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SRC a critical signaling mediator in FLT3 ITD positive AML

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For my family

Content

Abbreviations

α	alpha (anti)
β	beta
g	gamma
λ	wavelength
µg	10^{-6} gram
µl	10^{-6} liter
µmol	10^{-6} mol
µM (µmol/l)	10^{-6} mol/liter
fig.	figure
Abl	Abelson murine leukemia viral oncogene homolog 1
A. d.	Aqua destillata
ALL	acute lymphatic leukemia
AML	acute myeloid leukemia
APS	ammonium persulfate
AS	aminoacid
ATP	adenosin-5'-triphosphate
b	base
Bad	Bcl-2-associated death promoter
Bcl2	B-cell lymphoma 2
BCR	breakpoint cluster region
bp	base pairs
BSA	bovine serum albumine
C	Celsius

CBL	casitas B-lineage lymphoma
cDNA	complementary DNA
CML	chronic myeloid leukemia
CR	conserved region
CREB	cAMP-responsive element-binding protein
CSF	colony stimulating factor
DMEM	Dulbecco‘s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dNTP	2’-Desoxynukleosid-5’-triphosphat
dsDNA	doublestranded DNA
DTT	dithiothreitol
E	extinction
ECD	extracellular domain
E. coli	escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
ERK	extracellular signal regulated kinase
et al.	et alii
FACS	fluorescence activated cell scan
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FS	forward scatter
g	gravitation (9.81 m/s2)
GFP	green fluorescent protein
h	human/hour
HRP	horseradish peroxydase
Ig	immunglobulin
IL	interleukin
IRES	internal ribosomal entry site
Jak	janus kinase
kb	kilobase, 1000 base pairs

kD	kilodalton
LB	Luria-Bertani
lin	linear
log	logarithmic
LTR	long terminal repeat
m	murine
mA	milliampere
MAPK	mitogen activated protein kinase
mM (mmol/l)	10^{-3} Mol/liter
min	minute/s
miR	miR30 based shRNA
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	10^{-9} gram, nanogram
NLS	nucleare localisation sequence
nm	10^{-9} meter, nanometer
nM (nmol/l)	10^{-9} mol/liter
NaCl	sodium chloride
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PDK	pyruvat dehydrogenase kinase
PH	pleckstrin homolog
pH	pondus Hydrogenii
Ph^+	philadelphia-chromosom positive
PI	propidiumiodide
PI3-K	phosphoinositol 3-Kinase
PIP_2	phosphatidylinositol (3,4)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-triphosphate
PKB	proteinkinase B

PKC	proteinkinase C
PVDF	polyvinylidene fluoride
pY	phosphotyrosine
RNA	ribonucleic acid
RNAi	RNA-interference
Ras	rat sarcoma protein
Raf	rat fibrosarcoma protein
Raf-1	V-Raf-1 murine leukemia viral oncogene homolog 1
RT	reverse transkriptase/room temperature
RTK	receptortyrosinkinase
RT-PCR	reverse transkriptase polymerase chainreaction
s	second
SS	sideward scatter
SDS	sodium dodecyl sulfate
SH2	Src-homology 2
SH3	Src-homology 3
SHC	src homologous and collagen protein
shRNA	short hairpin RNA
siRNA	short interfering RNA
SOS	son of sevenless
STAT	signal transducer and activator of transcription
TAE	Tris-Acetate buffer
TEMED	N,N,N',N'-Tetramethylethylendiamine
Tris	tris(hydroxymethyl)aminomethane
U	unit
V	volt
Y	tyrosine
YFP	yellow fluorescent protein

1 Summary

Activating mutations of the receptor tyrosine kinase fms like tyrosine kinase (FLT3) are frequent in patients with acute mayloid leukemia (AML).[1,2] Two types of mutations are most common: Internal tandem duplications (ITD) of the juxtamembrane domain in approximately 25-30% of patients and point mutations within the second tyrosine kinase domain (TKD) in about 7% of AML patients.[3-5] While patients carrying the FLT3 ITD mutation have a significantly worse prognosis, FLT3 TKD mutations do not appear to influence the clinical outcome.[3]

Studies have shown that mice receiving a transplant of bone marrow expressing FLT3 ITD develop a myeloproliferative disease. In contrast, mice transplanted with FLT3 TKD infected bone marrow suffer from a lymphoid disease.[6-8] Thus, both FLT3 mutations seem to exert different biological functions. Interestingly, FLT3 ITD but not FLT3 TKD or FLT3 WT activates the STAT5 (signal transducer and activator of transcription 5) signaling pathway.[9] Therefore, STAT5 activation may be responsible for the observed differences in biology.[10,11]

This study investigated the signaling pathways leading to STAT5 activation down-stream of FLT3 ITD. FLT3 ITD does neither bind STAT5 directly nor activate the classical Janus kinase 2 (JAK2) pathway. Instead FLT3 ITD utilizes SRC to activate STAT5. Co-immunoprecipitations and GST pull-down experiments revealed a strong and exclusive interaction between SRC and FLT3 ITD, which is mediated by the SRC-SH2 domain. This interaction was absent in FLT3 TKD and FLT3 WT after ligand stimulation. Furthermore, the tyrosines 589 and 591 of FLT3 ITD appear to be essential for SRC binding and subsequent STAT5 activation. Specific SRC inhibitors or SRC shRNA blocked STAT5 activation and growth induced by FLT3 ITD but not FLT3 TKD. FLT3 ITD positive cells with a stable SRC

knockdown transplanted to syngenic mice led to a leukemic disease with a significant delayed onset and prolonged survival in comparison to the control group.

Based on these findings the effect of FLT3 inhibitor PKC412 (Midostaurin) was investigated in combination with the SRC inhibitor Dasatinib on FLT3 ITD and FLT3 TKD murine and human cell lines as well as on primary patient material.

In FLT3 ITD expressing murine myeloid 32D cells, the combination of an SRC inhibitor and FLT3 inhibitor showed additive effects on growth inhibition, apoptosis and activation of STAT5. In contrast, the SRC inhibitors had no additional effects on FLT3 TKD expressing cells. Accordingly, a strong additive effect of the SRC and FLT3 inhibitor could also be demonstrated in the FLT3 ITD positive human AML cell line MV4-11.

Finally, FLT3 ITD and FLT3 TKD positive primary human AML cells were investigated. Combination of SRC and FLT3 inhibitors led to significant additional growth inhibition of FLT3 ITD positive human cells. In contrast, no further growth reduction was detected when SRC was inhibited in primary AML cells expressing the FLT3 TKD mutation.

Together this study demonstrates that SRC is a crucial signaling mediator of FLT3 ITD but not of FLT3 TKD. Furthermore, SRC might be a pharmacologic target for the therapy of FLT3 ITD positive leukemia. Thus the combination of FLT3 and SRC inhibitors warrants further investigation in FLT3 ITD positive AML.

2 Introduction

2.1 The acute myeloid leukemia (AML)

Acute myeloid leukemia (AML) is a disease characterized by an accumulation of myeloblasts in the bone marrow and accounts for approximately 30% of all adult leukemia.[8,12-16] In most AML patients many different clonal chromosomal abnormalities such as reciprocal translocations, insertions, deletions and unbalanced translocations are found.[4] These chromosomal abnormalities include gains or losses of parts of or whole chromosomes, or translocations like t(8;21)/AML1-ETO (RUNX1/ RUNX1T1) or t(15;17)/PML-RARα.[17-19] The subclassification of AML is based on the cytogenetic alterations (WHO classification see fig. 2.2) as well as on the morphology and differentiation status of the cells (French American British (*FAB)* classification see fig.2.1).[20,21]

FAB subtype	Description	Comments
M0	Undifferentiated	Myeloperoxidase negative, myeloid markers positive
M1	Myelodysblastic without maturation	Some evidence of granulocytic differentiation
M2	Myelodysblastic with maturation	Maturation at or beyond promyelocytic stage of differentiation; can be divided into those wth t(8;21) and those without
M3	Promyelocytic	APL; most cases have t(15;17) PML-RARalpa or another translocation involving RARalpha
M4	Myelomonocytic	
M4 eo	Myelomonocytic with bone-marrow eosinophilia	Characterized by inversion of chromosome 16 involving CBFß,
M5	Monocytic	
M6	Erthyroeukaemia	
M7	Megakaryoblastic	GATA1 mutations in those associated with Down`s snydrome

Fig. 2.1: FAB classification of AML

AML with recurrent cytogenetic translocations	- AML with (8;21)(q22;q22); AML1/ETO - AML wit abnormal bne marrow eosinophils inv(16)(p13q22) or t(16;16)(p13;q22); CBFß/MYH11 - APL with t(15;17)(q22;q11-12) (PML/RARa) - and variants - AML with 11q23-(mixed-lineage leukemia) abnormalities
AML with multilineage dysplasia	- AML with prior myelodysplastic syndrome (MDS) - AML without prior MDS
AML and MDS, therapy related	- alkylating agent-related - epipodophyllotoxin-related - other types of therapy
AML not otherwise categorized	- AML, minimally differentiated (FAB M0) - AML without maturation (FAB M1) - AML with maturation (FAB M2) - Acute myelomonocytic leukemia (FAB M4) - Acute monocytic leukemia (FAB M5a, b) - Acute erythroid leukemia (FAB M6) - Acute megakaryocytic leukemia (FAB M7) - Acute basophilic leukemia - Acute panmyelosis with myelofibrosis
Acute biphenotypic leukaemias	

Fig. 2.2: WHO Classification of AML

2.1.1.1 Two hit hypothesis of AML pathology

The development of AML is described as a multi-step process in which the presence of at least two complementing mutations (fig. 2.3) is required. These alterations can be classified into two main groups.[1] The first group, referred to as class I mutations, consists of mutations of proteins including FMS-like tyrosine kinase 3 (FLT3) and c-KIT, which lead to reduced apoptosis and enhanced stem cell self-renewal.[22,23] On the otherhand, Class II mutations lead to repression of differentiation, as seen in gene fusions AML1-ETO and PML-RARα.[17,19]

The hypothesis of cooperation is supported by the fact that FLT3 ITD mutations are often found in combination with other mutations of the different class, e.g. in 40% of PML-RARα AML FLT3 is mutated. Furthermore, animal studies revealed that FLT3 ITD alone is not capable of inducing leukemia in a murine bone marrow transplantation model.[6,7,24]

Most class I mutations are found within the FLT3 receptor, either as FLT3 ITD or FLT3 TKD mutations. FLT3 ITD is associated with an unfavorable prognosis, thus, the clinical importance of FLT3 TKD mutations is up to now not fully understood.[3,22,25,26]

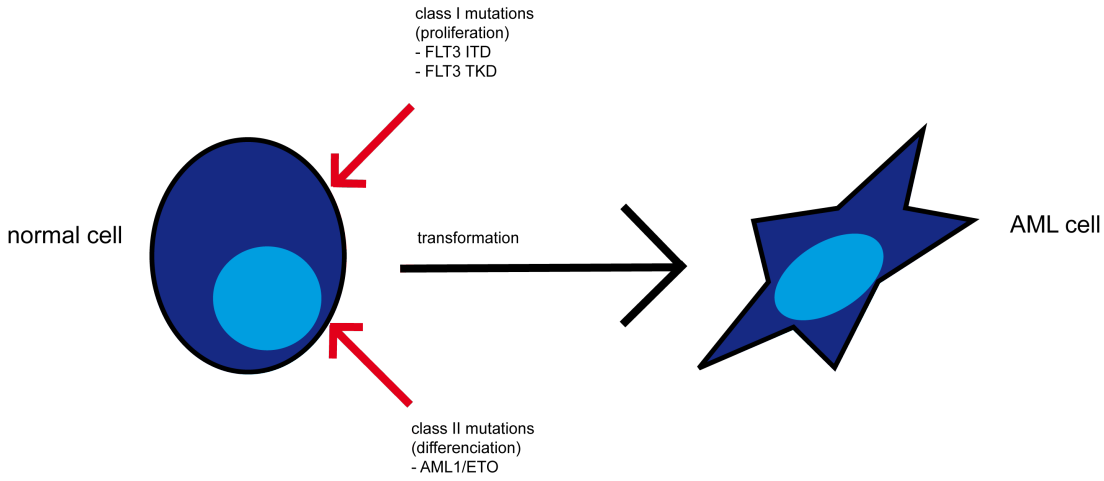

Fig. 2.3: Model of the two-hit hypothesis for the pathogenesis of the AML adapted from Gilliland et al. 2002

2.1.2 AML therapy

The clinical outcome of AML patients is still poor despite recent scientific progress in therapy. Although a complete remission can be achieved by conventional chemotherapy, patients frequently relapse.[27-29] At the moment bone marrow transplantation is the only option for curative treatment.[28,30] Due to the diversity of AML's cytogenetic and molecular background, there is intensive research on the way to develop new targeted therapy approaches.[31-33]

To improve the outcome of the chemotherapy a panel of inhibitors against proteins like FLT3 as well as anti-FLT3 antibodies have been developed and are at present evaluated in different phases of clinical trials.[34] In general, the compounds reduce the cell proliferation rate by up-regulating pro-apoptotic proteins.[10,35] Despite this effect, the therapeutic responses of the patients to single agents are considered modest and several current studies are therefore investigating the combination of FLT3 inhibitors and conventional chemotherapy.[28,36]

2.2 The protein FLT3

2.2.1 Structure and function of FMS-like tyrosine kinase 3

FLT3 is a membrane-bound receptor with an intrinsic tyrosine kinase domain. It is composed of an immunoglobulin-like extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane dimerization domain, and a highly conserved intracellular kinase domain, which is interrupted by a kinase insert (see fig. 2.3).[37]

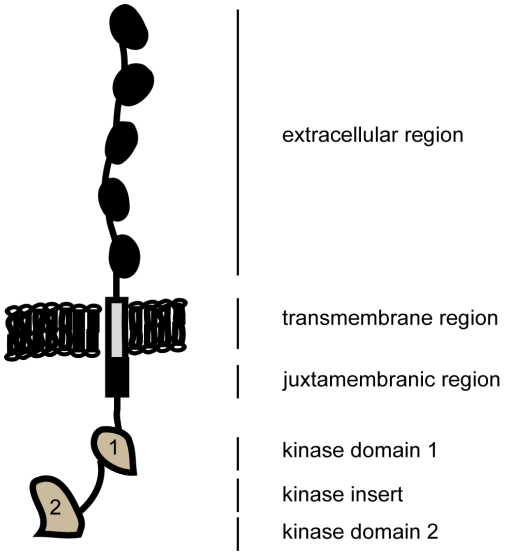

Fig. 2.3: Structure of FLT3

FLT3 belongs to the class III subfamily of receptor tyrosine kinases (RTK), which includes structurally similar members such as c-FMS, c-KIT and the PDGF receptor.

In the unstimulated state, the FLT3 receptor occurs in a monomeric unphosphorylated form with an inactive kinase part. Upon interaction with FLT3 ligand (FL) the receptor undergoes a conformational change, resulting in the unfolding of the receptor and the exposure of the dimerization domain, allowing receptor-receptor dimerization.[1,37] This receptor dimerization induces the activation of the tyrosine kinase domain and leads to phosphorylation of various sites in the intracellular domain (fig. 2.4).

The activated receptor in turn recruits a number of cytoplasmic proteins to form a complex with the intracellular domain. SHC-adaptor protein (SHC) proteins, Growth factor receptor-bound protein 2 (GRB2), GRB2-associated binder 2 (GAB2), SH2 domain-containing inositol phosphatase (SHIP), CBL, and CBLB-related protein (CBLB) are a few of

the adaptor proteins that interact with the activated FLT3 receptor.[38-42] Upon binding to the complex, the adaptor protein becomes activated, resulting in a cascade of phosphorylation reactions that culminates in the activation of downstream mediators, including mitogen-activated protein kinase (MAP kinase), signal transducers and activator of transcription (STAT) as well as members of the AKT/PI3 kinase signal transduction pathway.[7-9,43,44]

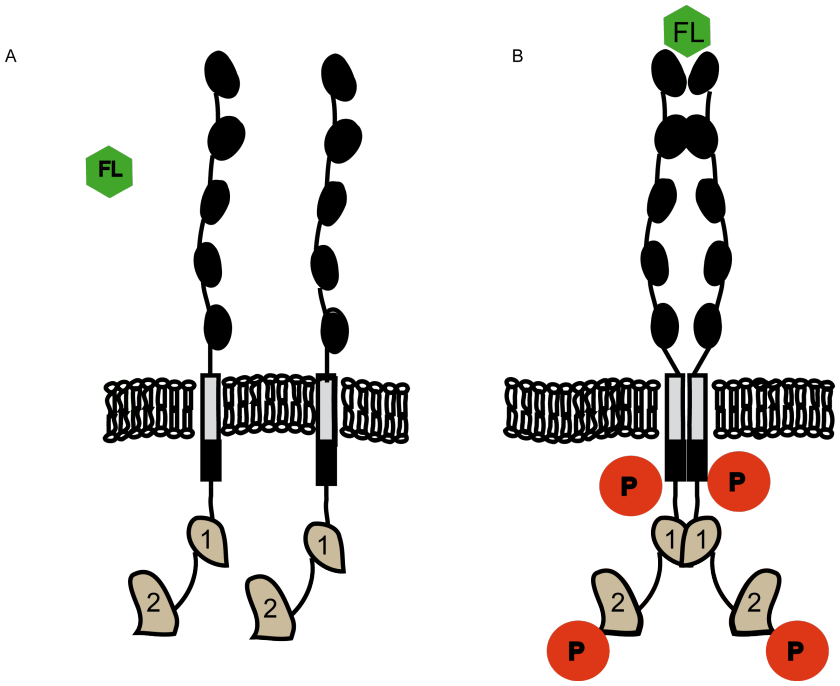

FIg. 2.4: FLT3 activation upon FL binding to the receptor.

