assume associated solid organ injuries and treat pain aggressively*, in *Avoiding Common Errors in the Emergency Department*, A.S.C. Amal Mattu, Stuart P. Swadron, Carrie Tibbles, Dale Woolridge, Editor. 2010, Lippincott Williams & Wilkins.

2. SR Downing, **Emmanouil P. Pappou**, N Ahuja, *Behavior and Dietary Modification in the Prevention of Colon Cancer*, in *Early Diagnosis and Treatment of Cancer Series: Colorectal Cancer*, N.A. Susan Gearhart, Editor. 2010, Saunders.

10 References

Akkina, R. K., R. M. Walton, et al. (1996). "High-efficiency gene transfer into CD34+ cells with a human immunodeficiency virus type 1-based retroviral vector pseudotyped with vesicular stomatitis virus envelope glycoprotein G." J Virol **70**(4): 2581-2585.

Andersson, S., D. L. Davis, et al. (1989). "Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme." J Biol Chem **264**(14): 8222-8229.

Anjos-Afonso, F., E. K. Siapati, et al. (2004). "In vivo contribution of murine mesenchymal stem cells into multiple cell-types under minimal damage conditions." J Cell Sci **117**(Pt 23): 5655-5664.

Arber, W. (1965). "Host-controlled modification of bacteriophage." Annu Rev Microbiol **19**: 365-378.

Asakura, A., M. Komaki, et al. (2001). "Muscle satellite cells are multipotential stem cells that exhibit myogenic, osteogenic, and adipogenic differentiation." Differentiation **68**(4-5): 245-253.

Aslan, H., Y. Zilberman, et al. (2006). "Osteogenic differentiation of noncultured immunoisolated bone marrow-derived CD105+ cells." Stem Cells **24**(7): 1728-1737.

Aubin, J. E., F. Liu, et al. (1995). "Osteoblast and chondroblast differentiation." Bone **17**(2 Suppl): 77S-83S.

Awad, H. A., D. L. Butler, et al. (1999). "Autologous mesenchymal stem cell-mediated repair of tendon." Tissue Eng **5**(3): 267-277.

Baxter, F. O., P. J. Came, et al. (2006). "IKKbeta/2 induces TWEAK and apoptosis in mammary epithelial cells." Development **133**(17): 3485-3494.

Bernard, P. and M. Couturier (1992). "Cell killing by the F plasmid CcdB protein involves poisoning of DNA-topoisomerase II complexes." J Mol Biol **226**(3): 735-745.

Beyer Nardi, N. and L. da Silva Meirelles (2006). "Mesenchymal stem cells: isolation, in vitro expansion and characterization." Handb Exp Pharmacol(174): 249-282.

Bianco, P. and P. Gehron Robey (2000). "Marrow stromal stem cells." J Clin Invest **105**(12): 1663-1668.

Bianco, P., M. Riminucci, et al. (2001). "Bone marrow stromal stem cells: nature, biology, and potential applications." Stem Cells **19**(3): 180-192.

Bianco, P. and P. G. Robey (2001). "Stem cells in tissue engineering." Nature **414**(6859): 118-121.

Birnboim, H. C. and J. Doly (1979). "A rapid alkaline extraction procedure for screening recombinant plasmid DNA." Nucleic Acids Res **7**(6): 1513-1523.

Bonizzi, G. and M. Karin (2004). "The two NF-kappaB activation pathways and their role in innate and adaptive immunity." Trends Immunol **25**(6): 280-288.

Boshart, M., F. Weber, et al. (1985). "A very strong enhancer is located upstream of an immediate early gene of human cytomegalovirus." Cell **41**(2): 521-530.

Bruder, S. P., N. Jaiswal, et al. (1997). "Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation." J Cell Biochem **64**(2): 278-294.

Bruder, S. P., A. A. Kurth, et al. (1998). "Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells." J Orthop Res **16**(2): 155-162.

Burns, J. C., T. Friedmann, et al. (1993). "Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells." Proc Natl Acad Sci U S A **90**(17): 8033-8037.

Caplan, A. I. (1991). "Mesenchymal stem cells." J Orthop Res **9**(5): 641-650.

Castro-Malaspina, H., R. E. Gay, et al. (1980). "Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny." Blood **56**(2): 289-301.

