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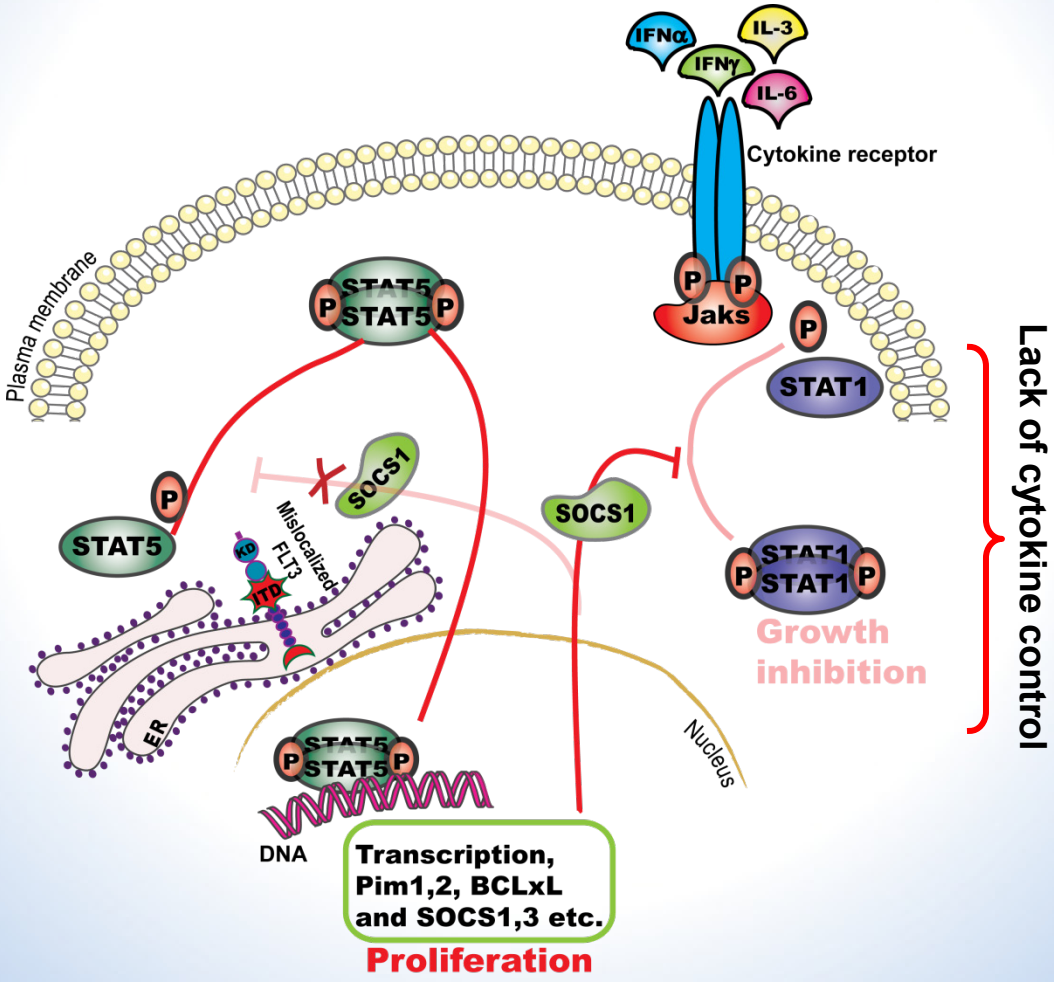
SOCS1 cooperates with FLT3-ITD by promoting the escape from external cytokine control



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Role of SOCS proteins in FLT3-ITD and BCR/ABL mediated leukemogenesis

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Summary

Acute myeloid/lymphoid leukemia is a fatal hematological malignancy characterized by accumulation of nonfunctional, immature blasts, which interferes with the production of normal blood cells. Activating mutations of receptor tyrosine kinases are common genetic lesions in leukemia. FLT3-ITD is a frequent activating mutation found in AML patients, leading to uncontrolled proliferation of leukemic blasts. FLT3-ITD directly activates STAT5, leading to the induction of STAT5 target gene expression like PIM kinases and SOCS genes. STAT5 and PIM kinases have been shown to play a crucial role in the FLT3-ITD mediated transformation. On the other hand, the role of SOCS proteins in FLT3-ITD mediated transformation has not been studied to date. SOCS proteins are part of a negative feedback mechanism that controls Jak kinases downstream of cytokine receptors. One of the SOCS family members, SOCS1 has been reported to suppress oncogenecity of several activating kinases implicated in hematologic malignancies. In this thesis the role of these SOCS proteins in FLT3-ITD mediated transformation (*in vitro*) and leukemogenesis (*in vivo*) is systematically explored.

Expression of FLT3-ITD in cell lines of myeloid (32D) and lymphoid (Ba/F3) origin, led to CIS, SOCS1 and SOCS2 expression. FLT3-ITD expression in primary murine bone marrow stem/progenitor cells led to a 59 fold induction of SOCS1 expression. Furthermore, FLT3-ITD positive AML cell lines (MV4-11, MOLM-13) show kinase dependent CIS, SOCS1, and SOCS3 expression. Importantly SOCS1 is highly expressed in AML patients with FLT3-ITD compared to healthy individuals. SOCS1 protein was expressed in FLT3-ITD transduced murine bone marrow stem cells and SOCS1 expression was abolished with kinase inhibition in MOLM-13 cell line. In conclusion, SOCS1 was highly regulated by FLT3-ITD in myeloid, lymphoid cell lines, in bone marrow stem/progenitors and in AML patient samples.

SOCS1 co-expression did not affect FLT3-ITD mediated signaling and proliferation, but abolished IL-3 mediated proliferation and protected 32D cells from interferon-α and interferon-γ mediated growth inhibition. FLT3-ITD expressing 32D cells showed diminished STAT1 activation in response to interferons (α and γ). Alone, SOCS1 strongly inhibited cytokine induced colony formation of bone marrow stem and progenitors, but not FLT3-ITD induced colony formation. Most importantly, in the

presence of growth inhibitory interferon-γ, SOCS1 co-expression with FLT3-ITD led to increased colony formation compared to FLT3-ITD alone. Taken together, FLT3-ITD induced and exogenously expressed SOCS1, shielded cells from external cytokines, signals, while not affecting FLT3-ITD induced proliferation/signaling.

In further experiments the *in vivo* effects of SOCS1 were studied in a bone marrow transplantation model. SOCS1 bone marrow transplants were unable to engraft/proliferate in mice. FLT3-ITD was shown to induce a myeloproliferative disease. Both control (empty vector), SOCS1 transplanted mice were normal and did not show any disease phenotype. FLT3-ITD alone and SOCS1 co-expressing FLT3-ITD developed either myeloproliferative disease or acute lymphoblastic leukemia with equal distribution. SOCS1 co-expression with FLT3-ITD led to a decreased latency. Mice transplanted with FLT3-ITD alone and SOCS1 co-expressing FLT3-ITD displayed enlarged spleens, liver and hypercellular bone marrow indicating infiltration of leukemic cells. Mice were also anemic and showed decreased platelet counts. Importantly SOCS1 co-expression particularly shortened the latency of myeloproliferative disease but not of acute lymphoblastic leukemia. In summary, in the context of FLT3-ITD, SOCS1 acts as a 'conditional oncogene' and cooperates with FLT3-ITD in the development of myeloproliferative disease. With these data we propose the following model: FLT3-ITD induces SOCS gene expression, which shields cells against proliferation and differentiation signals from cytokines, while not affecting FLT3-ITD mediated proliferative signals. This leaves cells under the dictate of FLT3-ITD thereby contributing to leukemogenesis.

Similar to FLT3-ITD, BCR/ABL (P190) (an oncogenic fusion kinase often found in acute lymphoblastic leukemia) induces SOCS gene expression in K562 and long-term cultured cells from patients with acute lymphoblastic leukemia. SOCS1 co-expression does not affect BCR/ABL mediated proliferation while abrogating IL-3 mediated proliferation. These findings suggest that SOCS proteins may play a general co-operative role in the context of oncogenes which aberrantly activate STAT3/5 independently of JAK kinases. This study reveals a novel molecular mechanism of FLT3-ITD mediated leukemogenesis and suggests SOCS genes as potential therapeutic targets.

Zusammenfassung

Akute Leukämien sind unbehandelt tödlich verlaufende, hämatologische Erkrankungen, bei denen es zu einer Anreicherung unreifer und funktionsloser Blasten im Knochenmark kommt, was wiederum mit der gesunden Hämatopoese interferiert. Ursache dieser Leukämien sind chomosomale Translokationen oder Funtionsgewinn- bzw. Funktionsverlustmutationen. Die Mehrheit dieser Mutationen tritt in Genen auf, die Proliferation und/oder Differenzierung regulieren. Im Rahmen dieser Dissertation wurde die Rolle einer dieser Mutationen der Rezeptortyrosinkinase FLT3 in der Leukämogenese bearbeitet, genannt FLT3-ITD (interne Tandemduplikation). FLT3 wird in frühen hämatopoetischen Stamm- und Progenitorzellen exprimiert und spielt eine wichtige Rolle für das Überleben und die Proliferation lymphatischer und myeloischer Linien. Die Bindung des FLT3 Liganden an den Wildtyp-FLT3 Rezeptor führt zur dessen Dimerisierung und Aktivierung, wobei diese Aktivierung wiederum unterschiedliche Signalwege wie z.B. PI3K/Akt und Erk aktiviert. FLT3-ITD ist eine aktivierende Mutation der FLT3 Rezeptortyrosinkinase, die bei etwa 25 % der AML-Patienten gefunden wird und zur unkontrollierten Proliferation leukämischer Blasten führt. Diese Mutation führt zu einer Ligand-unabhängigen, konstitutiv aktiven Form des Rezeptors, so dass die nachfolgenden Signalwege (PI3K/Akt, Erk und STAT5) permanent angeschaltet sind. Ein Genexpressionsprofil in FLT3-ITD exprimierenden 32D Zellen im Vergleich zu Ligand aktivierten Wildtyp-FLT3 exprimierenden Zellen zeigt eine erhöhte Expression von STAT5 Zielgenen wie PIM Kinasen und SOCS Proteinen. Interessanterweise aktiviert FLT3-ITD STAT5 dabei unabhängig von Jak und Src Kinasen und verleiht IL-3 abhängigen Zelllinien wie 32D und Ba/F3 ein Faktor unabhängiges Wachstum. Weiterhin verursacht eine FLT3-ITD Expression im Gegensatz zum Wildtyp-FLT3 in Maustransplantationsexperimenten eine myeloproliferative Erkrankung.

Vergleichbar mit FLT3-ITD ist BCR/ABL auch ein onkogenes Fusionsprotein, das durch eine Translokation zwischen den Chromosomen 9 und 22, bezeichnet als t(9;22), zustande kommt. Das BCR/ABL Protein wird in ca. 90 % aller chronischen myeloischen Leukämien (CML) und in 10-20 % aller akuten lymphatischen Leukämien (ALL) gefunden. Wie FLT3-ITD verleiht auch BCR/ABL 32D und Ba/F3

Zellen ein IL-3 unabhängiges Wachstum. Außerdem führt es zu einer konstitutiven Aktivierung der PI3K/AKT, Ras/MAPK und STAT5 Signalwege. Die BCR/ABL vermittelte Aktivierung von STAT5 erfolgt hierbei entweder durch direkte Phosphorylierung oder über die Src Kinase HCK. Im Knochenmarktransplantationsmodell induziert BCR/ABL eine myeloproliferative Erkrankung, die einer CML ähnelt.

Die Produktion hämatopoetischer Zellen unterliegt einer strengen Kontrolle durch hämatopoetische Zytokine. Die Zytokinrezeptoren besitzen keine intrinsische Kinaseaktivität. Die Signalweiterleitung erfolgt hier über Jak Kinasen. Auf diese Weise kontrollieren Zytokinrezeptoren wichtige Prozesse des Immunsystems und der Blutzellhomöostase. SOCS Proteine sind Teil eines negativen Rückkopplungsmechanismus, der die Aktivierung von STAT Proteinen durch Zytokinrezeptoren kontrolliert. SOCS Proteine binden an Jak Kinasen und inhibieren deren Kinaseaktivität über verschiedene Mechanismen, was zur Termination der Zytokinsignale führt. Von einem Mitglied der SOCS Familie, SOCS1, ist bekannt, dass es durch die Hemmung einiger aktivierender Kinasen bei hämatologischen Erkrankungen tumorsupressive Eigenschaften hat. Die Rolle der SOCS Proteine in der FLT3-ITD vermittelten Transformation ist bis heute nicht hinreichend analysiert. Insbesondere ist unklar wie transformierte Zellen der strengen Kontrolle des Zytokinnetzwerks entgehen können, das normalerweise die korrekte Funktion hämatopoetischer Zellen garantiert.

In der vorliegenden Arbeit wurde die Rolle von SOCS Proteinen in der FLT3-ITD vermittelten Transformation (*in vitro*) und Leukämieentstehung (*in vivo*) systematisch untersucht. Weiterhin wurde die SOCS Genexpression und die Folgen der BCR/ABL vermittelten Transformation analysiert.

Schlussfolgerung 1: FLT3-ITD induziert die Expression von SOCS Genen in Zelllinien und murinen Knochenmarkzellen; SOCS1 ist in Patienten mit FLT3-ITD induziert.

Zunächst wurden bestehende *microarray* Daten durch quantitative *real time* PCR validiert. Die Expression von FLT3-ITD in myeloischen (32D) und lymphatischen (Ba/F3) Zelllinien induziert die Expression von CIS, SOCS1 und SOCS2. In primärem murinen Knochenmarksstamm-/progenitorzellen führt die Expression von

FLT3-ITD zu einem sehr starken Anstieg der SOCS1 Expression (59-fach). Zudem zeigen Zelllinien, die von FLT3-ITD positiven AML-Patienten gewonnen wurden (MV4-11, MOLM-13), eine Kinase abhängige CIS, SOCS1 und SOCS3 Expression. Interessanterweise wird außerdem SOCS1 im Knochenmark von AML-Patienten mit FLT3-ITD Mutationen im Vergleich zu Kontrollen von gesunden Spender stark überexprimiert. Das SOCS1 Protein wird ferner in FLT3-ITD transduziertem murinen Knochenmark exprimiert und die Expression geht in MOLM-13 Zellen nach Behandlung mit Kinaseinhibitoren verloren. Zusammenfassend wird die Expression von SOCS1 in hohem Maße durch FLT3-ITD in myeloischen und lymphatischen Zelllinien sowie in hämatopoetischen Stamm- und Progenitorzellen und AML-Patienten hochreguliert.

Schlussfolgerung 2: Die SOCS1 Expression verhindert Zytokinrezeptor vermittelte Effekte aber nicht die FLT3-ITD induzierte Signalgebung und Proliferation.

Aufgrund der hohen SOCS1 Induktion durch FLT3-ITD im primären murinen Knochenmark und in Patientenproben wurde dessen Rolle in der FLT3-ITD vermittelten Transformation untersucht. Die Koexpression von SOCS1 beeinträchtigt nicht die durch FLT3-ITD induzierten Signalwege (MAPK/ERK, PI3K/AKT und STAT5) oder die Proliferation, jedoch unterbindet es die IL-3 vermittelte Proliferation und schützt 32D Zellen vor der Wachstumshemmung durch Interferon α und γ. So zeigten FLT3-ITD exprimierende 32D Zellen eine verminderte STAT1-Aktivierung durch Interferon α und γ. In einem kompetitiven Proliferationsassay wirkte die SOCS1 Expression alleine wachtumsinhibierend, koexprimiert mit FLT3-ITD trat allerdings der gegenteilige Effekt auf. Im murinen Knochenmark führte SOCS1 zu einer starken Hemmung der Zytokin induzierten Kolonienbildung hämatopoetischer Stamm- und Progenitorzellen, jedoch nicht der FLT3-ITD induzierten Kolonienbildung, was auf eine SOCS1 Resistenz von FLT3-ITD hindeutet. Interessanterweise führte SOCS1 in Anwesenheit des wachstumhemmenden Interferons γ zu einer Verstärkung der FLT3-ITD induzierten Kolonienbildung. Zusammenfassend führt die FLT3-ITD induzierte oder exogene SOCS1 Expression zu einer Abschirmung der Zellen von exogenen Zytokinsignalen, während die FLT3-ITD vermittelten, zellulären Effekte unbeeinflusst bleiben.

Schlussfolgerung 3: SOCS1 Expression verstärkt die myeloproliferative Erkrankung, die durch FLT3-ITD induziert wird: SOCS1 als „konditionales Onkogen".

Für FLT3-ITD wurde bereits die Entstehung einer myeloproliferativen Erkrankung und einer akuten lymphozytischen B- und T-Zellleukämie im murinen Knochenmark gezeigt. Im transgenen Mausmodell löst FLT3-ITD entweder eine myeloproliferative oder eine lymphatische Erkrankung aus.

In dieser Arbeit wurde die Rolle von SOCS1 in der FLT3-ITD vermittelten Leukämie im Knochmarktransplantationsmodell *in vivo* untersucht. Die Übertragung der *in vitro* gefundenen Effekte von SOCS1 auf FLT3-ITD in das Mausmodell *in vivo* zeigte, dass es bei der Transplantation von SOCS1 exprimierendem Knochenmark nicht zu einem Anwachsen oder zur Proliferation in der Maus kommt, was einen kompetitiven Wachstumsnachteil dieser Zellen demonstriert. SOCS1 und FLT3-ITD koexprimierende Transplantate zeigten jedoch eine höhere Proliferationsrate *in vivo* als ausschließlich Flt3-ITD exprimierende Zellen. Sowohl Kontroll- als auch SOCS1 transplantierte Mäuse waren unauffällig und ohne erkennbaren Phänotyp. Mäuse mit Flt3-ITD transduzierten oder FLT3-ITD und SOCS1 koexprimierenden Transplantaten entwickelten zu gleichen Teilen entweder eine myeloproliferative Erkrankung oder eine akute lymphatische Leukämie. Die Koexpression von SOCS1 mit FLT3-ITD führte zu einer verkürzten Latenzzeit. Mäuse mit FLT3-ITD transduzierten Transplantaten (mit oder ohne SOCS1) zeigten vergrößerte Milzen und Lebern sowie ein hyperzelluläres Knochenmark als Anhaltspunkt für eine Infiltration mit leukämischen Blasten. Ferner waren die Mäuse anämisch und hatte eine verminderte Thrombozytenzahl. Interessanterweise verkürzte die Koexpression von SOCS1 die Latenzzeit für die myeloproliferative Erkrankung, aber nicht für die akute lymphatische Leukämie. Zusammenfassend wirkt SOCS1 im Zusammenhang mit FLT3-ITD als konditionales Onkogen und kooperiert mit FLT3-ITD bei der Entstehung der myeloproliferativen Erkrankung.

Schlußfolgerung 4: SOCS Gene werden durch BCR/ABL induziert. SOCS1 Expression beeinflusst nicht die BCR/ABL vermittelte Transformation.

Eine Aktivierung von STAT5 wurde im Zusammenhang mit mehreren hämatologischen, malignen Erkrankungen, die durch onkogene Kinasen ausgelöst

wurden, beobachtet. Da BCR/ABL STAT5 stark aktiviert, wurde die SOCS Genexpression in BCR/ABL positiven Zelllinien und im Knochenmark mittels quantitativer PCR analysiert. Im Vergleich zu BCR/ABL negativen ALL Zellen wurde in BCR/ABL positiven, primären ALL Zellen eine Induktion von CIS, SOCS2 und SOCS3 beobachtet. Die Inhibition der ABL Kinaseaktivität in BCR/ABL positiven, primären ALL Zellen durch Zugabe von Imatinib führte zu einer Verminderung der SOCS Expression, was eine Kinase abhängige Expression dieser Proteine demonstriert. Ebenso konnte auch in der BCR/ABL positiven Zelllinie K562 durch die Inhibition der ABL Kinaseaktivität mittels Imatinib eine Reduktion von CIS, SOCS1 und SOCS3 demonstriert werden. Die Expression von BCR/ABL im primären murinen Knochenmark induzierte eine hohe Expression von CIS, SOCS1 und SOCS3. Die Koexpression von SOCS1 beeinflusste nicht die BCR/ABL vermittelte Proliferation, während aber die IL-3 vermittelte Proliferation unterdrückt wurde, was auf eine SOCS1 Resistenz von BCR/ABL deutet.

Mit diesen Ergebnissen schlagen wir ein Modell vor, bei dem FLT3-ITD die Expression von SOCS Genen induziert, welche wiederum die Zelle von pro- oder antiproliferativen sowie Differenzierungssignalen exogener Zytokine abschirmen, ohne die FLT3-ITD vermittelten Signale zu beeinflussen. So verbleibt die Zelle einzig unter der Kontrolle von FLT3-ITD, was zur Leukämieentstehung beiträgt. Dieses Model erklärt das ‚SOCS Paradoxon', in dem die scheinbar gegensätzlichen Ergebnisse der erhöhten Expression der Tumorsupressorproteine der SOCS Familie im Kontext mit onkogenen Kinasen wie FLT3-ITD stehen.

Die vorliegende Untersuchung zeigt einen neuen molekularen Mechanismus der FLT3-ITD vermittelten Leukämieentstehung. Mechanistisch setzen SOCS Proteine die externe Kontrolle durch Zytokine außer Kraft und wirken so positiv in der Leukämogenese. Dies deutet auf eine Rolle von SOCS Proteinen als mögliche therapeutische Zielstrukturen hin. Diese Beobachtungen legen nahe, dass SOCS Proteine generell mit Onkogenen kooperieren, die eine aberrante Aktivierung von STAT3/5 unabhängig von JAK Kinasen, induzieren.

1 Introduction

1.1 Hematopoiesis

Hematopoiesis is the continuous process through which all blood cell types are produced from the hematopoietic stem cell (HSC). HSCs emerge from the primitive ventral mesenchyme during early development. In the fetus, the sites of hematopoiesis include the yolk sac and the liver, whereas in the adult, the primary site of hematopoiesis is the bone marrow (Orkin and Zon, 2008; Weissman, 2000; Weissman et al., 2001).

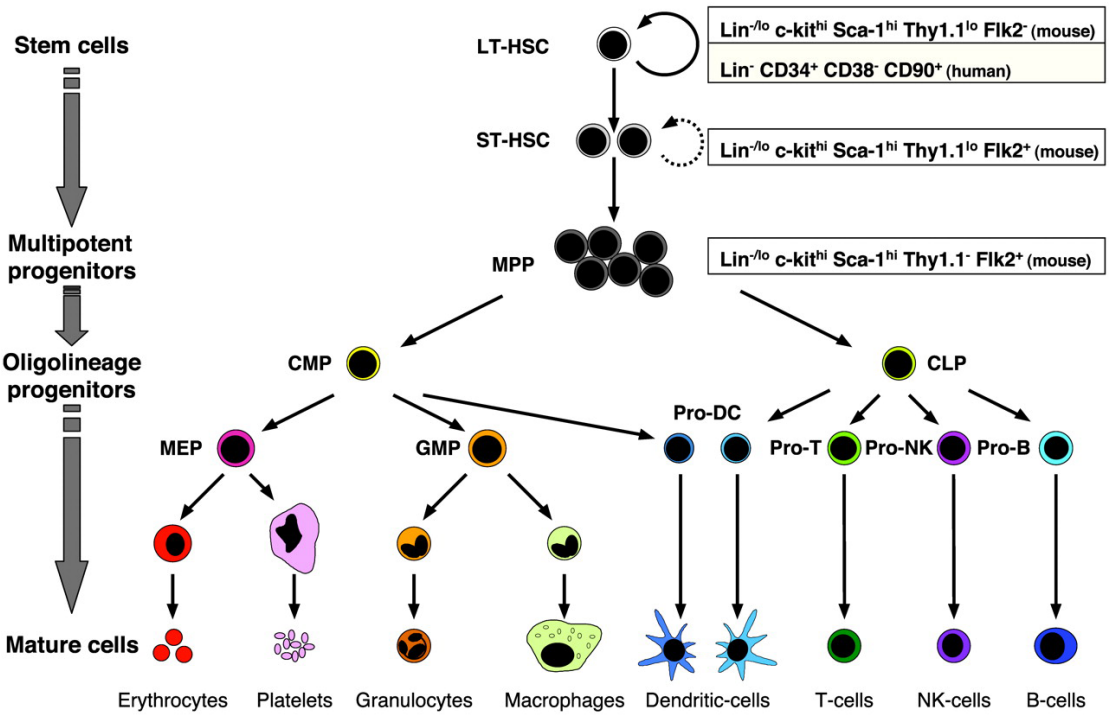

Figure 1: Hematopoietic-progenitor cell lineages and the hematopoietic hierarchy. HSCs can be divided into LT-HSCs, highly self-renewing cells that reconstitute an animal for its entire life span, or ST-HSCs, which reconstitute the animal for a limited period. ST-HSCs differentiate into MPPs, which do not or briefly self-renew, and have the ability to differentiate into oligolineage-restricted progenitors that ultimately give rise to differentiated progeny through functionally irreversible maturation (Passegue et al., 2003).

The blood consists of various types of cells which are divided into myeloid and the lymphoid cells. The myelo-erythroid lineage includes erythrocytes, which transport oxygen and carbon dioxide to and from the lungs to tissues, and platelets, which are important for blood coagulation and wound healing. Monocytes/granulocytes, migrate into the tissues and are involved in inflammation and phagocytosis. The

lymphoid cells include the B-lymphocytes, which provide immunity by the production of specific antibodies, and the T-lymphocytes, which eliminate cells that have foreign molecules on their surface. In a healthy individual the various types of blood cells are maintained at constant proportions throughout life.

