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New roles of STAT5 factors in chronic myeloid leukemia cell maintenance

Luana Casetti

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pour l'obtention du Diplôme de

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Soutenue
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par **Luana CASETTI**

**New roles of STAT5 factors in chronic myeloid leukemia
cell maintenance**

Thèse dirigée par
Mme le Docteur **Isabelle DUSANTER-FOURT**

JURY:

Mme le Professeur	Catherine LACOMBE	Président
M. le Professeur	Stefan CONTANTINESCU	Rapporteur
M. le Professeur	François-Xavier MAHON	Rapporteur
M. le Professeur	Philippe ROUSSELOT	Examineur
M. le Docteur	Jérôme KLUZA	Examineur

“Strive not to be a success, but rather to be of value”

Albert Einstein

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ABBREVIATIONS

AB :	Actin Binding	FACS :	Fluorescence-Activated Cell Sorting
ABC :	Adenosine triphosphate-Binding Cassette	FDA :	Food & Drug Administration
ABL :	Abelson murine Leukemia	FISH :	Fluorescent In Situ Hybridization
AGM :	Aorta-Gonad Mesonephros	Flt3-l :	Fms-related tyrosine kinase 3 ligand
AGP1 :	Alpha1-Acid Glycoprotein-1	FNIII :	Fibronectin type III
Allo-HSCT :	Allogenic Hematopoietic Stem Cell Transplantation	FOXO :	Forkhead box O
AML :	Acute Myeloid Leukemia	G-CSF :	Granulocyte-Colony Stimulating Factor
AP :	Accelerated Phase	GH :	Growth Hormone
BAD :	Bcl-2-Associated Death promoter	GLA :	Glutamic Acid
BCR :	Breakpoint Cluster Region	GM-CSF :	Granulocyte Macrophage- Colony Stimulating Factor
BCR-ABL :	Breakpoint Cluster Region- Abelson murine Leukemia	Grb2 :	Growth factor receptor bound protein 2
BM :	Bone Marrow	HIF :	Hypoxia-Inducible Factor
BP :	Blastic Phase	HIM :	Hematopoietic Inductive Microenvironment
CB :	Cord Blood	HLA :	Human Leukocyte Antigen
CCR :	Chemokine Receptor	HPV :	Human Papilloma Virus
CCyR :	Complete Cytogenic Response	HRR :	Homologous Recombination Repair
CLP :	Common Lymphoid Progenitor	HSC :	Hematopoietic Stem Cell
CML :	Chronic Myeloid Leukemia	HSP25 :	Heat-Shock Protein 25
CMP :	Common Myeloid Progenitor	HSPC :	Hematopoietic Stem and Progenitor Cell
CNL :	Chronic Neutrophilic Leukemia	ICAM :	Intercellular Adhesion Molecule
CP :	Chronic Phase	Ig :	Immunoglobulin
CXCL12 :	C-X-C motif Chemokine	IL :	Interleukin
DBD :	DNA Binding Domain	IM :	Imatinib Mesylate
DDsB :	DNA Double strand Break	INFAR :	IFN α / β -Receptor
EGF :	Epidermal Growth Factor		
EPO :	Erythropoietin		
ER :	Endoplasmic Reticulum		
ERK :	Extracellular Regulated Kinase		

JAK : Janus Kinase	PB : Peripheral Blood
JAK/STAT : Janus Kinase/Signal Transducers and Activator of Transduction	PCR : Polymerase Chain Reaction
JNK : Jun Kinase	PDGFR : Platelet-Derived Growth Factor Receptor
KO : Knock-Out	PHD : Prolyl-Hydroxylase
LG : Laminin G	PI3K : Phosphoinositide 3-Kinase
LTC-IC : Long Term Culture-Initiating Cell	PIAS : Protein Inhibitors of Activated STATs
MAPK : Mitogen Activated Protein Kinase	PLC : Phospho-Lipase C
M-bcr : Major breakpoint cluster region	PPR : Parathyroid hormone-related Peptide Receptor
m-bcr : Minor breakpoint cluster region	PRL : Prolactin
MDR : Multi-Drug Resistance	PtdSer : Phosphatidylserine
MGF : Mammary Gland Factor	PTP : Protein Tyrosine Phosphatase
MMP : Matrix Metalloproteinase	PV : Polycythemia Vera
MMR : Major Molecular Response	RacGAP : Rac GTPase Activating Protein
MMR : Mismatch Repair	RanBMP : Ran-Binding Protein in the Microtubule organization center
MSC : Mesenchymal Stem Cell	ROS : Reactive Oxygen Species
mTOR : Mammalian Target of Rapamycin	RTK : Receptor Tyrosine Kinase
NCK : Non-Catalytic region of tyrosine Kinase adaptor protein	RT-PCR : Reverse Transcriptase-Polymerase Chain Reaction
NER : Nucleotide Excision Repair	S/TK : Serine/Threonine Kinase
NK : Natural Killer	S6K : S6 Protein Kinase
NOD : Non-Obese Diabetic	SAPK : Stress Activated Protein Kinase
NOD-SCID : Non-Obese Diabetic-Several Combined Immunodeficiency	SCF : Stem Cell Factor
NOG : NOD/SCID/IL-2Rg null	SCID : Several Combined Immunodeficiency
NOX : NADPH Oxidase	SCN : Severe Congenital Neutropenia
NTS : Nuclear Translocation Signal	Sdf1 : Stromal derived factor
OCT1 : Organic Transporter 1	SH2 : Src-Homology 2
OLI : Oligomerization	SHBG : Sex Hormone Binding Globulin

SHP-1 : Src Homology region 2 domain-
containing Phosphatase-1

SOCS : Suppressors Of Cytokine
Signaling

STAT : Signal Transducers and
Activator of Transduction

TAM : Tyro3, Axe, Mez

TBLA : Trabecular Bone-Like Area

TK : Tyrosine Kinase

TKI : Tyrosine Kinase Inhibitor

TLR : Toll Like Receptor

TPO : Thrombopoietin

VEGF : Vascular Endothelial Growth
Factor

INTRODUCTION PART I

1. The Hematopoietic system

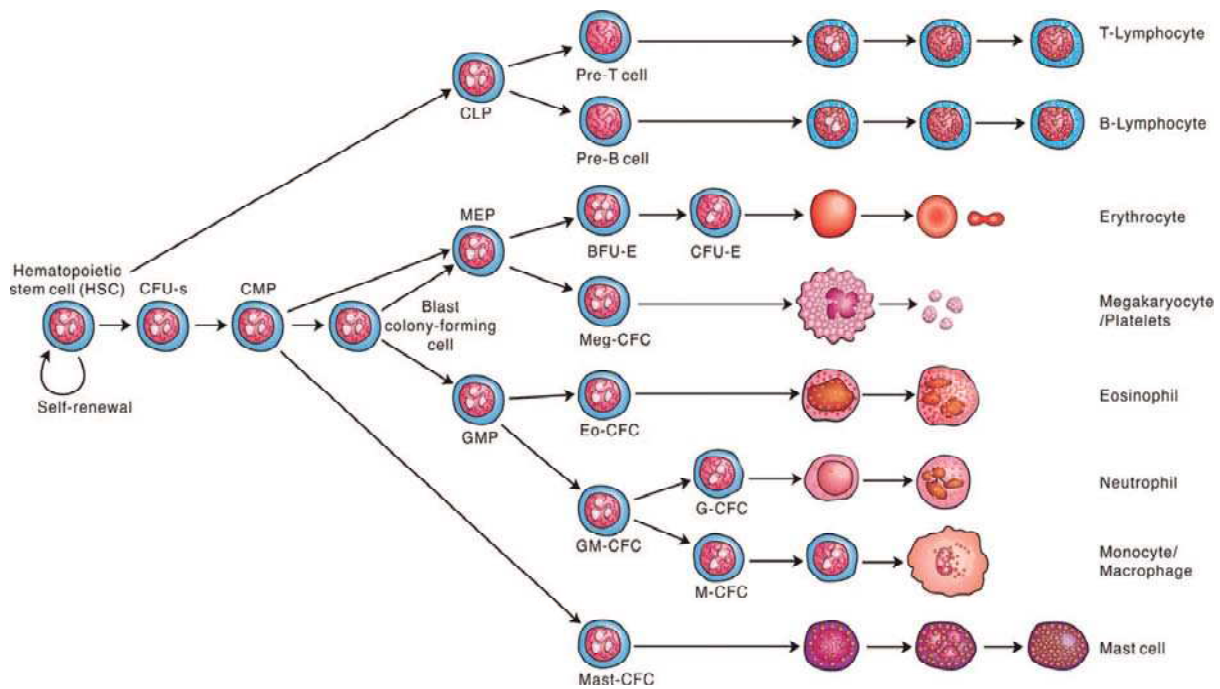
1.1 HEMATOPOIESIS AND HSCs

The hematopoiesis is the process that leads to the formation of all blood cells. These cells are required for a multitude of tasks, such as protection from infections, removal of damaged tissues but also to transport vital molecules such as oxygen throughout the body and to prevent bleeding. In a healthy adult person, approximately 10^{11} – 10^{12} new blood cells are produced every day in order to maintain steady state levels in the peripheral circulation.

All cellular blood components derive from **Hematopoietic Stem Cells (HSCs)**. HSCs are multipotent stem cells operationally defined by their capacity to reconstitute the entire blood system of a recipient and, at the same time, are capable of self-renewal. The self-renewal is the cellular process by which a stem cell divides into one mother cell that is identical to the original undifferentiated stem cell and may be either symmetric or asymmetric in its nature. A **symmetric self-renewing division** refers to the process whereby both daughter cells retain mother cell characteristics. This type of cell division expands the stem cell pool and is therefore thought to be important after transplantation or after hematopoietic injury. In the **asymmetric self-renewal division** the 2 daughter cells adopt different cell fates, resulting in only one cell maintaining stem cell property and the other one can commit to any of the alternative differentiation pathways that lead to the production of one or more specific types of blood cell.

Most of the mature blood cells have a limited life span, therefore HSCs are required throughout life to continuously replenish multilineage progenitors and precursors committed to individual hematopoietic lineages.

The process of hematopoietic differentiation is a hierarchical process that starts from the most immature HSCs and schematically leads to the generation of 2 kinds of progenitors with restricted potential: the Common Lymphoid Progenitor (CLP) and the Common Myeloid Progenitor (CMP). Progenitor cells can divide only a limited number of times; they are pushed to differentiate in their precursor cells whose fate is even more restricted and they will give rise to mature blood cells, such as mature erythroid, megakaryocytic, myeloid (monocyte/macrophage, eosinophil and neutrophil), mast, dendritic or lymphoid cells. (Fig.1)



From (Metcalf 2007)

Figure 1: Hematopoiesis scheme. Hematopoietic Stem Cell (HSC) is capable of self-renew and generates all the blood cells. Population cell numbers rise with increasing maturity. This diagram omits for simplicity natural killer and dendritic cells. Abbreviations: CFU-s (colony-forming unit spleen), CLP (common lymphoid progenitor), CMP (common myeloid progenitor), MEP (megacaryocyte-erythroid progenitor cell), GMP (granulocyte-macrophage progenitor cell), BFU-E (burst-forming unit-erythroid), Meg-CFC (megacaryocyte colony-forming cell), Eo-CFC (eosinophil colony-forming cell), GM-CFC (granulocyte-macrophage colony-forming cell), CFU-E (colony-forming unit-erythroid), G-CFC (granulocyte colony-forming cell), M-CFC (Monocyte-Macrophage colony-forming cell), Mast-CFC (Mast colony-forming cell).

1.2 HEMATOPOIETIC DEVELOPMENT

During mammal development there are different and sequential sites of hematopoiesis. Primitive hematopoiesis starts in the yolk sac and move later to the aorta-gonad mesonephros (AGM) region and to the fetal liver. Just before birth, cycling HSCs migrate from the liver to the developing bone marrow (BM), where they remain after birth.

The properties of HSCs in each site differ, probably reflecting different niches that support HSC expansion and/or differentiation and intrinsic characteristics of HSCs at each stage.

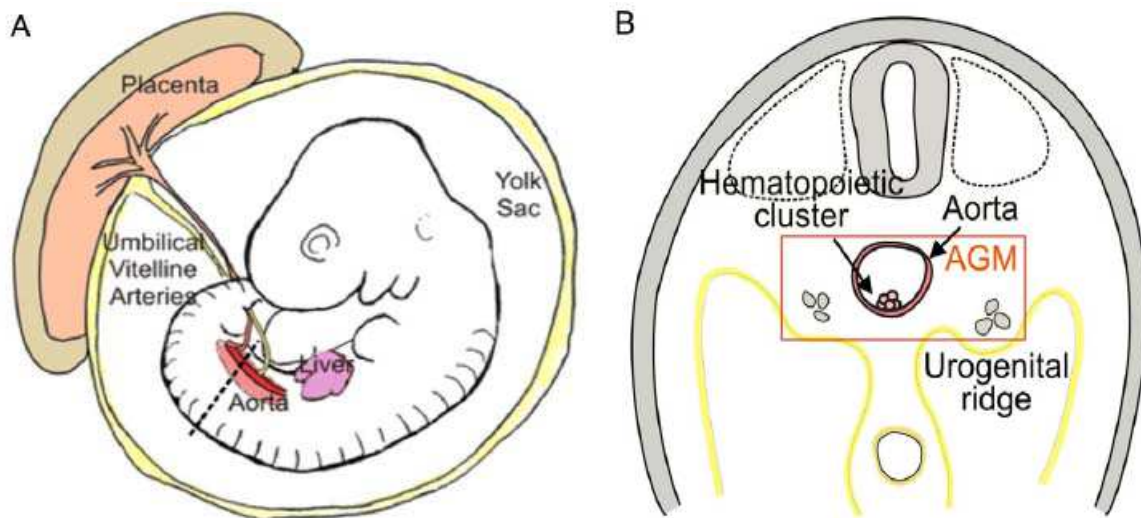
1.2.1 Embryonic hematopoiesis

De novo generation of hematopoietic cells starts shortly after mesoderm formation and continue through gestation. Hematopoiesis in embryo occurs in several tissues that include the yolk sac, the AGM region, the placenta and the liver (Fig.2A-2B). Embryonic hematopoiesis consists of three different waves which lead to the generation of HSCs.

- The **first wave** is called “**primitive hematopoiesis**” and it takes place in the yolk sac. This wave generates short-lived primitive nucleus bearing erythrocytes necessary to carry oxygen to the rapidly growing embryo. These cells co-localize with blood vessels, forming the so called “blood islands”. HSCs and vascular cells are believed to originate by a precursor cell called hemangioblast (Palis and Yoder 2001). These cells may be capable of producing both hematopoietic and endothelial cells. The first wave of primitive hematopoietic cell generation begins at embryonic day (E)7.5 in the mouse system and is highly conserved across vertebrate species, including men, where it begins at 16-20 days of gestation (Tavian and Peault 2005)
- The **second wave** of hematopoiesis is when the circulation starts and hematopoietic cells are detectable in the AGM region. These hematopoietic progenitors are functionally more complex than primitive progenitors; indeed they can produce erythroid, myeloid and lymphoid cells. At this development stage in AGM region, we can find the firsts definitive HSCs that are able to regenerate hematopoiesis in adult recipients (Medvinsky and Dzierzak 1996) and at this stage starts the so called “**definitive hematopoiesis**”.

- The **third wave** provides for the generation of fetal HSCs which are detectable in placenta, circulation and later in the fetal liver, which remains the main hematopoietic organ until birth.

From fetal liver HSCs migrate and colonize bone marrow just prior to birth. At this moment there is a switch from fetal to **adult hematopoiesis**: the high proliferative fetal program switches to the adult program, where HSCs are quiescent and mainly in G0/G1 cell cycle.



From (Kaimakis, Crisan and Dzierzak 2013)

Figure 2: Hematopoietic stem cell development in mouse embryo. Depiction of a mouse embryo at E10.5, at the time when the firsts HSCs are generated in the aorta. A) Sites generating hematopoietic cells are shown: the extraembryonic yolk sac and placenta, the intraembryonic aorta and liver, and the umbilical and vitelline arteries that connect the placenta and the yolk sac to the aorta. The dotted line indicates the transverse section showed in the panel B. B) Depiction of a transverse section with the AGM region in the rectangle. The AGM region is flanked on the dorsal side by the neural tube and the somites and on the ventral side by the gut and the peritoneum. A hematopoietic cluster is indicated on the ventral wall of the aorta.

Prior to birth, HSCs move to the BM where they engraft in small cavities of trabecular bone, close to the endosteal interface between bone and marrow. In adult humans, the major sites of hematopoiesis are the bones of axial skeleton (cranium, sternum, ribs and vertebrae) in addition to the ilium. In children, hematopoiesis occurs in the marrow of the long bones such as the femur and tibia, then it ceases between 5 and 7 years of age and the red (hematopoietic active) BM is replaced by yellow (hematopoietic inactive) adipose tissue. Moreover, in humans, contrary to mouse system, the spleen does not support hematopoiesis

after birth under steady-state conditions. However, extramedullary splenic hematopoiesis can occur in times of hematopoietic stress.

In humans, by four weeks after birth, HSCs stop cycling and acquire largely a **dormant phenotype**, in order to prevent cell exhaustion. In response to blood cell loss HSCs are able to enter in cycle and to replenish the hematopoietic system.

Adult hematopoietic hierarchy is the consequence of progressive HSC maturation culminating with the mature myeloid and white blood cells. Lineage specification largely takes place within the bone marrow as well; mature cells are then mainly found in blood circulation but also in thymus, spleen and lymph nodes.

1.2.3 Regulators of HSC generation

The hematopoietic fate is dictated by the microenvironment and by the intrinsic program of the cells. As it will be discussed later, there is a cross-talk between HSCs and the other cells that surround them, and they form together the microenvironment called “niche”. In the embryonic microenvironment, the underlying mesenchymal cells produce signaling molecules that impact directly or indirectly on emerging hematopoietic cells. These molecules regulate different signaling pathways in HSCs, such as BMP-TGF, JAK/STAT, Wnt and Notch pathways. The molecular mechanisms of these signaling pathways will be discussed below in the “Hematopoietic stem cell niche” paragraph.

In addition to the microenvironment, HSCs possess an intrinsic molecular program that specifies their stemness properties. It consists in the activation/repression of specific genes and signaling cascades that dictates the choice of the hematopoietic cell fate. A lot of intrinsic regulators of HSC generation have been described and the very first discovered come from genetic deletion experiments in mice. These 3 transcription factors are **Scl**, **Gata2** and **Runx1** and to date, they are the best known factors involved in HSC generation. Germ line deletion of each single gene leads to mid-gestation, embryonic lethality and profound anemia. Interestingly, these factors seem to work in complexes to regulate gene expression during hematopoietic development. Below, you can find a more detailed description of these crucial and more studied factors.

Scl/Tal

Scl/Tal1 is a basic helix–loop–helix (bHLH) transcription factor. It can form heterodimer complexes with E-proteins and binds specific DNA motifs by its bHLH domain,

in order to regulate gene expression. *Scl* is normally expressed in hematopoietic tissues of the developing embryo. *Scl* germ line deletion in mice show an early embryonic lethality at day E 9.5, with a completely absence of blood formation (Robb et al. 1996, Shivdasani, Mayer and Orkin 1995). Thus, *Scl* is an essential factor during primitive hematopoiesis (first wave) of the embryonic development.

Gata2

The *Gata2* transcription factor is a member of the Gata family of factors. There are 6 evolutionarily conserved proteins, *Gata1* to *Gata6*, and they have two highly conserved zinc finger (ZnF) domains. Only *Gata1*, 2 and 3 are relevant to the hematopoietic system: *Gata1* for the erythroid/megakaryocytic lineage and for mastocytes differentiation, *Gata2* for hematopoietic stem and progenitor cells, and *Gata3* for T-lymphocytes.

Gata2 is highly expressed in hematopoietic cells of the AGM region, fetal liver and placenta. *Gata2* knock-out (KO) mice exhibit embryonic lethality at day E10 and E10.5 because of a profound anemia. Moreover, in *Gata2* deficient mice, primitive hematopoietic progenitor numbers are decreased and no HSCs are produced (Ling et al. 2004, Tsai et al. 1994).

Runx1

Runx1/AML1 belongs to the transcription factor family that includes also *Runx2* and *Runx3*. It is often found in chromosomal rearrangement in acute myeloid leukemia (AML) cells. *Runx1* binds to regulatory elements of several genes and provides for tissue specific gene expression of molecules known to be important in hematopoiesis. Among them, there are the genes that encode for GM-CSF, IL3 and CSF1 receptor. Deletion of *Runx1* transcription factor in mice leads to embryonic lethality at E12.5. *Runx1* KO mice show normal morphogenesis and yolk-sac erythropoiesis, but they exhibit a block of HSCs generation in AGM region, a block of fetal liver hematopoiesis and a lack of all hematopoietic tissues (Wang et al. 1996, Okuda et al. 1996). These results suggest that *Runx1* regulate specific genes which are essential for definitive hematopoiesis of all lineages. Moreover, *Runx1* seems to act in concert with *Gata2*. Indeed, *Runx1*^{+/-} and *Gata2*^{+/-} single heterozygous mice are viable and present a normal hematopoietic blood profile, but *Runx1*^{+/-}:*Gata2*^{+/-} double heterozygous mice embryos die and fetal livers contain less hematopoietic progenitors than single heterozygous mice (Wilson et al. 2010). These findings show that *Runx1* and

Gata2 have a crucial role in hematopoietic development and that they can form a protein complex that regulate expression of genes essential for HSCs generation

Finally, the specific temporal and spatial sequence of extrinsic signals and the combination and/or the levels of intrinsic signals, play a role in the differential transcription factor expression and production of the distinct waves of hematopoietic cells in the developing embryo and steady as well as stress hematopoiesis in adults.

1.3 BIOLOGY OF HSCs

1.3.1 HSCs as quiescent cells: a protection mechanism that favors their resistance

Hematopoiesis is the tightly regulated process of blood formation. Short-lived mature blood cells are replenished every day by HSCs through a large number of hematopoietic progenies, as multipotent hematopoietic progenitors and more restricted lineage-committed progenitors. HSCs are the first identified and best characterized adult stem cells, and they have served as model for other kind of adult stem cells.

A common property of adult HSCs is **quiescence** in term of cell cycle. Quiescence and slow cell cycle progression are critical for sustaining self-renewing HSC compartment throughout life. Moreover, quiescence is responsible for the protection of HSC pool from various stress insults. Indeed, quiescent cells are protected from replication-associated mutation generation and they are also more resistant to radiation than other cells. This feature is due to expression of repair, anti-oxidant and anti-apoptotic machinery. Ito and colleagues demonstrated that the Ataxia Telangiectasia Mutated (ATM) gene, which maintains genomic stability in response to DNA damage, has an essential function in the reconstitutive capacity of HSCs, but is not as important for the proliferation or differentiation of progenitors. $Atm^{-/-}$ mice show progressive bone marrow failure resulting from a defect in HSC function that was associated with elevated reactive oxygen species (ROS). Treatment with anti-oxidative agents restored the reconstitutive capacity of $Atm^{-/-}$ HSCs, resulting in the prevention of bone marrow failure (Ito et al. 2004). These results show that the self-renewal capacity of HSCs

depends on inhibition of oxidative stress and its associated DNA damages which can alter genomic stability.

In addition, HSCs present a different metabolic status compared to their progenitors: they show **low oxidative phosphorylation** and **high glycolytic activity to synthesize ATP**, meaning that HSCs favor anaerobic enzymatic pathways to synthesize ATP. These data demonstrate that HSCs are in hypoxic conditions and are metabolically different from their progenitor cells (Fig.3). This specific metabolic status permits to the more immature stem cells to be protected throughout life and is responsible for their resistance to anti-cancer drug treatments and to stress insults such as oxidative and genotoxic insults.

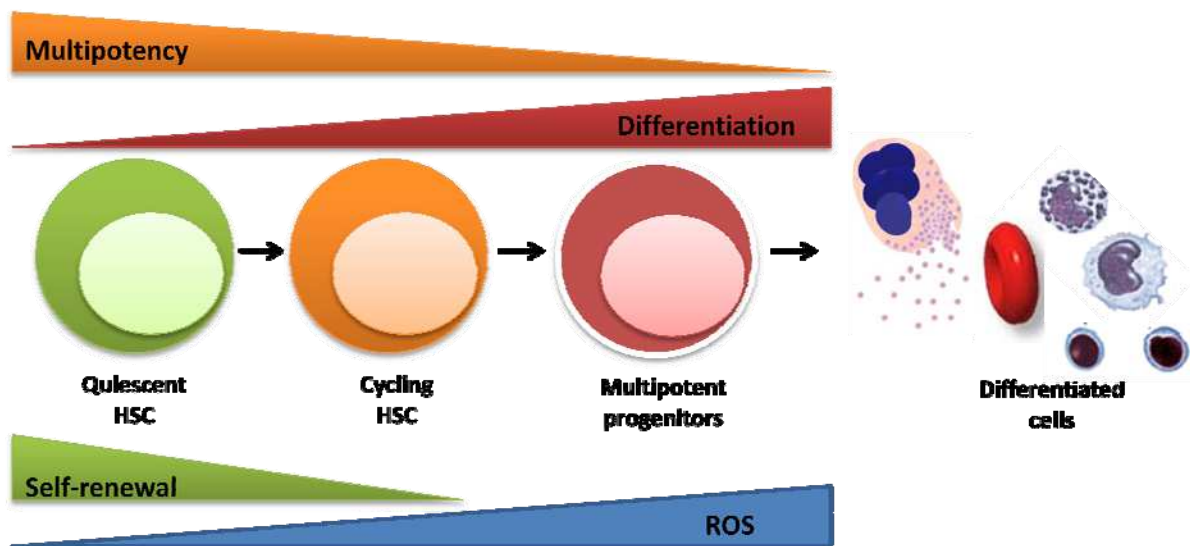


Figure3: cycling HSPCs have increased ROS levels. HSCs are quiescent and may proliferate to generate short-cycling HSCs that give rise to multipotent progenitors and differentiated cells. Most immature HSCs exhibit very low ROS levels and they are protected from oxidative insults. On the other side, more proliferative cells, such as cycling HSCs and progenitors, present increased ROS levels compared to immature HSCs. Low ROS in quiescent cells prevent oxidative stress and favor their resistance and long-live capacity.

1.3.2 Regulation of self-renewal and maintaining of HSCs

Hematopoiesis must be rapidly and meticulously regulated, in order to face to a variety of situations, ranging from normal homeostasis to acute blood loss, infection and to avoid over-production of mature cells. A wide variety of factors critical for HSC regulation have been identified, including cytokines, growth factors, transcription factors, chromatin modifiers and cell cycle regulators. All these factors have been long-time studied as they potentially represent a mean for maintaining HSCs *in vitro*, a feature that would have

significant impact on the collection of HSCs for transplantation and on gene therapy strategies. Below, some examples of both intrinsic and extrinsic regulators that have been shown to be important in adult HSC maintaining and self-renewal are reported.

1.3.2.1 Cell-intrinsic regulators of adult HSC maintaining

Valuable knowledge about factors influencing self-renewal, has been gained from gene manipulation studies, which have identified multiple proteins that play important roles in the regulation of HSC self-renewal, including transcription factors, epigenetic modifiers, and cell cycle regulators. The most studied genes are reported below.

Homeobox genes

One of the first genes described involved in HSC self-renewal is HoxB4. The homeobox (Hox) genes encode transcription factors that regulate embryonic body patterning and organogenesis. Several members of the homeobox gene family are expressed during hematopoietic differentiation. Over-expression of HoxB4 by retroviral infection of murine bone marrow cells leads to expansion of HSCs *in vivo* and *in vitro*, therefore appearing to be a positive regulator of HSC self-renewal (Sauvageau et al. 1995, Kros1 et al. 2003). Surprisingly, HoxB deficient mice do not exhibit any major defects in hematopoiesis, possibly because of the compensatory effects by HoxA and/or HoxC genes (Bijl et al. 2006).

It is also interesting to note that the over-expression of the HoxB4 paralogue HoxC4 in primitive hematopoietic cells also enhances the proliferation of both HSCs and committed progenitors, although the HoxC4's effect may be weaker than HoxB4's (Daga et al. 2000, Auvray et al. 2012). HoxB4 is a potent enhancer of primitive hematopoietic cell growth and this action may be explained by its interaction with c-Myc gene and the cell cycle machinery (Antonchuk, Sauvageau and Humphries 2001).

Ikaros

The Ikaros gene product is a zinc-finger transcription factor. Ikaros proteins modulate transcription by recruiting co-repressor complex to the promoter of target genes. Ikaros displays crucial functions in the hematopoietic system and mice homozygous for Ikaros dominant negative mutation (DNA-binding domain mutated) possess no measurable repopulating activity at all, conferring to Ikaros factors an essential role in the maintaining of the HSC pool (Nichogiannopoulou et al. 1999).

Tel/Etv6

The transcription factor Tel (Translocation Ets leukemia; also known as Etv6 [Ets variant gene 6]), the product of a locus frequently involved in translocations in leukemia, is a selective regulator of HSC survival. Following inactivation of Tel/Etv6, HSCs are lost in the adult bone marrow. Intriguingly, its inactivation in hematopoietic lineages does not affect neither their proliferation nor their survival (Hock et al. 2004), meaning that it acts selectively on immature hematopoietic cells.

Gfi1

Gfi1 (Growth factor independence 1) is a Zinc-finger repressor. When Gfi1 is deleted in mice, HSC frequencies are significantly reduced and Gfi1^{-/-} bone marrow cells are severely impaired in competitive long-term reconstituting abilities after transplantation. Gfi1 deficient bone marrow cells show a surprisingly high proportion of actively cycling HSCs, suggesting that Gfi1 restrains HSCs proliferation increasing their self-renewal ability (Zeng et al. 2004).

The Polycomb group proteins (PcG)

The Polycomb group proteins are a family of proteins that can remodel chromatin in order to epigenetically silencing gene expression. Members of the Polycomb repressor complex 1 (PRC1), especially Bmi1, have been implicated in HSCs self-renewal. Part of the mechanism by which PRC1 sustains HSC self-renewal is by repression of genes that promote lineage specification, cell death and cell cycle arrest (Park et al. 2003, Molofsky et al. 2003).

1.3.2.2 Cell-extrinsic regulators of HSCs

Adjacent cells and blood supply HSCs self-renewal and differentiation decision, bringing a number of cytokines, growth factors or hormones. Among these, Stem Cell Factor (**SCF**), Thrombopoietin (**TPO**), Fms-related tyrosine kinase 3 ligand (**Flt3-L**), Interleukin-11 (**IL-11**), Interleukin-3 (**IL-3**), Interleukin-6 (**IL-6**), Granulocyte Macrophage-colony stimulating factor (**GM-CSF**) and combination of these, have been used for *in vitro* HSC expansion. These cytokines allow short-term HSC maintenance for a few days but by themselves are not sufficient to sustain HSC self-renewal, leading to a progressive depletion of long-term repopulation capacity and differentiation of HSCs (Glimm and Eaves 1999, Le Goff, Le Hesran and Robert 1998). Detailed studies of purified murine HSCs have shown that

the receptors for TPO and SCF, c-mpl and c-kit respectively, are expressed on repopulating HSCs. Moreover, mice with genetic mutation in TPO or c-mpl genes have a dramatically reduction in HSCs, demonstrating a physiological role for TPO and its receptor in regulating HSC production and function (Solar et al. 1998, Kimura et al. 1998) .

The cocktail of SCF, Flt3, and TPO (mouse and humans) or IL-11 (mouse) is defined as the best cytokine cocktail medium supporting short-term Hematopoietic Stem and Progenitor Cells (HSPCs) proliferation and survival in *in vitro* cultures.