Chaffer, C. L. and R. A. Weinberg (2011). "A perspective on cancer cell metastasis." Science **331**(6024): 1559-1564.

Chen, C., C. Chou, et al. (2001). "Tumor necrosis factor alpha-induced activation of downstream NF-kappaB site of the promoter mediates epithelial ICAM-1 expression and monocyte adhesion. Involvement of PKCalpha, tyrosine kinase, and IKK2, but not MAPKs, pathway." Cell Signal **13**(8): 543-553.

Chen, F., Y. Lu, et al. (2006). "Loss of Ikkbeta promotes migration and proliferation of mouse embryo fibroblast cells." J Biol Chem **281**(48): 37142-37149.

Chen, F. E., D. B. Huang, et al. (1998). "Crystal structure of p50/p65 heterodimer of transcription factor NF-kappaB bound to DNA." Nature **391**(6665): 410-413.

Chen, L. B., X. B. Jiang, et al. (2004). "Differentiation of rat marrow mesenchymal stem cells into pancreatic islet beta-cells." World J Gastroenterol **10**(20): 3016-3020.

Cheng, L., P. Qasba, et al. (2000). "Human mesenchymal stem cells support megakaryocyte and pro-platelet formation from CD34(+) hematopoietic progenitor cells." J Cell Physiol **184**(1): 58-69.

Cohen, M., A. Meisser, et al. (2006). "Involvement of MAPK pathway in TNF-alpha-induced MMP-9 expression in human trophoblastic cells." Mol Hum Reprod **12**(4): 225-232.

Colter, D. C., R. Class, et al. (2000). "Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow." Proc Natl Acad Sci U S A **97**(7): 3213-3218.

Colter, D. C., I. Sekiya, et al. (2001). "Identification of a subpopulation of rapidly self-renewing and multipotential adult stem cells in colonies of human marrow stromal cells." Proc Natl Acad Sci U S A **98**(14): 7841-7845.

Conget, P. A. and J. J. Minguell (1999). "Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells." J Cell Physiol **181**(1): 67-73.

Cormack, B. P., R. H. Valdivia, et al. (1996). "FACS-optimized mutants of the green fluorescent protein (GFP)." Gene **173**(1 Spec No): 33-38.

Cramer, P., C. J. Larson, et al. (1997). "Structure of the human NF-kappaB p52 homodimer-DNA complex at 2.1 A resolution." Embo J **16**(23): 7078-7090.

Cramer, P. and C. W. Muller (1999). "A firm hand on NFkappaB: structures of the IkappaBalpha-NFkappaB complex." Structure **7**(1): R1-6.

Croy, C. H., S. Bergqvist, et al. (2004). "Biophysical characterization of the free IkappaBalpha ankyrin repeat domain in solution." Protein Sci **13**(7): 1767-1777.
D'Alessio, J. M. and G. F. Gerard (1988). "Second-strand cDNA synthesis with E. coli DNA polymerase I and RNase H: the fate of information at the mRNA 5' terminus and the effect of E. coli DNA ligase." Nucleic Acids Res **16**(5): 1999-2014.
D'Ippolito, G., S. Diabira, et al. (2004). "Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential." J Cell Sci **117**(Pt 14): 2971-2981.
da Silva Meirelles, L., P. C. Chagastelles, et al. (2006). "Mesenchymal stem cells reside in virtually all post-natal organs and tissues." J Cell Sci **119**(Pt 11): 2204-2213.
Dai, L. J., M. R. Moniri, et al. (2011). "Potential implications of mesenchymal stem cells in cancer therapy." Cancer Lett **305**(1): 8-20.
Danna, K. and D. Nathans (1971). "Specific cleavage of simian virus 40 DNA by restriction endonuclease of Hemophilus influenzae." Proc Natl Acad Sci U S A **68**(12): 2913-2917.
Davis, B. M., L. Humeau, et al. (2004). "ABC transporter inhibitors that are substrates enhance lentiviral vector transduction into primitive hematopoietic progenitor cells." Blood **104**(2): 364-373.
De Bari, C., F. Dell'Accio, et al. (2001). "Multipotent mesenchymal stem cells from adult human synovial membrane." Arthritis Rheum **44**(8): 1928-1942.
Delhase, M., M. Hayakawa, et al. (1999). "Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation." Science **284**(5412): 309-313.
Deng, W., Q. Han, et al. (2005). "Engrafted bone marrow-derived flk-(1+) mesenchymal stem cells regenerate skin tissue." Tissue Eng **11**(1-2): 110-119.
Devin, A., A. Cook, et al. (2000). "The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation." Immunity **12**(4): 419-429.
Di Nicola, M., C. Carlo-Stella, et al. (2002). "Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli." Blood **99**(10): 3838-3843.
DiDonato, J. A., M. Hayakawa, et al. (1997). "A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB." Nature **388**(6642): 548-554.
Digirolamo, C. M., D. Stokes, et al. (1999). "Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate." Br J Haematol **107**(2): 275-281.
Ding, D. C., W. C. Shyu, et al. (2011). "Mesenchymal stem cells." Cell Transplant **20**(1): 5-14.
Dominici, M., K. Le Blanc, et al. (2006). "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement." Cytotherapy **8**(4): 315-317.
Dull, T., R. Zufferey, et al. (1998). "A third-generation lentivirus vector with a conditional packaging system." J Virol **72**(11): 8463-8471.