2.2.2 FLT3 function in normal and malignant hematopoiesis

FLT3 is involved in numerous processes of normal hematopoiesis including transcription, proliferation and apoptosis.[45,46]

The protein is primarily expressed on committed myeloid and lymphoid progenitors with variable expression in the more mature monocytic lineage.[47,48] Its expression has further been described in lymphohematopoietic organs such as the liver, spleen, thymus, and placenta.[49,50]

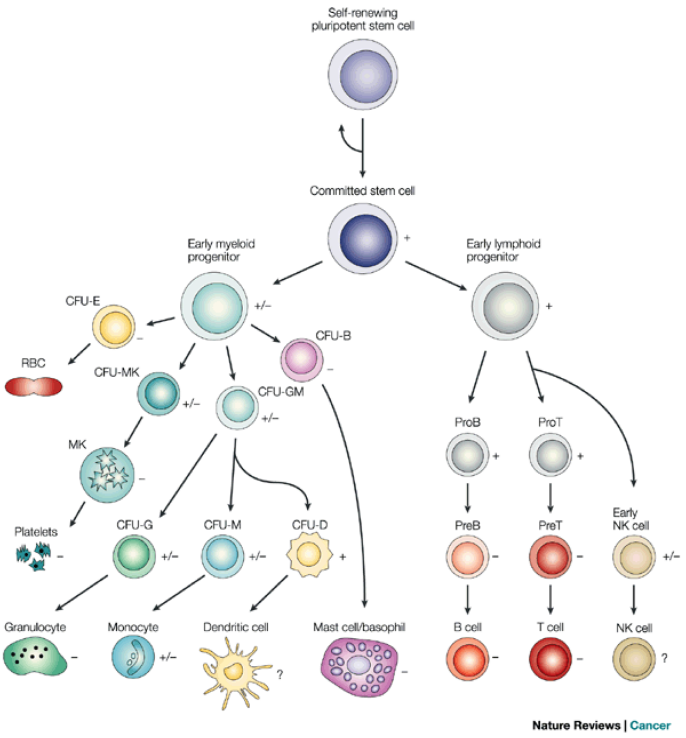

Fig. 2.5: Expression of FLT3 on hematological cells. [51]

Combinations of FL and other growth factors have been found to promote proliferation of primitive hematopoietic progenitor cells as well as more committed early myeloid and lymphoid precursors.[52-54] Furthermore FLT3 seems to mediate differentiation of the early progenitors, in which exposure of the hematopoietic progenitors to FL leads to monocytic differentiation without significant proliferation (fig. 2.5).[53]

Although FLT3 knockout mice have a subtle phenotype, as they have a reduced amount of lymphoid progenitors,[49] mice transplanted with FLT3 knockout cells displayed a more global disruption of hematopoiesis. Thus, the *in vitro* data and murine knockout models confirm a major role for FLT3 in normal hematopoiesis.[55]

2.2.3 Expression in hematologic malignancies

FLT3 is expressed in the majority of B-cell acute lymphocytic leukemia (ALL) and AML blasts (>90%).[48,52] Less frequently FLT3 receptors are also expressed in other hematopoietic malignancies like myelodysplasia (MDS), chronic myeloid leukemia (CML), T-cell ALL and chronic lymphocytic leukemia (CLL).[56,57-58]

2.2.4 Alterations of FLT3 in AML

Studies investigating the function of genomic alterations in the FLT3 gene in hematologic neoplasias identified two major groups of mutations, one within the juxtamembranic and another one in the kinase domains of the FLT3 gene (fig. 2.6).[59]

The first group consists of an internal tandem duplication of the juxtamembrane domain coding sequence in FLT3 (FLT3 ITD). This kind of mutation was first identified in patients with AML.[3,15,60] Other studies identified point mutations in the activation loop of the tyrosine kinase domain of the FLT3 receptor (FLT3 TKD).[61] Besides these, other groups have identified further point mutations in the activation loop, activating point mutations in the juxtamembrane domain and ITD mutations in the kinase domain.[62,63]

2.2.5 FLT3 internal tandem duplication mutations (FLT3 ITD)

FLT3 ITD mutations are the result of a duplication of a fragment within the juxtamembrane domain region, which is encoded by exons 14 and 15 of FLT3. FLT3 ITD mutations are amongst the most common mutations in hematologic malignancies, occurring in CML (5-10%), MDS (5-10%), and AML (15-35%) patients.[3,58,64]

The prevalence of FLT3 ITD is highly age-dependent, being rare in infant AML and most frequent in AML patients older than 55 years.[13] There is also considerable variety in size, and region of ITD involvement, ranging from 3 to >400 base pairs.[15,51]

In vitro studies have shown that FLT3 ITD promotes ligand independent receptor dimerization leading to autonomous phosphorylation and constitutive activation of the receptor, resulting in cytokine-independent cellular proliferation.[7,8,65]

The underlying mechanism by which FLT3 ITD leads to auto-dimerization is unknown, however, the juxtamembrane (JM) domain is thought to act as a negative regulatory domain by preventing the activation loop from adopting an active conformation, thus maintaining the receptor in an auto-inhibited state.[37] Three-dimensional structure analysis of the FLT3 receptor suggests that the duplication within the JM domain may disrupt the auto-inhibitory mechanisms, which normally prevent auto-dimerization in an unstimulated state, allowing receptor-receptor interaction to take place.[37,51]

FLT3 ITD is thought to promote proliferation via the activation of multiple signaling pathways including RAS/MAPK, STAT and the AKT/PI3 kinase pathways.[7,24] Grundler and

colleagues demonstrated that cytokine-independent cellular proliferation of FLT3 ITD transduced cells was mediated by RAS and STAT5 pathways.[7,8,24] Interestingly, the activation of STAT5 in FLT3 ITD positive cells highlighted that some of the effects of FLT3 ITD are unique to this kind of mutated receptor, as ligand-induced FLT3 WT or FLT3 TKD activation neither leads to FLT3 ITD like STAT5 activation nor STAT5 DNA binding.[51,66]

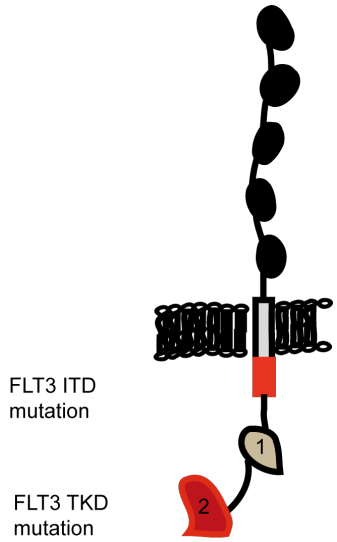

Fig. 2.6: The two major groups of FLT3 mutations.

2.2.6 FLT3 activation loop mutation (FLT3 TKD)

Point mutations within the tyrosine kinase domain are the second type of FLT3 mutations. The majority of these FLT3 alterations occur by an exchange of aspartic acid to tyrosine in codon 835 (D835Y).[3,61]

Although FLT3 TKD mutations promote autophosphorylation of the receptor, constitutive receptor activation and ligand-independent proliferation similar to FLT3 ITD, there are significant biological and biochemical differences between the two types of FLT3 mutations.[7,8,68]

Animal studies have shown that mice harboring FLT3 ITD primarily develop an oligoclonal myeloproliferative disorder, whereas mice harboring FLT3 TKD are more likely to develop oligoclonal lymphoid disorders.[6,7,24]

2.2.7 Clinical significance of FLT3 ITD

In 1999 Kiyoi and colleagues described FLT3 ITD mutations in AML patients, finding that approximately 25% of AML patients harbored these mutations.[69] Other studies have confirmed that the presence of FLT3 ITD is an independent prognostic factor for relapse and poor outcome of AML patients.[70]

Kottaridis et al. and Thiede et al. examined in 2002 the prevalence and prognostic significance of FLT3 ITD in a cohort of more than 850 adult patients.[3,25] These studies showed that AML patients with FLT3 ITD had a lower remission rate, higher relapse rate (RR), and an overall worse survival. Multivariate analyses found that FLT3 ITDs were the most significant prognostic factor with respect to the relapse rate (RR) and disease free survival (DFS) in AML.[4,26,58,60,71]

2.2.8 Signaling of FLT3 and activated pathways

Due to the two distinct phenotypes induced by FLT3 ITD and FLT3 TKD, much effort has been put into characterizing the downstream signaling of these classes of mutations.

Upon receptor binding canonical pathways like PI3 kinase and MAP kinase pathways are activated by FLT3 WT, FLT3 ITD and FLT3 TKD.[9,24,39,43,44,65-67] The most striking difference with regard to the signaling between FLT3 ITD and FLT3 TKD lies within the activation of the transcription factor STAT5.[7,43] Constitutively phosphorylated STAT5 has been verified not only in mice suffering from FLT3 ITD positive disease but also in primary AML blasts carrying the FLT3 ITD mutation.[1] In these cells inhibition of FLT3 leads to a reduction of pSTAT5 levels, clearly indicating a link between FLT3 ITD and STAT5 activation.[72,73]

Choudhary et al. 2009 demonstrated that the localization of the protein is crucial for the activation of downstream targets. FLT3 ITD, which in contrast to FLT3 TKD is strongly restricted to the endoplasmic reticulum, might therefore be able to activate STAT5 to a greater extent than FLT3 TKD.[74] The underlying mechanisms for this, however, are not clearly understood. STAT5 itself induces the activation of several downstream targets like proto-oncogene serine/threonine-protein kinase 1 (PIM-1), CDC25A, BCL2-associated death promoter (BAD) and B-cell lymphoma 2 (BCL-2). These proteins are known mediators of cell cycle progression and anti-apoptotic signaling.[75,76]

2.2.8.1 The map-kinase signaling pathway

The mitogen-associated protein kinase (MAPK)-pathway is one of the first kinase cascades, which has been investigated. There are five known MAPK families: ERK-1 (p44) and ERK-2 (p42), the JNK (c-JUN N-terminal kinase) family, the p38 MAPK family, ERK-3 and ERK-5.[77] ERK-1 and ERK-2 are activated and in turn activate a number of cytoplasmic and nuclear proteins by phosphorylation of tyrosine or threonine residues.

Several groups have shown that both wildtype and mutated forms of FLT3 are capable of activating the MAPK pathway and multiple mechanisms have been suggested.[41,78] One of them is through GRB2. Upon interaction of GRB2 with the tyrosine residues 768, 955 and 969 in FLT3, GRB2 becomes activated and induces ERK phosphorylation.[79]

2.2.8.2 The Pi3-kinase signaling pathway

The phosphoinositide 3 (PI3)-kinase pathway regulates various cellular processes involved in metabolism, proliferation and apoptosis of cells.[80,81] PI3-kinases are a family of lipid kinases that phosphorylate the 3′-hydroxyl group of phosphoinositides. Class I PI3Ks phosphorylate the membrane bound lipid phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3).[80,82]

The best-characterized downstream target of PI3K is the serine/threonine kinase AKT (also referred to as protein kinase B), a mediator of survival and proliferation.[83] When PI3K is activated, AKT localizes to the plasma membrane and becomes phosphorylated by 3-phosphoinositide-dependent kinase-1 (PDK1).[84]

For FLT3 it has been demonstrated that the human receptor does not contain a direct binding motif for of PI3K, whereas the murine receptor contains this motif and has been shown to directly associate with PI3K.[85]

Human FLT3 was found to phosphorylate GRB2-associated binding protein 1 (GAB1) and GRB2-associated binding protein 2 (GAB2), thus suggesting to activate the PI3K pathway indirectly through these intermediates, which is in line with the identification of tyrosine residues 768, 955 and 969 as GRB2/GAB2 association sites and mediators of downstream signaling via PI3K and AKT.[79]

2.2.9 FLT3 based AML treatment approaches

The FLT3 pathway is an obvious target for tyrosine kinase inhibitors (TKI) as FLT3 mutations are one of the most common mutations in AML.[3,86]

In vitro studies using nonspecific TKI found that these drugs block constitutive activation of FLT3 ITD and preferentially kill cells harboring FLT3 ITD mutations.[10] Subsequent studies identified numerous other potential compounds that also block FLT3 activation.[10,35,87-89]

PKC412 (Midostaurin) is a benzoylstaurosporine, which was initially developed as a vascular endothelial growth factor receptor inhibitor but also inhibits FLT3 receptor kinase.[34,90] A phase II trial examined PKC412 as a single agent in 20 high-risk AML patients with FLT3 ITD mutations. Although no complete remissions (CR) were observed, the autophosphorylation of the mutant receptor was blocked in most of the responding patients, indicating an *in vivo* response.[91]

Subsequently, PKC412 was combined with conventional chemotherapy (e.g. daunorubicin and cytarabine) in patients with de novo AML.[70] The acquired data suggest that FLT3 inhibitors as single agents have a limited potential in the treatment of AML, although their addition to conventional chemotherapy may provide therapeutic benefit for patients with FLT3 ITD.

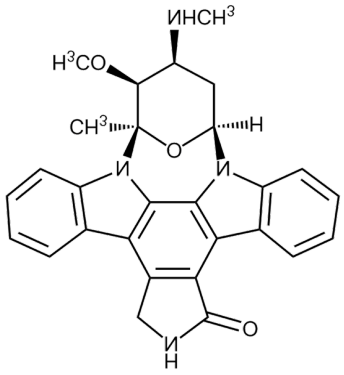

Fig. 2.7: Structure of PKC412

2.3 The SRC family kinases (SFK)

The SRC family of tyrosine kinases (SFKs) contains nine proteins: LYN, FYN, LCK, HCK, FGR, BLK, YRK, YES, and SRC.[92] Amongst these, SRC is the best-characterized and most frequently implicated in oncogenesis.[93] SRC encodes a non-receptor tyrosine kinase that is part of signaling cascades which mediate cellular proliferation, survival, migration, and angiogenesis.[94] It has been shown that numerous human malignancies display increased SRC expression and activity, suggesting that SRC may be intimately involved in oncogenesis.[95] Despite this, SRC alone is insufficient in transforming human cells *in vitro*, and so far only rare cases of activating SRC mutations have been identified in human cancers.[95]

2.3.1 SRC structure and function

Proteins of the SRC family have a highly conserved structure consisting of four SRC homology (SH) domains and a C-terminal segment containing a negative regulatory tyrosine residue (Y530).[92] Physiologically SRC exists in tightly regulated active and inactive conformations (fig. 2.8).[96] Negative regulation takes place through phosphorylation of Y530, leading to an intramolecular association between phosphorylated Y530 and the SH2 domain of SRC, thereby locking the protein in a closed conformation. Dephosphorylation of Y530 allows SRC to assume an open conformation. Full activity requires additional autophosphorylation of the Y419 residue within the catalytic domain. Loss of the negative-regulatory C-terminal segment has been shown to result in increased activity and transforming potential.[94]

The activity of SRC is regulated by a balance between kinases and phosphatases that act at the C-terminal Y530 residue (fig. 2.8).[92] Phosphorylation by C-terminal SRC kinase (CSK) and CSK homology kinase results in increased intramolecular interactions and leads to SRC inactivation. Several groups have shown the involvement of specific phosphatases in SRC activation.

Protein tyrosine phosphatase α (PTPα) and the SH-containing phosphatases SHP1/SHP2 are the most-studied proteins with SRC-specific dephosphorylation activity both *in vitro* and *in vivo*.[94] SRC can furthermore be activated by direct binding of focal adhesion kinase (FAK) and CRK-associated substrate (CAS) to the SH2 domain.[97]

Binding of these molecules activates SRC by disrupting inhibitory intramolecular interactions. Interestingly, both FAK and CAS are principal regulators of focal adhesion complex formation and actin cytoskeleton dynamics, essential processes for cell adhesion and migration.[95]

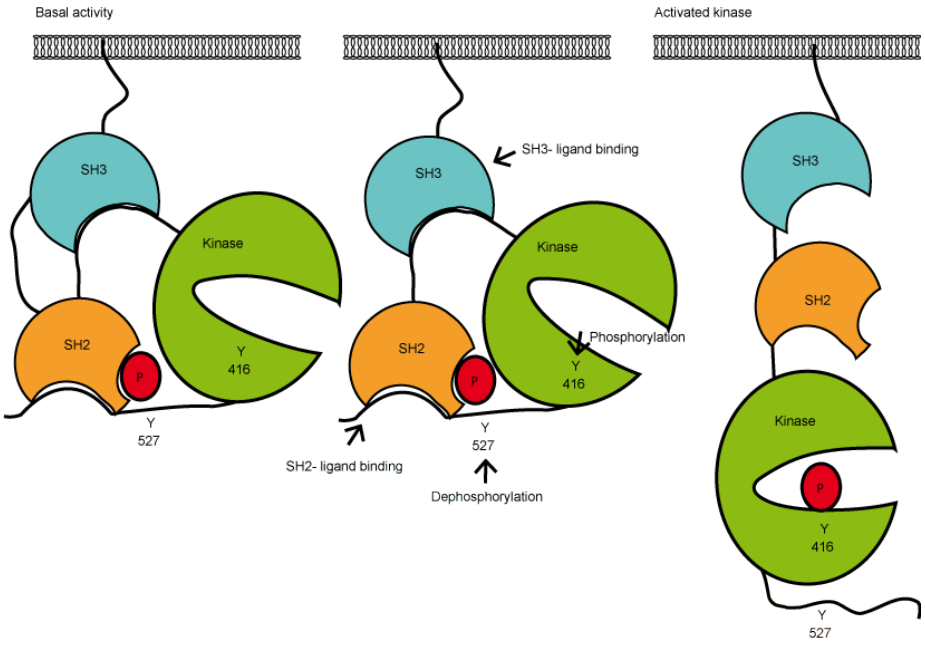

Fig. 2.8: Activation and subsequent conformational changes of SRC

2.3.2 Involvement of SRC in human cancers

Several research projects have shown that SRC plays an important role in the genesis and progression of multiple human cancer types, e.g. the carcinomas of the breast, gastrointestinal tract, lung and non-solid tumors like myeloproliferative disorders.[98-100] Moreover, studies using human neoplastic cell lines and *in vivo* murine models have demonstrated the complex network of SRC-interacting proteins that affect numerous signal-transduction pathways (Fig. 2.9).[76]

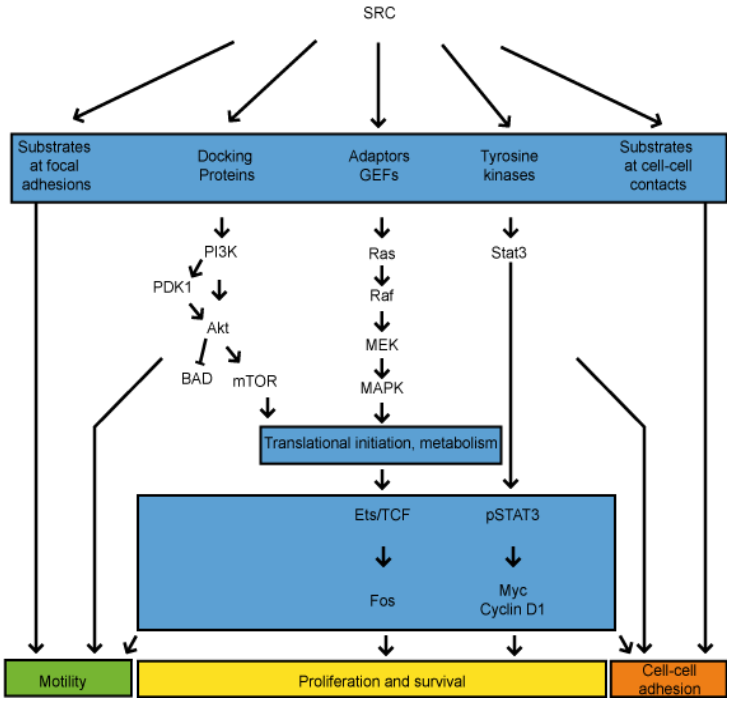

Fig. 2.9: Functions and the activated signaling pathways of SRC [94]

2.3.3 SFKs in leukemias, lymphomas, and myelomas

The majority of SFKs are expressed primarily in hematopoietic cells and are involved in signaling pathways, which regulate cell growth and proliferation. Several laboratory reports have indicated that myeloid cells primarily express LYN, HCK, and FGR.[76]

LYN may be involved in the growth, survival, and motility of various hematological malignancies. Interleukin 3 (IL-3)-induced up-regulation of LYN kinase activity may be mediated by the 120 kD common subunit of human IL-3 and granulocyte-macrophage

colony stimulating factor receptors. This evidence suggests that LYN kinase participates in early IL-3 initiated signaling events.[92]

Danhauser-Riedl et al. reported that the activity of SFK like LYN and HCK was increased in hematopoietic cells that expressed BCR-ABL. This interaction of BCR-ABL with SRC-kinases may be independent of SRC and ABL kinase activity.[98] In addition, expression of a kinase-defective mutant HCK in a cytokine dependent myeloid cell line significantly suppressed BCR-ABL-induced cytokine-independent cell growth.[101]

These and other results greatly influenced the development of second-line therapies for BCR-ABL positive CML. Amongst these agents Dasatinib is one of the most prominent as it is not only able to inhibit the fusion kinase but also inhibits SFK, leading to promising results in the therapy of BCR-ABL positive CML.[102]

2.3.3.1 SRC in FLT3 ITD positive AML

Studies have shown that SFKs play an important role in the signaling of BCR-ABL, which has been used to develop drugs for the treatment of BCR-ABL positive patients.[99] Based on the results of the described studies and on the results of other groups, which showed that members of the SFK are potential pharmacologic targets in other neoplastic malignancies, intensive research on the functions of this group of proteins in AML has been conducted.[103-105] Up to now the results are contradictory, as some findings suggest a central role of SFK within the signaling of FLT3 WT and FLT3 ITD whereas others point towards lesser or no involvement.[67,106-108]

2.3.4 SRC kinase inhibitors

The important role of SRC in numerous cancer types and other diseases triggered the interest in developing SRC inhibitors. SRC inhibitors can be classified as:

- pyrazolo[3,4-d]pyrimidines (e.g., PP1 and PP2)
- pyrrolo[2,3-d]pyrimidines (e.g., CGP76030 and CGP77675)
- pyrido-[2,3-d]pyrimidines (e.g., PD166-326, PD173955,and PD180970);
- quinolines (SKI606);
- indolinones (SU6656);
- dual SRC-ABL inhibitors (e.g., Dasatinib [N-(2-chloro-6-methylphenyl)-2-(6-[4-(2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide]);

- or dual LYN-ABL inhibitors (NS-187)

In this work, the inhibitor PD 166-326 and the SRC inhibitor Dasatinib were used and will thus be described in more detail.

