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XXIV CICLO

PROTEIN KINASE CK2: ANALYSIS OF ITS ROLE IN ACUTE MYELOID LEUKEMIA AND CONDITIONAL KO IN MOUSE HEMATOPOIESIS

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ABBREVIATIONS

AA	Aminoacid
ATP	Adenosine triphosphate
ATF6	activating transcription factor 6
BCL-2	B cell lymphoma 2
BSA	bovine serum albumin
Cdc37	cell division cycle protein 37
CFCs	colony-forming cells
CFU-G	colony forming unit- Granulocyte
CFU-GEMM	colony forming unit- Granulocyte, Erythrocytes, Monocytes, Megakaryocytes
CFU-GM	colony forming unit- Granulocyte-Macrophages
CFU-M	colony forming unit - Macrophages
CK2	protein kinase CK2
CK2β	β subunit of protein kinase CK2
CLP	progenitore linfoide comune
MEP	Megakaryocytes- erythroid progenitors
CMP	Common myeloid progenitor
C-terminal	carboxyl terminal
CTRL	control
dNTPs	deoxyribonucleotide
dpc	days post coitum
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
Hetero	heterozygous
FCS	fetal calf serum
FGF	fibroblast growth factor
floxed	flanked by loxP sites
GMPs	Granulocytes- Macrophages progenitors
GRP78	glucose-regulated protein 78
HSCs	hematopoietic stem cells
Hsp90	heat shock protein 90

Ig	immunoglobulin
IκB	Inhibitor κB
IRE1	inositol-requiring enzyme 1
KO	knockout
LMPPs	multipotent progenitors- lymphoid-primed
LT-HSCs	long-term hematopoietic stem cells
MAPK	mitogen-activated protein kinase
MDP	Macrophages-dendritic cells progenitor
MkEPs	Erythrocyte- Megakaryocytes progenitors
MPPs	Multipotential progenitros
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
N-terminale	amino terminale
PBS	phosphate buffer solution
PERK	PRKR-like endoplasmic reticulum kinase
PI3K	Phosphatidyl-inositol-3-phosphate kinase
PKB/Akt	protein kinase B/Akt
rpm	round per minute
SD	standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Polyacrylamide gel electrophoresis in SDS
ST-HSCs	short-term hematopoietic stem cells
TAE	Tris-acetate-EDTA
TBS	tris buffer solution (buffer solution Tris-HCl)
TCR	T cell receptor
Tm	melting temperature
Tris	tris(hydroxymethyl)aminomethane
UPR	unfolded protein response
WB	Western Blotting
WT	wild type

AMINO ACID ABBREVIATIONS

A	**Ala**	**Alanine**
C	**Cys**	**Cysteine**
D	**Asp**	**Aspartic acid**
E	**Glu**	**Glutamic acid**
F	**Phe**	**Phenylalanine**
G	**Gly**	**Glycine**
H	**His**	**Histidine**
I	**Ile**	**Isoleucine**
K	**Lys**	**Lysine**
L	**Leu**	**Leucine**
M	**Met**	**Methionine**
N	**Asn**	**Asparagine**
P	**Pro**	**Proline**
Q	**Gln**	**Glutamine**
R	**Arg**	**Arginine**
S	**Ser**	**Serine**
T	**Thr**	**Threonine**
V	**Val**	**Valine**
W	**Trp**	**Tryptophan**
Y	**Tyr**	**Tyrosine**
X	**generic amino acid**	

ABSTRACT

Hematopoiesis is a tightly controlled, hierarchically organized developmental process whereby hematopoietic stem cells (HSC) give rise to highly specialized, differentiated blood cells.

HSC are characterized by the capacity of both self-renewing and differentiating in downstream committed hematopoietic progenitor cells. In vertebrates, hematopoiesis in embryonic and fetal life occurs in primitive hematopoietic organs whereas in the adult organism the definitive site of blood cell production is the bone marrow (BM). HSC are quiescent cells that reside most of the time in G_0-G_1 phases of the cell cycle, dividing during their lifespan as much as it is needed to maintain their own pool and give rise to committed precursors.

Leukemia formation is characterized by the block of this process and accumulation of immature cells in the bone marrow and peripheral blood.

In particular, acute myeloid leukaemia (AML) is characterized by the accumulation of large numbers of abnormal cells that fail to differentiate into functional mature blood cells. Leukemic blasts have a limited, exhaustible proliferative potential, suggesting that, in order to maintain leukemia growth, a small subpopulation of leukemic stem cells (LSCs) must display an inexhaustible proliferative capacity and self-renewal potential. No definitive proof of LSCs was available, however, until Dick and colleagues showed that the engraftment of NOD/SCID mice with primary AML samples could only be accomplished using cells that were phenotypically similar to normal hematopoietic stem cells (HSCs) by expressing CD34 and lacking markers of lineage commitment such as CD38 .

HSCs and LSCs share common features: self-renewal, the capacity to differentiate, resistance to apoptosis, and limitless proliferative potential.

The pathway regulating these functional properties can be categorized into self-renewal, developmental, and miscellaneous pathways, each of which is governed by a distinct set of critical genes that have emerged from molecular profiling and can be associated with stemness.

In particular Wingless (Wnt)/β catenin and PI3K/AKT pathways are crucial for the control of both HSCs and LSCs biology, because they regulate proliferation, self renewal and differentiation of HSCs and they are involved in maintenance of LSCs.

Phosphorylation is one of the molecular mechanism responsible for the signal transduction modulation of these two pathways and some molecules belonging to these pathways are phosphorylated by serin-threonin kinase CK2. Thus, this protein can have an important role in the biology of both HSCs and LSCs.

CK2 is a pleiotropic and constitutive activated kinase, which has a tetrameric structure composed by two alpha catalytic subunits and two beta regulatory subunits, but these two components can work also separately. CK2 is essential for cell survival and proliferation, and is more and more evident its involvement in various aspects of tumor transformation. High levels of CK2 are found in different tumor type such as breast, lung, kidney cancer and also blood cancer. CK2 promotes the activity of proto-oncogenes (c-myc, C-Jun, A-Raf), drives cell cycle progression, stimulates beta-catenin activity, inhibits the onco-suppressors p53, PML and PTEN, and it can exert an anti-apoptotic effect through the inhibition of caspase activity.

For this reasons, the principal aim of our research project is obtaining experimental evidence of a role of CK2 in normal hematopoiesis and in LSCs through the analysis of AML cell lines and samples collected from patients, and of a conditional knockout mouse model for CK2β in the hematopoietic system.

As far as the role of CK2 in the hematopoiesis is concerned, our conditional KO mouse model for CK2β in the hematopoietic compartment, demonstrate that the regulatory subunit of CK2 has an important role in hematopoiesis. In particular, the KO of CK2β induces an impairment in hematopoiesis, especially in erythropoiesis. However, CK2β KO seems not to influence the HSCs pool but instead the hematopoietic progenitors.

As far as the analysis of LSCs from AML samples, our analysis performed both on AML samples derive from patients and on Kasumi-1 cell line demonstrated that CK2 does not affect the Wnt/ β catenin and HH pathways, whereas it is able to modulate the PI3K/AKT pathway. Moreover, we have demonstrated that CK2 is important in LSCs survival as its inhibition increases the apoptosis and potentiates the effects of Daunorubicin, a drug currently used for AML treatment in clinic.

Taken together, all our results indicate that CK2 possesses an important role both in hematopoiesis and in the biology of LSCs.

RIASSUNTO

L'ematopoiesi è un processo fisiologico, finemente e strettamente controllato, attraverso il quale si ha la produzione di tutte le cellule mature del sangue a partire da una piccola popolazione di cellule staminali, denominate cellule staminali ematopoietiche (HSC).
Le HSC si caratterizzano per la loro capacità di auto-rinnovarsi e di differenziare in cellule progenitrici maggiormente differenziate.
Nei vertebrati, l'ematopoiesi viene suddivisa in primitiva e definitiva: la prima avviene negli organi ematopoietici primitivi, mentre la seconda risiede nel midollo osseo delle ossa lunghe.
Le HSC sono cellule quiescenti, che per la maggior parte della loro vita si trovano nello stadio G_0/G_1 del ciclo cellulare, dividendosi solamente lo stretto necessario per auto mantenersi e per dare origine ai progenitori in grado poi di differenziare nelle cellule ematiche mature.
La formazione di una leucemia è caratterizzata dal blocco di questo processo e dall'accumulo di cellule immature nel midollo osseo e nel sangue periferico.
In particolare la leucemia mieloide acuta (AML) si caratterizza per l'accumulo di un gran numero di cellule anomale (blasti) incapaci di completare il processo di differenziamento in cellule mature del sangue. I blasti leucemici si caratterizzano per la loro limitata capacità proliferativa, e questo suggerisce che, per mantenere la crescita del clone leucemico, sia necessaria la presenza di una piccola popolazione di cellule in grado di auto rinnovarsi e proliferare. Questa popolazione viene definita come cellule staminali leucemiche (LSC).
La prova definitiva dell'esistenza delle LSC è stata fornita dagli studi condotti da Jonh Dick e colleghi in un modello di xenotrapianto in topi NOD/SCID, nei quali venivano introdotte cellule derivate da pazienti affetti da AML e aventi un fenotipo simile alle HSC normali (queste cellule infatti esprimono sulla loro superficie il marcatore CD34 ma mancano del marker CD38 e di tutti i marcatori di linea, come le cellule staminali ematopoietiche normali).
HSC e LSC posseggono caratteristiche comuni, quali la capacità di auto- rinnovarsi (*self-renewal*), di differenziare, di resistere all'apoptosi e un potenziale di proliferazione illimitato.

Tutte queste proprietà funzionali sono regolate a livello molecolare da molteplici vie del segnale, che vengono generalmente raggruppate nelle seguenti categorie: vie del segnale del *self-renewal*, dello sviluppo e miscellanei, ciascuno dei quali è controllato da un set distinto di geni il cui ruolo nel controllo e mantenimento della staminalità è stato determinato con una serie di studi di *profiling* molecolare.

Tra queste vie del segnale, le vie di Wnt/ β catenina, Hedgehog e PI3K/AKT posseggono un ruolo cruciale nel controllo della biologia sia delle LSC che delle HSC, dato che sono coinvolte nella regolazione dei processi di proliferazione, auto rinnovamento e differenziamento.

Uno dei meccanismi molecolari responsabile della modulazione della trasduzione del segnale di queste vie è la fosforilazione, e alcune molecole appartenenti a queste vie sono fosforilate dalla proteina chinasi CK2. Quindi questa proteina potrebbe rivestire un ruolo importante nella biologia sia delle HSC e delle LSC.

CK2 è una serin- treonin chinasi pleiotropica e costitutivamente attiva, che possiede una struttura tetramerica, composta da due subunità α ad attività catalitica e da due subunità β con attività regolatoria, ma questi due componenti possono lavorare anche indipendentemente l'una dall'altra. CK2 è essenziale per la sopravvivenza cellulare e proliferazione, ed è sempre più evidente il suo coinvolgimento in vari aspetti della formazione neoplastica. Infatti, elevati livelli di CK2 sono stati riscontrati in vari tipi di tumore, come il tumore al seno, al polmone, al rene ed ematologici. CK2 infatti promuove l'attività di proto-oncogeni (c-myc, C-Jun, A-Raf), regola la progressione del ciclo cellulare, stimola l'attività di β catenina , inibisce gli onco-soppressori p53, PML e PTEN e può esercitare un effetto anti-apoptotico attraverso l'inibizione delle caspasi.

Con il presente progetto di dottorato abbiamo pertanto cercato di ottenere prove sperimentali del ruolo svolto da CK2 sia nell'ematopoiesi normale, mediante lo studio di un modello murino di KO condizionale di CK2β nel tessuto ematopoietico, che nella condizione patologica di leucemia mieloide acuta, mediante lo studio di 10 casi di pazienti affetti da AML e con l'ausilio della linea cellulare Kasumi-1.

Per quanto concerne lo studio del ruolo di CK2 nell'ematopoiesi normale, il nostro modello di KO condizionale per CK2β nell'intero sistema ematopoietico ci ha permesso di determinare il ruolo fondamentale che tale proteina riveste in tale processo. In particolare il KO per CK2β ha un fenotipo letale, in quanto la mancanza di CK2β causa un grave deficit dell'ematopoiesi, in particolare nel processo di eritropoiesi. Tuttavia,

Tra le popolazioni analizzate solo i progenitori ematopoietici sembrano risentire del KO di CK2β, mentre le HSC non sembrano subire alterazioni nei topi KO.