Erices, A., P. Conget, et al. (2000). "Mesenchymal progenitor cells in human umbilical cord blood." Br J Haematol **109**(1): 235-242.
Fahim, M. R., S. M. Halim, et al. (2004). "Tumor necrosis factor alpha in patients with acute myocardial infarction." Egypt J Immunol **11**(1): 31-37.
Fehlmann, C., R. Krapf, et al. (1993). "Reverse transcriptase can block polymerase chain reaction." Clin Chem **39**(2): 368-369.
Ferrari, G., G. Cusella-De Angelis, et al. (1998). "Muscle regeneration by bone marrow-derived myogenic progenitors." Science **279**(5356): 1528-1530.
Franzoso, G., L. Carlson, et al. (1997). "Requirement for NF-kappaB in osteoclast and B-cell development." Genes Dev **11**(24): 3482-3496.
Friedenstein, A. J., R. K. Chailakhjan, et al. (1970). "The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells." Cell Tissue Kinet **3**(4): 393-403.
Friedenstein, A. J., S. Piatetzky, II, et al. (1966). "Osteogenesis in transplants of bone marrow cells." J Embryol Exp Morphol **16**(3): 381-390.
Galmiche, M. C., V. E. Koteliansky, et al. (1993). "Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway." Blood **82**(1): 66-76.
Gerondakis, S., M. Grossmann, et al. (1999). "Genetic approaches in mice to understand Rel/NF-kappaB and IkappaB function: transgenics and knockouts." Oncogene **18**(49): 6888-6895.
Gerstenfeld, L. C., T. J. Cho, et al. (2003). "Impaired fracture healing in the absence of TNF-alpha signaling: the role of TNF-alpha in endochondral cartilage resorption." J Bone Miner Res **18**(9): 1584-1592.
Ghosh, S. and M. Karin (2002). "Missing pieces in the NF-kappaB puzzle." Cell **109 Suppl**: S81-96.
Ghosh, S., M. J. May, et al. (1998). "NF-kappa B and Rel proteins: evolutionarily conserved mediators of immune responses." Annu Rev Immunol **16**: 225-260.
Gregory, C. A., H. Singh, et al. (2003). "The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow." J Biol Chem **278**(30): 28067-28078.
Gronthos, S., S. E. Graves, et al. (1994). "The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors." Blood **84**(12): 4164-4173.
Guttridge, D. C., C. Albanese, et al. (1999). "NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1." Mol Cell Biol **19**(8): 5785-5799.
Hanahan, D. (1983). "Studies on transformation of Escherichia coli with plasmids." J Mol Biol **166**(4): 557-580.
Haynesworth, S. E., M. A. Baber, et al. (1992). "Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies." Bone **13**(1): 69-80.
Hopkins, N. (1993). "High titers of retrovirus (vesicular stomatitis virus) pseudotypes, at last." Proc Natl Acad Sci U S A **90**(19): 8759-8760.
Hsu, H., H. B. Shu, et al. (1996). "TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways." Cell **84**(2): 299-308.