2.3.4.1 Dasatinib

Dasatinib (fig. 2.10) (N-[2-chloro-6-methylphenyl]-2-[6-(4-[2-hydroxyethyl)piperazin-1-yl]-2-methylpyrimidin-4-ylamino]thiazole-5-carboxamide) is a synthetic, small-molecule ATP-competitive inhibitor of SFK with broad spectrum activity against hematological and solid tumor cell lines.[102]

Structural analyses of ABL revealed that its SH3, SH2 and kinase domains adopt an assembled conformation similar to that of the SRC kinases.[109] This explains the effect that several compounds designed as SRC kinase inhibitors are also effective against BCR-ABL.

Compared to Imatinib, Dasatinib is >300-fold more potent against the wild-type BCR-ABL, and has little if any inhibitory effect on normal hematopoietic progenitors. It furthermore displayed *in vitro* and *in vivo* activity against 14 of 15 BCR-ABL mutants with T315I as the only exception.[102] In the clinic Dasatinib has been approved for the first line therapy of BCR-ABL positive CML and is the subject of several clinical trials for other neoplasias.

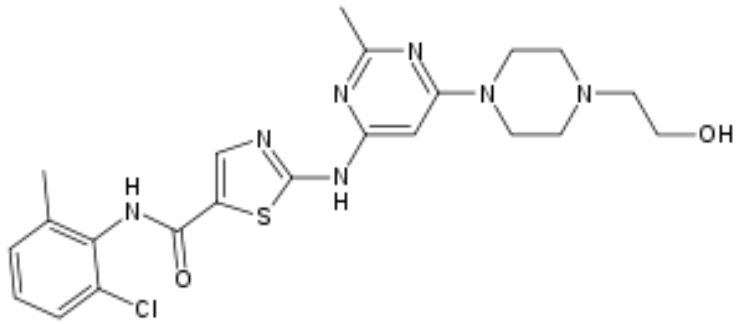

Fig. 2.10 Structure of Dasatinib Pyrido-pyrimidines

Pyrido-pyrimidines (pyrido-[2,3-d]pyrimidines) of which PD166-326 has been used in this work, are a group of small molecules inhibitors that act as antagonists of SRC. Further targets include PDGFR and EGFR and thus the specificity for SRC is significantly higher.[110]

In contrast to compounds like Imatinib, this group of SRC inhibitors binds near the ATP-binding site, thus blocking access of ATP. They are able to target both the active and

inactive configurations of ABL.[111] In preclinical experiments this compound has shown antileukemic activity in BCR-ABL positive CML inhibiting both SFK and the oncogene BCR-ABL.[111,112]

3 Aim of the study

The protein FLT3 is the most frequently mutated tyrosine kinase in patients suffering from AML. We and other groups could demonstrate that the two major classes of mutations, FLT3 ITD and FLT3 TKD, show differences with regard to biological and biochemical properties.[1,7] Apart from the phenotype induced in a murine bone marrow transplantation model, where FLT3 ITD transplanted mice develop a myeloid and FLT3 TKD transplanted mice a lymphoid hemato-oncological disease, the most striking difference lies within the activation of the transcription factor STAT5.[7,24] Despite intensive research the mechanisms of STAT5 activation by the receptor tyrosine kinase FLT3 ITD are still poorly understood.

Based on these facts the aim of the study was to identify signaling transduction molecules, which are activated by FLT3 ITD but not by FLT3 TKD. These proteins were then to be evaluated with regard to their involvement of STAT5 activation by FLT3 ITD. Additionally the reasons for the differences were to be addressed, with focus on the structural differences of FLT3 ITD and FLT3 TKD.

In the second part the biologic importance of the identified proteins is to be investigated using animal models and primary human material.

4 Results

4.1 Characterization of FLT3 ITD and FLT3 TKD signaling

The receptor tyrosine kinase FLT3 is the most frequently mutated kinase in the acute myeloid leukemia (AML).[1] In about 30% of all AML patients, alterations of this protein can be found.[3] It is therefore crucial to acquire insight into the signaling of FLT3 mutations in order to develop new therapeutic approaches for AML patients.

To investigate the signaling properties of FLT3 ITD and FLT3 TKD in an *in vitro* model, which mimics the *in vivo* situation, murine myeloid 32D cells were transduced with MIG-FLT3 constructs as well as MIG-empty (MOCK) vector. Afterwards, the cells were selected by growth factor deprivation or in case of FLT3 WT by FLT3 ligand (FL) stimulation. FACS analysis showed nearly 100% GFP positivity for all three cell lines (data not shown), assuring that all of the later used cells express the desired construct and are in line with the current opinion on the signaling properties of FLT3 ITD and FLT3 TKD.[1,7,44,66,113]

To address the question of different levels of STAT5 activation by the two major groups of mutations, 32D cells expressing FLT3 ITD or FLT3 TKD were starved for 6 h in serum free medium. Afterwards they were subject to lysis and separated by SDS-PAGE and consequently analyzed by western blot using pSTAT5 specific antibodies. In order to ensure equal loading the membranes were stripped and re-probed with STAT5 antibodies.

The experiments revealed a strong activation of STAT5 by FLT3 ITD but not FLT3 TKD (fig. 4.1).

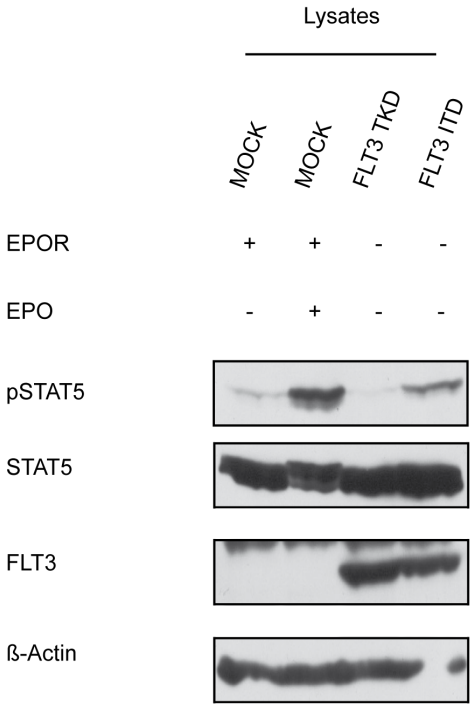

Fig. 4.1: Activation of STAT5 by FLT3 ITD and FLT3 TKD.

32D cells transduced with MIG-FLT3 ITD, MIG-FLT3 TKD or MIG-MOCK were analyzed with regard to their activation levels of STAT5.

To address the question whether or not the observed differences in activation levels of STAT5 are due to JAK2 activation, FLT3 ITD or FLT3 TKD positive cells were analyzed for JAK2 phosphorylation by western blot. Additionally MIG-MOCK transduced cells expressing the EPO-receptor were used as a control to induce JAK2 activation.

Neither FLT3 ITD nor FLT3 TKD appeared to activate JAK2 by phosphorylation. In contrast EPO stimulated MOCK transduced cells, expressing the EPO receptor, showed a strong activation of JAK2 (fig. 4.2). Re-probing the membranes with JAK2 antibody ensured equal loading of the samples.

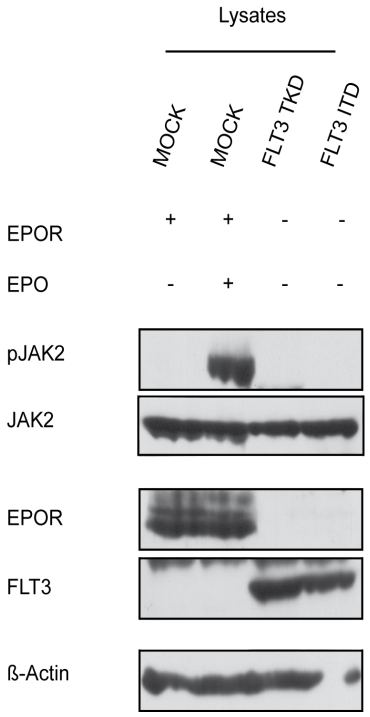

Fig. 4.2: Activation of JAK2 by FLT3 mutants. 32D cells expressing FLT3 ITD or FLT3 TKD, the cells were analyzed for JAK2 activation by immunoblot.

The cells were starved for 6 h in cytokine free medium and afterwards subjected to lysis and SDS-PAGE.

4.2 Testing of SFK as potential FLT3 ITD interaction partners

Having demonstrated the different level of STAT5 activation by the two major groups of FLT3 mutations, and furthermore shown that JAK2 is not involved in this process, in the next step other possible explanations for the observed differences in STAT5 activation were investigated.

There are several established models for the activation of STAT5 by receptor tyrosine kinases. One is the direct interaction between STAT5 and the receptor; another mechanism is the activation of JAK2, which in turn leads to increased STAT5 phosphorylation.[114,115] A third way by which STAT5 can be activated is through members of the SFK.[113] Since it has been shown that, FLT3 ITD does not directly interact with STAT5 and that FLT3 ITD does not activate JAK2, the aim was to study if an SFK might be the missing link between FLT3 ITD and STAT5.

To answer this question HEK293 cells were transfected with FLAG tagged FLT3 ITD or pCMV empty vector as a control (data not shown). The cells were harvested after 48 h

and lysed. To test for potential interactions with FLT3 ITD, GST tagged SH2 domains of several SFK (SRC, HCK, LCK and FYN) were purified and afterwards used in a pulldown experiment. The precipitates were subject to western blot analysis.

A strong interaction of FLT3 ITD and the SH2 domain of SRC could be observed. In contrast, no interaction between the other members of the SFK family and FLT3 ITD was detectable (fig 4.3).

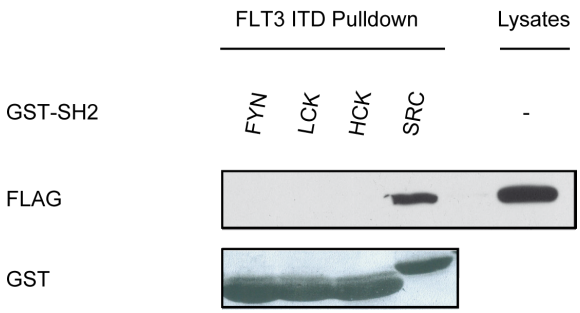

Fig. 4.3: Interaction of FLT3 ITD and SRC.

FLAG tagged FLT3 ITD was expressed in HEK293 cells. After a period of 48 h the cells were lysed and a pulldown experiment using the SH2 domains of different SFK was conducted.

4.3 Interaction of SRC and FLT3 WT, FLT3 ITD and FLT3 TKD

Based on the results that SRC interacts with FLT3 ITD the question raised was if SRC also binds to FLT3 WT or FLT3 TKD.

To this end, HEK293 cells were transfected with FLAG tagged FLT3 WT, FLT3 ITD or FLT3 TKD. The cells were either stimulated with FLT3 ligand (FL), as indicated, or left unstimulated before being harvested and lysed for further experiments. Following lysis, the cells were subject to a pulldown experiment using the SH2 domain of SRC fused to GST protein and coupled to glutathione beads.

While a strong interaction was observed between SRC-SH2 domain and FLT3 ITD, FLT3 WT and FLT3 TKD appeared to interact with SRC only weakly (fig. 4.4).

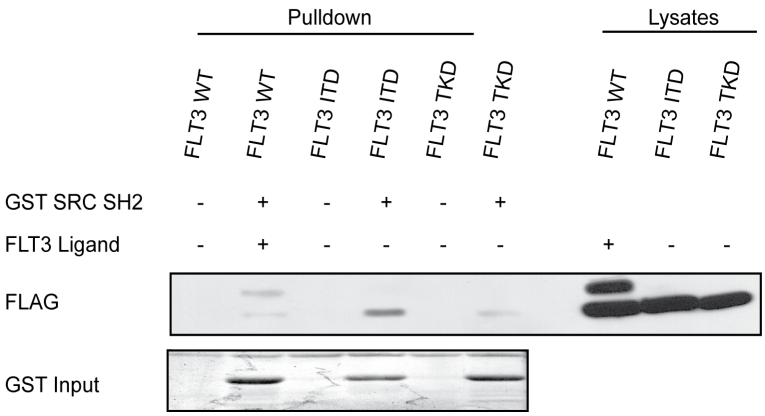

Fig. 4.4: Interaction of SRC and FLT3 WT, FLT3 ITD and FLT3 TKD.

FLAG tagged FLT3 WT, FLT3 ITD and FLT3 TKD constructs were expressed in HEK293 cells. After 48 h the cells were lysed and a pulldown experiment was conducted.

To ensure that the reduced interaction of FLT3 WT and FLT3 TKD with the SH2 domain of SRC was not due to impaired protein function, an immunoprecipitation of FLT3 WT, FLT3 ITD and FLT3 TKD was conducted. The respective proteins were expressed in HEK293 cells and afterwards subject to immunoprecipitation using FLAG specific antibodies. Since neither the phosphorylation nor the expression was impaired, the proper function of the constructs and therefore the reliability of the previously described results could be demonstrated (see fig. 4.5).

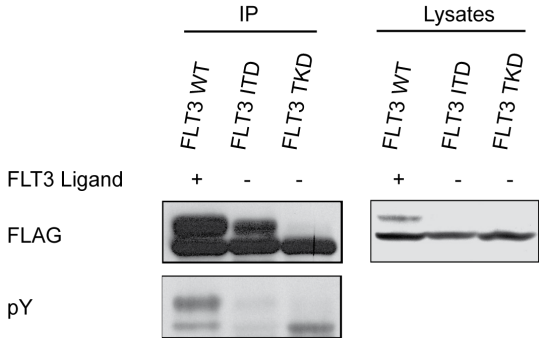

Fig. 4.5: Phosphorylation of FLT3 constructs.

FLAG tagged FLT3 constructs were expressed in HEK293 cells, lysed and subject to an immunprecipitation. To ensure that the observed differences in SRC binding are not due to impaired phosphorylation or expression.

4.4 Interaction of SRC and FLT3 ITD

To confirm the results from the GST-pulldown experiments in a physiological setting, co-immunoprecipitation of FLT3 WT, FLT3 ITD and FLT3 TKD was conducted. For this purpose the respective proteins were expressed in HEK293 cells, immunoprecipitated by a FLAG antibody and analyzed by western blot for FLT3 and co-immunoprecipitated SRC (fig. 4.6). As the interaction of SRC with receptor tyrosine kinases is dependent on the phosphorylation of the receptor, the phosphorylation of FLT3 was ensured by an immunoprecipitation followed by phospho-tyrosine western blot.

The SRC western blot showed an interaction of FLT3 ITD and SRC. In contrast no interaction between FLT3 WT (+/- ligand) or FLT3 TKD and SRC was detected.

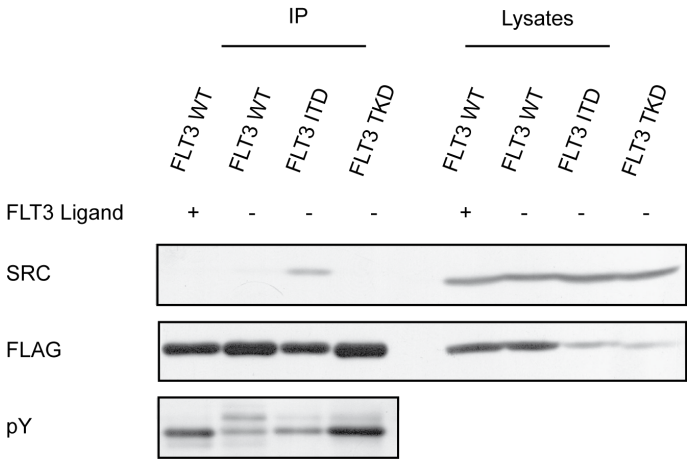

Fig. 4.6: Co-immunoprecipitation of FLT3 mutants and SRC.

The indicated FLAG tagged FLT3 constructs were transiently expressed in HEK293 cells. After 48 h the cells were lysed and a co-immunoprecipitation experiment was conducted.

4.5 Identification of Y589 and Y591 as binding sites of SRC and FLT3 ITD

As it has been shown that SRC interacts with tyrosine kinases via phosphorylated tyrosines[95] the possible interaction site of SRC and FLT3 ITD was to be determined. Other groups showed that the mutation of tyrosines within the FLT3 ITD protein changes the activation patterns of downstream targets like STAT5[113] and furthermore alters the FLT3 ITD induced phenotype in the murine bone marrow transplantation model.[116]

To verify if the tyrosines within FLT3 ITD are crucial for the physical interaction of SRC and the receptor, mutants of FLT3 ITD were checked for their interaction with SRC. As there are several tyrosine residues of interest within the protein, the interaction experiment was conducted with the mutation of FLT3 ITD in which the reduction of pSTAT5 was most pronounced. This is the case if the tyrosines Y589 and Y591 are substituted for phenylalanine (fig. 4.7). These constructs were kindly provided by K. Spiekermann.