These cytokines are present in blood and are also synthesized by HSC surrounding cells within the niche. These cells also secrete other HSC regulators which lead to the activation of proper signaling pathways in HSCs. Among these, there are the **Tie2-Ang1**, **BMP-TGF**, **Sdf1/CXCR4**, **Notch**, **Wnt** and **JAK/STAT** signaling pathways. The role of the niche and of these crucial factors in HSC pool maintaining will be discussed below in the “Hematopoietic stem cell niche” paragraph.

1.4 IDENTIFICATION AND ISOLATION OF HUMAN HSCs

The stem cell population constitutes only a small percentage of the total number of hematopoietic cells in bone marrow and blood. Identifying HSCs will allow for comprehension of their biology. Moreover, the availability of stem cells is extremely useful for bone marrow transplantation and for gene therapy. Since few decades ago, world-wide efforts have been done in order to isolate, characterize and biologically analyze HSCs.

1.4.1 The sources of human HSCs

The known sources of human HSCs are the **bone marrow (BM)**, the **umbilical cord blood (CB)** and the **peripheral blood (PB)**. In the BM, HSCs are rare cells representing the 0.01% (1 every 10.000 cells) of the all population. Even if the BM represents the source with the majority of HSCs, it has been known for decades that stem and progenitor cells circulate, in low percentage, also in the blood stream. Researchers have observed that HSCs can be mobilized to the blood stream upon granulocyte-colony stimulating factor (G-CSF) treatment, increasing their percentage in peripheral blood. Another source of HSCs recognized by

researchers since the late 1980s is the umbilical cord blood. This tissue is responsible for the support of the developing fetus during pregnancy, is delivered along with the baby and is usually discarded, so it is easier to be obtained compared to BM or PB. These features make the CB the most used sample as source of HSCs.

1.4.2 Experimental systems to identify HSCs

The characterization of human HSCs has been possible by making use of experimental systems that have permitted their identification on the basis of their biological properties. *In vitro* and *in vivo* approaches that permit the identification and isolation of human HSCs are summarized below.

In vivo models

The most stringent assay to identify cells with high stemness potential is by testing their blood system reconstitution capacity in irradiated mice. It consists in injecting stem and progenitor cells into recipient mice and evaluates their capacity to give rise to all blood lineages. However, it is important to underline that to study human hematopoiesis in *in vivo* models the host should not eliminate the xenograft via an immune reaction and, should provide a permissive microenvironment for engraftment and multilineage differentiation of donor cells. Immunodeficient mice present these criteria and have been modified to improve their model function. Early studies were done in **SCID (Severe Combined Immunodeficiency) mice** that lack of T- and B-cell defect (McCune et al. 1988). However, these mice still possess macrophages and NK cells that can mediate rejection of xenografts. More recently, SCID mice were crossed to non-obese diabetic (NOD) mice that display partially deficient NK cell, antigen-presenting cell, and macrophage functions. **NOD-SCID mice** are currently used in *in vivo* studies of hematopoiesis and they display a 10-20-times better engraftment capacity than the SCID mice. However, NOD-SCID mice engraftment can be monitored for about 6 months because of the limited life-span of these mice. More recently, the **NOD/SCID/IL-2Rg null (NOG) strain** was shown to exhibit significantly higher engraftment potential than other immunodeficient mouse strains (Shultz et al. 2005).

In vitro assay

In adults, the majority of primitive hematopoietic cells are located in the bone marrow, where they are in contact with a variety of cells and molecules that influence their quiescence,

self-renewal, and differentiation. The best and stringent *in vitro* assay of HSC activity is the **long term culture-initiating cell (LTC-IC)** assay and it was reported in 1989 by Sutherland and colleagues (Sutherland et al. 1989). This test has provided an approach for the investigation of the regulation and maintenance of HSCs under conditions that mimic the marrow microenvironment. LTC-IC approach provides proper evidence of HSC activity without recurring to *in vivo* models. In a first step, candidates HSCs are cultured on bone marrow-derived stromal cells, in a 30% serum medium for 5 weeks or longer. In a second step, cells are transferred in a semi-solid medium of methyl cellulose containing cytokines, for about 2 weeks. Each HSC will be able to give rise to multiple differentiated colonies. Finally, the total number of myeloid, erythroid and multilineage clonogenic progenitors is evaluated and used to provide a quantitative assessment of the number of LTC initiating cells originally added (Fig.4). This long-lasting test is now largely used and it is the most stringent assay to identify human most immature hematopoietic stem cells.

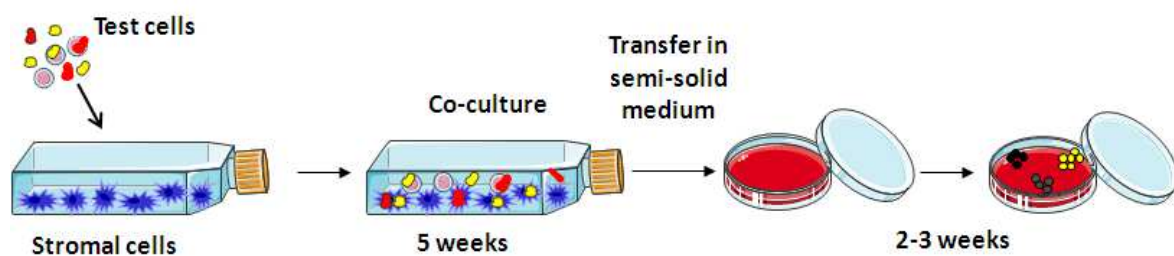


Figure 4: The LTC-IC *in vitro* assay to identify primitive hematopoietic cells. In the LTC-IC assay, stem and progenitor cells are co-cultured with bone marrow stromal cells for at least 5 weeks. Then, they are transferred in a semi-solid medium of methyl cellulose containing cytokines for about 2 weeks. The number of clonogenic progenitors raised is evaluated and used to provide an assessment of the number of LTC initiating cells.

1.4.3 Isolation of candidate human HSCs by their surface antigen markers

In vivo and *in vitro* studies together permitted the identification of a population of HSCs that was consequently characterized by the presence of different antigen markers. Detecting the expression of these marker panels allows separation of specific HSC-enriched cell populations via techniques like fluorescence-activated cell sorting (FACS).

Most of the studies on HSC characterization have been done in mice, but it is now clear that there are considerable differences in distribution of surface markers between human and mouse hematopoietic cells, which makes identification of human counterparts of mouse stem and progenitor cells more complicated. Importantly, **mouse LongTerm (LT)-HSCs are**

CD34^{-/low}, CD38⁺ and CD90 (Thy1)^{low}, whereas human HSCs are hCD34⁺, hCD38⁻ and hCD90⁺.(Iwasaki and Akashi 2007)

The major representative and most used marker for human hematopoietic stem and progenitor cells was the **CD34 antigen**. The role of CD34 in human HSPCs is poorly known; studies in mice in which human CD34 antigen was ectopically expressed on the cellular surface reveal a role for CD34 in adhesion to BM stromal cells, elucidating a possible role for CD34 in regulation and compartmentalization of stem cells (Healy et al. 1995). However, mutant mice do not exhibit any defect in peripheral blood counts and answer to hematopoietic stress as well as wild-type mice (Cheng et al. 1996). In conclusion, CD34 seems to not be essential for hematopoiesis mice. Moreover, the fact that mouse LT-HSCs in steady-state bone marrow do not express significant levels of mCD34 raised an important question of whether hCD34⁺ cells could mark all long-term self-renewing human HSCs. Human CD34⁺ cell population is a heterogeneous population made of stem cells but also of progenitor cells. Among the CD34⁺ cells, just the 1-10% does not present any marker of lineage differentiation (Lin⁻) as CD3, CD4, CD8, CD11b and CD14. Oppositely, the majority of CD34⁺ cells (90–99%) co-express the CD38 antigen, and this subset contains most of the lineage-restricted progenitors. Indeed, **CD34⁺CD38⁻** cells and not CD34⁺CD38⁺ cells are highly enriched for LTC-IC (Petzer et al. 1996) and contain NOD-SCID- repopulating cells (Bhatia et al. 1997). However, although most human HSCs are described as CD34⁺, the existence of CD34⁻ HSCs in human and rhesus monkey have been described (Goodell et al. 1997). Recently, Anjos-Afonso and colleagues showed that in addition to CD34⁺ HSPC population, there is a rare CD34⁻ population with severe combined immune-deficiency repopulating capacity. This **CD34⁻CD38⁻CD93^{hi}** population contains cells that not only function as HSCs, but that can also be placed above CD34⁺CD38⁻ population in hematopoietic hierarchy (Anjos-Afonso et al. 2013). Thus, CD34⁻CD38⁻CD93^{hi} population contain more bona fide HSCs than CD34⁺CD38⁻ population and the CD34 marker might be used as a positive marker anymore, but rather as a negative marker of most immature HSCs in addition to CD38 negative and CD93 positive markers.

Another well-established marker for HSCs enrichment is the **CD90 (Thy-1)** antigen, which is present in humans and not in mice (Baum et al. 1992). Other markers as the **CD45RA negative marker** was assumed to be specific for the most immature human HSC population (Majeti, Park and Weissman 2007).

In conclusion, it was supposed that a purified human HSC LTC-IC capable to engraft immune-compromised mice and to generate all the blood lineages is mostly a Lin⁻

CD34⁺CD38⁻ cell. However, recent evidences seem to favor the hypothesis that most immature HSCs possess the CD34 negative marker and that CD34⁻CD38⁻CD93^{hi} population can be placed at the top of hematopoietic hierarchy.

1.5 HEMATOPOIETIC STEM CELL NICHE

HSCs reside as rare cells in the bone marrow and depend on their microenvironment for regulation of self-renewal and differentiation. This microenvironment, which consists of hematopoietic and non-hematopoietic (stromal) cells, is a specific anatomic location called **niche**. A lot of efforts have been done to decipher the interplay between a hematopoietic cell and its niche. A historical perspective starting from the first concept of niche formulated in 1971 until the notions we have nowadays on this complicated but intriguingly system is reported below.

1.5.1 Historical perspective on the hematopoietic stem cell niche

The first experiments evidencing the concept of niche were performed in 1968 in Trentin's laboratory. They demonstrated that both bone marrow and spleen stromal cells have an active role in the regulation of differentiation of HSCs into all blood lineages type. Moreover, they demonstrated that marrow and spleen organ stroma are geographically segmented into microenvironments and that in spleen stroma the predominant microenvironment dictates erythroid differentiation, whereas in marrow stroma the predominant microenvironment dictates neutrophilic granuloid differentiation. The hematopoietic organ stroma were thereby termed **hematopoietic inductive microenvironments (HIMs)** (Trentin 1971).

Trentin formulated the concept of HIMs as a differentiation inducing microenvironment. This observation was expanded by Schofield in 1978. Schofield observed that HSCs need to reside in the bone marrow to retain their infinite potential, whereas those homed to the spleen were more restricted to their capacity to sustain hematopoiesis. He proposed that, in addition to a differentiation microenvironment, there was also a specific hematopoietic microenvironment which fixed the stem cells in place and prevented their maturation, allowing the stem cells to proliferate and retain their stemness. Schoefield

formulated that **“The stem cell is seen in association with other cells that determine its behavior”** (Schofield 1978). The concept that stem cells fate is determined by surrounding cell structure is nowadays referred to as the **stem cell microenvironment or the stem cell niche**.

In 2003, two reports published on Nature journal demonstrated by *in vivo* experiments in two different mouse models, that bone-forming osteoblasts were critical component of the niche, being capable of influencing HSC size and number (Calvi et al. 2003, Zhang et al. 2003). However, few years later, Kiel and colleagues suggested the role of another hematopoietic stem cell niche, which was rather formed by the endothelial cells within the bone marrow (Kiel et al. 2005).

Nowadays, it is clear that numerous elements of the BM intersect each other to appropriately regulate HSC fate choices and that it is inappropriate to say that HSCs are regulated just by osteoblast or by endothelial cells. Many other cells interplay with HSC and regulate their fate, as monocyte-macrophages, megakaryocytes and nervous system cells. Understanding how niche regulate HSCs is becoming more and more attractive for stem cell regenerative therapies, where cell proliferation and differentiation must be controlled *in vitro*, so that sufficient quantity of the proper stem cells can be produced prior to being introduced back into the patients.

1.5.2 Cell type forming HSC niche

The stem cell niche is the functional and anatomical ‘node’ that allows integration of signals from the periphery into the appropriate stem cell behavior. Osteoblasts and endothelial cells of the bone marrow niche have been long time studied, as the two major components of the niche that support maintenance, proliferation and differentiation of HSCs. Two different niches were proposed: the endosteal niche, where HSCs are closed to osteoblasts, and the perivascular niche, where HSCs are closed to vascular endothelium in marrow sinuses. Much has been debated over the distinction of a single niche, yet, to this day, consensus has not been reached in determining either over the other. This is because the periosteal region of the BM is a sponge-like trabecular complex structure where dormant HSCs are influenced not only by osteoblasts and vasculature but also by other stromal structures in quite close proximity (Fig.5). To date, it is in fact known that in addition to osteoblasts and endothelial cells, there are also reticular stromal cells, macrophages, megakaryocytes, adipocytes, regulatory T-cells and nervous system cells as key component of the BM niche, regulating

HSCs. Therefore, the concept of the BM niche should be viewed as a **conjoined influence of different cellular components**.

The most studied cell types present in the BM niche and how they are able to regulate HSC maintenance are discussed below.

Osteoblasts and mesenchymal stem cells (MSCs)

Osteoblasts are mononucleated cells that are responsible for the bone formation. Bone tissue undergoes a constant process of remodeling and is constantly being reshaped by osteoblasts, which produce matrix and minerals, and osteoclasts, that are responsible for the resorption of the tissue.

Different cells of the osteoblastic lineage are present in the bone marrow: mesenchymal stem cells (MSCs) (the multipotent stromal cells that give rise to osteogenic lineages), osteoprogenitor cells, osteoblast and osteocytes.

Osteoblasts are usually found in layer along the endosteum (the connective tissue around the medullary cavity) and the periosteum (the membrane that lines the outer surface of the bone). *In vitro* results obtained in 1996 seem to favor the hypothesis that human osteoblast can support HSCs in *ex vivo* culture system (Taichman, Reilly and Emerson 1996). Based on these observations, Zhang and colleagues and Calvi and colleagues examined the osteoblast regulation of the niche by two different *in vivo* approaches. Zhang and colleagues made use of a mouse model in which the Bone Morphogenetic Protein Receptor1A (BMPR1A) was conditionally deleted. They found that in these mice there was an ectopic formation of a trabecular bone-like area (TBLA) and an increase of N-Cadherin-positive osteoblasts. The formation of the TBLA correlated with an increase of the number of HSCs and these HSCs were associated with osteoblast by N-Cadherin (Zhang et al. 2003). Calvi and colleagues examined the effects of the constitutively activated parathyroid hormone-related peptide receptor (PPR) under the control of a specific osteoblast-collagen promoter. They found that in PPR transgenic mice there was a size increase of the trabecular bone which correlated with an increased number of osteoblasts and an increased number of HSCs in conjunction with the trabecular bone. Moreover, they found that these HSCs had a more activated Notch1 pathway than HSCs of wild-type mice. (Calvi et al. 2003).

However, studies in which osteoblast number is decreased do not show a reduction in HSCs. Vinjic and colleagues made use of a mouse model in which deletion of osteoblast was possible by the expression of thymidine kinase under the control of an osteoblast-specific promoter and the consecutive treatment with gancyclovir. In gancyclovir-treated mice there

was a decreased of marrow size and a decreased number of HSCs in the bone marrow. However, they found an increased extramedullary hematopoiesis in spleen and in the liver, resulting in an increase in peripheral HSCs and active primary *in vitro* hematopoiesis (Visnjic et al. 2004).

Recently, also mesenchymal stem cells (MSCs) have been shown to play a role in HSC regulation by secretion of specific factors. For instance they express, in association with osteoblasts, the tissue inhibitor of metalloproteinase-3 (TIMP3), to influence not only HSC quiescence and fate determination, but also normal bone formation and maintenance (Shen et al. 2010, Nakajima et al. 2010). Moreover, agrin, a proteoglycan involved in neuromuscular junctions, is expressed by MSCs and differentiated osteoblasts, while its receptor, alpha-dystroglycan, is expressed by HSCs. Agrin was shown to support the proliferation of hematopoietic progenitor cells and a microenvironment devoid of agrin results in progenitor cell apoptosis as well as reduced hematopoiesis (Mazzon et al. 2011).

In conclusion, both MSCs and osteoblasts contribute to HSC regulation in the endosteal niche of the bone marrow.

The endothelial cells

In bone marrow, endothelial cells form a barrier between the marrow and the blood circulation. They are the site of entrance of all blood cells that leave the blood circulation to enter into bone marrow and the site of exit for cells that are ready to enter the blood stream.

A decade ago it was shown that endothelial cells regulate proliferation of hematopoietic progenitor cells and long-term culture initiating cells by elaboration of lineage-specific cytokines in *in vitro* system (Rafii et al. 1995). These results were then validated by the use of human brain endothelial cells (Chute et al. 2002) and heart, lung and liver endothelial cells (Li et al. 2004). Moreover, *in vivo* confocal imaging of bone marrow mice revealed that HSCs can localize to specific subset of the marrow microvasculature where cells persist or increase in number (Sipkins et al. 2005). Finally, another group found that the majority of HSCs are located in the sinusoidal endothelium region and that only the 16% of these cells localize at the periendosteal region (Kiel and Morrison 2006).

Endosteal monocytes and macrophages

Also monocytes and macrophages can importantly contribute to HSC regulation in the BM niche. In a study of G-CSF mobilization of HSCs from the BM, Winkler and colleagues found that there was a depletion of endosteal monocytes or macrophages. *In vivo* depletion of

macrophages, recapitulated the HSC mobilization into blood (Winkler et al. 2010). Reduced endosteal monocyte-macrophage populations coincide with reduced levels of HSPC-active factors, like CXCL12, in the niche, providing a potential mechanism of how these myeloid cells function in HSC regulation (Chow et al. 2011). Together, these results establish that bone marrow monocytes and macrophages are pivotal to maintain the endosteal HSC niche and that the loss of such monocytes/macrophages leads to the egress of HSCs into the blood.

Cells of the nervous system

In 2011 Yamazaki and colleagues proposed that also particular nervous system cells can play a role in niche regulation of HSCs. They found that non-myelinating Schwann glial cells in BM are TGF-beta-producing cells and express HSC niche factor genes. Moreover, they are in contact with a substantial proportion of HSCs. A nerve denervation reduces the number of these active TGF-beta-producing cells and leads to rapid loss of HSCs from BM. Thus, they propose that glial cells are components of BM niche and maintain HSC quiescence by regulating activation of latent TGF-beta (Yamazaki et al. 2011).

Reticular stromal cells

CXCL12 abundant reticular (CAR) cells are a population of reticular cells, which express CXCL12 at high amounts, with several long processes and scattered throughout adult BM niche. Most cells expressing high amounts of CXCL12 are not in close proximity to the bone surface but rather they are scattered throughout the trabecular space of the BM cavity (Sugiyama et al. 2006). When BM CXCL12-expressing cells are ablated *in vivo*, HSC number and size decrease, they are more quiescent and they express myeloid genes (Omatsu et al. 2010). Thus, CAR cells are required for proliferation of HSCs as well as maintenance of HSCs in an undifferentiated state thanks to their ability to express CXCL12 and consequently to retain HSCs in BM, preventing their mobilization to vasculature.

Megakaryocytes

Another mature cells type with key HSC regulatory roles are the megakaryocytes (MKs). TPO, through its receptor c-mpl, favors platelet productions from MKs as well as HSC self-renewal. MKs control the availability of TPO and in an environment where the number of MKs is increased, like in Myb or p300 mutated mice, TPO is less available and HSCs show an increase in cell cycle and a decrease in number (de Graaf et al. 2010).

In conclusion, the bone has a peculiar role in hematopoiesis regulation and it is composed of different kind of cells that create a cross-talk with HSCs and impact their residency and function. Research is evolving to better understand how these cells are anatomically and functionally organized, in a 3D structure, and how the BM architecture changes with disease. If the BM niche can be mimicked *in vitro*, this will not only allow for HSC expansion and differentiation *in vitro*, but will also increase understanding and ability to manipulate HSCs for more robust engraftment and mobilization. Although more research is required to ascertain how they interact with each other, MSCs, osteoblasts, nerves, reticular stromal cells and endothelial cells, as well as macrophages and megakaryocytes should all be considered key components of the HSC niche, playing a critical role in regulating HSC fate.

1.5.3 Microenvironment-dependent signals which regulate hematopoiesis

HSCs are normally quiescent cells that reside in the bone marrow. In response to hematopoietic stress, they are able to divide, to differentiate and to replenish the hematopoietic system. The different choices of a HSC are regulated by its microenvironment and this tight regulation involves different microenvironment-dependent signaling pathways. The most important are described below.

Tie2-Ang1 signaling pathway and N-Cadherin

Tie2-Angiopoietin1 (Ang-1) signaling pathway is required to maintain HSC quiescent at the endosteal surface. Indeed, HSCs that express the tyrosin kinase receptor Tie2 are quiescent and anti-apoptotic; the interaction of these cells with the Tie2-ligand, Ang-1, permit the maintaining of *in vivo* long-term repopulating activity of HSCs. Moreover, Ang1 enhances the ability of HSCs to remain quiescent and to adhere to the bone (Arai et al. 2004).

One target of Tie2 signaling is N-Cadherin; this adhesion protein was shown to be implicated in niche-HSC interaction too. Over-expression of N-Cadherin in HSCs promotes quiescence and preserves HSC activity during serial bone marrow transplantation. Moreover, N-Cadherin knock-down inhibits the HSC localization to the endosteal surface of bone marrow and decreases long term engraftment. Oppositely, N-Cadherin knocked down cells in the spleen do not exhibit any activity defects, meaning that this adhesion molecule is essential for proper HSC regulation in bone marrow niche (Arai et al. 2012).

VEGF

The vascular endothelial growth factor (VEGF) is a stimulator of angiogenesis and a key mediator of the interaction between osteoblasts and vascular endothelial cells. VEGFR2 is the main receptor expressed by the osteoblasts, osteoclasts as well as endothelial cells within the bone marrow. VEGF can couple osteogenesis and angiogenesis by stimulating survival and proliferation of endothelial cells and osteoblastic lineage commitment of osteoprogenitors (Maes et al. 2010). VEGF was also shown to be essential for HSC maintaining by an autocrine loop control (Gerber et al. 2002) and for the correct hematopoietic niche formation (Chan et al. 2009).

BMP-TGF pathway

Bone Morphogenetic Proteins (BMP) is a group of growth factors that interact with their receptors (BMPR) at the cellular surface and activate mobilization of the SMAD proteins. BMP4 is expressed in cells of the hematopoietic stem cell microenvironment, including osteoblasts, endothelial cells and megakaryocytes. BMP4 is implicated in stromal cell regulation of embryonic HSC in the AGM region (Durand et al. 2007) as well as adult HSC *in vivo* (Goldman et al. 2009). One receptor of BMP is BMPR1 and it is found to be highly expressed by osteoblasts. As described before, BMPR1 deficiency strongly decreases the number of HSCs capable of repopulating activity (Zhang et al. 2003).

Sdf1/CXCL12 and CXCR4

Stromal derived factor (Sdf1), also called C-X-C motif chemokine (CXCL12), is a chemotactic molecule produced by stromal cells and its receptor, CXCR4, is highly expressed on earliest HSCs.

Sdf1 is capable to regulate the attachment of the HSCs to the niche and consequently to modify the number of HSCs in the blood circulation, by regulating soluble-kit release via the matrix metalloproteinase1 (MMP1) (Heissig et al. 2002). Moreover, Sdf1/CXCR4 signaling is not only essential to confine HSCs to their proper niche, but also to maintain these cells in a quiescent status (Nie, Han and Zou 2008).

Notch pathway

The Notch receptor is a single-pass transmembrane receptor protein, composed of a large extracellular domain and a smaller intracellular domain. In mammalian, members of the

Delta-like (DLL1, DLL3, DLL4) and Jagged (JAG1, JAG2) families serve as ligands for Notch signaling receptors.

In murine hematopoietic stem cells, LSK (Lin⁻, Sca1⁺, c-Kit⁺) cells, an over-expression of the active intracellular Notch1 domain is responsible for self-renewal (Varnum-Finney et al. 2000). Moreover, *in vitro* results obtained from cell line cultures, show that the interaction between Jagged1(JAG1) ligand and Notch1 sustains the long-term maintenance of hematopoietic progenitor cells and impairs differentiation, suggesting a role in cell fate decision for JAG1 and Notch signaling pathway (Li et al. 1998). These *in vitro* results were strengthened by *in vivo* data in which Notch activation was shown to have a role in lymphoid over myeloid lineage commitment (Stier et al. 2002). More recently, it has been shown that Notch1 is expressed on HSCs and Notch ligands as DLL1 and JAG1 are expressed by osteoblastic cells, meaning that Notch1 could be a crucial mediator of HSC-niche crosstalk (Nobta et al. 2005, Calvi et al. 2003). Notch signaling seems to be very important for niche dependent HSC regulation.

Wnt pathway

Wingless (Wnt) signaling pathway is activated by the binding of a Wnt-protein to a Frizzled family ligand. Two different Wnt downstream signaling pathways may exist: the β -catenin-dependent canonical and non-canonical pathway. Both canonical and non-canonical pathway promotes self-renewal of HSCs in an intrinsically or extrinsically way, respectively (Luis et al. 2009, Murdoch et al. 2003). Moreover, blocking Wnt signaling in the niche, by the expression of a Wnt inhibitor under the control of a specific osteoblastic promoter, increases the number of proliferating HSCs and reduces their ability to reconstitute the hematopoietic system of irradiated recipient mice. This effect is microenvironment-dependent because if these HSCs issue from transgenic mice are transplanted in wild type mice, they are completely capable to reconstitute hematopoiesis (Fleming et al. 2008). These findings show that Wnt/ β -catenin activity is crucial for the cross-talk between HSC and their niche and the consequently maintain of HSCs.

The JAK/STAT pathway

Last but not least, another molecular pathway involved in HSC maintenance by the niche is the JAK/STAT (Janus family kinase–signal transducer and activator of transcription) pathway. It is a common downstream pathway of extracellular signaling (cytokine, growth

factors, hormones...) and plays critical role in transmitting a variety of biological functions by regulating transcription of target genes.

This pathway will be discussed in greater detail in the last paragraph.

Figure 5: Schematic picture of the HSC microenvironment. HSCs are located in a sponge-like trabecular structure and there is a conjoined influence of different cellular compartments. Among these there are osteoblasts, endothelial cells, mesenchymal stem cells (MSC), CXCL-12 abundant reticular cells (CAR), macrophages, megakaryocytes and adipocytes. Under steady state, they all interact with HSCs by secreting specific factors such as TGF-beta, CXCL-12 and Ang-1, and they promote the maintaining of their stemness properties. Once HSCs start cycling to produce new hematopoietic differentiated cells, they move to blood vessels, where they cross the endothelial barrier and reach the blood stream.

1.5.4 Hypoxia and reactive oxygen species (ROS)

As discussed before, the proper interaction between HSCs and bone marrow niche is crucial to prevent cell exhaustion from excessive proliferation. In addition, the niche protects HSCs from stress, like accumulation of reactive oxygen species (ROS) and DNA damages. Indeed, in the bone marrow niche there is an O₂ gradient from below 1% in hypoxic niche to

6% in sinusoidal cavity. In 2007, Parmar and colleagues showed, by making fractions of the bone marrow and using the hypoxic marker pimonidazole, that long-term repopulating cells are located predominantly at the lowest end of the O₂ gradient (Parmar et al. 2007). This finding leads to the observation that the level of oxidative stress influences the HSC function. Indeed, mice which lack ATM activity, the crucial factor for maintaining of genomic stability, show progressive bone marrow failure resulting from an excessive accumulation of ROS – and DNA damages- in HSCs (Ito et al. 2004).

The heterodimeric transcription factor hypoxia-inducible factor (HIF)-1 constitutes the major molecular response to hypoxia. It consists of two basic helix-loop-helix subunits: an oxygen-sensitive HIF-1 α subunit and an oxygen-insensitive HIF-1 β subunit. At normal oxygen levels, above 5%, hydroxylation of the proline residues 402 and 564 of HIF-1 α by specific HIF-prolyl-hydroxylase (PHDs) enables binding of an E3 ubiquitin ligase which leads to degradation of HIF-1 α by the proteasome. In contrast, in hypoxic conditions, at oxygen level below 5%, hydroxylation is inhibited, leading to stabilization of HIF-1 α . It seems that hypoxic conditions enhance “stemness” by HIF-1 α -mediated activation. In HIF-1 α -deficient mice, HSCs lose their cell cycle quiescence and HSC number decrease during various stress settings including bone marrow transplantation, myelosuppression, or aging (Takubo et al. 2010). Stemness maintenance by HIF-1 α involves regulation of genes involved in cell-cycle control as well as protection to harmful effects of mitochondrial ROS. HSCs have elevated HIF-1 α expression corresponding to low mitochondrial activity, and increased glycolysis activity instead of mitochondrial oxidative phosphorylation (Simsek et al. 2010).

2. The Chronic Myeloid Leukemia (CML)

2.1 WHAT IS CANCER?

An adult human being has approximately 10^{14} living cells in the body. Normally, cells grow, divide and die in an orderly way. During the early years of a person life, cells grow and divide faster, in order to produce the new tissues of which an individual is composed. After the person becomes an adult, most of the cells continue to divide just to replace the damaged or dyed cells. Cancer develops when cells lose their capacity to control their proliferation. Instead of dying, cancer cells keep on growing and form other cancer cells. Moreover, cancer cells acquire the capacity to move and invade other tissues different from the tissue where they originated. Indeed, they are able to enter into blood or lymph vessels, to move throughout blood stream and finally colonize new parts of the body, in a process called metastasis. Thus, cancer cells differ from normal cells essentially for their capacity to proliferate out of control and to invade new tissues of the body.

2.1.1 Leukemia

Leukemia is a kind of cancer characterized by an uncontrolled proliferation and development of hematopoietic cells. There are different kinds of leukemia, depending on clinical and pathological characteristics.

The first division is about its acute or chronic forms:

- the **acute** leukemia is characterized by an increase proliferation of immature blood cells, which are blocked in their ability to mature the way they should. It leads to a rapid progression and accumulation of functionally defective leukemic cells and, without treatment, most patients live only few months. Acute leukemia is the most common leukemia in children.
- the **chronic** leukemia is characterized by the progressive accumulation of more mature blood cells which exhibit some functionality. Normally, chronic

leukemia takes months and years to progress. It mostly occurred in aged people but can also occur in young people.

The second division concerns which kind of blood cell is affected in the leukemic process:

- if leukemic process takes place in a cell that expresses lymphoid markers, the leukemia is called “**lymphocytic**” or “**lymphoblastic**”, depending on its chronic or acute form, respectively.
- if the transforming cell express myeloid markers it is called “**myeloid**” or “**myelogenous**”.

As results of these subgroups there are 4 different major types of leukemia: **acute myeloid leukemia (AML)**, **chronic myeloid leukemia (CML)**, **acute lymphoblastic leukemia (ALL)** and **chronic lymphocytic leukemia (CLL)**. These 4 main groups of leukemia are further classified into subtypes based on specific features of cells.

2.2 THE CML DISEASE

The “**chronic myeloid leukemia**”, is called by several other names, like “**chronic myelogenous leukemia**”, “**chronic myelocytic leukemia**” and “**chronic granulocytic leukemia**”.

CML is a clonal myeloproliferative disorder of the primitive hematopoietic stem cell, characterized by the clonal expansion of terminally differentiated myeloid cells. The main observation in CML patients is a proliferation of mature granulocytes (neutrophils, eosinophils and basophils) and of their precursors.