HSCs are functionally defined by their unique ability to self-renew and to differentiate to form all mature blood cell types. Based on how long they can repopulate lethally irradiated mice, HSCs are divided into either long-term HSC (LT-HSC) or short-term HSC (ST-HSC) (Morrison and Weissman, 1994). These HSCs subsequently form multi-potent progenitors (MPPs) that generate committed progenitors of different lineages, the common myeloid progenitor (CMP) for the myelo-erythroid lineage and the common lymphoid progenitor (CLP) for the lymphoid lineage. The CMP in turn gives rise to the granulocyte-macrophage progenitor (GMP) and the megakaryocyte-erythrocyte progenitors (MEP). These progenitors will give rise to all mature blood cells. Thus the hematopoietic hierarchy is composed of stem cells, committed progenitors and their progeny and mature blood cells of all lineages (Figure 1) (Orkin and Zon, 2008; Weissman, 2000; Weissman et al., 2001).

1.2 Leukemia

Leukemia is the malignant growth of the progenitor cells from which mature blood cells develop. The underlying process of development of leukemia is called leukemogenesis. Leukemias are often broadly classified as ‘acute’ and ‘chronic’, based on the duration for the disease to manifest and progress. Acute leukemias often progress very rapid and can be fatal within few weeks to months. Chronic leukemias progress gradually over a period of years of time. Leukemias are classified depending on the type of cells that are abnormally growing: for example myeloid or lymphoid leukemias.

Leukemia are classified based upon whether the leukemia is acute versus chronic and myeloid versus lymphoid that is:

- Acute myeloid leukemia (AML)
- Chronic myeloid leukemia (CML)
- Acute lymphoblastic leukemia (ALL)

- Chronic lymphoblastic leukemia (CLL)

As the present study mainly deals with the acute leukemias, these types are explained in more detail.

1.2.1 Acute myeloid leukemia (AML)

AML is characterized by the malignant transformation of myeloid stem/progenitor cells in the bone marrow, which are incapable of normal differentiation and maturation, resulting in 'blast' cells. As a consequence, production of normal marrow cells is perturbed, leading to a deficiency of red cells, blood-clotting platelets and normal infection-fighting white cells. Since normal hematopoiesis is organized hierarchically, AML may arise in a stem cell capable of differentiating into cells of erythroid, granulocytic, monocytic and megakaryocytic lineages. The FAB (French-American-British) classification system divides AML into 9 subtypes based on maturation-stage, the type of cell from which the leukemia developed.

M0: Minimally differentiated

M1: Myeloblastic leukemia without maturation

M2: Myeloblastic leukemia with maturation

M3: Hypergranular promyelocytic leukemia

M3v: Microgranular promyelocytic leukemia

M4: Myelomonocytic leukemia

M4 variant: increase in marrow eosinophils

M5: Monocytic leukemia

M6: Erythroleukemia

M7: Megakaryoblastic leukemia

More recently the FAB classification has been replaced by the WHO (World Health Organization) system based on the cytogenetic, chromosomal abnormalities (Harris et al., 1999; Vardiman et al., 2009). The WHO also set the diagnosis of AML to be at least 20% of the cells identified in the blood or bone marrow as blasts of myeloid origin. Myeloproliferative disease is characterized by increased accumulation of mature myeloid cells in the peripheral blood and bone marrow.

1.2.2 Acute lymphoblastic leukemia (ALL)

ALL is characterized by abnormalities of the lymphoid cell precursors leading to excessive accumulation of leukemic lymphoblasts (either B or T cells) in the bone marrow and other organs, in particular the spleen and liver. ALL most commonly

affects children, particularly those between 3 and 10 years of age. It accounts for about 85% of all childhood leukemias. It also affects older adults. FAB classification of ALL has three subtypes. The FAB classification has been revised and replaced by WHO (Harris et al., 1999; Vardiman et al., 2009).

L1: Small homogeneous, high nuclear: cytoplasmic ratio, small nucleoli

L2: Larger, pleomorphic, low nuclear: cytoplasmic ratio, prominent nucleoli

L3: Larger, vacuolated basophilic cytoplasm, large vesicular nucleus, large nucleoli; resemble Burkitt's lymphoma cells

1.3 Two hit model of leukemogenesis

Development of leukemia is multi-step process. Majority of the mutations found in AML patients, when expressed in mouse develops a pre-leukemic condition. Studies in mice using genetic alterations found in AML patients have shown that a minimum of two genetic alterations (genetic hits) is required for pre-leukemic myeloproliferation to manifest into AML disease (Kelly et al., 2002a; Kim et al., 2008; Schessl et al., 2005). This hypothesis has been called the "two-hit model" of AML (Brandts et al., 2007; Dash and Gilliland, 2001; Kelly and Gilliland, 2002).

The model suggests that there are two classes of mutations that are required for the development of AML. Class I mutations confer increased survival and proliferative advantage to the cells for example FLT3-ITD, N- or K-RAS, constitutively active c-kit. Class II mutations block differentiation, for example AML1/ETO, PML/RARα, inv(16) or transcription factors like C/EBP alpha (Figure 4). Supporting this hypothesis, in a clinical setting, FLT3-ITD mutations are often found together with other chromosomal translocations (described in detail above in FLT3 mutations subheading). FLT3-ITD cooperates with PML-RARα, AML1-ETO and Inv(16) to develop AML in mouse models (Kelly et al., 2002a; Kim et al., 2008; Schessl et al., 2005).

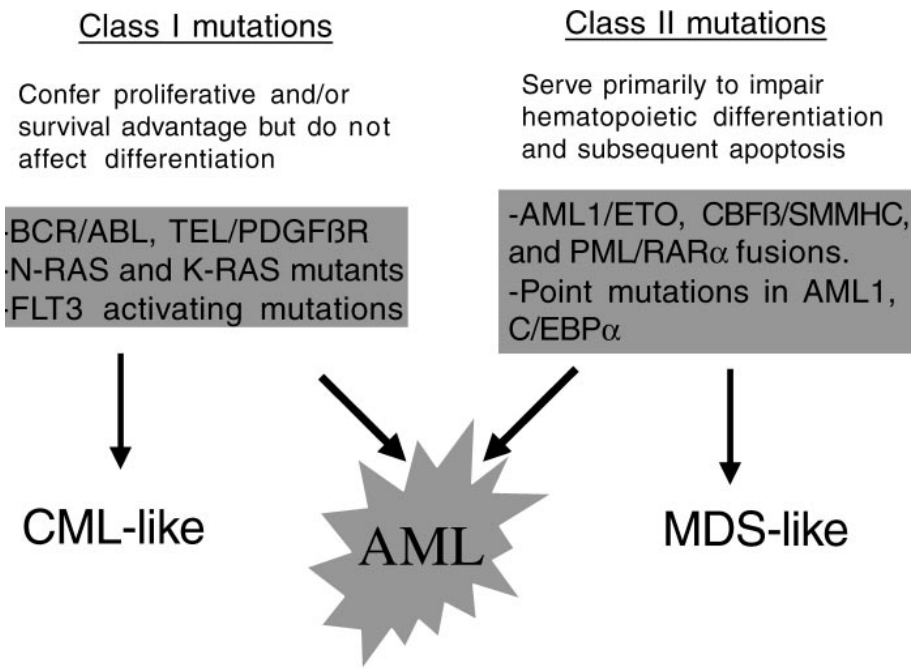

Figure 2. Two hit model of AML development. This model hypothesizes that AML is the consequence of collaboration between at least two broad classes of mutations. Class I mutations that cause a CML-like disease (when expressed alone). Class II mutations that lead to MDS when expressed alone. (Figure adopted from (Kelly and Gilliland, 2002)).

1.4 Oncogenic tyrosine kinases implicated in leukemia

Numerous receptor tyrosine kinases and non-receptor tyrosine kinases have been implicated in leukemia. Activating mutations of FLT3, c-kit and JAK2, oncogenic fusion proteins formed due to chromosomal translocations like Tel-PDGFR-β, Tel-Abl, BCR/ABL are some important examples (Blume-Jensen and Hunter, 2001).

1.5 FLT3 (FMS-like tyrosine kinase 3) receptor

FLT3 (FMS-like tyrosine kinase 3) is a receptor tyrosine kinase, is also called fetal liver kinase-2; Flk-2 or Stem cell Tyrosine Kinase-1; STK-1. FLT3 belongs to RTK subclass III and it is highly homologous to other subclass III members like the M-CSF (macrophage colony stimulating factor) receptor PDGF (platelet derived growth factor) receptor and c-kit receptor (Blume-Jensen and Hunter, 2001; Meshinchi and Appelbaum, 2009; Stirewalt and Radich, 2003). FLT3 is composed of an immunoglobulin-like extracellular ligand-binding domain, a transmembrane domain, a juxtamembrane dimerization domain, and a highly conserved intracellular kinase domain interrupted by a kinase insert (Blume-Jensen and Hunter, 2001; Meshinchi and Appelbaum, 2009; Stirewalt and Radich, 2003). Binding of FLT3 ligand (FL) to extracellular domain of the receptor lead to receptor activation and

downstream signaling.

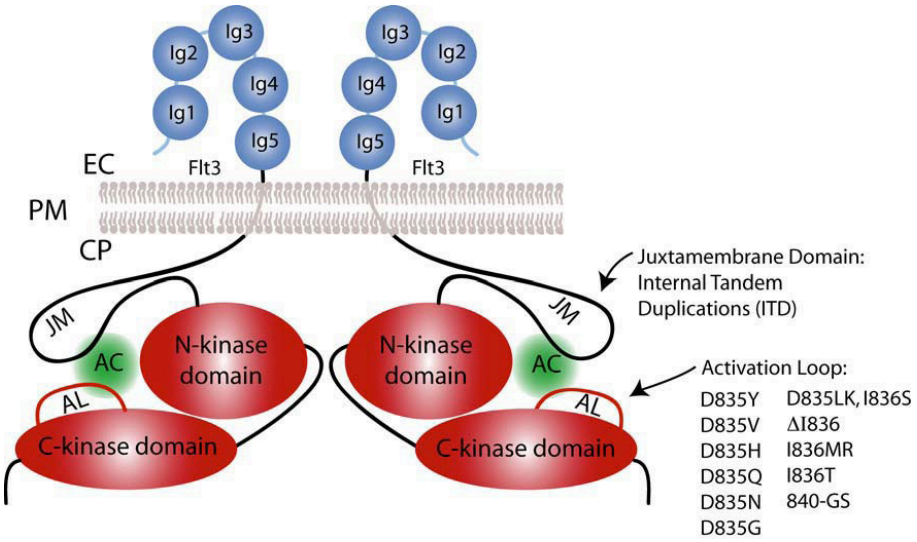

Figure 3: Structure of FLT3 and its activating mutations. The FLT3 receptor is comprised of an extracellular ligand-binding domain, consisting of five immunoglobulin-like domains, a transmembrane, and an intracellular split kinase domain. Binding of a FL to the FLT3 receptor induces dimerization of two monomers leading to auto-phosphorylation of the juxtamembrane tyrosines and activation of the receptor molecules. The oncogenic ITD mutations in FLT3 are clustered in juxtamembrane region whereas TKD mutations are present in the activation loop of the C-terminal kinase lobe.

The predicted molecular weight of FLT3 protein based on its primary sequence is about 110 kDa. However, western blot analysis of FLT3 results in the two bands of 130-143 kDa and 155-160 kDa. This difference in the calculated and actual molecular weight of FLT3 is due to its N-glycosylation (Lyman et al., 1993; Maroc et al., 1993).

1.5.1 Expression and functions of FLT3 receptor in normal hematopoiesis

A very small portion (about 2%) of adult human bone marrow is FLT3 positive; these cells represent early progenitor or stem cells (Rosnet et al., 1996). Analysis of FLT3 expression on human hematopoietic cell lines shows that most myeloid, monocytic, pro-B and pre-B cell lines are FLT3 positive, while erythroid, NK and T cell lines are negative (Brasel et al., 1995; DaSilva et al., 1994; Meierhoff et al., 1995; Rosnet et al., 1996). In mice up-regulation of FLT3 expression is accompanied by a loss of self-renewal capacity of stem cells and those FLT3 positive cells have a prominent lymphoid reconstitution potential (Adolfsson et al., 2001; Christensen and Weissman, 2001). Outside of the hematopoietic system, FLT3 could be detected in brain, placenta, testis, lymph nodes, thymus, and in the liver of adult mice (deLapeyriere et al., 1995; Rosnet et al., 1996).

FLT3 has been shown to serve important functions in early hematopoietic progenitor proliferation and survival as well as in macrophage and dendritic cell differentiation (Gilliland and Griffin, 2002; Stirewalt and Radich, 2003). Mice with homozygous deletion of FLT3 receptor were healthy and showed normal peripheral blood counts (Mackarehtschian et al., 1995). However, the numbers of early B-cell progenitors in bone marrow of these animals were reduced compared to normal mice. In addition, stem cells isolated from *FLT3* knockout mice are deficient in their ability to repopulate lymphoid and myeloid compartments when introduced into lethally irradiated animals (Mackarehtschian et al., 1995).

1.5.2 FLT3 ligand

The FLT3 ligand (FL) is a type I transmembrane protein, which is proteolitically cleaved to form a soluble homodimeric protein. Although FL is a weak growth stimulator of proliferation, it strongly synergizes with other hematopoietic growth factors and interleukins (Broxmeyer et al., 1995; Lyman and Jacobsen, 1998).

1.5.3 FLT3 mutations in leukemia

FLT3 is over-expressed in several hematologic malignancies, including acute myeloid leukemia (AML), B-precursor cell acute lymphoblastic leukemia (ALL) and chronic myeloid leukemia (CML) in lymphoid blast crisis suggesting increased FLT3 expression may play a role in the survival or proliferation of leukemic blasts of bothmyeloid and lymphoid origion (Carow et al., 1996; Drexler, 1996).

Activating mutations of FLT3 are present in about one third of AML cases, making FLT3 one of the most frequently mutated gene found in AML (Gilliland and Griffin, 2002; Nakao et al., 1996; Schnittger et al., 2002; Stirewalt and Radich, 2003). FLT3 mutations are also found in a small number of cases with myelodysplastic syndrome (MDS) or ALL (Armstrong et al., 2004; Horiike et al., 1997; Yokota et al., 1997). Two classes of FLT3 mutations have been described. First, internal tandem duplications (ITD) in exon 14 and/or exon 15 of the receptor were observed in about 25% of AML patients. They lead to the insertion of several amino acids in the juxtamembrane domain (Figure 2) (Nakao et al., 1996; Steudel et al., 2003; Stirewalt and Radich, 2003; Thiede et al., 2002). Second, small deletions or insertions in the activation loop (AL) of the tyrosine kinase domain occur in 7% of AML patients (*FLT3-TKD*) (Figure 2) (Abu-Duhier et al., 2001; Thiede et al., 2002;

Yamamoto et al., 2001). Most commonly, *FLT3-TKD* mutations involve substitution of aspartic acid 835 (D835) by other residues (Thiede et al., 2002). Deletion of I836, substitution of N841 or Y842 or insertions of single amino acids in the activation loop have also been found (Figure 2) (Jiang et al., 2004; Kindler et al., 2005; Spiekermann et al., 2002a; Thiede et al., 2002). Both these above mentioned mutations lead to constitutive activation of the receptor.

FLT3 activating mutations are often prevalent in patients with normal karyotype in leukemias (Abu-Duhier et al., 2001; Schnittger et al., 2002; Thiede et al., 2002). *FLT3-ITD* has been detected in all FAB subtypes of AML, with the highest reported frequency in the M3 and M5 subtype, and less frequently in the M2 subtype (Gilliland and Griffin, 2002; Stirewalt and Radich, 2003; Thiede et al., 2002). The prevalence of *FLT3-ITD* in patients with AML increases with age, ranging from 5–15% in pediatric patients to 25–35% in adults. Most studies in patients with AML have found that *FLT3-ITD* is a strong, independent predictor of poor clinical outcome compared to *FLT3-TKD* mutations (Abu-Duhier et al., 2001; Iwai et al., 1999; Kiyoi et al., 1999; Kondo et al., 1999; Schnittger et al., 2002; Thiede et al., 2002).

1.5.4 Activation of FLT3-WT and FLT3-ITD receptors

Wild-type FLT3 receptor is expressed on the cell surface as a monomer and is inactive in the absence of its ligand FL (Weiss and Schlessinger, 1998). Binding of FL induces receptor dimerization and leads to the auto-phosphorylation of the receptor. Ligand-induced dimerization of receptors is thought to expose tyrosine autophosphorylation sites and to stabilize the active conformational state, which further enhances the activation of the receptors (Turner et al., 1996; Weiss and Schlessinger, 1998). The crystal structure of the auto-inhibited form of FLT3 has been published (Griffith et al., 2004). The normal juxtamembrane (JM) domain, interacts with the kinase domain, buries the catalytic centre, and thus stabilizes the inactive kinase conformation. Ligand-induced dimerization and conformational changes lead to phosphorylation of two key JM tyrosines 589/591. This event destabilizes the conformation of the JM domain and allows the JM domain to move away from the active centre of the kinase, leading to its activation. ITD mutations in the FLT3 JM domain destroy the conformational integrity of the JM domain, whereby the JM domain can no longer inhibit the kinase and allows the ligand-

independent activation of the receptor (Griffith et al., 2004; Hubbard, 2004).

1.5.5 Signaling and biological differences of FLT3-WT and FLT3-ITD

Activation of the FLT3-WT receptor by FL results in the rapid phosphorylation of several components of the SHC/Ras/Raf/MEK/ERK pathway and PI3K/Akt pathways. FL stimulation is a prerequisite for the activation of FLT3-WT receptor. In contrast FLT3-ITD mutations constitutively activate SHC/Ras/Raf/MEK/ERK and PI3K/Akt pathways in a ligand independent manner. FLT3-ITD, in addition to these pathways, also activates STAT5 and induces expression of STAT5 target genes like Pim1, Pim2, SOCS2 and SOCS3 (Choudhary et al., 2005; Kiyoi et al., 2002; Levis et al., 2002; Mizuki et al., 2000; Schmidt-Arras et al., 2005). The detailed signaling differences of wild type receptor and its mutant forms are given in Figure 3. Retroviral expression of FLT3-ITD confers factor independent growth for IL-3 dependent 32D and Ba/F3 cell lines and support colony formation in semisolid media (Choudhary et al., 2005; Mizuki et al., 2000). FLT3-ITD induces myeloid differentiation block by suppression of the myeloid transcription factors c/EBPα and Pu1 expression. FLT3-ITD also induces block of c/EBPα function by hyperphosphorylation (Mizuki et al., 2000; Radomska et al., 2006). FLT3-ITD also confers resistance to externally induced apoptosis in 32D and Ba/F3 cells (Choudhary et al., 2005).

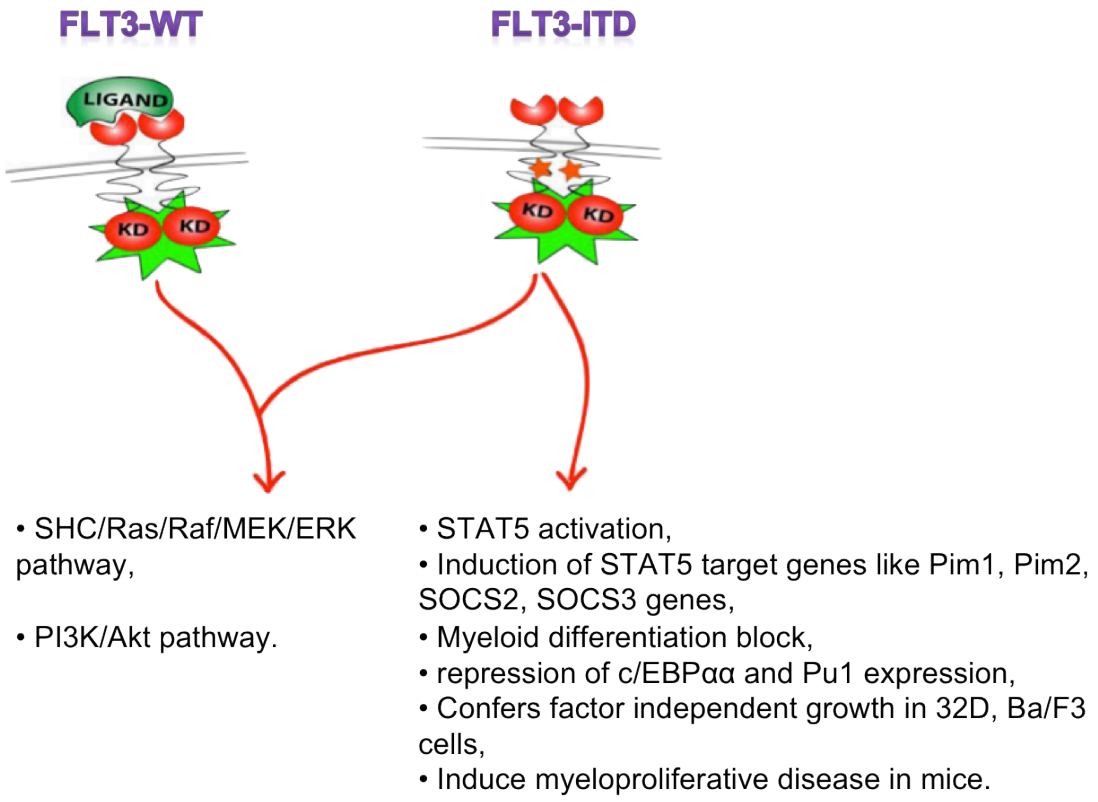

Figure 3: Signaling and biological differences of FLT3-WT and FLT3-ITD. Activation mutant of FLT3 (FLT3-ITD) activates similar to wild type receptor activates Ras/MAPK/ERK and PI3K pathways. Contrasting to FLT3-WT, FLT3-ITD activates STAT5, suppresses myeloid transcription factors and leads to a myeloproliferative disease in mice.

1.5.6 Mouse models for FLT3-ITD

Injection of 32D cells expressing FLT3-ITD induced rapid development of a leukemia-type disease in syngeneic mice (Mizuki et al., 2000). Bone marrow transplantation with FLT3-ITD in mice leads to development of a myeloproliferative disease (Grundler et al., 2005; Kelly et al., 2002b). Transgenic mice expressing FLT3-ITD under the control of VAV hematopoietic promoter induced either a myeloproliferative disease or a lymphoid disease (Lee et al., 2005). In a knock-in mouse model, FLT3-ITD developed myeloproliferative disease with a resemblance to chronic myelomonocytic leukemia (CMML) (Lee et al., 2007). In contrast, another knock-in mouse model for FLT3-ITD mice developed a myeloproliferative (Li et al., 2008).

1.6 The Philadelphia Chromosome (BCR/ABL)

The Philadelphia chromosome is a shortened chromosome 22 resulting from a balanced translocation of the long arms of chromosome 9 and chromosome 22: t

(9; 22). This translocation occurs in about 90% of CML patients and in 10-20% of ALL patients.

1.6.1 BCR/ABL signaling

The BCR/ABL fusion protein has a constitutive tyrosine kinase activity of Abl, which is required for BCR/ABL mediated transformation. The oligomerization domain of Bcr contributes to BCR/ABL mediated transformation (McWhirter et al., 1993). Imatinib mesylate is a ABL kinase inhibitor that can selectively inhibit BCR/ABL-induced *in vitro* cell growth and *in vivo* tumor formation in mice from cell lines expressing BCR/ABL but not from control cell lines (Druker et al., 1996).

BCR/ABL activates multiple signaling pathways which contribute to increased survival, proliferation and transformation (reviewed by (Wong and Witte, 2004). BCR/ABL constitutively activate STAT5 (prominently) and STAT1 and STAT3 to a less extent (Ilaria and Van Etten, 1996). BCR/ABL mediated STAT5 target genes A1 and Pim kinases play an important role in BCR/ABL mediated survival (Adam et al., 2006; Nieborowska-Skorska et al., 2002). BCR/ABL mediated STAT5 activation is shown to be direct and/or via HCK (Src family kinase) (Ilaria and Van Etten, 1996; Klejman et al., 2002).

BCR/ABL also activates PI3K/Akt via its direct association with the p85 subunit of PI3K (Wong and Witte, 2004).The Ras/MAPK pathway is activated by BCR/ABL via Grb2/Gab2 and this activation is dependent of tyrosine 177 (an auto-phosphorylation site on BCR/ABL) (Wong and Witte, 2004). Gab2, being a critical mediator of PI3K/Akt and Ras/MAPK downstream of BCR/ABL, plays important role in BCR/ABL mediated leukemogenesis (Sattler et al., 2002).

1.6.2 Mouse models for BCR/ABL

Retroviral expression of BCR/ABL in mouse bone marrow cells and transplantation has been shown to induce a myeloproliferative disorder (MPD) that closely resembled to CML (Daley et al., 1990; Kelliher et al., 1990). Since the transgenic expression BCR/ABL is embryonically lethal, a tetracycline responsive promoter driven BCR/ABL transgenic mice were generated. Using this system BCR/ABL expression in B-lymphocyte and megakaryocytic precursors developed B-ALL and megakaryocytic myeloproliferative disease respectively (Huettner et al., 2003;

Huettner et al., 2000). When the BCR/ABL is expressed in hematopoietic stem cells, using the mouse stem cell leukemia (SCL) enhancer, mice developed a chronic-phase CML-like disease (Koschmieder et al., 2005). These mouse models provide significant insights into molecular pathogenesis of BCR/ABL mediated leukemogenesis and served as models for testing the newly developed drugs (Ren, 2005; Wong and Witte, 2004).