Hu, Y., V. Baud, et al. (2001). "IKKalpha controls formation of the epidermis independently of NF-kappaB." Nature **410**(6829): 710-714.
Huber, M. A., N. Azoitei, et al. (2004). "NF-kappaB is essential for epithelial-mesenchymal transition and metastasis in a model of breast cancer progression." J Clin Invest **114**(4): 569-581.
Huber, M. A., H. Beug, et al. (2004). "Epithelial-mesenchymal transition: NF-kappaB takes center stage." Cell Cycle **3**(12): 1477-1480.
Huber, M. A., H. J. Maier, et al. (2010). "BI 5700, a Selective Chemical Inhibitor of IκB Kinase 2, Specifically Suppresses Epithelial-Mesenchymal Transition and Metastasis in Mouse Models of Tumor Progression." Genes Cancer **1**(2): 101-114.
Izumi, M., H. Miyazawa, et al. (1991). "Blasticidin S-resistance gene (bsr): a novel selectable marker for mammalian cells." Exp Cell Res **197**(2): 229-233.
Jacobs, M. D. and S. C. Harrison (1998). "Structure of an IkappaBalpha/NF-kappaB complex." Cell **95**(6): 749-758.
Jiang, J., K. Tian, et al. (1997). "Kinetics of plasma cytokines and its clinical significance in patients with severe trauma." Chin Med J (Engl) **110**(12): 923-926.
Jiang, Y., B. N. Jahagirdar, et al. (2002). "Pluripotency of mesenchymal stem cells derived from adult marrow." Nature **418**(6893): 41-49.
Johnson, C., D. Van Antwerp, et al. (1999). "An N-terminal nuclear export signal is required for the nucleocytoplasmic shuttling of IkappaBalpha." Embo J **18**(23): 6682-6693.
Jorgensen, C., F. Djouad, et al. (2003). "Engineering mesenchymal stem cells for immunotherapy." Gene Ther **10**(10): 928-931.
Kadivar, M., S. Khatami, et al. (2006). "In vitro cardiomyogenic potential of human umbilical vein-derived mesenchymal stem cells." Biochem Biophys Res Commun **340**(2): 639-647.
Kadiyala, S., R. G. Young, et al. (1997). "Culture expanded canine mesenchymal stem cells possess osteochondrogenic potential in vivo and in vitro." Cell Transplant **6**(2): 125-134.
Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.
Kang, X. Q., W. J. Zang, et al. (2005). "Fibroblast growth factor-4 and hepatocyte growth factor induce differentiation of human umbilical cord blood-derived mesenchymal stem cells into hepatocytes." World J Gastroenterol **11**(47): 7461-7465.
Kang, Y. J., E. S. Jeon, et al. (2005). "Role of c-Jun N-terminal kinase in the PDGF-induced proliferation and migration of human adipose tissue-derived mesenchymal stem cells." J Cell Biochem **95**(6): 1135-1145.
Karin, M. and Y. Ben-Neriah (2000). "Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity." Annu Rev Immunol **18**: 621-663.
Karin, M., T. Lawrence, et al. (2006). "Innate immunity gone awry: linking microbial infections to chronic inflammation and cancer." Cell **124**(4): 823-835.
Karin, M., Y. Yamamoto, et al. (2004). "The IKK NF-kappa B system: a treasure trove for drug development." Nat Rev Drug Discov **3**(1): 17-26.