The hallmark of CML is the presence of the **Philadelphia (Ph) chromosome** (Fig.6). The Ph chromosome was first discovered and described in 1960 by Peter Nowell from the University of Pennsylvania and David Hungerford from the Fox Chase Cancer Center and was therefore named as the city where both the facilities are located. The Ph chromosome is generated from a reciprocal chromosome translocation between chromosome 9 and chromosome 22. The break on chromosome 9 (region q34) leads to a mutation of *Abelson murine leukemia (ABL)* gene, while the break on chromosome 22 (region q11) involves the *Breakpoint Cluster Region (BCR)* gene. This chromosome translocation is designated as t(9;22)(q34;q11). Therefore, the mutated *ABL* gene moves to chromosome 22 and fuses with

the remaining portion of the *BCR* gene, creating an elongated chromosome 9 and a truncated chromosome 22, which is the Ph chromosome (Rowley 1973). The molecular consequence of this translocation is the leukemia-causing fusion oncogene *BCR-ABL*, which in turn translates into a BCR-ABL oncoprotein.

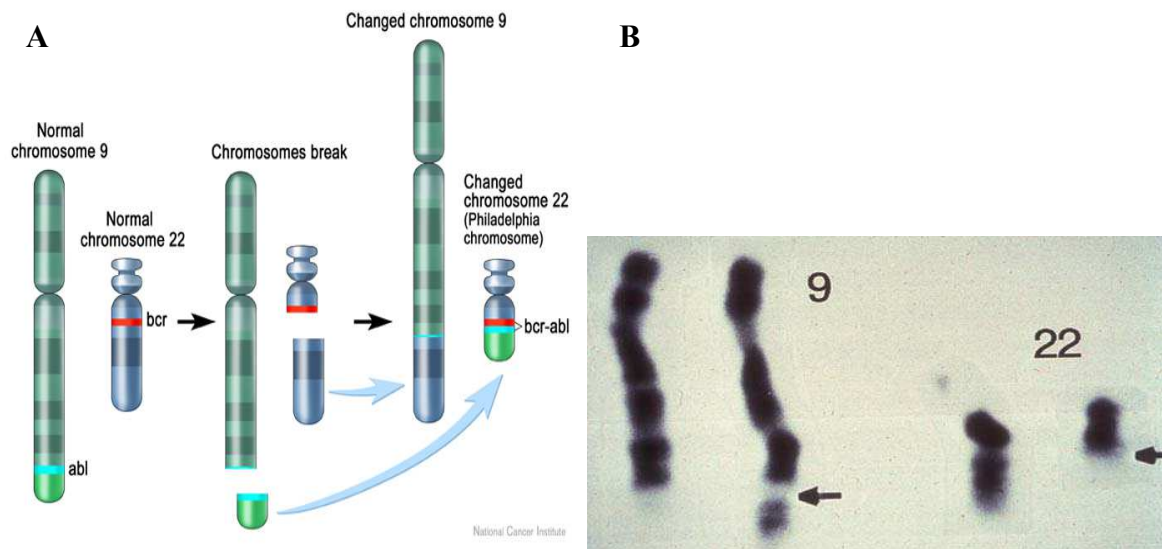


Figure 6: the t(9;22) that generates the Philadelphia chromosome. (A) schematic representation of the t(9;22). First of all, there is a break of a part of the long arm of the chromosome 9 that includes the *ABL* gene, and another break of the long arm of the chromosome 22 at the level of *BCR* gene. After the chromosome break, there is a reciprocal translocation of the *ABL* gene-included region of the chromosome 9 on the chromosome 22, resulting in the Ph chromosome. (B) Karyotype of an individual with CML. A chromosome preparation of cells at the metaphase stage of mitosis was stained with GIEMSA that binds AT-base pairs. The Ph chromosome (right arrow) results smaller than its counterpart. Left arrow indicates portion of chromosome 22 translocated on chromosome 9.

2.3 THE BCR-ABL ONCOGENE

More than 95% of patients with CML have *BCR-ABL* gene in their leukemic cells. Thus, *BCR-ABL* can be seen as the hallmark of CML. However, it is not exclusively to CML because it can be found in 10-20% of adults and 2-5% of children with acute lymphoblastic leukemia, as well as in occasional cases of acute myeloid leukemia, lymphoma, myeloma and chronic neutrophilic leukemia (CNL), which is a more benign variant of CML (Melo 1996). The precise location of breakpoint in *BCR* and in *ABL*, and thus the composition of the fusion BCR-ABL protein, determines the differences in the disease phenotype.

2.3.1 Structure of the various *BCR-ABL* fusion genes

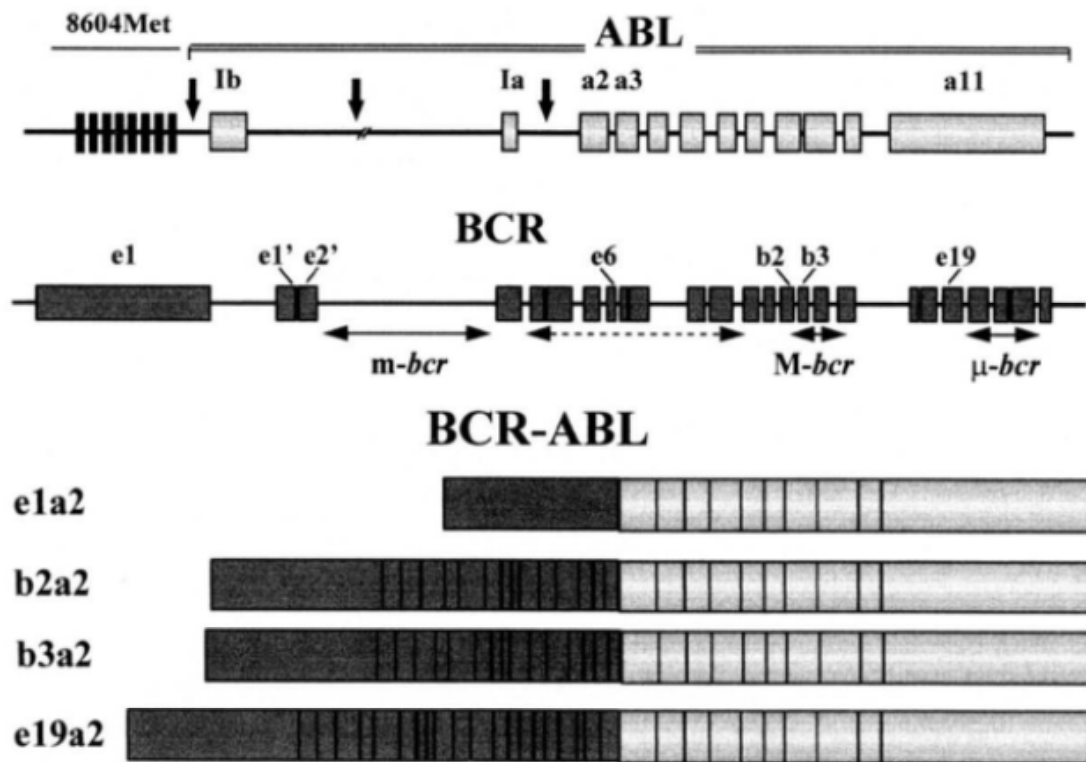
The *BCR-ABL* oncogene is located on the shorter derivative 22 chromosome. Depending on the precise location of the fusion, the molecular weight of BCR-ABL protein varies between 190, 210 or 230 kDa. For this reason BCR-ABL protein is usually called **p190**, **p210** or **p230**.

In figure 7 there is a schematic representation of the different transcripts that can be generated depending on the different breakpoints.

- *ABL* gene breakpoints: there are 3 potential breakpoints in *ABL* gene and they can occur in a >300kb region in the 5' end of the gene, either upstream the first exon Ib, between exon Ib and Ia, or downstream of exon Ia.
- *BCR* gene breakpoints: in the vast majority of CML patients and in one third of ALLs, the breakpoint in *BCR* gene is found within a 5.8 kb region known as the major breakpoint cluster region (M-bcr). In the M-bcr region there are 5 exons historically named b1 to b5, now known to be exons 12 to 16 of the *BCR* gene. The breakpoint can be found either in exon b2 or b3. In addition to these breakpoints which are found in the majority of CML cells, there are two other breakpoints: one in the first exon e1 within the minor breakpoint cluster region (m-bcr) and another in the exon e19 within the μ -bcr region.

Regardless of the position of the breakpoint of *ABL* gene, four different transcripts of *BCR-ABL* gene can be found, depending on the breakpoint position of the *BCR* gene. These different transcripts are:

- **e1a2 transcript** which encodes for a 190 kDa BCR-ABL protein (**p190**), found in very rare cases of CML and AML and in two thirds of ALLs.
- **b2a2 transcript** which encodes for a 210 kDa BCR-ABL protein (**p210**), found in more than 95% of CML and in one third of ALLs.
- **b3a2 transcript** which encodes for a 210 kDa BCR-ABL protein (**p210**), found in more than 95% of CML and in one third of ALLs.
- **e19a2 transcript** which encodes for a 230 kDa BCR-ABL protein (**p230**), found in CNL cells.



From (Melo 1996)

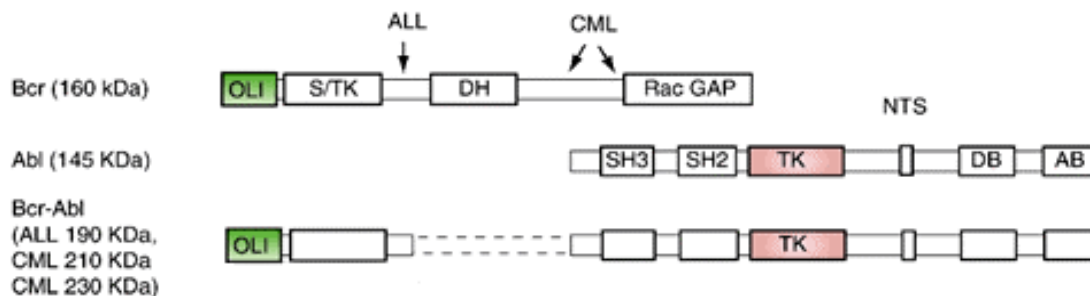
Figure 7: Schematic representation of the BCR and ABL genes disrupted in the t(9;22) . Exons are represented by boxes and introns by connecting lines. The 3 potential breakpoints in ABL gene are indicated with arrows and are located either upstream exon Ib, between exons Ib and Ia, or downstream exon Ia. The BCR gene contains 25 exons and two putative alternative exons (e1' and e2'). The breakpoints in BCR usually occur within one of three breakpoint cluster regions (bcr), the location and probable extents of each are shown by the three double-headed horizontal arrows. In exceptional cases the BCR breakpoints fall between m-bcr and M-bcr, within the region indicated by the double-headed dashed-line arrow. The lower half of the figure shows the structure of the various BCR-ABL mRNA transcripts which are formed in accordance with the position of the breakpoint in BCR.

2.3.2 BCR-ABL fusion proteins

BCR-ABL oncoprotein is not only a cancer marker, but also the causative lesion of CML. This indispensable role of BCR-ABL was demonstrated for the first time in 1990 by Daley and colleagues. They infected murine bone marrow cells with a retrovirus encoding p210 BCR-ABL followed by transplantation of infected cells into irradiated mice. Recipients developed several hematologic malignancies and, among them, there was a myeloproliferative syndrome closely resembling the initial chronic phase of the CML (Daley, Van Etten and Baltimore 1990).

BCR-ABL fusion protein is formed, as described above, by a head-to-tail fusion of the *BCR* and *ABL* genes. BCR-ABL proteins contain the N-terminal 927 or 426 amino acids for BCR in CML (p210) or Ph+ ALL (p190), respectively.

The human BCR protein contains 1.271 amino acids and multiple domains, including the oligomerization domain (OLI), the serine/threonine kinase domain (S/TK), the domain homologous to the human Dbl and yeast Cdc24 proteins (DH) and the domain with the Rac GTPase-activating protein (RacGAP). The ABL protein belongs to the non receptor family of tyrosine kinase and contains 1.097 amino acids. ABL protein includes: the Src-homology domain 3/2 (SH3/SH2), which bind to phosphotyrosine residues or proline-rich sequences, respectively, the tyrosine kinase (TK) domain, the nuclear translocation signal (NTS), the DNA binding domain (DBD) and the actin binding (AB) motif (Fig. 8). The two domains essential for transforming activity are the OLI from BCR and the TK from ABL.



From. (Zhao et al. 2002)

Figure 8: Structural and functional domains of BCR, ABL and BCR-ABL. The two domains essential for transforming activity—OLI from BCR and TK from ABL—are illustrated in green and red, respectively. Depending on the site of the breakpoint in the *BCR* gene, the fusion protein can vary in size, from 190 to 230 kDa. Each fusion protein contains the same portion of ABL protein but differs in the length of BCR portion. The breakpoints in BCR that give rise to CLL or ALL are indicated by arrowheads.

Oligomerization of BCR-ABL is essential for oncogenicity and this involves the N-terminal oligomerization domain. This domain presents a coiled-coil segment and two BCR-ABL monomers can dimerize by swapping their N-terminal helices. Followed by oligodimerization, two dimers stack onto each other to form an active tetramer that exhibit transforming activity (Zhao et al. 2002, McWhirter, Galasso and Wang 1993).

BCR-ABL fusion protein results in a constitutively active tyrosine kinase. Fusion to BCR unmarks the domain of actin binding at the C-terminal region of ABL, resulting in a massive localization of BCR-ABL in the cytoplasm.

2.3.3 Signaling pathways regulated in BCR-ABL cells

A crucial feature for BCR-ABL activity is oligomerization by their N-terminal coiled coil domains and auto-phosphorylation on its Tyr 177. This Tyr phosphorylation leads to maximal BCR-ABL kinase activity and is responsible for CML progenitor expansion, proliferation and survival. Blocking BCR-ABL-Tyr 177 mediated signaling enhances the effect of Imatinib, a BCR-ABL inhibitor, on progenitor survival. (Chu et al. 2007). The most relevant signaling pathways are represented in figure 9.

- **RAS pathway**: upon ligand binding and dimerization, activated Receptor Tyrosine Kinases (RTKs) recruit SH2 and SH3 containing adaptor molecules like Grb-2 into signaling complexes with Rat sarcoma (RAS) exchange factors at the cell membrane. These complexes trigger the accumulation of the active GTP-bound form of RAS. Similar to RTKs, BCR-ABL also activates RAS. The mechanism for BCR-ABL-dependent RAS activation involves at least 3 different adaptor proteins: **Grb-2**, **Gab-2** and **CRKL**. Each of these 3 factors can form a complex with BCR-ABL and can link BCR-ABL to RAS. Dominant mutants of Grb-2 can reverse the BCR-ABL transformed phenotype (Gishizky, Cortez and Pendergast 1995). Gab-2 adaptor also plays a role in BCR-ABL transforming activity and murine bone marrow Gab-2 knock-down cells are resistant to transformation by BCR-ABL (Sattler et al. 2002). Finally, CRKL adaptor seems to have a non-overlapping function with Grb-2 and it is the most prominent phosphor-protein in clinical CML cells and it is sufficient to recapitulate much of the activity of BCR-ABL (Nichols et al. 1994).
- **MAPK pathways**: a primary signaling event following RAS activation is activation of mitogen activated protein kinase (MAPK) signaling pathways. Three MAPK cascades have been well characterized: the extracellular regulated kinase (**ERK**), the stress activated protein kinase (**SAPK**) and the jun kinase (**JNK**) pathways. All of the 3 signaling pathways are activated in BCR-ABL expressing cells (Raitano et al. 1995).

- **PI3-kinase:** Phosphoinositide 3-kinase (PI3K) is a member of the phosphoinositide kinase superfamily that functions in the regulation of phosphoinositide lipid metabolism and generation of lipid second messengers involved in signal transduction. PI3K is a heterodimeric protein composed of a p85 regulatory subunit and a p110 catalytic subunit that is capable to phosphorylate both lipids and proteins. The p85 subunit is phosphorylated in BCR-ABL expressing cells (Gotoh et al. 1994) and form complexes with BCR-ABL through interaction with CBL and CRKL (Sattler et al. 1996). PI3K activation results in phosphorylation of **AKT**, which functions in: suppression of the activity of Forkhead box protein O (**FOXO**) transcription factors (Naka et al. 2010), proteosomal degradation of **p27** through upregulation of SKP2 (Agarwal et al. 2008) and activation of mammalian target of rapamycin (**mTOR**) which leads to enhanced protein translation and proliferation (Markova et al. 2010). All these molecule regulations participate to in increased survival and proliferation of BCR-ABL cells.

- **JAK/STAT pathway:** BCR-ABL is able to activate JAK/STAT pathway in hematopoietic cells. In CML cells, it has been shown that among the STAT factors, STAT5 and STAT3 are constitutively activated by BCR-ABL (Klejman et al. 2002). STAT5 is not only a BCR-ABL target factor, but also a pivotal protein for CML cell maintaining. Both STAT3 and 5, enhance **Bcl-xL** expression by direct binding on its promoter. (Dumon et al. 1999, Fujio et al. 1997). B-cell lymphoma extra Large (Bcl-xL) is a transmembrane molecule in the mitochondria. It is a member of the Bcl-2 family proteins and it acts as a pro-survival anti-apoptotic factor by preventing the release of mitochondrial contents such as cytochrome C which would lead to caspase activation and apoptosis. Bcl-xL anti-apoptotic network is not essential for development and maintenance of CML, but is required for disease progression in patients undergoing blastic transformation (Harb et al. 2013). The implication of STAT5 factor in CML development will be discussed in detail in the next chapter.

- **Molecules involved in cell adhesion:** CML cells migrate and adhere abnormally. This feature is due to the BCR-ABL down-regulation of some genes,

including the focal adhesion protein **Paxillin** (Salgia et al. 1995), **β 1-integrin** (Lundell et al. 1996), **L-selectin**, **intercellular adhesion molecule-1 (ICAM-1)**, and the **chemokine receptor CCR7**. CCR7 impairments are associated with impaired chemotaxis toward its ligands CCL19 and CCL21 (Jongen-Lavrencic et al. 2005).

- **Wnt/ β -catenin signaling:** Wnt/ β -catenin activity is crucial for the cross-talk between HSC and their niche and the consequently maintain of HSCs. Aberrant Wnt/ β -catenin signaling pathway is implicated in the genesis of various cancers. It has been reported that genetic inactivation of the β -catenin gene in mice impairs the development of BCR-ABL induced CML (Hu et al. 2009, Zhao et al. 2007). More recently, Heidel and colleagues showed that β -catenin is activated and required for CML stem cell maintenance in mice (Heidel et al. 2012). These findings suggest that this is another crucial signaling pathway which plays a role in BCR-ABL cell maintaining.

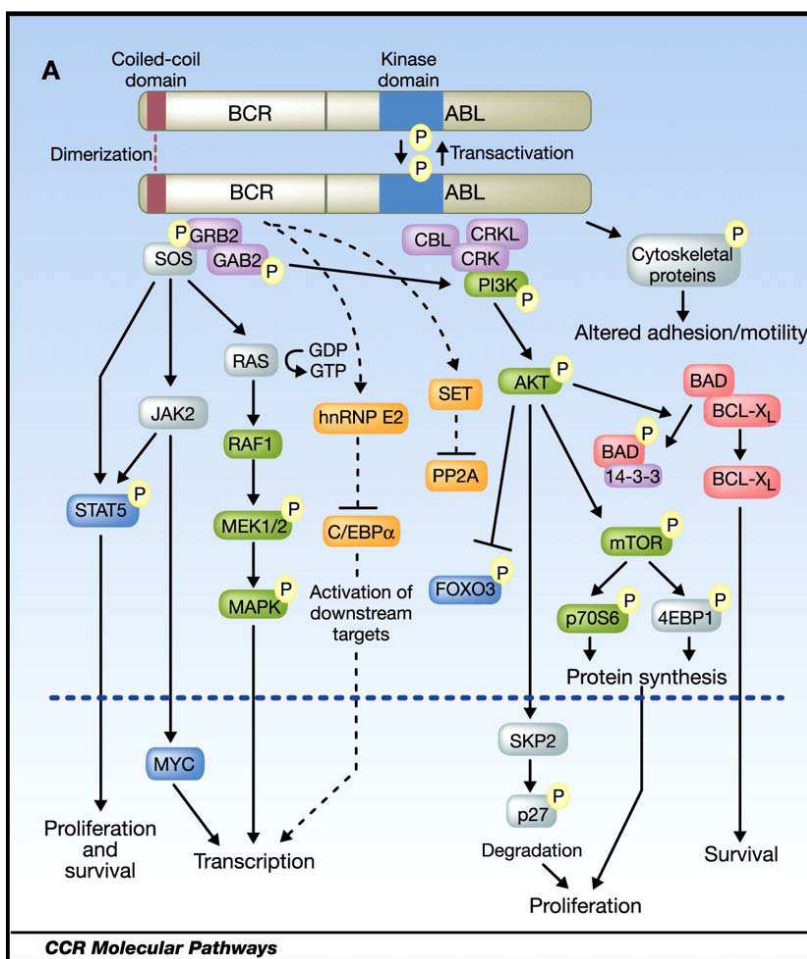


Figure 9: The BCR-ABL signalling network. Dimerization of BCR-ABL triggers autophosphorylation events that activate the kinase and generate docking sites for intermediary adapter proteins (purple) such as Grb-2. BCR-ABL-dependent signalling facilitates activation of multiple downstream pathways that enforce enhanced survival, inhibition of apoptosis, and perturbation of cell adhesion and migration. A subset of these pathways and their constituent transcription factors (blue), serine/threonine-specific kinases (green), and apoptosis-related proteins (red) are shown. A few pathways that were more recently implicated in CML stem cell maintenance and BCR-ABL-mediated disease transformation are shown (orange). Of note, this is a simplified diagram and many more associations between BCR-ABL and signalling proteins have been reported.

Adapted from (O'Hare et al. 2011)

2.3.4 BCR-ABL and promotion of DNA mutations

DNA damage involves DNA break(s) or mutation(s). When it is not correctly repaired, DNA lesions and mutations lead to alteration in the genome and, when overrepresented, to genomic instability. The clonal evolution and the accumulation of genetic aberrations is a feature of CML cells for the transition from chronic phase to blast phase and accelerated phase. In early 1990s, biologists hypothesized that BCR-ABL can be involved in genomic instability. Laneuville and colleagues showed, by infecting myeloblastic cell line 32D with a retroviral vector expressing BCR-ABL p210, that BCR-ABL is directly responsible for the accumulation of genetic instability and promotes the emergence of clones with a proliferative advantage (Laneuville et al. 1992). Several groups confirmed Laneuville observations in murine CML models and primary CML cells (Salloukh and Laneuville 2000, Koptyra et al. 2008, Skorski 2008). The alteration induced by BCR-ABL appear to be random than specific, consistent with a general “mutator phenotype”.

One of the causes of DNA damages is the **production of Reactive oxygen species (ROS)** which can enter to the nucleus and generate DNA lesions. BCR-ABL is able to induce chromosomal instability by generating ROS which in turn cause oxidative damages and mutations (Kim et al. 2005, Sattler et al. 2000). In addition, DNA damage can occur also because of the **over-proliferation of CML cells**, as polymerases themselves can generate some errors during DNA replication.

Single nucleotide alterations are repaired by mismatch repair (MMR) or by nucleotide excision repair (NER). Strand breaks are repaired either by high-fidelity homologous recombination (HRR) when a sister chromatid is available as a template (during the S or G2 phase of the cell cycle) or by non-homologous end joining (NEHJ) repair. Homologous recombination is a more fidelity reparation machinery compared to the NEHJ.

- **BCR-ABL and MMR**: BCR-ABL, in both murine and primary CML cells, decreases MMR activity. This action is BCR-ABL dependent, as Imatinib treatment revert MMR activity to control level (Stoklosa et al. 2008).
- **BCR-ABL and NER**: the activity of BCR-ABL on NER activity is controversial and it seems to depend on cellular context. Indeed, BCR-ABL decreases NER activity in BaF3, MO7E and 4Q2+ cells and sensitize the cells to ultraviolet (UV)-induced damages (Canitrot et al. 2003, Laurent et al. 2003). However,

in all other cell lines tested, the presence of BCR-ABL increases NER activity. In all cases, IM restores NER activity to control level, indicating that is strikingly BCR-ABL dependent (Sliwinski et al. 2008).

- **BCR-ABL and HRR:** cells expressing BCR-ABL show enhanced HRR efficiency, but the repair is unfaithful (Slupianek et al. 2006, Nowicki et al. 2004). Indeed, the number of repair events in BCR-ABL cells is the same compared to control cells, but there is a 100-fold increase of mutations in BCR-ABL expressing cells. Among the proteins involved in HRR, there is RAD51. **RAD51** was found to be increased in BCR-ABL cells after irradiation. In addition, BCR-ABL was found to associate with RAD51 and phosphorylate it on its Tyr residue, stimulating the formation of RAD51/RAD52 complexes (Chen et al. 1999).
- **BCR-ABL and NEHJ:** in both CML cell lines and primary cells, it has been shown that BCR-ABL increases NEHJ activity but the fidelity of repair is compromised (Gaymes, Mufti and Rassool 2002). They also demonstrated the implication of Ku70 and Ku80 protein in this repair.

In conclusion, BCR-ABL expressing cells are subjected to accumulation of mutated DNA. BCR-ABL enhances genomic instability by producing ROS and by interacting with some reparation machineries which favor mutation-proned DNA reparation and cause accumulation of DNA damages.

2.4 MANIFESTATION, DIAGNOSES AND STAGING OF CML

Usually, the CML disease is asymptomatic and it is initially found on routine physical examination or blood tests. Common signs of CML in chronic phase result in anemia and splenomegaly. Patients can show weight loss, fatigue and easy satiety. Patient's peripheral blood presents a high level of white cell count, in particular of granulocytes, and a decreased level of red blood cells. Platelet count can be decreased or increased, depending on the severity of the pathology. Once identified the blood cell count aberration, the diagnosis of

CML relies on of the detection of the Ph chromosome by fluorescent in situ hybridization (FISH) or on the detection of *BCR-ABL* mRNA by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

2.4.1 The phases of CML

CML can be classified into 3 different disease phases: **chronic phase (CP)**, **accelerated phase (AP)** and **blastic phase (BP)**. Most often, CML is diagnosed in CP. The progression from CP, to AP or BP results from additional genetic alterations in leukemic cells.

- **CP**: approximately 85% of patients are in CP at the time of diagnosis (*BCR-ABL* gene or transcript detection). The duration of CP is variable and depends on how early the disease was diagnosed as well as the therapy used. In the absence of treatment, the disease progresses to AP.

- **AP**: most patients evolve into AP before BP. In AP, patients may present worsening anemia, splenomegaly and increased white blood cell count. The blast count in the blood and bone marrow usually starts augmenting (10-19% of blasts in blood or bone marrow) and is further elevated in BP. In this phase there is usually an accumulation of chromosome aberration, in addition to Ph chromosome. Patients may lose their sense of well-being and other complications may follow. Drug treatment usually becomes less effective in this stage.

- **BP**: this is the final phase in the evolution of CML, with rapid progression and short survival. BP presents as an acute leukemia, the number of blasts dramatically increases in marrow and blood and there is a loss of terminal differentiation capacity, resulting in red cell, platelet and neutrophil number very low. Patients can experience episode of bleeding and infections.

2.4.2 Incidence of CML pathology

Most of the cases of CML occur in adults and it is a rare disease among adult leukemias, with a median age at the diagnosis of 65 years old. The frequency of CML increases with age, from about less than 1 in 100,000 people until about 40 years, to about 2 in 100,000 people at 55 years, to about 9 in 100,000 people at 80 years and older. A small number of children can also develop CML (www.seer.cancer.gov)

2.5 TREATMENT FOR CML AND CLINICAL RESISTANCES

2.5.1 Imatinib, the first generation TKI

Until the early 1980s, CML therapy was based on busulfan, cytarabine or hydroxyurea, which had a negligible effect on the natural course of the disease. In 1983 Talpaz and colleagues started the first studies on human interferon-alpha ($IFN\alpha$) for the management of CML and they showed that $IFN\alpha$ induced cytogenetic response and that it was more durable and reproducible than those induced by chemotherapy (Talpaz et al. 1983, Talpaz et al. 1987). By 1990, it was discovered that BCR-ABL tyrosine kinase activity is crucial for transforming properties of BCR-ABL cells, thus many studies have been performed in order to block BCR-ABL activity and to limit the disease. In 1996, the first tyrosine kinase inhibitor (TKI) was developed, the **Imatinib mesylate (IM) (Gleevec, Novartis Pharmaceutical Corporation, NJ, USA)** and few years later, in 2001, its safety and efficacy in CML patients was shown, leading to the use of IM as first-line therapy for CML treatment (Druker et al. 1996) (Druker et al. 2001). IM has revolutionarily changed the outcome for CML patients, with much higher rate of survival.

IM acts via competitive inhibition at the ATP-binding site of BCR-ABL protein, which results in loss of phosphorylation of downstream signaling proteins involved in BCR-ABL signal transduction (Fig.10). IM efficiently inhibits BCR-ABL but is not specific, indeed it blocks also the platelet-derived growth factor receptor (PDGFR) and the c-kit tyrosine kinase activity (Druker and Lydon 2000). Still, 75% of IM-treated patients presented a complete cytogenetic response (CCyR), with Ph chromosome undetectable in bone marrow

and blood cells by cytogenetic tests. However, as soon as IM-treatment is stopped, CML comes out, resulting in molecular relapse of BCR-ABL in 50% of patients (Rousselot et al. 2007). The clinical relapse of patients followed by IM discontinuation was later explained by the presence of BCR-ABL mRNA in the most immature stem/progenitor cell population, which is refractory to IM treatment and is responsible for the residual disease (Chomel et al. 2011). Moreover, some patients fail or are intolerant to IM treatment and primary resistance to IM can occur in 15% of patients (Druker et al. 2001). For all these reasons, there was the need to develop second generation TKIs, like Dasatinib and Nilotinib.

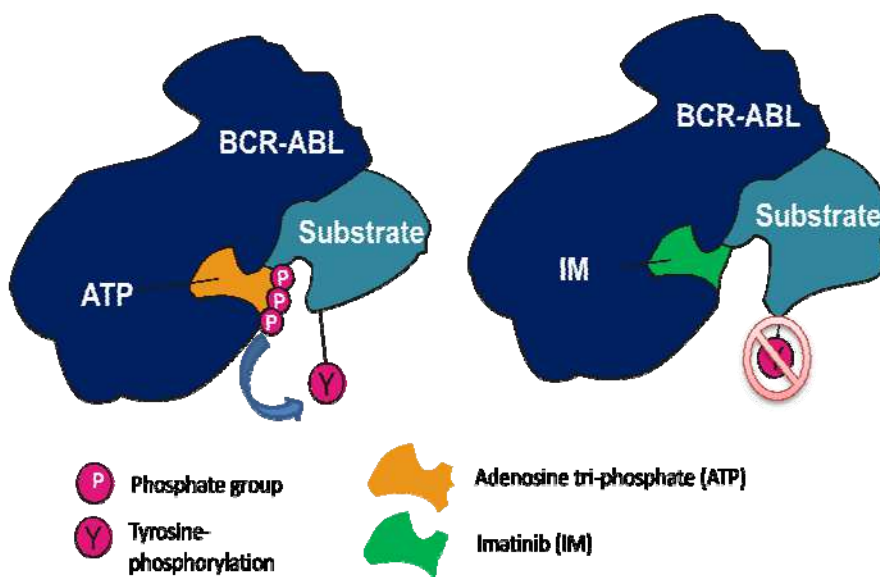


Figure 10: Mechanism of action of Imatinib. The active site of BCR-ABL has an ATP binding site which permits the transfer of the terminal phosphate from ATP to tyrosine residues on target proteins. Imatinib works by competitive binding to the ATP binding site, locking it in an inhibited conformation and therefore inhibiting BCR-ABL activity.

2.5.2 Mechanisms of resistance to IM

The apparition of IM in 2001 has revolutionized the prognosis of CML. However, some patients experience a lack of response to treatment at the beginning of therapy or a treatment failure after an initial response. Studies of relapsed patients after IM treatment showed that BCR-ABL signaling is often reactivated at the time of resistance (Gorre et al. 2001). Therapeutic resistance to TKIs can be classified as primary or secondary, depending on whether an initial decline in disease levels are observed or not.