1.7 Physiological cytokine/JAK/STAT signaling pathway

The survival and differentiation of hematopoietic stem and progenitor cells as well as the function of mature blood cells are under control of cytokines. Unlike RTKs (receptor tyrosine kinases), receptors for cytokines do not have intrinsic kinase activity; instead they utilize JAK kinases (Janus kinases) for signal transduction. Binding of cytokines to their cognate receptors changes the conformation and promotes the binding of JAKs. JAKs in turn phosphorylate the receptors as well as other intracellular proteins like STATs (Signal Transducers and Activator of Transcription). Upon phosphorylation STAT proteins form dimers and translocate into the nucleus, functioning as transcription factors to induce gene expression. There are 4 JAKs namely JAK1-3 and Tyk-2, and 7 STATs: STAT1-4, STAT5A, STAT5B and STAT6. Specificity of cytokine signaling is largely determined by the combination of activated JAKs and STATs. Depending on the JAK/STAT member combinations and cellular context, cytokines exert a variety of functions includes survival/proliferation, differentiation and apoptosis/growth arrest (Figure 5).

Stimulation of IFNα and IFNγ receptor, leads to STAT1 and STAT2 activation by receptor associated JAK1, JAK2 and Tyk2. IFNα and IFNγ activated STAT1 induce expression of several genes involved in growth arrest, apoptosis, anti-proliferative and antiviral effects (Bromberg et al., 1996; Chin et al., 1997; Chin et al., 1996; Horvath and Darnell, 1996). On the other hand IL-3, IL-5, G-CSF, and GM-CSF mediated proliferative functions and neutrophilic differentiation is mediated mainly by STAT5 (Dong et al., 1998; Ilaria et al., 1999; Mui et al., 1995). IL-6 and G-CSF induced STAT3 activation plays a key role in the process of differentiation of macrophages and neutrophilic granulocytes respectively (Nakajima et al., 1996; Shimozaki et al., 1997). Thus, functional specificity of cytokines is achieved through the activation of STATs and their transcriptional targets.

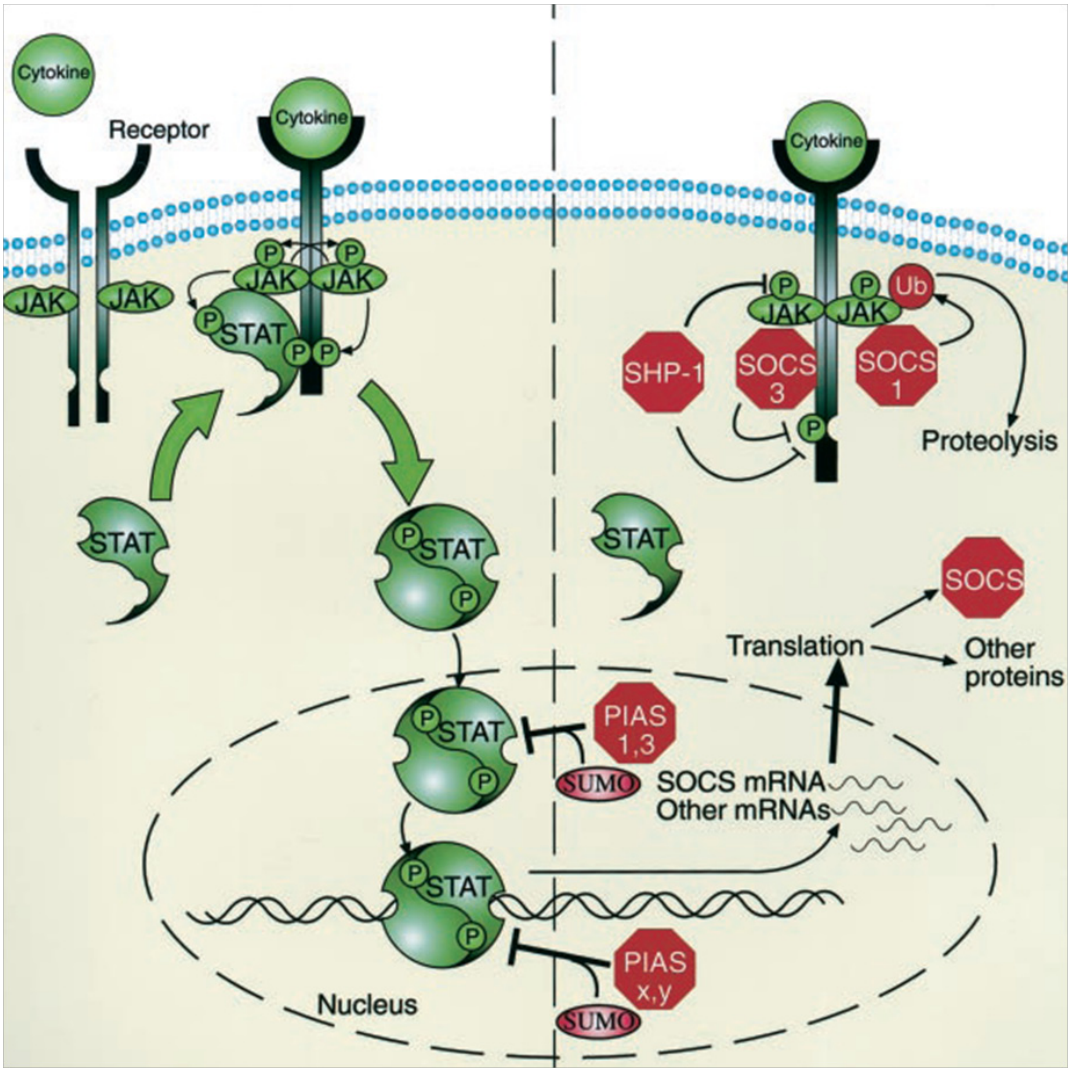

Figure 5: Cytokine/JAK/STAT signaling pathway. Cytokines exert various functions like survival/ proliferation, differentiation and growth inhibition through Janus kinases mediated, STAT protein activation. Activated STATs form dimers and translocate into the nucleus leading to transcription and expression of different target genes important for either of the above-mentioned functions. JAK/STAT pathway is in tight regulation by negative feedback mechanisms (SOCS, PIAS and PTPs). Figure adopted from (Wormald and Hilton, 2004).

Cytokine signaling in hematopoiesis and the biological implications of JAK/STAT pathway are extensively reviewed elsewhere (Baker et al., 2007; Levy and Darnell, 2002; O'Shea et al., 2002).

Activation of physiological JAK/STAT signaling pathway is under tight control by three major classes of negative regulators: SOCS (suppressors of cytokine signaling), PIAS (protein inhibitors of activated STATs) and PTPs (protein tyrosine phosphatases), which ensure a proper function of these pathways (Greenhalgh and Hilton, 2001; O'Shea et al., 2002; Wormald and Hilton, 2004). SOCS proteins are the major focus of the thesis and are explained in detail below.

1.8 Oncogenic JAK/STAT pathway

As discussed above activating mutations in kinases for example TEL-JAK2, TEL-ABL, FLT3-ITD and BCR/ABL etc. are frequently found in leukemia (Blume-Jensen and Hunter, 2001). Several components of the JAK/STAT pathway are often deregulated in leukemia. For example, activating point mutations JAK2 like V617F, are associated with myeloid leukemias such as polycythemia vera, essential thrombocytopenia, idiopathic myelofibrosis (James et al., 2005; Kralovics et al., 2005; Levine et al., 2005). Similarly, increased activation of STAT3 and STAT5 which promote cell proliferation and survival were observed in leukemia patients (Benekli et al., 2003; Chai et al., 1997; Frank et al., 1997; Gouilleux-Gruart et al., 1996; Spiekermann et al., 2002b). STAT5 also play important role in the development of leukemia by several oncogenes. STAT5 is required for the development of myelo- and lympho-proliferative disease by Tel-JAK2 in mice (Schwaller et al., 2000). Constitutively active STAT5 promoted human stem cell self-renewal and erythroid differentiation (Schuringa et al., 2004). Bone marrow transplantation of constitutively active STAT5 led to myeloproliferative disease in mice (Moriggl et al., 2005).

As the major focus of the present work is FLT3-ITD and BCR-ABL, a more detailed description of STAT5 pathway in the context of these two oncogens is given below. FLT3-ITD induces strong STAT5 activation and its target genes in 32D cell line. STAT5 plays an important role in FLT3-ITD mediated transformation. Expression of dominant negative form of STAT5 in 32D-FLT3-ITD cell lines stunted the colony formation (Mizuki et al., 2000). The enforced expression of FLT3-ITD in human $CD34^+$ resulted in increased self-renewal, formation of early CAs (Cobblestone areas) and enhanced erythropoiesis of these cells in STAT5 dependent manner (Chung et al., 2005). STAT5 target genes Pim1 and Pim2 play an important role in FLT3-ITD mediated transformation (Adam et al., 2006; Kim et al., 2005; Mizuki et al., 2003). Importantly, FLT3-ITD directly activates STAT5 independent of JAK or Src kinase, this activation is resistant to over expression of potent JAK kinase inhibitors SOCS1 and SOCS3, suggesting Jak kinases are not required for its transformation. Similarly, Tyk2 is dispensable for FLT3-ITD mediated myeloproliferative disease in mice (Nakajima et al., 2006).

Constitutively active STAT3 is found in multiple myeloma (MM), anaplastic large T cell lymphoma (ALCL). Aberrant STAT3 is also associated with erythroleukemia, acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia, acute myeloid leukemia (AML), chronic myeloid leukemia (CML), megakaryocytic leukemia (Catlett-Falcone et al., 1999; Epling-Burnette et al., 2001; Kirito et al., 2002; Zamo et al., 2002). STAT3 and STAT5 are constitutively activated by BCR/ABL. BCR/ABL activates STAT5 via Hck (a member of Src family kinase) (Ilaria and Van Etten, 1996; Ren, 2005). It is has also been showed that STAT5 is directly activated by BCR/ABL (Carlesso et al., 1996). The role of STAT5 in BCR/ABL mediated leukemogenesis remains controversial. Retroviral transduction of BCR/ABL into bone marrow induces a CML-like MPD in STAT5 deficient mice (Sexl et al., 2000). Inactivation of STAT5 with siRNA in primary CML samples impairs Ph^+ myeloid colony formation. Fetal liver hematopoietic progenitors from STAT5 knockout mice failed to induce leukemia in recipient mice after retroviral transduction with BCR/ABL (Hoelbl et al., 2006; Ye et al., 2006).

1.9 SOCS proteins

Being part of cytokine/JAK/STAT pathway, Suppressors Of Cytokine Signaling (SOCS) play a major role in the regulation of cytokine responses. There are eight members present in SOCS family, namely SOCS1-7 and CIS (cytokine-inducible SH2 domain containing protein). The CIS gene was first identified as an immediate early response gene induced in hematopoietic cells in response to IL-2, IL-3 and EPO (erythropoietin) (Yoshimura et al., 1995). Later, three different groups identified SOCS1 as a novel JAK regulatory protein (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). Based on sequence homology 6 additional SOCS proteins were cloned (Hilton et al., 1998; Masuhara et al., 1997; Starr et al., 1997). All SOCS family members have a three-part domain structure, a central SH2 domain, an amino-terminal domain of variable length and divergent sequence, and a carboxy-terminal 40-amino-acid module, known as the SOCS box.

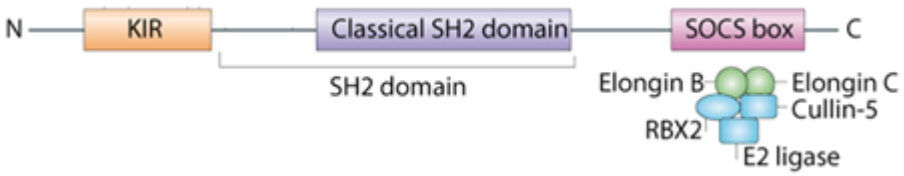

Figure 6: Domain structure of SOCS proteins. SOCS Figure adopted from (Yoshimura et al., 2007).

The SOCS box interacts with elongin B and elongin C, cullin-5 and RING-box-2 (RBX2) that recruit E2 ubiquitin transferase. Thereby, SOCS family proteins probably function as E3 ubiquitin ligases and mediate the degradation of proteins that are associated. In addition to the E3 ligase activity, SOCS1 and SOCS3 are shown to inhibit JAK tyrosine kinase activity directly through their kinase inhibitory region (KIR) that seems to function as a pseudosubstrate.

1.9.1 Expression of SOCS proteins

SOCS proteins are often expressed at low level to undetectable levels in resting/unstimulated cells. SOCS proteins are upregulated rapidly in response to a broad range of cytokines and in turn, control the duration and intensity of cytokine responses by blocking various aspects of the signaling pathways. SOCS proteins down-modulate the signaling pathway, which was responsible for their own expression and hence it is a part of negative feedback mechanism (Figure 5). The pattern of SOCS expression by a particular cytokine varies according to the cell type or tissue. The induced SOCS proteins can attenuate signaling of various cytokines and may be involved in inhibitory cross talk between different cytokine systems. SOCS can be induced by stimuli other than cytokines, including growth factors, chemokines, hormones, pathogens or any foreign materials like CpG DNA or LPS (Baetz et al., 2004; Crespo et al., 2000; Dalpke et al., 2001; Dogusan et al., 2000; Krebs and Hilton, 2003; Leong et al., 2004; Stoiber et al., 1999). Various cytokine induced SOCS expression, and cytokines inhibited by different SOCS members are given in table1.

1.9.2 CIS

CIS is induced by cytokines that activate STAT5, such as Epo, GH, PRL, IL-2 and IL-3 and CIS induction is reported to suppress signaling cascades by masking the STAT5 binding sites in their receptors (Aman et al., 1999; Dif et al., 2001; Endo et

al., 2003; Hansen et al., 1999; Matsumoto et al., 1997; Ram and Waxman, 1999; Verdier et al., 1998; Yoshimura et al., 1995). In fact, binding of CIS to erythropoietin receptor (EpoR) induces proteasome-dependent degradation of the receptor (Verdier et al., 1998).

	Induced by	**Inhibits signaling of**
CIS	IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-12, IL-13, Epo, LIF, G-CSF, GM-CSF, TPO, IFNα, IFNγ,	Epo, G-CSF, IL-2, IL-3
SOCS1	Epo, LIF, G-CSF, GM-CSF, IFNα/β, IFNγ, IL-2, IL-3, IL-4, IL-6, IL-13	Epo, TNFα, OSM, TPO, IFNα/β, IFNγ, IL-2, IL-3, IL-4, IL-6, IL-7, IL-12, IL-15
SOCS2	EPO, G-CSF, GM-CSF, TNFα, LIF, IFNα, IFNγ, IL-1, IL-2, IL-3, IL-4, IL-6, IL-9, IL-10,	LIF, IFNγ, IL-6
SOCS3	Epo, GM-CSF, M-CSF, G-CSF, TPO, TNFα, LIF, IFNα, IFNγ, IL-1, IL-6, IL-9, IL-11, IL-12, IL-13,	Epo, OSM, LIF, IFNα/β, IFNγ, IL-2, IL-3, IL-4, IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-10, IL-11

Table 1: List of cytokines, which induce SOCS expression and cytokines that are affected by SOCS expression. (Table modified from (Alexander, 2002)).

CIS is also shown to bind to GHR and leads to its internalization and degradation (Landsman and Waxman, 2005; Ram and Waxman, 1999). CIS knockout mice are phenotypically normal; this was probably due to functional compensation by other SOCS proteins. But, CIS transgenic mice have impaired mammary gland development and reduced numbers of natural killer (NK); these phenotypic features are highly similar to STAT5 knockout mice, supporting CIS to be a specific negative regulator of STAT5 signaling (Matsumoto et al., 1999; Teglund et al., 1998).

1.9.3 SOCS1

SOCS1 directly binds to all JAK members, thereby inhibiting their kinase activity (Endo et al., 1997; Naka et al., 1997). SOCS1 targets the phosphorylated tyrosine1007, in the activation loop of JAK2. Kinase activation of JAK2 involves phosphorylation of Y1007. Although SH2 domain of SOCS1 is sufficient for JAK2 interaction, as mentioned above, the KIR domain is required for high affinity binding

and efficient inhibition of JAK2 activity (Yasukawa et al., 1999). Importantly, SOCS1 is shown to bind directly to the type I and type II IFN receptors, which is why SOCS1 shows efficient inhibitory effect on IFN signaling (Fenner et al., 2006; Qing et al., 2005).

Studies on SOCS1 In vitro shows its implications on a wide range of cytokines that include GH, Epo, PRL, IL-3, IL-6, IFNα/β, IFNγ and IL-4 (Adams et al., 1998; Dif et al., 2001; Hansen et al., 1999; Song and Shuai, 1998). The potent inhibitory effects exhibited by SOCS1 are due to its ability to inhibit the kinase activity of JAK kinases. In addition SOCS1 induces the ubiquitination and destruction of VAV, JAK2 and the TEL-JAK2 oncogene in a SOCS box dependent fashion (De Sepulveda et al., 2000; Frantsve et al., 2001; Kamizono et al., 2001; Ungureanu et al., 2002). SOCS1 also facilitates ubiquitin-mediated degradation MyD88 Adaptor-Like (Mal), thereby leading to the suppression of Mal-dependent p65 phosphorylation and transactivation of NF-κB (Mansell et al., 2006; Ryo et al., 2003).

Very recently, the nuclear localization signal of SOCS1 has been identified; this signal is only present in SOCS1 among other family members. SOCS1 deficient of a nuclear localization signal could still inhibit IFNγ, but there is decreased IFNγ-mediated CD54 expression (Baetz et al., 2008). This suggests many unidentified new roles for SOCS1. The Role of SOCS1 in cancer is discussed below.

SOCS1 knockout mice die within 7 to 20 days after birth, with a phenotype of stunted growth, fatty degeneration of the liver, low mature B cells and small thymus, monocytic infiltration of major organs (Naka et al., 1998; Starr et al., 1998). These multiple deregulations of the immune system were attributed to uncontrolled IFNγ signaling leading to constitutive STAT1 activation. In support of this, knocking out IFNγ gene or administration of neutralizing anti-IFNγ antibodies prevented phenotype of SOCS1 knockout mice (Alexander et al., 1999; Marine et al., 1999b). SOCS1 conditional knockout mice demonstrated an inhibitory role for SOCS1 on IL-2 or IL-7 (Chong et al., 2003; Cornish et al., 2003). SOCS1 transgenic mice are characterized by defective thymocyte development and perturbed homeostasis of T cells (Fujimoto et al., 2000).

1.9.4 SOCS2

SOCS1 interaction with phosphotyrosine motifs on the GHR, PRLR, EGFR, EpoR and LR has been reported. SOCS2 act as an ubiquitin ligase and appeared to be crucial for the negative regulation of GH signaling (Eyckerman et al., 2001; Goldshmit et al., 2004; Greenhalgh et al., 2005; Lavens et al., 2006; Pezet et al., 1999). Mice lacking SOCS2 exhibit gigantism associated with increases in bone and body length and enhanced weight of organs due to uncontrolled growth hormone induced STAT5 (Greenhalgh et al., 2002; Metcalf et al., 2000).

1.9.5 SOCS3

Although SOCS3 is structurally very similar to SOCS1, it exerts a very different effect. SOCS3 is known to interact with gp130, LR, EpoR, LIFR, IL-6R, IL-12R and GHR (Bjorbak et al., 2000; Hortner et al., 2002; Lehmann et al., 2003; Ram and Waxman, 1999; Yamamoto et al., 2003). Deletion of the SOCS box of SOCS3 in transgenic mice leads to impaired regulation of G-CSF signaling and response to inflammatory stimuli, establishing a role for the SOCS box in the *in vivo* actions of SOCS3 (Boyle et al., 2007). SOCS3 not only controls the magnitude of IL-6 signaling but also shapes IL-6 responses. IL-6 loses its pro-inflammatory function and elicits immunosuppressive actions in SOCS3 deficient macrophages (Yasukawa et al., 2003). Also, IL-6 strongly activates STAT1, thereby inducing the expression of IFN-responsive genes in SOCS3 deficient macrophages (Croker et al., 2003; Lang et al., 2003).

Both SOCS3 knockout and transgenic mice die *in utero*. The lack of SOCS3 leads to uncontrolled LIF signaling in placental tissue. Transgenic SOCS3 expression in mice results in defective fetal liver erythropoiesis (Marine et al., 1999a; Roberts et al., 2001; Takahashi et al., 2003). Studies in conditional SOCS3 knockout mice demonstrated that SOCS3 is an important negative regulator of GCSF (Croker et al., 2004; Kimura et al., 2004) and IL-6 (Croker et al., 2003; Lang et al., 2003; Yasukawa et al., 2003).

1.9.6 SOCS4-7

SOCS4, 5, 6 and 7 are the least studied among other SOCS family members SOCS4 and SOCS5 are highly up regulated upon EGF (epidermal growth factor) stimulation and leads to EGFR (epidermal growth factor receptor) degradation

(Kario et al., 2005). SOCS6 is induced by insulin, SCF (stem cell factor or Kit Ligand) and it interacts with the insulin receptor, KIT receptor (Bayle et al., 2004; Li et al., 2004; Mooney et al., 2001). SOCS7 can suppress PRL, GH and leptin signaling by interacting with STAT5 or STAT3 and preventing their nuclear translocation (Martens et al., 2005).

1.10 SOCS proteins in cancer

SOCS1 has been shown to have tumor suppressor properties. SOCS1 blocks the oncogenicity of several hematopoietic specific oncogenes such as the constitutively active form of the KIT receptor, TEL-JAK2, v-ABL, oncogenic form of VAV and partially BCR/ABL (De Sepulveda et al., 2000; Rottapel et al., 2002). Inactivating mutations of SOCS1 were found in Hodgkin lymphoma and were associated with higher JAK2 expression, STAT6 activation and nuclear accumulation of phospho-STAT5 (Mottok et al., 2007; Weniger et al., 2006). The SOCS1 gene is found to be hyper-methylated in hepatocellular carcinoma, multiple myeloma, pancreatic cancer and liver carcinomas (Galm et al., 2003; Komazaki et al., 2004; Yoshikawa et al., 2001) Constitutive activation of the JAK-STAT pathway in several hematological malignancies including leukemia and lymphoma are also associated with SOCS down regulation (Galm et al., 2003; Watanabe et al., 2004; Weniger et al., 2006). Since SOCS proteins appear to have tumor suppressor functions that need to be bypassed for transformation to occur (Limnander et al., 2004). CIS and SOCS1 expression with BCR/ABL reduced its oncogenic potential (Rottapel et al., 2002; Tauchi et al., 2001). This implies that the forced expression of SOCS might be beneficial for the treatment of some malignancies (Frantsve et al., 2001; Rottapel et al., 2002).

SOCS expression is induced by oncogenic kinases BCR/ABL and FLT3-ITD. SOCS2 overexpression was clearly found to correlate with advanced stages of chronic myeloid leukemia (CML) and this expression was abrogated by imatinib treatment (Schultheis et al., 2002; Zheng et al., 2006) Mizuki et al. also found SOCS2 and SOCS3 to be up regulated by FLT3-ITD in 32D cells (Mizuki et al., 2003).

2 Objectives

2.1 Hypothesis

FLT3-ITD, an activating mutation of FLT3, is one of the most frequent mutations found in AML patients. Unlike FLT3-WT, FLT3-ITD activates STAT5 and its targets Pim kinases and SOCS proteins, which play a key role in transformation (Mizuki et al., 2000). Importantly STAT5 target genes like Pim kinases play a crucial role in FLT3-ITD mediated transformation (Adam et al., 2006; Grundler et al., 2009). FLT3-ITD directly activates STAT5 independently of JAK or Src kinases therefore the activation is resistant to physiological negative feedback mediated SOCS proteins (Choudhary et al., 2007). Hence we hypothesize that these FLT3-ITD-induced SOCS genes, lead to escape from external cytokine control, thereby promoting the leukemogenesis. SOCS proteins are known for their growth inhibitory functions; especially SOCS1 is shown to be a tumor suppressor in the context of several oncogenes (Rottapel et al., 2002; Yoshimura et al., 2007). The role of SOCS proteins in FLT3-ITD mediated leukemogenesis remains unexplored. Hence studying the role of SOCS proteins in the molecular pathogenesis of FLT3-ITD may provide their prognostic importance and help to find new therapeutic targets.

2.2 Specific objectives

- To study FLT3-ITD induced SOCS gene expression in primary bone marrow and in blasts from AML patients.
- To study the role of SOCS proteins in FLT3-ITD mediated transformation *in vitro* and leukemogenesis *in vivo*.
- To determine if SOCS proteins play a general role in the context of other oncogenes such as BCR/ABL.