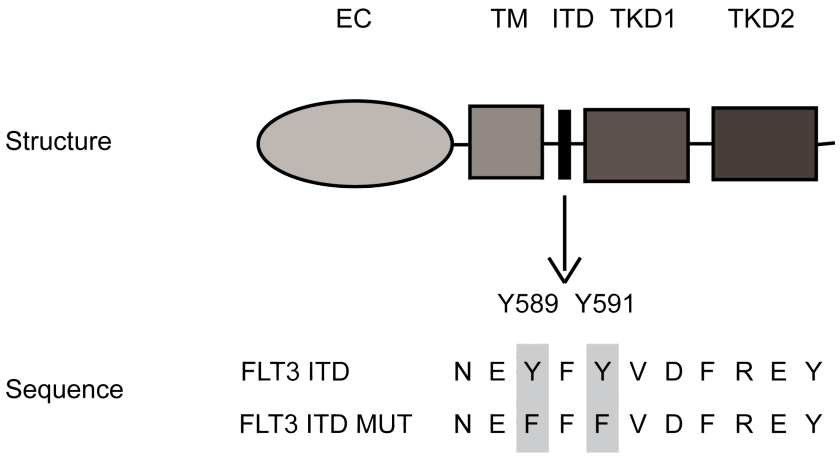

Fig. 4.7: The structure of mutated or unmutated FLT3 ITD.

The graphic shows the structure of FLT3 ITD and the sequence of the duplication within FLT3. The lower part depicts the sequence of the mutated part of FLT3 ITD in which the tyrosines 589 and 591 were substituted by phenylalanines.

To address if Y589 and Y591 are crucial for the interaction of FLT3 ITD and SRC, FLT3 ITD and FLT3 ITD Y589F/Y591F were transiently expressed in HEK293 cells. After 48 h the cells were harvested and subjected to lysis followed by a pulldown experiment using the SH2 domain of SRC bound to a GST fusion protein. To assess an interaction, SDS-PAGE was conducted, followed by immunoblot

The interaction between FLT3 ITD and SRC was interrupted if Y589 and Y591 were substituted for phenylalanine (fig. 4.8 A). Furthermore reduced activation of pSTAT5 was found when FLT3 ITD Y589F/Y591F was expressed in the HEK293 cells (fig. 4.8 B).

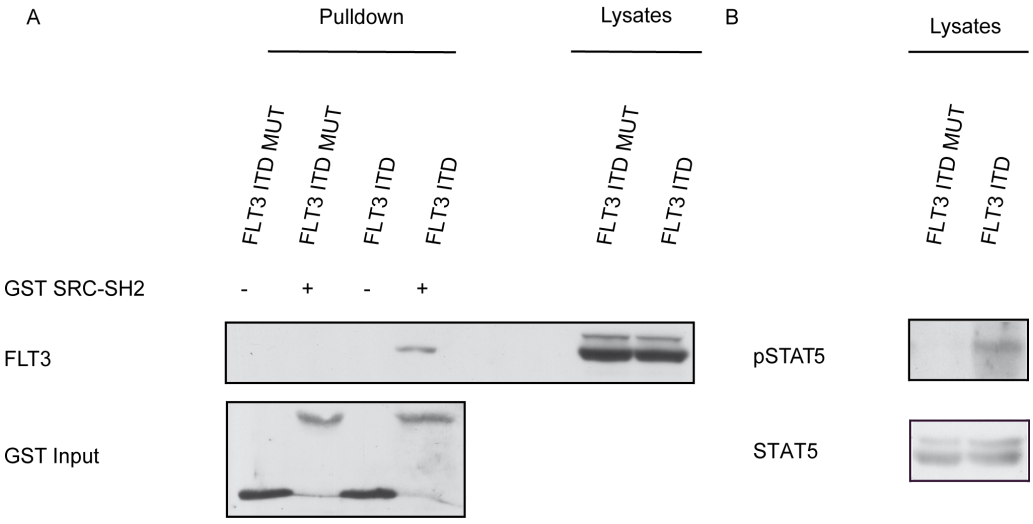

Fig. 4.8: Interaction of FLT3 ITD SRC via the SH2 domain of SRC is abolished if Y589 and Y591 are mutated (A). Furthermore this mutation reduces the activation of STAT5 (B).

HEK293 cells were transfected with FLT3 ITD or FLT3 ITD 589/591. After 48 h the cell were starved and subject to a GST-pulldown. In parallel the lysates were analyzed with regard to pSTAT5 levels by western blot.

4.6 Y589 and Y591 show different patterns of phosphorylation in FLT3 WT, FLT3 ITD or FLT3 TKD

Given the evidence that the phosphorylation-status of stimulated FLT3 WT and unstimulated FLT3 ITD or FLT3 TKD varies significantly[7,24], phosphospecific antibodies for Y589 and Y591 were obtained from Lars Rönnstrand (Lund University) to investigate the phosphorylation status of these specific sites.

BaF3 cells were retrovirally transduced with FLT3 WT, FLT3 ITD or FLT3 TKD and selected by growth factor deprivation. Before lysis and western blot, the cells were starved for 6 h in serum free medium. Whole phosphorylation and specific phosphorylation of Y589 and Y591 in FLT3 was analyzed. To ensure equal expression of FLT3, the membranes were stripped and re-probed with FLT3 antibody.

To investigate the role of ligand stimulation, all three forms of FLT3 were stimulated with FL for the indicated period of time. This stimulation only had an effect on the phosphorylation of FLT3 WT and showed no effect on FLT3 ITD or FLT3 TKD phosphorylation.

In summary, for FLT3 ITD a strong Y589 and Y591 phosphorylation was detected, regardless of ligand stimulation. In contrast, the tyrosine residues were much more weakly phosphorylated in FLT3 WT and FLT3 TKD.

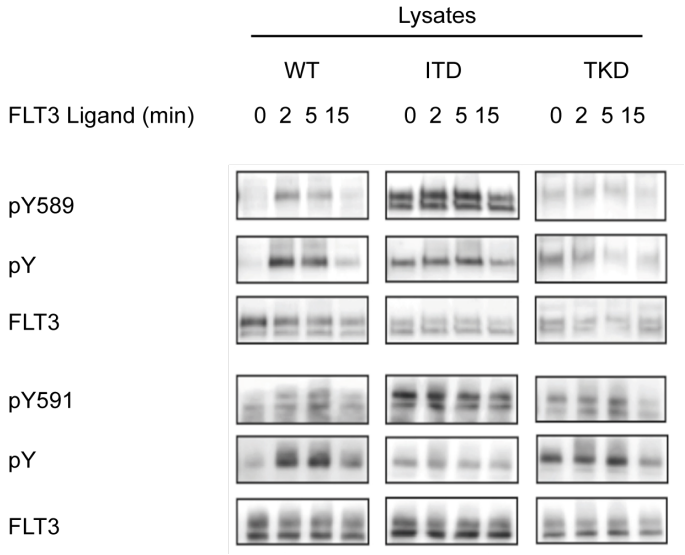

Fig. 4.9: Y589 and Y591 are stronger phosphorylated in FLT3 ITD than in FLT3 WT and FLT3 TKD.

BaF3 cells expressing FLT3 WT, FLT3 ITD or FLT3 TKD were used to investigate the phosphorylation of the Y589 and Y591, which are thought to be potential SRC binding sites. Lars Rönnstrand and his group from Lund University kindly provided these results.

4.7 The inhibitor PD166-326 has growth inhibitory effects on FLT3 ITD cells

To address if the tyrosine kinase SRC has any biological relevance in FLT3 ITD positive cells besides interacting with FLT3 ITD, the Pyrido[2,3-d]pyrimidine inhibitors PD166-326, PD173-952 and PD180-970 were tested for their effect on FLT3 WT, FLT3 ITD and FLT3 TKD expressing cells. As the compound PD166-326 turned out to be the most effective SRC inhibiting substance, the following experiments were conducted using this substance.

First the effect of PD166-326 on FLT3 ITD and FLT3 TKD expressing 32D cells was examined. 32D cells were retrovirally transduced with the respective constructs and selected by growth factor deprivation. Afterwards, the cells were incubated for 48 h with the indicated

concentrations of PD166-326 and their proliferative activity was analyzed using the MTT assay.

Strikingly the compound affected only FLT3 ITD positive cells up to a concentration of 125 nM. At this inhibitor concentration the proliferation was reduced by 55 % compared to untreated control cells. In contrast FLT3 TKD expressing cells were not affected regarding their proliferative activity (fig. 4.10). To ensure that the observed effect was not due to any unspecific cytotoxic effect of the compound, the FLT3 ITD positive cells were stimulated with IL3. The inhibitory effect of PD166-326 on the FLT3 ITD positive cells could be reversed through the addition of this cytokine.

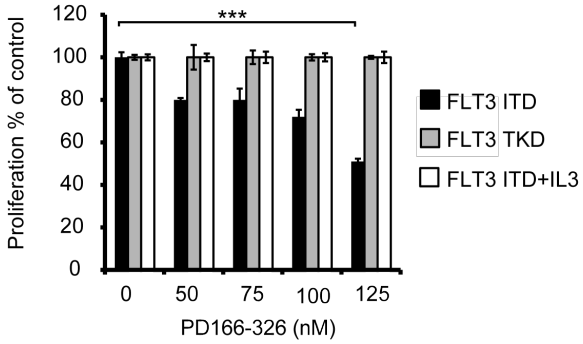

Fig. 4.10: The SRC inhibitor PD 166-326 inhibits the proliferation of FLT3 ITD but not FLT3 TKD expressing 32D cells.

32D cells were incubated for 48 h with the indicated concentrations of PD 166-326 with or without IL3 and afterwards investigated regarding their proliferative activity using the MTT assay. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates. One representative of at least three independent experiments is shown. Statistical analysis was conducted ***= p< 0,001;**= p<0,01; *= p value < 0,05; NS (no significance)= p>0,05

4.8 PD166-326 leads to reduced pSTAT5 levels in FLT3 ITD cells through SRC inhibition

To assess if the observed growth inhibitory effect of PD166-326 on FLT3 ITD positive 32D cells is due to SRC inhibition, an immunoprecipitation of SRC was conducted. 32D cells expressing FLT3 ITD were generated as previously described and incubated with the indicated concentrations of PD166-326 in serum free medium for 6 h. The cells were harvested, lysed and subjected to western blot analysis.

In order to monitor the SRC inhibition by PD166-326 an immunoprecipitation followed by phospho-tyrosine blot was performed. In parallel an immunoprecipitation of FLT3 was utilized to rule out an inhibition of FLT3 by the compound.

The inhibitor treatment led to a reduction of pSTAT5 levels in a dose-dependent manner showing complete reduction of the pSTAT5 signal between 50 and 75 nM of the inhibitor PD166-326. To ensure equal loading, the membranes were stripped and re-probed with STAT5 antibody revealing no significant differences in STAT5 activation (fig. 4.11).

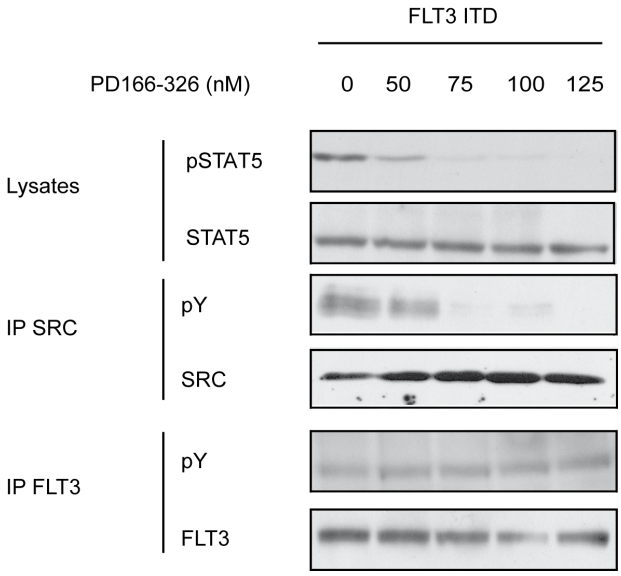

Fig. 4.11: SRC inhibition reduces FLT3 ITD dependent activation of STAT5 in 32D cells.

32D cells expressing FLT3 ITD were treated with indicated concentrations of PD166-326 for 6 h and analyzed regarding the activation of STAT5, SRC and FLT3.

To control if cytotoxic effects induced the observed effects on the FLT3 ITD down-stream targets, the cells were treated with the same concentrations of inhibitors and in addition stimulated with IL3. The experiments showed equal pSTAT5 level up to the maximum inhibitor concentration of 125 nM, ruling out any cytotoxic effects (fig. 4.12).

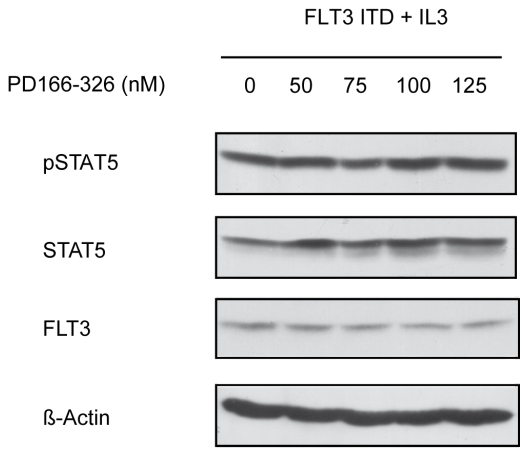

Fig. 4.12: Dose dependent reduction of pSTAT5 level by PD166-326 can be reversed by stimulation of the cells with IL3.

32D cells expressing FLT3 ITD were incubated for 6 h in serum free medium with the indicated concentrations of inhibitors and additionally stimulated with IL3.

4.9 Combination PD166-326 and PKC412 has additive inhibitory effects on FLT3 ITD positive cells

Most of today's chemotherapy regimes are composed of several cytostatic drugs, in order to achieve the maximum clinical outcome. The inhibitor PKC412 is known to inhibit FLT3 WT and its mutants and is therefore used in several clinical trials of acute myeloid leukemia.[91] The positive results of these studies encouraged us to investigate the effect of PKC412 and PD166-326 in combination on FLT3 ITD and FLT3 TKD expressing cells.

To this end FLT3 ITD and FLT3 TKD expressing 32D cells were assessed with regard to their proliferation activity after 48 h of treatment with PD166-326 and PKC412 alone or in combination. Analysis of the acquired data showed the expected growth inhibition of FLT3 ITD and FLT3 TKD expressing cells by PD166-326 and PKC412 (fig. 4.13). The combination of both substances led to an additive reduction of proliferation of 20% on FLT3 ITD expressing cells. In contrast FLT3 TKD positive cells were not affected by the combination of the compounds.

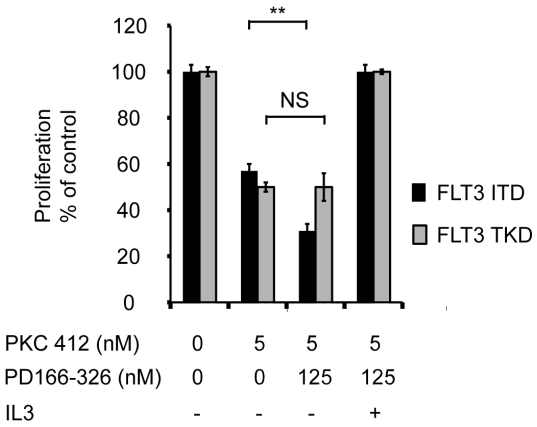

Fig. 4.13: The combination of PKC412 and PD166-326 leads to additive growth inhibitory effects on FLT3 ITD cells.

32D cells expressing FLT3 ITD or FLT3 TKD were cultured in the presence of different inhibitor concentrations and combination and evaluated for their proliferative activity. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates. One representative of at least three independent experiments is shown. Statistical analysis was conducted ***= p< 0,001;**= p<0,01; *= p value < 0,05; NS (no significance)= p>0,05

4.10 The biochemical effect of PKC412 and PD166-326 combination on downstream targets of FLT3 ITD

Since the combination of the two inhibitors proved to effectively inhibit the proliferative activity of FLT3 ITD positive 32D cells, the underlying mechanisms remained to be investigated.

To assess the molecular biological effect of the inhibitor combination, FLT3 ITD positive 32D cells were incubated for 6 h in the present of the indicated inhibitors in serum free medium. Afterwards the cells were harvested, lysed and subjected to SDS-PAGE before being analyzed by immunoblot.

The combination of both substances caused a significant reduction in pSTAT5 level (fig. 4.14).

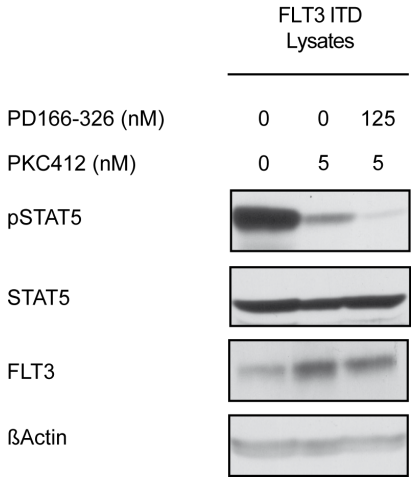

Fig. 4.14: Combination of PKC412 and the SRC inhibitor PD166-326 leads to a reduction of pSTAT5 level in FLT3 ITD positive cells.

32D cells stably expressing FLT3 ITD were incubated in serum free medium with the indicated concentration of the inhibitors for 6 h.

4.11 siRNA mediated down-regulation of SRC leads to reduced proliferation of FLT3 ITD positive cells

As inhibitors are prone to off-target effects in the next step a more specific method was used. The siRNA-mediated down-regulation of target genes is a well-established and versatile method to investigate the function of target genes under *in vivo* like conditions.[117-

[119] By using the BIOPREDsi algorithm of the Novartis Institutes of BioMedical Research, an SRC specific shRNA was designed and cloned into a pSUPER-puro vector, ensuring a stable expression of the siRNA. The plasmid encoding for the shRNA was transduced into FLT3 ITD and FLT3 TKD expressing 32D cells. After transfection, the cells were selected for 3-5 days using puromycin and IL3. As control, 32D cells expressing a luciferase shRNA was generated and used in the same manner.[120]

First the impact of SRC downregulation on FLT3 ITD and FLT3 TKD expressing 32D cells was assessed by MTT assay. To to so the cells were kept in culture and their proliferative activity was measured after 48 h of IL3 deprivation but in the presence of puromycin.

The down-regulation of SRC (fig 4.16) led to a significant reduction of proliferative activity in FLT3 ITD cells. In contrast SRC down-regulation had no effect on FLT3 TKD positive 32D cells (fig. 4.15).