Per quanto riguarda invece lo studio del ruolo di CK2 nelle cellule staminali leucemiche, l'analisi affettuata sia su campioni prelevati da pazienti affetti da LMA che sulla linea cellulare Kasumi-1 ha evidenziato come CK2 sia in grado di modulare l'attività della via del segnale di PI3K/AKT, mentre le vie di Hedgehog e Wnt/ β catenina non sembrano influenzate in modo rilevante da CK2.

Inoltre, studi condotti combiando l'inibitore di CK2, CX-4945, da solo o in combinazione con Daunorubicina (un chemioterapico correntemente usato nella terapia della LMA), ha evidenziato come la mancanza dell'attività di CK2, non solo aumenti il tasso di apoptosi nelle LSC ma sia in grado di potenziare l'effetto citotossico della Daunorubicina, rendendole maggiormente sensibili all'azione del farmaco.

Concludendo, i risultati elencati sopra dimostrano come CK2 possegga un ruolo essenziale sia nell'ematopoiesi che nella biologia delle LSC.

1. INTRODUCTION

1.0 THE HEMATOPOIETIC SYSTEM

The hematopoietic hierarchy

Hematopoiesis in vertebrates is a complex, hierarchically organized developmental process by which all of the different blood cell types are generated and maintained throughout the life span of an organism [1]. Hematopoiesis is paradigmatic in that it is an ontogenetic process whereby differentiated, highly specialized cells originates through an ordinate maturation program from undifferentiated hematopoietic stem cells (HSCs). HSC are endowed with the capacity of self-renewing (i.e., giving rise to identical HSC progeny, in order to maintain the HSC pool) as well as starting a multi-step differentiation process that ultimately results in the production of differentiated, mature blood cells [1].

HSCs resides primarily within the bone marrow during adulthood and they are thought to reside within HSC niches, which are specialized microenvironments created by supporting cells that express membrane-bound and secreted factors that promote HSC maintenance (survival and self-renewal), and that regulate HSC migration, quiescence and differentiation. Within the bone marrow, many HSCs reside at, or near, the endosteum and endosteal cells secrete factors that promote HSC maintenance. Therefore, the endosteum has an important role in promoting HSC maintenance and regulating HSC function. Other bone-marrow cells probably also have important roles in the creation of HSC niches. Many HSCs reside adjacent to sinusoidal blood vessels in the bone marrow and perivascular cells also secrete factors that regulate HSC maintenance [2].

HSCs can also be found at low levels in extramedullary tissues such as the spleen and liver throughout adult life. When bone-marrow hematopoiesis is impaired by age, cancer or myeloablation, expanded numbers of HSCs can engage in extramedullary hematopoiesis in the spleen. [2].

HSCs reside at the top of the hematopoietic hierarchy and give rise to multipotent progenitors (Figure 1). The initial events during HSCs differentiation process include the transition from long term self-renewing HSCs (LT-HSC) to short-term self-renewing HSC (ST-HSC) that are responsible for the formation of the multipotent progenitors (MPPs), characterized by a more limited self-renewal capacity. It is

believed that cells originated from MPPs lose multipotency, because of the beginning of the commitment process at different cell lines [3].

The term "progenitors" include all dividing cells that display a certain differentiation potential. In the case of hematopoiesis the definition is more specific, and it is referred to the *in vitro* identification of cells that differentiate after cytokines stimulation: colony forming units granulocytes-macrophages (CFU-GM), and colony forming unit-erythroid (CFU-E) constitute some examples.

Multipotent progenitors in turn give rise to oligo-potent progenitors, which possess more restricted developmental potential. This appears to represent a branching point in the hematopoietic hierarchy with the common lymphoid progenitor (CLP) giving rise to mature lymphoid effectors cells including B, T, dendritic, and natural killer (NK) cells but lacking the potential to form myelo-erythroid cells. On the other hand common myeloid progenitor (CMP) subsets are capable of giving rise to mature myelo-erythroid effectors cells yet lacking the capacity to form lymphoid progeny: granulocytes-macrophages progenitors (GMPs) differentiate into monocytes/macrophages and granulocytes (but they are also able to generate dendritic cells), and megakaryocytes/erythrocytes progenitors (MEPs) give rise to megakariocytes/ platelates and erythrocytes. Such oligo-potent progenitors in turn give rise to more lineage-restricted progenitors (Pro-B, ProT, Pro-NK), from which all of the mature blood cells eventually arise.

The stages in primary B-cell development are defined by the sequential rearrangement and expression of heavy- and light-chain immunoglobulin genes.

The development of a B-lineage cell proceeds through several stages marked by the rearrangement and expression of the immunoglobulin genes. The stem cell has not yet begun to rearrange its immunoglobulin (Ig) gene segments; they are in the germline configuration. The earliest B-lineage cells are known as pro-B cells. They are derived from pluripotent hematopoietic stem cells and are identified by the appearance of cell-surface proteins characteristic of early B-lineage cells. Rearrangement of the immunoglobulin heavy-chain locus takes place in pro-B cells; D_H to J_H joining at the early pro-B cell stage is followed by V_H to DJ_H joining at the late pro-B cell stage.

Productive VDJ_H joining leads to the expression of an intact μ heavy chain, which is the hallmark of the next main stage of development, the pre-B cell stage. The μ chain in large pre-B cells is expressed intracellularly and possibly in small amounts at the cell surface, in combination with a surrogate light chain, to form the pre-B-cell receptor.

Expression of the pre-B-cell receptor signals the cell to halt heavy-chain locus rearrangement and production of the surrogate light chain, and to divide several times before giving rise to small pre-B cells, in which light-chain rearrangements begin. Once a light-chain gene is assembled and a complete IgM molecule is expressed on the cell surface, the cell is defined as an immature B cell.

All development up to this point has taken place in the bone marrow and is independent of antigen. Immature B cells now undergo selection for self-tolerance and subsequently for the ability to survive in the peripheral lymphoid tissues. B cells that survive in the periphery undergo further differentiation to become mature B cells that express IgD in addition to IgM. These cells, also called naive B cells until they encounter their specific antigen, recirculate through peripheral lymphoid tissues, where they may encounter and be activated by the appropriate foreign antigen.

T lymphocytes develop from a common lymphoid progenitor in the bone marrow that also gives rise to B lymphocytes, but those progenitor cells destined to give rise to T cells leave the bone marrow and migrate to the thymus, which provides a unique microenvironment for T-cell development. The differentiation program of T cells is similar to that of B cell, including the sequential rearrangement of antigen receptor gene segments. Developing T cells pass through a series of stages that can be distinguished by the expression of the CD3,T-cell receptor complex proteins, and the co-receptor proteins CD4 and CD8. In the case of T cells, development is compartmentalized, with different types of stromal cells in the thymic cortex and medulla. Most steps in T-cell differentiation occur in the cortex of the thymus. The thymic medulla contains mainly mature T cells. Lymphocyte development is accompanied by extensive cell death, reflecting intense selection and the elimination of those cells with inappropriate receptor specificities [4].

NK cells have been traditionally classified as cells belonging to innate immunity, as they don't need a previous event of sensitization against their targets, despite they constitute the third major lymphocytic population of cells. The development of NK cells occurs mainly in the bone marrow, but some studies indicate that liver, spleen and lymph nodes as other potential site of maturation. After having left the bone marrow, competent NK cells respond to possible infections and activate mechanisms of anti-tumor immuno-surveillance [5].

Monocytes and dendritic cells are different groups of mononucleated phagocytes, that originate in the bone marrow. Dendritic cells are specialized in presenting antigens to T

lymphocytes. They originate from the CMP, that also give rise to monocytes and macrophages. Dendritic cells are generated continuously by circulating precursors with short life-span, the so called pre-DC, that are present in bone marrow, peripheral blood and lymphatic organ [6]. Monocytes, after their entrance in blood stream, are able to differentiate in dendritic cells or macrophages during an inflammation status, migrating to peripheral tissue [7].

In adult mammals, granulopoieisis occurs primarily in the bone marrow, with expansion to extramedullary sites in time of increased demand or with bone marrow disease.

Committed neutrophil, eosinophil and basophil precursors are originated by CMP and undergo a similar maturation sequence within the bone marrow [8].

Thrombopoieisis is sustained by MEPs, that first differentiate in megakariocyte progenitors with the subsequent maturation in functional platelets. This process takes place in specialized niche in the bone marrow, where megakariocytes resides near the vascular endothelium [9].

Although questions remain regarding the absolute lineage potential of the different hematopoietic progenitor subsets and their relationship to one another, there is wide consensus that the sequential differentiation of HSCs through progenitors to fully differentiated blood cells is a primarily irreversible process under normal physiological steady-state conditions [10].

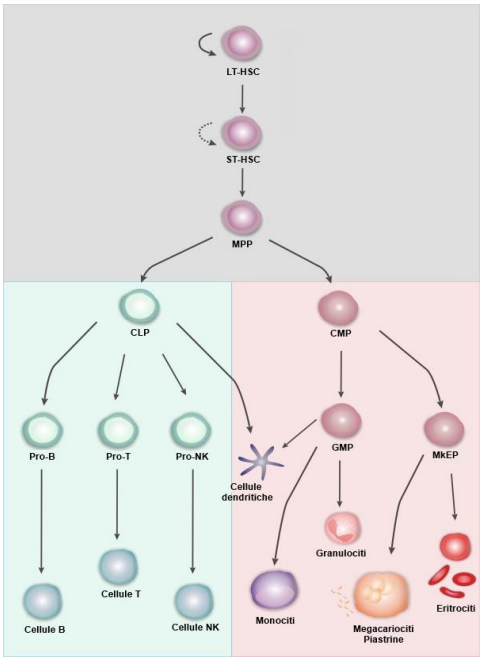

Figure 1: The hematopoietic hierarchy in adult organism. LT-HSC = Long-term HSC; ST-HSC = short-term HSC; MPP = Multipotent progenitor; CLP = Common lymphoid progenitor; Pro-B = B cell progenitors; Pro-T = T cell progenitors; Pro-NK = Natural killer cells progenitor; CMP = Common myeloid progenitors; GMP = granulocytes- macrophages progenitor; MkEP = Megakaryocyets/erythroid progenitor (Modified from Luc S *et al*., 2008).

Ontogeny of hematopoietic system

The original pool of HSCs is formed during embryogenesis in a complex developmental process that involves several anatomical sites (the yolk sac, the aorta-gonad-mesonephros region, the placenta and the fetal liver), after which HSCs colonize the bone marrow at birth (Figure 2).

In mammals, hematopoiesis can be divided in two steps: primitive and definitive hematopoiesis. The first one takes place in the yolk sack and is equal to embryonic hematopoiesis. The definitive hematopoiesis (fetal and adult hematopoiesis) starts with the colonization of fetal liver by the HSCs, previously generated in an area that surrounds the dorsal aorta, the so called aorta-gonad-mesonephros region (AGM). At birth, another wave of migration of HSCs shifts hematopoiesis in the bone marrow, where resides the definitive hematopoiesis [11].

During postnatal life, a steady state is established in which HSC pool size is maintained by the regulation of HSC self-renewal and differentiation. This is possible because the bone marrow contains specialized niches in which the multipotency of HSCs is conserved through cell divisions, while their progeny are directed towards lineage differentiation. During homeostasis, most adult HSCs are quiescent and divide only rarely to maintain an appropriate quantity of differentiated blood cells and to renew the HSC pool [12].

In the adult organism the differentiation process is generally unidirectional, whereas, in the embryo there is the production of several hematopoietic progenitors and specialized blood cells before t he appearance of HSCs [13].

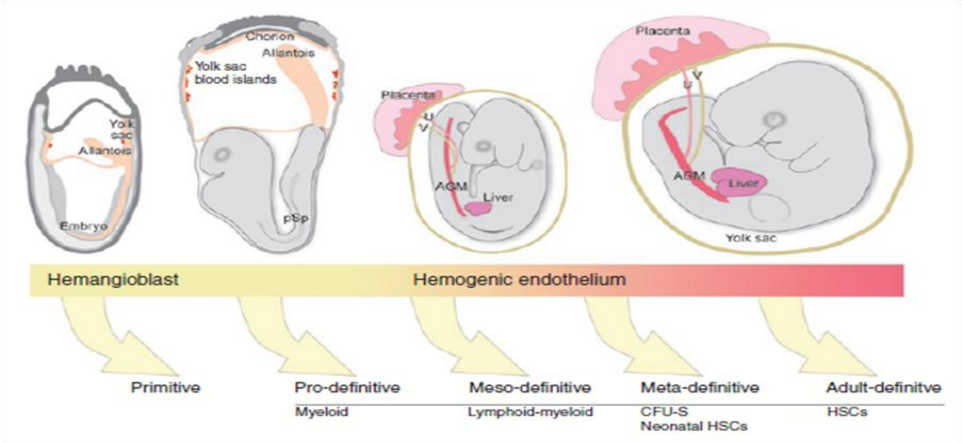

Figure 2: Generation of hematopoietic cells in mouse, in temporal progression. U = umbilical artery; V = vitelline artery [14].