3 Materials and Methods

3.1 Materials

3.1.1 Instruments and apparatus

FACS Machine	FACScan and FACS Canto II Becton Dickinson, Heidelberg
Cytocentrifuge	Thermo Shandon, Pittsburgh, US
Cytofunnel	Shandon, Pittsburgh, USA
Irradiation of mice-Betatron 500A	Siemens, Munich, Germany
Magnetic cell separation	Miltenyi, Bergisch Gladbach, Germany
Agarose gel apparatus	Biometra, Goettingen, Germany
SDS-PAGE apparatus	XCell SureLock™ Mini-Cell, Invitrogen, Karlsruhe, Germany
Gene Pulser system (electroporation)	Bio-Rad Laboratories, Munich, Germany
Axio-Cam up-right Microscope	Zeiss, Goettingen, Germany
Fast Real-Time PCR System (7900HT)	Applied Biosystems, Foster City, CA

3.1.2 Kits

Plasmid mini-prep Kit	Zymo Research, Freiburg, Germany
Gel-DNA recovery Kit	Zymo Research, Freiburg, Germany
Plasmid-Maxi-prep Kit	Genomed, Loehne, Germany
TRIzol-RNA isolation Kit	Invitrogen, Karslruhe, Germany
Luminol reagent	Santa Cruz Biotechnology (Santa Cruz, CA)
Bone marrow lineage cell depletion kit	Miltenyi, Bergisch Gladbach, Germany
Sca-1 isolation kit	Miltenyi, Bergisch Gladbach, Germany

3.1.3 Chemokines and cytokines

rmG-CSF	Peprotech, (Rocky Hill, NJ, USA)
rmSCF	Peprotech, (Rocky Hill, NJ,USA)
rmIL-6	Peprotech, (Rocky Hill, NJ,USA)
rmIL-3	Peprotech, (Rocky Hill, NJ, USA)
rmIFNα	R&D systems(MinneapolisUSA)
rmIFNγ	Sigma Aldrich (Taufkirchen, Germany)

3.1.4 Enzymes

Calf intestinal phosphatase (CIP)	New England Biolabs, Frankfurt, Germany
Gateway LR clonase enzyme mix	Invitrogen, Karslruhe, Germany
Klenow-Fragment DNA-polymerase-I	NEB, Frankfurt, Germany
Restriction endonucleases	NEB, Frankfurt, Germany
RNAse	Sigma, Steinheim, Germany
T4 DNA-ligase	NEB, Frankfurt, Germany
Taq-DNA-polymerase	Biosystems, Weiterstadt, Germany
Proteinase K	Stratagene, La Jolla, USA

3.1.5 Primary antibodies used for western blotting

Mouse anti-p-STAT5- Y694	Biotechnology (Lake Placid, NY)
Anti-phosphoErk1/2-T202/Y204	Cell Signaling (Beverly, MA)
Anti-phosphoFLT3-Y591	Cell Signaling (Beverly, MA)
Anti-phosphoSTAT1-Y701	Cell Signaling (Beverly, MA)
Anti-phosphoAkt-S473	Cell Signaling (Beverly, MA)
Anti-Erk1/2	Santa Cruz Biotechnology (Santa Cruz, CA)

Anti-STAT5a/b	Santa Cruz Biotechnology (Santa Cruz, CA)
Anti-GFP	Santa Cruz Biotechnology (Santa Cruz, CA)
Anti-Pim1	Santa Cruz Biotechnology (Santa Cruz, CA)
Anti-Pim2	Santa Cruz Biotechnology(Santa Cruz, CA)
Anti-β Actin	Sigma Aldrich (Taufkirchen, Germany)
Anti-SOCS1	MBL (Freiburg, Germany).

3.1.6 Secondary antibodies

Anti-mouse IgG-HRP	Jackson immune research Labs Inc. Hamburg, Germany
Anti-rabbit IgG-HRP	Jackson immune research Labs Inc. Hamburg, Germany

3.1.7 FACS antibodies

Rat-anti- mouse-PE-Gr-1	(Becton Dickinson, Heidelberg, Germany)
Rat-anti- mouse-PE-Mac-1	(Becton Dickinson, Heidelberg, Germany)
Rat-anti- mouse-PE-B220	(Becton Dickinson, Heidelberg, Germany)
Mouse-anti-PE-IgG $_{2a,}$ *K*	(Becton Dickinson, Heidelberg, Germany)
Mouse-anti-PE-IgG $_{2b,}$ *K*	(Becton Dickinson, Heidelberg, Germany)

Rat –anti-Mouse-PE-Sca-1	(Becton Dickinson, Heidelberg, Germany)
Rat-anti- mouse-PE-kit	(Becton Dickinson, Heidelberg, Germany)
Rat-anti- mouse-PE-CD3e	(Becton Dickinson, Heidelberg, Germany)
Rat-anti- mouse-PE-CD4	(Becton Dickinson, Heidelberg, Germany)

3.1.8 Cell culture media and reagents

BSA solution	Sigma, Steinheim, Germany
Chloroquine	Sigma, Steinheim, Germany
DMEM, -High glucose (4.5g/l)	Invitrogen, Karlsruhe, Germany
DMSO	Sigma, Steinheim, Germany
FBS	Gibco BRL, Paisley, Schottland
Ficoll separating solution	Biochrom, Berlin, Germany
HBSS	Gibco, Karlsruhe, Germany
HEPES solution	Gibco, Karlsruhe, Germany
Imatinib	Novartis, Basel, Switzerland
PKC412	Novartis, Basel, Switzerland
IMDM	Gibco, Karlsruhe, Germany
L-Glutamine	Gibco, Karlsruhe, Germany
Methylcellulose (M3434, M3231)	StemCell Technologies, Vancouver, Canada
PBS	Gibco, Karlsruhe, Germany
Penicillin-Streptomycin solution	Gibco, Karlsruhe, Germany

Retronectin	Takara, Shiga, Japan
RPMI 1640	Gibco, Karlsruhe, Germany
Trypan blue stain (0.4%)	Gibco, Karlsruhe, Germany
Trypsin-EDTA, 0.25% solution	Gibco, Karlsruhe, Germany
β-mercapto-ethanol	Fluka , Deisenhofen, Germany

3.1.9 Bacteria

E. coli (DH5α, BL21, JM83, DB 3.1)	Invitrogen, Karlsruhe, Germany

3.1.10 Cell lines

32D cells	IL-3 dependent mouse (C3H/HeJ strain) bone marrow progenitor cell line
Ba/F3 cells	IL-3 dependent mouse (Balb/c strain) pro-B cell line, established from peripheral blood
Platinum-E cells	293T cell based ecotropic retroviral packaging cell line
SC1 cells	Mouse fibroblast cell line
MV4-11 cells	FLT3-ITD positive acute monocytic leukemia (AML-M5) cell line established from a patient
MOLM-13 cells	FLT3-ITD positive acute myeloid leukemia (AML-M5a) cell line established from a patient
K-562 cells	BCR/ABL positive cell line established from a chronic myeloid leukemia (CML-blast crisis) patient

Sup-B15 cells	BCR/ABL positive cell line established from bone marrow of an acute lymphoblastic leukemia (precursor B cell-ALL) patient

3.1.11 Long-term cultured primary lymphoblastic leukemia cells from patients

3.1.11.1 Ph^+ Patients (Philadelphia chromosome positive):

BV, CM, WD, KW, PH and VB (these are the patient initials) (Nijmeijer et al., 2009).

3.1.11.2 Ph^- Patients (Philadelphia chromosome negative):

VG, KR, RL, SK, CR and HP (these are the patient initials) (Nijmeijer et al., 2009).

3.1.12 Mice

Female Balb/c mice (aged between 4-8 weeks) were purchased from Charles River Laboratories GmbH in Munich, Germany.

3.1.13 Patient samples

Samples were collected from patients enrolled in a treatment optimization trial in Germany. Control total bone marrow samples were obtained from healthy donors. Written informed consent was obtained from all individuals. The ethics committee of the Medical Faculty of the University of Muenster approved the use of patient samples.

3.2 Methods

3.2.1 Cell culture

32D, Ba/F3 cell lines were cultured in RPMI1640 medium supplemented with 10% FCS, 10%WEHI conditioned medium (IL-3 source) and 1% Penicillin and streptomycin.
MV4-11, MOLM-13, K562 and Sup-B15 cell lines were cultured in RPMI1640 medium supplemented with 10% FCS and 1% Penicillin and streptomycin.

Platinum-E and SC-1 cell lines were cultured in DMEM medium supplemented with 10% FCS and 1% Penicillin and streptomycin.

BV, CM, WD, KW, PH, VB, VG, KR, RL, SK, CR and HP cells were cultured in IMDM medium supplemented with 1 µg/mL bovine insulin, 0.5 µM β-mercapto-ethanol, 200 µg/mL Fe3+-saturated human apo–transferrin, 0.6% human serum albumin, 2.0 mM L–glutamine and 20 µg/mL cholesterol as described previously (Nijmeijer et al., 2009).

3.2.2 RNA preparation, quantitative PCR (polymerase chain reaction)

The TRIzol method was used for RNA isolation as described by the manufacturer (Invitrogen). RNA was accurately quantified using spectrophotometer. Equal amounts of RNA were used for making cDNA according to the protocol provided by manufacturer (Fermentas). Prior to cDNA synthesis, all RNA samples were treated with DNAse to prevent genomic DNA contamination. Real time quantitative PCR is done using probe master mix from Fermentas. Endogenous control assay kits from Eurogentech for GAPDH, 18S rRNA, RNA polymerase IIa, beta Actin were used for normalization of the expression. A list of all the primers and probes used is given below. Unless stated otherwise, all the probes used in the study were 5'-FAM and 3'BHQ1 labelled.

Murine CIS:

Probe	-	TCTATTACAGCCAGCGAGGCCCGG
Primer Forward (FW)	-	CGGGAATCTGGGTGGTACTG
Primer Reverse (REV)	-	ACCCTCCGGCATCTTCTGTA

Murine SOCS1:

Probe	-	TGGCGCGCATCCCTCTTAACCC

Primer (FW)	-	CCGTGGGTCGCGAGAAC
Primer (REV)	-	AAGGAACTCAGGTAGTCACGGAGTAC
Murine SOCS2:		
Probe	-	CAGGCCCAGAAGCCCCACGG
Primer (FW)	-	TCCAGATGTGCAAGGATAAACG
Primer (REV)	-	GGTTTGGTCAGGTACAGGTGAAC
Murine SOCS3:		
Probe	-	ACTGTCAACGGCCACCTGGACTCCT
Primer (FW)	-	CCACCCTCCAGCATCTTTGT
Primer (REV)	-	CAGGCAGCTGGGTCACTTTC
Murine SOCS4:		
Probe	-	CACTCCCTTAATCCGGACGTTCCCC
Primer (FW)	-	TGTTCTTTGAGCCGCTCTTGT
Primer (REV)	-	TGCAAATATGCTGCAAGGAAA
Murine SOCS5:		
Probe	-	CTGTGCCGCAGAGATCCCTCAAGTG
Primer (FW)	-	TTTCCAGGCGGAACCAAA
Primer (REV)	-	TGTCTTTCTCGATGCTGATTTCA
Murine SOCS6:		
Probe	-	TGGCCTCTCGGGCCCAGACTT
Primer (FW)	-	AACCCAATCCAAAGGAACTTCA
Primer (REV)	-	CGAACACTTTCGGCCATGT
Murine SOCS7:		
Probe	-	ACCCCACGCCCCAGATGCGT
Primer (FW)	-	TTGCAGTCTTTCCCCCTACCT
Primer (REV)	-	ACGCTCTGATGGGAGCAATT
Human CIS:		
Probe	-	TCTGCTGTGCATAGCCAAGACCTTCTCC
Primer (FW)	-	CAGACAGAGAGTGAGCCAAAGGT
Primer (REV)	-	ACCCCAATACCAGCCAGATTC

Human SOCS1:		
Probe	-	CTTAGCGTGAAGATGGCCTCGGGA
Primer (FW)	-	CCAGCGGAACTGCTTTTTC
Primer (REV)	-	GCTGCCATCCAGGTGAAAG
Human SOCS2:		
Probe	-	ACCAAACCGCTCTACACGTCAGCACC
Primer (FW)	-	GAACGGCACTGTTCACCTTTATC
Primer (REV)	-	GCCTACAGAGATGCTGCAGAGA
Human SOCS3:		
Probe	-	CCTTCAGCTCCAAGAGCGAGTACCAGC
Primer (FW)	-	CCAGCCTGCGCCTCAA
Primer (REV)	-	CTTGCGCACTGCGTTCAC
Human SOCS4:		
Probe	-	TTCCAGAAAAAGAAACAAACCCAAA TGGGA
Primer (FW)	-	GAAGATAGTGATATGGATTCCGATGA
Primer (REV)	-	TGTTTCCAACTGCAGGATTTCA
Human SOCS5:		
Probe	-	ACTACCTCTTCTCTGTGAGCTTCCGCCG
Primer (FW)	-	CAGGGACTCTGCGCAAGAG
Primer (REV)	-	CGGGCATGCAGGGATCT
Human SOCS6:		
Probe	-	CGTGCCAGGAGCAAGCCAATTCA
Primer (FW)	-	TCCACGACCTCCAGTCTGAGA
Primer (REV)	-	ATTATGAGAAGCCGAGCTCTTCA
Human SOCS7:		
Probe	-	AGCACCTTTGCAGATTCCGGATACGA
Primer (FW)	-	CCCGATTCAGCAATGTCAAA
Primer (REV)	-	AGAGGTTTAGGCAGTGGGAGATC

3.2.3 Plasmids and Cloning

pMY-IRES-GFP

A MMLV/MESV based retroviral vector was a kind gift from T. Kitamura and is very useful in producing high titer retrovirus.(Kitamura et al., 2003).

pMY-SOCS1-IRES-GFP

Murine SOCS1 cDNA is cloned into pMY-IRES-GFP BamHI and KpnI sites.

pMY-FLT3-ITD-IRES-GFP

Human FLT3-ITD cDNA is cloned into pMY-IRES-GFP in NotI site.

pMY-SOCS1-T2A-FLT3-ITD-IRES-GFP

SOCS1-T2A PCR product with 5' SacII and 3' Cla1 was directly cloned into pMY-FLT3-ITD-IRES-GFP in frame to generate pMY SOCS1-T2A-FLT3-ITD-IRES-GFP. T2A is a self-cleaving peptide sequence that enables the cleavage of SOCS1 and FLT3-ITD by a ribosomal skip mechanism without affecting the translation either proteins (Szymczak et al., 2004).

pMY-SOCS1-FLAG-IRES-GFP

Quick-change mutagenesis was performed using the primers (sequences are given below) to introduce FLAG tag, STOP codon and a Not1 site on C-terminus of SOCS1. KpnI and NotI released fragment of SOCS1-FLAG is cloned into pENTR1 vector. pENTR1A SOCS1-FLAG was digested with BamH1, Not1 and then finally ligated into pMY-IRES-GFP.

Flag-SOCS1 STOP Not I forward primer:
CCTTCCCCTTCCAGATCTGAGCGGCCGCGAGGGCAGAGG

Flag-SOCS1 STOP Not I forward primer:
CCTCTGCCCTCGCGGCCGCTCAGATCTGGAAGGGGAAGG

pMY-BCR/ABL-HA-IRES-GFP

Oligos for HA sequence with restriction sites, SacII on 5' and FseI site on the 3' end were annealed and ligated inframe with BCR/ABL into pENTR-BCR/ABL vector (kind gift from Martin Ruthardt's group) digested with SacII and FseI enzymes. BCR/ABL-HA fragment is released by digesting with EcoRI and ligated into pMY-

IRES-GFP (that is digested EcoR1 and 5' dephosphorylated with calf intestinal alkaline phosphatase).

SacII HA FseI forward primer:

GGATCTGATGCCGCGGATTATGGCATACCCATACGATGTTCCAGATTACGCGG CCGGCCTGGCGTGCGA

SacII HA FseI reverse primer:

TCGCACGCCAGGCCGGCCGCGTAATCTGGAACATCGTATGGGTATGCCATAA TCCGCGGCATCAGATCC

pMY-SOCS1-FLAG-T2A-BCR/ABL-HA-IRES-GFP

SOCS1-FLAG-T2A with SacII site on 5' side and FseI site on 3' side was PCR amplified and cloned in-frame into SacII and FseI linearized pENTR-BCR/ABL-HA to create pENTR-SOCS1-FLAG-T2A-BCR/ABL-HA-IRES-GFP. Finally, SOCS1-FLAG-T2A-BCR/ABL-HA is released using EcoRI site and ligated into pMY-IRES-GFP vector (that is digested EcoR1 and 5' dephosphorylated with calf intestinal alkaline phosphatase), generating pMY-SOCS1-FLAG-T2A-BCR/ABL-HA-IRES-GFP.

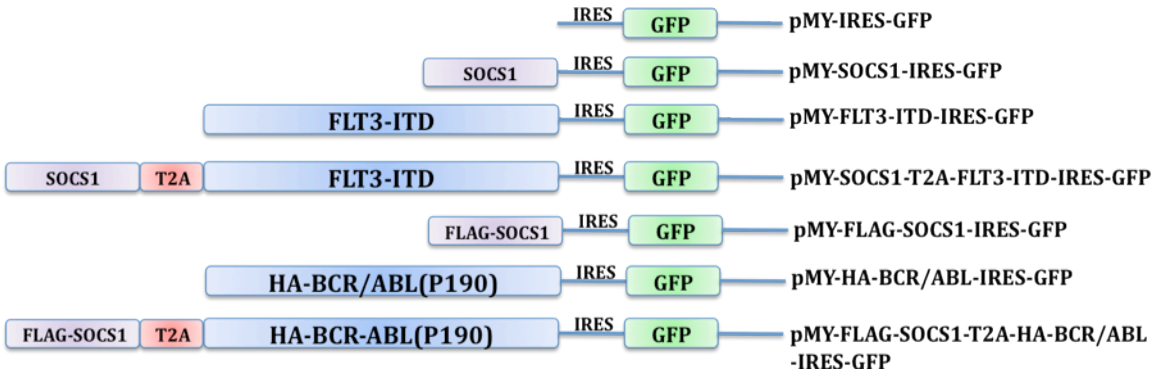

Figure 7: Schematic depiction of various plasmid vectors used in the study.

3.2.4 Generation of stable 32D and Ba/F3 cell lines

32D and Ba/F3 cells were transduced with pMY-empty (GFP control), FLT3-ITD, SOCS1-T2A-FLT3-ITD retroviral supernatants in the presence of polybrene three times a day for two consecutive days. Then cells were sorted for GFP. Sorted bulk-cultures were used for analyzing proliferation assay and signaling. These cells were also used to study Interferon mediated STAT1 activation.

3.2.5 3[H]-thymidine incorporation assay

A total of 2 x 10^4 32D cells were starved from IL-3 and incubated in medium containing 0.5% FCS for 12 hours in 200µL medium in a 96-well plate. Subsequently, cells were placed in medium with 10% FCS, supplemented with the indicated concentrations of 100 nM PKC412, 2 ng/mL IL-3, 1000 U/mL IFNα and 100 ng/mL IFNγ. The PKC412, wherever used, was added 1 hour prior to addition of growth factors. Addition of radio-labeled 3(H)-thymidine and measurement of incorporation was done as previously described (Choudhary et al., 2005). Each data point represents the mean and the SD of 3 independent experiments and each experiment is the average of quadruplicates of every condition.

3.2.6 Competitive proliferation assay by FACS

32D cells were tranduced with control (GFP), SOCS1, FLT3-ITD and SOCS1-T2A-FLT3-ITD retrovirus. These mixed population of transduced (GFP) and un-transduced cells (no GFP) were either cultured in the presence or absence of IL-3. Selective advantage or disadvantage of transduced cells was monitored by measuring the percentage of GFP positivity by FACS.

3.2.7 Stimulation of cells

Stable cell lines (mentioned above) were washed three times with PBS and starved overnight in RPMI medium containing 0.5% serum. The next morning cells were either stimulated for 15 minutes with IFNα (1000 U/mL) or with IFNγ, (100 ng/mL) or left unstimulated. Protein lysates were made as described below.

3.2.8 Western blotting

Cells were harvested and washed once with ice-cold PBS and lysed with buffer containing 50 mM HEPES (N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)), 10% glycerol, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM EGTA (ethylene glycol tetra acetic acid), 50 µM ZnCl2, 25 mM NaF, proteinase inhibitors, 1 µM pepstatin, and 1 mM sodium orthovanadate. After incubation on ice for 30 minutes, cell lysates were centrifuged at 14000 rpm for 10 minutes at 4°C. Equal amounts of protein was resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon P membranes (Millipore, Bedford, MA) and probed with

the indicated antibodies. Immunodetection was done using luminol reagent from Santa Cruz Biotechnology.

3.2.9 Flow cytometry

Single-cell suspensions of peripheral blood or tissue, bone marrow samples were prepared and red blood cells were lysed prior to analysis. Nucleated cells were pre-incubated with Fc-block and subsequently stained with phycoerythrin (PE)-conjugated anti-CD19, anti-B220, anti-Gr-1, anti-CD117 (KIT) antibodies and analyzed on FACS Scan.

3.2.10 Primary murine bone marrow

Balb/c mice were injected with 150 µg of 5-flurouracil / gram body weight of the mouse and after 3 days mice were sacrificed and bone marrow were harvested. Cells were pre-stimulated overnight in Iscove modified Dulbecco medium (IMDM medium) supplemented with 20% FCS with mSCF (50 ng/ml), mIL-3 (10 ng/ml), hIL-6 (10 ng/ml) and 100µM β-mercapto-ethanol.

3.2.11 Retroviral supernatants and transduction of murine bone marrow

All retroviral vectors (pMY empty, pMY mSOCS1, pMY FLT3-ITD, pMY SOCS1-T2A-FLT3-ITD were transfected into the Plat-E packaging cell line with lipofectamine and Plus reagent (Invitrogen) according to manufacturer instructions. Supernatants were collected after 36 hours for every 12 hours until 60 hours. The retroviral supernatants were tested for titer on SC1 cells. Retroviral supernatants with equal titers were coated on RetroNectin pre-coated 6-well plates according to manufacturer instructions. The bone marrow cells were seeded overnight in equal numbers in corresponding wells and transferred to freshly coated wells for 2 consecutive days. The cells were FACS-analyzed for GFP expression.

3.2.12 Colony assay of murine bone marrow cells

Retrovirally transduced bone marrow was sorted for GFP. These GFP+ cells were washed three times with PBS and 2000 cells were plated in 1 mL of methylcellulose per dish in triplicates. Methylcellulose was either supplemented with IFNγ (100 ng/mL) (Methocult #3231) or contained 50 ng/mL of mSCF, 10 ng/mL of mIL-3 and 10 ng/mL of hIL-6 (Methocult #3534). Colonies were counted after 12 days and equal number of cells (2000 cells/ml) from colony assay were serially replated.

3.2.13 Transplantation of murine bone marrow and assessment of mice

The bone marrow was resuspended in PBS and 2 x 10^4 GFP-positive cells (at least 1.5X 10^5 propidium iodide negative cells in total) were injected into the tail veins of lethally irradiated (8 Gy) female Balb/c recipient mice. In total, the groups were pMY (empty vector, n = 7), pMY SOCS1 (n = 10), pMY FLT3-ITD (n = 6) and pMY SOCS1-T2A-FLT3-ITD (n = 10). Blood counts of the peripheral blood of transplanted animals were monitored on day 10, 20 and 30 on a Hemavet 950 (DREW Scientific, Oxford, Connecticut, USA) and by FACS analysis for GFP expression. All moribund mice were sacrificed and peripheral blood, bone marrow and spleen cells were analyzed for GFP and for CD19, CD117 and Gr-1 surface markers. At the end of the experiment (day 120) all remaining mice were sacrificed and analyzed. The experimental protocols were reviewed and approved by the local Committee on Animal experimentation.

3.2.14 Cytospin preparations and Wright Giemsa staining

Cytospins of single cell suspensions were performed by resuspending cells in PBS at a concentration of 2-6x10^5 cells per 200 µl. The cells were permanently fixed on glass slides by centrifugation at 500 rpm for 10 minutes and subsequently air-dried. Modified Wright Giemsa staining was performed by immersing the slides in May-Gruenwald solution stain for 5 minutes. This was followed by immersing the slides in 1:50 diluted Giemsa stain for 1 hour. Slides were dipped in water to remove excess stain between the two staining steps and after the staining procedure and air-dried. These slides were observed under the inverted light microscope (as described below).

3.2.15 Histology and Microscopy

Organs were formalin fixed, paraffin embedded and 4-6µm sections were stained with hematoxylin and eosin stain (H&E) for histopathologic analysis. The representative images were acquired an Axio Cam camera (Zeiss) and Axiovision 4.0 software (Zeiss) at room temperature with Zeiss upright microscope system (Zeiss, Thornwood, NY), the original magnification of 50X and 400X for spleen and 100X and 400X for liver and sternum bone marrow.

3.2.16 Software and Statistics

The CellQuest Version 3.1(f) and FACS Diva (BD Biosciences, Palo Alto, CA) were used for FACS experiments. Calculations of survival curve, t-tests were performed using GraphPad Prism version 5 (Graph Pad Software, San Diego California, USA). The ABI Prism 310 sequencing software (Applied Biosystems, Foster City, CA) was used for sequencing and analysis of sequences. Primers were designed using the Primer3 program, Whitehead Institute, Massachusetts Institute for Technology, MA. Quantification of relative mRNA expression was performed using SDS version 2.3 software (Applied Biosystems, Foster City, CA). Primers probes for quantitative real time PCR were designed using Primer express Version 3 (Applied Biosystems, Foster City, CA). Bone marrow/ blood smears and histological images were acquired and processed using Axiovision 4.0 software (Zeiss, Thornwood, NY). Microsoft office 2007, Endnote version X2, Adobe illustrator creative suite 4 were used to make figures, write thesis and manuscript.

4 Results

4.1 Role of SOCS1 in FLT3-ITD mediated leukemogenesis

4.2 Retroviral expression of FLT3-ITD in 32D, Ba/F3 and murine bone marrow induced SOCS gene expression

Previous reports have shown that FLT3-ITD activates STAT5 and induces its target genes like SOCS2 and SOCS3 expression in a microarray experiment (Mizuki et al., 2003). To confirm previous results and to further analyze the induction SOCS gene expression by FLT3-ITD, mRNA expression of SOCS family members in 32D, Ba/F3 and in primary murine bone marrow retrovirally expressing FLT3-ITD was comprehensively analyzed.