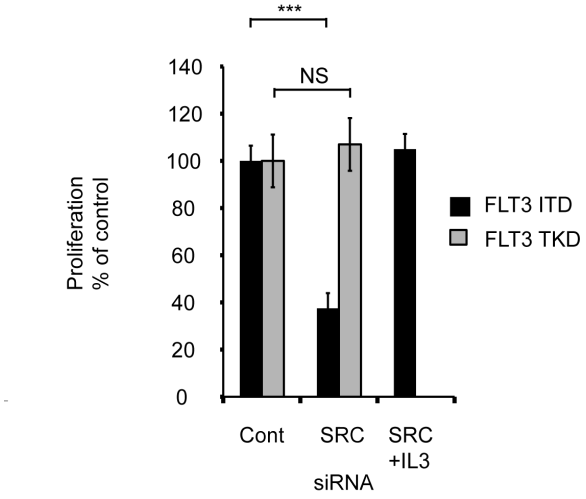

Fig. 4.15: shRNA mediated downregulation of SRC leads to decreased proliferative activity in FLT3 ITD expressing 32D cells.

32D cells expressing a SRC siRNA or a control siRNA against luciferase were assessed for their proliferative activity after 48 h. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates. One representative of at least three independent experiments is shown. Statistical analysis was conducted ***= p< 0,001;**= p<0,01; *= p value < 0,05; NS (no significance)= p>0,05

4.12 Assessment of the biochemical effect of SRC down-regulation on FLT3 ITD and FLT3 TKD positive cells

Having shown that reduced expression of SRC has a biological effect on FLT3 ITD but not on FLT3 TKD cells in the next step, the impact of reduced SRC expression on the biochemical properties of the cells was addressed.

32D cells expressing either FLT3 ITD or FLT3 ITD were transduced with a control shRNA or a SRC-specific shRNA and kept for 6 h in serum free medium, harvested and subjected to analysis by western blot for SRC expression.

Upon down-regulation of SRC, the pSTAT5 level in FLT3 ITD positive 32D cells was remarkably reduced. The additional evaluation of established FLT3 ITD signaling conductors, such as AKT and ERK, revealed no significant impairment (fig. 4.16 and data not shown). In FLT3 TKD cells the reduction of SRC expression did not affect signaling pathways like AKT and ERK (fig. 4.16 and data not shown). To rule out any cytotoxic effect of the siRNA on the cells, the FLT3 ITD positive 32D cells expressing either the SRC siRNA or the control siRNA were stimulated with IL3 and their status of STAT5 activation was analyzed. Upon IL3 treatment the observed reduction of pSTAT5 levels was reversed (fig. 4.17).

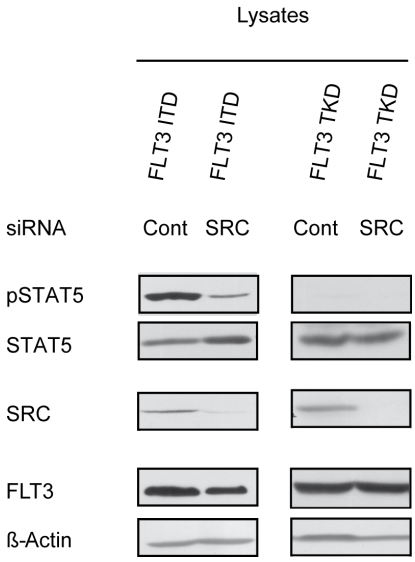

Fig. 4.16: Reduced SRC expression leads to a reduction of STAT5 phosphorylation in FLT3 ITD cells.

32D cells stably expressing FLT3 ITD or FLT3 TKD and siRNA against Luciferase (control) or SRC were analyzed for the activation of STAT5 and SRC expression.

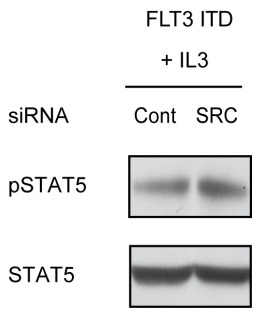

Fig. 4.17: SRC siRNA mediated pSTAT5 reduction in FLT3 ITD cells can be reversed by IL3 stimulation.

32D cells, retrovirally transduced with FLT3 ITD and the indicated siRNA, were stimulated with IL3 and analyzed by western blot.

4.13 The effect of reduced SRC expression on FLT3 ITD induced leukemia in mice

As the previously described experiments verified the importance of SRC for the signal transduction in FLT3 ITD positive 32D cells, in the following part the effect of SRC down-regulation on FLT3 ITD mediated leukemogenesis was assessed. Therefore, a mouse-model was applied that made use of 32D cells expressing FLT3 ITD and the SRC specific or control siRNA. Upon injection of the FLT3 ITD or FLT3 TKD positive 32D cells, the animals succumbed to a lethal hematological disease within a few weeks. As previously described, the cells were intravenously injected into recipient mice and the animals monitored by peripheral blood analysis for GFP positive cells and sacrificed if moribund.[63]

Reduced SRC expression led to a prolonged survival in FLT3 ITD transplanted animals, with both cell lines causing a lethal hematopoietic disease (fig. 4.18). In contrast to the control group (median survival of 32 days), the mice injected with cells expressing the SRC siRNA had a prolonged latency (median survival of 48 days). Animals transplanted with FLT3 TKD positive cells expressing SRC siRNA or Luciferase siRNA did not develop a lethal leukemia like disease for over 140 days (data not shown).

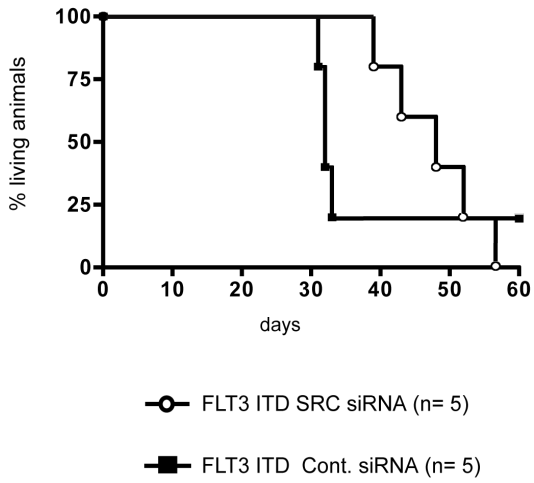

Fig. 4.18: SRC expression is crucial for a FLT3 ITD positive leukemia-like disease in mice.

Kaplan Meier plot of C3H mice injected with 32D FLT3 ITD cells stably expressing either control siRNA or a SRC siRNA. One of three independent experiments is shown.

4.14 LCK is not involved in the signal transduction of FLT3 ITD

To verify the specificity of the approach, in the next step an shRNA against LCK was designed. An LCK shRNA or a control shRNA against luciferase were transduced into 32D cells expressing FLT3 ITD. Afterwards the cells were selected as previously described using puromycin. First, the cells proliferative activity was measured after 48 h and simultaneously analyzed. The reduction of LCK expression had no effect on the proliferative activity of the cells, with them showing slightly higher values than the luciferase siRNA expressing control cells (fig. 4.19). Consistent with these findings, the down-regulation of LCK was efficient and showed no effect on the phosphorylation of STAT5 in FLT3 ITD positive 32D cells (fig. 4.20).

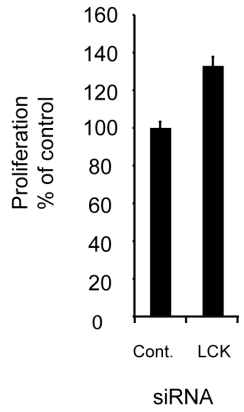

Fig. 4.19: LCK down-regulation does not affect the proliferation of FLT3 ITD positive 32D cells.

FLT3 ITD positive 32D cells expressing either control or LCK siRNA were assessed for their proliferative activity after 48 h. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates.

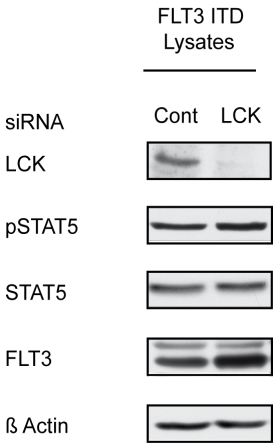

Fig. 4.20: Reduced LCK expression has no effect on the activation of STAT5 in FLT3 ITD positive 32D cells.

32D cells stably expressing FLT3 ITD and control or LCK siRNA were kept in cytokine free medium for 6 h and afterwards analyzed by western blot.

4.15 Combination of PKC412 and Dasatinib has inhibitory effects on human and murine FLT3 ITD positive cells

Up to this point the described experiments were conducted in murine cell lines as well as in animals. Thus the question needed to be addressed if the observed results reflect the situation in human AML cells. To investigate this FLT3 ITD or FLT3 TKD positive human and, as control, murine cell lines were treated with the FLT3 inhibitor PKC412 alone or in combination with Dasatinib.

FLT3 ITD positive human MV4-11 cells and 32D cells expressing FLT3 ITD and TKD were cultured in the presence of PKC412 or PKC412 and Dasatinib for 48 h. Then, the proliferative activity measured by an MTT assay.

The inhibitor PKC412 led to the reduction of proliferative activity. The combination of PKC412 and Dasatinib led to an additive inhibitory effect on the FLT3 ITD expressing cells regardless of human or murine origin (fig. 4.21).

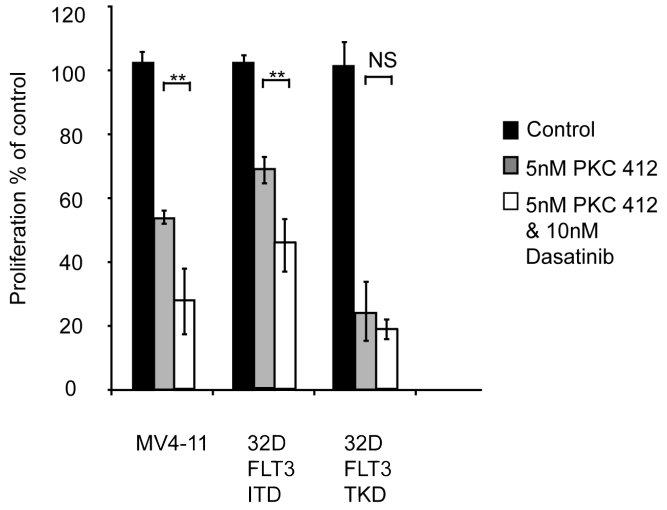

Fig. 4.21: Combination of PKC412 and Dasatinib has additive growth inhibitory effects only on FLT3 ITD positive cells.

MV4-11 cells or 32D cells expressing FLT3 ITD or FLT3 TKD were incubated with the indicated concentrations of inhibitors an analyzed with regard to their proliferative activity after 48 h. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates. One representative of at least three independent experiments is shown. Statistical analysis was conducted ***= p< 0,001;**= p<0,01; *= p value < 0,05; NS (no significance)= p>0,05

4.16 Biochemical effect of the combination of PKC412 and Dasatinib on MV4-11 cells

Having demonstrated a reduction of proliferative activity of MV4-11 cells by PKC412 and Dasatinib treatment, in the next step the biochemical effects were analyzed. Therefore, the cells were incubated with the indicated concentrations of inhibitors for 6 h in serum free medium, harvested, lysed and subjected to western blot.

The combination of both compounds led to an increased reduction of pSTAT5 (fig. 4.22) closely correlating with the observed effects from the proliferation assay.

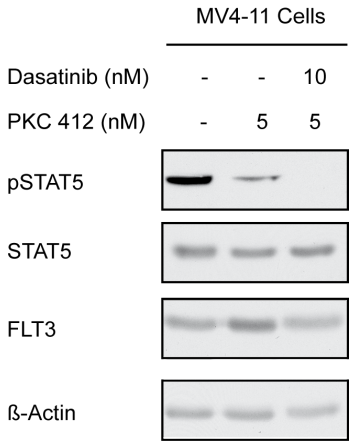

Fig. 4.22: Combination of PKC412 and Dasatinib leads to additive biochemical effects on FLT3 ITD positive MV4-11 cells.

MV4-11 cells were cultured in the presence of the indicated inhibitor concentrations for 6 h in serum free medium and analyzed by western blot.

4.16.1 Combination of PKC412 and Dasatinib increases apoptosis in MV4-11 cells

To assess the effect of the inhibitor combination with regard to apoptosis, MV4-11 cells were exposed to several concentrations of PKC412 and Dasatinib. The cells were harvested at daily intervals, and Annexin V/PI (propidium iodide) staining was carried out. MV4-11 cells showed a response to both inhibitors (fig. 4.23). Furthermore, the combination of both inhibitors led to an additive increase of apoptosis at doses as low as 5 nM (PKC412) and 100 nM (Dasatinib).

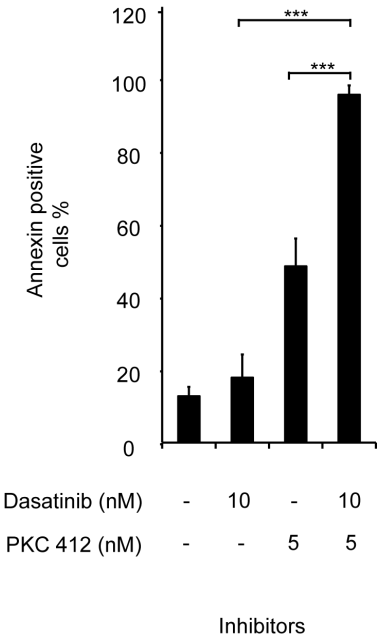

Fig. 4.23: Increased apoptosis of MV4-11 cells by the combination of PKC412 and Dasatinib.

MV4-11 cells were treated with indicated inhibitor concentrations and analyzed for apoptosis by Annexin V/PI staining. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates. One representative of at least three independent experiments is shown. Statistical analysis was conducted ***= p< 0,001;**= p<0,01; *= p value < 0,05; NS (no significance)= p>0,05

4.17 Dasatinib and PKC412 show additive inhibitory effects on primary human AML cells

In the next step it was assessed if the acquired results can also be observed in patients. FLT3 ITD and FLT3 TKD positive patient samples were obtained and separated using the Ficoll method. After 48 h of cultivation in the presence of PKC412 and Dasatinib the cells were tested for their proliferative activity by MTT assay.

Strikingly, the combination of both inhibitors had an additive inhibitory effect on cells of the FLT ITD patient, although this was not as pronounced as observed in 32D or MV4-11 cells (fig. 4.24). In contrast FLT3 TKD positive cells showed only a decrease when treated with PKC412 and no additive effect for the combination with Dasatinib was observed (fig. 4.24).

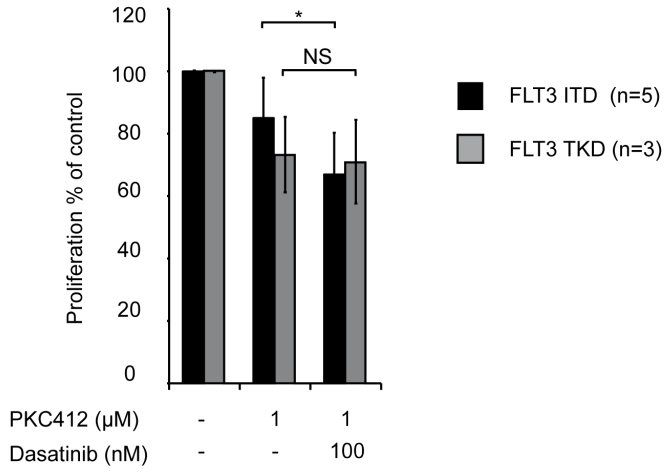

Fig. 4.24: Combination of Dasatinib and PKC412 leads to a reduction in proliferation of FLT3 ITD positive patient sample cells.

Peripheral blood cells of FLT3 ITD or FLT3 TKD positive AML patients were treated with the indicated concentrations of inhibitors and assessed for their proliferative activity after 48 h. Data are presented as percentage of DMSO treated cells and represent values +/- SD of triplicates. One representative of at least three independent experiments is shown. Statistical analysis was conducted ***= p< 0,0001;**= p<0,001; *= p value < 0,005; NS (no significance)= p>0,005

5 Discussion

5.1 Identification of potential FLT3 ITD interacting partners

FLT3 is a membrane-bound receptor, which is composed of an immunoglobulin-like extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane dimerization domain, and a highly conserved intracellular kinase domain that is subdivided by a kinase insert.[1,37]

The protein is involved in numerous processes of normal hematopoiesis, including transcription, proliferation and apoptosis.[59] Combinations of FL and other growth factors have been found to promote proliferation of primitive hematopoietic progenitor cells as well as more committed early myeloid and lymphoid precursors.[53,54] FLT3 furthermore seems to mediate differentiation of early progenitors. In these cells exposure to FL leads to monocytic differentiation without significant proliferation.[53] FLT3 knockout mice have a subtle phenotype displaying reduced b-cell precursors, with mice transplanted with FLT3 knockout cells suffering from a more global disruption of hematopoiesis.[55,121]

In AML patients FLT3 is the most frequently mutated receptor tyrosine kinase and alterations can be found in about 30% of all AML patients.[3] There are two major types of mutations: FLT3 ITD and FLT3 TKD mutations.[15,61] FLT3 ITD is a group of mutations characterized by a duplication within the juxtamembrane region of the receptor of varying length.[51] Patients harboring this kind of FLT3 mutation appear to have less favorable outcomes.[3,25] In contrast, point mutations within the kinase domain (FLT3 TKD) are less frequent and appear to have no effect on the clinical outcome of the patients.[1,4]

The two classes of mutations not only differ in their clinical outcome and underlying molecular changes, they furthermore show significant disparities with regard to their biological transforming potential.[7]

Our and other groups have shown that FLT3 ITD induces an MPD-like disease in a murine bone marrow transplantation model.[6,7,24] In contrast FLT3 TKD transplanted mice succumb to a lymphoid hematological disease.

Molecular biological analysis of cell lines stably expressing FLT3 ITD or FLT3 TKD of both human and murine origin revealed further differences within the downstream signaling of these two mutation classes.[8,122] Most intriguing were the differences with regard to STAT5 activation, which was strongly induced in FLT3 ITD expressing cells but only marginally in FLT3 TKD positive cells,[43,66,122] suggesting that STAT5 activation and further STAT5 target gene modulation could be particularly important for FLT3 ITD mediated AML carcinogenesis.

Currently, there are several established models for the activation of STAT5 by receptor tyrosine kinases.[115] Firstly, through direct interaction between STAT5 and the receptor; secondly, through the protein JAK2, which leads to increased STAT5 phosphorylation;[76] or thirdly, via adaptor molecules such as members of the SFK.[123]

It has been shown that FLT3 ITD neither directly interacts with STAT5 nor does it activate JAK2.[113] Since other groups demonstrated that the activation of STAT5 in solid and hematopoietic malignancies is mediated by SFK,[101,124-126] this work focused on the investigation of the importance of this group of proteins in the signal transduction of FLT3 ITD and FLT TKD.[113,124-126]

To do so, an approach was used in which different members of the SFK family were tested for their physical interaction with FLT3 ITD. In these experiments an interaction between FLT3 ITD and SRC was observed.