Primitive hematopoiesis

Hematopoiesis occurs at multiple sites within the embryo and in extra embryonic tissues. The phase of blood cell production, referred to as *primitive hematopoiesis*, is responsible for producing blood elements during the earliest stage of embryogenesis. Primitive hematopoiesis takes place in the visceral yolk sac beginning at approximately E7.0. Thus primitive hematopoietic cells are among the earliest distinct tissues to differentiate in the embryo. Formation of primitive cells declines rapidly after E11. The visceral yolk sac or extra embryonic splanchnopleure (the term for a structure in which mesoderm and endoderm are directly apposed) arises from the migration of extra embryonic mesoderm streaming from the caudal primitive streak along the inner surface of visceral endoderm. The mesodermal cells committed to initiate and support hematopoiesis have been termed *hemangioblasts* because the contiguity of primitive hematopoiesis and vasculogenesis in both space and time suggest that primitive hematopoietic and endothelial cells in the yolk sac share a common ancestor. Hemagioblasts arise as undifferentiated cells at the primitive-streak stage and commit to produce a particular cell lineage before blood island formation. These pluripotent cells also can differentiate into other mesenchyme-derived tissues.

Between E7.5 and E9, hemangioblasts form multiple aggregates termed *blood islands*. Each blood island contains a central core of unattached inner hemangioblast (hematopoietic progenitors) surrounded by a rim of spindle-shaped outer hemangiblast (endothelial cells). Nucleated erythroid cells are first recognized in the core of blood

islands at E8.0 and are evident circulating in the cardiovascular system starting E8.25, where they continue to divide until approximately E13.0.

The majority of cells produced during primitive hematopoiesis are of the erythroid lineage. Committed erythroid colony-forming cells arrive in the yolk sac at approximately E7.25. These cells expand until E8.0 and then differentiate into primitive erythroblasts; all colony forming cells have regressed completely by E9.0, which corresponds approximately to the earliest phase of definitive erythropoiesis.

Recent studies suggest that other hematopoietic cell lineages also are generated in the yolk sac during this primitive stage of hematopoietic development. Primitive lymphoid precursors and even some adult stem cells evolve at E7.5 and subsequently seed other sites of hematopoiesis, including AGM region, umbilical vessels, and liver. Primitive macrophages have been identified in the yolk sac by E8.0 to E9.0 (Figure3) [15].

Definitive hematopoiesis

The second stage of blood cell production, termed *definitive hematopoiesis*, is thought to rise primarily from the AGM. The AGM domain is the main source of mesenchimal-derived, definitive HSCs that will serve the developing animal during late gestation and post-natal life. Initiation of definitive hematopoiesis ranges between E8.5 and E9.25, with the definitive HSCs evident in the AGM by no later than E10. Peak production of HSC in the AGM occurs between E10.5 and E11.5, at which time they comprise almost 10% of all AGM cells. Regardless of their original site of the novo synthesis, HSCs migrate to seed other locations that support definitive hematopoiesis: embryonic liver, followed by embryonic thymus, fetal spleen, and bone marrow (in that order). These latter destinations do not produce HSCs de novo but rather contain niches suitable for expansion of newly arrived HSCs. The embryonic liver is colonized first, apparently because it shares many molecular and functional similarities with the yolk sac. It provides the major locus for definitive hematopoiesis from E12 to E16. The first HSCs to enter the liver are pluripotent and can form any type of hematopoietic cell.

Embryonic thymus and fetal spleen are seeded either from the liver or AGM, or both, beginning about E13 for thymus and E15 for spleen. The thymus typically accepts only those HSCs that are committed to make T cells, whereas the population of intra-hepatic B cell progenitors exhibits a reverse trend. Most types of definitive hematopoietic cells in the spleen arise from precursors cells that commit to a specific lineage before leaving

the liver. Multi-potent HSCs entering the spleen cease proliferate and differentiate into mature macrophages. These cells may regulate intra-splenic erythropoiesis.

The bone marrow first receives HSCs from hepatic depots at about E16. Thereafter, the allocation of colony-forming hematopoietic precursors shifts from a primarily hepato-centric localization at E18 through more dispersed distribution (bone marrow, liver, and spleen in approximately equal number) at P2 to a profile favoring bone marrow and to a lesser extent spleen at P4 and after. Thus bone morrow, liver and spleen function cooperatively to regulate hematopoiesis (Figure 3) [15].

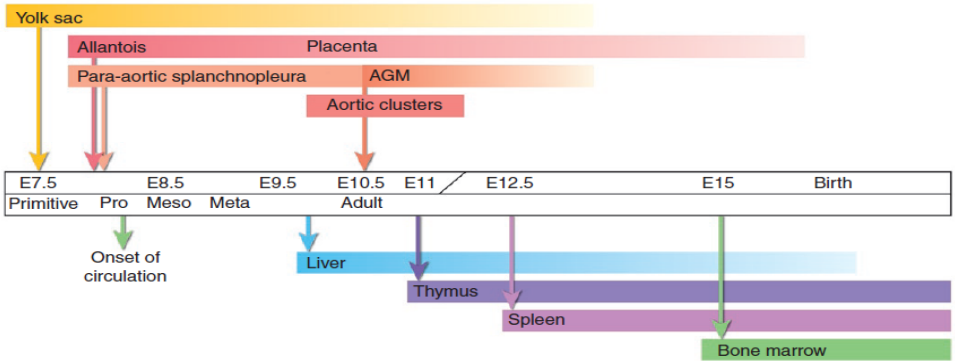

Figure 3: Timeline of hematopoietic events in the mouse conceptus. Arrows above indicate the onset of specific hematopoietic cell generatio and/or appearence; arrows below indicate the earliest time of colonization of the secondary hematopoietic territories

Erythroid differentiation

The red cells of mammals are unique in the animal kingdom as they circulate as enucleated cells. In contrast, the fully mature red cells of birds, amphibians, and fish remain nucleated . A century ago, examination of mammalian embryos revealed the presence of distinct nucleated and enucleated red cells . The continuous circulation of small, enucleated erythrocytes during fetal and postnatal life (‘definitive’ erythropoiesis) was distinguished from ‘primitive’ erythropoiesis, characterized by the transient circulation of large, nucleated red cells that originate in the yolk sac.

Examination of embryonic and fetal blood cell morphology, however, revealed that primitive red cells undergo a synchronous wave of maturation in the bloodstream, and that late-stage primitive erythroblasts in the mouse embryo complete their maturation by enucleating and continuing to circulate for several more days as erythrocytes,

undermining the dogma for which the principal difference between primitive and definitive erythrocytes is the presence or absence of the nucleus [16].

Erythropoiesis is the proliferation and progressive differentiation of HSCs into hemoglobinized, red blood cells (RBCs). RBCs originates from HSC in a stepwise manner, wherein each step includes cell division and differentiation, and is initiated and regulated by specific humoral, microenviromental, cell surface, and trascription factors.

A multipotent HSC first differentiates into a common myeloid progenitor (CMP), which is then capable of differentiating into granulocyte/macrophages progenitor or a megakariocyte/erythroid progenitor (MEP). CMPs and MEPs in humans and mice can be identified by their specific cell surface phenotype. Differentiation from CMP to MEP is associated with the expression of various gene-specific transcription factors, growth factor receptors, and functional proteins, and is influenced by a variety of growth factors.

MEPs differentiate into burst-forming units-erythroid (BFU-Es). These cells are identified by their growth from a single cell to several thousand cells in culture. The colony-forming units-erythroid (CFU-E) differentiate from BFU-E. In culture, each CFU-E divides into 8-32 cells. Thus, BFU-E have a higher proliferative potential than more differentiate CFU-E. These progenitor cells differentiate into precursors cells that can be recognized as erythroid cells based on morphologic characteristic. The earliest precursor is proerythroblast: this is a large cell with deeply basophilic cytoplasm, a round centrally located nucleus and one or more prominent nucleoli. Proerythroblast undergo a series of differentiation steps that result in a progressive decrease in cell size, and a gradual condensation of nuclear chromatin. The basophilic erythroblast is similar to the proerythroblast except that it does not contain a nucleolus. Subsequent stages are called polychromatic erythroblast and orthochromatic erythroblast. These cells have increasing amounts of hemoglobin; basophilic erythroblasts have the lowest hemoglobin concentration, and therefore still have deeply basophilic cytoplasm. Orthochromatic erythroblasts have the most hemoglobin, and dense, homogeneous nuclear chromatin.

At this point the nucleus is extruded to produce reticulocytes.

Reticulocytes than exit the bone marrow, and they still contain residual mitochondria, Golgi membranes, ribosomes, and microtubular components, and can have approximately 35% more volume than mature RBCs. During maturation in the circulation, reticulocytes gain hemoglobin and lose organelles, membrane surface area, volume, and numerous cell surface proteins and became mature erythrocytes (Figure 4).

All precursors except orthochromatic erythroblast are capable of division [17]. The stages of erythroid differentiation are summarized in figure 4.

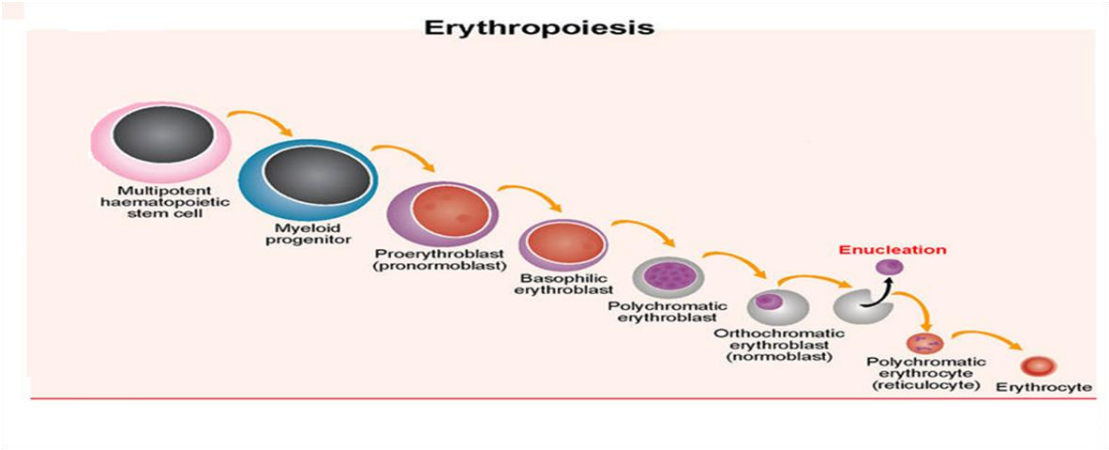

Figure 4: Erythropoiesis: scheme of differentiation

1.2 ACUTE MYELOID LEUKEMIA

Hematopoiesis, as previously described, is a tightly controlled, hierarchically organized developmental process whereby hematopoietic stem cells (HSC) give rise to highly specialized, differentiated blood cells.

Leukemia formation is characterized by the block of this process and accumulation of immature cells in the bone marrow and peripheral blood [18].

Acute myeloid leukemia (AML), in particular, represents a group of clonal hematopoietic stem cell disorders in which both failure to differentiate and over-proliferation in the stem cell compartment result in accumulation of non-functional cells termed myeloblasts. AML represents 30% of all acute leukemias affecting preferentially adults from 18 to 60 years; its incidence increases with age with a peak at 50 years. The symptoms of AML are caused by replacement of normal bone marrow with leukemic cells: the early signs of AML include fever, weakness and fatigue, reduction of weight and appetite, and pains in the bone or joints. Other signs include red spots in the skin, easy bleeding due to platelet loss, decreased resistance to infection linked to neutrophil loss and anemia.

AML progress rapidly and is typically fatal within weeks or months if left untreated. AML generally involve the bone marrow and the diagnosis of AML need the presence at least of 20% of blasts in this compartment; in some cases, AML cells may spread to peripheral blood and other organs, such as the liver and spleen. In the 1970s, a group of French, American, and British leukemia experts divided acute myeloid leukemias into

subtypes, M0 through M7, on the basis of morphology, special stains, cytogenetics, and cell surface markers; this method was called FAB classification [19] (Table 1).