4.2.1 Ectopic expression FLT3-ITD induced CIS, SOCS1-2 expression in hematopoietic cell lines

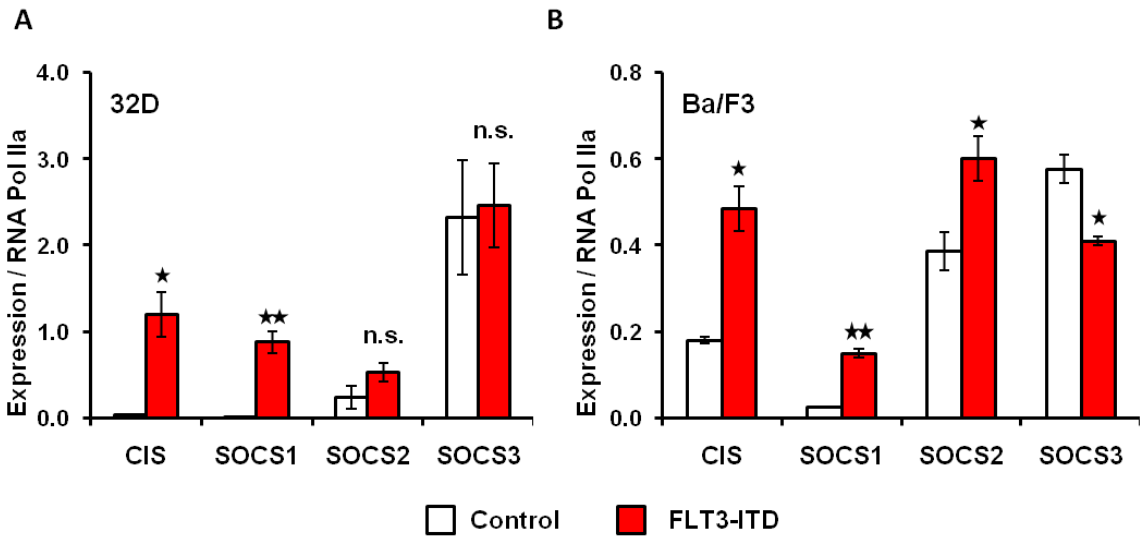

Figure 8: FLT3-ITD induced CIS, SOCS1-3 gene expression in 32D, Ba/F3 cell lines. 32D and Ba/F3 stable cell lines expressing either FLT3-ITD or an empty GFP vector (control) were starved overnight of IL-3 and FCS. The expression of CIS, SOCS1-3 transcripts is measured by quantitative PCR as describe in methods. (A) Normalized expression of CIS, SOCS1-3 in 32D-FLT3-ITD cell line and (B) Normalized expression of CIS, SOCS1-3 in Ba/F3-FLT3-ITD cell line. Standard deviations are presented as error bars. (* $p<0.05$, ** $p<0.01$)

32D and Ba/F3 cell lines were transduced with a pMY-FLT3-ITD-IRES-GFP or an empty vector (pMY-IRES-GFP). Retroviral vector -pMY in combination with platinum-E cells has been shown to produce high titer virus and the IRES driven GFP expression enabled tracking and purification of transduced cells (Kitamura et al., 2003; Morita et al., 2000) In all experiments, GFP expressing empty vector transduced cells were used as controls. Cells were washed of IL-3 and were

cultured overnight in 0.1% FCS, RNA was extracted and relative expression of CIS, SOCS1, SOCS2 and SOCS3 was measured by real-time PCR. In both 32D and Ba/F3 cell lines, FLT3-ITD induced high expression of CIS, SOCS1 and SOCS2 compared controls (Figure 8A and 8B). Less SOCS3 expression is observed in Ba/F3-FLT3-ITD cells than the control Ba/F3 cells (Figure 8B). These results indicate that FLT3-ITD leads to the expression of SOCS genes in myeloid and lymphoid cell lines, confirming our previous microarray results.

4.2.2 SOCS1 expression was highly induced by FLT3-ITD in murine bone marrow

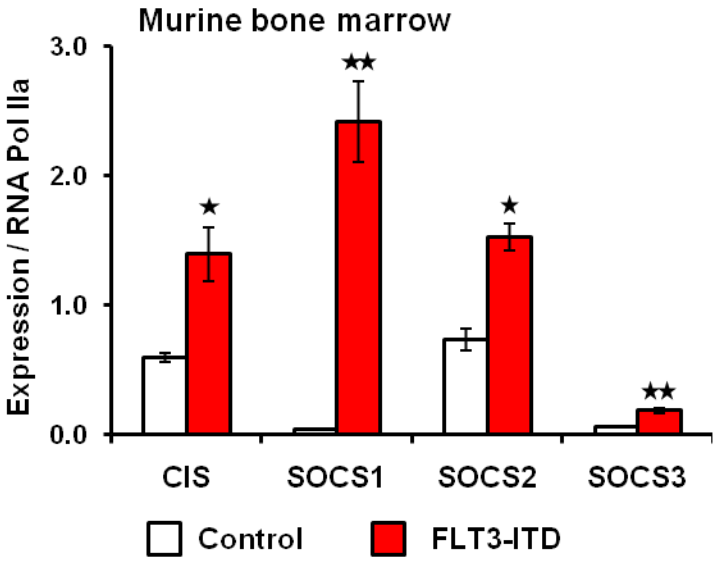

Figure 9: Retroviral expression of FLT3-ITD in murine bone marrow stem/ progenitor cells induced very high expression of SOCS1 and moderate expression of CIS, SOCS2 and SOCS3. Murine bone marrow stem and progenitor cells were transduced with FLT3-ITD or an empty GFP vector (control) and sorted for GFP. Cells were starved overnight of cytokine cocktail. The expression of CIS, SOCS1-3 transcripts was measured by quantitative PCR as described in methods section. Normalized gene expression is plotted with standard deviations as error bars. (* $p<0.05$, ** $p<0.01$)

Next, SOCS gene expression by FLT3-ITD in primary murine bone marrow was analyzed. For this purpose, bone marrow enriched for stem / progenitor cells (5-FU treated) was transduced with either control or with FLT3-ITD and compared the relative mRNA expression of SOCS genes was compared by RT-PCR. As shown in Figure 9, FLT3-ITD expression led to very high induction of SOCS1 (59 fold) and a moderate induction of CIS (2.3 fold), SOCS2 (2 fold) and SOCS3 (3.2 fold) mRNA expression compared to control. Further substantiating these RT-PCR results, SOCS1 protein can be detected in primary murine bone marrow by FLT3-ITD

(Figure 9B). These results clearly demonstrate SOCS1 to be the most regulated SOCS gene by FLT3-ITD in bone marrow stem / progenitor cells.

4.3 Kinase dependent expression of SOCS genes in FLT3-ITD$^+$ AML derived cell lines

After SOCS gene expression in cell lines and in primary bone marrow stem cells by FLT3-ITD was observed, the requirement of FLT3-ITD kinase activity for the induction of SOCS genes were determined.

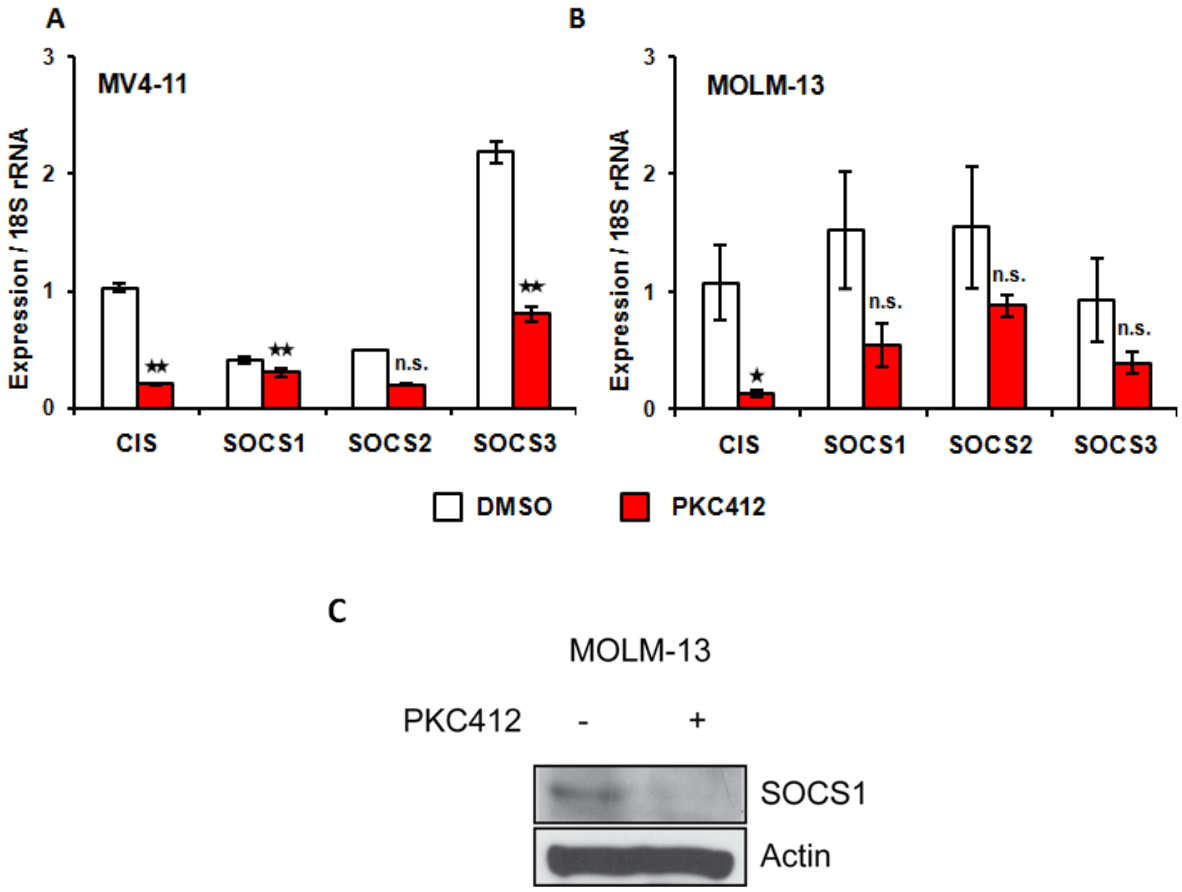

Figure 10: FLT3-ITD kinase activity is required for CIS, SOCS1-3 mRNA expression and SOCS1 protein expression in AML patient-derived cell lines.
Real time quantitative PCR analysis of CIS, SOCS1-3 mRNA expression in (A) MV4-11 and (B) MOLM-13 cell lines. MV4-11 and MOLM-13 cells were incubated overnight with the FLT3 kinase inhibitor PKC412 (100 ηM) or with solvent control (DMSO) as indicated. Normalized RNA expression is plotted with standard deviation as error bars. (C) Endogenous SOCS1 protein is decreased upon kinase inhibition. Western blot analysis of MOLM-13 cells either treated with PKC412 (100 ηM) or with DMSO. Actin blot served as protein loading control. (* $p<0.05$, ** $p<0.01$)

To test this, two AML-derived FLT3-ITD positive cell lines MV4-11 and MOLM-13 were cultured overnight with or without PKC412 (a FLT3 specific kinase inhibitor) and relative mRNA levels of SOCS members were measured. Both MV4-11 and

MOLM-13 cell lines showed decreased CIS, SOCS1, SOCS2 and SOCS3 expression upon FLT3 inhibition with PKC412 (Figure 10A and 10B). In agreement with RT-PCR results, in MOLM-13 cells, SOCS1 protein expression is decreased to an undetectable level upon PKC412 treatment (Figure 10C). In MV4-11 and in 32D cells over expressing FLT3-ITD, SOCS1 expression could not be detected on protein level (data not shown).

4.4 SOCS1 is highly expressed in FLT3-ITD$^+$ AML patient bone marrow

As SOCS1 expression is highly up-regulated by FLT3-ITD in primary murine bone marrow and in AML derived FLT3-ITD positive cell lines next, SOCS1 and SOCS3 expression in bone marrow samples from AML patients is determined. SOCS1, but not SOCS3 was significantly highly induced in FLT3-ITD positive AML patient bone marrow compared to CD34 positive healthy bone marrow samples (Figure 11A and 11B).

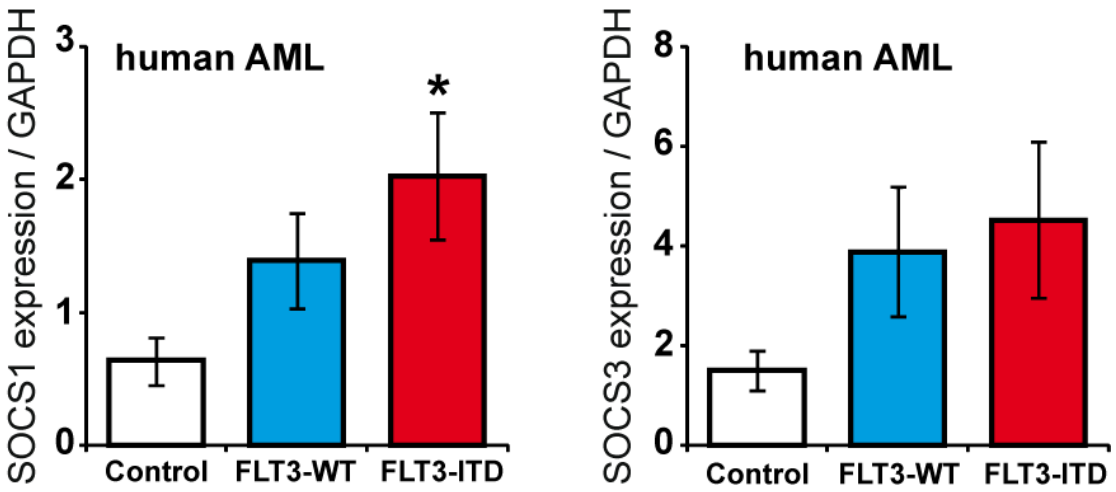

Figure 11: SOCS1 and SOCS3 mRNA is highly expressed in AML patient bone marrow. Bone marrow samples from a cohort of 77 AML patients (38 of FLT3-ITD and 39 FLT3-WT) and 7 healthy CD34+ donors were analyzed for the expression of (A) SOCS1 and (B) SOCS3. The normalized mRNA expression is plotted with standard errors as error bars. (* $p<0.05$)

Taken together, SOCS genes are induced by FLT3-ITD in a kinase dependent manner in cell lines and murine bone marrow. Particularly SOCS1 mRNA and protein were highly up-regulated by FLT3-ITD in murine bone marrow stem cells and finally, very importantly SOCS1 mRNA was expressed significantly high in FLT3-ITD positive AML-patient bone marrow.

4.5 Role of SOCS1 in FLT3-ITD mediated transformation *in vitro*

Various reports have shown that SOCS1 could block the oncogenicity of several hematopoietic specific oncogenes like the constitutively active form of the KIT receptor, TEL-JAK2, v-ABL, oncogenic form of VAV and partially BCR/ABL (De Sepulveda et al., 2000; Rottapel et al., 2002). Given the tumor suppressor nature of SOCS1, its role was systematically studied in FLT3-ITD mediated transformation *in vitro* and *in vivo.*

4.5.1 SOCS1 co-expression with FLT3-ITD

Given the tumor suppressor nature of SOCS1, it is expected that SOCS1 co-expression might block or reduce the oncogenicity of FLT3-ITD. As described in materials and methods, a retroviral vector with IRES driven GFP expression that simultaneously expresses SOCS1 and FLT3-ITD was made and it enables tracking and isolation of cells.

4.5.2 SOCS1 co-expression did not affect FLT3-ITD mediated signaling pathways

32D cells retrovirally transduced with SOCS1-T2A-FLT3-ITD indeed have SOCS1 and FLT3 protein expression. Completely cleaved, well separated FLT3 and SOCS1 as shown by western blot at their respective molecular weights and that amount FLT3 expression was equal to the expression of FLT3 in 32D cells expressing FLT3-ITD alone (Figure 12). Two bulk cultures of SOCS1-T2A-FLT3-ITD were used to avoid the possibility of clonal selection. SOCS1 co-expression does not affect the auto-phosphorylation of FLT3-ITD, PI3K/Akt, MAPK/ERK, and STAT5 pathways as evidenced by similar phosphorylation levels of FLT3, Akt, ERK and STAT5 compared to FLT3-ITD alone (Figure 12).

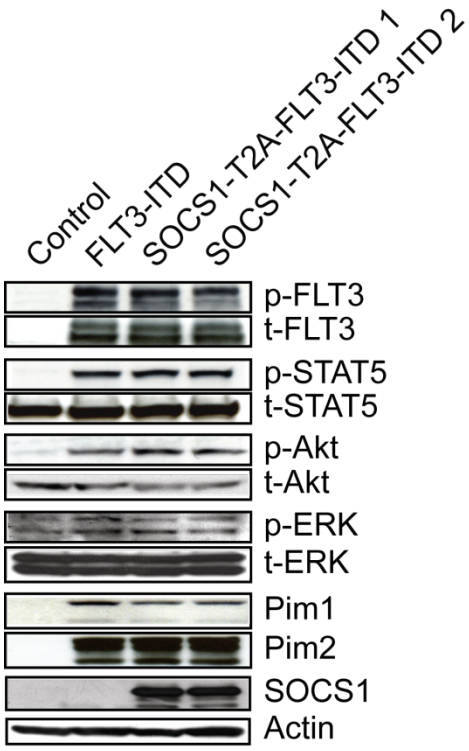

Figure 12: SOCS1 co-expression does not inhibit FLT3-ITD activated signaling. Western blot analysis of retrovirally transduced stable 32D cells expressing FLT3-ITD or SOCS1-T2A-FLT3-ITD or control (empty vector). Cells were washed 3 times with sterile PBS and cultured overnight in 0.5% FCS and lysates were made. Equal amounts of total cell lysates were resolved by SDS-PAGE and blotted on to PVDF membranes. These membranes were incubated with indicated phospho-specific antibodies that represent activation of a particular pathway. Subsequently, membranes were reprobed for the total antibodies and actin to ensure equal loading. Two independent bulk cultures (1 and 2) of SOCS1-T2A-FLT3-ITD were used to exclude clonal artifacts.

Equal levels of Pim1 and Pim2 proteins (STAT5 targets) in both FLT3-ITD and SOCS1-T2A-FLT3-ITD could also be observed. This experiment clearly indicates that SOCS1 could not affect any major FLT3-ITD mediated signaling pathways.

4.5.3 SOCS1 co-expression did not affect FLT3-ITD mediated proliferation but abrogated IL-3 mediated proliferation and protected from Interferon mediated growth inhibition

Next the functional consequences of SOCS1 co-expression on (i) FLT3-ITD mediated proliferation, (ii) IL-3 mediated proliferation, (iii) IFNα / IFNγ mediated growth inhibition, (iv) IFNα and IFNγ signaling were analyzed.

DNA synthesis was measured by 3[H]-thimidine incorporation in 32D cells expressing FLT3-ITD, SOCS1-T2A-FLT3-ITD and GFP control, in the presence or absence of IL-3, IFNα, IFNγ and PKC412 (a FLT3 specific tyrosine kinase inhibitor). As shown in Figure 13A, SOCS1 did not inhibit FLT3-ITD mediated DNA synthesis.

As expected, when FLT3-ITD kinase activity (PKC412) is inhibited, co-expression of SOCS1 severely impaired IL-3-induced proliferation of 32D cells (Figure 13A). Importantly, when cells were cultured with growth inhibitory cytokines (IFNα and IFNγ), SOCS1 co-expression led to increased proliferation (Figure 13A), indicating that SOCS1 expression could protect FLT3-ITD cells from growth inhibitory cytokines while not altering FLT3-ITD mediated proliferation.

In a competitive proliferation assay, retroviral transductions with GFP control, SOCS1, FLT3-ITD and SOCS1-T2A-FLT3-ITD were performed and compared the percentage of GFP-positive (i.e. transduced) versus GFP-negative (non-transduced) cells in the absence of IL-3 (Figure 13B) and in the presence of IL-3 (Figure 13C). In the absence of IL-3, the control and the SOCS1 transduced 32D cells rapidly died (undetectable GFP expression) (Figure 6B), 32D cells transduced with FLT3-ITD or SOCS1-T2A-FLT3-ITD grew out to a homogeneous GFP^+ population at a similar rate. This demonstrated that SOCS1 co-expression has no influence on FLT3-ITD induced cell proliferation (Figure 13B). In the presence of IL-3, 32D cells expressing SOCS1 rapidly died, leaving no detectable GFP+ cells (Figure 13C). In contrast, the percentage of GFP-positivity remained constant in GFP control, FLT3-ITD, SOCS1-T2A-FLT3-ITD transduced cells (Figure 13C). This implies that although SOCS1 expression inhibited IL-3 dependent proliferation of 32D cells, the FLT3-ITD dependent proliferation was resistant to SOCS1 inhibition.

Taken together these experiments in 32D cell line show that FLT3-ITD induced SOCS1 could not affect FLT3-ITD activated signaling and proliferation, while abolishing the IL-3 induced proliferation and protecting from Interferons (IFNα and IFNγ) mediated growth inhibition.

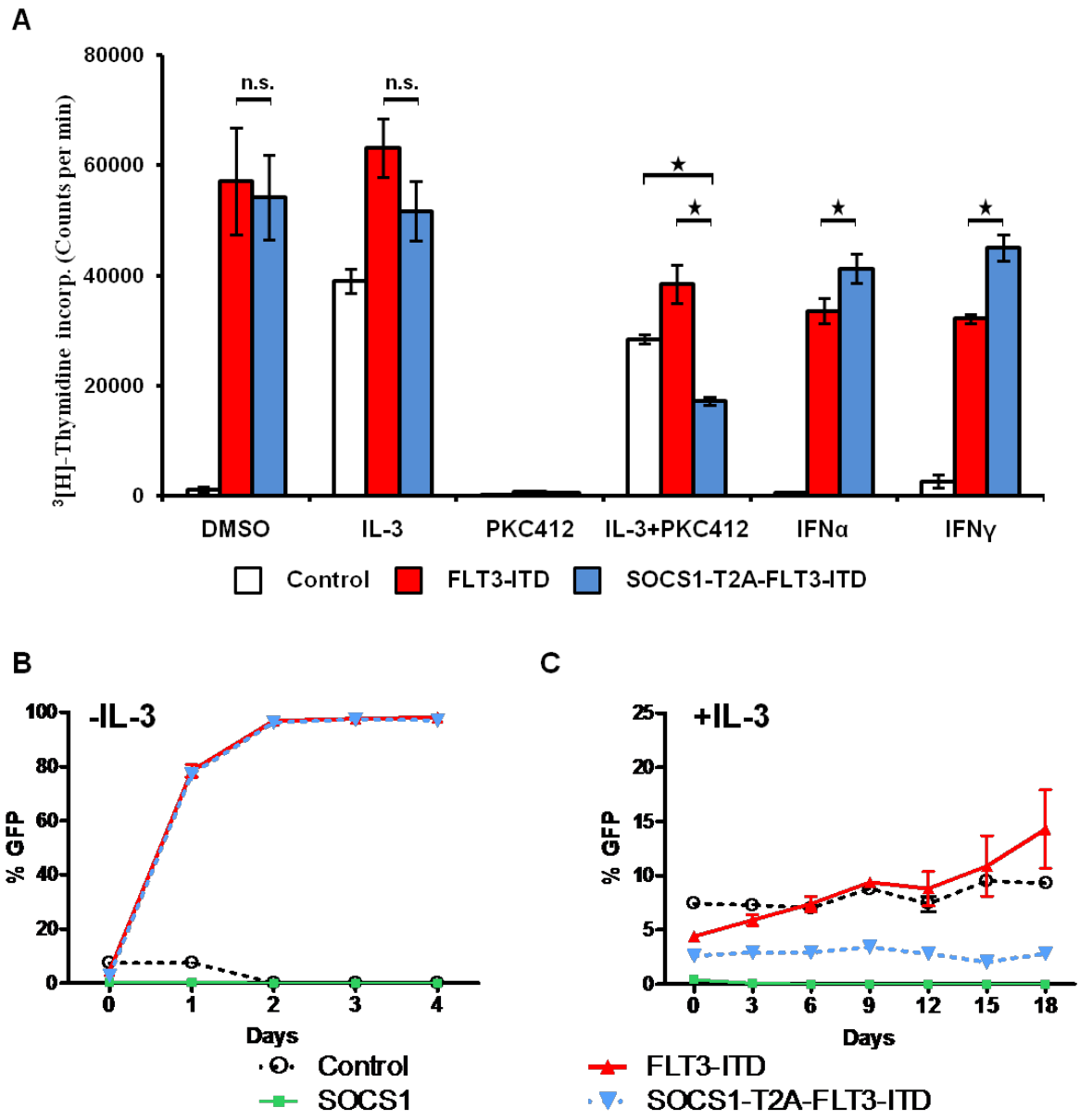

Figure 13: Biological consequences of SOCS1 co-expression with FLT3-ITD. (A) Proliferation assay by measurement of DNA synthesis using ^{3}[H]-thymidine incorporation. The stable 32D cells expressing the indicated constructs were analyzed for their proliferation by ^{3}[H]-thymidine incorporation as described in materials and methods. The data is plotted as a graph with the mean of quadruplicates and standard deviation as error bars. (B and C) Competitive proliferation assays of 32D cell lines expressing control (GFP), SOCS1, FLT3-ITD and SOCS1-T2A-FLT3-ITD. One day after retroviral transduction, 32D cells were washed 3 times with PBS and cultured without IL-3 (B) or with IL-3 (C) and the percentage of GFP is measured by FACS at 24 hours intervals. The percentage of GFP is plotted with standard deviation as error bars. (* $p<0.05$)

4.5.4 Interferon alpha and Interferon gamma induced STAT1 activation is diminished by FLT3-ITD

Implication of increased SOCS1 expression by FLT3-ITD on IFNα and IFNγ mediated STAT1 activation, which is a key mediator of anti-proliferative function downstream of IFNα and IFNγ receptors was studied. 32D cells expressing either

GFP control or FLT3-ITD or SOCS1-T2A-FLT3-ITD, were examined for STAT1 phosphorylation after IFNα or IFNγ stimulation. In response to IFNα and IFNγ, cells expressing FLT3-ITD showed less STAT1 phosphorylation compared to control, which was further diminished by SOCS1 co-expression (SOCS1-T2A-FLT3-ITD) (Figure 14).