In order to investigate if the association of SRC is exclusive for FLT3 ITD, the physical interaction between SRC and FLT3 WT, FLT3 ITD and FLT3 TKD was determined by GST pull down and co-immunoprecipitation. The interaction between SRC and FLT3 ITD appeared to be mutually exclusive.

These results are in line with the observations of several groups, which report strong activation of SFK in primary AML cells.[106,127] In contrast, Choudhary et al. claim that the activation of STAT5 by FLT3 ITD is not mediated through activation of SFK, subsequently postulating a direct interaction of FLT3 ITD and STAT5.[44] These observed differences might be due to the different material and experimental settings.

5.2 The role of Y589 and Y591 for the interaction of FLT3 ITD and SRC

The group of Griffith et al. showed that the tyrosine rich juxtamembrane region of FLT3 has a crucial role in modulating the activity of FLT3 WT.[37] It is believed that this stretch exerts inhibitory effects on spontaneous phosphorylation of the inhibitor. Their findings further suggest that it may be possible that ITD mutations within the JM domain of FLT3 result in a change of FLT3 structure leading to constitutive activation of the kinase and exposure of Y589 and Y591.

In FLT3 WT, these amino acids have been shown to be important for the FL-dependent activation, as substitution of the four tyrosines Y589, Y591, Y597 and Y599 abrogated the proliferation of stimulated FLT3 WT cells.[128] Thus the molecular mechanisms in which these amino acids are involved are not understood so far.[40,113,116]

To investigate the role of the tyrosines Y589 and Y591 in the context of SRC and FLT3 ITD interaction, we obtained a FLT3 ITD mutant from Karsten Spiekermann's group. In this mutant Y589 and Y591 were substituted for phenylalanine. They report that the simultaneous substitution of Y589 and Y591 completely abolishes the transforming potential of FLT3 ITD when expressed in BaF3 cells and furthermore leads to a significant reduction of STAT5 activation within the cells.[113]

Other groups could show that the juxtamembrane tyrosine residues analogous to Y589 and Y591 in other members of related receptor tyrosine kinases have significant functions in the regulation of the protein.[40,116] For example in PDGFRb, another class 3 receptor tyrosine kinase, the mutation of Y579 and Y581 led to a decrease in kinase activity.[129-130] In addition, phenylalanine substitutions of the corresponding juxtamembrane tyrosines in c-KIT also impaired kinase activity.[131]

Based on the findings of other groups, which showed that SH2-containing molecules like SFK recruit to tyrosines within the juxtamembranic domain in other Class III RTKs, we investigated if Y589 and Y591 are involved in the interaction of FLT3 ITD and SRC.[132,133]

Using the phosphodeficient mutants of FLT3 ITD interaction, experiments investigating physical association of FLT3 ITD and the SH2 domain of SRC were conducted. The interaction of FLT3 ITD and SRC was abrogated upon mutation of both tyrosines. In addition the pSTAT5 levels were significantly reduced upon substitution of the amino acids.

These findings point towards a crucial role of Y589 and Y591 for FLT3 ITD and SRC interaction. Furthermore the results might help to explain the substantial change in the phenotype of FLT3 ITD transplanted mice reported by Rocnick et al.[116]

5.3 The phosphorylation of Y589 and Y591 differs between FLT3 WT and mutants

It is an established model that upon receptor stimulation by ligand binding, the activated FLT3 in turn induces biochemical processes mediating target gene expression, protein trafficking or cell cycle progression.[132] This is done by the recruitment of downstream targets, so-called cytoplasmic protein kinases (PTK) like the SFK.

The interaction of the PTK with the receptor protein can take place via specific domains e.g. the SRC-homology-domain2 (SH2) and SRC-homology-domain 3 (SH3). These domains are further embedded in other sequences, which are responsible for the binding specificity of the SH2 domains.[133,134] SH2 domains are known to recognize phosphotyrosine rich sequences with a high affinity, making proteins bearing a SH2 domain explicitly involved in the signaling of receptor tyrosine kinases.[135-137]

In 2009, Razumovskaya et al. published a work in which they investigated the phosphorylation status of several tyrosines within FLT3 WT, FLT3 ITD and FLT3 TKD. By using phospho-tyrosine specific antibodies they were able to detect new phosphorylation sites, of FLT3 WT, FLT3 ITD and FLT3 TKD.

Analysis of the tyrosine residues Y589 and Y591 using the antibodies created by Razumovskaya et al. revealed striking differences in the degree of phosphorylation between FLT3 ITD and FLT3 TKD. In FLT3 ITD the tyrosines are much stronger phosphorylated than in FLT3 TKD or FLT3 WT. This data reveals strong differences in phosphorylation of Y589 and Y591 and also gives a potential explanation for the strong interaction of SRC with FLT3 ITD, supporting our results, which underline the importance of these amino acids in this interaction.

5.4 The effect of reduced SRC expression on FLT3 ITD and FLT3 TKD positive cells

RNA interference is a naturally occurring mechanism of gene silencing that was first discovered in nematodes.[118] The mechanism of RNA interference can be found in almost every multicellular organism and is thought to be evolved as a defense mechanism against viral dsRNA.[138] There are currently three mechanisms for achieving RNA interference: through small interfering RNA, which is double strand RNA of 21 or 22 nucleotides in length, short hairpin RNA (shRNA), which is named after its hairpin structure, or microRNA (miR), endogenous non-coding RNA of 22 nucleotides length.[119]

One of the most challenging parts of siRNA usage is the appropriate delivery into and expression in the cells. There are several published methods utilizing synthetic polymers, lipid polymers and viral vectors.[139,140]

In cancer research RNAi has been used for the reduced expression of genes *in vivo*, as this allows a better assessment of the specific gene of interest. Many pre-clinical studies are being established to investigate the feasibility of RNAi usage for the therapy of several cancers, but these trials are in their early stages.[138,141- 143]

In this project an shRNA against SRC was used to determine the impact of its down-regulation on FLT3 ITD and FLT3 TKD expressing cells. As the desired experiments required long-term expression of the interfering RNA, an shRNA model was used in which the sequence was cloned into a pSUPER-puro vector, which enabled efficient retroviral transduction and consecutive selection of the target cells.

The effective downregulation showed strong molecular biological and biological effects on FLT3 ITD but not on FLT3 TKD positive cells, thus arguing for a role of SRC in the signaling of FLT3 ITD but not for FLT3 TKD.

5.5 The influence on SRC down regulation on FLT3 ITD induced leukemia in mice

In the murine bone marrow transplantation model FLT3 ITD and FLT3 TKD induced two different phenotypes. Animals transplanted with bone marrow expressing FLT3 ITD develop a myeloproliferative disease whereas FLT3 TKD transplanted animals suffer from a lymphoid disease.[7,24]

This mouse model was used by Rocnick et al. 2006[116] to investigate the impact of mutations within the tyrosine residues of the juxtamembranic region of FLT3 ITD. They transplanted mice with bone marrow expressing either FLT3 ITD or FLT3 ITD deficient for phosphorylation of Y589 and Y591 and subsequently showed that the mice do not develop the myeloproliferative hematologic disease that unmutated FLT3 ITD usually induces.

Another broadly used method is the transplantation of 32D cells in C3H mice.[107,144] If transplanted with FLT3 ITD positive cells, the animals develop a leukemia-like disease within a few weeks.[63] Interestingly, animals receiving 32D cells positive for FLT3 TKD do not develop any disease. The reason for this phenomenon remains unclear though it might be due to the reduced transforming potential of FLT3 TKD.[10]

In this project the 32D cell based mouse model was used to assess the impact of RNAi induced SRC down-regulation on the FLT3 ITD and FLT3 TKD driven leukemia-like disease in mice. Animals, which received FLT3 ITD positive cells, developed a lethal leukemia-like disease within 4 weeks. Interestingly the shRNA-mediated reduction of SRC expression within FLT3 ITD positive cells increased the median survival of the mice by more than two weeks. FLT3 TKD transplanted mice did not develop any disease.

In conclusion these data indicate that SRC is crucial for the development of the leukemia-like disease induced by FLT3 ITD.

5.6 FLT3 mutants respond differently to SRC inhibitors and inhibitor combinations

The FLT3 protein is an attractive target for tyrosine kinase inhibitors (TKIs) as FLT3 mutations are one of the most common mutations diagnosed in AML patients.[1]

PKC412 is a benzoylstaurosporine, which was developed as a vascular endothelial growth factor receptor inhibitor but also inhibits the FLT3 receptor kinase.[90] In studies, PKC412 was used as a single agent[91] or combined with conventional chemotherapy such as daunorubicin and cytarabine.[70] The results suggest a possible benefit for patients with FLT3 ITD if PKC412 is combined with standard chemotherapy.

The important role of SRC in numerous cancers and other diseases triggered the interest in developing SRC inhibitors.[99] Pyrido-pyrimidines (pyrido-[2,3-d]pyrimidines) are a group of small molecule inhibitors that act as antagonists of SRC.[110] PD166-326, the

compound that is used in this project, has been proven to effectively inhibit SFK in BCR-ABL positive leukemic murine bone marrow cells.[111]

In the experiments the SRC inhibitor PD166-326 led to a reduction of proliferative activity in FLT3 ITD but not of FLT3 TKD expressing cells, suggesting an important role of SRC in the signaling of FLT3 ITD. The analysis further revealed a reduction of SRC and STAT5 activation in FLT3 ITD positive cells. In addition, the effect of PKC412 and PD166-326 on FLT3 ITD and FLT3 TKD expressing cells was assessed and showed exclusively additive growth inhibitory effects on FLT3 ITD but not on FLT3 TKD cells.

These data not only underline the significant role of SRC in the mechanisms by which FLT3 ITD activates STAT5 but also point towards beneficial effects of combinatory treatment with PKC412 and an SRC inhibitor to target the proliferation of FLT3 ITD positive cells.

Up to now there has not been much data available on the effect of SRC inhibitors on FLT3 WT, FLT3 ITD and FLT3 TKD expressing cells. Okamoto et al. 2007[107] used PP2, a pan SFK inhibitor, and observed its effect on the growth of FLT3 WT and FLT3 ITD expressing cells. They detected a reduction of STAT5 activation coherent with a reduction in SFK phosphorylation. In contrast Choudhary et al.[44] using the same compound at identical concentrations could not see any effect on STAT5 activation. These disparities are interesting and most likely explainable by different experimental settings and material used. As all these experiments were performed *in vitro* in murine cell lines, further experiments are needed to verify if the SRC inhibition has the same effect on human FLT3 ITD positive cells.

5.7 SRC as a therapeutic target in human AML therapy

Currently, the treatment of AML patients can be regarded as unsatisfying. Despite the fact that a complete remission can be achieved by treating the patients using chemotherapy agents like anthracyclins and cytarabine, they frequently relapse.[28]

As the therapeutic responses are considered modest, the combination of FLT3 inhibitors and conventional chemotherapy is currently investigated in several trials.[89,145] Recently, new FLT3 inhibitors have been developed using alternative approaches to inhibit FLT3.[146]

Hitherto there is only few data on SRC inhibitors for the treatment of AML patients, especially FLT3 ITD positive patients. Ozawa et al. reported in 2007 on constitutive activation SFK in human AML cell lines and showed increased phosphorylation in some

samples of AML patients.[127] In addition pre-clinically used SRC inhibitors like PP2 were tested on different AML cell lines and samples and showed growth reducing effects on some of them.[106] This is in line with our findings, which saw growth reduction by Dasatinib (a dual SRC ABL inhibitor), which has been approved for clinical use in CML treatment.[102] The findings of Dos Santos et al. support our hypothesis, as they were able to detect increased SFK phosphorylation in several AML patient samples.[106]

In this study the effect of Dasatinib in combination with the FLT3 inhibitor PKC412 was assessed. In the human FLT3 ITD positive cell line MV4-11 the SRC inhibitor Dasatinib led to a reduction in proliferation; this reduction was increased by the combination of Dasatinib and PKC412. The analysis of the cells revealed a decreased phosphorylation of the downstream signal component STAT5.

Using the primary patient material the effect was reproducible on cells of a FLT3 ITD positive patient. In contrast, SRC inhibition using Dasatinib did not lead to any detectable reduction in proliferative activity using the cells of a patient harboring the FLT3 TKD mutation.

Taken together these data demonstrate that human AML cells expressing FLT3 ITD are more sensitive to SRC inhibitors like Dasatinib than FLT3 TKD positive cells. This can be explained by interaction of SRC and FLT3 ITD, which subsequently leads to the activation of STAT5. This observation needs to be followed up by further experiments, although still indicates a potential therapy option for FLT3 ITD positive AML patients.

6 Material

6.1 Reagents

Acrylamide/Bisacrylamide Gel 30	*Carl Roth, Karlsruhe*
Agarose	*Carl Roth, Karlsruhe*
Aluminum hydroxide gel	*Sigma-Aldrich, Taufkirchen*
Ammonium chloride	*Fluka, Taufkirchen*
Ammonium hydrogen carbonate	*Sigma-Aldrich, Taufkirchen*
Ammonium persulfate (APS)	*Sigma-Aldrich, Taufkirchen*
Ampicillin	*Sigma-Aldrich, Taufkirchen*
Aqua ad injectabilia, steril	*Braun, Melsungen*
Bacto™ Agar	*BD Biosciences, Heidelberg*
Bacto™ Tryptone	*BD Biosciences, Heidelberg*
Bromphenolblue	*Sigma-Aldrich, Taufkirchen*
BSA, Fraction V	*Carl Roth, Karlsruhe*
Calcium chloride	*Merck, Darmstadt*
Calcium hydrogen carbonate	*Sigma-Aldrich, Taufkirchen*
Chloroform	*Sigma-Aldrich, Taufkirchen*
Complete™ Protease Inhibitor Cocktail	*Roche Diagnostics, Penzberg*
Diethylpyrocarbonate(DEPC)	*Fluka, Taufkirchen*
Dimethyl sulfoxide (DMSO)	*Sigma-Aldrich, Taufkirchen*
Dinatrium hydrogen phosphate	*Merck, Darmstadt*
Dithiothreitol (DTT)	*Promega, Heidelberg*
dNTP-Mix, 10 mM	*Fermentas, St. Leon-Rot*

Glacial acetic acid	*Carl Roth, Karlsruhe*
Ethanol	*Riedel-de Haën, Taufkirchen*
Ethidium bromide	*Carl Roth, Karlsruhe*
Ethylendiamin-N,N,N′,N′-tetraacetic acid (EDTA)	*Fluka, Taufkirchen*
Formaldehyde, 37%	*Sigma-Aldrich, Taufkirchen*
Glucose	*Carl Roth, Karlsruhe*
Glutaraldehyde 2.5%	*Fluka, Taufkirchen*
Glutathione-Sepharose	*Pharmacia Biotech, Freiburg*
Glycerol	*Fluka, Taufkirchen*
Glycerol-2-phosphate	*Sigma-Aldrich, Taufkirchen*
Glycine	*Merck, Darmstadt*
HEPES	*Fluka, Taufkirchen*
Isopropanol	*Merck, Darmstadt*
Isopropyl-β-D-Thiogalactopyranosside (IPTG)	*Sigma-Aldrich, Taufkirchen*
Lipopolysaccharides (LPS)	*Sigma-Aldrich, Taufkirchen*
Magnesium chloride	*Carl Roth, Karlsruhe*
Methanol	*Merck, Darmstadt*
Milk powder	*Fluka, Taufkirchen*
N,N-Dimethylformamid	*Merck, Darmstadt*
Permeabilization buffer (10x)	*eBioscience, San Diego, USA*
Phosphat buffered saline (PBS), 10X	*Biochrom AG, Berlin*
PMSF	*Roche, Penzberg*
Polybrene	*Sigma-Aldrich, Taufkirchen*
Propidium iodide	*Sigma-Aldrich, Taufkirchen*
Sodium acetate	*Fluka, Taufkirchen*
Sodium azide	*Sigma-Aldrich, Taufkirchen*
Sodium chloride	*Carl Roth, Karlsruhe*
Sodium citrate	*Fluka,Taufkirchen*
Sodium dihydrogen Phosphat	*Merck, Darmstadt*
Sodium dodecyl sulfate (SDS)	*Carl Roth, Karlsruhe*
Sodium fluoride	*Fluka, Taufkirchen*
Sodium hydroxide	*Merck, Darmstadt*

Sodium ortho vanadate	*Sigma-Aldrich, Taufkirchen*
Sodium pyrophosphate	*Fluka, Taufkirchen*
Tetramethylethylendiamine (TEMED)	*Fluka, Taufkirchen*
Tris(hydroxymethyl)aminomethane (TRIS)	*Carl Roth, Karlsruhe*
Triton X-100	*Sigma-Aldrich, Taufkirchen*
Tween 20	*Fluka, Taufkirchen*
Xylol cyanol	*Sigma-Aldrich, Taufkirchen*

6.2 Medium and supplements for cell culture

β-Mercaptoethanol, 50mM	*Gibco/Invitrogen, Karlsruhe*
DMEM, cell culture medium	*PAA, Pasching, Austria*
FCS Gold	*PAA, Pasching, Austria*
G418 (Neomycin)	*Calbiochem, Darmstadt*
Penicillin and streptomycin 100x	*PAA, Pasching, Austria*
HANK's BSS	*PAA, Pasching, Austria*
L-Glutamine, 100x	*Gibco/Invitrogen, Karlsruhe*
IL-3	*R and D, Karlsruhe*
Hygromycin	*Calbiochem, Darmstadt*
Diptheria Toxin (DT)	*Calbiochem, Darmstadt*
Lipofectamin™2000, Transfection reagent	*Invitrogen,Karlsruhe*
MethoCult®	*StemCellTechnologies, Canada*
Opti-Mem®	*Gibco/Invitrogen, Karlsruhe*
PBS, 10 x, sterile	*PAA, Pasching, Austria*
RPMI 1640 (high Glucose, Glutamine)	*PAA, Pasching, Austria*
Trypsin-EDTA, 10 x	*PAA, Pasching, Austria*

6.3 Enzymes

Alkaline phosphatase	*Fermentas, St. Leon-Rot*
Restriction endonucleases	*Fermentas, St. Leon-Rot*

Pfu DNA-polymerase	*Fermentas, St. Leon-Rot*
Taq DNA-polymerase	*Fermentas, St. Leon-Rot*
T4 DNA-polymerase	*Fermentas, St. Leon-Rot*
T4 DNA-ligase	*Fermentas, St. Leon-Rot*