In **primary resistance** (also referred to as “**refractoriness**”), patients exhibit a lack of efficacy to TKI treatment from the start of therapy. The causes of primary resistances are listed below.

- ***IM plasma levels***

IM-resistance can be caused by a low concentration of the drug in the cells. This low concentration can be due to an accumulation of IM in the plasma because of the binding to the plasma protein alpha 1-acid glycoprotein-1 (AGP1), which is a reactant that binds drug molecules at a 1:1 molar ratio. These binding results in a lower concentration in the plasma of the active drug decreased IM available to enter into the cells and consequently limited therapeutic activity (Gambacorti-Passerini et al. 2000)

- ***Intracellular uptake of IM***

The amount of IM that enters the target cell is a direct function of the balance between influx and efflux transport. The adenosine triphosphate-binding cassette (ABC) transporter ABCB1 (also called multi-drug resistance 1 (MDR1)) is a transmembrane protein that mediates multidrug resistance in multiple neoplasia through regulation of the efflux of chemotherapeutic agents. Interestingly, MDR1 is over-expressed in cells of patients in BP-CML and has been linked to the development of IM resistance (Mahon et al. 2000). Moreover, in a study of patients who failed to attain MCR, it was shown that they over-express ABCB1 (Galimberti et al. 2005).

In addition to drug efflux regulation, IM resistance can be also linked to the knock-down of IM-influx transporter expression. The human organic transporter1 (hOCT1), is a crucial regulator of IM availability. Even if a correlation between hOCT1 expression and disease progression or resistance has not been established, there is a 2 times down-regulation of hOCT1 expression in patients after IM-treatment and this could explain another mechanism of resistance (Crossman et al. 2005).

- ***CML stem cell quiescence***

As discussed before, CML differentiated cells are sensitive to IM treatment and readily die under IM. However, CML stem cells are not affected by IM exposure and are therefore responsible for residual disease that causes CML persistence. These cells are insensitive to IM despite inhibition of BCR-ABL activity because they do not depend on BCR-ABL for their survival and therefore they are not eliminated after IM exposure

(Corbin et al. 2011). This suggests that CML stem cell survival depends on other signals which derive from their microenvironment niche, like cytokines or other extracellular signals.

- ***Clonal evolution***

In CML disease, like in other cancers, there is a clonal evolution, or rather an accumulation of genetic events like new DNA mutations or translocation. The most frequent are trisomy 8, isochromosome 17, which consists of an abnormal chromosome 17 with two identical long arms due to duplication of the long arm and loss of the short arm, loss of chromosome 17p region and *BCR-ABL* mutation (which will be discussed below). Other genetic aberrancies are trisomy 21, trisomy 17, trisomy 19, and deletion 7 (Johansson, Fioretos and Mitelman 2002).

- ***Intracellular signaling pathways alteration***

Among the mechanisms of resistance to TKIs which involve the deregulation of BCR-ABL down-stream signaling pathways there is the up-regulation of SRC family kinases expression. The SRC family encompasses 9 cytoplasmic non receptor kinases: SRC, FYN, YES, BLK, YRK, FGR, HCK, LCK and LYN. BCR-ABL activates LYN, HCK and FGR in B-lymphoblastic leukemia (Hu et al. 2004). In particular, **LYN** was found over-expressed in TKI resistant cell lines and primary cells (Mahon et al. 2008). More recently, 2 additional tyrosine kinases were found to be over-activated in resistant CML cells: the spleen tyrosine kinase **Syk** and the receptor tyrosine kinase **Axl**; they form a complex with LYN kinase and with the participation of the adaptor CDCP-1 (Gioia et al. 2011). Axl belongs to the TAM receptor family, which includes also Mer and Tyro3 receptors. Axl was originally cloned from DNA of two patients with CML (O'Bryan et al. 1991) and is over-expressed in many human cancers and its increased expression correlates with advanced tumor stage and chemoresistance (Linger et al. 2010).

Secondary resistance (also referred to as “**acquired resistance**”), results after the initial achievement of response to TKI lasting for a period of time of variable length.

- ***BCR-ABL point mutations***

The most common mechanism of resistance in CML patients (50%-90%) is due to point mutations on BCR-ABL kinase domain which is the target of IM binding and activity. More than 100 point mutations encoding for single amino acids substitutions that confer resistance to IM have been described. Among them, the most frequent are the T315I (discussed before), Gly250, Tyr253, Glu255, Thr315, Met351, and Phe359, which account for 60% to 70% of all mutations.

- ***BCR-ABL over-expression***

Another mechanisms of resistance BCR-ABL dependent that accounts for 10% of CML resistance, consists of the amplification of BCR-ABL proteins associated with the amplification of the fusion gene.

Resistance can be further classified into **hematologic** (lack of normalization of peripheral blood counts), **cytogenetic** (persistence of Ph chromosome), and **molecular** (persistence of BCR-ABL transcript by RT-PCR).

2.5.3 Nilotinib and Dasatinib, the second generation TKIs

After the discovery that IM treatment may lead to clinical resistance, second generation TKIs were developed. Second generation drugs are intended to have decreased resistance and intolerance than Imatinib.

- **Dasatinib (Sprycel, Bristol-Myers Squibb):** is a second generation TKI that was initially approved in 2007 by Food and Drug Administration (FDA). In 2010 it was also approved as first line therapy of CML. Dasatinib inhibits BCR-ABL and also Src tyrosine kinase activity. Its BCR-ABL inhibitory action is 350 times more potent than IM *in vitro*. Moreover, it is efficient also in IM-resistant CML cells (Lombardo et al. 2004, Tokarski et al. 2006, O'Hare et al. 2005). To test the efficiency of Dasatinib, the DASISION international trial evaluated the effects of IM vs Dasatinib in random newly diagnosed patients with CML-CP for an 18 months-follow up. They found that a higher percentage of Dasatinib-treated patients present CCyR (77% with Dasatinib vs 66% with IM) after a 12 months-follow up. Moreover, Dasatinib was able to induce more major molecular response (MMR), defined as no detectable

BCR-ABL transcript by RT-PCR compared to IM group (Kantarjian et al. 2010).

- **Nilotinib (Tasigna, Novartis Pharmaceutical Corporation, NJ, USA):** it was approved with Dasatinib as first-line CML therapy in 2010 by FDA. Nilotinib is a structural analog of IM and its BCR-ABL-affinity *in vitro* is 50 time stronger than IM (Weisberg et al. 2005). In ENESTnd international trial on random patients with CML-CP treated either with Nilotinib or IM, it was found that Nilotinib decreases the percentage of patients that progress to AP or BP and that in a follow up of 12 months, the percentage of Nilotinib-treated patients with MMR was higher than the percentage of IM-treated patients (43% vs 22%) (Saglio et al. 2010).

To date, current guidelines recommend the 3 TKIs as first-line therapy for treatment of newly diagnosed CML and, given the excellent outcome and long-term survival with these TKIs, the use of chemotherapy agents is no longer recommended.

CML is definitely the first and true model of the success of targeted therapies and the recommendation is to continue TKI treatment permanently. However, there are emerging problems concerning the long-term tolerability of TKIs, especially for young patients and there is an increase of drug resistance due to the widespread of TKIs. Moreover, there is an economic burden of these expensive drugs. These observations together, raise the need to discontinue TKI treatment and CML continues to be more than ever a model for cancer therapy.

2.5.4 New therapeutic agents

There are specific BCR-ABL mutations that confer resistance to TKIs treatment. One of the most frequent is T315I, which results in the substitution at the position 315 from a threonine (T) to an isoleucine (I). Therefore, new agents have been developed and are presently in course of study. Among them, there is the Ponatinib which has been shown to inhibit BCR-ABL, including T315I mutant, as well as other tyrosine kinases, including FLT3, PDGFR, VEGF and c-kit (O'Hare et al. 2009).

Other novel agents include DCC-2036 which binds the area responsible for the change between inactive and active BCR-ABL protein (Chan et al. 2011), or the Bosutinib, a dual

Src/ABL kinase inhibitor (Cortes et al. 2011), or HG-7-85-01 which uses a modified nilotinib-dasatinib hybrid structure to avoid gatekeeper mutations, and GNF-2, an allosteric ABL inhibitor that has been shown to be effective in combination with ATP-competitive ABL inhibitors (O'Hare et al. 2011).

2.5.5 Targeting CML stem cells

In the majority of patients on IM treatment, residual disease is detectable and only a few achieve a complete molecular response, defined as no detectable BCR-ABL transcript by RT-PCR, and even fewer maintain this molecular response upon discontinuation of treatment (Rousselot et al. 2007). The inability of IM to eliminate all leukemia cells allows **disease persistence** or **residual disease**. Persistence is due to the presence of CML stem cells that are not fully addicted to BCR-ABL kinase activity. Indeed, *BCR-ABL* transcript remains detectable in the most immature CML stem cell population (Chomel et al. 2011). This limitation of BCR-ABL inhibitors demands for alternative ways eliminate the leukemic stem cell clone. Targeting cell energy providers such as mitochondria metabolism, genome wide regulators, or BCR-ABL downstream signaling pathways have been new options. These include Wnt- β -catenin pathway (Zhang et al. 2013), Hedgehog pathway (Dierks et al. 2008) or STAT5 factors (Hoelbl et al. 2010), histone deacetylase inhibitors (Zhang et al. 2010) or the use of BMS-214662, a farnesyl transferase inhibitor, that induces mitochondrial apoptosis of CML stem cells, without affecting normal HSCs survival (Pellicano et al. 2009).

2.5.6 Allogenic hematopoietic stem cell transplantation (Allo-HSCT)

Briefly, the Allo-HSCT is the transplantation of multipotent hematopoietic stem cells, usually derived from bone marrow or cord blood of a healthy individual that have a human leukocyte antigen (HLA) that matches with those of the CML patient. The number of patients undergoing to Allo-HSCT has dramatically decreased after the advent of IM and second generation TKIs. However, it has an important role for primary resistant patients, for patients that evolve to BP and for patient harboring secondary BCR-ABL mutations that are resistant to first and second generation TKIs.

2.6 MODELS TO STUDY CML

2.6.1 *In vitro* models: CML cell lines and primary cells

The first CML-derived leukemia cell line was K562. It was established in 1970 from the pleural effusion of a 53-year-old woman with CML in blast crisis (Lozzio and Lozzio 1975). Few years later, in 1975, the NALM-1 cell line was established from the peripheral blood of a 3-years-old girl in lymphoid blast crisis (Minowada et al. 1977). Since these first cell lines, more than 40 Ph⁺ CML cell lines have been reported. Among these, there are the LAMA84, MEG01 and KU812. Different cell lines model the different types of CML leukemias that carry different BCR-ABL fusions as well as different chromosomal rearrangements in addition to the Ph chromosome. Specifically, LAMA84 cell line was established from the peripheral blood of a 29-year-old woman with CML one month after onset of myeloid-megakaryocytic blast crisis; MEG01 cell line was established from the bone marrow of a 55-year-old man with CML in megakaryoblastic crisis.

In addition to CML cell lines, also primary cells from CML patients may be used. Primary CML stem cells are normally purified from peripheral blood of patients at the moment of diagnosis. Nearly all CD34⁺CD38⁻ HSC-enriched cells present in patient bloodstream are BCR-ABL expressing stem cells

2.6.2 *In vivo* models: a new CML mouse models

To more easily study CML stem cells, a mouse model was developed and it was used until few years ago. Murine cells were transduced with retroviral vectors that express BCR-ABL (p210) and followed by transplantation into irradiated mice (Li et al. 1999, Pear et al. 1998). BCR-ABL-expressing cells trigger a myeloproliferative disorder, but leukemia is quite fulminant, associated with pulmonary hemorrhage and death within 4 weeks. This model may be more representative of AP-CML than a CP-CML. Even if the disease development of these mice does not represent exactly the disease progression in humans, this model has been the standard to study molecular mechanisms involved in CML disease until 2005. In 2005, Koschmieder and colleagues proposed a transgenic mouse model of BCR-ABL leukemogenesis in which BCR-ABL gene is expressed under the control of a Tetracycline-

regulated 3' enhancer of the murine stem cell leukemia (SCL) gene allowing targeted BCR-ABL expression in stem and progenitor cells (Koschmieder et al. 2005). SCL gene encodes a helix-loop helix transcription factors which is expressed in the very immature HSC in addition to erythroid cells, mast cells, megakaryocytes and multipotent progenitor cells. Thus, in SCL-tTA/BCR-ABL mice, tetracycline exposure enhances BCR-ABL expression in stem and progenitor cells. Expression of BCR-ABL results in a chronic myeloproliferative disorder with chronic neutrophilia and leukocytosis, splenomegaly, extramedullary hematopoiesis and invasion of non-hematopoietic organs by myeloid cells. Mice have an overall median survival of almost 50 days, with few mice dying after 100 days upon BCR-ABL induction. Moreover, reversion of BCR-ABL expression rescues the leukemia phenotype. These features closely resemble the natural course of human CML without treatment and this mouse model allows for the study of CML development and maintenance *in vivo*.

2.7 MICROENVIRONMENTAL REGULATION OF CML CELLS

CML stem cells, as well as normal HSCs, reside in specific functional niche within the bone marrow. This microenvironment provides critical signals to regulate HSC numbers and quiescence and support HSC preservation. In 1995, Bhatia and colleagues raised the hypothesis that abnormal stromal functions in CML may in part sustain the hematopoietic abnormalities observed in CML. They made use of marrow stromal macrophages from patients with CML, which are derived from the BCR-ABL positive clone. They showed that CML marrow stroma has a reduced ability to support growth of normal progenitors whereas growth of CML progenitors is not impaired (Bhatia et al. 1995).

Using CML cell lines, it was shown that BM stromal cells or extracellular matrix may sustain growth or protect leukemic cells from the effect of chemotherapy and small molecule-targeted therapies (Matsunaga et al. 2003, van der Kuip et al. 2001, Zhang, Groffen and Heisterkamp 2007a). Jin and colleagues showed that IM treatment increases CXCR4-mediated migration of CML cell lines to BM MSCs and results in increased cell cycle arrest and survival of quiescent cells (Jin et al. 2008).

The new CML mouse model reported above, the SCL-tTA/BCR-ABL mouse, has allowed to better investigate the role of BM microenvironment on CML stem cells *in vivo*. Recent reports, in which authors made use of this mouse model, have shown that

hematopoietic **niche has a protective role on CML stem cells from TKI mediated cell death and depletion.** Last year, Zhang and colleagues, found that in BCR-ABL mice there is a reduction of leukemic stem cells in the BM and an increased egress of these cells to the spleen, with an enhanced proliferation in the spleen. They showed a reduction in BM CXCL12 levels as a mechanism underlying impaired leukemic stem cells homing and retention in CML BM. Indeed, CXCL12 expression was reduced not only in BM of BCR-ABL mice but also in CML patients. Moreover, they showed that altered CML BM microenvironmental function contributes to suppression of normal HSCs and provides a selective advantage to leukemic stem cells. The expression of several other cytokines was increased in BM of BCR-ABL mice, like IL-1 α and β and TNF- α as supporting reduced proliferation and growth of normal compared to CML stem cells. Moreover, although IM treatment corrects several abnormalities in cytokine and chemokine expression in CML cells, it does not completely restore CXCL12 levels in the BM of BCR-ABL mice, indicating that it does not completely reverse leukemia-associated changes in the microenvironment (Zhang et al. 2012a).

More recently, the same team showed that BM-derived MSCs protect CML stem and progenitor cells from TKI-mediated cell death and depletion and that this protection involves the N-Cadherin-mediated adhesion pathway. N-Cadherin is expressed by human stem and progenitor cells, but in a lower level in CML cells compared to normal cells. Moreover, N-Cadherin expression is increased when they adhere to MSCs, corresponding to an increased association of N-Cadherin and activation of Wnt- β -catenin signaling, which is the highest in IM-treated cells (Zhang et al. 2013).

These results together indicate that CML BM microenvironment plays an active role in the maintaining of CML cells and in resistance to TKI treatment. Novel molecular mechanisms proposed, as the Wnt- β -catenin signaling pathway, permit to find new therapeutic treatment which target the microenvironment where CML stem cells reside, rather than CML cells themselves. However, when I started my PhD project in 2010, few notions were known about niche-dependent regulation of CML cells.

3.

The JAK/STAT signaling pathway and STAT5 factors

The Janus Kinase/Signal Transducers and Activator of Transduction (JAK/STAT) pathway is a signaling cascade used in animals, from humans to flies. In mammals, the JAK/STAT pathway is one of the principal signaling mechanisms for a wide variety of cytokines and growth factors. JAK activation regulates cell proliferation, differentiation, migration, apoptosis and many other cellular processes. These cellular events are critical to a variety of biological functions like hematopoiesis, immune development, mammary gland development and lactation, adipogenesis, sexually dimorphic growth and many others.

In mammals, 4 JAK and 7 STAT proteins have been identified: **JAK1**, **JAK2**, **JAK3**, **TYK2** and **STAT1**, **STAT2**, **STAT3**, **STAT4**, **STAT5A**, **STAT5B** and **STAT6**.

In vitro and *in vivo* studies using knock-out mice, have indicated that in hematopoietic cells JAK/STAT pathway is activated by different cytokines such as IL-2 and IL-7 which act on lymphoid cells, IL-3, IL-5, GM-CSF and G-CSF on myeloid cells, EPO for erythroid cells, FLT3 ligand, stem cell factor and TPO which act on the most immature hematopoietic stem compartment as well as on selective progenitors.

3.1 JAK AND STAT PROTEIN STRUCTURES

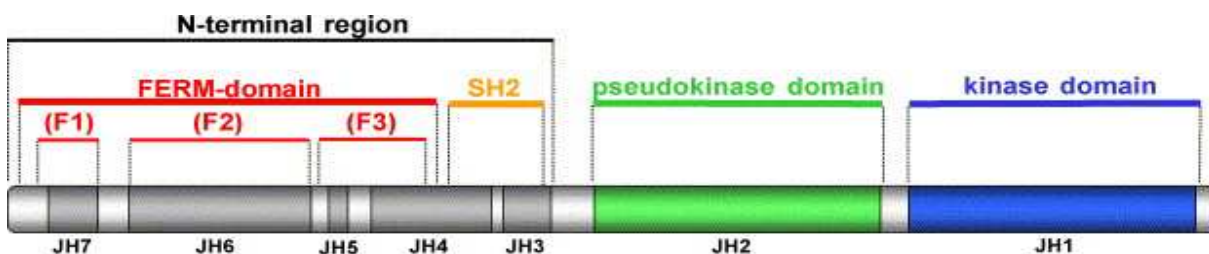
3.1.1 The JAK kinases

JAK proteins are a family of intracellular, non receptor tyrosine kinase and they are structurally conserved. JAKs range from 120 to 140 kDa and have seven defined regions of homology called **Janus Homology domains 1 to 7 (JH1-JH7)** (Fig.11).

- **JH1** domain is situated at the C-terminal region of JAKs. It is the **kinase domain** which is essential for the enzymatic activity of JAKs. This region contains conserved tyrosine (Y) necessary for JAK activation (e.g. Y1038/Y1039 in JAK1, Y1007/Y1008 in JAK2, Y980/Y981 in JAK3, and Y1054/Y1055 in

Tyk2). Once JAK is phosphorylated on one of its Tyr residues, it changes its conformation in order to facilitate kinase activity and substrate binding.

- **JH2** is a “**pseudokinase domain**”. This domain is quite structurally similar to the kinase domain, but lacks many of the characteristics essential to conventional kinase activity. JH2 is involved in the regulation of the activity of the JH1 domain.
- **JH3** domain and a part of **JH4** domain share some homology with the Src-homology2 (**SH2**) domain that allows docking to phosphorylated Tyr residues.
- The N-terminal region is composed of the **JH4**, **JH5**, **JH6** and **JH7** domains. This region is called the **FERM (four-point-one, ezrin, radixin, moesin) domain**, which is a widespread protein module involved in localizing protein to the plasma membranes. In JAK factors, this domain is implicated in JAKs association with cytokine receptors.



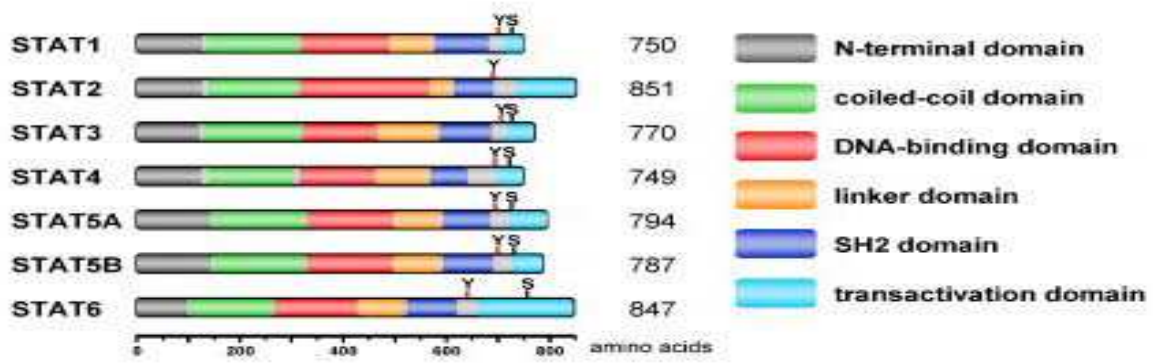
Adapted from (Haan et al. 2006)

Figure 11: The structure of JAKs. JAK proteins present 7 Janus Homology domains (JH1-JH7). The JH1 is the kinase domain, the JH2 is the pseudokinase domain which seems to regulate the kinase domain, the JH3 and a part of the JH4 are part of the Src Homology 2 (SH2) domain and the JH4, JH5, JH6 and JH7 domains at the N-terminal region form the FERM (four-point-one, ezrin, radixin, moesin) domain which is involved in association with other JAKs, cytokine receptors and other kinases

3.1.2 The STAT factors

STAT proteins are transcriptional factors that are present in both cytoplasmic and nuclear cell compartments. They have a mass of approximately 80-100 kDa and are structurally conserved and essential for carrying out multiple cellular functions in response to extracellular cytokines and growth factors signals. STATs are usually activated by JAK kinases. They present 6 domains (Fig.12).

- at the N-terminal region there is the **N-ter-dimerization domain (ND)** which is the less conserved domain among the STATs and it is responsible for some STAT dimer-dimer interactions and protein partnerships.
- the **coiled-coil domain (CC)** is responsible for many protein-protein interactions.
- the **DNA binding domain (DBD)**, which is situated between the amino acids 350 and 500. It is responsible for the binding to DNA. Most STAT dimers recognize an 8- to 10-base pair inverted repeat DNA element with a consensus sequence of 5'-TTCN₂₋₄GAA-3'. Differential binding affinity of a particular activated STAT dimer for a single target DNA sequence is determined by the number of nucleotides between TTC and GAA (Ehret et al. 2001) (Table 1).
- the **linker domain (LD)** (aa 500-600) connects the DBD to the C-terminal region.
- the **SH2 domain** (aa 600-690) which targets the proteins to specific tyrosine-phosphorylated peptide sequences within their binding partners, thereby regulating a wide range of intracellular signaling events.
- the **transactivation domain (TA)** at the C-terminal region. This domain contains a crucial Tyr residue that is essential to STAT DNA-binding and transcriptional activation. It also includes other residues that can be phosphorylated upon stimuli and regulate STAT biological activities. The TA domain varies greatly among the STATs, with the highest homology found between STAT1, STAT3 and STAT4.



Adapted from (Mohr et al. 2012)

Figure 12: Conserved domains of STATs. STAT proteins share a common domain structure with an N-terminal domain followed by a coiled-coil domain, a DNA-binding domain, a linker domain, an SH2 domain and finally a C-terminal transactivation domain. The tyrosine residue that becomes phosphorylated upon activation is located between the SH2 and the trans-activation domain.

	N	High affinity BS	Low affinity BS
STAT1	3, (2)	TTCN ₃ GAA	TTAN ₃ TAA, TTGN ₃ CAA
STAT2	3	TTCN ₃ GAA	
STAT3	3	TTCN ₃ GAA	TTAN ₃ TAA, TTTN ₃ AAA, TTGN ₃ CAA
STAT4	3	TTCN ₃ GAA	TTAN ₃ TAA
STAT5	3, (4)	TTCN ₃ GAA	TTAN ₃ TAA
STAT6	4	TTCN ₄ GAA	

N= number of nucleotides between TTC and GAA

BS= binding sequences

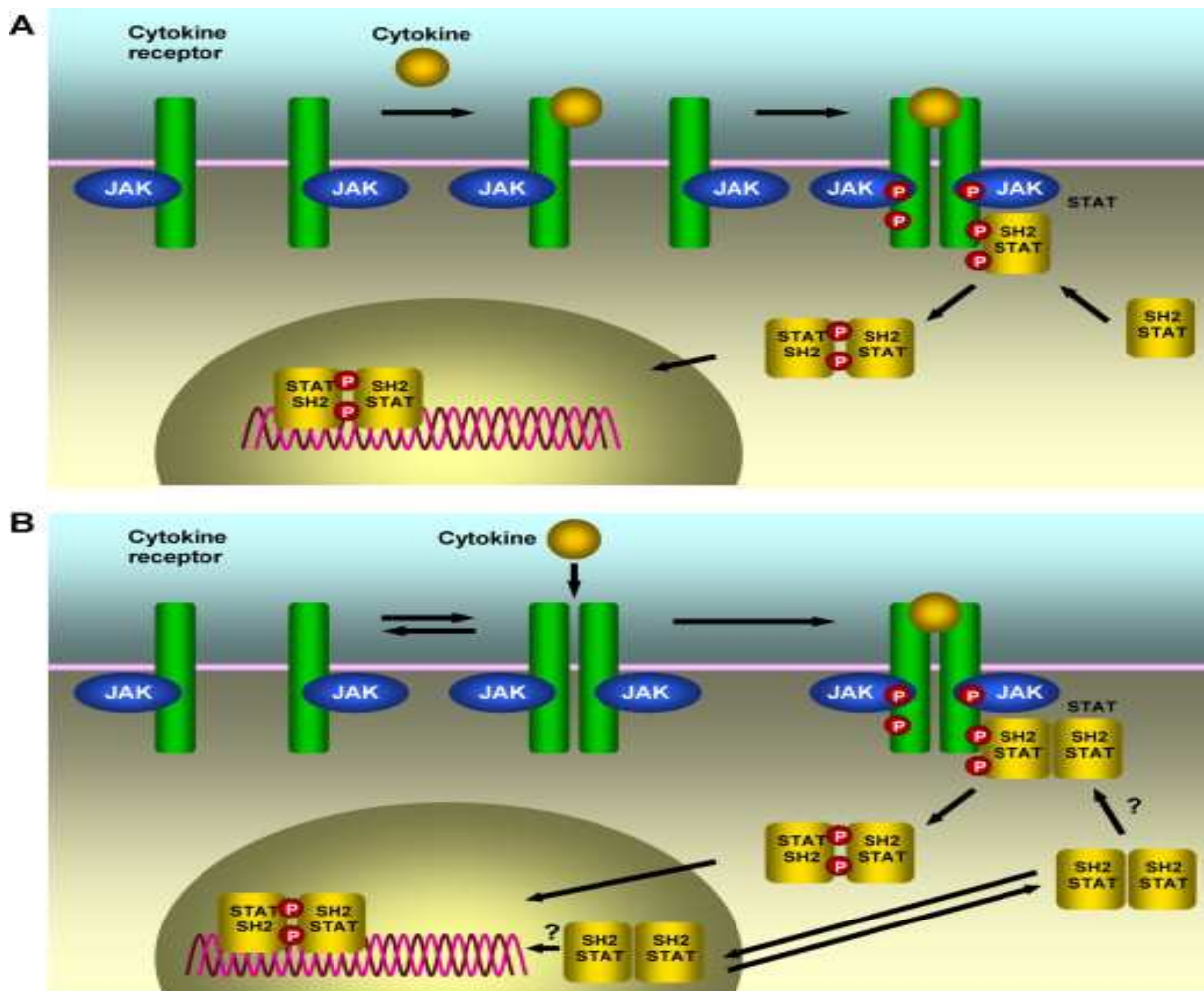
Table 1: Preferential STAT responsive elements described in the literature. The sequence specificity of different STAT proteins is very similar but not identical. STAT6 differs from the other STATs in its reference for sites in which the two palindromic halves are separated by 4 rather than 3 nucleotides. STAT1 can bind N₂ and N₃ sites. STAT5 can bind N₄ sites but that its affinity for N₃ sites is much higher.

3.2 CANONICAL AND NON-CANONICAL JAK/STAT SIGNALLING

Canonical JAK/STAT signaling, as formulated in early 1990s (Darnell, Kerr and Stark 1994), describes the basic features of this signaling pathway but recent works reported the existence of others non-canonical pathways (Fig.13).

3.2.1 Canonical JAK/STAT signaling

STAT factors are initially present in inactive forms in the cytoplasm, while JAK kinases are constitutively associated with the cytoplasmic membrane-proximal regions of various receptors. Upon ligand binding on the receptors, JAK kinases trans-phosphorylate each other on their Tyr residues and, at the same time, they phosphorylate Tyr residues in the cytoplasmic domain of the receptor. This phosphorylation leads to the recruitment of STAT monomers via recognition of the receptor phospho-tyrosines by the STAT SH2 domains. Once they are bound to the receptors, STAT factors are phosphorylated by receptor-bound JAKs. Thereafter, phosphorylated STATs are detached from the receptor and become homodimerized or heterodimerized through reciprocal intermolecular SH2 domain interactions. The phospho-STAT dimers are then able to translocate to the nucleus, to bind the GAS responsive element on DNA and to activate or repress gene transcription (Fig.13A). Cessation of signaling involves dephosphorylation of DNA-bound STAT dimers within the nucleus and export back to the cytoplasm.



From (Mohr et al. 2012)

Figure 13: The canonical and non-canonical JAK/STAT signalling pathway. (A) The main features of canonical JAK/STAT signaling are dimerization or multimerization of receptors after ligand-binding. Then STAT monomers dimerize upon phosphorylation by receptor-bound JAKs and they enter to the nucleus (when phosphorylated). (B) Non-canonical JAK/STAT signalling includes preformed dimers of receptors as well as STAT monomers. Unphosphorylated STATs in the nucleus may contribute to the regulation of gene expression and may also have role without the nucleus.

3.2.2 Non-canonical JAK/STAT signaling

Despite the prevalence of the canonical model, it has now been firmly established that non-canonical JAK/STAT signaling may exist (Fig.13B). One of the very first evidence for non-canonical pathways came from the observations that Tyr kinases different from the JAKs, such as SRC kinases, can activate STAT factors through canonical Tyr-phosphorylation (Levy and Darnell 2002). In addition, non-phosphorylated STATs have been shown to exhibit biological activities.

First, cytokine receptor might dimerize in absence of ligands, or ligands could just stabilize a pre-formed receptor dimer. As proposed for the receptors, there are strong evidences for STAT dimers in absence of canonical tyrosine phosphorylation (Braunstein et al. 2003, Novak et al. 1998, Stancato et al. 1996).

Second, while in canonical JAK/STAT signaling STAT dimers enter to the nucleus in response to activation by Tyr-phosphorylation, in the non-canonical model a fraction of latent STATs is consistently located in the nucleus without being phosphorylated on Tyr, as a result of a steady state of a constant nuclear import and export, as it was shown for STAT1 (Chatterjee-Kishore, 2000), STAT3 (Yang, Liao, G&D 2007), STAT5B (Zeng et al. 2002), for STAT5A (Iyer and Reich 2008) and for STAT6 (Chen and Reich 2010) and STAT2 (Frahm, Hauser and Koster 2006). These non-phosphorylated STAT factors may also contribute to gene regulation. Stark and colleagues have pioneered the demonstration that non-phosphorylated STAT1 and STAT3 have transcriptional effects in the nucleus through complexes formed with IRF1, NFkB or other transcription factors (Chatterjee-Kishore et al. 2000, Yang et al. 2005, Yang et al. 2007). The steady state distribution, as well as the shuttling dynamics, differs between individual STATs.