Table 1. FAB classification of AML

FAB subtype	**Name**	**% of adult AML patients**	**Prognosis compared to average for AML**
M0	Undifferentiated acute myeloblastic leukemia	5%	Worse
M1	Acute myeloblastic leukemia with minimal maturation	15%	Average
M2	Acute myeloblastic leukemia with maturation	25%	Better
M3	Acute promyelocytic leukemia (APL)	10%	Best
M4	Acute myelomonocytic leukemia	20%	Average
M4 eos	Acute myelomonocytic leukemia with eosinophilia	5%	Better
M5	Acute monocytic leukemia	10%	Average
M6	Acute erythroid leukemia	5%	Worse
M7	Acute megakaryoblastic leukemia	5%	Worse

The FAB classification system is useful and is still commonly used but it does not take into account many of the factors that are known to impact prognosis such as cytogenetic aberration and other genetic defects. The World Health Organization (WHO) has

proposed a newer system that includes some of these factors to try to help better classify cases of AML [20].

The WHO classification system divides AML into several broad groups:

1) AML with certain genetic abnormalities

- AML with a translocation between chromosomes 8 and 21
- AML with a translocation or inversion in chromosome 16
- AML with changes in chromosome 11
- APL (M3), which usually has translocation between chromosomes 15 and 17

2) AML with multilineage dysplasia (more than one abnormal myeloid cell type is involved)

3) AML related to previous chemotherapy or radiation

4) AML not otherwise specified (includes cases of AML that do not fall into one of the above groups; similar to the FAB classification)

- Undifferentiated AML (M0)
- AML with minimal maturation (M1)
- AML with maturation (M2)
- Acute myelomonocytic leukemia (M4)
- Acute monocytic leukemia (M5)
- Acute erythroid leukemia (M6)
- Acute megakaryoblastic leukemia (M7)
- Acute basophilic leukemia
- Acute panmyelosis with fibrosis
- Myeloid sarcoma (also known as granulocytic sarcoma or chloroma)

5) Undifferentiated or biphenotypic acute leukemias (leukemias that have both lymphocytic and myeloid features). Sometimes called ALL with myeloid markers, AML with lymphoid markers, or mixed lineage leukemias.

The evolution of the classification system in AML from morphology to cytogenetic/genetic-based reflects the recognition of the importance of subtype-specific biology. The two major prognostic factors in newly diagnosed AML, patient age and chromosome status, form the basis of important treatment decisions.

According to the *European Leukemia Network (ELN)* guidelines, young adults are generally subdivided in three groups according to the risk of factor: favorable, intermediate or adverse (table 2) [21] [22]. A complex karyotype, that is present in 10-12% of patients, has a significant association with an unfavorable prognosis and it has been defined as the presence of three or more chromosomal abnormalities except for t(8;21), inv(16), t(16;16) and t(15;17). An important characteristic of cases with a complex karyotype is the loss of 17p and/or mutation of p53, present in two-thirds of the cases.

Recently, a new cytogenetic category was proposed to distinguish LMA with very unfavorable prognosis, that is the monosomial karyotype, defined by the presence of a single monosomy (except for the isolated loss of X or Y) in association with at least another monosomy or structural chromosomal abnormality (all but CBF-AML).

It is important to observe that as the age increases it increase also the unfavorable chromosomal abnormalities and this explains in part the negative prognostic value of age.

Table 2: Group of risk related to cytogenetical and molecular data

RISK GROUP	SUBGROUPS
Favorable	LMA with t(8;21)(q22;q22); RUNX1-RUNX1T1 LMA with inv(16)(p13;q22) o t(16;16)(p13;q22); CBFB-MYH11 NPM1 mutated without FLT3-ITD (normal karyotype) CEBPA mutated (normal karyotype)
Intermediate-I	NPM1 mutated and FLT3-ITD (normal karyotype) NPM1 *wild type* and FLT3-ITD (normal karyotype) NPM1 *wild type* senza FLT3-ITD (normal karyotype)
Intermediate-II	t(9;11)(p22;q23); MLLT3-MLL cytogenetic aberration unclassifiable as favorable or adverse
Unfavorable	LMA with t(6;9)(p23;q34); DEK-NUP214 LMA with inv(3)(q21;q26) o t(3;3)(q21;q26); RPN1-EVI1 t(v;11)(v;q23); riarrangement of MLL -5 o del(5q); -7; complex karyotype

While cytogenetic analysis is an important part of the diagnostic evaluation in all patients, outcome risk has been difficult to define for patients presenting without chromosome aberrations. However, recent studies have discovered several molecular

markers that allow for the definition of outcome risk, even in patients with cytogenetically normal AML (CN-AML): a group that comprises 40–50% of patients with newly diagnosed disease.. As a result of the identification of these mutations, patients with CN-AML are recognized as a diverse group with distinct clinical outcomes (Walker A, Marcucci G.,Molecular Prognostic Factors in cytogenetically normal acute myeloid leukemia, Expert Rev Hematol 2012 Oct;5(5):547-58. doi: 10.1586/ehm.12.45). Among these mutations some example are represented by the mutation of NPM1, CEBPA, FLT3, alone or in combination. Patients with normal karyotype bearing internal tandem duplication (ITD-FLT3) have a worse prognosis as compared to patients without it [22], [23].

Several studies have highlighted that the genotype " NPM1 mutated without ITD-FLT3" represent a favorable prognostic marker, which is associated with a major frequency of complete remission, and a better relaps free survival and overall survival.

The presence of mutations of CEBPA in patients with normal karyotype is classified as a favorable prognostic marker [21], [22], [23].

The therapeutic approach to adults aged 18-60 years classically involves separate treatment phases. The first one consists of induction chemotherapy in which the goal of myelosuppression is to empty the bone marrow of all hematopoietic elements (both benign and malignant) and to allow the repopulation of the marrow with normal cells, thereby yelding remission (<5% marrow blasts).

Cytarabine (AraC) is the cornerstone of induction therapy and consolidation therapy for AML. Consolidation therapy comprises treatment with additional courses of intensive chemotherapy after the patient has achieved a complete remission (CR), usually with higher doses of the same drugs that were used during the induction period. Despite substantial progress in the treatment of newly diagnosed AML, 20% to 40% of patients do not achieve remission with the standard induction chemotherapy, and 50% to 70% of first CR patients are expected to relapse within 3 years. The optimum strategy at the time of relapse, or for patients with the resistant disease, remains uncertain. Allogenic stem cell transplantation has been established as the most effective form of anti-leukemic therapy in patients with AML in first or subsequent remission [24]. However, this approach carries a high degree of initial mortality and a significant degree of long-term morbidity in the form of chronic graft-versus-host disease (GVHD). Chemotherapy-based approaches with or without autologous stem cell rescue can be performed relatively safely, but there remains a high chance for disease recurrence [25].

There is a problem linked to the common use of agents directed toward the inhibition of proliferation because leukemia arises in the reserve hematopoietic stem cells, which can only be eliminated by total bone marrow ablation. Differentiation therapy is an alternative approach to leukemia treatment, which does not directly kill the proliferating cells but induces them to mature and die [26]. New therapies have been approved for clinical trials that include agents promoting differentiation such as azacitidine, a DNA hypomethylating agent, or using histone deacetylase inhibitors valproic acid (VPA) [27]**,** and all trans retinoic acid**.** Other new therapies include small molecules that inhibit signal transduction (i.e thymidine kinase inhibition) and antibody-directed cytotoxicity (anti-CD-33 immunotoxin).

Genetic alterations in AML

Human leukemia, like all cancers, results from multiple mutations that lead to abnormalities in the expression or function of gene products that affect the delicate balance among proliferation, differentiation, and apoptosis. Leukemias are characterized by acquisition of recurring genetic aberrations and chromosomal translocations. These last ones include loss of function mutations in transcription factors that are required for normal hematopoietic development. However they are not sufficient to cause leukemia and appear to be one hit in the multistep pathway. Recent studies indicate that activating mutations, that confer prolipherative and survival signals to the progenitors cells, could represent "the second hit", thus cooperating with loss of function mutations in the transcription factors towards uncontrolled proliferation and impaired differentiation.

- Chromosomal translocations. They are the most common aberration in AML and in the majority of cases are balanced rearrangements. To date more than 700 recurrent non-random translocations have been identified: different studies have provided evidence that gene rearrangements correlate with specific tumor phenotypes, but at the meantime they uncover the presence of a shared molecular plateau that translocations use to transform cells. Indeed these translocations lead to the expression of fusion proteins that show a common structural and functional theme: they are usually composed of a transcriptional factor, that retains the DNA-binding motifs of the wild-type protein, and of a

non correlated protein, that is able to interact with a corepressor complex, thus altering the expression of target genes necessary for myeloid development [28]

- The most prevalent fusion protein are:
  - t(15;17) PML-RARα found in 95% of acute promyelocytic leukemia, that will be discussed in more detail in the next paragraph;

  - t(6;21) AML1-ETO where AML1 is a transcription factor crucial for hematopoietic differentiation and ETO is a protein with transcriptional repressor activity;

  - Inv(16) CBFβ-MYH11 where the first aminoacid of core binding factor β are fused to the c-terminal region of a smooth muscle myosin heavy chain;

  -MLL rearrangements. MLL is implicated in leukemias of various types: acute linfoblastic leukemia (ALL), in AMLs, biphenotypic ALs, and infant leukemias. In general the prognosis is poor. MLL protein can fuse to one of more 50 partners, resulting in a MLL-fusion protein that acts as a potent oncogene [29].

- Mutations in transcription factors involved in myelopoiesis and granulopoiesis such as PU.1, GATA-1, c-EBPα [30].

Other genetic defects are activating mutations that confer proliferative and survival signals:

- <u>Receptor tyrosine kinases</u> which become constitutively active, such as c-KIT and FLT3. This last one is found in 15-42% of patients with AML and is associated with a poor prognosis. Two types of mutations results in constitutive activation of the receptor: internal tandem duplications and missense point mutations.

- <u>Other signalling molecules:</u>
  -RAS proteins involved in the transduction through receptor tyrosine kinases: the mechanism of activation is constituted by point mutations in the GTP binding site and occur at a frequency of 25% in AML;

  -STAT proteins in particular STAT3 and STAT5;

-over-expression of anti-apoptotic genes of the BCL-2 family;

- alterations in the genes that control cell cycle: loss of Rb, overexpression of cyclin D and cyclin E, increased activity of cdk kinases [31]**.**

Leukemic stem cells and the leukemic microenviroment

During the last years, it is become more and more evident the hypothesis that a neoplasm is constituted from an heterogeneous population of cells, in which the proliferation capacity is a property of a specific subpopulation of cells: the leukemic stem cells (LSCs). As HSC (their healthy counterpart), LSCs are present in a very small percentage, they can divide in an asymmetric manner, and they are in a quiescent state for the majority of their life.

The discovery of these cells has revolutionized the concepts and the approaches to cancer therapies.

The vast majority of therapies now used for the treatment of AML is aim at destroying only the bulk of the tumor, with a mechanism targeting cells in active replication. For this reason, they are able to induce an effective temporary clinical remission, but they are not as much effective in maintaining a long term and stable remission.

In fact, cancer stem cells are resistant to chemotherapeutic treatment as they do not replicate, and for this reason LSCs are able to induce disease relapses.

Therefore, the identification and characterization of these aberrant cells could allow the production of specific and selective drugs.

AML is a heterogeneous disease, both biologically and clinically, in which a number of distinct genetic abnormalities have been described. However, despite this heterogeneity, early pioneering studies demonstrated that only the most primitive Lin-CD34+CD38- fraction of AML cells and not the more mature Lin-CD34+CD38+ or CD34- populations were capable of transferring disease to NOD/SCID mice. In the recipient mice, the CD34+CD38- cells differentiated into leukemic blasts and recapitulated the phenotype of the disease observed in the patient. Furthermore, these cells were able to reconstitute and give rise to AML in secondary recipients, indicating self-renewal of the LSC in the primary recipients [32].

Thus, in a similar way to normal hematopoiesis, it was demonstrated that AML is arranged as a loose hierarchy in which a small population of self-renewing leukemic stem cells (LSC) give rise to a large population of more mature leukemic blasts which lack self-renewal capacity.

Several studies have revealed that genetic rearrangements, fusion gene, mutations, aberrantly activation of signaling pathway and transcription factors induce the formation of a neoplastic cell with stemness properties.

According to a model recently proposed, the principal responsible for the origin of LSC is constituted by all the oncogenic events that leads to the transformation of the normal counterpart [33].

In some cases, LSCs can originate from HSCs through a process of carcinogenesis that requires several steps. However, these events can be defined pre-leukemic, as they causes the acquisition of new properties that give proliferation and survival advantages as compared to normal cells, but these cells alone are not able to induce disease.

The establishment of new genetic alteration and the lost of differentiation capacity must associate with the pre-leukemic events to originate leukemia.

Due to the rarity of this events, only the stem compartment, the only one which is able to self-renew for all the life-span of an individual, can go through this kind neoplastic transformation.

However, some mutations are able alone to induce the neoplastic phenotype, leading to the acquisition of self-renewal capacity to cells without this feature (for example precursors cells), or conferring to HSC the ability to proliferate without control.