Kern, S., H. Eichler, et al. (2006). "Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue." Stem Cells **24**(5): 1294-1301.
Kim, Y. S., H. J. Park, et al. (2009). "TNF-alpha enhances engraftment of mesenchymal stem cells into infarcted myocardium." Front Biosci **14**: 2845-2856.
Kimura, M., A. Takatsuki, et al. (1994). "Blasticidin S deaminase gene from Aspergillus terreus (BSD): a new drug resistance gene for transfection of mammalian cells." Biochim Biophys Acta **1219**(3): 653-659.
Kjems, J., M. Brown, et al. (1991). "Structural analysis of the interaction between the human immunodeficiency virus Rev protein and the Rev response element." Proc Natl Acad Sci U S A **88**(3): 683-687.
Koc, O. N. and H. M. Lazarus (2001). "Mesenchymal stem cells: heading into the clinic." Bone Marrow Transplant **27**(3): 235-239.
Kon, T., T. J. Cho, et al. (2001). "Expression of osteoprotegerin, receptor activator of NF-kappaB ligand (osteoprotegerin ligand) and related proinflammatory cytokines during fracture healing." J Bone Miner Res **16**(6): 1004-1014.
Kopen, G. C., D. J. Prockop, et al. (1999). "Marrow stromal cells migrate throughout forebrain and cerebellum, and they differentiate into astrocytes after injection into neonatal mouse brains." Proc Natl Acad Sci U S A **96**(19): 10711-10716.
Kozak, M. (1987). "An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs." Nucleic Acids Res **15**(20): 8125-8148.
Krampera, M., S. Glennie, et al. (2003). "Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide." Blood **101**(9): 3722-3729.
Kuznetsov, S. A., P. H. Krebsbach, et al. (1997). "Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation in vivo." J Bone Miner Res **12**(9): 1335-1347.
Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(5259): 680-685.
Landy, A. (1989). "Dynamic, structural, and regulatory aspects of lambda site-specific recombination." Annu Rev Biochem **58**: 913-949.
Le Gouill, C., J. L. Parent, et al. (1994). "Analysis of recombinant plasmids by a modified alkaline lysis method." Anal Biochem **219**(1): 164.
Lee, C. I., D. B. Kohn, et al. (2004). "Morphological analysis and lentiviral transduction of fetal monkey bone marrow-derived mesenchymal stem cells." Mol Ther **9**(1): 112-123.
Lever, A. M., P. M. Strappe, et al. (2004). "Lentiviral vectors." J Biomed Sci **11**(4): 439-449.
Lin, K. T., S. H. Yeh, et al. (2005). "Epigenetic activation of alpha4, beta2 and beta6 integrins involved in cell migration in trichostatin A-treated Hep3B cells." J Biomed Sci **12**(5): 803-813.
Ling, L., Z. Cao, et al. (1998). "NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser-176." Proc Natl Acad Sci U S A **95**(7): 3792-3797.
Liss, B. (2002). "Improved quantitative real-time RT-PCR for expression profiling of individual cells." Nucleic Acids Res **30**(17): e89.

Liu, Y. P., O. V. Dovzhenko, et al. (2004). "Maintenance of pluripotency in human embryonic stem cells stably over-expressing enhanced green fluorescent protein." Stem Cells Dev **13**(6): 636-645.
Maier, H. J., U. Schmidt-Strassburger, et al. (2010). "NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells." Cancer Lett **295**(2): 214-228.
Majumdar, M. K., M. A. Thiede, et al. (1998). "Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells." J Cell Physiol **176**(1): 57-66.
Malek, S., D. B. Huang, et al. (2003). "X-ray crystal structure of an IkappaBbeta x NF-kappaB p65 homodimer complex." J Biol Chem **278**(25): 23094-23100.
Malim, M. H., J. Hauber, et al. (1989). "The HIV-1 rev trans-activator acts through a structured target sequence to activate nuclear export of unspliced viral mRNA." Nature **338**(6212): 254-257.
Manchester, K. L. (1995). "Value of A260/A280 ratios for measurement of purity of nucleic acids." Biotechniques **19**(2): 208-210.
Mason, J. M., D. A. Grande, et al. (1998). "Expression of human bone morphogenic protein 7 in primary rabbit periosteal cells: potential utility in gene therapy for osteochondral repair." Gene Ther **5**(8): 1098-1104.
Matsudaira, P. (1987). "Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes." J Biol Chem **262**(21): 10035-10038.
May, M. J., F. D'Acquisto, et al. (2000). "Selective inhibition of NF-kappaB activation by a peptide that blocks the interaction of NEMO with the IkappaB kinase complex." Science **289**(5484): 1550-1554.
McClure, M. O., M. Marsh, et al. (1988). "Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism." Embo J **7**(2): 513-518.
Mehrhof, F. B., R. Schmidt-Ullrich, et al. (2005). "Regulation of vascular smooth muscle cell proliferation: role of NF-kappaB revisited." Circ Res **96**(9): 958-964.
Mercurio, F., H. Zhu, et al. (1997). "IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation." Science **278**(5339): 860-866.
Miki, T., J. A. Park, et al. (1992). "Control of segregation of chromosomal DNA by sex factor F in Escherichia coli. Mutants of DNA gyrase subunit A suppress letD (ccdB) product growth inhibition." J Mol Biol **225**(1): 39-52.
Miller, B. S. and E. Zandi (2001). "Complete reconstitution of human IkappaB kinase (IKK) complex in yeast. Assessment of its stoichiometry and the role of IKKgamma on the complex activity in the absence of stimulation." J Biol Chem **276**(39): 36320-36326.
Miller, D. G., M. A. Adam, et al. (1990). "Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection." Mol Cell Biol **10**(8): 4239-4242.
Minguell, J. J., A. Erices, et al. (2001). "Mesenchymal stem cells." Exp Biol Med (Maywood) **226**(6): 507-520.
Miyoshi, H., U. Blomer, et al. (1998). "Development of a self-inactivating lentivirus vector." J Virol **72**(10): 8150-8157.