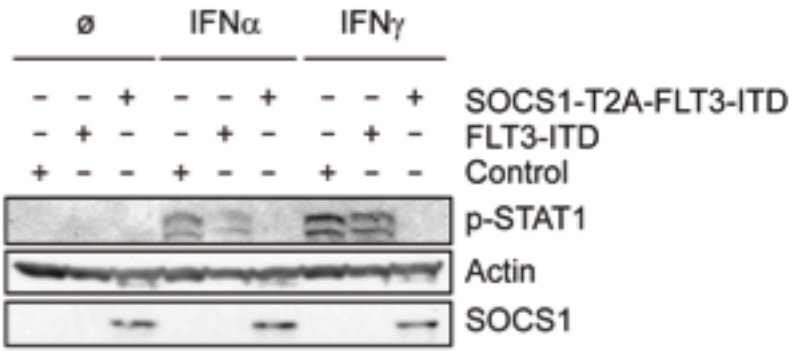

Figure 14: IFNα and IFNγ mediated STAT1 activation is diminished in 32D-FLT3-ITD cells, possibly due to FLT3-ITD induced SOCS1 expression. 32D cells expressing empty vector (control), FLT3-ITD or SOCS1-T2A-FLT3-ITD were either stimulated with IFNα (1000 U/mL) and IFNγ (100 ng/mL) for 10 minutes or left unstimulated. Equal amounts of protein is resolved on SDS-PAGE then blotted on to PVDF membrane. STAT1 activation is measured as indicated with phospho-specific STAT1 antibody, actin is the loading control.

4.5.5 SOCS1 co-expression with FLT3-ITD protected primary bone marrow from Interferon gamma

Experiments with 32D cells hinted that SOCS1 co-expression leads to inhibition of external cytokine cues, hence the implication of SOCS1 induction/co-expression, on growth inhibitory cytokines were analyzed in a more biologically meaningful setting (i.e. murine bone marrow stem and progenitor cells).

Stem and progenitor cell enriched (5-FU treated) bone marrow was retrovirally transduced with GFP control, SOCS1, FLT3-ITD, and SOCS1-T2A-FLT3-ITD then isolated for GFP by FACS. First, the relative expression of SOCS1 mRNA in the GFP sorted bone marrow stem and progenitors cells was checked. As shown in Figure 15A, a comparable amount of SOCS1 mRNA is expressed in both SOCS1 and FLT3-ITD transduced bone marrow stem progenitors. And SOCS1-T2A-FLT3-ITD had about 5-fold higher SOCS1 mRNA expression compared to FLT3-ITD (Figure 15A). Next, FLT3 and SOCS1 protein expression was analyzed in GFP sorted bone marrow progenitors. As shown in Figure 15B, amount of FLT3 protein

expression in both FLT3-ITD and SOCS1-T2A-FLT3-ITD was equal. FLT3-ITD induced SOCS1 protein (running at a slightly lower molecular weight) and retrovirally co-expressed SOCS1 protein (running at a slightly higher molecular weight, due to the extra amino acids from the T2A peptide cleave) could be detected by western blot. This indicates endogenous SOCS1 induced by FLT3-ITD is much less in amount compared to that of exogenously expressed SOCS1. Although the level of SOCS1 mRNA expression in FLT3-ITD transduced bone marrow and SOCS1 transduced bone marrow is equal, SOCS1 protein can be detected only in FLT3-ITD bone marrow by western blot.

The above-mentioned bone marrow cells were measured for their clonogenic growth. Colony forming assays were performed in methylcellulose containing pro-proliferative cytokines (IL-3, IL-6, SCF) and in anti-proliferative cytokine (IFNγ). As indicated in Figure 15C (left panel), colony growth was severely impaired in bone marrow expressing SOCS1 in methylcellulose containing IL-3, IL-6 and SCF, but this was reversed with FLT3-ITD co-expression, once again ascertaining FLT3-ITD resistance to inhibitory effects SOCS1 in bone marrow stem cells. Of note, both FLT3-ITD and SOCS-T2A-FLT3-ITD made comparable number of colonies.

However, in the presence of IFNγ, an anti-proliferative cytokine, SOCS1-T2A-FLT3-ITD made significantly higher numbers of colonies compared to FLT3-ITD (Figure 15C, right panel). This is because of the higher expression of exogenous SOCS1 in SOCS1-T2A-FLT3-ITD, which led to efficient protection from growth inhibitory function of IFNγ compared to the relative small amount of endogenous SOCS1, which is induced by FLT3-ITD. SOCS1 is known to inhibit IFNγ by inhibiting JAK kinases downstream of IFNγ receptor (Endo et al., 1997; Fenner et al., 2006). Neither GFP control nor SOCS1 could form colonies.

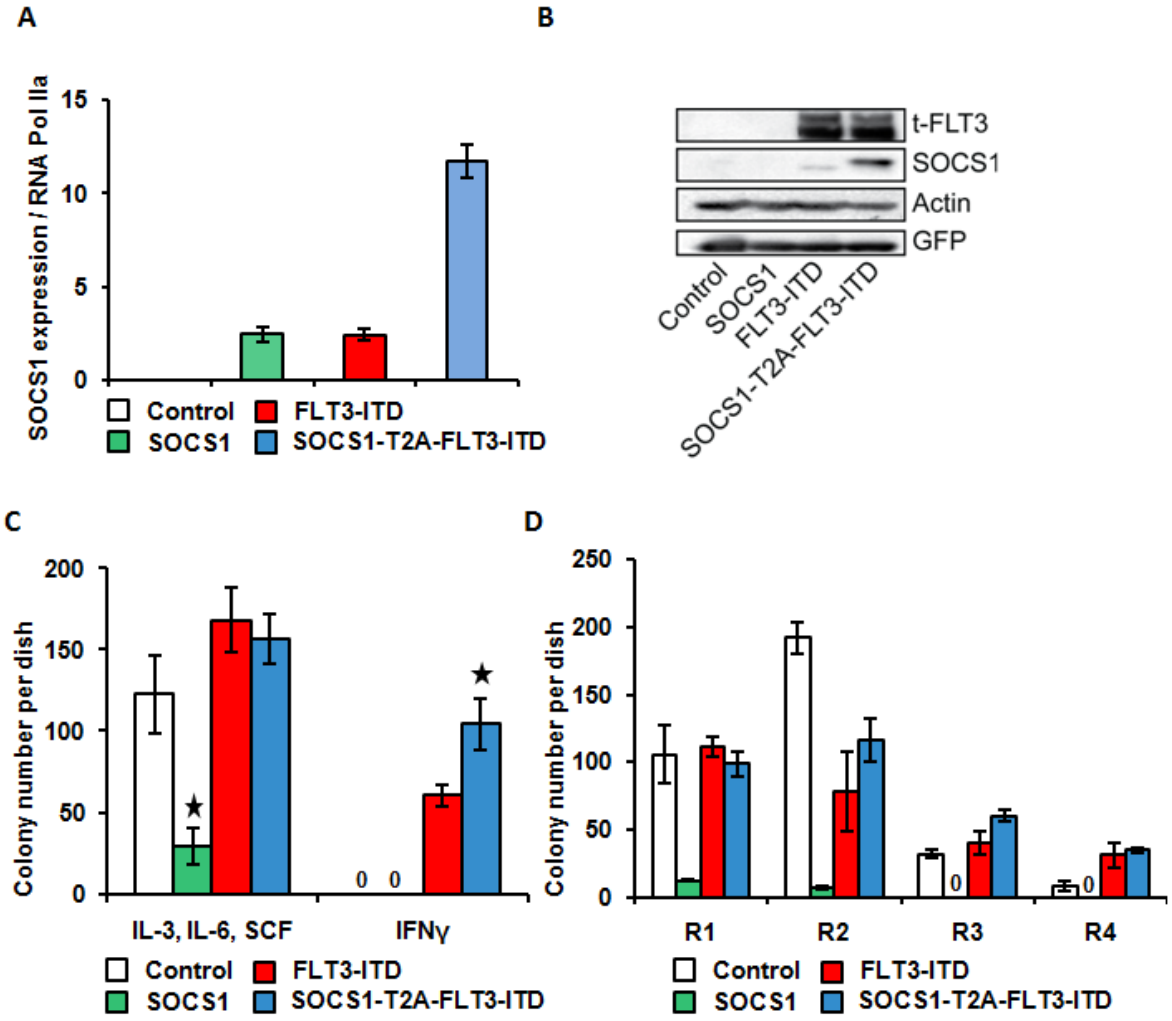

Figure 15: SOCS1 co-expression with FLT3-ITD in murine bone marrow stem/ progenitor cells conferred resistance to IFNγ inhibitory effects. Murine bone marrow Stem / progenitor cells were transduced with empty GFP vector (control), SOCS1, FLT3-ITD and SOCS1-T2A-FLT3-ITD and sorted for GFP. These cells were starved of cytokine cocktail and FCS overnight, then (A) SOCS1 mRNA expression and (B) FLT3, SOCS1 and GFP protein expression were measured by quantitative PCR and western blot respectively. Actin, GFP blots ensure equal protein loading. (C) Sorted GFP cells were washed three times with PBS and plated at a density of 2000 cells/mL in triplicates in either methylcellulose medium containing IL-3, SCF, IL-6 or in methylcellulose medium containing IFNγ. Colony numbers were counted after 12 days and mean colony number from triplicates was plotted with standard deviation as error bars. (D) Serial replating of colony assays in methylcellulose containing IL-3, SCF and IL-6. (* $p<0.05$)

Taken together these data show that FLT3-ITD induced SOCS1 expression in primary bone marrow protected cells from external growth inhibitory cytokines (IFNγ).

Although it was shown that FLT3-ITD does not increase replating activity of primary bone marrow (Li et al., 2008)), it was tested whether SOCS1 co-expression could alter this phenomenon. A similar replating efficiency of SOCS1-T2A-FLT3-ITD as

FLT3-ITD was observed, suggesting that SOCS1 does not influence the replating capacity of FLT3-ITD (Figure 15D).

4.6 Role of SOCS1 expression in FLT3-ITD induced leukemogenesis *in vivo*

FLT3-ITD has been shown to induce a myeloproliferative disease in a murine bone marrow transplantation model (Grundler et al., 2005; Kelly et al., 2002b) and either a myeloproliferative or lymphoid disease in a transgenic mouse model (Lee et al., 2005; Li et al., 2008). The role of SOCS1 in FLT3-ITD mediated leukemogenesis is studied in a murine bone marrow transplantation model.

4.6.1 SOCS1 co-expression enhanced FLT3-ITD bone marrow engraftment and proliferation

Bone marrow transduction and transplantations were done as described in the materials and methods section. 5-FU treated bone marrow stem and progenitor cells were transduced retrovirally with GFP control, SOCS1, FLT3-ITD and SOCS1-T2A-FLT3-ITD. The transduced bone marrow cells were transplantated into lethally irradiated recipient mice. After the transplantation, peripheral blood parameters of all animals were monitored for the engraftment and for the development of leukemic symptoms at a regular interval of 10-days. If mice fail to engraft the transplants they will die shortly after transplantation, but none of the animal were dead immediately after transplantation indicating successful bone marrow transplantation.

As shown in Figure 16A, the percentage of GFP expressing cells in the peripheral blood from control mice remained constant over a period of 30 days, while there were no detectable GFP expressing cells present in SOCS1 transplanted mice by 20 days. This clearly demonstrates a competitive growth disadvantage of SOCS1 transplants in the latter group. On the other hand, the percentage of GFP positive cells in FLT3-ITD or SOCS1-T2A-FLT3-ITD transplanted mice were significantly increased from day 10 to day 30, with an increase of 9.3% to 54% and 13.9% to 67.3% respectively ($p<0.05$, Figure 16A).

Similarly, WBC counts of FLT3-ITD mice (3.3×10^3/µL on day 10 to 4.3×10^4/µL on day 30) and SOCS1-T2A-FLT3-ITD mice (1.2×10^3/µL on day 10 to 4.8×10^4/µL on day 30) showed a significant increase compared to control mice ($p<0.05$, Figure 16B). Both the increase of GFP positive cells and WBC in the FLT3-ITD and the

SOCS1-T2A-FLT3-ITD mice indicate a competitive growth advantage of these cells. These mice also show signs of leukemia such as lower hemoglobin content and PLT count when compared to the control or SOCS1 (Figure 16C and 16D).

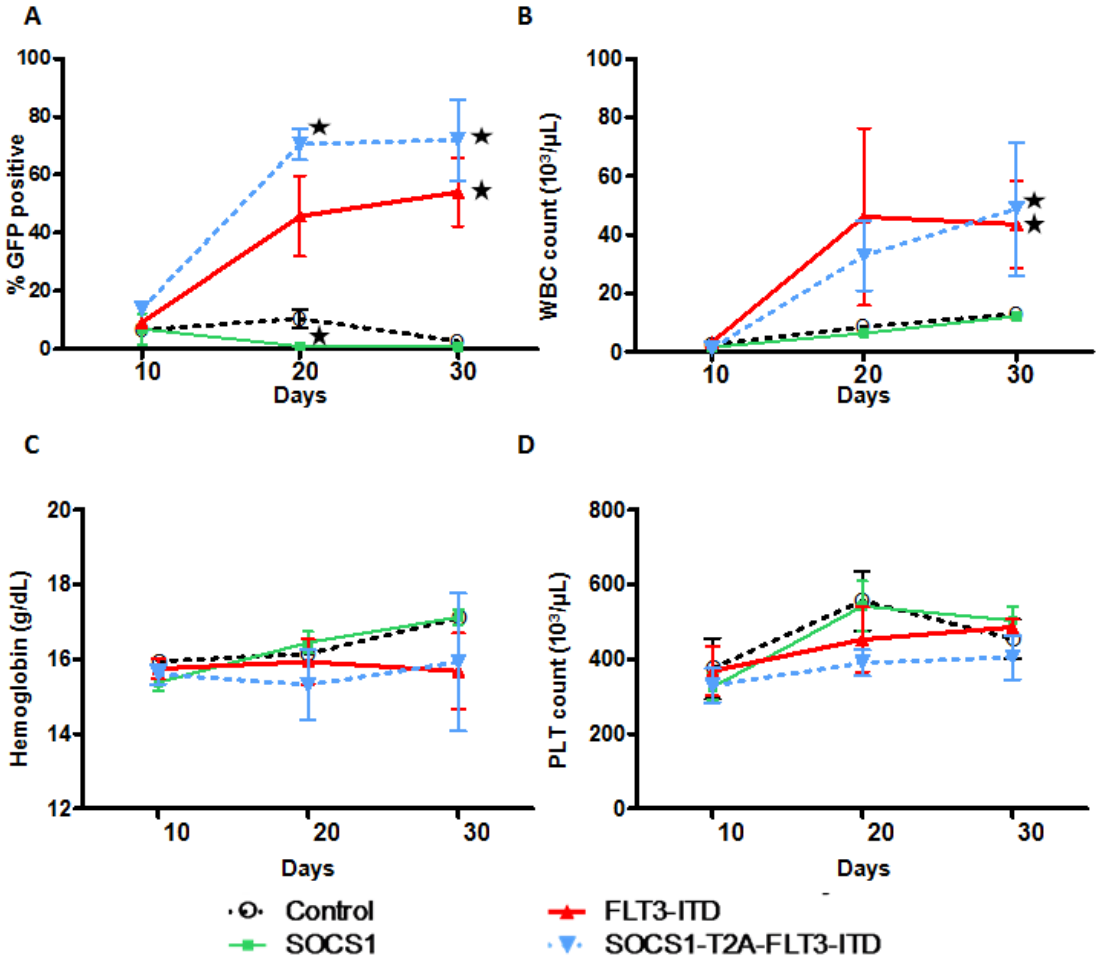

Figure 16: Post-transplantation follow-up of mice for engraftment and development of leukemia. Bone marrow transduction and transplantation was performed as described in methods section. All transplanted mice were bled at 10-day intervals. The peripheral blood cell counts (WBC, platelet, and hemoglobin content) were measured by Haemavet and GFP expression was measured by flow cytometry. The mean values from each different group of mice were plotted as (A) percentage GFP, (B) WBC count (x 1000/µL), (C) Hemoglobin content (g/dL) and (D) Platelets count (x 1000/µL) versus time in days of post transplantation.

In agreement with the post-transplantation follow-up, the percentage of GFP expressing cells in the control mice at the end of the experiment (day 120) was comparable to the percentage of GFP at day 10, and there was no detectable GFP in SOCS1 mice at day 120 (Table1). Similarly both FLT3-ITD and SOCS1-T2A-FLT3-ITD mice had high percentages of GFP+ cells and higher WBC counts at the time of death. Detailed analysis of peripheral blood from control, SOCS1 mice (after

120 days) and FLT3-ITD, SOCS1-T2A-FLT3-ITD mice (at the time of death) are given in Table 2.

4.6.2 SOCS1 co-expression accelerated the onset of FLT3-ITD induced myeloproliferative disease and acute lymphoblastic leukemia

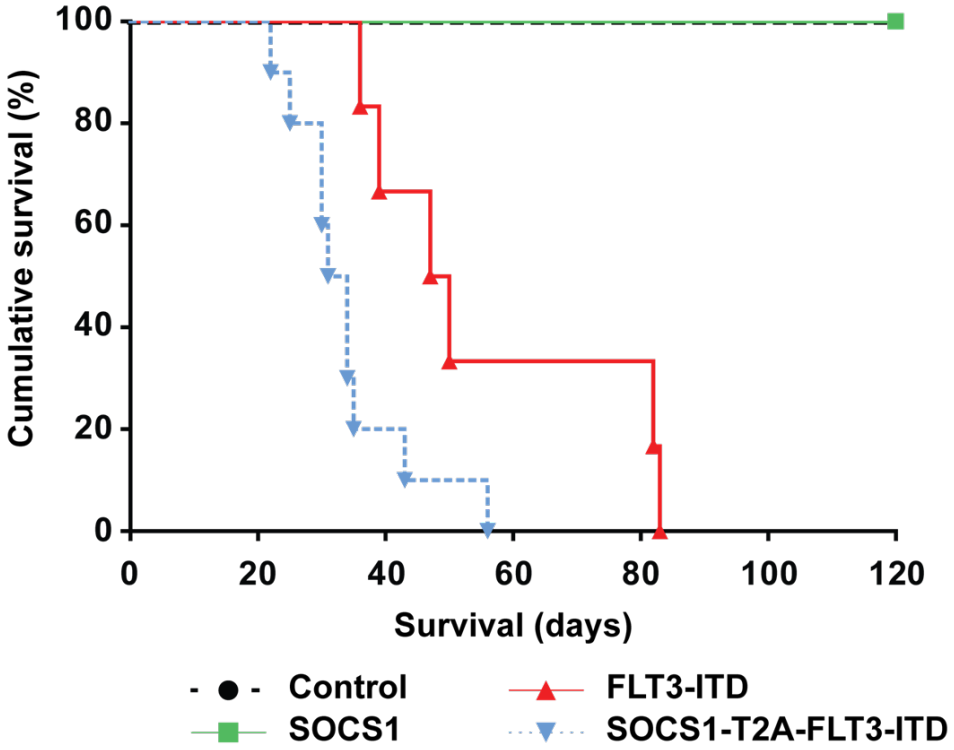

Figure 17: SOCS1 accelerates FLT3-ITD induced disease onset in murine bone marrow transplant model. Kaplan-Meyer plot displaying survival post transplantation, no mice received transplant of control or SOCS1 died during the experiment. The mean survival of mice transplanted with FLT3-ITD was 56d ± 21d; with SOCS1-T2A-FLT3-ITD was 34d ± 9.6d ($p = 0.01$). All animals from FLT3-ITD and SOCS1-T2A-FLT3-ITD died before the end of the experiment.

Importantly, none of control mice or SOCS1 mice developed a disease phenotype until the end of the experiment (i.e. 120 days). All the mice transplanted with FLT3-ITD and SOCS1-T2A-FLT3-ITD developed either a myeloproliferative disease (MPD) or an acute lymphoblastic leukemia (B-ALL) with 100% penetration. Mice which received a FLT3-ITD transplant died with a median survival of 48.5 days (range 36-83) and those which received a SOCS1-T2A-FLT3-ITD transplant died with a median survival of 32.5 days (range 22-56). Three of 6 FLT3-ITD mice developed MPD (72.5% of Gr1+ peripheral blood cells) and 3 out of 6 developed B-ALL (96.4% of CD19+ peripheral blood cells) (Table 2). Similarly, 5 out of 10

SOCS1-T2A-FLT3-ITD mice developed MPD (64% of Gr1+ peripheral blood cells) and 5 out of 10 developed B-ALL (85.4% CD19+ peripheral blood cells) (Table 2). Although SOCS1 co-expression did not alter the disease phenotype, it significantly accelerated the disease development (p = 0.01, Figure 17).

	No. mice (n)	Phenotype	WBC (10^3/ µL)	Hb (g/dL)	PLT (10^3/ µL)	% GFP$^+$	% CD19$^+$	% GR1$^+$
Control	7	none	6.9 (4.6-11.2)	16 (11.4-17.5)	499 (335-1203)	5 (0.9-6.1)	11.2 (7.4- 23.7)	22.1 (11.8-26.7)
SOCS1	10	none	6.2 (4.5-16.6)	14.9 (12.9-16.9)	256 (131-2612)	0.7 (0-2.2)	9.7 (0.5-21.6)	27.8 (19.3-40.1)
FLT3-ITD	6	3 MPD	36.8 (36.8-42.1)	12 (9.6-14.3)	574 (497-651)	48.1 (9.2-87.1)	3.4 (1.1-4.8)	72.5 (45.4-88.8)
		3 ALL	86.1 (47.5-86.1)	14 (11.0-16.6)	441 (282-466)	75 (39.3-87.8)	96.4 (23.0-97.4)	1.2 (0.2-54.8)
SOCS1-T2A-FLT3-ITD	10	5 MPD	34.5 (11.1-61.9)	14.6 (10.4-17.3)	321 (227-539)	73.6 (61.2-90.7)	5.9 (2.3-19.9)	64 (60.7-90.2)
		5 ALL	16.8 (3.4-76.0)	15.8 (13.7-17.0)	365 (312-463)	83.3 (21.6-97.6)	85.4 (77.2-93.6)	5 (3.1-7.1)

Table 2: Peripheral blood analysis of transplanted mice at the time of death.
Mice transplanted with bone marrow expressing FLT3-ITD or SOCS1-T2A-FLT3-ITD were sacrificed when moribund, while mice transplanted with bone marrow expressing control GFP or SOCS1 were sacrificed at the end of the experiment at day 120. Peripheral blood analysis included white blood cell count (WBC), hemoglobin content (Hb), platelets (PLT), the % of GFP expression and surface expression of lineage specific markers CD19 (immature B cell) and Gr1 (granulocytes). MPD: myeloproliferative disease; ALL: acute lymphoblastic leukemia. Values are presented as median (range).

	No. analyzed, Phenotype	Bone marrow				Splenocytes			
		% GFP$^+$	% CD19$^+$	% GR1$^+$	% CD117$^+$	% GFP$^+$	% CD19$^+$	% GR1$^+$	% CD117$^+$
Control	7, no phenotype	1.4 (0.7-6.7)	19.9 (7.7-27.2)	66.8 (41.6-82.1)	12.8 (4.0-22.6)	2.3 (0.8-11.4)	42.6 (23.6-53.0)	18.7 (8.8-24.2)	4.4 (1.1-9.1)
SOCS1	10, no phenotype	0.3 (0.0-1.4)	33.4 (12.1-43.1)	58.5 (40.6-85.3)	7.2 (0.3-18.5)	0.3 (0.0-1.4)	56.2 (24.1-61.4)	11.4 (1.4-25.9)	3.2 (0.2-4.3)
FLT3-ITD	2, MPD	34.5 (21.8-47.1)	27.2 (8.2-46.3)	53.8 (41.1-66.4)	6.2	53.1 (45.4-60.7)	13.6 (1.9-25.2)	50.2 (33.7-66.7)	7.4
	2, ALL	63.6 (36.6-90.6)	83 (69.7-96.2)	1.6 (1.3-1.8)	0.8 (0.2-1.5)	53.7 (26.1-81.2)	44.8 (13.3-76.3)	4.7 (2.4-6.9)	5.3 (3.1-7.5)
SOCS1-T2A-FLT3-ITD	1, MPD	22.6	5.1	56.2	2.5	47.8	2.6	39.0	2.6
	4, ALL	81.8 (43.8-96.0)	89.2 (74.6-97.2)	9.6 (6.7-19.3)	1.2 (0.2-3.8)	90 (83.0-93.4)	90.6 (87.4-92.1)	6.7 (5.4-7.5)	4 (2.5-6.0)

Table 3: Bone marrow and splenocyte analysis of transplanted mice at the time of death. Mice transplanted with bone marrow expressing FLT3-ITD or SOCS1-T2A-FLT3-ITD were sacrificed when moribund, while mice transplanted with bone marrow expressing control GFP or SOCS1 were sacrificed at the end of the experiment at day 120. Bone marrow and spleen cells were analyzed for GFP positivity to monitor infiltration of transplants and for surface expression of lineage specific markers CD19 (immature B cells), GR1 (granulocytes) CD117 (KIT receptor). MPD: myeloproliferative disease; ALL: acute lymphoblastic leukemia. Values are presented as median (range).