Third, nuclear non-phosphorylated STATs were shown to associate with and regulate heterochromatin stability. This role in heterochromatin was described for the STAT ortholog STAT92E in *D. Melanogaster*. Non-phosphorylated STAT92E is localized on heterochromatin in association with Heterochromatin Protein 1 (HP1), and is required for stabilizing HP1 localization and histone H3 Lys 9 methylation (H3mK9). However, activation by phosphorylation reduces heterochromatin-associated STAT92E, causing HP1 displacement and heterochromatin destabilization (Shi et al. 2008). Similar activities have been recently reported for STAT5A in human cells (Hu et al. 2013)

Fourth, despite the best documented function of STATs is gene transcription control, in the non-canonical model some STATs exert other non-canonical functions outside of the

nucleus. Some of these non-canonical functions, which are independent of Tyr-phosphorylation, have been well described for STAT3. Indeed, STAT3 has been described as a regulator of oxidative phosphorylation within mitochondria, where it interacts with complex I and II of the respiratory chain. The absence of STAT3 in mitochondria compromises the function of the respiratory chain. For this mitochondrial function of STAT3, instead of the canonical Tyr phosphorylation, the serine 727 phosphorylation is required. (Wegrzyn et al. 2009, Gough et al. 2009). Another example of non-canonical functions of STATs has been described for STAT5A in CML. In this disease STAT5A is massively localized in the cytoplasm of CML cells where it associate with the p85 subunit of the PI3K (Harir et al. 2007) or the Src family kinases (Chatain et al. 2013). In addition, non-phosphorylated STAT5A, and to a lesser extent STAT5B, has been shown to be associated with the Golgi apparatus and the rough endoplasmic reticulum in vascular cells, where it preserves the structure and function of these organelles (Lee et al., 2012).

These non-canonical functions are described in figure 14 and open a wide field for future research on JAK/STAT pathway and underline the difficulty of describing in few words a simple molecular mechanism for this pathway.

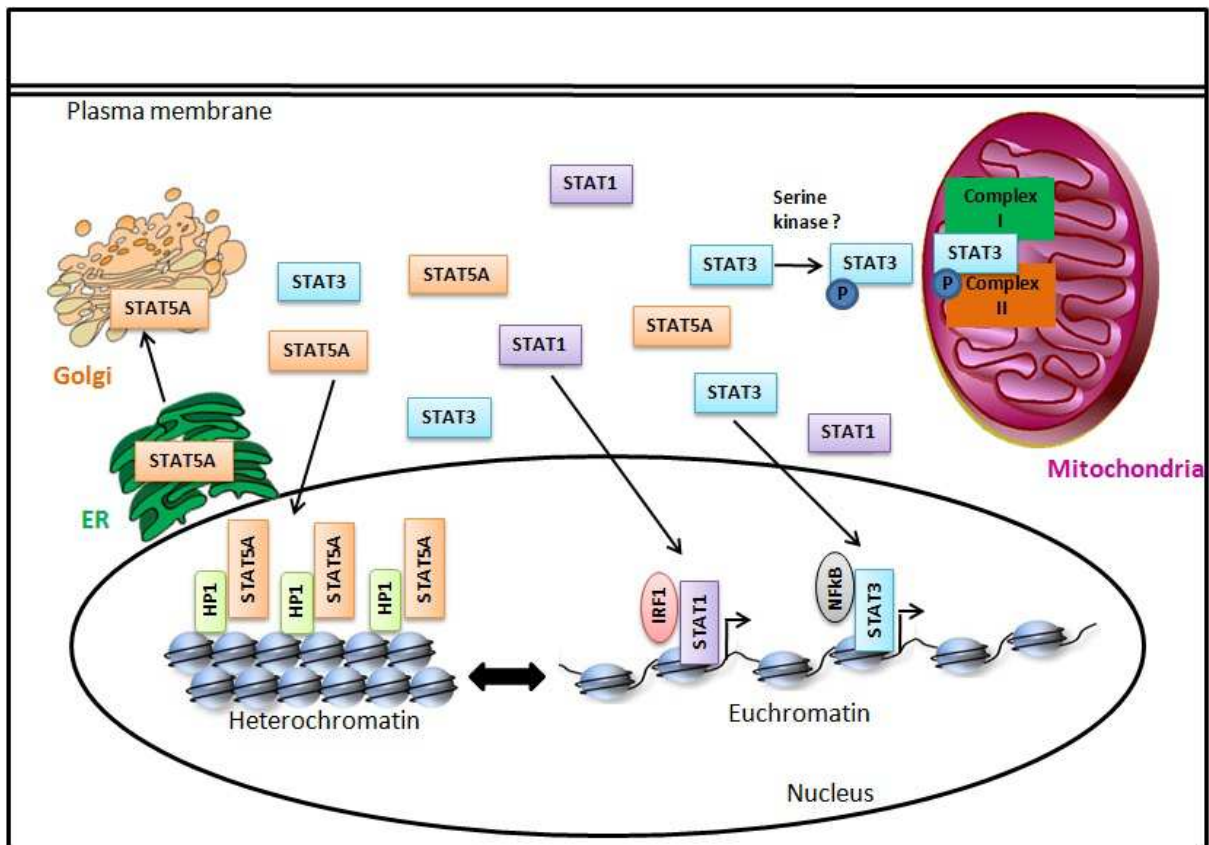


Figure 14: The non-canonical JAK/STAT signalling pathway. STAT factors can exert cellular functions even in the absence of classical phosphorylation on Tyr or Ser residues. Moreover, in addition to their role as transcription factors, they can have different roles in the cytoplasm, as it has been shown for STAT3 in the mitochondria and for STAT5A in the Golgi apparatus and in endoplasmic reticulum. STAT factors can be found also in the nucleus in absence of Tyr phosphorylation and have a role in heterochromatin stability and in gene transcription.

3.3 NEGATIVE REGULATORS OF JAK/STAT PATHWAY

There are three major classes of JAK/STAT pathway-negative regulators: the suppressors of cytokine signaling (SOCS), the protein inhibitors of activated STATs (PIAS) and the protein tyrosine phosphatase (PTPs) (Greenhalgh and Hilton 2001).

- the **PTPs** reverse the activity of JAKs through dephosphorylation. Among the PTPs, the best characterized is the **Src homology region 2 domain-containing phosphatase-1 (SHP-1)**. SHP-1 contains two SH2 domains and can bind either to phosphorylated JAKs or to phosphorylated receptors to facilitate dephosphorylation of these activated signaling molecules. Other tyrosine phosphatases, such as the hematopoietic transmembrane phosphatase

CD45, have a role in regulating JAK/STAT signaling cascade through a subset of receptors.

- **SOCS** proteins are a family of 8 members (**SOCS1-SOCS7** and **CISH**) containing an SH2 domain and a SOCS box at the C-terminus. In addition, a small kinase inhibitory region located N-terminal to the SH2 domain, has been identified for SOCS1 and SOCS3. The SOCS factors are responsible for the end of JAK/STAT signaling: activated STATs stimulate transcription of SOCS genes and the resulting SOCS proteins bind phosphorylated JAKs and the receptors to turn off the pathway. SOCS factors can affect their negative regulation by 3 different mechanisms: 1) by binding phosphotyrosine on the receptors they block recruitment of signal transducers to the receptors, 2) by binding directly to JAKs or to the receptors to inhibit specifically the JAK kinase activity, 3) by interacting with the elongin BC complex and cullin2 and facilitating the ubiquitination of JAKs.
- the third class of negative regulators is the **PIAS** family proteins, composed of **PIAS1, PIAS3, PIASx** and **PIASy**. These proteins have a Zn-binding RING-finger domain in the central portion, a well-conserved SAP (SAF-A/Acinus/PIAS) domain at the N-terminal region and a less-well-conserved carboxyl domain. PIAS proteins bind to activated STAT and prevent them from binding DNA. PIAS proteins seem to act by sumoylating STAT factors and leading them to degradation.

3.4 STAT TARGET GENES

Many target genes of STATs have been identified. These genes change depending on cellular context and on STAT factors. However, there are some genes which are shared by multiple STAT transcription factors and which are mostly involved in negative regulation of JAK/STAT signaling, such as the SOCS and the PTP factors, in order to avoid the continuously activation of the signal cascade. Some other identified target genes are involved in cell proliferation and survival (for STAT3-6). Among the genes involved in the cell proliferation there are: Cyclin D1, Cyclin D2, Cyclin D3, Cyclin E1, cyclin-dependent kinase

inhibitor 1A (Cdkn1A or p21) and the transcription factor c-myc. For the genes involved in survival there are the anti-apoptotic factors Bcl-x1, survivin and myeloid cell leukemia-1 (mcl-1), the apoptotic factors Bcl-2 and Fas receptor, and the oncogenes Pim-1 and Pim-2.

3.5 STAT5 FACTORS

STAT5 was discovered in 1994 as a transcription factor regulating milk protein expression. It was initially called Mammary Gland Factor (MGF) (Schmitt-Ney et al. 1992). However, based on its similarity to the family of STAT factors, it was renamed appropriately STAT5. In 1995, three groups independently cloned the mouse homolog, now named STAT5A, and a closely related protein, called STAT5B (Azam et al. 1995, Liu et al. 1995, Mui et al. 1995). Later, it turned out that both STAT5A and STAT5B are rather ubiquitously expressed and activated by a plethora of cytokines and growth factors, including prolactin (PRL), GH, EPO, TPO and several interleukins.

3.5.1 STAT5 molecular structures

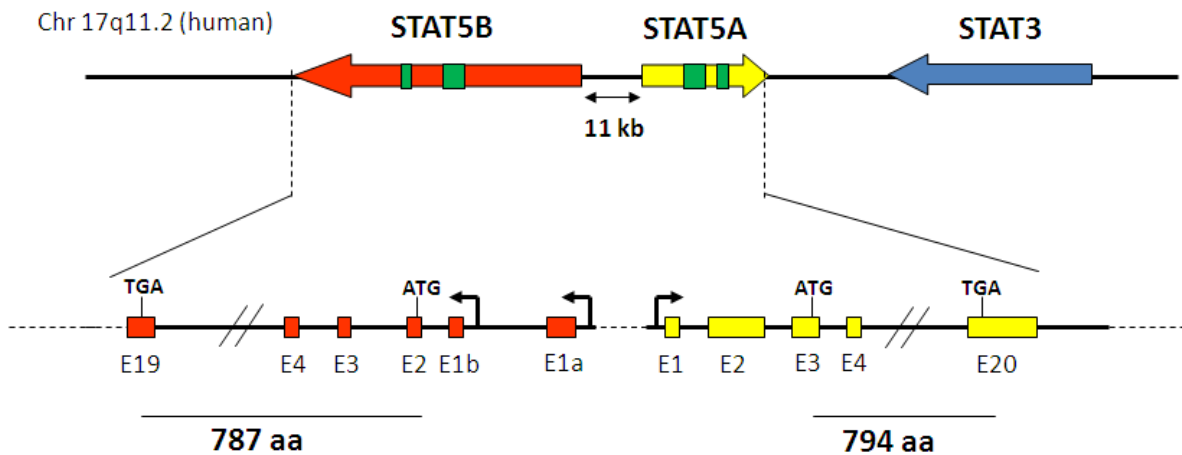
STAT5A and STAT5B present a high similarity, of 96%, at the protein level. They are encoded by two juxtaposed genes which have two different promoters and the transcriptional start sites are within 11kb of each other. *STAT5A* and *STAT5B* are located on chromosome 11 (in mouse) and chromosome 17 (in humans), in a locus that also contains the *STAT3* gene. The identity and the chromosomal localization suggest that the two genes result from recent gene duplication. In humans, the *STAT5A* gene is composed of 20 exons, while *STAT5B* is composed of 19 exons. The ATG codon is located in exon 3 for *STAT5A* and in exon 2 for *STAT5B*, while the stop codon is in exon 20 for *STAT5A* and in exon 19 for *STAT5B*. Moreover, *STAT5A* presents a single start site, situated at -642 bp from ATG, whereas *STAT5B* presents 2 alternative first exons (E), the E1a and the E1b. The *STAT5B* E1a is ubiquitously expressed, whereas the E1b is expressed only in some tissues, such as placenta, spleen and brain (Ambrosio et al., 2002) (Fig.15A).

STAT5A and STAT5B proteins contain respectively 794 and 787 amino acids and the highest degree of divergence is found in the C-terminal trans-activation domain. Serine and Tyrosine target of phosphorylation are localized at the C-terminal region, nearby the trans-activation domain and are (Fig. 15B):

- **Tyr 694, Ser 725 and Ser 779** for **STAT5A** (Friedbichler et al. 2010);
- **Tyr 699, Ser 731 and Ser 193** for **STAT5B** (Mitra et al. 2012).

STAT5A and STAT5B homodimers share similar core TTCN₃GAA GAS motifs. However, STAT5A differs from STAT5B for tetramer formation and STAT5A tetramers can bind to tandemly linked GAS motifs not retaining a consensus TTCN₃GAA motif (Soldaini et al. 2000). Stable tetramer formation of STAT5A is mediated through the N-terminal domain of STAT5A which allows dimers oligomerization. Tetramerization-deficient STAT5A proteins have a more limited set of binding sites than tetramerization-competent STAT5s, indicating an essential role for the N-terminal domain (John et al. 1999).

A



B

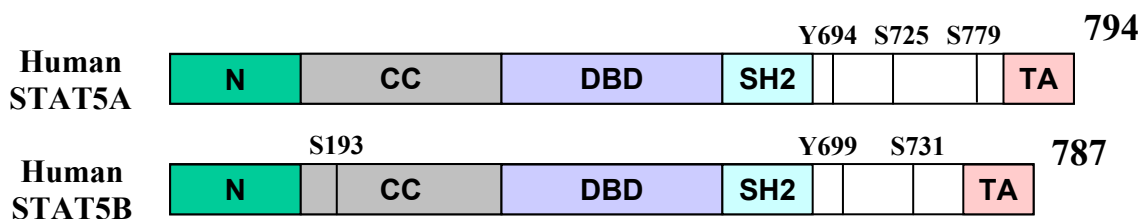


Figure 15: Human STAT5 factors. A) STAT5A and STAT5B genes are located on chromosome 17 in humans and they are encoded by two different genes which present two different promoters. B) Functional domains of human STAT5A and STAT5B factors: N: N-terminal domain, CC: coiled-coil domain, DBD: DNA binding domain, SH2: Src homology-domain for the dimerization, TA: transactivation domain. The tyrosine (Y) and Serine (S) marked represent the targets of phosphorylation.

3.5.2 Known roles for STAT5A and STAT5B

Although STAT5A and STAT5B gene promoters might share certain regulatory elements, cell-preferential transcriptional patterns have emerged (Liu et al. 1995). While STAT5A is prevalent in mammary tissue, STAT5B is predominant in muscle and liver. Other tissues, such as heart and salivary gland, have an equivalent distribution of STAT5A and STAT5B. The differential roles for STAT5A and STAT5B described in the literature are summarized in Table 2.

Lessons from mice

In 1997, mice with disabling mutations in the genes encoding STAT5A or STAT5B were described. Mice lacking the individual genes were viable and displayed different defects. It was shown that **STAT5B deletion leads to a loss of sexually differentiated responses associated with the sexually dimorphic pattern of GH secretion**. Sexual dimorphism was lost with male characteristic body growth rate and male-specific liver gene expression decreasing to wild type female levels in STAT5B^{-/-} males (Udy et al. 1997). On the other hand, **STAT5A KO showed that it is the principal and obligate mediator of mammary gland development and differentiation during pregnancy**, whereas STAT5B KO did not affect gland mammary (Liu et al. 1997). Moreover, STAT5A^{-/-} showed decreased levels of T cells whereas STAT5B^{-/-} had decreased levels of Natural Killer (NK) cells.

The first double STAT5A/5B^{null/null} mice displayed immunological defects but not as severe as anticipated from mutation in the various cytokine receptors that signal through the JAK2/STAT5 pathway. Indeed, these mice expressed STAT5A/5B factors truncated at the N-terminal level, which were still able to form active dimers but not tetramers; these mice were therefore renamed STAT5A/5B^{ΔN/ΔN}. **Almost all STAT5A/5B truly doubly deficient mice, STAT5A/5B^{null/null}, die perinatally; the few surviving STAT5A/5B^{null/null} mice exhibit severe combined immunodeficiency, they failed to develop T, B and NK cells, they present anemia and leukopenia** (Yao et al. 2006, Hoelbl et al. 2006). Furthermore, it was shown that these mice exhibit **depleted long-term HSC pool** (Wang et al. 2009).

An artificial mutant STAT5A-1*6 was generated by PCR-driven random mutagenesis. It presents two amino acid substitutions: one is located upstream of the putative DNA binding domain (H299R) and the other is located in the trans-activation domain (S711F). The

mutant *STAT5A-1*6* was constitutively –although weakly- phosphorylated on tyrosine residues, localized in the nucleus, and was transcriptional active (Onishi et al. 1998). **Introduction of active STAT5A-1*6 in embryonic stem cells facilitates hematopoietic differentiation generating ES-derived HSCs.** These HSCs display long-term self-renewal characteristics *in vitro* and contribute to hematopoietic reconstitution *in vivo* (Schuringa et al. 2004).

Lessons from humans

Disabling mutations in the STAT5B gene were identified in six patients with severe growth retardation (Kofoed et al. 2003), indicating a central role for STAT5B in GH-mediated post-natal growth. **In contrast to mice, 5 of the 6 patients with reduced size were females, suggesting that in humans STAT5B activity is not restricted to sexual dimorphism.** In addition, these patients showed immunological defects.

In cervical cancers associated with human papilloma virus (HPV) infections, STAT5B is over-expressed, whereas STAT5A expression is down-regulated. Moreover, STAT5B expression pattern is associated with the severity of the disease (Sobti et al. 2010).

In cells that express the NPM1-ALK oncogenic tyrosine kinase, STAT5A is methylated at its promoter level and so its transcription is inhibited; when STAT5A expression is restored by a DNA methyltransferase inhibitor, it can bind NPM1-ALK and enhance its inhibition. This suggests a tumour suppressor key role for STAT5A and not for STAT5B in these cells (Zhang et al. 2007b).

Moreover, in humans, STAT5A and STAT5B have been shown to interact with different and specific co-factors. For instance, **in human breast cancer cells, c-Myb interacts with STAT5A and not with STAT5B;** here c-Myb potentiates STAT5A-driven gene expression, possibly acting as a STAT5A co-activator (Fang, Rycyzyn and Clevenger 2009).

In 2004, Schuringa and colleagues introduced the artificial constitutive active STAT5A-1*6 mutant (presented before) into human stem and progenitors cells derived from umbilical cord blood. They demonstrated that such **exogenous expression of an activated STAT5A in CD34⁺ cells results in enhanced self-renewal.** Furthermore, it drives the expression of a number of proto-oncogenes and cytokines, as well as a number of erythroid specific genes, **favoring erythroid over myeloid differentiation and providing a long-term proliferative advantage for erythroid progenitors.** In contrast, **STAT5A/5B down-**

regulation promotes megakaryocytes expansion from CD34⁺ cells and impairs the development of erythroid progenitors (Olthof et al. 2008).

In conclusion, mounting evidence in mouse and in humans seems to favor the hypothesis of a different role for STAT5A and for STAT5B, which depends on the cellular context and on patho-physiological states.

	Mice	Humans
STAT5A	<ul style="list-style-type: none"> - mammary gland development during pregnancy - hematopoietic differentiation of embryonic stem cells - reduced lymphocyte number 	<ul style="list-style-type: none"> - tumor suppressor role in NPM1-ALK expressing lymphome - self-renewal enhancer of CD34+ HSCs - central role in erythroid over myeloid differentiation of HSCs
STAT5B	<ul style="list-style-type: none"> -sexual dimorphism in male controlled by GH secretion - defect in NK cell function 	<ul style="list-style-type: none"> - central role in GH-mediated post-natal growth - crucial role in cervical cancers associated with HPV infections, liver cancers, it contributes to squamous cell tumorigenesis and it enhances the transforming potential of v-Src.

Table 2: STAT5A and STAT5B differential roles described in the literature for humans and mice.

3.5.3 STAT5 in pathophysiology

STAT5 is activated by a plethora of cytokines, growth factors, hormones and some other cytoplasmic kinases. The interest of STAT5 in cancer studies comes from the initial observations of its activation in many human cancers. Mutations in signaling pathways acting upstream of STAT5 proteins and consequently aberrant phosphorylation of STAT5 on tyrosine and serine residues, is the best-documented mechanism of STAT5 activation in human cancers, especially in hematopoietic diseases. In addition to these aberrations, some other defects causing the over-activation of STAT5 can involve the negative regulatory control of STAT5 activation mediated by tyrosine phosphatases, such as SOCS, PIAS and PTPs.

3.5.3.1 Hematopoietic neoplasms

In 1996, Van Etten and colleagues reported that STAT5 is constitutively activated in BCR-ABL-induced **CML** cells. Other studies similarly observed over-activation of STAT5 in acute leukemia patient samples (Ilaria and Van Etten 1996, Van Etten 2007, Gouilleux-Gruart et al. 1997). Over-activation of STAT5 was also linked to the presence of oncogenic over-active tyrosine kinases, such as mutated aberrant kinase FLT3-internal tandem duplication (called **FLT3-ITD**) or the **Kit (D816V)** mutant, **TEL/PDGFβR** or **Tel/Jak2** fusion proteins, and shown to participate to leukemic cell growth. In addition, 40% of patients with **severe congenital neutropenia (SCN)** carry mutations in the gene encoding the G-CSF receptor that truncate part of the cytoplasmic domain of the receptor, resulting in constitutive activation of STAT5 and development of AML (Germeshausen, Ballmaier and Welte 2007). Among the hematopoietic malignancies, there is also the **Polycythemia Vera (PV)** myeloproliferative disease, which presents a mutation on JAK2 (the V617F mutation) that has been associated with constitutive activation of STAT5 (Wernig et al. 2006).

In contrast to STAT5 oncogenic role, STAT5A was reported to act as tumor suppressor in a subset of **T-cell lymphoma** where STAT5A, but not STAT5B, is epigenetically silenced by the NPM1-ALK tyrosine kinase. In this cellular context, when STAT5A expression is restored leads to inhibition of NPM1-ALK expression (Zhang et al. 2007b).

Finally, STAT5A exhibits selective tetramerization potential and a unique Serine residue, Ser-779, which is absent in STAT5B. Both STAT5A characteristics have been linked to leukemic transformation (Friedbichler et al. 2011, Moriggl et al. 2005, Soldaini et al. 2000).

3.5.3.2 Solid cancers

STAT5 proteins are important not only for the development of hematopoietic diseases, but also of several solid cancers. For instance, STAT5 was suggested to have a dual role in **breast cancer** cells. On one hand STAT5 promotes malignant transformation in mammary epithelial cells and is important for cancer initiation (Vafaizadeh et al. 2010). On the other hand, STAT5 activity correlates with a better prognosis for patient survival since it indicates mammary epithelial cell differentiation and delays metastatic progression (Wagner and Rui 2008).

Persistent STAT5 activity, often in association with GH and Prolactin expression, is found also in the majority of **hormone refractory prostate cancers**. Moreover, studies in cell line models show that over-phosphorylation of STAT5 on Tyr residues, is responsible for the maintaining of the malignant phenotype (Tan et al. 2008). JAK/STAT pathway is frequently activated in **lung and head and neck cancers** and STAT5 contributes to the growth and formation of malignant cells and to resistance to EGFR inhibitors (Koppikar et al. 2008). In liver cancer, there is activation of STAT5B factor and in patient samples STAT5B activation was found to be correlated with the stage of tumor. STAT5B promotes aggressiveness of **liver cancer** cells through induction of epithelial-mesenchymal transition and promotion of tumor metastasis (Lee et al. 2006). STAT5 activation was also found in several patient with **melanoma** and its activation contributes to resistance to interferons therapy and cell survival (Wellbrock et al. 2005). Furthermore, activated STAT5 was found in **intraductal papillary mucinos neoplasms** (Kataoka et al. 2007), **glioblastoma** (Liang et al. 2009), **colon carcinoma** (Xiong et al. 2009), **ovarian carcinoma** (Chen et al. 2004) and, just STAT5B but not STAT5A, in **cervical cancers** after HPV infection (Sobti et al. 2010).

4.

STAT5 factors in CML development and maintaining

4.1 STAT5 AS A CRUCIAL FACTOR FOR CML: A HISTORICAL PERSPECTIVE

4.1.1 First results obtained in *in vitro* models

Van Etten and colleagues provided the first evidence of STAT5 implication in CML disease. They made use of BaF3 (lymphoid) and FDCP1 (myeloid) cell lines transformed by the isoform p210 of BCR-ABL and showed that STAT5 phosphorylation was dependent of BCR-ABL. However, they did not find STAT5 to immunoprecipitate with BCR-ABL, suggesting that STAT5 activation by BCR-ABL was an indirect activation and raising the question of how STAT5 was phosphorylated (Ilaria and Van Etten 1996).

In 1999, Nieborowska-Skorska and colleagues found that STAT5-BCR-ABL induced phosphorylation and activation was mediated by a mechanism that requires the BCR-ABL SRC homology (SH2) domain and the proline-rich binding site of the SH3 domain (Fig.8). BCR-ABL mutants deleted in both SH3 and SH2 domains completely abolished the ability to activate STAT5. Moreover, they found that STAT5 is required for leukemogenesis using a dominant active mutant of STAT5B. They assessed that STAT5B has a role in protection from apoptosis and induction of growth factor-independent proliferation in a BCR-ABL dependent manner (Nieborowska-Skorska et al. 1999).

STAT5 activation was linked with cell cycle progression and inhibition of apoptosis. Among the genes which play a pivotal role in these processes and which are regulated by STAT5 in CML there are Cyclin D1, Bcl-x1, myc (de Groot et al. 2000). Moreover, it was shown that STAT5 cooperates with p85 subunit of PI3K and Ras protein to promote its oncogenic activity (Santos et al. 2001).

4.1.2 First results obtained in *in vivo* models

The first experiment to prove the crucial role for STAT5 in *in vivo* mice was performed by Ye and colleagues in 2006. They expressed p210 BCR-ABL in primary murine cells deficient for STAT5 and they found that these cells were defective in inducing CML-like illness in recipient mice. Importantly, reconstitution of wild-type mice with BM cells co-expressing BCR-ABL and dominant negative STAT5 profoundly reduced the incidence of CML. These findings indicated that STAT5 plays an important role for the pathogenesis of the CML-like illness also *in vivo* models (Ye et al. 2006). Then, Hoelbl and colleagues confirmed and expanded these *in vivo* results. By using a tumor-specific gene-deletion approach, they dissected the role of STAT5, and also of STAT3, in BCR-ABL-induced leukemia maintenance. They found that both are required for the initial transformation by BCR-ABL, but, once established, only STAT5 is crucial for viability and proliferation of leukemic myeloid cells. Furthermore, the deletion of STAT5 in leukemic cells resulted, as it was already shown *in vitro*, in G0/G1 cell cycle arrest followed by apoptosis. The mere expression of a single STAT5 target gene, such as c-myc, Bcl-xl or Cyclin F1, cannot replace STAT5 expression (Hoelbl et al. 2010).

In conclusion, BCR-ABL cells are addicted to STAT5 for initiation and maintaining in the leukemia state and, since its discovery, STAT5 has drawn the attention of a lot of biologists and physicians as a potential drug target.

4.1.3 STAT5 as a new therapeutic target: the use of Pimozide

Targeting STAT5 has become an attractive approach to overcome TKI resistances. In 2011, Nelson and colleagues performed a high throughput screen based on STAT5 transcriptional activity and found a chemical compound known to be safe in humans and to inhibit the activity of STAT5, the Pimozide. Pimozide is a FDA-approved drug in the United States for the treatment of Tourette syndrome and appears to work by blocking a dopamine receptor. Pimozide inhibits STAT5 expression and phosphorylation on Tyr, by a mechanism that results unknown. However, treatment of CML cell lines, such as KU812 and K562, and CML primary cells from BM with Pimozide induces apoptosis and cell cycle arrest in 48 hours. Furthermore, Pimozide synergizes with Imatinib and Nilotinib to decrease STAT5 phosphorylation and induce apoptosis also of TKI-resistant CML cells (Nelson et al. 2011).

Thus, Pimozide is a potent STAT5 inhibitor that is effective in models of CML. The efficacy of Pimozide against resistant CML mutants and its synergic effects in combination of Imatinib and Nilotinib, suggest that STAT5 inhibition might have beneficial effects in the treatment of CML.

4.2 STAT5 PHOSPHORYLATION IN CML: WHAT'S NEW

Using murine CML models combined with inducible ablation of JAK2, Hantschel and colleagues showed that initial myeloid transformation and leukemia maintenance is independent of JAK2. In BCR-ABL positive cells there is an uncoupling of the canonical JAK2/STAT5 signaling. Thus, pharmacological targets need to focus on STAT5 itself and not on JAK2 (Hantschel et al. 2012). However, how STAT5 is phosphorylated in CML cells is a central unresolved question. Some data suggest that JAK2-independent phosphorylation of STAT5 could be mediated by SRC kinases. In 32D cell line, a reduced expression of SRC is responsible for the decreased phosphorylation of STAT5. These results were obtained in cell lines, but they were never reproduced in other *in vitro* or *in vivo* models.

Understanding of STAT5 activation remains crucial to find new possible therapeutic targets for those patients resistant or intolerant to TKI treatment.

4.3 STAT5 AND TKIs RESISTANCE

STAT5 implication in TKIs resistance emerged from a study of Wang and colleagues. They found that IM- and Nilotinib-resistant cell lines and primary cells exhibit an elevated mRNA and protein levels of GM-CSF. GM-CSF autocrine secretion mediated resistance to IM and Nilotinib by activating JAK2/STAT5 pathway activation and consequently circumventing the need for BCR-ABL signaling to maintain survival (Wang et al. 2007). They were the first to reveal a correlation between elevated STAT5 phosphorylation and TKIs resistance. In 2010, as the time I started my PhD research, nothing more was known about STAT5 implications in TKIs resistance and their roles and molecular mechanisms, as well as their expression in TKI resistance, has become more and more attractive.

4.4 STAT5 AND CELLULAR LOCALIZATION IN CML CELLS

The evidence of a cytoplasmic localization of STAT5 factors emerged in CML cells, where STAT5 factors were found to be localized in specific cytoplasmic structures called podosome. Podosomes are structures involved in cell adhesion and migration. This specific STAT5 localization in CML cells was found to be mediated by the Src kinase HCK, which is activated by BCR-ABL (Poincloux et al. 2007). These findings were confirmed and expanded by Harir and colleagues. Using primary cells, they found that persistent STAT5 phosphorylation is detected mainly in the cytoplasm of patients with CML and AML. Moreover, they showed that oncogenic activation of STAT5 triggers its association with PI3K and the scaffolding protein Gab2, and that activation of this pathway may be involved in the leukemic potential of constitutive active STAT5 molecules (Harir et al. 2007).