Mochizuki, H., J. P. Schwartz, et al. (1998). "High-titer human immunodeficiency virus type 1-based vector systems for gene delivery into nondividing cells." J Virol **72**(11): 8873-8883.
Muraglia, A., R. Cancedda, et al. (2000). "Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model." J Cell Sci **113 (Pt 7)**: 1161-1166.
Nagasaki, T., E. Ishimura, et al. (1999). "Alphav integrin regulates TNF-alpha-induced nitric oxide synthesis in rat mesangial cells--possible role of osteopontin." Nephrol Dial Transplant **14**(8): 1861-1866.
Nakahara, H., V. M. Goldberg, et al. (1991). "Culture-expanded human periosteal-derived cells exhibit osteochondral potential in vivo." J Orthop Res **9**(4): 465-476.
Naldini, L., U. Blomer, et al. (1996). "Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector." Proc Natl Acad Sci U S A **93**(21): 11382-11388.
Naldini, L., U. Blomer, et al. (1996). "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector." Science **272**(5259): 263-267.
Nelson, J. A., C. Reynolds-Kohler, et al. (1987). "Negative and positive regulation by a short segment in the 5'-flanking region of the human cytomegalovirus major immediate-early gene." Mol Cell Biol **7**(11): 4125-4129.
Neth, P., M. Ciccarella, et al. (2006). "Wnt signaling regulates the invasion capacity of human mesenchymal stem cells." Stem Cells **24**(8): 1892-1903.
Noth, U., A. M. Osyczka, et al. (2002). "Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells." J Orthop Res **20**(5): 1060-1069.
Orosz, A., I. Boros, et al. (1991). "Analysis of the complex transcription termination region of the Escherichia coli rrnB gene." Eur J Biochem **201**(3): 653-659.
Pahl, H. L. (1999). "Activators and target genes of Rel/NF-kappaB transcription factors." Oncogene **18**(49): 6853-6866.
Pasparakis, M., T. Luedde, et al. (2006). "Dissection of the NF-kappaB signalling cascade in transgenic and knockout mice." Cell Death Differ **13**(5): 861-872.
Pfeifer, A. (2004). "Lentiviral transgenesis." Transgenic Res **13**(6): 513-522.
Pittenger, M. F., A. M. Mackay, et al. (1999). "Multilineage potential of adult human mesenchymal stem cells." Science **284**(5411): 143-147.
Prasher, D. C., V. K. Eckenrode, et al. (1992). "Primary structure of the Aequorea victoria green-fluorescent protein." Gene **111**(2): 229-233.
Prigent, M., I. Barlat, et al. (2000). "IkappaBalpha and IkappaBalpha /NF-kappa B complexes are retained in the cytoplasm through interaction with a novel partner, RasGAP SH3-binding protein 2." J Biol Chem **275**(46): 36441-36449.
Rayet, B. and C. Gelinas (1999). "Aberrant rel/nfkb genes and activity in human cancer." Oncogene **18**(49): 6938-6947.
Regnier, C. H., H. Y. Song, et al. (1997). "Identification and characterization of an IkappaB kinase." Cell **90**(2): 373-383.
Reiser, J., G. Harmison, et al. (1996). "Transduction of nondividing cells using pseudotyped defective high-titer HIV type 1 particles." Proc Natl Acad Sci U S A **93**(26): 15266-15271.