4.6.3 Spleen, liver and bone marrow infiltration in FLT3-ITD or SOCS1-T2A-FLT3-ITD transplanted mice

All moribund mice transplanted with either FLT3-ITD or SOCS1-T2A-FLT3-ITD showed enlarged spleens (Figure 18A). Spleen weights of FLT3-ITD and SOCS1-T2A-FLT3-ITD were significantly higher compared to control mice (Figure 18B). Similarly, a significant increase in liver weights in the SOCS1- T2A-FLT3-ITD mice compared to control mice was observed, whereas the FLT3-ITD mice had slightly enlarged livers which were not statistically significant (Figure 18C). FLT3-ITD and SOCS1-T2A-FLT3-ITD transplanted mice showed higher WBC or nucleated cells in their peripheral blood (blood smears), compared to SOCS1 and control mice (Figure 19A and Table 2).

Leukemia progression is marked by the infiltration of blasts in various organs and therefore sacrificed moribund mice were assessed for these signs by making histopathological sections of fixed organs and immunostaining followed by microscopy. Histological examination of spleens and livers from FLT3-ITD and SOCS1-T2A-FLT3-ITD demonstrate blast infiltrations and destruction of the natural

organ architecture (Figure 19B). Furthermore, there was a clear increase in bone marrow cellularity in both groups of mice compared to controls, with a loss of bone marrow adipocytes (Figure 19B). The detailed FACS analysis of spleen, bone marrow infiltrates is shown in Table 3. All the mice from both FLT3-ITD and SOCS1-T2A-FLT3-ITD, which suffered from MPD, exhibited spleen and bone marrow infiltrated with Gr1/GFP positive cells. Similarly those mice which suffered from B-ALL, had spleen and bone marrow infiltrated with CD19/GFP positive cells (Table 3).

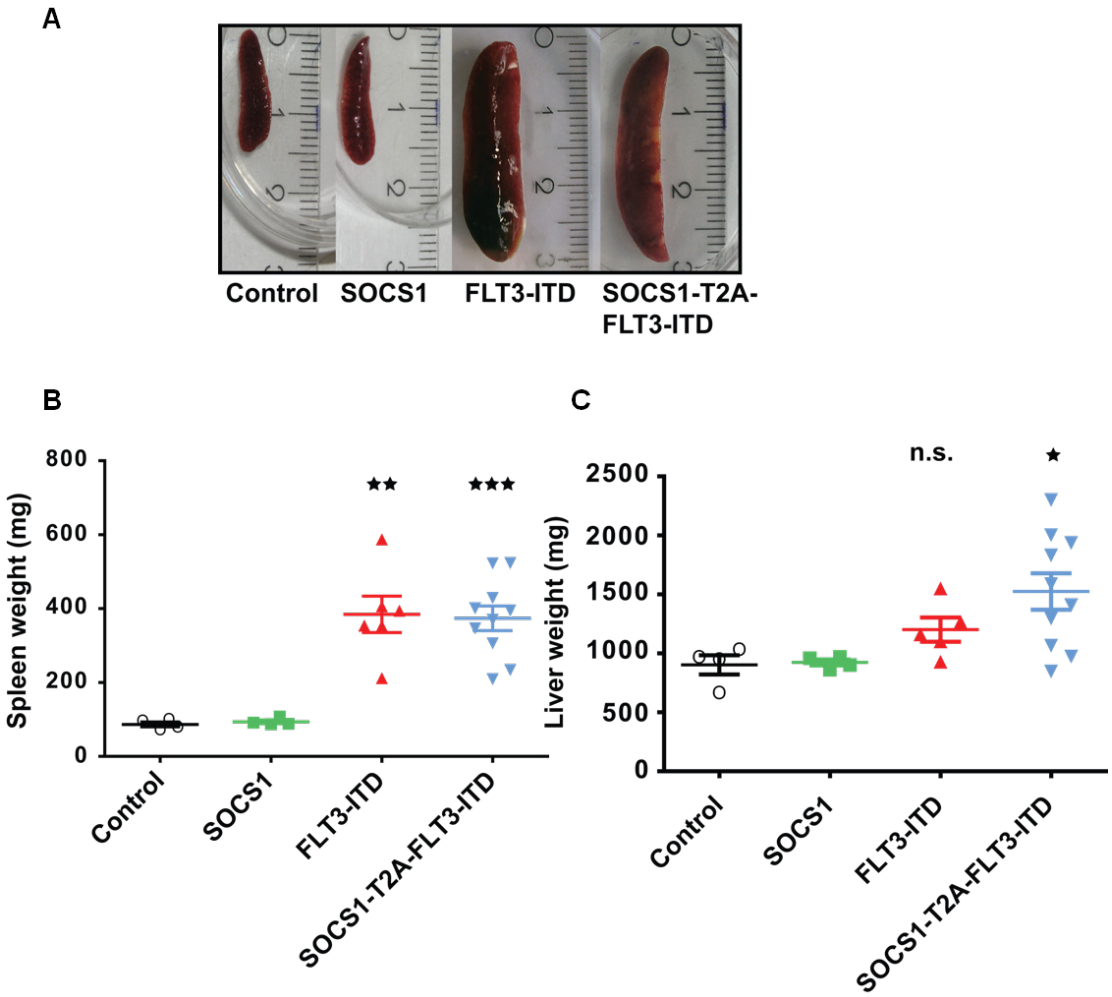

Figure 18: Mice transplanted with FLT3-ITD and SOCS1-T2A-FLT3-ITD displayed enlarged spleen and liver. (A) Representative spleens from control, SOCS1 groups (120d post-transplantation) and from moribund mice of FLT3-ITD and SOCS1-T2A-FLT3-ITD groups, indicating normal or enlarged spleens, respectively. Analysis of spleen weights (B) and liver weights (C) at the time of death or at the end of experiment (120d). Mice received transplant of FLT3-ITD and SOCS1-T2A-FLT3-ITD have enlarged spleens compared to control. (* $p<0.05$, ** $p<0.01$, ***$p<0.001$)

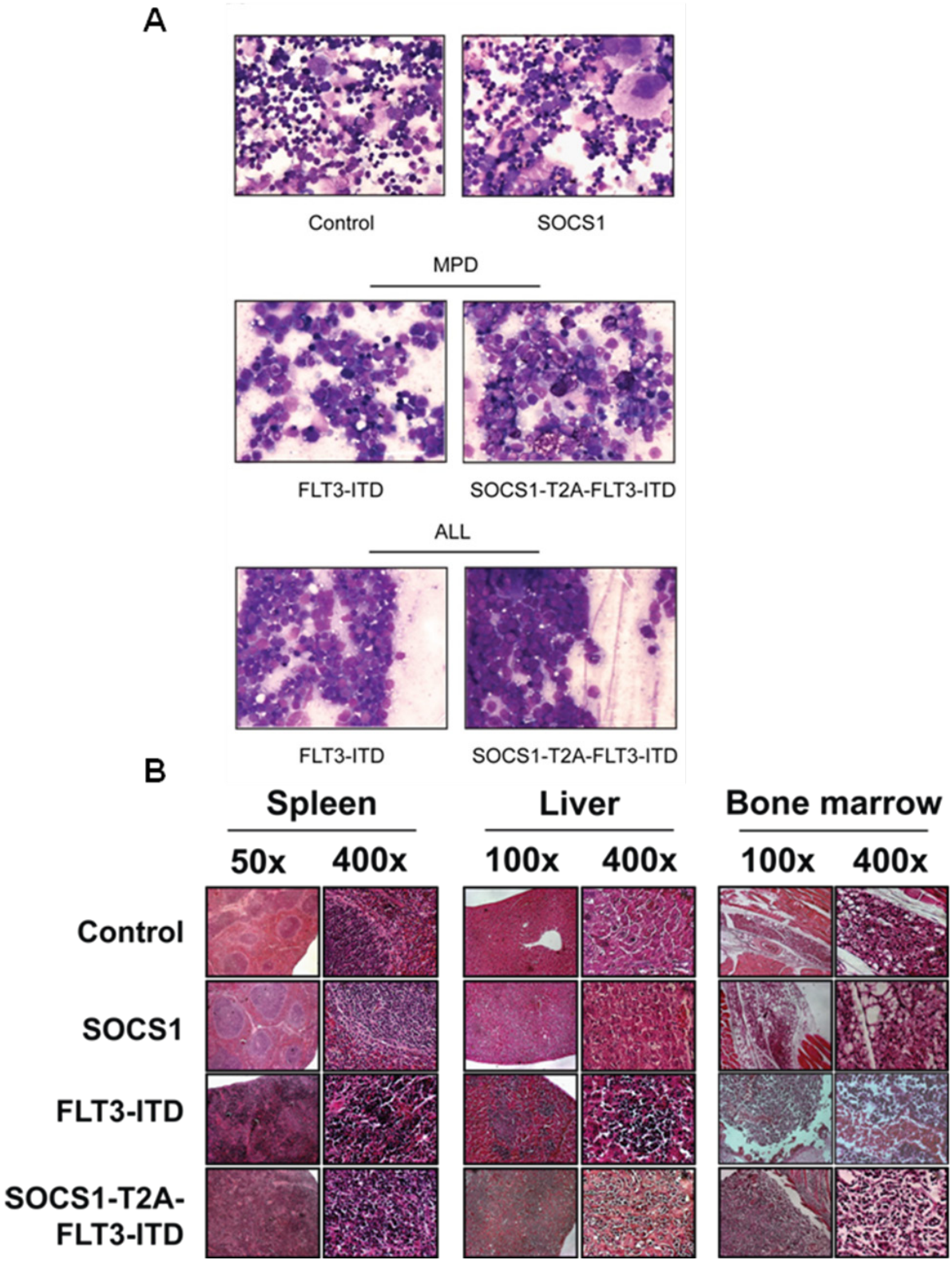

Figure 19: Histology of transplanted mice. (A) Representative bone marrow smears from 120d post-transplantation control and SOCS1 or from FLT3-ITD and SOCS1-T2A-FLT3-ITD moribund mice, indicating a normal or high number of immature myeloid / lymphoid blasts respectively.(B) Hematoxylin and eosin staining (H&E) of tissues (spleen, liver) from control and SOCS1 mice and leukemic FLT3-ITD and SOCS1-T2A-FLT3-ITD mice. Data are representative of one of 6 moribund FLT3-ITD animals and one of 10 moribund SOCS1-T2A-FLT3-ITD mice.

4.6.4 SOCS1 co-operates with MPD development

Interestingly, when survival was analyzed with respect to the phenotype and latency, a surprising correlation was observed: SOCS1 co-expression significantly shortened the latency of FLT3-ITD induced myeloproliferative disease phenotype ($p = 0.01$, Figure 19 left panel), while the latency of FLT3-ITD induced B-cell acute lymphoblastic leukemia disease phenotype was not affected ($p = 0.85$, Figure 19 right panel). This clearly shows that SOCS1 co-expression with FLT3-ITD accelerates the myeloproliferative disease but not acute lymphoblastic leukemia (Figure 20A).

4.6.5 Model for SOCS1 co-operation with FLT3-ITD

Together these data provide a model in which mutant FLT3-ITD up-regulates SOCS family proteins particularly SOCS1 which inhibits pro and anti-proliferative as well as differentiation signals from cytokines. By abrogating these external signals, cells expressing FLT3-ITD are under the dictate of pro-proliferative and survival signals transmitted downstream of the oncogenic receptor. This model may explain `SOCS paradox`, the seemingly contradictory finding of increased expression of SOCS1 with tumor suppressor function, in context of oncogenic kinase FLT3-ITD (Figure 20B).

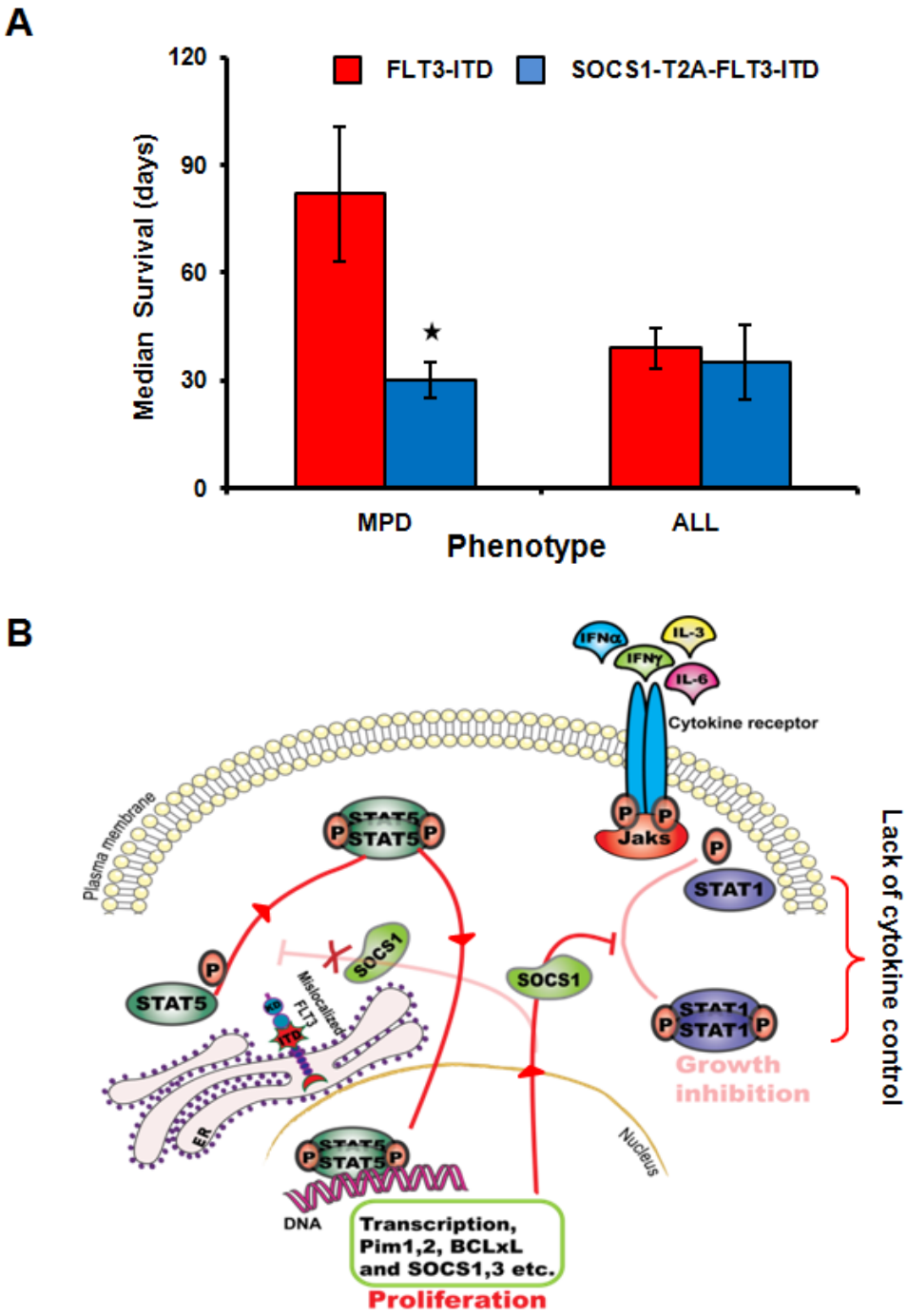

Figure 20: SOCS1 play important role in FLT3-ITD mediated myeloproliferative disease.
(A) Comparison of median survival of mice developed MPD (FLT3-ITD = 82d ± 18.8d, SOCS1-T2A-FLT3-ITD = 30d ± 4.8d) and ALL (FLT3-ITD = 39d ± 5.7d, SOCS1-T2A-FLT3-ITD = 35d ± 10.3d). On the Y-axis the median survival (in days +/- standard deviation) is plotted, and separately shown for MPD and ALL phenotype. (B) Mechanistic model for co-operation of SOCS1 in FLT3-ITD mediated leukemogenesis. Constitutively active oncogenic FLT3-ITD directly phosphorylates STAT5. Upon phosphorylation STAT5 dimerizes and translocates into nucleus and induces transcription of STAT5 target genes, which includes SOCS1. FLT3-ITD induced SOCS1 expression neither affects FLT3-ITD mediated proliferative signals nor the STAT5 activation, while it terminates cytokine. Thereby, SOCS proteins cooperate with FLT3-ITD by “shielding” the cell from cytokine control (for example IFNα, IFNγ, IL-3 and IL-6).

4.7 Role of SOCS proteins in BCR/ABL mediated leukemogenesis

4.8 SOCS gene expression in patient derived ALL cells

4.8.1 CIS, SOCS2, SOCS3 were highly expressed in BCR/ABL positive, long-term cultures of primary acute lymphoblastic leukemia patient samples

BCR/ABL is an oncogenic fusion protein associated with acute lymphoblastic leukemia and chronic myeloid leukemia. BCR/ABL also highly activates STAT5 similar to FLT3-ITD and additionally STAT3. SOCS genes are known STAT3 and STAT5 target genes. Since it was already observed that SOCS1 and SOCS3 are highly expressed in AML patient bone marrow, their expression was analyzed in long-term cultures of primary cells from patients with acute lymphoblastic leukemia.

Quantitative real-time PCR results show high CIS, SOCS2 and SOCS3 expression in BCR/ABL positive patient cell lines compared to BCR/ABL negative patient cell lines. Although SOCS4, SOCS5, SOCS6 and SOCS7 mRNA was detected in ALL patient cell lines, their expression was independent of the presence or absence of BCR/ABL (Figure 21). Surprisingly, a high SOCS1 expression in BCR/ABL negative cell lines was observed (Figure 21).

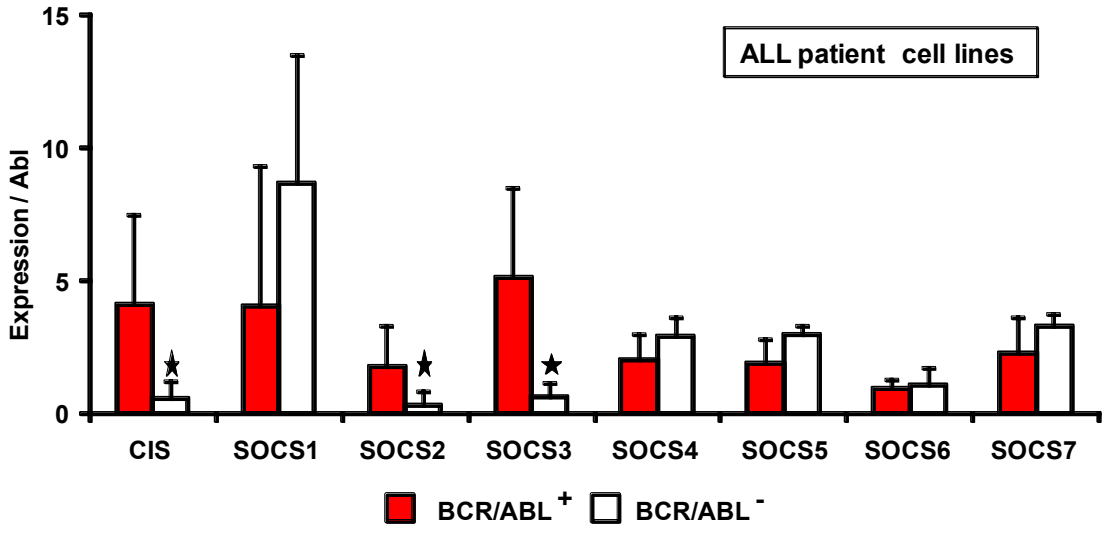

Figure 21: CIS, SOCS2 and SOCS3 are highly expressed in BCR/ABL positive ALL patient cell lines. Long-term cultures cell lines from BCR/ABL positive patients (BV, CM, WD, KW, PH and VB) and BCR/ABL negative patients (VG, KR, RL, SK, CR and HP) were analyzed for CIS, SOCS1-SOCS7 mRNA expression by real time quantitative PCR as described in methods section. Normalized SOCS gene expression of BCR/ABL positive cell lines is plotted comparing to BCR/ABL negative cell lines (standard deviations as error bars).

4.8.2 CIS, SOCS2 and SOCS3 expression is Abl kinase dependent in long-term cultures of primary acute lymphoblastic leukemia patient samples

The observed high CIS, SOCS2, SOCS3 expression in BCR/ABL positive ALL cell lines was further tested to determine Abl kinase dependence. For this purpose, BCR/ABL positive cell lines were treated overnight with Abl specific kinase inhibitor imatinib (1 μM) followed by SOCS gene expression as measured by quantitative PCR. Solvent treated cell lines were used as a control. As shown in Figure 22, Abl inhibition led to decreased expression of CIS, SOCS2 and SOCS3 mRNA in all cell lines, whereas Abl inhibition had varying effects on SOCS1 expression in different cell lines. As expected SOCS4, SOCS5, SOCS6 and SOCS7 expression was not influenced by the inhibition of Abl (Figure 22).

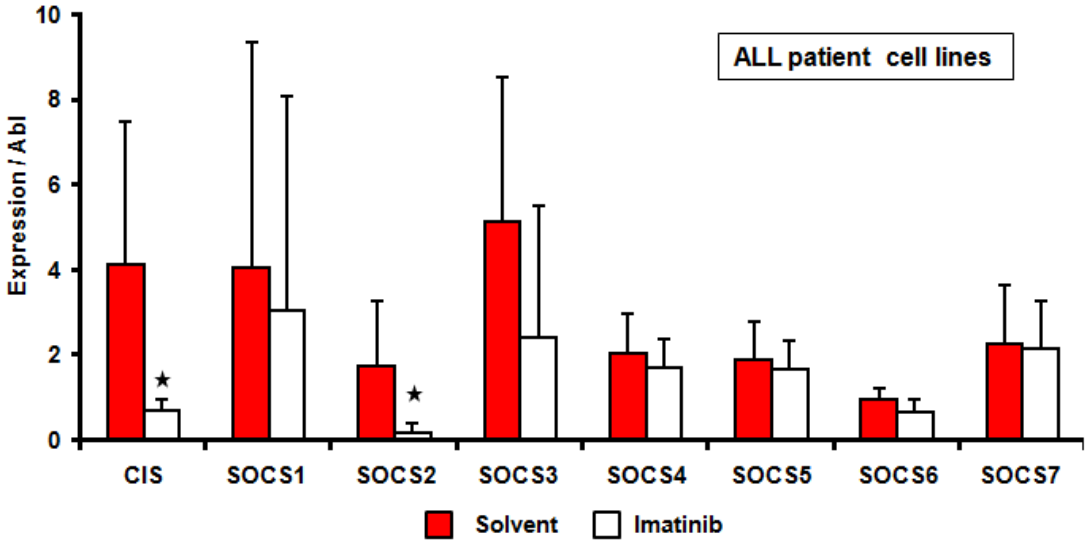

Figure 22: Kinase dependent expression of CIS, SOCS2 and SOCS3 in BCR/ABL positive ALL patient cell lines. Long-term cultured cell lines from BCR/ABL positive patients (BV, CM, WD, KW, PH and VB) were treated overnight with 1 μM imatinib (Abl specific kinase inhibitor) or with solvent control and CIS, SOCS1-SOCS7 mRNA expression is analyzed by real time quantitative PCR as described in the methods section. Normalized SOCS gene expression of imatinib treated cell lines is plotted with standard deviations as error bars.

4.9 Kinase dependent expression of CIS, SOCS1 and SOCS3 in K-562 cell line

SOCS gene expression was tested in the K-562 cell line, which is used extensively to study BCR/ABL signaling pathways. K-562 cells were treated overnight with either Abl kinase inhibitor imatinib (2 μM) or with a solvent control, SOCS mRNA expression was measured by quantitative PCR. As shown in Figure 23, CIS, SOCS1 and SOCS3 mRNA is highly expressed in K-562 cells and their expression

is diminished with Abl kinase inhibition. SOCS4, SOCS5, SOCS6 and SOCS7 expression is very low and their expression is not affected by Abl inhibition.