Meanwhile, this mutations alter the differentiation capacity, with the subsequent development of leukemia.

Among this events, it can be cited the genetic fusion that hits MLL transcriptional factors (mixed lineage leukemia) that control genes important in cell proliferation, for example MLL-ENL (11-19 leukemia) and MLL-GAS7 fusion [34].

Thanks to this leukemic events, LSCs can originate not only from HSC but also from more differentiated progenitors.

This explains the presence of different subpopulation of LSCs between patients [35] (Figure 5).

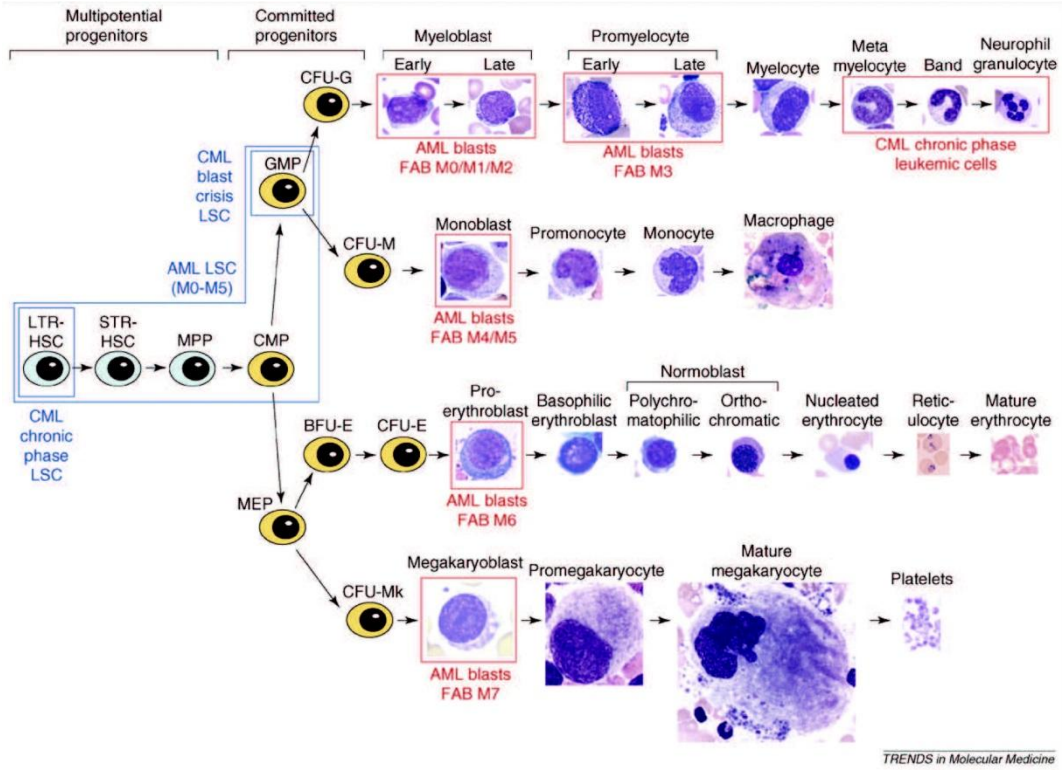

Figure 5. Hematopoieisis and origine of leukemic blasts and LSCs.

Quiescent long term repopulating HSC (LTR-HSC); in active replication short term repopulating HSC (STR-HSC), and multipotential progenitors (MPP) are blue-stained at the left. The common progenitors: common myeloid progenitors (CMP), granulocyte-macrophages progenitors (GMP), megakaryocytes-erythroid progenitors (MEP) and colony forming unit granulocyte (CFU-G), macrophage (CFU-M), erythroid (CFU-E) and megakaryocyte (CFU-Mk) are stained in orange in the middle of the picture. At the right it is shown the morphology of terminal differentiated cells. Within the red boxes are represented the different blasts that can be identified in acute and chronic AML according to the FAB classification. In this picture is not represented the common lymphoid progenitor (CLP) that give rise to B and T lymphocytes and NK cells.

Due to the complexity and the importance of the bone marrow microenvironment in maintaining the homeostasis of hematopoiesis, it is reasonable to infer that alteration of the microenvironment can be fundamental in sustain leukemogenesis. Several experimental evidence support this hypothesis.

For example, it has been demonstrated that the expression of CXCL12 in the vascular niche is reduced in the zone that are infiltrated by cancer cells, whereas high levels of CXCR4 are described in AML associated with an unfavorable [36], [37].

The adhesion to the stroma and to fibronectin mediated by integrins VLA-4 and -5 seems to be maintained as in the healthy counterpart; however LSCs seem to possess an increase migration capacity that make them able to distance themselves from factors

that inhibits the cell growth or from signals that promote quiescence and that are secreted by osteoblasts and stromal cells [38], [39].

Alteration of metalloproteases and of their inhibitors could be associated to migration and invasiveness of neoplastic cells.

Microenvironment can also influence the response to therapy: experimental models of AML have demonstrated that the release of asparagine synthetase by mesenchimal cells, causes resistance to asparaginase treatment, whereas signals that promote the quiescence of LSCs will cause refractariety to therapy [40]

The knowledge of the microenvironment, of its constituents and of their aberrant activities opens new way for novel therapies, that do not target directly leukemic cells but instead alters the microenvironment that sustain them.

These new therapeutic targets could be adhesion molecule, receptors, signaling pathway, etc. For example, in some AML model was seen that the treatment with the monoclonal antibody against CD44, blocs the progression of the disease and induces blasts differentiation. It was hypothesized that the mechanism underlying this effect is the destruction of LSC-niche interaction [41].

1.3 WNT/ β catenin, Hedgehog, PI3K/AKT signaling pathways, and their role in hematopoiesis and leukemogenesis

Wnt/β catenin signaling pathway and its regulation

The WNT signal transduction cascade controls myriad biological phenomena throughout development an adult life of all animals.

When interacting with target cells, Wnt proteins bind heterodimeric receptor complex, consistintg of a Frizzled (Fz) and an LRP5/6 protein. The ten mammalian Fz proteins are seven-transmembrane domain receptor proteins and have large extracellular N-terminal cystein-rich domains that provide a primary platform for Wnt binding [42].

In the absence of Wnt, its transcriptional regulator, β catenin, is degraded, preventing its nuclear translocation: the cytosolic serin-threonin kinase CK1 α/δ and GSK3 α/β, phosphorilate β catenin, thus permitting its bind to the destruction complex constituted by the proteins Axin, APC, WTX that is responsible for the degradation of β catenin itself.

In particular, Axin coordinates he first phosphorylation of β catenin at serin 45 made by CK1α, followed by the phosphorylation made by GSK3 at threonin 41, serin 33 and 37. The specific phosphorylation allows the ubiquitination of β catenin made by βtrcp, a E3 ubiquitin ligase, and its then degraded by proteasome.

Mutations of β catenin at level of threonin 41, serins 45, 37, 33 are frequently present in cancer cells: in these cases β catenin cannot be control anymore.

Instead, after the binding of Wnt to its receptor, LPR6 is phosphorylated by CK1 α/δ and by GSK3 α/β. Axin is sequestered by the phosphorylated receptor, the degradation complex falls apart, β catenin is stabilized and can translocate to the nucleus.

A new model based on studying endogenous destruction complex components [43]. In the absence of Wnt, the destruction complex resides in the cytoplasm, where it binds, phosphorylates, and ubiquitinates β catenin by βtrcp. The proteasome recycles the complex by degrading βcatenin. Wnt induces the association of the intact complex with phosphorylated LRP. After binding to LRP, the destruction complex stills captures and phosphorylates b-catenin, but ubiquitination by βtrcp is blocked. Newly synthesized β catenin accumulates.

Independently to the model, the protein stabilization induced with this mechanism seems to be not sufficient to allow the nuclear translocation; it is believed that Wnt activates also the Rac1 GTPase pathway promoting an additional phosphorilation of β catenin with a subsequent nuclear translocation.

In the nucleus, TCF/LEF (*T-cell factor/lymphocyte- enhancer- binding factor*) is bound to DNA and is maintained in the inactive state by the repressor Groucho. The activation of the pathway happens through the binding of Wnt ligands to the transmembrane receptor complex, constituted by the receptor Frizzeled (Frz) and the coreceptor Lrp5/6. This interaction determins the activation of GSK3β by the protein Dishevelled (Dvl). When Wnt ligands are present, β catenin is no more address to proteasome, but migrate into the nucleus, where it binds to a transcription factor belonging to the TCF/LEF family and activates the expression of its target genes in cooperation with other nuclear proteins [44]. This is called the canonical Wnt signaling pathway.

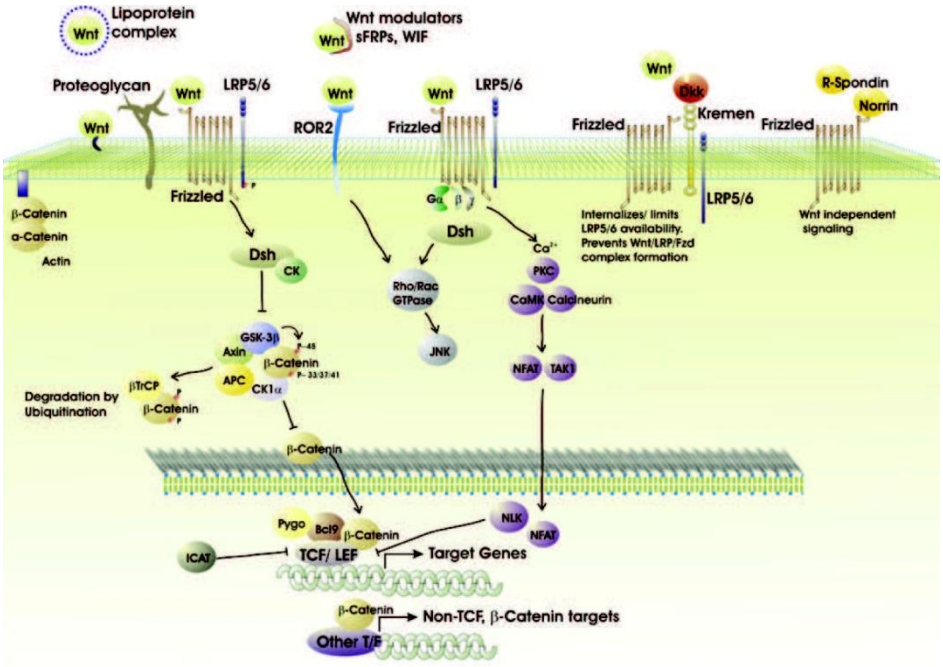

Figure 6. Wnt/β catenin signaling pathway

Wnt is also able to activate genetic transcription even through signaling pathway that do not activate β catenin; this several and less known pathway are defined as non canonical and often they do not need the binding of Wnt to the receptor Frz. Among this pathway, particularly important for hematopoiesis, is the Ca^{2+} dependent pathway activated by Wnt5a. After the binding of Wnt5a to the receptor Frz, phospholipase C, p38 kinase and phosphodiesterase 6 are activated and they induce an intracellular increase in Ca^{2+} concentration. This lead to the activation of protein kinase 2 and of calmodulin dependent kinase II that cause the final activation of NF-AT-calcineurin dependent trascription factors [45].

The regulation of Wnt/ βcatenin pathway is complex and is exert at different levels. The binding of Wnt to its receptor can be inhibited by Frizzeled- related secreted protein and by WNT inhibitory protein, that are both able to bind Wnt preventing its binding to the receptor. Proteins belonging to Dickkopf (DKK) family and WISE/SOST antagonize Wnt signaling, through the binding of LRP5/6 or disrupting the binding between Frz and LRP5/.

To inhibitors are opposed the action of agonists of Wnt pathway: Norrin and R-sponding, that activate Frz receptor even when the Wnt ligands are not present.

CK1 α/δ and GSK3 inhibit the pathway through the phosphorylation of β catenin, Axin and APC and thus stabilizing the destruction complex. On the other hand, serin-

threonin phosphatase PP1dephoshorylates AXIN and/or APC causing the falling apart of the destruction complex, whereas the sern- threonin phosphatase PP2a dephosphorylates β catenin blocking its degradation.

CK1ε isoform is rapidly activated after Wnt binding in order to phosphorylate, together with GSK3, LRP6 and Dvl, thus promoting the falling apart of the destruction complex. Dvl seems also to be responsible of the Wnt-induced CK1ε activation through a positive feedback mechanism.

Additional key role are played by the oncosuppressors Axin and APC that are strictly controlled through a complex system of phosphorylation and dephosphorylation. APC controls Axin promoting its destruction in order to maintain constant levels of phosphorylated β catenin.