5. Objectives

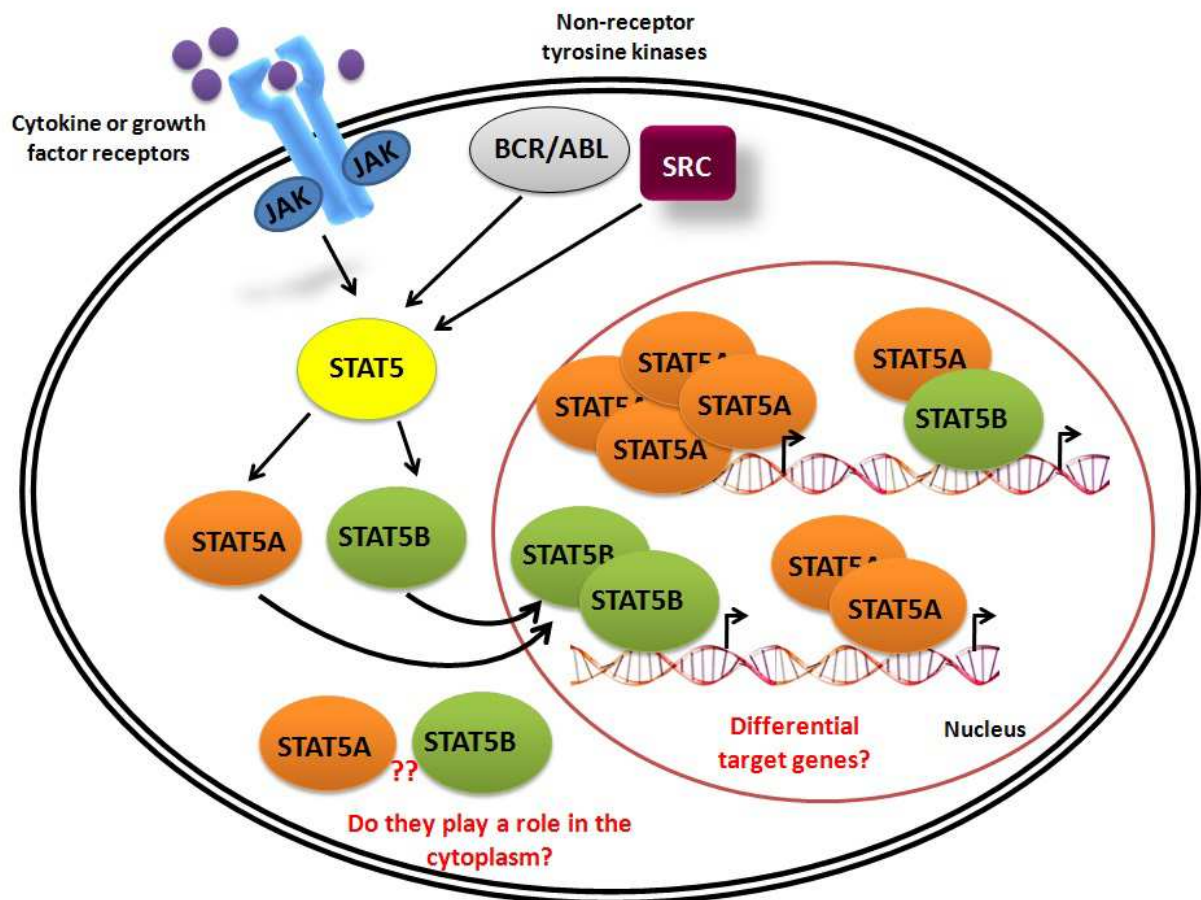


Figure 16: STAT5 activation and questions arisen on STAT5A and STAT5B mode of actions

STAT5 activation can be mediated by JAK kinases which are associated to cytokine or growth factor receptors and by some non-receptor tyrosine kinases, such as SRC and BCR-ABL. STAT5 activation is a crucial feature of BCR-ABL expressing cells, however there has been no clear data studying a differential effect that might be induced by STAT5A or STAT5B in either the normal HSC context or in CML. Moreover, the molecular mechanisms of STAT5s, outside their implication in CML cell survival and consequently their regulation of proliferative and anti-apoptotic target genes, remain poorly known.

For these reasons, at the beginning of my PhD project, I focused on the differential roles of STAT5A and STAT5B factors in normal and CML cell.

RESULTS

PART I

Differential contributions of STAT5A and STAT5B to stress protection and tyrosine kinase inhibitor resistance of chronic myeloid leukemia stem/progenitor cells.

EXPERIMENTAL DESIGN

To differentiate STAT5A and STAT5B activities in human HSCs, we identified small hairpin RNAs (shRNAs) that differentially knock-down the two STAT5s. We chose to perform an RNA interference based strategy because over-expression of STAT5A in CML cells resulted to be toxic and to lead to cell apoptosis, complicating this study. Moreover, we used a shRNA approach because it provides specific and long-lasting gene silencing, without clonal selection.

The shSTAT5 RNA sequences were found by the Ambion software. These shRNAs were then introduced in lentiviral vectors expressing the Green Fluorescent Protein (GFP) or the truncated CD4 receptor reporter genes to transduce cord blood CD34⁺, CML CD34⁺ and CML cell lines (K562, LAMA84 and MEG01). As control, we used an empty lentiviral vector or a vector expressing a shRNA directed against the luciferase RNA. To assess shRNA efficiency we disposed of specific antibodies which selectively recognize STAT5A, STAT5B or both STAT5A/5B. STAT5A and STAT5B protein levels were always checked by Immunoblot; 10% SDS-polyacrilamide gels allowed for a differential migration of STAT5A and STAT5B. Immunoblot analyses showed that one shRNA, hereafter called shSTAT5A (shS5A), strongly decreased STAT5A protein levels without affecting STAT5B, as compared to not-transduced cells or control shRNA expressing cells. Another shRNA, renamed shSTAT5B (shS5B), strongly inhibited STAT5B protein expression, but also weakly inhibited STAT5A. A third shRNA, shSTAT5A/5B, knocked-down both STAT5A and STAT5B proteins. shRNAs neither change STAT1, STAT3 or STAT6 expression, nor STAT1/3 phosphorylation on Tyr (Fig.17). Furthermore, we always checked specific shRNA activities by rescue experiments. We restored STAT5s expression in STAT5s KD cells by introducing murine *STAT5s* cDNA which are not recognized by our shSTAT5s. We were always able to prove that the specific shSTAT5s activities we found was due to proper inhibition of STAT5 and that they were not an shRNA side effect.

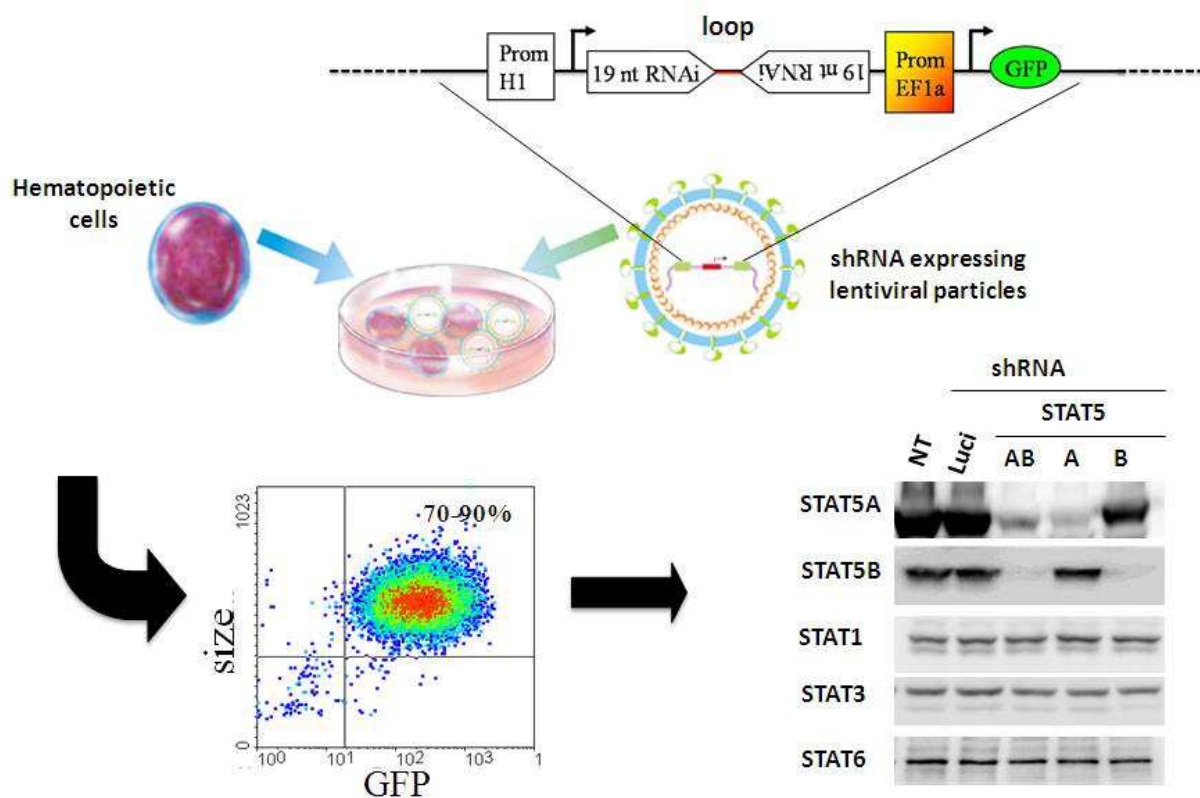


Figure 17: shRNA lentiviral vectors and workflow. shRNA expressing vectors encoding for the GFP or CD4 reporter genes were transduced in hematopoietic cells (cord blood CD34⁺, CML CD34⁺ and CML cell lines). Transduction efficiency was assessed by Fluorescent Activated Cell Sorting (FACS) and cells were sorted when required. shRNA proper inhibition was assessed by Immunoblot experiments.

To investigate STAT5A and STAT5B activities, we made use of primary CD34⁺ cells from healthy cord blood, peripheral blood and bone marrow and CD34⁺ cells from CML patients at diagnosis or after acquired resistance to IM. To study their molecular mechanisms, we made use of 3 different CML cell lines, K562, LAMA84 and MEG01.

STAT5A and STAT5B activities were first evaluated by analyzing growth properties of shSTAT5 expressing normal and leukemic cells. We could immediately ascertain that the 2 factors had different incidences on maintaining of normal and leukemic cell survival under standard condition, resulting in STAT5B have a crucial role. The shSTAT5A did not affect cell survival and this allowed the study of STAT5A inhibition in other contexts outside survival.

Second, we subjected shSTAT5A cells to exogenous oxidative stress and investigated their ability to respond to this kind of insult. These experiments shed new light on the activity and molecular mechanisms of STAT5 factors in normal and leukemic cells, resulting in STAT5A had novel role as anti-oxidant factor.

Third, given the importance of STAT5A to face oxidative stress, we wondered if it could play a role also in the protection to drug insult. We analyzed the effects of STAT5 KD in a context of IM-resistance and we evidenced another new role for STAT5A as important mediator of drug resistance.

These results together, allowed in April 2013 the publication of my first PhD paper on Cancer Research journal. A more detailed summary of the paper is on the next page.

SUMMARY ARTICLE I

The CML is a clonal hematopoietic stem cell disorder characterized by the t(9:22) genetic translocation and expression of the oncogenic tyrosine kinase BCR-ABL . A first BCR-ABL Tyrosine Kinase Inhibitor (TKI), Imatinib (IM, Novartis), was identified that inhibits proliferation of BCR-ABL expressing hematopoietic cells and leads to disease remission. However, *BCR-ABL* mRNA remains detectable in the most immature HSCs (Chomel et al. 2011) and discontinuation of IM results in clinical relapse (Rousselot et al. 2007).

More than ten years ago it was shown that STAT5 transcription factors play a crucial role in the CML pathogenesis reporting that STAT5 is constitutively activated in human primary CML cells (Ilaria and Van Etten 1996). The pivotal role of STAT5 in CML was confirmed in STAT5 KO mice few years ago. In this study authors demonstrated that STAT5 is not only required for disease initiation but also for leukemia maintenance (Hoelbl et al. 2010). However, the specific contributions of the two related STAT5 genes, STAT5A and STAT5B, were unknown.

We used an RNA interference based strategy to analyze STAT5A or STAT5B expression in normal and CML cells.

We showed that STAT5A/5B double knock-down triggers normal and CML cell apoptosis and suppresses long-term clonogenic potential of immature HSCs which are known to be resistant to TKI treatment and responsible for residual disease. STAT5A and STAT5B exhibited similar pro-survival activity, but STAT5A loss alone was ineffective at impairing growth of both normal and CML CD34⁺ cells under standard conditions. In contrast, STAT5A loss was sufficient to enhance Reactive Oxygen Species (ROS) in presence -and also in absence- of H₂O₂ stimuli. Moreover, we found that basal ROS enhancement in shS5A-cells is in part generated by NADPH oxidases. ROS augmentation is mostly correlated with enhanced DNA damages. Indeed, shS5A cells present an increased number of γ H2AX and RAD51 foci in the nucleus, which are involved in DNA double strand breaks reparation. We looked for STAT5A target genes in CML cells and found that the cellular oxygen sensor Prolyl-Hydroxylase 3 (PHD3), which regulates the degradation of Hypoxia Induced Factors (HIF), is negatively regulated by STAT5A but not by STAT5B. STAT5A-dependent PHD3 regulation does not require the trans-activator domain of STAT5A, suggesting that STAT5A contributes

to stress resistance through unconventional mechanisms, in a non-transcriptional-dependent manner. This observation fits with STAT5A massive cytoplasmic localization in CML cells found by our group and others (Chatain et al. 2013).

We further investigated the expression of STAT5A and STAT5B in primary WT and CML cells and we found that STAT5A/STAT5B expression ratio increases with the progression of the pathology. This suggested that STAT5A may play a role in the resistance to therapeutic treatment. Therefore, we selected for IM-resistant stem/progenitor cells that did not present over-expression or over-activation of BCR-ABL. In contrast to primary cells at diagnosis, IM-resistant cells showed enhanced STAT5A dependence, by being sensitive to STAT5A single knock-down.

Collectively, our findings highlight the pro-survival, stress protection and drug resistance roles of STAT5 factors, providing new understanding for medical treatment of CML patients. STAT5A specific inhibition could limit CML resistance to TKIs treatment without affecting normal hematopoietic cells.

ARTICLE I

Differential Contributions of STAT5A and STAT5B to Stress Protection and Tyrosine Kinase Inhibitor Resistance of Chronic Myeloid Leukemia Stem/Progenitor Cells

Luana Casetti^{1,2}, Séverine Martin-Lannerée^{1,2}, Imen Najjar^{1,2}, Isabelle Plo³, Sylvie Augé^{1,2}, Lydia Roy⁴,
Jean-Claude Chomel⁵, Evelyne Lauret^{1,2}, Ali G. Turhan⁵, and **Isabelle Dusanter-Fourt**^{1,2}

Differential Contributions of STAT5A and STAT5B to Stress Protection and Tyrosine Kinase Inhibitor Resistance of Chronic Myeloid Leukemia Stem/Progenitor Cells

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Abstract

STAT5 fulfills essential roles in hematopoietic stem cell (HSC) self-renewal and chronic myeloid leukemia (CML), a prototypical stem cell malignancy. However, the specific contributions of the two related genes STAT5A and STAT5B have not been determined. In this study, we used a RNAi-based strategy to establish participation of these genes to CML disease and persistence following targeted therapy. We showed that STAT5A/STAT5B double-knockdown triggers CML cell apoptosis and suppresses both normal and CML HSC long-term clonogenic potential. STAT5A and STAT5B exhibited similar prosurvival activity, but STAT5A attenuation alone was ineffective at impairing growth of normal and CML CD34⁺ cells isolated at diagnosis. In contrast, STAT5A attenuation was sufficient to enhance basal oxidative stress and DNA damage of normal CD34⁺ and CML cells. Furthermore, it weakened the ability to manage exogenous oxidative stress, increased p53 (TRP53)/CHK-2 (CHEK2) stress pathway activation, and enhanced prolyl hydroxylase domain (PHD)-3 (EGLN3) mRNA expression. Only STAT5A and its transactivation domain-deficient mutant STAT5AA749 specifically rescued these activities. STAT5A attenuation was also active at inhibiting growth of CML CD34⁺ cells from patients with acquired resistance to imatinib. Our findings show that STAT5A has a selective role in contributing to stress resistance through unconventional mechanisms, offering new opportunities to eradicate the most primitive and tyrosine kinase inhibitor-resistant CML cells with an additional potential to eradicate persistent stem cell populations. *Cancer Res*; 73(7); 2052–8. ©2013 AACR.

Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell (HSC) malignancy characterized by the unique t(9;22) chromosome translocation. This generates the BCR-ABL oncogene, a constitutively activated tyrosine kinase, which is the primary oncogenic event causing CML (1). This has made CML a model for targeted therapies by the use of first-generation (Imatinib mesylate, IM) and second-generation tyrosine kinase inhibitors (TKI). Currently, TKI administration leads rapidly to complete cytogenetic remission and major molecular response in the majority of patients with CML. However, BCR-ABL mRNA remains

detectable in the most immature stem/progenitor cells despite long-term TKI therapy, discontinuation of IM in patients with complete molecular remission results in molecular relapse in 60% patients, and clinical resistance to IM also occurs in approximately 15% patients (2, 3). These observations highlight the needs to approach mechanisms of CML persistence.

Activation of STAT5 is a signaling hallmark of CML, reported more than a decade ago (4). STAT5 plays a central role in the maintenance of murine and human HSCs from healthy donors and patients with several types of acute leukemia (5). Inhibition with a dominant negative mutant of STAT5 and STAT5 knockdown impair survival of BCR-ABL-expressing cell lines and short-term clonogenic potential of primary CML progenitors, respectively (6, 7). *In vivo* transplantation of BCR-ABL-expressing murine hematopoietic progenitor cells induces an acute so-called "CML-like" leukemia in recipient animals, which almost disappears in STAT5-deficient background. Moreover, STAT5 overexpression rendered v-ABL⁺ murine BM cells resistant to IM (8). These observations showed that STAT5 is essential to the induction of CML-like leukemia by exogenous BCR-ABL and provides resistance to IM in murine models. Thus, there is considerable interest in delineating STAT5 contribution to normal and CML HSC maintenance in human cells.

Authors' Affiliations: ¹Inserm U1016, Institut Cochin; ²Université Paris Descartes, Sorbonne Paris Cité, Paris; ³Inserm U1009, Villejuif; ⁴Inserm CIC0802, CHU; and ⁵Inserm U935, Université and CHU, Poitiers, France

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L. Casetti, S. Martin-Lannerée, and I. Najjar contributed equally to this work.

Corresponding Author: Isabelle Dusanter-Fourt, Institut Cochin, 22 rue Méchain 75014 Paris, France. Phone: 33-1-40-51-64-04; Fax: 33-1-40-51-64-54; E-mail: isabelle.dusanter@inserm.fr

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STAT5 consists of 2 separate proteins, STAT5A and STAT5B, which are encoded by 2 closely related genes. Most studies have made use of *STAT5A* and *STAT5B* double deficient mice to get insight into STAT5's functions. However, single gene knock-out mouse models have reported nonoverlapping functions of the 2 STAT5s that have been linked to restricted expression of a single STAT5 in a few tissues, gene dosage effect, or differential biochemical properties of the 2 factors (9, 10).

In this study, in an attempt to approach mechanisms of CML persistence, we tracked human STAT5 activities by differentially knocking down the 2 STAT5 genes in normal and CML HSC/progenitor cells, using a RNA interference strategy.

Materials and Methods

Cell lines and primary samples

Umbilical cord blood or adult peripheral blood samples from healthy donors and CML patients were collected after informed consent, in accordance with the Declaration of Helsinki. Patient samples were collected at time of initial diagnosis or after >18 months of IM (Novartis) treatment. Mononuclear cells were isolated by density-gradient separation on Ficoll-Paque Plus (GE Healthcare). CD34⁺ cells were isolated using a positive magnetic bead selection procedure (Miltenyi Biotec) and were maintained in IMDM medium supplemented with 15% BIT (Stem cell Technologies), stem cell factor (100 ng/mL; Amgen), Flt3 ligand (100 ng/mL), interleukin-3 (50 ng/mL), and thrombopoietin mimetic peptide (10 nmol/L; Sigma-Aldrich) (4F-medium). For long-term culture assays, CD34⁺ cells were first transduced in 4F-medium and transferred 24 hours later on MS5 murine stromal cells in LTC-IC conditioned medium before fluorescence-activated cell sorting (FACS)-sorting GFP⁺ cells at day 4. GFP⁺ cells were maintained on MS5 cells for 1 or 5 weeks before cell plating for colony assays using standard procedures. IM (1.5 μ mol/L) was added to the medium of IM-resistant cells. The resulting colony-forming cells (CFC) were scored after 14 days of incubation. CML cell lines (MEG01, LAMA84, and K562) were purchased from cell depository banks. IM-resistant LAMA84 or MEG01 cells were generated using escalating concentrations of IM (from 0.05 to 1 μ mol/L) over 4 months. Resistant cells were maintained in IM (1 μ mol/L)-supplemented medium.

Lentiviral constructs, transductions

The shC, shS5A, shS5B, and shS5A/B sequences (see Supplementary Information) were inserted downstream of the HI promoter into pTRIPAU3-EF1 α , which encodes green fluorescent protein (GFP) or human CD4 as reporters. Murine *STAT5A* or *STAT5B* cDNA were introduced into pTRIPAU3-EF1 α ahead of IRES_{EMCV}-GFP sequence. VSV-G-pseudotyped lentiviral particles were generated as described (11). About 85% to 98% and 70% to 90% transduction with CML cell lines and CD34⁺ cells, respectively, were obtained as measured by GFP expression. Cells were FACS-sorted when required. All subsequent assays were done at day 7 posttransduction. For rescue assays, cells were transduced with shRNA/CD4 encoding vectors and maintained in culture for 4 days before adding the

cDNA/GFP rescue vectors. Assays were done at day 3 to 5 after second transduction.

Cytofluorometric analysis

Cell viability and cell cycle were assessed after staining with AnnexinV/7AAD or propidium iodide (PI). Cell oxidative stress was assessed by incubating cells for 15 minutes at room temperature in the dark in the presence of dihydroethidium (DHE, 5 μ mol/L; Life Technologies) and, where indicated, cyclosporine A (20 μ mol/L; Novartis). Cells were preincubated with H₂O₂ for 6 hours or VAS2870 (Sigma) for 1 hour. Flow cytometry was conducted on FACS-Canto II or Accuri (Becton Dickinson) using anti-CD34-PC5, anti-CD4-PC7, AnnexinV-PE, 7AAD, PI reagents, all from BD Biosciences.

Western blot analysis

Cell extracts were analyzed by standard procedures using commercially available primary and peroxidase-labeled secondary antibodies (see Supplementary Information). Protein signals were detected by chemiluminescence (GE Healthcare) and quantified using a Fuji-LAS4000 image analyzer.

Immunofluorescence

Immunofluorescence assays were conducted by standard procedures using anti- γ H2Ax (Cell Signaling Technology) or anti-RAD51 (Pharmingen) antibody and Alexa 546-conjugated anti-rabbit Ig.

Quantitative PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen); quantitative PCR (qPCR) was conducted using SYBR Green Kit on a Lightcycler (Roche). Data were normalized to *HPRT* mRNA expression.

Statistical analysis

Data were expressed as mean \pm SEM. Student *t* test was used for statistical analysis.

Results and Discussion

STAT5 is required for long-term maintenance of normal and CML HSCs

We identified 3 small hairpin RNAs (shRNA) that differentially knockdown human STAT5A and STAT5B factors, thereafter named shS5A, shS5B, and shS5A/B. These were stably introduced into 3 CML cell lines (K562, LAMA84, and MEG01) using lentiviral vectors that coencode the GFP dye, and immunoblot analyses of the transduced cells were conducted (Fig. 1A and Supplementary Fig. S1). ShS5A decreased *STAT5A* gene expression only, with no impact on STAT5B, as compared with untransduced (uT) or control shRNA (shC)-expressing cells. ShS5B inhibited STAT5B and partially STAT5A expression, whereas shS5A/B inhibited both STAT5s. Our attempts to select for other STAT5A- or STAT5B-restricted shRNAs have been unsuccessful. The shRNAs did not change STAT1/3/6 expression or STAT1/3 tyrosine phosphorylation (Fig. 1A and Supplementary Fig. S1 and data not shown), as opposed to the compensatory STAT1/3 activation observed in some murine STAT5A/B double deficient cells (9). Such compensations may

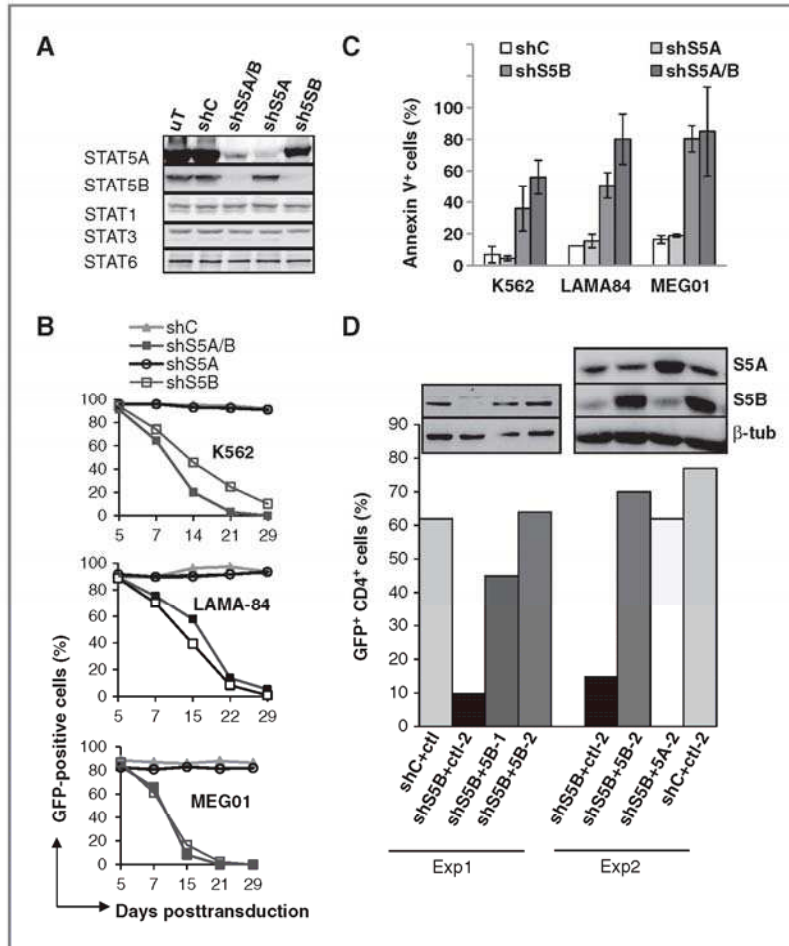


Figure 1. STAT5 knockdown suppresses CML cell growth. A, immunoblot analysis of the indicated proteins in CML (K562) cells at day 7 post-shRNA/GFP lentiviral transduction. ShC, control shRNA directed against luciferase; uT, untransduced cells. B, FACS analysis of GFP expression in CML (K562, LAMA84, MEG01) cells as a function of time post-shRNA/GFP transduction (day 0). One representative experiment is shown ($n = 4$). C, FACS analysis of Annexin V labeling in the indicated CML cells at day 7 post-shRNA/GFP transduction. The percentage of Annexin V⁺ cells is indicated (mean \pm SEM, $n = 4$). D, FACS analysis of GFP and CD4 expression in CML (K562) cells upon double (shRNA/CD4 and cDNA/GFP) transduction. ctl, empty/GFP lentiviral vector; numbers indicate the multiplicity of infection used for ctl/cDNA vectors. Inset, immunoblot analysis of STAT5A (S5A), STAT5B (S5B), and β -tubulin (β -tub) expression in the FACS-analyzed doubly transduced CML (K562) cells. β -Tubulin served as a loading control. Cells were analyzed at day 5 after second transduction. Two independent experiments (Exp) are shown ($n = 4$).

need complete STAT5A/B knockout to occur. Growth properties of the transduced cells were evaluated by analyzing GFP⁺ cells along time of culture (Fig. 1B and Supplementary Fig. S2). ShS5A and shC had no impact on CML cell growth, regardless of the virus-to-cell ratio used. In contrast, shS5B⁺ and shS5A/B⁺ CML cells progressively disappeared. Expression of sh5B and shS5A/B had no impact on cell-cycle phase transitions (data not shown); however, it induced cell apoptosis, as assessed by AnnexinV staining and poly(ADP-ribose) polymerase-1 cleavage (Fig. 1C and Supplementary Fig. S1). ShS5B activity was confirmed by conducting rescue experiments. Murine *STAT5B* which is insensitive to shS5B reversed STAT5B expression to endogenous levels in shS5B⁺ cells (Fig. 1D, inset); it simultaneously rescued shS5B⁺ cell survival, as assessed by the maintenance of mSTAT5⁺shS5B⁺ doubly transduced cells in culture (Fig. 1D). These observations assessed that shS5B activities were not "off-target" effects. Murine *STAT5A* was then introduced in place of *STAT5B* and

similarly rescued shS5B⁺ cell growth (Fig. 1D). These results indicated that the 2 STAT5s commonly sustain CML cell survival. Because shS5A—which inhibited STAT5A only—was inactive at altering CML cell growth, as opposed to shS5B which inhibited both STAT5B and STAT5A, the data further suggested that CML cell growth is sensitive to STAT5 gene dosage.

We next examined STAT5 activities in the context of primary CML stem/progenitor cells. ShRNAs were introduced in CML CD34⁺ cells that were then assayed for their clonogenic potential. ShS5B strongly impaired the short-term clonogenic properties of CML CD34⁺ cells ($P < 0.01$), whereas shS5A had no or only weakly positive effects (Fig. 2A). Also, shS5B⁺—but not shS5A⁺—CD34⁺ CML cells showed strongly reduced long-term colony initiating cell (LTC-IC) potential, the most stringent *in vitro* assays of human HSCs (Fig. 2B). We extended these experiments to normal (wt) CD34⁺ cells collected from umbilical cord (CB) and adult peripheral (PB) blood samples.

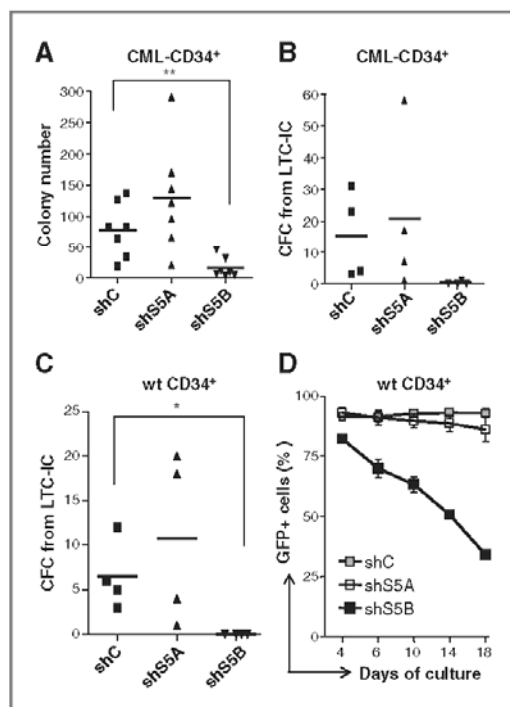


Figure 2. STAT5 is essential for long-term HSC maintenance. **A**, CFC assays were done on 7 newly diagnosed CML CD34⁺ blood samples upon shRNA/GFP transduction and GFP sorting. Results are expressed as the number of clones per 10⁵ initial GFP⁺CD34⁺ cells (**, $P < 0.01$). **B**, LTC-IC assays of shRNA/GFP⁺ CD34⁺ CML cells; total CFC from LTC-IC were scored; CD34⁺ CML cells were isolated from blood samples at diagnosis ($n = 4$). **C**, long-term colony-initiating cell (LTC-IC) assays of shRNA/GFP⁺ peripheral blood CD34⁺ cells. Total CFC from LTC-IC were scored (*, $P < 0.05$). **D**, FACS analysis of GFP expression in cord blood CD34⁺ cells as a function of time post-shRNA/GFP transduction (mean \pm SEM, $n = 3$). Wt, cells collected from healthy donors.