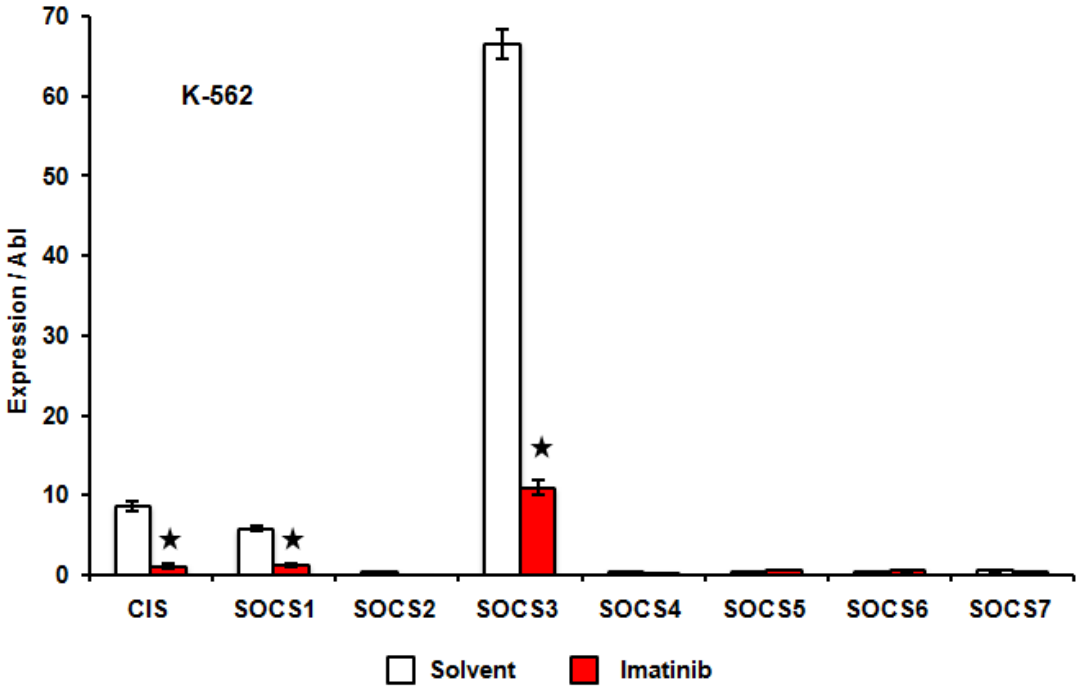

Figure 23: Kinase dependent expression of CIS, SOCS2 and SOCS3 in K-562 cell line. K-562 cells were treated overnight with 2 μM imatinib (Abl specific kinase inhibitor) or with solvent control and CIS, SOCS1-SOCS7 mRNA expression is analyzed by real time quantitative PCR as described in the methods section. Normalized SOCS gene expression of imatinib treated cell lines is plotted in comparison to solvent control treated cell lines (standard deviations as error bars).

4.10 Ectopic expression of BCR/ABL in murine bone marrow induced SOCS gene expression

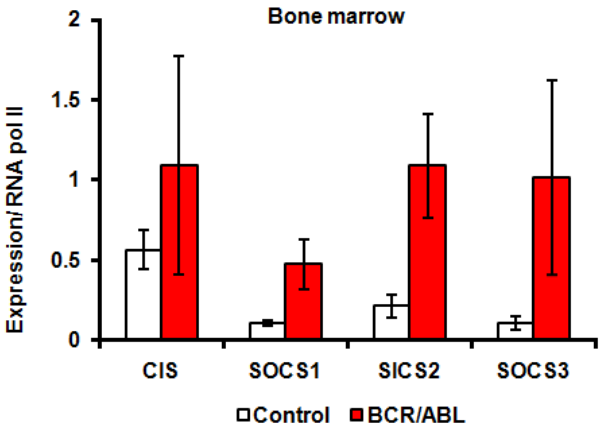

Figure 24: BCR/ABL induces SOCS gene expression in murine bone marrow. Murine bone marrow stem and progenitor cells were transduced with BCR/ABL or an empty GFP vector (control) and sorted for GFP. Cells were starved overnight of cytokine cocktail. The expression of CIS, SOCS1-3 transcripts was measured by quantitative PCR as described in methods section. Normalized gene expression is plotted with standard deviations as error bars.

Next, SOCS gene expression by BCR/ABL in primary murine bone marrow was analyzed. Lineage depleted bone marrow cells expressing either control or BCR/ABL were starved from serum and cytokines and SOCS gene expression was measured by quantitative PCR. As shown in Figure 24, BCR/ABL expressing bone marrow cells have higher CIS, SOCS1-3 expression compared to control.

4.11 SOCS1 did not affect BCR/ABL mediated proliferation, while abrogating IL-3 mediated proliferation

SOCS1 is expressed in a kinase dependent manner in K-562 cell line, and three of 6 BCR/ABL positive ALL cells. The consequence of SOCS1 co-expression with BCR/ABL in a Ba/F3 cell line (a pro-B cell line) was tested. Ba/F3 cells expressing either BCR/ABL (p190) or a vector control were retro-virally transduced with SOCS1 or a GFP control. These mixed populations of transduced (green) and un-transduced (no green) cells were either cultured in the presence or absence of IL-3. GFP expression was followed at regular intervals by FACS. Surprisingly, over-expression of SOCS1 had no effect on growth of BCR/ABL expressing cells and SOCS1 transduced BCR/ABL cells grew at the rates similar to GFP transduced cells, as the proportion of SOCS1 transduced GFP positive cells remained constant over time (Figure 25A). In contrast, over expression of SOCS1 severely inhibited IL-3 mediated growth of control Ba/F3 cells and resulted in a drastic reduction of SOCS1 positive cells, suggesting a selective inhibitory effect of SOCS1 on IL-3 but not on BCR/ABL mediated growth of Ba/F3 cells (Figure 25A and 25B). Similar to earlier reports using FDCP cells (Bourette et al., 2001), SOCS1 transduced Ba/F3 cells underwent rapid apoptosis in the presence of IL-3.

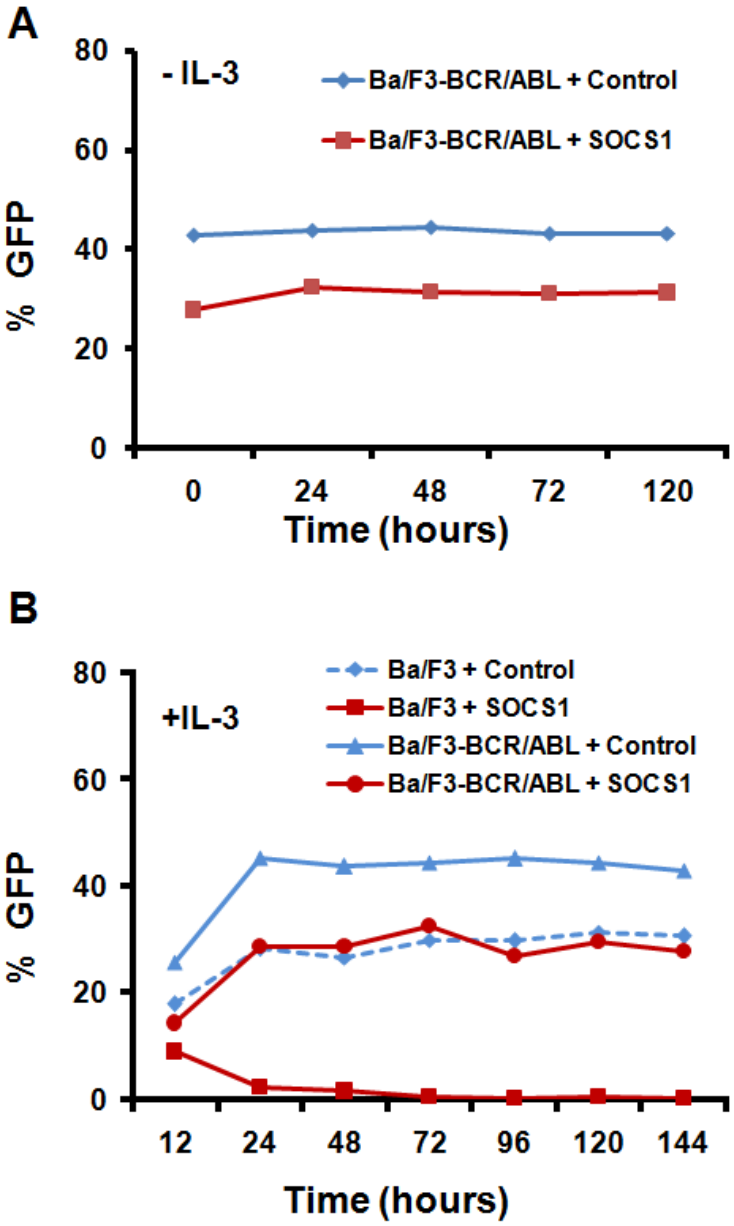

Figure 25: SOCS1 inhibits IL-3 but not BCR/ABL mediated proliferation. (A) SOCS1 does not inhibit growth of BCR/ABL expressing cells. Ba/F3-FLT3-ITD cells were retrovirally transduced SOCS1 and empty GFP vector (control). Mixed populations of transduced and un-transduced cells were washed three times with PBS and were further grown in the presence or absence of IL-3 and the fate of transduced cells were monitored by measuring the proportion of GFP positive cells every day by flow cytometry.

5 Discussion

Aberrant activation of oncogenic RTKs and deregulation of downstream JAK-STAT signaling cascades have been described for almost all hematological malignancies studied to date. Particularly, constitutive activation of STAT3 and STAT5 are frequently found in leukemia (Benekli et al., 2003; Garcia and Jove, 1998). FLT3-ITD, a frequent activating mutation in AML also activates STAT5 and induces its target genes like PIM kinases and SOCS genes (Mizuki et al., 2003).

Hematopoietic cytokines regulate steady state production of blood cells and their proper function. Physiological responses of cytokines mediated majorly by JAK-STAT pathway. Interestingly cytokines and oncogenes share several components of JAK-STAT pathway. Of which, important are SOCS genes, physiological negative regulators of cytokines were induced by oncogenes raising important question whether they are mediating immune escape of transformed cells. It remained unclear how transformed cells escape the tight regulatory cytokine networks that normally control the cell cycle, survival and lineage choice of hematopoietic stem and progenitor cells (Rieger et al., 2009).

Here we showed that SOCS genes, mainly SOCS1, a tumor suppressor gene was highly induced by FLT3-ITD. Expression of SOCS1 with FLT3-ITD inhibited external cytokine signals and aggravated FLT3-ITD mediated myeloproliferative disease with a reduced latency. This work suggests that SOCS proteins (SOCS1 as an example) are being part of escape mechanism from cytokine control in FLT3-ITD mediated leukemogenesis. *In vitro* data also suggests a similar mechanism is true in the context BCR/ABL, an activated kinase similar to FLT3-ITD.

FLT3-ITD induces SOCS1 expression

Numerous biological differences between FLT3-ITD and FL-stimulated FLT3-WT have been described to date, including the striking ability of FLT3-ITD to cause colony growth in semi-solid methylcellulose – as an *in vitro* surrogate for transformation – while FL-stimulated FLT3-WT does not (Brandts et al., 2007; Hayakawa et al., 2000; Kiyoi et al., 2002; Mizuki et al., 2000; Mizuki et al., 2003; Rocnik et al., 2006; Stirewalt and Radich, 2003) Importantly, FLT3-ITD (but not ligand-activated FLT3-WT) activates STAT5 and induces expression of its target genes (Mizuki et al., 2003).). Also, constitutive STAT5 has been reported in about

70% of AML blasts (Birkenkamp et al., 2001). By analyzing global phosphoproteomic changes induced by FLT3-ITD, we have recently found that mislocalization of FLT3-ITD to the endoplasmic analysis causes aberrant STAT5 activation (Choudhary et al., 2009). We have now performed a systematic expression analysis of the STAT5 target genes CIS, SOCS1, SOCS2 and SOCS3 (Figures 8-11). These experiments consistently demonstrated elevated CIS and SOCS1 expression in human FLT3-ITD positive AML cell lines in a kinase-dependent manner (Figure 10 A and B), in murine lymphoid and myeloid cell lines overexpressing FLT3-ITD (Figure 8 A and B) and in retrovirally transduced primary murine bone marrow (Figure 9). This was also the case for SOCS2 and SOCS3 in most experiments. Importantly, the analysis of human AML samples has revealed a significant increase of SOCS1 (but not SOCS3) expression in samples with FLT3-ITD expression compared to FLT3-WT$^+$ AML samples (Figure 11 A and B). Importantly, decreased SOCS1 protein expression was also shown upon FLT3-ITD kinase inhibition (Figure 10C). These data demonstrate a consistent pattern of increased SOCS1 expression in the presence of FLT3-ITD, and – to a lesser degree – of other SOCS family members. Similar observations were made in TEL-Jak2 (Monni et al., 2001) and v-Abl overexpressing cell lines (Limnander et al., 2004).

SOCS1 does not affect FLT3-ITD mediated transformation or signaling but abolished cytokine signaling

We have previously shown that FLT3-ITD phosphorylates STAT5 directly, independently of Jak kinase family members (Choudhary et al., 2007). We therefore analyzed the biological effects of SOCS1 overexpression in the presence or absence of FLT3-ITD by use of a T2A constructs which allows for expression of both SOCS1 and FLT3-ITD in the same target cell (Szymczak et al., 2004). In the 32D cell line model, SOCS1 overexpression protected FLT3-ITD from the antiproliferative effects of INFα and IFNγ (Figure 13A) and abrogated the IFN-mediated STAT1 phosphorylation (Figure 14), as SOCS1 is known to inhibit STAT1 activation downstream of IFNα, IFNβ and IFNγ (Alexander et al., 1999; Fenner et al., 2006). Also, IFNα- and IFNγ-induced STAT1 activation was diminished in 32D cells expressing FLT3-ITD alone, presumably due to low level expression of endogenous SOCS1 (Figure 14).

As previously shown (Choudhary et al., 2007), FLT3-ITD was resistant to SOCS1 overexpression, while expression of SOCS1 alone causes a rapid loss of cells that cannot be rescued by IL3-treatment (Figure 13 B and C). Also, in the absence of FLT3-ITD, we could not obtain 32D cells stably expressing SOCS1 (data not shown), consistent with similar observation made in the IL-3 dependent, myeloid cell line FDC-P1 (Bourette et al., 2001). In agreement with these findings, co-expression of SOCS1 with FLT3-ITD did not alter constitutively activated signaling pathways when compared to FLT3-ITD alone, as measured by p-AKT, p-ERK and p-STAT5 levels (Figure 12).

In order to address the role of SOCS1 in a more physiological setting, further experiments were performed in primary murine hematopoietic stem and progenitor cells (Figures 15-20). As seen by others for M-CSF (Bourette et al., 2001) and G-CSF (van de Geijn et al, 2004), overexpression of SOCS1 led to smaller (data not shown) and fewer colonies under pro-proliferative conditions with IL3, IL6 and SCF, when compared to controls (Figure 15C). In the presence of FLT3-ITD, however, co-expression of SOCS1 did not affect colony growth under these conditions (Figure 15C, left part). Importantly, under the conditions with IFNγ, co-expression of SOCS1 with FLT3-ITD led to significantly more colonies when compared to FLT3-ITD alone (Figure 15C, right part). Due to the fact that FLT3-ITD induces SOCS1 expression (Figure 15A and B), colony formation was observed in the presence of IFNγ, however when SOCS1 is overexpressed (SOCS1-T2A-FLT3-ITD) colony formation is further enhanced in a dose-dependent manner (Figure 15C). As SOCS1 has been shown to be the major inhibitor of IFNγ signaling (Alexander et al, Cell 1999), this data suggests that co-expression of (either endogenous or exogenous) SOCS1 protects FLT3-ITD from the anti-proliferative and possibly deleterious effects of IFNγ. These findings contrast to previous reports which have demonstrated that SOCS1 can inhibit transformation by TEL-JAK2 and by oncogenic VAV through their ubiquitination and proteosomal degradation (Frantsve 2001, Rottappel 2002, De Sepulveda 2000), suggesting that SOCS1 can act as a substrate-specific recognition component of the E3 ubiquitin ligase complex (Kamizono et al JBC, 2001).

It had previously been shown that FLT3-ITD overexpression in primary bone marrow does not increase replating efficiency.(Lee et al., 2007) We postulated that

the cytokine escape mechanism in SOCS1-overexpressing cells may contribute to self-renewal of hematopoietic stem cells; however, replating assays showed no significant differences between FLT3-ITD alone and SOCS1/FLT3-ITD conditions (Figure 15D).

SOCS1 significantly shortened the latency of FLT3-ITD induced myeloproliferative disease *in vivo*

We next asked whether co-expression of SOCS1 would affect the transforming phenotype of FLT3-ITD *in vivo* and made again use of our FLT3-ITD and SOCS1-T2A-FLT3-ITD constructs, which leads to similar FLT3-ITD protein levels (Figure15B). It was previously been shown that in bone marrow transplantation models and in knock-in models FLT3-ITD leads to the development of a myeloproliferative disease or lymphoid disease.(Grundler et al., 2005; Kelly et al., 2002b; Lee et al., 2005; Lee et al., 2007)

As shown in the post-transplantation follow-up, mice transplanted with either FLT3-ITD or SOCS1-T2A-FLT3-ITD expressing bone marrow revealed significantly higher GFP-positive cells and higher WBC counts, with a tendency towards lower hemoglobin and platelet levels (Figure 16). In addition, the biological advantage of GFP-positive cells appeared the most pronounced in the SOCS1/FLT3-ITD arm. On the contrary, as expected, SOCS1-expressing cells rapidly disappeared from the peripheral blood, demonstrating a pronounced biological disadvantage (Figure 16A). The survival data demonstrates a clearly more aggressive phenotype in those mice co-expressing both SOCS1 and FLT3-ITD, with a shorter latency in MPD development (Figure 20A) and increased liver weight compared to FLT3-ITD alone (Figure 18C). Disease was manifested in peripheral blood, spleen, liver and bone marrow (Table 2 and 3). The mice in both groups developed either MPD or ALL with a 100% penetrance, whereas mice transplanted with SOCS1 transduced cells did not develop any disease. Previously, in a similar study, co-expression of SOCS1 prolonged the latency of disease by TEL-JAK2 *in vivo* in a bone marrow transplant model. This was mainly attributed to the degradation of TEL-JAK2 and inhibition JAK kinase activity leading to diminished ERK activation by exogenously expressed SOCS1(Frantsve et al., 2001). In contrast to this, in our model we neither observed degradation of FLT3-ITD nor diminished ERK activation by SOCS1 co-expression. Thus, SOCS1 functions in a context-dependent manner.

This is due to the fact that FLT3-ITD directly activates STAT5 and does not activate JAK kinases, which is a prerequisite for SOCS1 mediated degradation. (Choudhary et al., 2007) When we carefully analyzed median survival and phenotypes, decreased median survival in SOCS1 co-expressing group was exclusively contributed by MPD phenotype but not by ALL phenotype suggesting SOCS might play an important role in the myeloid compartment. Taken together our data provide a proof of principle that induction of SOCS proteins by FLT3-ITD is a mechanism to escape the external cytokine control and ultimately contributing to the development of leukemogenesis. We think this may be a general mechanism applicable to many other oncogenes that activate the STAT5 pathway and for which JAK kinase activity is indispensible for the transformation, such as BCR/ABL and TEL-PDGFβR.

BCR/ABL induces SOCS expression

Published results suggest that BCR/ABL mediated STAT5 activation may not need Jak2 and that it is mediated majorly by HCK or by direct phosphorylation (Ilaria and Van Etten, 1996; Klejman et al., 2002). CIS, SOCS2 and SOCS3, STAT5 target genes have been shown to be expressed in BCR/ABL$^+$ CML patients (Schultheis et al., 2002; Takahashi et al., 2003; Tauchi et al., 2001). Similar to previous finding in CML patients, here we found increased expression of CIS, SOCS2 and SOCS3 in BCR/ABL positive long-term cultured ALL cells compared to BCR/ABL negative long-term cultured ALL cells (Figure 21). Further in BCR/ABL positive long-term cultured ALL cells CIS, SOCS2 and SOCS3 expression is decreased upon imatinib treatment (Figure 22). Kinase activity of BCR/ABL is required for CIS, SOCS1 and SOCS3 expression in K562, a BCR/ABL positive leukemic cell line (Figure 23). Ectopic expression of BCR/ABL in murine bone marrow stem cells led to increased expression of CIS, SOCS1 and SOCS3 genes (Figure 24). In summary SOCS genes (CIS, SOCS1-3) expression is driven by BCR/ABL in primary patient cell lines and in murine bone marrow stem cells.

BCR/ABL mediated transformation resistant to SOCS1

We hypothesized that, BCR/ABL mediated STAT5 activation is resistant to SOCS proteins because it is not dependent on Jak2 and hence SOCS1 may not affect BCR/ABL mediated transformation. To test our hypothesis, we transduced Ba/F3 cells expressing BCR/ABL with SOCS1 retrovirus. We found that SOCS1 over-

expression neither inhibited nor reduced BCR/ABL mediated proliferation, showing that BCR/ABL is resistant to SOCS1 mediated growth inhibition (Figure 25A). However, as expected, IL-3 mediated proliferation is inhibited by SOCS1 (Figure 25B).

Previously, CIS, SOCS1 and SOCS2 expression was shown to reduce but not inhibit the transformation potential of BCR/ABL (Rottapel et al., 2002; Schultheis et al., 2002; Tauchi et al., 2001). In our experiments we did not observe reduced growth of SOCS1 transduced BCR/ABL cells. In agreement with our results, another study found that SOCS1 expression did not affect transformation potential of BCR/ABL (Frantsve et al., 2001). Our data suggest that the SOCS gene expression in BCR/ABL transformed cells may shield cells from cytokine control such as IL-3 (Figure 25B). To support our hypothesis, CML patients, who responded poorly to IFNα, had increased SOCS3 expression. Hence we conclude that SOCS gene expression by BCR/ABL may have clinical implications concerning cytokine therapy.

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7 Eidesstattliche erklärung

EIDESSTATTLICHE ERKLÄRUNG

Ich erkläre hiermit ehrenwörtlich, daß ich die dem Fachbereich Biochemie, Chemie und Pharmazie zur Promotionsprüfung eingereichte Arbeit mit dem Titel

“Role of SOCS proteins in FLT3-ITD and BCR/ABL mediated leukemogenesis”

im Zentrum der Inneren Medizin, Medizinische Klinik II, Hämatologie/Oncologie des Universitätsklinikums Frankfurt bei Prof. Dr. Hubert Serve und unter Leitung von Prof. Dr. Rolf Marschalek mit Unterstützung von Dr. Christian Brandts ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe.

Ich habe bisher an keiner in- oder ausländischen Biologischen Fakultät ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende Arbeit als Dissertation vorgelegt.

Frankfurt, den 23-07-2010

Pavan Kumar Reddy, NG.

Abbreviations

μC	Microcurie
3[H]	Tritium thymidine
AL	Activation loop
ALL	Acute lymphoid leukemia
AML	Acute myeloid leukemia
ATP	Adenosine tri phosphate
BM	Bone marrow
BSA	Bovine serum albumin
CA	Constitutively active
CAs	Cobblestone areas
cDNA	Complementary DNA
CIS	Cytokine induced Src homology 2 protein
CML	Chronic myeloid leukemia
CSF	Colony stimulating factor
CT	C-terminus
DMEM	Dulbecco's modified eagle medium
IMDM	Iscove´s modified dulbecco´s medium
dNTP	Deoxyribonucleotide triphosphate
DTT	dithiothreitol
E. coli	Escherichia coli
ECL	Enhanced chemiluminiscence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFP	Enhanced green fluorescent protein
EGTA	Ethyleneglycol-bis (β-aminoethyl)-N,N,N',N'-tetraacetic Acid
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FACS	Fluorescent activated cell sorter
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate

FL	FLT3 ligand
Flk2	Fetal liver kinase 2
Flt	FMS-like tyrosine kinase
GAM	Goat anti-mouse
GAR	Goat anti-rabbit
Gy	Gray
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
IF	Immunofluorescence
IFNα	Interferon alpha
IFNγ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal ribosome entry site
ITD	Internal tandem duplication
JAK	Janus associated kinase
JM	Juxtamembrane
kDa	Kilodalton
KI	Kinase insert
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MDS	Myelodysplastic syndrome
MnCl2	Manganese chloride
NaCl	Sodium chloride
NaF	Sodium fluoride
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PE	Phycoerythrin
pH	Potential hydrogen
PI3-K	Phosphoinositol 3-kinase
PI	Propidium Iodide
PKC	Protein kinase C
PM	Plasma membrane

PTB	Phospho tyrosine binding
PVDF	Polyvinylidene fluoride
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
RT	Reverse transcription
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SCF	Stem cell factor
SDS-PAGE	Sodium dodecyl (lauryl) sulfate-polyacrylamide gel electrophoresis
SH	Src homology
SOCS	Suppressor of cytokine signaling
SP	Signal peptide
STAT	Signal transducer and activator of transcription
STK	Stem cell tyrosine kinase
TKD	Tyrosine kinase domain
TM	Transmembrane
WEHI	Walter and Elisa Hall Institute
WT	Wild-type
ZnCl2	Zinc chloride

Curriculum Vitae

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Publications

1. Pavankumar Reddy NG, Bülent Sargin, Chunaram Choudhary, Stefan Stein, Manuel Grez, Carsten Müller-Tidow, Wolfgang E. Berdel, Hubert Serve, Christian H. Brandts. **FLT3-ITD induced SOCS1 inhibits cytokine signaling and accelerates the development of myeloproliferative disease**. *Manuscript* submitted to *Blood* journal.

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Symposia and Poster/Oral presentations

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4. Poster presentation: **14th European Hematology Association (EHA)** Annual meeting 4-7 June 2009, Berlin Germany.
5. Poster presentation: SFB-684 symposium on *'Molecular Mechanisms of normal and malignant hematopoiesis',* 2- 4 April 2009, Munich, Germany.
6. Poster presentation: **9th ADNAT and 30th *ISHG*** *joint annual International symposium on 'Molecular Medicine and health',* 20-23 February, 2005 in Centre for Cellular and Molecular Biology (CCMB), Hyderabad, India.
7. Poster presentation: International symposium *on 'Advances and challenges in Reproductive Health Research in the Post Genomic Era',* 9-12 January. 2005, National Institute for Research in Reproductive Health **(*NIRRH),*** Mumbai, India.
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