Reyes, M., T. Lund, et al. (2001). "Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells." Blood **98**(9): 2615-2625.
Ries, C., V. Egea, et al. (2007). "MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines." Blood **109**(9): 4055-4063.
Roe, T., T. C. Reynolds, et al. (1993). "Integration of murine leukemia virus DNA depends on mitosis." Embo J **12**(5): 2099-2108.
Rogers, J. J., H. E. Young, et al. (1995). "Differentiation factors induce expression of muscle, fat, cartilage, and bone in a clone of mouse pluripotent mesenchymal stem cells." Am Surg **61**(3): 231-236.
Rothwarf, D. M. and M. Karin (1999). "The NF-kappa B activation pathway: a paradigm in information transfer from membrane to nucleus." Sci STKE **1999**(5): RE1.
Ruster, B., S. Gottig, et al. (2006). "Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells." Blood **108**(12): 3938-3944.
Sabatini, F., L. Petecchia, et al. (2005). "Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities." Lab Invest **85**(8): 962-971.
Sakon, S., X. Xue, et al. (2003). "NF-kappaB inhibits TNF-induced accumulation of ROS that mediate prolonged MAPK activation and necrotic cell death." Embo J **22**(15): 3898-3909.
Sambrook J., R. D. W. (2001). Molecular Cloning: A Laboratory Manual Cold Spring Harbor Lab Press.
Sanchez-Ramos, J., S. Song, et al. (2000). "Adult bone marrow stromal cells differentiate into neural cells in vitro." Exp Neurol **164**(2): 247-256.
Sanders, D. A. (2002). "No false start for novel pseudotyped vectors." Curr Opin Biotechnol **13**(5): 437-442.
Sanger, F., S. Nicklen, et al. (1977). "DNA sequencing with chain-terminating inhibitors." Proc Natl Acad Sci U S A **74**(12): 5463-5467.
Sato, Y., H. Araki, et al. (2005). "Human mesenchymal stem cells xenografted directly to rat liver are differentiated into human hepatocytes without fusion." Blood **106**(2): 756-763.
Sellner, L. N., R. J. Coelen, et al. (1992). "Reverse transcriptase inhibits Taq polymerase activity." Nucleic Acids Res **20**(7): 1487-1490.
Sen, R. and D. Baltimore (1986). "Multiple nuclear factors interact with the immunoglobulin enhancer sequences." Cell **46**(5): 705-716.
Senftleben, U., Z. W. Li, et al. (2001). "IKKbeta is essential for protecting T cells from TNFalpha-induced apoptosis." Immunity **14**(3): 217-230.
Shi, S., S. Gronthos, et al. (2002). "Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression." Nat Biotechnol **20**(6): 587-591.
Silverman, N. and T. Maniatis (2001). "NF-kappaB signaling pathways in mammalian and insect innate immunity." Genes Dev **15**(18): 2321-2342.
Simmons, P. J. and B. Torok-Storb (1991). "CD34 expression by stromal precursors in normal human adult bone marrow." Blood **78**(11): 2848-2853.
Smith, H. O. and K. W. Wilcox (1970). "A restriction enzyme from Hemophilus influenzae. I. Purification and general properties." J Mol Biol **51**(2): 379-391.