ShS5B—but not shS5A—similarly suppressed LTC-IC potential of wtCD34⁺ cells and further inhibited CD34⁺ cell growth upon *in vitro* culture in a minimal 4-cytokine serum-free growth medium (Fig. 2C and D). Altogether these data indicated that STAT5 activities sustain long-term maintenance of both healthy and CML HSCs. These findings clearly extend previous reports and show for the first time in the context of CML stem cells, the crucial role of human STAT5 in HSC maintenance. BCR-ABL activity was shown to provide expansion of CML progenitors/precursors in a potentially STAT5-dependent manner (6). However, recent reports showed that primitive CML HSCs exhibit a BCR-ABL-independent phenotype that mimics normal microenvironment-dependent HSCs (12); these CML HSCs are refractory to TKI treatment and sustain residual disease. Our findings now indicate that STAT5 may mediate such crucial stroma-derived signals. STAT5 thus could be a novel attractive therapeutic target to eliminate the most immature BCR-ABL-independent CML HSCs.

STAT5A protects cells from stress

Because shS5A did not affect CML cell apoptosis under standard conditions, we used this tool to address additional STAT5 roles. shRNA⁺-CML cell lines and -wtCD34⁺ cells were subjected to oxidative stress by short hydrogen peroxide (H₂O₂) treatment and intracellular reactive oxygen species (ROS) were evaluated by dihydroxyethidium (DHE) staining. shS5A⁺-CML and -wtCD34⁺ cells exhibited enhanced ROS levels in the presence—and also in absence—of H₂O₂, as compared to controls. Coincubating DHE with cyclosporine A, a drug efflux inhibitor, improved ROS detection. Basal production of ROS increased along time after shS5A transduction, which was partially suppressed by the NADPH oxidase (Nox)-restricted inhibitor VAS2870, in absence of mitochondria dysfunctions (Fig. 3A and Supplementary Fig. S3A and S3B). ROS trigger genomic stress with basal p53-mediated DNA damage response (DDR), histone H2AX phosphorylation on Ser139, and the assembly of γ H2AX and RAD51 positive DNA repair foci. ShS5A⁺-CML cell lines and -wtCD34⁺ cells showed an increased proportion of cells with γ H2AX⁺ and RAD51⁺ repair foci, as compared with shC⁺ cells, without RAD51 expression change (Fig. 3B and Supplementary Fig. S3C). As another source of stress, wtCD34⁺ cells were allowed to proliferate at very high concentration for a few days, without medium change. Cell stress response was analyzed by probing activation of the well-known ATM-ATR/CHK/p53 stress-signaling pathway. Overconcentrated shS5A⁺ cells showed a strong increase in p53 expression, which was present under its active [pSer15]-p53 form; cells also exhibited enhanced [pThr68]-CHK2 level (Fig. 3C). Activation of this p53/CHK2 stress-response pathway was not detectable by this approach in normally growing shS5A⁺ cells. Overall, these data indicated that shS5A enhances cell oxidative/replicative/genomic stress, as compared with controls, and Nox activity contributes to this action. Nox plays critical roles in the production of ROS in leukemic cell lines and normal CD34⁺ cells; ROS activate STAT5 that associates with the central Nox regulator Rac1 (13, 14). STAT5A may thus participate to a negative regulatory loop that limits cellular ROS. Among ROS cellular sensors is the oxygen-dependent prolyl hydroxylase domain (PHD) family. All 3 shS5A⁺-CML cell lines showed enhanced PHD3 expression as compared to controls. This activity was reversed upon shS5A-resistant murine STAT5A and transcription-deficient mutant STAT5A Δ 749, but not STAT5B, expression; it was not duplicated by shS5B that weakly inhibits STAT5A (Fig. 3D). Collectively, our results indicated that STAT5A exhibits the restricted property to limit CML and normal stem/progenitor cell stress, independently of its canonical transcriptional activity. In line with these data, STAT5 downregulates ROS production in pre-B leukemic cell lines, in absence of detectable STAT5 tyrosine phosphorylation; oncogenic activation of STAT5 shows preferential cytoplasmic localization and function in myeloid leukemias including CML cells (15, 16). Moreover, SRC/ABL kinases differentially affect nuclear translocation of STAT5A and STAT5B; accumulation of ROS correlates with reduced STAT5A—but not STAT5B—activity in aged macrophages; STAT5A shows differential

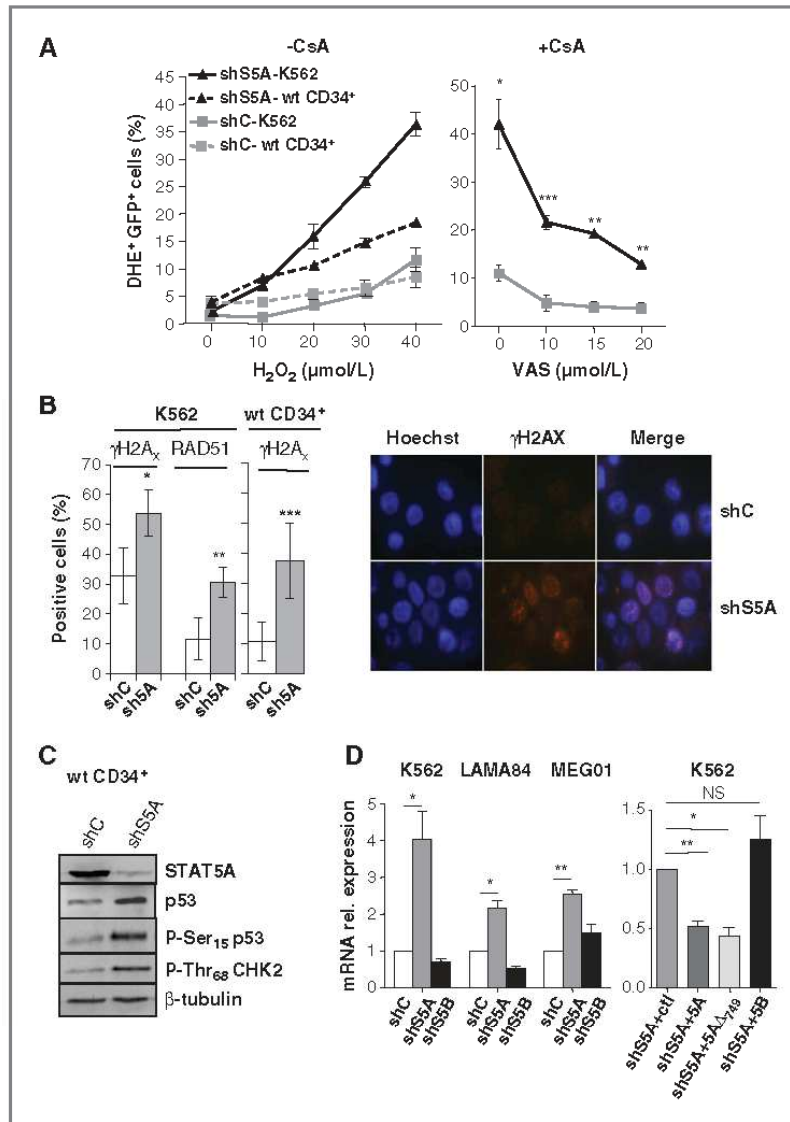


Figure 3. STAT5 protects normal and CML cells from stress. **A**, FACS analysis of DHE labeling in the indicated shRNA/GFP⁺ cells following H₂O₂ (6 hours) or VAS2870 (1 hour) pretreatment. DHE was coincubated with cyclosporine A (CsA, 20 μmol/L) where indicated (mean ± SEM, n = 4). **B**, immunofluorescence microscopy of γH2Ax⁺ and RAD51⁺ foci in the indicated shRNA/GFP⁺ sorted cells. The percentage of positive cells (>3 foci per cell) is presented (mean ± SEM, n = 5; left, one representative experiment in transduced GFP⁺CD34⁺ cells). **C**, immunoblot of the indicated proteins in overproliferating shRNA/GFP⁺ cells at day 7 post-CB CD34⁺ cell transduction. β-Tubulin served as a loading control. **D**, qRT-PCR analysis of PHD3 transcripts in the indicated shRNA⁺ CML cell lines at day 7 post-shRNA transduction. Results are normalized to HPRT mRNA and expressed relative to PHD3 levels in shC⁺ cells (left) or shS5A⁺empty (ctrl) vector⁺ doubly transduced cells (right; n = 3, mean ± SEM). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

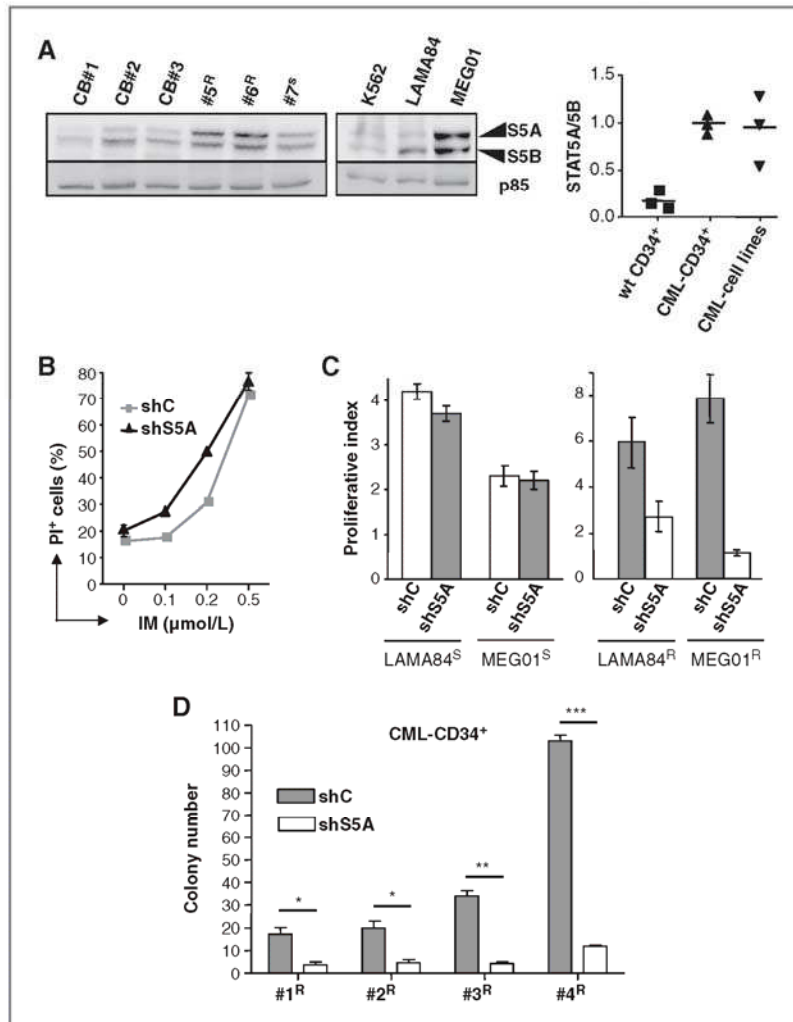
tetramerization potential and selective posttranslational modifications (9, 17, 18).

TKI-resistant CML cells show enhanced STAT5 dependence

Leukemic cell growth relies on proper oxidative stress control. STAT5A-to-STAT5B ratios were enhanced in all CML cell lines and patient cells as compared to normal counterparts (Fig. 4A). STAT5A antistress activity may further help CML cells face exogenous insults. ShC⁻ and shS5A⁺CML cells were treated with increasing concentra-

tions of IM for 2 days. As compared to untreated cells, shS5A reduced cell viability of IM-treated cells (Fig. 4B). Stable IM-resistant cells were further generated from 2 CML (LAMA84 and MEG01) cell lines by increasing IM concentration in culture medium over 4 months. The resistant cells did not exhibit overexpressed or overactive BCR-ABL, increased multidrug resistance pump activity, and overexpressed or overactive STAT5 (Supplementary Fig. S4). However, shS5A greatly affected growth of IM-resistant but not parental CML cells (Fig. 4C). Secondary IM-resistant patients were further selected that (i) show no BCR-ABL overexpression or

Figure 4. IM-resistant CML cells show enhanced STAT5 dependence. A, immunoblot analysis of the indicated proteins in normal and CML cells. Samples were from 3 CB CD34⁺ cell lots, 2 CML CD34⁺ cells from secondary IM-resistant patients with no BCRABL overactivity, 1 CML CD34⁺ cells at diagnosis, and the 3 CML (K562, LAMA84, and MEG01) cell lines. Long electrophoretic runs and STAT5 A/B antibody were used. STAT5A and STAT5B signals were quantified for each sample, expressed as STAT5A/STAT5B ratios and grouped as indicated. B, FACS analysis of PI labeling in shRNA/GFP⁺ CML (LAMA84) cells after a 2-day treatment with imatinib mesylate (IM) at the indicated concentration. C, proliferative index of parental (LAMA84^S; MEG01^S) and IM-resistant CML (LAMA84^R; MEG01^R) cells during a 5-day culture, between day 7 and day 12 post-shRNA transduction (mean ± SEM, n = 3). D, colony assay of shRNA/GFP⁺ secondary IM-resistant leukemic CD34⁺ cells post-shRNA transduction and GFP sorting (mean ± SEM, n = 3). Results are expressed as the number of CFC per 2 × 10³ initial GFP⁺CD34⁺ cells (*, P < 0.05; **, P < 0.01; ***, P < 0.001).



overactivity (no BCR-ABL mutations); (ii) exhibit IM-resistant CD34⁺ cell clonogenic properties. These IM-resistant CD34⁺ cells did not express enhanced STAT5A or STAT5B mRNA levels and STAT5A/STAT5B protein ratios (Fig. 4A and Supplementary Fig. S5). They, however, showed decreased clonogenic potential upon STAT5A knockdown (Fig. 4D), as opposed to CML CD34⁺ cells at diagnosis (Fig. 2A). Altogether, our data indicated that IM-resistant cells have acquired enhanced STAT5-dependence, as compared to their sensitive counterparts, by being sensitive to STAT5A single knockdown. STAT5A has been identified as 1 of the 4 predictors of secondary IM resistance in CML (19). IM-resistant CML cells exhibit increased susceptibility to oxidant treatment because of mitochondrial dysfunction and high endogenous ROS levels (20). Our findings now indicate

that human STAT5A could be one of these novel attractive therapeutic targets to hit ROS oversensitive CML cells and counteract development of long-term resistance to targeted therapy.

Collectively, our findings reveal STAT5s as doubly active prosurvival and stress protective factors, in primary normal and CML hematopoietic stem/progenitor cells; they further show that IM-resistance is associated with enhanced STAT5 dependence of patient stem/progenitor cells. These data highlight the central role of human STAT5s as pleiotropic oncogenic mediators of potential clinical importance in the treatment of CML and its recurrence. Our results could guide new strategies, based on STAT5 inhibition, for targeting drug-resistant CML stem cell population. These novel STAT5 activities may also favor the maintenance, development, and

possible drug resistance of other known STAT5-dependent myeloproliferative syndromes, leukemia, as well as solid tumors (e.g., hepatocarcinoma, prostate cancer).

Disclosure of Potential Conflicts of Interest

A.G. Turhan: honoraria from speakers bureau, Bristol Myers Squibb. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: I. Dusanter-Fourt

Development of methodology: L. Casetti, S. Martin-Lannerée, I. Najjar, I. Pio, S. Augé, E. Lauret, A. G. Turhan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Casetti, I. Najjar, L. Roy, A. G. Turhan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Casetti, S. Martin-Lannerée, I. Najjar, J.-C. Chomel
Writing, review, and/or revision of the manuscript: I. Najjar, E. Lauret, A. G. Turhan, I. Dusanter-Fourt

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Martin-Lannerée, I. Najjar, Sylvie Augé

Study supervision: I. Dusanter-Fourt

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Supplementary methods.

Lentiviral constructs, transduction. Oligonucleotides targeting firefly luciferase (shC, 5' CGTACGCGGAATACTTCGA-3'), human STAT5A (shS5A, 5'-GTACTACACTCCTGTGTG-3'), STAT5B (shS5B, 5'-GCAACAGGCCCATGACCTA-3') and STAT5A/B (shS5A/B, 5'-GGAGAACCTCGTGTTCCTG-3') were inserted downstream of H1 promoter into pTRIPAU3-EF1 α which encodes green fluorescent protein (GFP) or human CD4 as reporters. Lentiviral particles were all titrated on K562 cells at day 3 post-transduction using GFP flow cytometry analysis. CML cell lines and CD34⁺ cells were transduced with shRNA encoding vectors at a multiplicity of infection (MOI) of 4 and 10, respectively, in a single-step transduction protocol. At day 4-5, GFP⁺ cells were sorted when required. All cells were analyzed at day 7-8 post virus addition, except otherwise stated. For rescue assays, cells were first transduced with shRNA/CD4 encoding vectors and maintained for 4 days in culture before adding the indicated cDNA/GFP rescue vectors at the indicated MOI. Cells were then analyzed at day 3-5 post 2nd transduction.

Immunoblots and antibodies. Immunoblot of the indicated proteins were performed on total cell extracts, unless otherwise specified. Cytoplasmic and nuclear extracts were performed as previously described (1). Primary antibodies used for immunoblotting were STAT5 (#9363), P-Tyr694-STAT5 (#9351), STAT1 (#9172), STAT3 (#9132), P-Thr68 CHK2 (#2197), γ H2Ax (#2577), PathScan Bcr/Abl Activity Assay (#5300) from Cell Signalling Technology, STAT5A (sc-1081), STAT5B (sc-1656), STAT5A/B (sc-835), STAT6 (sc-981), c-Abl (sc-131), β -tubulin (sc-9104), p53 (sc-126), poly (ADP-ribose) polymerase (PARP)-1 (sc-74470), RAD51 (sc-8349) from Santa Cruz Biotechnology, β -actin (A5441) from Sigma-Aldrich, and P-Ser₁₅-p53 from Merck. Anti-p85 PI3 kinase antibody was kindly provided by Dr P. Mayeux (Paris, France).

RNA

Total RNA was extracted using the RNA Mini Kit (Ambion); the first-strand of the cDNA was synthesized using the Maxima First Strand cDNA Synthesis kit (Fermentas), and QPCR analysis was performed using LightCycler® FastStart DNA Master^{PLUS} SYBRGreen I kit on a Lightcycler (Roche). The primers used were: STAT5A forward 5'-CGAGTGCAGTGGTGAGATCC-3', STAT5A reverse 5'- TCCTCTGTACGGACTCTGC-3', STAT5B forward 5'-GGCAGAGTCGGTGACAGAAG-3', STAT5B reverse 5'-GGCTCTGCAAAAGCATTGTC-3', PHD3 forward 5'-ATCGACAGGCTGGTCCTCTA-3'; PHD3 reverse 5'- GGATCCCAC-CATGTAGCTTG-3'; HPRT forward 5' TGACTGCAAAACAATGCA 3'; HPRT reverse 5' GGCCTTTTCACCAGCAAGCT. Data were normalized using *HPRT* mRNA expression. Similar results were obtained using *cyclophilin A* and *GAPDH* transcripts as two other references.

Supplemental Reference

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Supplemental Figure Legends

Supplemental Figure 1. shS5A, shS5B and shS5A/B differentially affect STAT5 expression in CML and normal stem/progenitor cells. Immunoblot analysis of the indicated proteins in the indicated cells at day 7 post shRNA/GFP lentiviral transduction. ShC stands for control shRNA directed against luciferase; uT stands for untransduced cells; normal (wt) CD34⁺ cells were purified from cord blood samples. Black vertical lines separate samples that were run on the same gel but were not contiguous.

Supplemental Figure 2. Characteristics of CML cells following addition of the indicated shRNA encoding lentiviral particles. FACS analysis of Forward Scatter (FSC), Side Scatter (SSC) and GFP expression in CML (K562) cells along days post viral transduction (day 0). The percentages of living (see indicated morphology gate) and GFP⁺ cells are indicated. Black lines separate GFP signal from autofluorescence of untransduced cells.

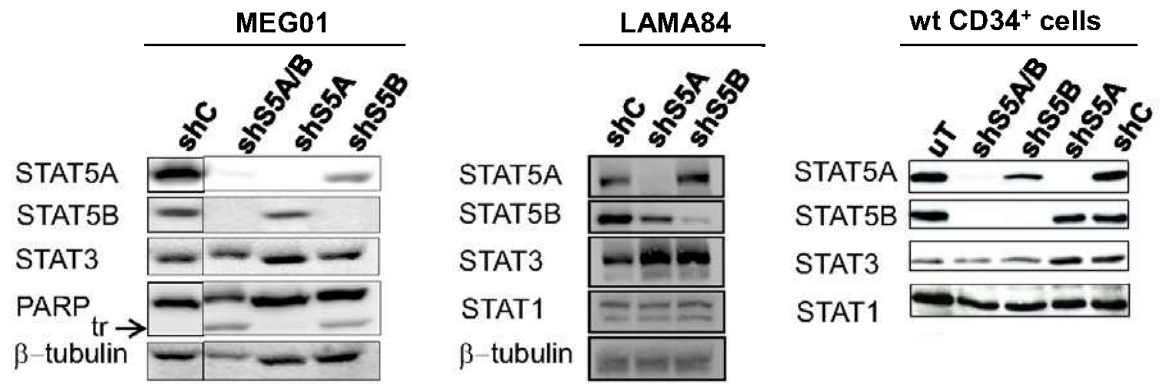
Supplemental Figure 3. shS5A enhances ROS which depends on NADPH oxidase activity in CML cells. (A) FACS analysis of DHE labeling in CML (K562) cells as a function of time post shRNA/GFP lentiviral transduction (day 0). DHE was co-incubated with cyclosporine A (20 μ M) for 15 min before flow cytometry analysis; one representative experiment is shown; the percentage of DHE positive cells is indicated. Black lines separate DHE signal from cell autofluorescence. (B) FACS analysis of mitochondria transmembrane potential in shRNA/GFP⁺ CML (K562) cells in the absence or presence of VAS2870 (VAS). Cells were pre-incubated with VAS2870 (15 μ M) for 1hr before being incubated for 15 min at room temperature with tetramethylrhodamine, methyl ester (TMRM, 40 nM), a cell permeant red-orange fluorescent dye that is readily sequestered by active mitochondria. The percentage

of TMRM positive cells is indicated; one representative experiment is shown. (C) Western blot analysis of the indicated proteins in shRNA⁺ CML (K562) cells.

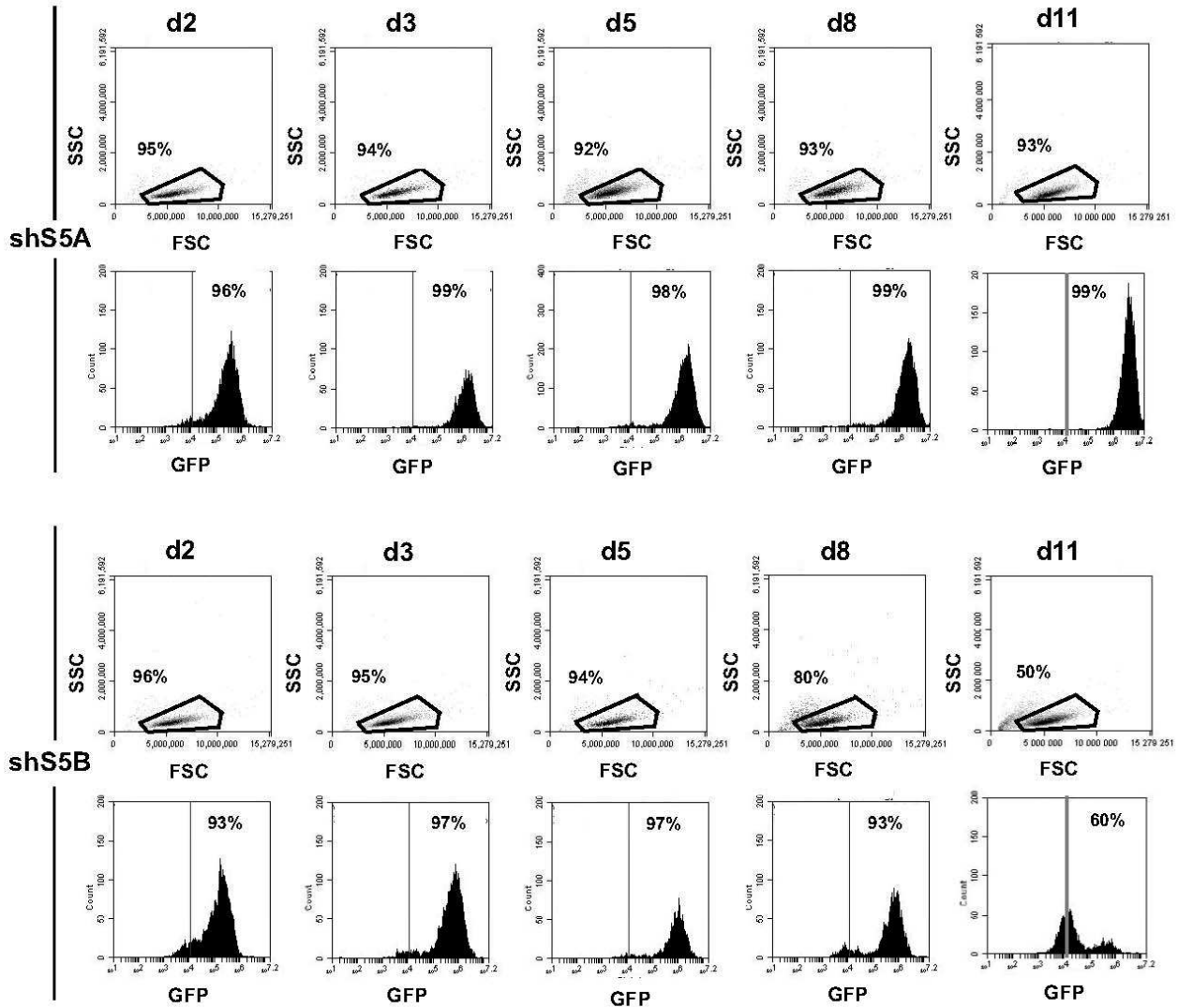
Supplemental Figure 4. Characteristics of IM-sensitive and -resistant CML cells. (A) Immunoblot analysis of the indicated proteins in parental IM-sensitive (S) and IM-resistant (R) CML (LAMA84, MEG01) cells using total (T), cytoplasmic (C) and nuclear (N) extracts, as indicated. (B) FACS analysis of multidrug resistance pump activity. 3,3'-Diethyloxacarbocyanine iodide (DiOC2(3), 50 nM), a substrate for multidrug resistance pump MDR1 and its relatives, was incubated for 30 min with IM-resistant cells in the absence (black line) or presence (dot line) of the multidrug resistance pump inhibitor cyclosporine A (CsA, 20 μ M) before flow cytometry analysis. Daunorubicin-resistant CML (K562) cells that expressed enhanced MDR pumps have served as positive control (pos.control). Grey peaks show basal cell autofluorescence.

Supplemental Figure 5. Expression of *STAT5A* and *STAT5B* mRNA in normal and CML CD34⁺ stem/progenitor cells. QRT-PCR of *STAT5A* and *STAT5B* transcripts in wt and CML CD34⁺ cells. Wt CD34⁺ cells were isolated from 2 CB, 2 PB and two bone marrow samples; CML CD34⁺ cells were from blood samples collected at diagnosis (CML^S) or upon secondary resistance to IM in absence of BCR-ABL overactivity (CML^R). Results were normalized to HPRT mRNA and were grouped as indicated.

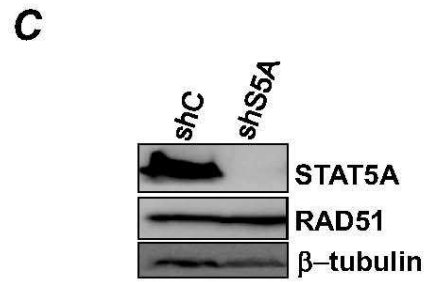
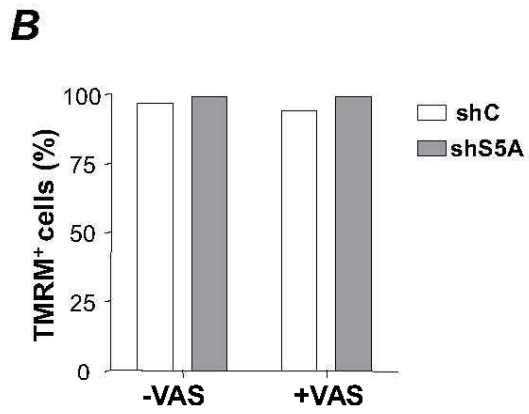
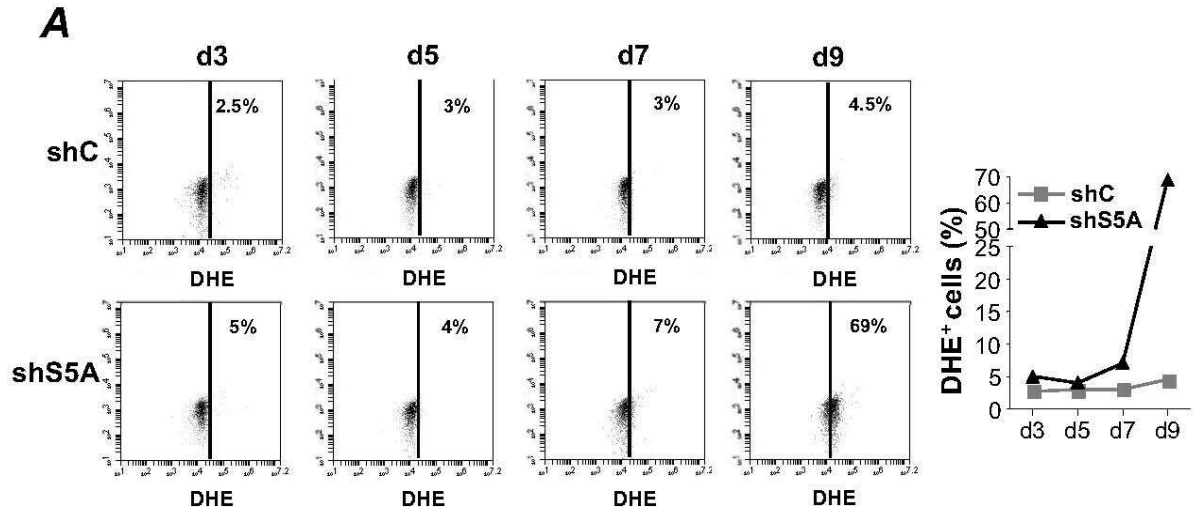
Supplemental Figure 1



Supplemental Figure 2

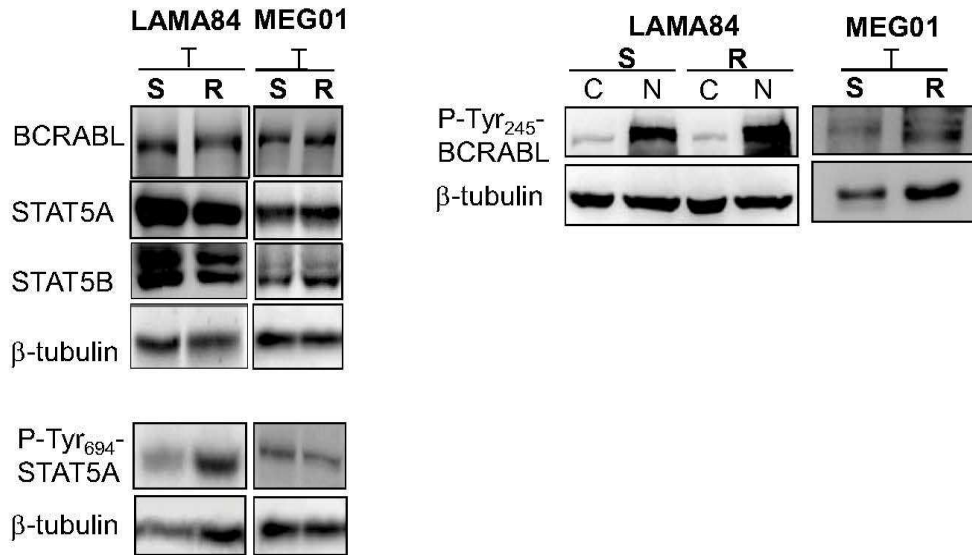


Supplemental Figure 3

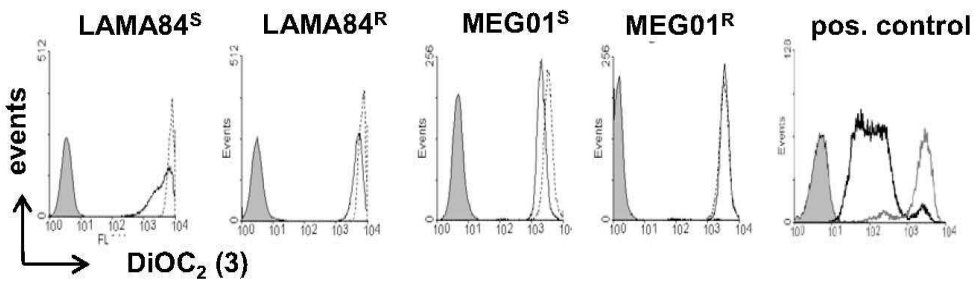


Supplemental Figure 4

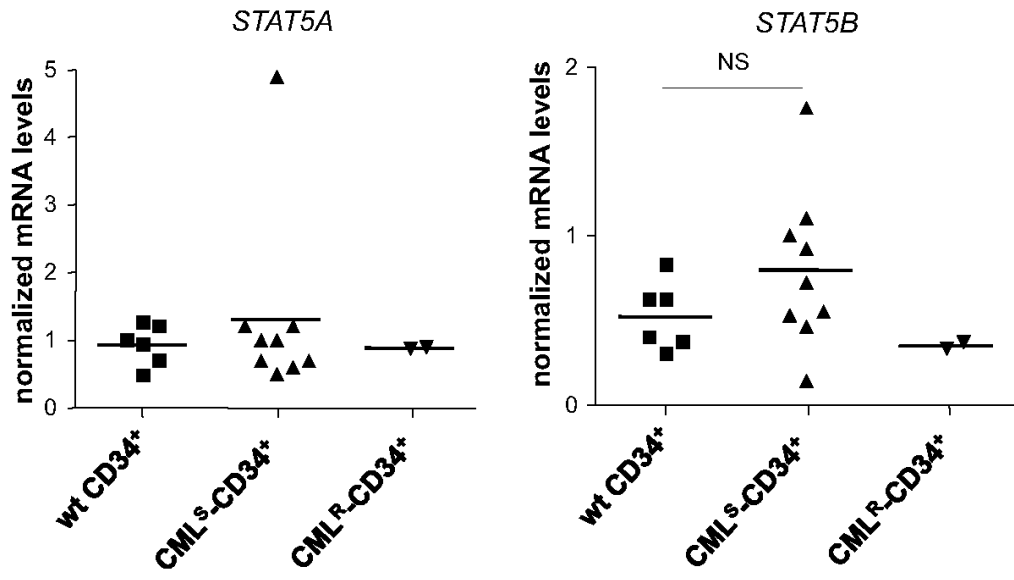
A.