β catenin activity is controlled also at nuclear level through antagonists as Chibby and ICAT that can degrade it, preventing its binding to co-activators and promoting the nuclear exportation, or through TCF and LEF-1 transcription factors that do not have the β catenin binding domain.

Several β catenin co-activators have been identified, for example BCL9, Pygopus, Mediata, p300/CBP and TRRAP/TIP60 histone acetyltransferase , MLL1/2 histone methyltransferase, the ATPase family SWI/SNF for chromatin remodelling, and the PAF1 transcription elongation complex.

The signaling is regulated also through modifications of transcription factors β catenin associated: CK1ε phosphorylate TCF-3, whereas CK2 phosphorylates LEF-1, enhancing their binding to β catenin and thus inhibiting the binding to the repressor Groucho.

Even the cytosolic proteins, described before, participate to the nuclear regulation of the pathway: APC inhibits transcription acting on chromatin; GSK3 and β-Trcp have an inhibitory effect, whereas Dvl promote the β catenin binding to the c-Sun transcription factor.

β catenin itself can activate or inhibit the transcription of fundamental factors [46].

Wnt/β catenin pathway in hematopoiesis

The discovery that Wnt genes were expressed in hematopoietic tissues, has promoted several studies in order to understand the role of Wnt in hematopoiesis.

Studies conducted on mice transfected with a constitutive activated β catenin gene, demonstrated that a considerable increase both of HSCs number and of their ability of

stably and correctly reconstitute the hematopoietic process in mice where the bone marrow was destroyed [47].

However, subsequent studies demonstrated that HSCs bearing a constitutive activated β catenin, even if they retain their multipotency ant their ability to self-renew, they were able to differentiate only when β catenin levels were down-regulated. Moreover, HSCs go through gradual depletion, with subsequent loss of the staminal pool of cells [48].

Additional studies, demonstrated an enormous increase of proliferation, the block of differentiation and an increase level of apoptosis in HSC in which APC was deleted [49]. Clearly, these cells presented high levels of β catenin and a considerable activation of nt signaling pathway.

Surprisingly knockout (KO) mice for β and γ catenin presented normal HSCs. However, in these cells the activity of Wnt pathway was still present, demonstrating that despite the activity of β catenin can be substituted by some other homolog protein, Wnt pathway is fundamental in the HSC development [50].

Another recent study that has examined specific deletion of β catenin in hematopoietic stem cells has demonstrated that these cells are deficient in long-term growth and maintenance [51].

Other studies have demonstrated an antagonism between the canonical pathway activated by Wnt3a and the non canonical pathway activated by Wnt5a: Wnt3a inhibits the HSCs differentiation through undifferentiated lymphoid cells, whereas Wnt5a promote it. Moreover Wnt5a seems to be involved in the ability of adult HSC to self-renew [52].

All these experimental evidence have highlighted the crucial role of the canonical Wnt/βcatenin signaling pathway in maintaining the ability of HSC to self-renew to the detriment of differentiation.

It was also hypothesized a role for Wnt in the homing process and in the regulation of the position of the stem cell and of progenitors within the niche. Transfecting HSCs with a constitutive activated β catenin or increasing the activation of canonical Wnt pathway [53], cells presented a dramatic reduction of adhesion molecule.

However, it is relevant to remember that Wnt is a morphogen, the activity of which is dependent on its concentration; so the real determinant in the pathway activation and of its role in the staminal cell fate is not Wnt itself, but instead the microenvironment in which the staminal cell resides. Indeed, the concentration of Wnt, the presence of agonist and antagonist that modulate the cell response to the morphogen, the interaction

between Wnt pathway and other signaling pathways depend all to the microenvironment in which the cell resides. Only the combination of all these aspects determines the real response of the cell to the Wnt signals.

Role of Wnt/β catenin in leukemogenesis

An increasing number of clinical and experimental evidence sustain the fundamental role of Wnt/β catenin as a lekemogenesis promoter. Aberrant activations of the pathway are often present in AML: overexpression, gain of function or loss of function mutations, deletions or epigenetic modification are responsible for this aberrant activation.

For example, both in some AML and in some acute lymphoblastic leukemias (ALL) the transcription of the gene associated with the Frz secreted receptor, antagonist of the Wnt pthway, is inhibited because of the ipermethylation of the gene promoter [54]. Some mutations of Flt3, often found in AML patients, induce an increase of Frz receptor with the subsequent increase activation of the pathway [55].

The blast crisis in acute myeloid leukemia, characterized by an enormous increase of cellular proliferation and by a block of differentiation, is characterized by an activation of Wnt pathway not present in the previous stages of the disease.

In a recent work, using a syngeneic retroviral model of MLL-AF9 induced acute myeloid leukemia (AML), the authors have identified 2 different stages of leukemia progression, propagated by “pre-LSCs” and established leukemia (LSCs) and compared the homing properties of these distinctive entities to that of normal HSCs. The homing and microlocalization of pre-LSCs was most similar to long-term HSCs and was dependent on cell-intrinsic Wnt signaling. In contrast, the homing of established LSCs was most similar to that of committed myeloid progenitors and distinct from HSCs. Although osteoblast-derived Dickkopf-1, a potent Wnt inhibitor known to impair HSC function, dramatically impaired normal HSC localization within the bone marrow, it did not affect pre-LSCs, LSC homing, or AML development [56].

All these evidence sustain the hypothesis that the aberrant activation of canonical Wnt pathway can be responsible for the acquisition of staminal properties by the neoplastic cells, as the limitless proliferation capacity and the impossibility to differentiate.

Hedgehog signaling pathway and its regulation

Hedgehog (HH) signaling pathway plays an essential role in the development of a lot of organs. In adult tissues it regulates the homeostasis and the repair process [57].

The activation of this pathway depends on the binding of Hedgehog (HH) to its transmembrane receptor PTCH (patched). In mammalian cells can be found three different ligands: Sonic Hedgehog (SHH), Indian Hedgehog (IHH) present in the hematopoietic tissue, and Desert Hedgehog (DHH) and two receptors: PTCH2 and PTCH2, the expression of which differs according to the tissue.

When HH is not present, PTCH inhibits smoothened (SMO), a transmembrane protein responsible for the signal transduction from the receptor to the cytoplasmic proteins, till the final effectors that are the Gli (*glioma transcription factor)* transcription factors: Gli1 is an activator, Gli3 is a repressor, Gli2 can both activate or inhibit the pathway.

In the absence of signal, Gli is located in the cytoplasm and is bound to a complex of regulatory proteins composed by the kinase Fused (Fu), the suppressor of Fused (SuFu), the protein kinase A (PKA), GSK3 and CK1. This protein complex is phosphorylated and then deprived of its N-terminal transcription activation domain through proteolytic cleavage. The so cleaved factor enters the nucleus where it acts as a transcriptional repressor. After the binding of HH to PTCH, SMO is no more inhibited, so it can be internalize and, together with Fu, phosphorylates Gli, stabilizing it and allowing its enters into the nucleus (Fig 7).

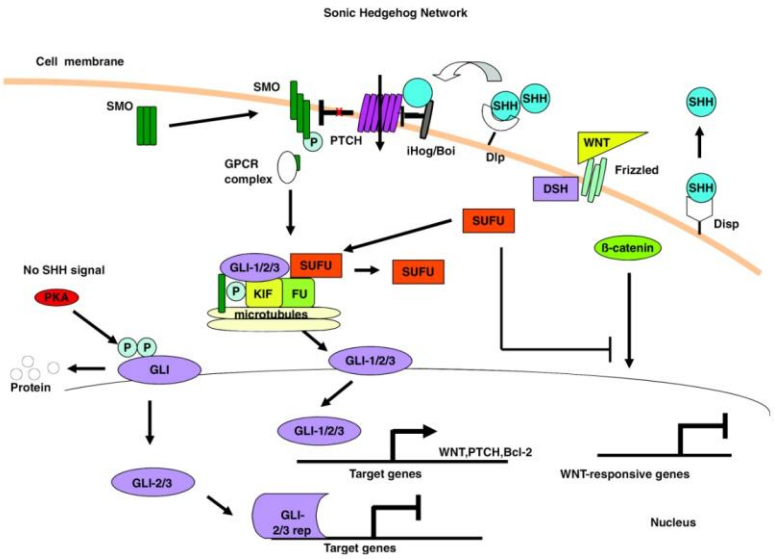

Figure 7. Sonic/Hedgehog signalimg pathway

However, HH is a morphogen and that means that its action is determined in a concentration-dependent manner.

Intermediate levels of HH leads to the binding with Gli and SuFu, thus blocking its action; whereas high levels of HH causes the stabilization of Gli and its entrance into the nucleus[58] .

In table 3 are listed the genes, the expression of which is regulated by HH. It is important to notice that, among them, there are genes involved in cell proliferation, angiogenesis, apoptosis, cellular adhesion, in tissue development and genes that participate in the signal transduction, suggesting a fine regulation of the pathway even at the genetic level [58].

Gene Name	Gene Abbreviation	Function	Regulation
Osteopontin[87]	SPP1	Cell adhesion, apoptosis, ossification	Activated
Snail[75,88]	SNAI1	Transcription, Cartilage development	Activated
TIMP metallopeptidase inhibitor 3[88]	TIMP3	Apoptosis	Activated
Parathyroid hormone receptor 1[88]	PTHR1	G protein coupled receptor	Activated
Cyclin D1[88]	CCND1	Cell proliferation	Activated
Patched[89]	PTCH	Cell proliferation	Activated
Cyclin D2[87]	CCND2	Cell proliferation	Activated
Hexokinase 1[90]	HK1	Glycolysis	Activated
Protein kinase c, epsilon[88]	PRKCE	Signal transduction, apoptosis	Activated
Clusterin[88]	CLU	Apoptosis, immune response	Activated
Forkhead box F1[91]	FOXF1	Transcription	Activated
Forkhead box M1[92]	FOXM1	Transcription, stress response	Activated
Forkhead box E1[93]	FOXE1	Transcription	Activated
Vascular endothelial growth factor[88]	VEGF	Angiogenesis, cell migration	Activated
Insulinlike growth factor 2[94]	IGF2	Cell proliferation, skeletal development	Activated
Nuclear receptor subfamily 2, group F, member 2[95]	NR2F2	Transcription	Activated
B-cell CLL/lymphoma 2[25,26]	BCL2	Apoptosis, cell proliferation	Activated
Fibroblast growth factor10[88,91]	FGF10	Organogenesis, cell-cell signaling	Repressed/ activated (cell type specific)
TSC22 domain family, member 1[87]	TSC22D1	Transcription, apoptosis	Repressed
Target of myb1[87]	TOM1	Endocytosis	Repressed
Plakoglobin[87]	JUP	Cell adhesion	Repressed

Tabel 3. Target genes of the Hedgehog signaling pathway

This pathway is strictly control at different levels. Fu is a positive regulator of the pathway, stabilizing Gli and inhibiting its ubiquitination, whereas SuFu works both at cytoplasm level, inhibiting the nuclear translocation of Gli, and at nuclear level, promoting the genetic silencing of HH target genes with the recruitment of histone-deacetylase . On the other hand, PKA, GSK3 and CK1 phosphorylate Gli, tagging it for degradation, whereas the transmembrane protein *Hedgehog interacting protein* bins to HH but it is not able to activate the pathway [58].

Hedgehog pathway and hematopoiesis

The role of HH in hematopoiesis is still somehow uncertain and controversial [59].
In embryonic hematopoiesis, IHH, expressed by the endodermic cells, promotes the differentiation of the common progenitor of the endothelial and hematopoietic cells (hemangioblast) to the development of committed progenitors directed exclusively to the endothelial or hematopoietic lineage. The role of HH in the adult hematopoiesis is more controversial; several studies conducted on different key proteins of HH pathway in the same animal model have produced different results, only partially explainable by the different experimental conditions. Heterozygous mice for PTCH ($PTCH^{+/-}$) showed an increased activity of HH pathway and an expansion of the staminal compartment, with a subsequent more rapid recovery after the treatment with 5-FU (5-fluorouracil) and with an increased ability of HSC to be[. However, while for some authors HSCs went to a progressive depletion [60], according to other authors their long-term proliferation capacity were retained [61].
Additional studies conducted also in mouse model demonstrated that if PTCH is deleted only in HSCs, there were no particular alteration in the staminal compartment; if the KO of PTCH was made in more tissues, HSCs displayed an increased motility and proliferation, without impairing their long-term proliferation capacity [62].
All together, this results indicate that HH have an important role in promoting the self-renewal of the stem cells, even though it does not have a direct activity on HSC but rather on the bone marrow microenvironment.
The activity of SMO was also studied with apparently opposing results. SMO KO mouse cells, using the Mxe/Cre system, do not present significant differences as compare to the normal counterpart; whereas KO mice produced with the Vav-Cre system displayed HSCs with reduced growth capacity [63].
Considering that Mxe-Cre system create a KO model only in the adult hematopoietic cells, while Vav-Cre system induced the KO during the embryonic development, thus involving different cell lines, these results prove again the importance of HH pathway more in the microenvironment than on single HSCs.
Furthermore induced experimental down-regulation of Gli has led to a reduction of HSC and myeloid progenitors proliferation and differentiation, thus confirming the role of HH in the cellular expansion.