6.4 Membranes

PVDF membrane (Immobilon P)	*Millipore, Schwalbach*

6.5 Antibodies

D, donkey	R, rabbit	M, mouse	R, rat	G, goat

α-β-Actin (AC-15)		M		Sigma-Aldrich, Deisenhofen
α-Akt1/2	R			Santa Cruz, Heidelberg
α-m-FLT3 ICD	R			Upstate Biotechnology, USA
α-p-FLT3	R			Cell Signaling, Frankfurt/Main
α-m-FLT3 ECD			G	R&D Systems, Wiesbaden
α-ERK, p44/p42 MAP Kinase		R		Cell Signaling, Frankfurt/Main
α-LCK (H-6)	R			Santa Cruz, Heidelberg
α-SRC (44)	R			Cell Signaling, Frankfurt/Main
α-pSRC (Tyr416)		M		Cell Signaling, Frankfurt/Main
α-Phospho-Akt (Ser473)	R			Cell Signaling, Frankfurt/Main
α-Phospho-ERK, p44/p42 MAPK	R			Cell Signaling, Frankfurt/Main
α-Phospho-STAT5 A/B (18E5)		M		Tom Wheeler, Hamilton, New Zealand
α-Phospho-Tyrosin (4G10)		M		Upstate Biotechnology, USA ,
α-Phospho-Tyrosin (pY20)		M		PharMingen, San Diego, USA
α-Phospho-mFLT3 ITD-Y589R				L. Rönnstrand, Sweden
5 α-Phospho-mFLT3 ITD-Y591		R		L. Rönnstrand, Sweden
α-STAT5 A/B (C-17)K				Santa Cruz, Heidelberg
α-rabbit IgG, HRP-conjugated		E		Amersham, Braunschweig

α-mouse IgG, HRP-conjugated	E	Amersham, Braunschweig

6.6 Material and kits

Cell Line Nucleofector® Kits	*Lonza Cologne, Köln*
CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTT)	*Promega, Mannheim*
CL-XPosure™ Film	Thermo Scientific, Karlsruhe
DC Protein Assay,	*Bio-Rad, München*
NucleoSpin® Plasmid Kit	*Machery-Nagel, Düren*
NucleoSpin® Extract II Kit	*Machery-Nagel, Düren*
NucleoBond® Plasmid Maxi Kit	*Machery-Nagel, Düren*
Platinum® SYBR® Green qPCR SuperMix UDG	*Invitrogen, Karlsruhe*
QIAshredder homogenizer Kit	*Qiagen, Hilden*
Rapid DNA Ligation Kit	*Fermentas, St. Leon-Rot*
RevertAid™ H Minus First Strand cDNA Kit	*Fermentas, St. Leon-Rot*
RNeasy Mini Kit	*Qiagen, Hilden*
SuperScript™ II Rnase H^-Reverse Transcriptase	*Invitrogen, Karlsruhe*
SuperSignal West Pico, Dura und Femto	*Perbio Science, Bonn*
Trizol® Reagent	Invitrogen, Karlsruhe

6.7 DNA and protein marker

PageRuler™ Prestained Protein Ladder	*Fermentas, St. Leon-Rot*
GeneRuler™ 1kb Plus Ladder	*Fermentas, St. Leon-Rot*
GeneRuler™ 100 bp DNA Ladder	*Fermentas, St. Leon-Rot*
GeneRuler™ Ladder Mix	*Fermentas, St. Leon-Rot*

6.8 Instruments

ABI PRISM® 7700	*Applied Biosystems, Foster City, USA*
Binocular Stemi 2000-CS	*Zeiss, Jena*
Binocular-lamp LL1500 LCD	*Zeiss, Jena*
Centrifuge J2-HS, Rotor JA-14	*Beckman, Fullerton, USA*
Centrifuge 5417R	*Eppendorf, Hamburg*
Centrifuge 5415D	*Eppendorf, Hamburg*
Centrifuge Megafuge R 1.0	*Thermo Scientific, Karlsruhe*
CO2-incubator SW J 500 TV BB	*Nunc, Wiesbaden*
Cryo- Mr. Frosti Nalgene	*Thermo Scientific, Karlsruhe*
current generator PowerPac HC™, PowerPac 200, PowerPac 25	*Bio-Rad, München*
FACS EPICS® XL	*Beckman-Coulter, Krefeld*
Elisa Reader Sunrise™	*Tecan, Crailsheim*
Film processor Optimax	*Protec, Oberstenfeld*
Gel electrophoresis chamber	*Biometra, Göttingen*
Incubator-shaker Innova 4000	*New Brunswick Scientific, Edison, USA*
Incubator-shaker Certomat® BS-1	*B. Braun, Melsungen*
Microscope Axiovert 40 CFL	*Zeiss, Jena*
Mini Protean® Tetra Cell	*Bio-Rad, München*
Multi-Gel Long gel electrophoresis chamber	*Biometra, Göttingen*
Neubauer counting chamber	*Reichert, New York, USA*
Nucleofector® Elektroporator	*Lonza Cologne, Köln*
PCR-Thermocycler Primus	*MWG-Biotech, Ebersberg*
pH-Meter inoLab®	*WTW, Weilheim*
Refrigerated Incubator-Shaker Innova	*New Brunswick Scientific, Edison, USA*
Scale BP 221S	*Satorius, Göttingen*
Scale LC 1200 S	*Satorius, Göttingen*

Shaker WT 12	*Biometra, Göttingen*
Sterile work bench, Laminar-Flow 1.8	*Holten, Gydewang, Dänemark*
Sub-Cell® Model 192	*Bio-Rad, München*
TE Series Transphor Electrophoresis Unit	*Hoefer, Holliston, USA*
Thermomixer comfort	*Eppendorf, Hamburg*
Ultra-Turrax T8	*IKA®-Werke, Staufen*
UV-Lamp TI 2	*Biometra, Göttingen*
UV Stratalinker 2400	*Stratagene, La Jolla, USA*
Vortex Mixer 7-2020	*neoLab, Heidelberg*
Waterbath 1083	*GFL, Burgwedel*

6.9 Oligonucleoitides

The nucleotides are listed in 5'-3' orientation and were purchased from *Eurofins MWG Operon, Ebersberg*.

6.9.1 ShRNA Sequences

SRC siRNA 1
5' GGCTGCAGATTGTCAATAA 3'
SRC siRNA 2
5' GGTTCACCATCAAGTCGGA 3'
Luciferase siRNA
According to Baumgart et al. 2010
LCK siRNA
5' CCTTCAACTTCGTGGCGAA 3'

6.10 DNA-constructs

MSCV MIG-R1	J. Miller and W. Pear, Philadelphia, USA[147]
MSCV MIG-R1 mFLT3 WT	R. Dechow
MSCV MIG-R1 mFLT3 ITD	R. Dechow
MSCV MIG-R1 mFLT3 TKD	R. Dechow
pcDNA 3.1/Zeo(+)	Invitrogen, Leek, Netherlands
pcDNA 3.1/Zeo(+)-mFLT3-WT	R. Dechow
pcDNA 3.1/Zeo(+)-mFLT3-ITD	R. Dechow
pcDNA 3.1/Zeo(+)-mFLT3 D838Y	R. Dechow
	Thermo Scientific, Karlsruhe
pCMV-flag 4c	Clontech Laboratories Inc., USA
pCMV-mFLT3 WT-flag 4c	R. Dechow
pCMV-mFLT3 ITD-flag 4c	R. Dechow
pCMV-mFLT3 TKD-flag 4c	R. Dechow
pSuper.retro.puro	Oligo Engine, USA
pSUPER.retro.puro-control siRNA	T. Dechow
pSUPER.retro.puro-c-SRC siRNA 1	
pSUPER.retro.puro-c-SRC siRNA 2	
pSUPER.retro.puro-LCK siRNA	
MSCV-IRES-EYFP	K. Spiekermann, LMU Munich, Germany
MSCV-IRES-EYFP-mFLT3 ITD	K. Spiekermann, LMU Munich, Germany
MSCV-IRES-EYFP-mFLT3 ITD 589/591	K. Spiekermann, LMU Munich, Germany

6.11 Bacterias

Escherichia Coli DH5α™: *Invitrogen, Karlsruhe*

F-F80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rK-, mK+), *pho*A, *sup*E44, l-*thi*-1, *gyr*A96, *rel*A1

6.12 Cell lines

32D	murine, IL-3-dependent cell line, isolated from C3H/HeJ mice
BaF3	murine, IL-3-dependent, Pro-B-cell line, isolated from Balb/C mice
MV4-11	human, acute monocytic leukemia cell line (FAB M5), isolated from a 10 year old boy, positive for FLT3 ITD [148]
NIH3T3	murine, fibroblastic cell line
HEK293	(Phoenix E) murine, retroviral, ecotrope packaging-cell line (Helfervi rus-frei); 293T (humane embryonale kidney) cells, stably expressing moloney GagPol-IRES-Lyt2-construct under the control of the RSV-promotor (selection with Hygromycin B), further expresses moloney ecotropic envelope gene under the control of the CMV-promotor (selection with diphterie toxin)

The cell lines 32D, BaF3 and NIH3T3 were obtained from the Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (Bereich Menschliche und Tierische Zellkulturen) in Braunschweig, Germany. Phoenix E were a kind gift of Gary P. Nolan, Stanford, USA.

6.13 Mice

Mice strain:

C3H *Harlan Winkelmann, Borchen*

6.14 Materials used in the animal experiments

Animal fudder	*Altromin, Lage*
DiscarditTM II syringe, sterile	*Becton-Dickinson, Heidelberg*
FalconTM cell strainer (100 µm)	*Becton-Dickinson, Heidelberg*
Infrared lamp	*Petra, Burgau*
Mice cage IVC	*Tecniplast, Hohenpeißenberg*
Object slides (ISO Norm 8037/1)	*Menzel Gläser, Braunschweig*
Sterican syringe, sterile (27G 3/4)	*B. Braun, Melsungen*
S-Monovette®, EDTA	*Sarstedt, Nümbrecht*
Sub-Q, sterile PP syringe, 1 ml, 26G 1/2	*Becton-Dickinson, Heidelberg*
Vet abc Blood Counter	*Scil, Viernheim*

6.15 Solutions and Buffer

Amidoblack staining	0.2% naphtol blue black 25% isopropanol 10% glacial acetic acid
Amidoblack destaining buffer	25% isopropanol 10% glacial acetic acid
Coomassie-staining	0.25% Coomassie-blue 45% methanol 10% glacial acetic acid
Coomassie-destaining	5% methanol 10% glacial acetic acid
DEPC-H_2O:	0.1% DEPC in A.d. O.N after autoclave
DNA-Loading buffer (6X):	30% glycerol (v/v)

	0.25% bromphenol blue (w/v) 0.25% xylolcyanol 50 mM EDTA in A.d.
FACS-buffer:	0.1% BSA in PBS
Luria-Bertani (LB) Medium	1% bacto tryptone 0.5% bacto beef extract 1% NaCl in A.d. With 1 M NaOH (pH 7.0)
Lysis buffer:	10 mM Tris/HCl (pH 7.5) 130 mM NaCl 5 mM EDTA 0.5% Triton X-100 20 mM Na_2HPO_4/NaH_2PO_4 (pH 7.5) 10 mM sodium pyrophosphate (pH 7) 1 mM sodium orthovanadate 20 mM sodium fluoride 1 mM glycerol-2-phosphate 1 Protease-Inhibitor Cocktail tablet Ad 10 ml A.d.
Lysis buffer P50:	10 mM Tris/HCl (pH 7.5) 1 mM calium acetate 1.5 mM magnesium acetate 2 mM DTT 1 Protease Inhibitor Cocktail tablet Add for 10 ml A.d.

RBC (*Red Blood Cell*) lysis buffer:	150 mM NH_4Cl 1 mM $KHCO_3$ 0,1 mM Na_2EDTA, pH 7.3 in A.d.
RNA loading buffer (5X):	0.2% bromphenol blue 4 mM EDTA 7.2% formaldehyde (37%) 20% glycerol 3% formamide 40% RNA running buffer in DEPC-H_2O
Separating buffer for SDS-PAGE (4X):	0.5 M Tris (pH 6.8) 0.4% SDS in A.d.
SDS-electrophoresis buffer:	25 mM Tris 192 mM glycine 0.1% SDS in A.d.
SDS-loading buffer (2X):	1 M Tris/HCl (pH 6.8) 200 mM DTT 4% SDS 0.2% bromphenol blue 20% glycine in A.d.
SSC-buffer (20X)	0.3 M Sodium citrate HCl (pH 7.0) 3 M NaCl
TAE-buffer (10X):	0.4 M Tris 1.1% acetic acid 2% 0.5 M EDTA (pH 8) in A.d.

TNE-buffer:	10 mM Tris (pH 8) 100 mM NaCl 1 mM EDTA
Transfer buffer:	25 mM Tris 192 mM glycine 0.1% SDS in A.d. 20% methanol in A.d.
Stacking gel buffer SDS-PAGE (4X):	1.5 M Tris (pH 8.8) 0.4% SDS in A.d.

7 Methods

7.1 Molecular biology techniques

7.1.1 Polymerase chain reaction

Through the use of the polymerase chain reaction it is possible to in vitro synthesize nucleotide sequences as well as create mutations.[149] At the beginning (initiation phase) of an amplification the template DNA is denatured through heating up to temperatures of 95°C. Subsequently the PCR reaction mixture is cooled, which leads to an annealing of the template and the previously designed primer (annealing phase). In the third step, which is called elongation step, a DNA polymerase binds to the edge of single and double strand DNA and starts to synthesize new DNA along the single DNA strand. Besides the ability to synthesize DNA, is the Pfu-polymerase capable of proof reading and repairing the synthesized DNA.[150]. The reaction is conducted in a thermo cycler, which automatically regulates the temperature and therefore enables the scientist to initiate and terminate the reaction.

7.1.2 Digestion of DNA with restriction enzymes

Restriction enzymes were first discovered in bacteria, and are thought to have evolved as defense mechanism against viruses. This kind of enzymes is capable of cutting double stranded DNA at specific recognition sides. In this work these enzymes were used in the

process of siRNA cloning. Therefore the enzymes were chosen according to their restriction side and used as described in the manufacturers manual.

7.1.3 Agarose Gel Electrophoresis

To determine the size of DNA fragments, to estimate DNA quantity and to separate and isolate the DNA fragments, agarose gel electrophoresis can be used. Therefore the concentration of the agarose gel is adjusted according to the size of the DNA fragments. Consequently the correct amount of agarose (Gibco BRL, Germany) is added to a measured quantity of electrophoresis buffer (1 x TBE). This mixture is heated in a microwave until the agarose is dissolved. Afterwards ethidium bromide solution [0.5 µg/ml] is added and the solution is poured into a gel chamber.

Before loading, the samples are mixed with 6x loading buffer and loaded into the slots of the gel. Then the DNA fragments are visualized under a UV light. To determine the size of the DNA fragments, a marker with defined fragment sizes is used.

TAE-buffer (1 x): 40 mM Tris; 1 mM EDTA; 20 mM ethanoic acid; pH 8.0
DNA-buffer (6x): 30% glycerol (v/v), 50 mM EDTA in a.d.

7.1.4 Dephosphorylation of 5'-phosphate residues of DNA using calf intestine phosphatase

In order to linearize DNA plasmids (cutting), restriction enzymes are frequently used. Thus after the linearization the two ends which were created during the enzymatic cutting process, tend to spontaneously religate. As this is an unwanted process during cloning, the cutted plasmid can be dephosphorylated using the calf intestine phosphatase. This enzyme removes the 5' phosphate residues from the DNA ends and therefore prevents the process of religation, as this only takes place if the 5' phosphate residues are intact.

As the phosphatase operates under the same conditions as the restriction enzymes it can be added to the digestion mix for 30 min after the restriction. Its inactivation is induced by an increase of the incubation temperature from 37° C to 65° C for 10 min.

7.1.5 Gel extraction

To extract DNA fragments from the gel, they are cut out using a scalpel. In order to prevent UV light induced mutations of the DNA the exposure is kept as short as possible during the cutting process. To extract the DNA out of the agarose gel, the „NucleoSpin® Extract II Kit“ is used according to the manufacturers recommendations. Afterwards the DNA can be stored at -20°C.

NucleoSpin® Extract II Kit

7.1.6 Ligation

During the process of cloning, the insert is integrated into the target vector. This process is catalyzed by the T4-ligase. Experiments have shown, that for proper functioning of the ligation process, the ratio of insert to vector should not exceed 3:1. Therefore the concentration of the DNA is measured using the NanoDrop machine. After the measurement, the ligation is performed according to the manufacturers recommendations. A control experiment is conducted in parallel, in which the insert DNA is substituted by water. Further more a transformation of the ligation mixture is afterwards carried out in competent bacteria.

Rapid Ligation Kit

7.2 Working with bacteria

7.2.1 Preparation of the competent bacteria

Over-night culture of E. coli was prepared, then the cells were transferred in LB medium and grown up to OD_{600} = 0.3-0.4 (over night). After this time period, the cells are centrifuged at 4000-5000 rpm for 10 min at 4°C and the pellet are resuspended in 15 ml ice-cold TSB-medium. 100 µl aliquots of cells are transferred in Eppendorf-tubes and frozen down immediately. The aliquots are stored at -80°C.

7.2.2 Transformation of competent bacteria

The term transformation of competent bacteria refers to the process of non-viral DNA uptake by bacteria. E. coli are usually unable to take up sufficient DNA, therefore they are treated with high concentrations of Ca^{2+} or Rb^{2+} a process which destabilizes the membrane and increases the DNA uptake capacity of the bacteria. To further increase the uptake of DNA, the bacteria are subjected to a heat shock.

To perform a transformation using the heat shock method, the DNA of interest (maximum volume of 10 µl) are mixed with 100 µl of competent bacteria, which needs to be thawed on ice. The mixture is incubated for 20min on ice and afterwards incubated for 45 sec at 42°C.

Afterwards the mixture was once again incubated on ice for 2 min and then spread on LB-plates and incubated for 12 h at 37°C.

7.2.3 Plasmid-preparation

Plasmids are double stranded DNA molecules, which can be multiplied in bacteria and yeast independent of the chromosomal DNA. During the project the following methods were used to isolate the DNA: Mini Prep and Maxi Prep.

7.2.3.1 Mini-preparation

This protocol is designed for purification of up to 20 µl of high-copy plasmid DNA from 5 ml overnight cultures of E. coli in LB (Luria-Bretani) medium. Pelleted bacterial cells are resuspended in 250 µl of Buffer P1 and transferred to a microfuge tube. 250 µl of Buffer P2 were added. 350 µl of Buffer N3 are added and tubes subject to centrifugation for 10 min. The supernatants are then applied to the QIAprep column by pipetting and centrifugation for 30 sec. QIAprep spin column are washed by adding 0.75 ml of Buffer PE and centrifuged for 30 sec and then for an additional 1 min to remove residual wash buffer. Further QIAprep spin column are placed in a clean 1.5 ml microfuge tubes. To elute DNA, 50 µl of Buffer EB (10mM Tris-Cl, pH 8.5) were added to the columns and centrifuged for 1 min.

Mini Kit QIAGEN Miniprep Kit

7.2.3.2 Maxi-preparation

A single colony is picked from a freshly streaked selective plate and incubated first in 2-5 ml LB medium containing the appropriate selective antibiotic for 1 hour, then in 100 ml for additional 12-16 hours at 37°C with vigorous shaking (300rpm).

After that the bacterial cells are harvested by centrifugation at 6000xg for 15 min at 4°C and resuspended in 10 ml Buffer P1. Then 10 ml Buffer P2 is added and incubated at room temperature for 5 min. After that 10 ml chilled Buffer P3 is added to the lysate and whole mix is poured into the barrel of the QIAfilter cartridge and incubated at room temperature for 10 min.