B.



Supplemental Figure 5



GENERAL DISCUSSIONS AND PERSPECTIVES

My PhD work contributed to the knowledge of CML oncogenic processes and the role of STAT5 factors in these processes. By using an RNA interference based strategy in both normal and leukemic cells, we could demonstrate that:

- ❖ STAT5 factors are required to maintain CML stem and progenitor cells, which are known to be independent of BCR-ABL signaling and responsible for residual disease.
- ❖ STAT5A exhibits a new role in CML physiopathology as an anti-oxidant factor. STAT5A inhibits the expression of the cellular oxygen sensor PHD3 and this regulation does not depend on STAT5A transcriptional-activator domain.
- ❖ STAT5A contributes to TKI secondary resistance, with resistant cells becoming addicted to STAT5A for their survival.
- ❖ STAT5A regulates 2 new target genes in CML cells: the receptor tyrosine kinase Axl and its ligand, Gas6.
- ❖ Axl contributes to oxidative stress regulation, and favors IM-resistance of CML cells, thus mimicking STAT5A activities.
- ❖ BCR-ABL-activated STAT5A triggers a strong Gas6 down-regulation, which is not observed in healthy cells. Gas6 dysregulation may contribute to CML leukemic properties.

STAT5 factors and CML stem and progenitor cell maintenance. The presence of BCR-ABL has made CML disease a model for targeted therapies. Few decades ago, patients were treated with interferon- α , then the identification of the first BCR-ABL inhibitor, Imatinib, has completely revolutionized the outcome for CML patients. However, Imatinib does not cure CML disease and patients must be life-long treated. Moreover, some patients can exhibit primary or develop secondary resistances to Imatinib. Thus, second generation tyrosine kinase inhibitors (TKIs) have been developed, such as Nilotinib and Dasatinib, which are much more efficient than Imatinib and can overcome some Imatinib (primary and secondary) resistances. However, none of the three TKIs is able to completely destroy the most immature BCR-ABL expressing cells. Indeed, the most immature CML cell population does not depend on BCR-ABL signaling for its survival (Corbin et al. 2011, Hamilton et al. 2012) and *BCR-ABL* mRNA remains detectable in these TKI-refractory leukemic stem cells (Chomel et al. 2011). CML stem cells reside in the bone marrow niche and this

microenvironment provides critical signals that sustain HSC survival and support CML stem cell preservation (Bhatia et al. 1995, Zhang et al. 2013). In the literature, STAT5 activity and contribution in CML was analyzed in different models, such as CML cell lines, cell lines supplied with an exogenous BCR-ABL and mouse models where BCR-ABL was introduced into hematopoietic progenitors that were engrafted. These models allowed the characterization of STAT5 factor activities in CML cells, however very few studies have used patient progenitors. Our data has made STAT5 one central mediator of niche-dependent pro-survival messages that sustain both normal and leukemic primary stem cells (Figure 24). During our studies, two separate groups showed, in *in vitro* systems, that blocking JAK-mediated extrinsic survival signals in primary CML, in the context of the BM microenvironment, restore sensitivity of CML cells to TKIs (Nair et al. 2012, Traer et al. 2012), suggesting that niche-mediated signals activate STAT5 in primary CML independently of BCR-ABL activity. Targeting CML stem cells to eradicate disease persistence is a new field of interest and some other pathway have been proposed as potential therapeutic targets, such as the Wnt- β -catenin pathway (Zhang et al. 2013) and the Hedgehog pathway (Dierks et al. 2008). Whether STAT5 and these other signaling pathways work separately, or converge on common intracellular transducer(s) that integrate niche-dependent information remains to be studied.

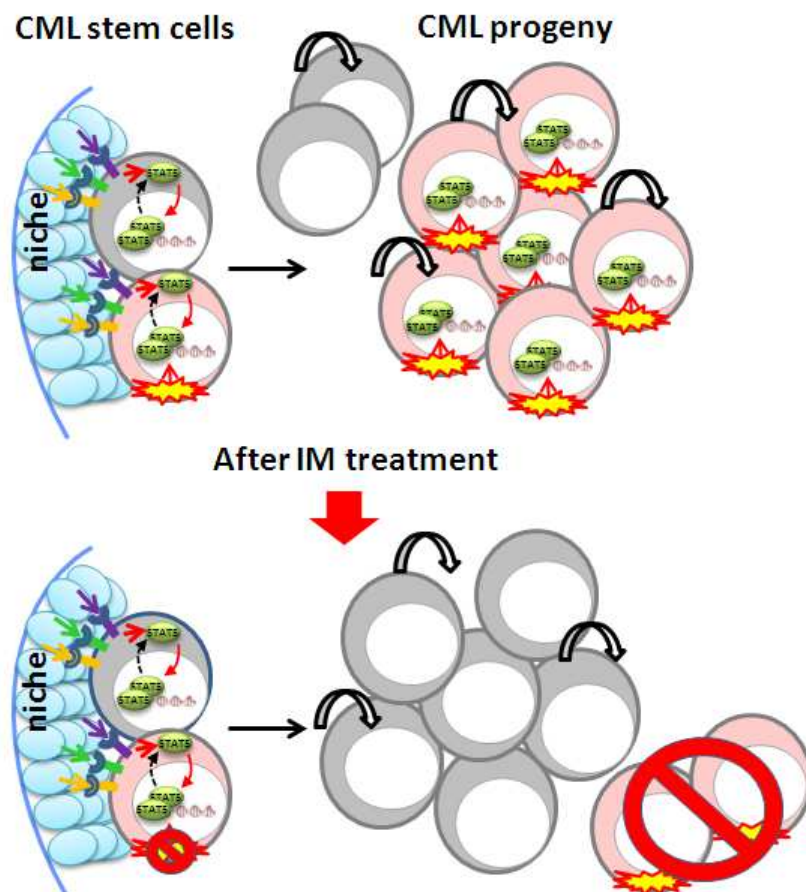


Figure 24: activation of STAT5 by the niche is responsible for CML stem cell survival. The bone marrow niche, where normal and leukemic hematopoietic stem cells reside, activates STAT5 signaling. This results in a constitutive activation of STAT5 by niche-dependent signals. When cells are treated with Imatinib, the mature CML progeny, which depends only on BCR-ABL activity, is lost. However, the most immature BCR-ABL population, which presents a basal activation of STAT5, can still survive and give rise to CML progeny again.

A new role for STAT5A as anti-oxidant factor. Previously, STAT5 activation was linked with cell cycle progression and inhibition of apoptosis in CML cells and other cancer cells. These are well established features of STAT5 factors. We now show that, in addition to well-known anti-apoptotic and proliferative effects, STAT5 has another major role as negative regulator of oxidative stress in normal HSCs and in CML cells. Although STAT5 is ubiquitous and involved in many oncogenic processes, its anti-oxidant activity was similarly recently proposed in human leukemic pre-B cell lines (Cholez et al. 2012). In these cells, anti-oxidative property of STAT5 was linked to DJ-1, GSH synthetase and transaldolase activities. We did not observe any STAT5-dependent regulation of these factors in healthy or in CML primary stem progenitor cells, but instead we show that STAT5A decreases NADPH-oxidase (NOX) ROS-generated levels (Figure 25). Such discrepancy may be linked to the leukemic cell models used. Another group, in line with our data, showed a link between STAT5 activity and NOX generated ROS in AML cells that present the FLT3-ITD mutation; in these cells, FLT3 driven H₂O₂ production is mediated by p22phox, a component of the NOX complex, and is critical for STAT5 signaling (Woolley et al. 2012). Oxygen is a critical component in stem cell biology; hypoxia protects HSCs from ROS-mediated DNA damages and premature senescence, sustaining HSC long life (Ito et al. 2004, Mohyeldin, Garzon-Muvdi and Quinones-Hinojosa 2010). Cancer cells are permanently proliferating and show increased ROS levels, thus recent studies suggest that this biochemical property of cancer cells can be exploited for therapeutic benefits (Trachootham et al. 2009). In line with this observation, recently Kluza and colleagues have shown that TKI-resistant CML cells present a mitochondrial dysfunction responsible for a higher generation of ROS compared to TKI-sensitive cells; thus, mitochondria dysfunction may be exploited for selective therapeutic intervention (Kluza et al. 2011). Our observations of STAT5A down-regulation of cell oxidative stress, suggest that this action may limit DNA and protein damages generated by the high toxic ROS levels present in leukemic cells, thus protecting these cells. This observation complements the pro-survival activity of STAT5 factors previously reported and highlights the pleiotropic oncogenic role of STAT5 in CML cells. This makes STAT5 factors ideal tools to eradicate CML stem cells. However, last year Warsch and colleagues reported, as opposite to our data, that STAT5 factors actually enhance oxidative stress in CML cells. They inhibited or over-expressed both STAT5A and STAT5B in different murine cell lines transfected with an exogenous BCR-ABL or used also in K562 cells. They showed that, in all these cells, when STAT5s are over-expressed there is an accumulation of ROS and, on the other hand, when they are lost ROS levels decrease (Warsch et al. 2012). This STAT5 pro-oxidant action

may be the consequence of the well-described STAT5 KD-induced cell death already shown by our group and others. (Nieborowska-Skorska et al. 1999, de Groot et al. 2000). Moreover, exogenous oncogene over-expression triggers abnormally strong intracellular signaling pathways and cell over-proliferation; such an increased proliferate rate clearly generates overwhelming levels of ROS which may all change STAT5 overall activities. These observations could reconcile our data with their.

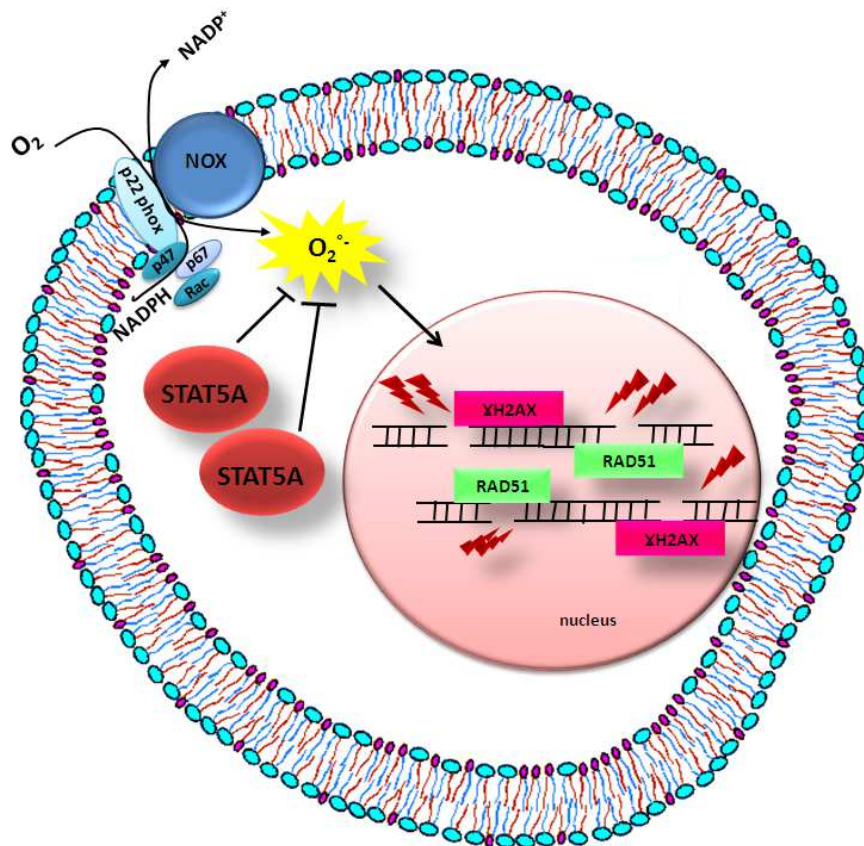


Figure 25: STAT5A negatively control NOX generated ROS levels. NADPH-oxidase (NOX) complexes are located at the cell membrane and generate superoxide anions by transferring electrons from NADPH inside the cells to oxygen. Cytoplasmic STAT5A decreases NOX-generated ROS levels and consequently the number of DNA damage breaks, leading to a decreased formation of γ H2Ax and RAD51 repair foci.

STAT5A dependent stress control is mediated by a non-canonical transcription domain-independent signaling. We have also shined light on molecular mechanisms by which STAT5A regulates oxidative stress. The best known STAT5 signaling pathway involves its phosphorylation on Tyr residues which leads to dimers or tetramers formation and consequently to their translocation to the nucleus, followed by their direct binding to DNA motifs (GAS elements) and the transcriptional regulation of target gene expression. We now

report evidence that STAT5A regulates the cellular oxygen sensor *PHD3* mRNA even in absence of its trans-activator domain, in a possible transcription-independent way. This observation completely fits with a recent finding reported this year by Chatain and colleagues. They identified for the first time different localization for STAT5A and STAT5B in CML cells, resulting in STAT5A being massively cytoplasmic and STAT5B being massively a nuclear factor (Chatain et al. 2013). Very few STAT5A quantities were detected in the nucleus of CML cells, suggesting a new non-nuclear role for STAT5A -different from gene transcription- in these cells. This different distribution, and our data, suggests that STAT5A may work as a cytoplasmic regulator of oxidative-stress. Notably, mounting evidence favors the hypothesis of additional non-nuclear STATs roles, as it was shown for STAT3 within the mitochondria (Wegrzyn et al. 2009, Gough et al. 2009) or for STAT5 in rough endoplasmic reticulum (ER) and Golgi apparatus (Lee et al. 2012). STAT5 localization in rough ER or Golgi apparatus could explain our observation of STAT5A-dependent non-transcriptional *PHD3* mRNA regulation: STAT5A could affect *PHD3* mRNA stability by increasing transcript degradation and reducing its lifetime. However, the small amount of STAT5A in the nucleus may still be sufficient to regulate *PHD3* at the transcriptional level. The *PHD3* promoter contains 4 STAT binding sites (GAS): one in the proximal promoter region (-760), while the others are located more distant from the transcription start site (-4678, -5840 and -8076). The presence of GAS elements at the promoter level suggests that *PHD3* expression may be transcriptionally regulated by few nuclear STAT5A in a direct way; it can maybe form dimers with STAT5B and use the trans-activator domain of STAT5B to regulate *PHD3* gene expression. However, STAT5A could also cooperate with other transcription factors and regulate *PHD3* in an indirect way, without directly binding to GAS sites, as it was shown for Oct-1 (Magne et al. 2003) . Further experiments are needed to clarify whether oxidative stress down-regulation involves STAT5A nuclear or cytoplasmic activity and whether it goes through canonical STAT5 Tyr-phosphorylation. To this purpose we are developing two mutant murine *STAT5A* expressing lentiviral vectors: one which presents an amino acid substitution on the canonical target Tyr responsible for STAT5A activation (Tyr694Phe) which is normally required for STAT5 nuclear localization, and another which presents two amino acid substitutions in the DNA binding domain (Glu437Ala, Glu438Ala) that prevent STAT5A binding to DNA. These tools will allow us to determine if STAT5A-dependent gene transcription is necessary for its role as negative regulator of *PHD3* expression or not.

STAT5A supplies CML cells with growth advantage to resist not only to oxidative stress but also to drug treatment. During my PhD work, different groups showed a correlation between TKI resistance and STAT5 phosphorylation levels in CML cell lines and primary cells. Increased levels of STAT5 activation was reported to counteract TKI therapy. Warsch and colleagues showed that this can happen by two different mechanisms: the high STAT5 protein levels observed in human patients after prolonged disease may shield cells from TKI-dependent cell death, but also in the meantime may favor BCR-ABL mutations, genomic instability and generation of resistant clones (Wang et al. 2007, Warsch et al. 2011, Warsch et al. 2012). Moreover, inhibiting STAT5 factors by treating TKI-resistant cell lines with Pimozide, strongly decreases their survival (Nelson et al. 2011). However, the way pimozide is working and whether it only inhibits STAT5 remains to be established. Few years ago, Zhang and colleagues performed a transcriptomic study in which they identified STAT5A, and not STAT5B, as one of four predictors of secondary IM resistance compared to primary resistance in CML cells from patients (Zhang et al. 2009). In line with all these data, our findings now indicate that human STAT5A plays a crucial role in favoring development of irreversible long-term resistance to drug treatment of immature CML stem cells which may be linked to its anti-oxidant properties.

Collectively, these findings highlight the central role of ubiquitously expressed STAT5s as multipotent oncogenic mediators of potential clinical importance in the treatment of CML and its recurrence. Each of these activities may also participate to the maintenance/development of other known STAT5-dependent leukemia and tumor process. We showed that STAT5A may exhibit non-nuclear activities (see also STAT5A cellular distribution) which are not shared by STAT5B. This differential role may be explained by different possible post-translational modifications of STAT5 factors. Indeed, even if STAT5A and STAT5B exhibit high similarity at the protein level (96%), they present different target of phosphorylation, such as Serine 779 for STAT5A and Serine 193 for STAT5B. In the literature, differential post-translational modifications of STAT5A and STAT5B are poorly described; in mammary gland of Hirosaki Hairless Rat, STAT5A is massively nuclear, whereas STAT5B is cytoplasmic and this differential distribution is due to an *O*-linked *N*-acetylglucosamination of STAT5A which leads to its translocation to the nucleus (Nanashima et al. 2005). Thus, STAT5A and STAT5B post-translational modifications could explain the functional diversity of these two factors and therefore their different interaction with protein partners. To this purpose, we also decided to look for STAT5A mechanism of action by searching STAT5A

partners. We are presently analyzing, by Stable Isotope Labeling with Amino acid in Cell culture (SILAC) proteomic approach, the different STAT5A and STAT5B partners present in STAT5A or STAT5B immunoprecipitates using quantitative mass spectrometry following immunoprecipitation experiments. This kind of approach will allow the identification of new possible partners and mechanisms that lead to the different roles of STAT5A and STAT5B that we found in normal and CML cells.

ANNEXES



NOUVELLE

STAT5, une nouvelle cible thérapeutique contre les récurrences de la leucémie myéloïde chronique ?

Luana Casetti, Séverine Martin-Lannerée, Imen Najjar, Isabelle Dusanter-Fourt

Inserm U1016, Institut Cochin,
Université Paris Descartes,
22, rue Méchain,
75014 Paris, France.
isabelle.dusanter@inserm.fr

> La leucémie myéloïde chronique (LMC) représente un cas d'école en cancérologie : c'est le premier cancer à avoir été relié à une anomalie chromosomique, la translocation t(9;22). Cet échange entre les chromosomes 9 et 22 dans une cellule

souche hématopoïétique (CSH) aboutit à la formation du gène de fusion *BCRABL* (BCR pour *breakpoint cluster region* et ABL pour *abelson*), dont le produit est une protéine à activité tyrosine kinase, constitutivement activée et responsable

de la maladie. La découverte de *BCRABL* a conduit à l'identification d'un premier inhibiteur chimique de *BCRABL*, l'imatinib mesylate (IM, ou Glivec), et à la mise en place en 2001 de la première thérapie anticancéreuse ciblant uniquement la

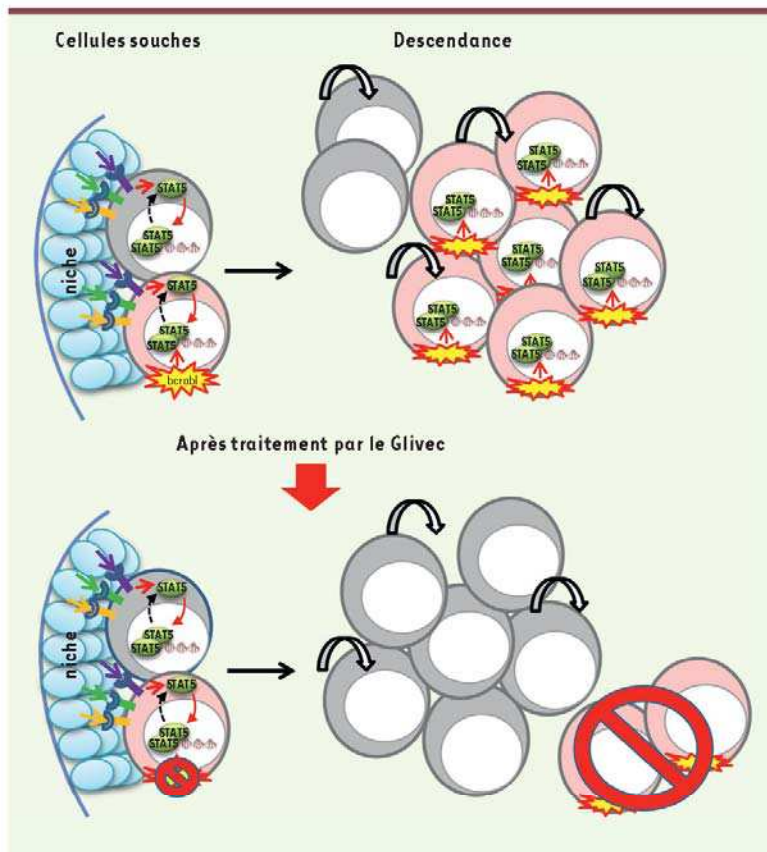


Figure 1. Relation entre la niche hématopoïétique et l'activation de STAT5 dans la LMC. Le microenvironnement (niche) des cellules souches hématopoïétiques active STAT5 qui maintient ces cellules en vie et les protège de divers stress garantissant ainsi leur pérennité. En présence de traitement anti-BCRABL (Glivec), STAT5 n'est plus activée par BCRABL, ce qui conduit à la mort des cellules leucémiques. Seules les cellules souches leucémiques sont protégées par l'activité de STAT5 issue du microenvironnement avec lequel elles sont directement en contact, contrairement aux cellules plus matures.

cellule tumorale (on parle d'une thérapie ciblée). L'IM/Glivec bloque sélectivement l'activité de BCRABL, et, par conséquent, la survie et la multiplication des cellules tumorales, conduisant à la rémission de la maladie. Cependant, des récurrences surviennent chez une majorité des patients lors de l'arrêt du traitement, indiquant la persistance de cellules souches leucémiques capables de provoquer la rechute de la maladie (on parle de maladie résiduelle). Ces cellules sont intrinsèquement réfractaires à l'inhibiteur anti-BCRABL. Par ailleurs, environ 15 à 20 % des patients développent au cours du traitement des

résistances à l'inhibiteur. Ces observations pointent la nécessité de découvrir les mécanismes qui sous-tendent ces différentes résistances et mettent en échec une guérison totale.

Les molécules STAT : fonctions dans les cellules souches hématopoïétiques

Les cellules du sang ont une durée de vie très limitée et doivent être en permanence renouvelées grâce à l'activité des CSH. Celles-ci s'adaptent aux besoins de l'organisme en fonction des informations qu'elles reçoivent des cellules de leur microenvironnement ou « niche », sous la forme de facteurs de

croissance, cytokines et autres signaux. Cette niche assure aux CSH survie et protection contre divers stress, garantissant leur pérennité. Les informations de la niche sont relayées dans les CSH par un panel de molécules-messagers qui vont guider en conséquence le devenir de ces cellules. STAT5 (*signal transducer and activator of transcription 5*) est un de ces messagers. La molécule STAT5 existe à l'état latent et inactif dans la cellule et est activée à la réception de certaines informations à la surface de la CSH. Cette activation se traduit par une phosphorylation sur un acide aminé tyrosine particulier de STAT5, ce qui favorise la formation de paires de STAT5 capables de rejoindre le noyau de la cellule et de modifier directement l'expression de certains gènes cibles. STAT5 fait ainsi la navette entre la surface de la cellule - qui réceptionne les informations externes - et les gènes de la cellule dont elle adapte le niveau d'expression. L'expression de BCRABL dans les cellules de LMC déclenche la phosphorylation - et donc l'activation - permanente de STAT5, ce qui entraîne une croissance cellulaire continue, de caractéristique tumorale [1]. STAT5 désigne deux molécules à 96 % identiques, STAT5A et STAT5B, codées par deux gènes différents dont on a longtemps cru qu'ils étaient interchangeables pour l'essentiel de leurs activités [2].

Notre équipe a cherché à savoir si ces deux gènes jouaient effectivement le même rôle dans les propriétés des cellules souches de LMC. Des ARN interférents induisant la perte d'expression de STAT5A uniquement, ou de STAT5A et STAT5B simultanément, ont été identifiés et introduits de manière stable par le biais de vecteurs lentiviraux dans une population cellulaire (CD34⁺) enrichie en CSH humaines. La perte conjointe de STAT5A et STAT5B induit la perte des CSH cultivées en présence de cellules de leur microenvironnement naturel (la niche). Cette perte touche aussi bien les CSH issues de patients leucémiques que celles d'individus sains. Ainsi, les



facteurs STAT5 permettent de maintenir en vie les CSH saines comme les cellules souches leucémiques, qui sont réfractaires au Glivec. Les CSH leucémiques ne sont donc pas tributaires de l'activité BCRABL mais de STAT5 pour leur survie, et seront préservées lors d'un traitement anti-BCRABL par le Glivec.

STAT5A : un rôle protecteur contre le stress cellulaire

Si la perte des deux facteurs STAT5 conduit à l'élimination des CSH saines et malades, la seule perte de STAT5A n'a pas d'impact sur la survie de ces cellules, ce qui reflète l'activité redondante des facteurs STAT5A et STAT5B pour cette fonction précise. Nous avons posé la question de l'implication des facteurs STAT5 dans d'autres activités cellulaires essentielles au bon fonctionnement des CSH. Les CSH saines doivent résister aux différentes sources de stress rencontrées, qui pourraient altérer leur patrimoine génétique et donc les propriétés biologiques transmises à toute leur descendance. La croissance massive des cellules leucémiques, quant à elle, nécessite une bonne élimination de composants toxiques issus de leur prolifération incontrôlée qui, sinon, détériore la cellule leucémique elle-même et déclenche sa mort. Nous avons analysé les conséquences de la seule perte d'expression de STAT5A sur les capacités de résistance des CSH à divers types de stress, tels que l'exposition à une source de réactifs oxygénés (H_2O_2) ou à une densité cellulaire excessive. L'absence de STAT5A accroît le stress de la CSH normale et de la CSH leucémique, comme l'attestent la présence de réactifs oxygénés supplémentaires, l'activation de la voie canonique de réponse au stress - la voie p53 -, et

la présence de foyers de cassure de l'ADN génomique. La perte de STAT5A augmente également le niveau de base de stress cellulaire en l'absence de stimulus. Ces données suggèrent qu'une des fonctions, jusque là inconnue, de STAT5A est d'aider la CSH à endiguer les conséquences de l'exposition à diverses sources de stress, qu'elles soient exogènes ou endogènes. Des expériences supplémentaires ont montré que cette fonction de protection contre le stress de STAT5A n'est pas partagée par STAT5B. De plus, cette fonction semble être indépendante d'une régulation directe de l'expression de gènes. Ces observations élargissent les domaines d'intervention de la molécule STAT5A et posent la question de(s) mécanisme(s) qui sous-tendent cette nouvelle activité.

STAT5A : un facteur de survie clé pour les cellules souches leucémiques résistantes au Glivec

Une des sources majeures de stress à laquelle la cellule leucémique est confrontée est le traitement antitumoral qui induit son élimination. Notre équipe a analysé l'impact de l'activité de STAT5A sur les cellules leucémiques qui ont développé une résistance au Glivec. Ces processus de résistance sont souvent liés à l'émergence de formes mutantes de BCRABL qui ne sont plus inhibées par le traitement anti-BCRABL. Notre équipe s'est intéressée aux patients atteints de LMC qui ont développé une résistance au Glivec en l'absence de mutation dans le gène BCRABL et elle a analysé les conséquences de la seule perte de STAT5A dans les cellules leucémiques. Contrairement aux CSH normales et aux cellules souches leucémiques testées au moment du diagnostic, pour lesquelles la perte de STAT5A n'a pas de répercussion sur la

croissance, les cellules de LMC résistantes au traitement sont devenues entièrement tributaires de STAT5A pour leur survie : la perte de STAT5A induit leur mort. Ces données confortent des travaux de la littérature qui ont identifié STAT5A - et non STAT5B - comme l'un des quatre gènes, avec les deux gènes codant les canaux transmembranaires ABCB1 et ABCC1 (qui favorisent le rejet de drogues hors de la cellule) et le gène codant le facteur RUNX3, dont l'expression corrèle avec l'acquisition de résistances secondaires au traitement par le Glivec [3] (Figure 1). L'ensemble de ces données montrent que les facteurs STAT5, par leur double capacité à maintenir en vie les CSH saines ou leucémiques et leur faculté de les protéger contre différentes formes de stress cellulaire, pourraient constituer de nouvelles cibles thérapeutiques pour éradiquer les rares cellules souches leucémiques à l'origine de la maladie résiduelle et limiter le développement de résistances aux traitements anti-BCRABL [4]. ♦

STAT5, a new therapeutic target against the relapse of chronic myelogenous leukemia?

LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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