Finally, HH is important also for mature and terminal differentiate cells: KO mice for PTCH display increased apoptosis of pre-B cells, block of maturation of T cell at bone marrow level and apoptosis of $CD4^+CD8^+$ T cells because of the lack of the support of the microenvironment (cit 52,54 tesi fede). The deletion of SMO causes thymic atrophy and proliferation, maturation and survival defects of the T cells [64].

Role of Hedgehog in leukemogenesis

So far, little is known about the role of HH pathway in the development of AML. However, some clinical evidences sustain its importance. It was seen that HH and GLI1 are expressed in stem cells and leukemic blasts [65], whereas recently it was demonstrated that the self-renewal of B cell LLA progenitors is reduced both *in vitro* and *in vivo* after the treatment with SMO inhibitors [66].
Anyway, even when the HH pathway is altered, the inhibition of this pathway alone is not sufficient to block LSCs, being only one of all the alteration present in this cells.
However the rational of using this pathway a therapeutic target can be found in the attempt to weaken LSCS, thus reducing their ability to give pathology [67].

Regulation of PI3K/AKT signaling pathway

Phosphoinositide 3- kinase/AKT/mTOR (*mammalian target of rapamycin)* is fundamental for the control of several cellular processes, like cell cycle progression, transcription, translation, differentiation, apoptosis, cellular motility and metabolism [68].
Among the multiple components of the phosphoinositide 3- kinase (PI3K) family, the isoform 1 seems to be the most frequently involved in the signaling activated by growth factors or pro-survival stimuli. Indeed, after the interaction between these growth factors with their specific tyrosin-kinase transmembrane receptor, that leads to the autophosphotyation of the receptor and the subsequent binding of PI3K to the receptor. This binding allows the localisazion of the kinase near the plasma membrane with the subsequent phosphorylation of phosphatidylinositol 4-5) bisphosphate (PIP_2) to phosphatidylinositol (3-4-5) triphosphate (PIP_3), an important lipid mediator that is able to interact with and recruit proteins having the *pleckstrim-homology-domain (PH).* Among these proteins, the serine- threonine kinase AKT that is activated after the

phosphorylation in serine 308. The isoforms 1 and 2 are involved in blocking apoptosis and cell growth. AKT inhibits apoptosis through the direct phosphorylation of key components of the apoptosis cascade, as BAD, a member of Bcl2 family proteins, that promote apoptosis binding and so antagonizing the action of the antiapoptotic components belonging to its family (Bcl-2 and Bcl-xL). AKT phosphorylates BAD in serine 136, causing its retention in the cytoplasm, thus preventing its interaction with Bcl-2 or Bcl-xL.

Another target of AKT are the SAPKs (*stress-activated protein kinases*), a group of proteins that are involved in the activation of apoptosis after the exposure to ionizing radiation, heat shock or osmotic stress. In this case, AKT phosphorylates and activates ASK-1, a protein involved in the proapoptotic signal induced by JNK (a member of SAPKs), thus preventing apoptosis. Moreover AKT phosphorylates and inhibits caspase 9.

AKT promotes cell survival through the phosphorylation of transcription factors that control pro- and anti-apoptotic genes expression. Among the target molecules involved in the block of apoptosis, there is the FoxO transcription factor family genes. The phosphorylation of FoxO members, mediated by AKT, alters the intracellular localization of this proteins.

When AKT is inactive, FoxO proteins are usually localized in the nucleus, where they promote the transcription of target genes with pro- apoptotic activity. Moreover FoxO3, one of the principal member of the FoxO family, when activated regulates its own transcription and that of the other members of the FoxO family protein (FoxO1 and FoxO4). The activation of PI3K/AKT axis leads to the exportation of this transcription factors from the nucleus to the cytoplasm, where they are degraded by the proteasome. In this way AKT promotes cell growth and survival [69].

AKT is also able to activate the nuclear factor kB (NF-kB), another transcription factor involved in the regulation of proliferation, apoptosis and cell survival. NF-kB activity is modulated through its association with a co-factor, IkB, that inhibits it by sequestering NF-kB in the cytoplasm. AKT phosphorylates and activate IKKs kinases, that in turn phosphorylate IkB, leading to its degradation through the proteasome. Consequently, NF-kB is free to go into the nucleus. IKKs itselfs are also able to inhibit FoxO members. Moreover, AKT increases the transcriptional activity of p65Rel, a subunit of NF-kB (fig 7?).

AKT modulate cell growth phosphotylating mdm2 in serines 166 and 186 of the nuclear translocation domain, leading to the traslocation of the protein from the cytoplasm to the nucleus. At this level mdm2 can bind p53, thus inhibiting its transcriptional activity and tagging it for proteasome degradation. Through this mechanism, AKT participates to the modulation of cell cycle, DNA repair, angiogenesis, senescence and apoptosis [70]

Another down-stream target of AKT is mTOR (*mammalian Target of Rapamycin*), a serine- threonine protein kinase highly conserved that possess an essential role in the modulation of cell growth and proliferation [71]. Indeed, mTOR is able to regulate the synthesis of key proteins, for example pRB, p27KIP, cyclin D1, c-myc and STAT3 and recent studies have also demonstrated its involvement in the cell death process. AKT is responsible of mTOR activation through a multi-step complex mechanism.

Down-stream of mTOR we can find 4E-BP1(*4E-Binding Protein* 1) and p70S6K p70S6K (*p70 Ribosomal S6 Kinase*). The phoshorylation of 4E-BP,1 mTOR mediated, inhibits its capacity to bind the transcription initiation factor 4E (eIF-4E), a protein that controls protein synthesis and induces the transcription of genes involved in cell cycle control. On the other hand, mTOR mediates also the phosphorylation and the subsequent activation of p70S6K, that in turn phosphorylates the ribosomal protein S6, making it able to participate to protein synthesis (Figure 8).

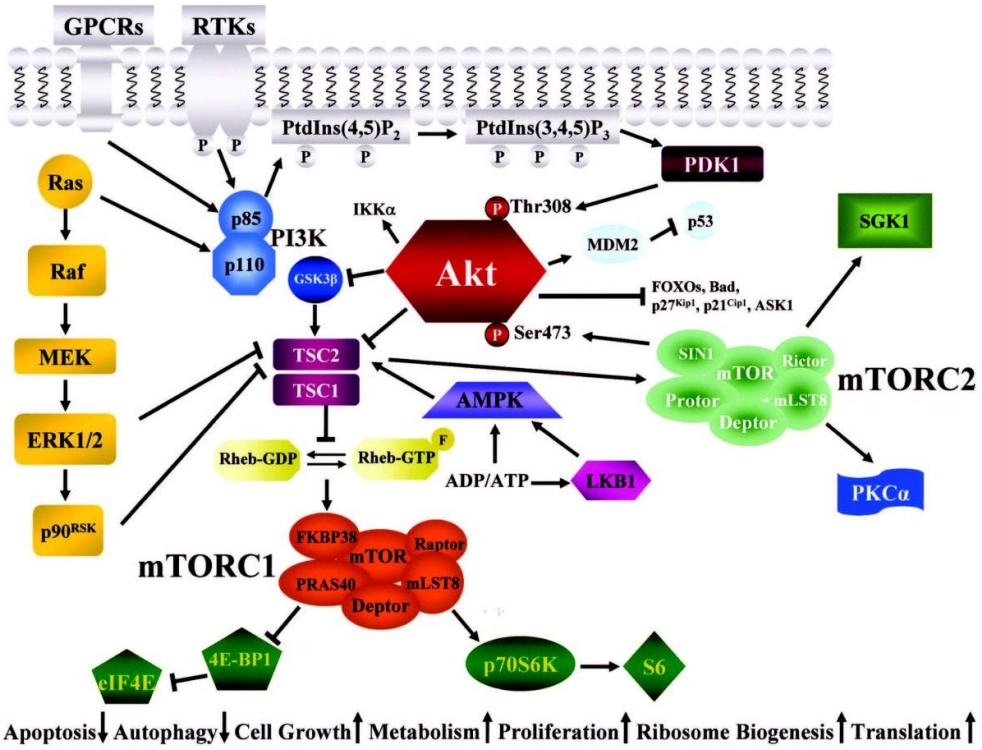

Figure 8. di PI3K/AKT/mTOR signaling pathway

AKT activity is modulated by a complex network of regulatory proteins that interacts directly with its PH, kinasic or C-terminal domain.

The onco-suppressor PTEN (*Phosphatase and tensin homology*), is an important negative regulator of the pathway. Part of its regulatory role on AKT pathway is made through its lipidic phosphatase activity: it dephosphorylates PIP_3 to PIP_2, thus blocking AKT activation.

PI3/AKT pathway promotes angiogenesis through the stabilization of HIF-1 factor, that in turn induces the expression of VEGF (*vascular endothelial growth factor*). PTEN, because of its inhibitory effect on AKT, has a role in the inhibition of angiogenesis [72].

PI3K/AKT pathway in hematopoiesis

The correct regulation of the activity of PI3K is fundamental for the survival of HSC.

In PTEN knockout mice HSC are unable to maintain themselves because PTEN deletion promotes HSC proliferation. However this led to HSC depletion via a cell-autonomous mechanism, preventing these cells from stably reconstituting irradiated mice [73].

Moreover, PI3K is involved in B lymphocytes development and AKT regulates the development of hematopoietic progenitors.
FoxO factors, that are inhibited by AKT, are involved in the maintenance of HSCs [74].

Role of PI3K/AKT signaling pathway in leukemogenesis

In the last years several evidence of aberrant activation of this pathway in patients with AML, while experiments conducted on murine HSCs have revealed its leukemic potential [73].
LSC without PTEN showed a limitless proliferation capacity, as opposed of their healthy counterpart, and this ability was retain during time [73].
At clinical level, 50 to 80% of AML patients present an activating phosphorylation on AKT. This patients are characterized by a reduced overall survival as compared to patients that do not have this activation [74].
The explanation could be found in that cells with this pathway activated present a major expression of an ABC(*ATP-binding cassette*) transmembrane transporter, that is able to promote the extrusion of drugs used in cancer therapy [75]. However, a recent study sustains that the activation of this pathway can be consider a favorable prognostic marker, because it promote the entrance in S phase of LSCs, thus rendering these cells more susceptible to therapy [76].
Besides the activation of AKT, there are other activation mechanisms found for this pathway in AML patients.
About 75% of patients bear an inactivating phosphorylation of PTEN, that is associated with high levels of phosphorylated AKT, that results in a reduced patients survival.
Recent studies have also demonstrated that the activation of PI3K/AKT axis could be due to mutations of Flt-3, a tyrosine-kinase receptor, principally expressed in hematopoietic cells, that is important during all the differentiation process of these cells. Flt-3 activates AKT through the signaling pathway dependent by PI3K and Ras [77]. Furthermore, about 15-20% of AML patients bear point mutations that affected N-RAS or K-RAS that are able to abolish their intrinsic GTPase activity, that causes the stimulation of the PI3K/AKT axis. In another 20-30% of cases were found mutations of the tyrosine-kinase receptor c-kit, the receptor of the stem cell factor, that lead to un up-regulation of PI3K/AKT pathway [78].

Moreover, it was recently proposed another mechanism of activation of this pathway in leukemic cells that is based on the action of VEGF, an potent proangiogenic molecule that activate PI3K/AKT and that is secreted also by leukemic blasts.
It was also seen that the interaction between LSC and the extracellular matrix, through the binding of β1 integrin on neoplastic cells and stromal fibronectin, is able to activate the pathway, that in turn increases the binding between CXCRL12 and CXCR4 [78]. All this experimental results demonstrate the importance of this pathway in the regulation of leukemogenesis.

1.4 PROTEIN KINASE CK2

Protein kinase CK2 (formerly known as casein kinase II) is a highly conserved and expressed serine/threonine kinase. It is now abundantly clear that it is a promiscuous enzyme as a diverse and somewhat bewildering array of more than 300 potential substrates have been identified. CK2 participates in a wide variety of cellular processes including cell proliferation, survival and differentiation [79]. There is an increasing body of evidence indicating that CK2 is involved in protein kinase networks controlling cell cycle progression and cellular responses to stress including ultraviolet light, heat shock, TNFα. Furthermore, abnormally high levels of CK2 have been observed in various types of cancer both in solid tumours (breast, prostate, lung, kidney, neck and head) and haematological malignancies (AML, multiple myeloma, burkitt lymphoma, acute myeloid leukemia in blast crisis). Based on this involvement in transformation and tumorigenesis, CK2 has recently attracted attention as a potential therapeutic target[80].