Sonenshein, G. E. (1997). "Rel/NF-kappa B transcription factors and the control of apoptosis." Semin Cancer Biol **8**(2): 113-119.
Southern, J. A., D. F. Young, et al. (1991). "Identification of an epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with different biological characteristics." J Gen Virol **72 (Pt 7)**: 1551-1557.
Takahashi, A., F. Higashino, et al. (2003). "EWS/ETS fusions activate telomerase in Ewing's tumors." Cancer Res **63**(23): 8338-8344.
Takeuchi, S., K. Hirayama, et al. (1958). "Blasticidin S, a new antibiotic." J Antibiot (Tokyo) **11**(1): 1-5.
Theiss, A. L., J. G. Simmons, et al. (2005). "Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2." J Biol Chem **280**(43): 36099-36109.
Thiery, J. P., H. Acloque, et al. (2009). "Epithelial-mesenchymal transitions in development and disease." Cell **139**(5): 871-890.
Tocci, A. and L. Forte (2003). "Mesenchymal stem cell: use and perspectives." Hematol J **4**(2): 92-96.
Totsugawa, T., N. Kobayashi, et al. (2002). "Lentiviral transfer of the LacZ gene into human endothelial cells and human bone marrow mesenchymal stem cells." Cell Transplant **11**(5): 481-488.
Trono, D. (1998). "When accessories turn out to be essential." Nat Med **4**(12): 1368-1369.
Trono, D. (2000). "Lentiviral vectors: turning a deadly foe into a therapeutic agent." Gene Ther **7**(1): 20-23.
Vogel, W., F. Grunebach, et al. (2003). "Heterogeneity among human bone marrow-derived mesenchymal stem cells and neural progenitor cells." Haematologica **88**(2): 126-133.
Vogelstein, B. and D. Gillespie (1979). "Preparative and analytical purification of DNA from agarose." Proc Natl Acad Sci U S A **76**(2): 615-619.
Vollweiler, J. L., S. P. Zielske, et al. (2003). "Hematopoietic stem cell gene therapy: progress toward therapeutic targets." Bone Marrow Transplant **32**(1): 1-7.
Wakitani, S., T. Goto, et al. (1994). "Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage." J Bone Joint Surg Am **76**(4): 579-592.
Wei, C. M., M. Gibson, et al. (1981). "Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1." J Virol **39**(3): 935-944.
Widera, D., I. Mikenberg, et al. (2006). "Tumor necrosis factor alpha triggers proliferation of adult neural stem cells via IKK/NF-kappaB signaling." BMC Neurosci **7**: 64.
Wilfinger, W. W., K. Mackey, et al. (1997). "Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity." Biotechniques **22**(3): 474-476, 478-481.
Wolf, K., C. Schulz, et al. (2002). "Tumour necrosis factor-alpha induced CD70 and interleukin-7R mRNA expression in BEAS-2B cells." Eur Respir J **20**(2): 369-375.

Woronicz, J. D., X. Gao, et al. (1997). "IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK." Science **278**(5339): 866-869.

Wu, M., H. Lee, et al. (1996). "Inhibition of NF-kappaB/Rel induces apoptosis of murine B cells." Embo J **15**(17): 4682-4690.

Yamaguchi, H., C. Yamamoto, et al. (1965). "Inhibition of protein synthesis by blasticidin S. I. Studies with cell-free systems from bacterial and mammalian cells." J Biochem (Tokyo) **57**(5): 667-677.

Young, H. E., T. A. Steele, et al. (2001). "Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors." Anat Rec **264**(1): 51-62.

Young, R. G., D. L. Butler, et al. (1998). "Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair." J Orthop Res **16**(4): 406-413.

Zandi, E., D. M. Rothwarf, et al. (1997). "The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation." Cell **91**(2): 243-252.

Zhang, S. Q., A. Kovalenko, et al. (2000). "Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKgamma) upon receptor stimulation." Immunity **12**(3): 301-311.

Zhang, X. Y., V. F. La Russa, et al. (2002). "Lentiviral vectors for sustained transgene expression in human bone marrow-derived stromal cells." Mol Ther **5**(5 Pt 1): 555-565.

Zhang, X. Y., V. F. La Russa, et al. (2004). "Transduction of bone-marrow-derived mesenchymal stem cells by using lentivirus vectors pseudotyped with modified RD114 envelope glycoproteins." J Virol **78**(3): 1219-1229.

Zhu, H., N. Mitsuhashi, et al. (2006). "The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix." Stem Cells **24**(4): 928-935.

Zufferey, R., T. Dull, et al. (1998). "Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery." J Virol **72**(12): 9873-9880.

Zufferey, R., D. Nagy, et al. (1997). "Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo." Nat Biotechnol **15**(9): 871-875.

Zuk, P. A., M. Zhu, et al. (2001). "Multilineage cells from human adipose tissue: implications for cell-based therapies." Tissue Eng **7**(2): 211-228.

Zvaifler, N. J., L. Marinova-Mutafchieva, et al. (2000). "Mesenchymal precursor cells in the blood of normal individuals." Arthritis Res **2**(6): 477-488.