After filtration of the cell lysate into a 50 ml tube, 2.5 ml Buffer ER is added and incubated on ice for 30 min. A QIAGEN-tip 500 is equilibrated by applying 10 ml Buffer QBT. Afterwards the filtered lysate is placed to the QIAGEN-tip and the QIAGEN-tip is washed with 2 x 30 ml QC buffer. DNA elution is performed with 15 ml Buffer QN. DNA is precipitated by adding 10.5 ml room-temperature isopropanol and centrifuged at 15,000 x g for 30 min at 4°C. DNA pellet is washed with 5 ml of endotoxin-free, room-temperature 70% ethanol and centrifuged at 15,000 x g for 10 min. The pellet is air-dried for 5-10 min, and redissolved in a suitable volume of endotoxin-free Buffer TE.

Maxi Kit	QIAGEN Maxi Kit

7.2.4 Sequencing

The sequencing of the DNA-constructs was performed by *GATC Biotech* in Konstanz.

7.3 Cell culture

7.3.1 Culturing of suspension cells

The suspension cells BaF3, 32D and MV4-11 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin and streptomycin + 1% L-glutamine.

Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% humidity. Cells were seeded at a density of 4 x 10^4/ml growth medium in the presence or absence of murine IL-3 (0,2 ng/ml) and inhibitors as indicated.

7.3.2 Culturing of adherent cells

Phoenix and NIH/3T3 were cultured in DMEM-medium. The cells were split in daily (phoenix) intervals in a ratio of 1:1. For splitting the old medium was removed, the cells washed with 1x PBS and subsequently incubated with trypsin for 3 min.

The trypsin/EDTA solution enables the scientist to enzymatically detach the cells from the surface on which they are cultivated on. After the described incubation with the trypsin/EDTA solution, the cells are washed off the culturing device (e.g. flask) with medium. The cell suspension is then centrifuged and the cells pelleted. The cell pellet can be resuspended in fresh medium and the cells plated in desired density.

DMEM-medium: DMEM, 2 mM glutamine, 1% antibiotic solution, 10% FCS
1 x PBS: 10% 10 x PBS in A. d.
1 x Trypsin/EDTA: 10% Trypsin EDTA in 1x PBS

7.3.3 Freezing of cells

For long-term storage, the cells can be stored in liquid nitrogen. This enables the scientist to later thaw the cells again in order to cultivate them. Therefore the 1-10 x 10^6 cells are resuspended in 1ml of medium and mixed in a ratio of 1:1 with freezing medium. Afterwards the cells are transferred into a -80°C freezer for 24 h and then stored in liquid nitrogen.

Freezing medium 2x: 20% FCS; 80% DMSO

7.3.4 Thawing of cells

For optimal recovery rapid thawing of the cells is essential. Therefore the cells are thawed and subsequently diluted with PBS or culture medium to a ratio of 1:20 and

centrifuged at 1500 rpm for 3 minutes. The supernatant is discarded and the pellet resuspended in fresh culture medium.

7.3.5 Cell quantification and evaluation of viability

For exact and successful experiments it is crucial for the scientist to determine the exact number of cells used in the experiments. Therefore the cells are stained with trypan blue, a dye that can only penetrates through the wall of dead or dying cells does and therefore does not stain viable cells.

Afterwards the suspension is transferred into a Neubauer counting chamber and the cells are counted under a microscope.

7.3.6 Starving of the cells prior lysis

To analyze the phosphorylation of specific signaling pathways it is necessary to starve the cells before lysis. The cells were washed twice with PBS and cultivated subsequently in starving medium. For 32D and BaF3 cells a maximum starvation time of 6 h was applied.

Starving medium: RPMI-1640 or DMEM, 0% FCS, 50 U/ml Penicillin, 50 U/ml Streptomycin

7.3.7 Stimulation of cells with cytokine/ligand before cell lysis

For stimulation of cells with cytokines (IL3 or FLT3 ligand), the cells are incubated with a defined quantity of cytokine/ligand for 10 minutes. Then the cells are centrifuged at 1500 rpm at 25°C to pellet the cells.

7.3.8 Transfection

Transfection describes the process of introducing genetic material into eukaryotic cells. There are several different methods for transduction, lipofection, nucleofection and retroviral transduction. Lipofection is most efficient in adherent cells, whereas nucleofection and transduction are commonly used for suspension cells. In this work, lipofection is used

for HEK293 cells. 32D or BaF3 cells are transduced using either nucleofection or retroviral transduction.

7.3.8.1 Lipofection

The lipofection of phoenix cells using Lipofectamine™ 2000 was performed according to the manufacturers protocols. To generate virus, 2 million phoenix cells were plated on 6 cm plates. 24 h later the medium was replaced by fresh medium. In parallel 0.5 ml Opti-MEM®I with 20 µl Lipofectamine™ 2000 and 0.5 ml Opti-MEM®I with 10 µg of specific DNA is mixed. After 5 minutes of incubation both solutions are mixed and incubated for another 20 minutes at RT. This final mixture was then added to the phoenix cells.

Lipofectamine™ 2000
Opti-MEM®I Serum reduced medium

7.3.8.2 Nucleofection

The nucleofection method of Amaxa combines electroporation and cell type specific buffers in order to ensure efficient transfection. There are several different programs and buffers available.

To transfect the target cells with this method, 1-2 x 10^6 cells are resuspended in the specific Amaxa kit solution (Amaxa, Köln, Deutschland) and mixed with 2-5 µg of plasmid DNA. This mixture is processed into a specific cuvette and placed into the nucleofector machine. The machine in turn runs a cell line specific program. Afterwards the cells are resuspended in 500 µl of medium and kept in culture for a minimum period of 24 h.

Nucleofector®-Device
32D and BaF3 cells: Cell Line Nucleofector® Kit V, program X-01

7.3.9 Virus production and retroviral transduction

7.3.9.1 Production of virus

Phoenix cells are human embryonic kidney cells and derived from the cell line 293T. They were generated by the introduction of two plasmids, which encode for the capsid

protein (gag), the reverse transcriptase (pol) and the envelope protein (env). Depending on the species of the target cells either ecotropic (e, murine) or amphotrophic (a, human) phoenix cells should be used.

As retroviral vectors express the packaging signal Ψ, transfected genetic information will be packed into the virus particles synthesized and excreted by the phoenix cells.

For virus production, 2 million phoenix cells are plated on a 6 cm plate and transfected using Lipofectamine™ 2000 as previously described. 24 h after transfection the medium is changed. From this time point on the medium is changed every 12 h up to four times. In contrast to normal cell culture, the old medium is not discarded as it contains the virus particles. After removing the medium with the virus particles, it is filtered und stored at 4°C.

7.3.9.2 Retroviral infection of 32D and BaF3 cells

To retrovirally transfect 32D or BaF3 cells, the spin-infection method was used. Therefore 50000 cells were resuspended in 500 µl of RPMI medium with IL3, mixed with 2 ml of filtered virus containing medium and put into a 24-well-plate. To ease the binding of the virus to the cell membrane, polybrene is added (4 µg/ml). Afterwards the cells are centrifuged for 90 minutes at 32 °C and 2400 rpm. This procedure is repeated 3 times every 12 h.

Virus titration

To test the titer of the produced virus, NIH/3T3 are infected with the virus collected from the transfected phoenix e cells. For this purpose, 2.5 x 10^4 NIH/3T3 cells are plated on a 6-well-plate in DMEM medium. After 14 h the virus is pipetted on to the cells in dilutions of 1:20, 1:200 and 1:500. In order to increase the transduction polybrene is added as previously described. After 24 h the medium is changed, additional 24 h later the cells are detached from the plates by trypsinization and analyzed by FACS.

The calculation of the titer is calculated by multiplying 5x10^4 with the dilution factor of the cells and the percentage of GFP positive cells. The titer CFU/ml equals the mean value of the three determines values.

7.3.10 FACS-analysis

The efficiency of the transduction can be estimated by flow cytometry if the transduced vector encodes a fluorescent protein (e.g. GFP). The vector MSCV MIG-RI, which is used in this study encodes for the protein GFP. Due to the design of the vector the expression of the GFP correlates closely with the protein of interest encoded by the vector. It is therefore possible to measure indirectly the expression of the encoded protein by determining the GFP expression in a flow cytometer (FCM).

The FCM has an additional benefit. It measures the forward and sideward scatter of the transduced cells and is therefore able to provide information on the size and granularity of the cells. In order to perform this measurement, the 1 x 10^6 of the cells are resuspended in PBS are filtrated (40 µm) and measured in the flow cytometer.

7.3.11 Selection of transduced cells

As the transfection efficiency of the retroviral transduction is rarely above 90%, certain methods have been developed to increase the percentage of transduced cells up to nearly 100%.

One possibility, if the vector encodes a fluorescent protein, is the sorting of the cells by FACS. Another is the selection of the cells using antibiotics like G418 or puromycin. Though this is only feasible if the transduced plasmid contains genes, which make the expressing cells resistant against the specific antibiotic. In this work puromycin is used for 32D and BaF3 cells in a concentration of 2 µg/ml for 2 to 7days.

A third method is selection by growth factor deprival, which can only be done if the cells after the transduction express a pro-proliferative protein.

7.3.12 Preparation of cell lysates

Before lysis, the cells are harvested and either stored at -80°C or directly resuspended in lysis buffer and incubated on ice for 20 minutes. Afterwards the cells are centrifuged at 14000 rpm for 15 minutes at 4°C. The supernatant is transferred into a new tube and the concentration of the proteins can be determined.

Afterwards the lysates are immediately resuspended in SDS-buffer and heated at 95°C for 5 minutes in order to denature the proteins within the lysates. Post „cooking“ the lysates can be stored at -80°C or used directly for SDS-PAGE.

Lysis-buffer:	10 mM Tris/HCl, pH 7.5; 130 mM NaCl; 5 mM EDTA; 0.5% Triton
X-100;	20 mM Na_2HPO_4/NaH_2PO_4, pH 7.5; 10 mM sodium pyrophosphate,
pH 7;	1 mM sodium orthovanadate; 20 mM sodium fluoride;
	1 mM glycerol-2-phosphate; 1 protease inhibitor cocktail tablet/10 ml
	a.d
SDS-buffer (2x):	M Tris/HCl, pH 6.8; 200 mM DTT; 4% SDS; 0.2% bromphenole blue;
	20% glycine in a.d.

7.3.13 GST purification and GST binding studies

Wild type SRC, HCK, FYN and YES SH2 domains were cloned into pGEX4T.1 vector to obtain a GST- fusion construct. Recombinant proteins were expressed in and purified from BL-21 strain (Invitrogen) (according to the manufacturers manual). GST fusion proteins were incubated for 3 h with glutathione-agarose beads in binding buffer (150 mM NaCl, 50mM Tris-HCl pH 8.0, and 5mM EDTA) at 4°C. After being washed three times in binding buffer, the beads were incubated with their potential binding proteins for 3 h at 4°C. The beads were then washed three times and afterwards bound proteins were eluted with sample buffer.

7.3.14 Immunoprecipitation of proteins from cellular lysates

In this approach, specific antibody is added to the cellular lysate to bind the protein of interest. Antibody-protein complexes are subsequently precipitated using beads. Bacterial proteins A and G which have specific binding sites for Fc-parts of antibodies, covalently coupled to cross-linked agarose, are usually used to precipitate protein-antibody complexes.

Cellular lysates are mixed with about 1 µg of antibody in pre-chilled 1.5 ml tubes on ice. Volumes of the mixture are adjusted to 200-300 µl to obtain equal protein concentrations in each sample. The tubes are incubated for 3 hours to overnight at 4°C with constant rotation. 25-30 µl of Protein A or G coupled agarose beads are added to each sample and the tubes are incubated for additional 60 minutes at 4°C with constant rotation. Following the incubation, the immuno-complexes were pelleted by centrifugation and washed 3 times with ice-cold lysis buffer. After the last washing step, loading buffer is added to the samples

which were then heated at 95°C for 5 minutes. Samples are analyzed immediately by SDS-PAGE or frozen at -80°C SDS-gel electrophoresis.

7.3.15 SDS-PAGE Electrophoresis

The SDS-PAGE method enables the scientist to separate proteins according to their size. The gel that is used consists of a stacking and a separating gel. According to the size of the investigated protein, the separating gel content of polyacrylamide is changed (5-15 %).

The gels can be purchased or made by the scientist himself. Therefore a longstanding protocol is used. First the separating gel is cast and coated with isopropanol until it is polymerized. Upon successful polymerization of the separating gel the isopropanol is removed and the stacking gel is cast on top of it. Before the stacking gel is polymerized, a comb is inserted into the stacking gel to form pockets for the probes which are to be loaded alter on. After the polymerization of the stacking gel, the comb is removed and the gel is into the electrophoretic apparatus, which is filled with electrophoresis buffer. The probes filled into the pockets and a current of 25-140 V is applied to the chamber.

As a control a protein marker is loaded in addition to the samples. After the probes have run through the gel, they are transferred onto PVDF membranes.

Separating gel:	5-15 % polyacrylamide-solution; 375 mM Tris/HCl, pH 8,8; 0,1 % SDS; 0,3 % APS; 0,1 % TEMED
Stacking gel:	5 % polyacrylamide-solution; 12,5 mM Tris/HCl, pH 6,8; 0,1 % SDS; 0,3 % APS; 0,1 % TEMED
SDS-Electrophoresisbuffer:	25 mM Tris; 192 mM Glycin; 0,1 % SDS in a.d.

7.3.16 Western blot

Western blot describes the transfer of proteins within a gel onto a protein binding membrane. Therefore a PVDF membrane is activated in methanol and placed on the gel between two sheets of whatman paper. The gel and its surrounding material are placed into the transfer chamber in which 1 A is applied to transfer the proteins onto the membrane. It is important to know the proteins size as the time of transfer depends on it (1 min per kDa).

Transferbuffer:	25 mM Tris; 192 mM glycin; 0,1 % SDS; 20 % methanol in a.d.

7.3.17 Immunhistochemical detection of proteins

The process in which proteins are detected by specific antibodies is called immunhistochemistry. As the protein is stuck on the membrane, this membrane is washed with PBST-buffer and afterwards blocked in milk to reduce the unspecific binding.

Afterwards the primary antibody is prepared according to the manufacturers recommendations. The membrane is incubated in this antibody for 12 h at 4°C. Next the antibody is removed, the membrane washed 3 times with PBST-buffer and incubated for 30 min at RT with a secondary antibody against the F_c fragment of the primary antibody. This secondary antibody is conjugated with horseradish peroxidase (HRP).

In the next step the membrane is washed and incubated with HRP substrate (*Super-Signal West*). The substrate is processed by the HRP, a process, which results in a fluorescent signal, which can be detected with a hyper film.

PBS-Tween-(PBST)-buffer:	0,1 % Tween 20 in PBS
Blocking buffer:	10 % skim milk powder in PBS-Tween-buffer; 5 % BSA in PBS-Tween-Puffer
SuperSignal West	

7.4 Biological assays

7.4.1 Apoptosis assay using Annexin-V apoptosis detection kit

Annexin-V is a calcium-dependent phospholipid binding protein that has a great affinity for phosphatidylserine. In normal cells phosphatidylserine is present in the inner leaflet of the plasma membrane. In contrast the outer leaflet contains mostly neutral phospholipids. If cells undergo apoptosis, the amount of phosphatidylserine in the outer leaflet of the membrane increases.

Therefore, Annexin-V binds apoptotic but not normal cells. To discriminate between apoptotic and necrotic cells, staining with the vital dye 7-Amino-actinomycin (7-AAD) or propidium iodine (PI) was performed in parallel to Annexin-V labeling.

Cells are pelleted by centrifugation, washed with PBS and resuspended in 100 µl of Annexin-V binding buffer at the concentration of 1 X 10^6 cells/ml. 5 µl of Annexin-V conjugated to PE and 5 µl of 7-AAD or PI are added to the cells. Cells are mixed gently and incubated for 15 minutes at room temperature in the dark. The cells were analyzed by flow cytometry within 2-3 hours. All cells, which were Annexin-V positive, were considered apoptotic.

Apoptosis detection kit Bio Vision

7.4.2 Proliferation assay

Proliferation is measured using an MTS (3-(4,5 dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)-based method by absorption of formazan at 490 nm (CellTiter 96; Promega, Madison, WI). Measurements are performed as triplicates after 72 and 96 hours of culture without cytokines.

CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay

7.5 Animal experiments

7.5.1 Animal care

The animals are kept in specially designed cages (IVC System, Tecniplast) in numbers of 4-6 animals. The company Altromin supplied sterile food and water; water bottles were exchanged twice weekly.

The mice facilities are specially designed rooms and operated by trained staff. A night and day rhythm of 12 hours (each phase) is ensured. The hygienic conditions and standards are approved by the FELASA.

7.5.2 Preparation of the cells and mice prior to transplantation

The 32D cells no longer than two weeks in culture are controlled with regard to their biochemical characteristics. Which included FLT3 ITD and FLT3 TKD expression as well as the downregulation of the target gene SRC.

Afterwards the cells are counted and 1 million in a volume of 1million/100 µl of Hank's medium cells are injected into the tail veins of CH3 mice.

Prior to this, C3H (Harlan) mice in the age of 4-6 weeks are kept in the mice facilities for a maximum period of 7 day to ensure the animals to adapt to their new surrounding.

After the injection the animals are returned into their cages and monitored.

Hank's Balanced Salt Solution

7.5.3 Drawing of blood

During the experiment, blood is being taken from the mice to monitor the development of the leukemia in the animals. To do so the amount of leukocytes, erythrocytes, thrombocytes and the Hb as well as the Hkt are being measured weekly.

Prior to the analysis of the blood, the mice are warmed with an infrared lamp and fixed in a specially designed device. Afterwards blood is taken from one of the tail veins, either the dorsal or lateral, using a Sub-Q PP syringe. An S-Monometer® is used to collect the blood for later analysis.

7.5.4 Monitoring of the transplanted mice

To detect the development of the expected hematological disease as early as possible, the blood of the animals is analyzed once weekly after the transplantation. To do so 20 µl of blood are being analyzed using the VetABC-Blood-Counter.

8 Literature

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9 Figures

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11 Publications

"SRC is a crucial signaling mediator and potential therapeutical target in FLT3 ITD but not in FLT3 TKD positive AML" (manuscript in revision)

Hannes Leischner , Rebekka Grundler, Corinna Albers, Lars Rönnstrand, Karsten Spiekermann, Katharina Götze, Christian Peschel and Justus Duyster

„SRC Is a Critical Signaling Mediator in FLT3-ITD Positive AML“; 51st ASH Annual Meeting and Exposition, New Orleans 2009

Hannes Leischner , Rebekka Grundler, Corinna Albers, Anna Lena Illert, Karsten Spiekermann, Christian Peschel and Justus Duyster

“SRC a critical signaling mediator of FLT3 mutants in AML“; DGHO Meeting, Mannheim 2009

Hannes Leischner, Rebekka Grundler, Corinna Albers, Anna Lena Illert, Karsten Spiekermann, Christian Peschel and Justus Duyster,