CK2 structure.

CK2 has typically been viewed as a tetrameric complex consisting of two catalytic subunits (38-42kDa in mammals) and two regulatory subunits (27kDa in mammals) (Fig.10). However the catalytic subunit can perform its activity also as monomer in the absence of the regulatory counterpart. CK2 was distinguished among other protein kinase for its ability to phosphorylate serine or threonine residues that are proximal to acidic amino acid. Pinna and colleagues defined a minimal consensus sequence for phosphorylation by CK2, however there are sites that are efficiently phosphorylated by CK2 despite of the absence of this consensus sequence. CK2 has also the ability to

phosphorylate tyrosine residues, although the kinetic parameters for this phosphorylation are much less favourable than those in ser/thr residues[81].

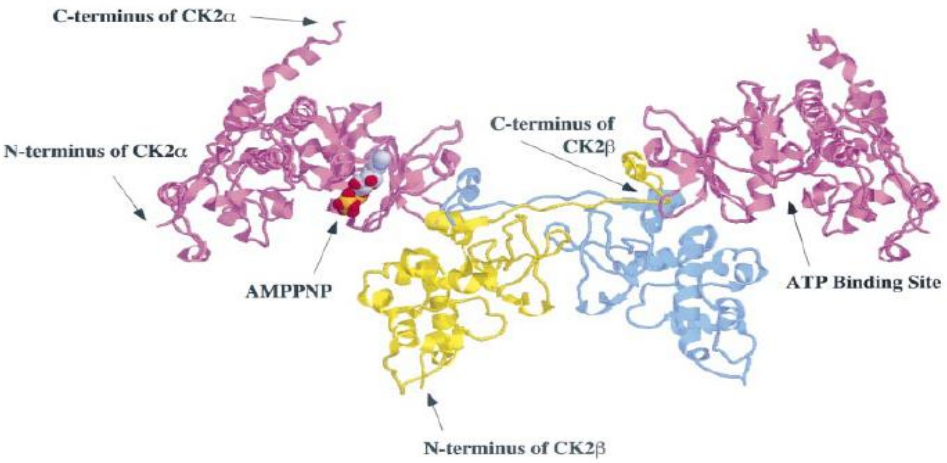

Figure 9. Ribbon diagram illustrating the high-resolution structure of tetrameric CK2.

- **CK2α.** In humans, two different forms of its catalytic subunits (designated CK2α and CK2α') which are encoded by distinct genes, were initially characterized. With exception of their unrelated C-terminal domains, these two isoforms are very similar to one another exhibiting approximately 90% identity within their catalytic domain. Recently a third isoform, CK2 α", was identified, that is almost completely identical to CK2α; the only distinguishing feature lies in the completely distinct C-terminal domain. It is known that the different CK2 isoforms are closely related and show considerable functional overlap; indeed, a knockout of the gene encoding CK2α' in mice results in variable offspring when heterozigous mice are bred to homozygosity, suggesting that CK2α has the capacity to compensate for CK2α' in the context of viability. However the male are sterile and display defect in speramatogenesis, demonstrating that the functional compensation is not absolute. There is also evidence for functional specialization of the individual CK2 isoforms in yeast, mice and mammals and there may also be differences in the subcellular localization of CK2α and CK2α' [81]

- **CK2β.** In contrast of the catalytic isoforms of CK2, only one known form of the regulatory subunit β has been identified in mammals, but multiple forms have been identified in other organisms, such as *Saccharomyces cerevisiae*. CK2β is remarkably conserved among species and x-ray crystallography studies have determined that a dimer of the CK2 β subunits forms the core of the CK2 tetramer[81].

  A large proportion of CK2β has been shown to be phosphorylated at an autophosphorylation site consisting of serine 2, 3 and 4 at its N-terminus.

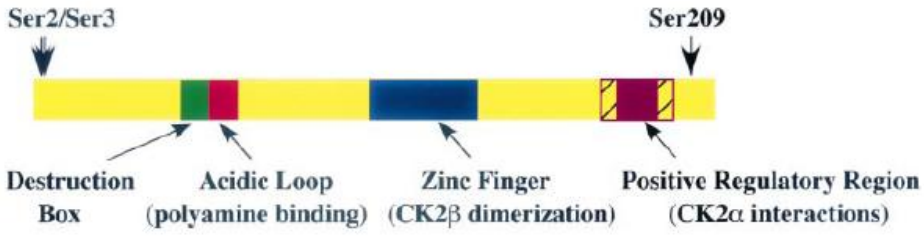

Figure 10. The regulatory CK2β subunit. Linear representation of CK2β, illustrating the main elements within its amino acid sequence.

It was hypothesized that this autophosphorylation could be mediated by an intermolucular reaction through the formation of higher order CK2 structures and it could enhance CK2β stability. CK2β is also phosphorylated at S^{209} near its C-terminus, a residue which is phosphorylated in a cell-cycle dependent manner by $p34^{cdc2}$ (Fig.10).

-It is particularly intriguing that CK2β has motifs that have been previously characterized as motifs that regulate cyclin degradation. Indeed, this sequence is similar to the amino acid motif called destruction box that plays a key role in the specific degradation of cyclin B at the end of mitosis[82].

X-ray crystallography revealed the importance of the zinc-finger region: this sequence is characterized by four cysteine residues wich mediate the interaction allowing the CK2β dimer to form the core of the CK2 holoenzyme. CK2β

dimerization precedes catalytic subunit binding and it is a prerequisite for CK2 tetramer formation.

-C-terminal region is responsible for the ability of CK2β to enhance and stabilize CK2 activity.

-One additional important sequence is the acidic loop: it has been identified as the site on CK2 that binds polyamines which are known to stimulate CK2 activity *in vitro* . [81]

Challenge to the traditional view of CK2.

While consideration of CK2 as a tetrameric complex remains relevant, significance evidence has emerged to challenge the view that its individual subunits exist exclusively within these complexes. Indeed a lot of data indicate that the regulatory CK2β subunit exists and performs functions independently of CK2 tetramers. In particular:

-X-ray crystallography revealed that the CK2α and CK2β interface was relatively small and flexible; this result raises the possibility that CK2 tetramers are subject to disassembly and reassembly[82].

-Expression of CK2. Relatively little is known about how either CK2α or CK2β expression is regulated. Earlier studies had shown that CK2β protein was sythesized in excess of the catalytic subunit, underling a lack of coordinated expression. Several reports have also revealed an unbalanced expression of the two subunits in different tissues. For example the level of CK2β in testis was significantly higher in comparison to the level of CK2α.
The intriguing demonstration that aberrantly high levels of CK2β have also been observed in tumors, highlights the importance of understanding the dynamic role of CK2β both within the context of the CK2 holoenzyme and as an independent protein [82].

-Localization of CK2 subunits. Immunofluorescence studies confirmed that the catalytic and regulatory subunits of CK2 are not exclusively co-localized. While the majority of both subunits were localized to nuclear fraction, a major proportion of CK2α was tightly bound to nuclear components whereas CK2β was only loosely associated with other nuclear components. In addition, it was demonstrated in mammalian cells that all the

three subunits of CK2 were localized to the smooth endoplasmic reticulum and the Golgi complex, instead only CK2α and CK2α' could be detected in the rough endoplasmic reticulum. In addition to confirming the predominantly nuclear and moderately cytoplasmic localization of both CK2α and CK2β, these studies showed that both nuclear import and export of CK2 subunits are regulated independently and can result in rapid changes of their steady-state distribution. However, when associated in stable holoenzyme complex, the two subunits are dynamically retarget in the cytoplasm. Moreover they demonstrated that the binding of fibroblast growth factor 2 (FGF-2) to the holoenzyme provokes its nuclear accumulation, supporting the concept of a signal-mediated localization, which may result in a sophisticated regulation of the kinase.

CK2 beta functions.

Over the last decade a plethora of CK2β-specific interaction partners have been identified through studies performed *in vitro* and *in vivo.* Some of these proteins have undergone more extensive validation allowing for their classification as either CK2 dependent or CK2 independent partners of CK2β.

-CK2-dependent binding partners are proteins that interact with the tetrameric CK2 through binding sites of CK2β. Within CK2 complex a major role of CK2β appears to be substrate docking or recruitment where it brings the substrate protein and the catalytic subunit into close enough proximity to facilitate the phosphorylation reaction. A second function of CK2β appears to involve transmission of regulatory signals provided by other proteins in manner that could be analogous to that seen with polyamines. FGF-2 exemplifies this, as binding of FGF-2 to CK2β stimulates CK2 activity.

Thus, these two functions of CK2β modulates the ability of CK2 to phosphorylate specific cellular targets**.**

- CK2β independent binding partners are proteins that interact with CK2β in the absence of catalytic CK2 subunits. These proteins include A.-Raf, c-Mos, and Chk1, that are ser/thr protein kinases containing sequences reminiscent of the CK2β binding region present in the CK2 catalytic subunit. In the case of A-Raf-CK2β interaction it was demonstrated that the presence of CK2α abolishes the activation observed with CK2β, suggesting that CK2α was competing with A-Raf for binding to CK2β. Interestingly,

while in the case of c-Mos, the interaction with CK2β leads to down-regulation of the latter, inducing mitotic arrest in rapidly dividing embryonic cells; in the case of A-Raf and Chk1, the kinase activity is enhanced upon interaction with CK2β [83].

Regulation of CK2 in cells.

The traditional view of CK2 looks at this protein as a constitutive active [84]. and unregulated kinase, nevertheless, several studies support the idea that there are distinct mechanisms contributing to the physiological regulation of CK2:

- the first one is represented by the CK2β subunit that influences CK2 recruitment of the substrate and CK2 localization; moreover, it was demonstrated that the presence of the destruction box in CK2β, and consequently its degradation through proteasome, determines the oscillation of CK2 activity during cell cycle[82].
- Phosphorylation of CK2: several works indicate that phosphorylation is not absolutely required to activate CK2 in a manner analougous to that seen with MAP kinases. However they do not exclude the possibility that phosphorylation partecipates to some degree in aspects of CK2 regulation. Examination of CK2, isolated from mammal cells, has led to the identification of a number of physiological phosphorylation sites on both CK2α and CK2β. Indeed CK2β is phosphorylated at its autophosphorylation site and at Ser^{209}, a residue that is phosphorylated in a cell-cycle dependent manner. Autophosphorylation of CK2β could indirectly regulate CK2 activity. CK2α is phosphorylated in a cell-cycle dependent manner at four sites within its unique c-terminal domain even if these sites do not appear to effect a dramatic change in the catalytic activity of the kinase[81]. CK2 can also be phosphorylated by the Scr-family protein tyrosine kinases, by c-Abl tyrosine kinase and by the pathological counterpart Bcr-Abl fusion protein (typical of chronic myeloid leukemia). In this last context CK2 activity is inhibited by phosphorylation [85].
- Protein-protein interaction: it has been shown that CK2 interacts with proteins such as FGF-1, FGF-2, HSP90 (heat shock protein 90) and the cochaperonine cdc37 that may directly alter or stabilize its catalytic activity. CK2 also interacts with tubulin, FAF-1 and cKIP-1, that could be involved in the targeting of CK2 to specific sites or structures within cells. There are three tumor suppressors that

bind and inhibit CK2 activity: p53 interacts with the β subunit affecting its function; in a similar way also p21WAF1 binds toCK2 β[86]; adenomatous polyposis coli protein (APC) inhibits CK2 through the interaction with α subunit[87]..

- Role of small molecules in CK2 regulation: CK2 is inhibited by negatively charged compounds such as heparin and activated by positively charged compounds, including polyamine [81].

CK2 functions.

CK2 always behaves as an antiapoptotic agent implying on different cellular functions, signalling pathways and biochemical reactions which ultimately cooperate to promote cell survival (Fig.12).

- CK2 is a multisite regulator of different signalling pathways that are potentiated by phosphorylation:

  -NFkB: this transcription factor is normally sequestered in the cytosol by the binding to its inhibitor IkB. CK2 acts at different levels: it phosphorylates IkB promoting its degradation through proteasome, increases the expression of IKK kinases, phosphorylates p65 subunit of NFkB increasing its transcriptional capability.

  -Wnt pathway: in the presence of wnt, the destruction complex which targets β-catenin to the proteasome is inhibited by the stabilizing protein dishevelled (Dvl). CK2 phosphorylates Dvl and β-catenin promoting their stabilization, and TCF/LEF, facilitating its association to partner molecules;

  -PI3K/Akt: here again CK2 operates as multisite regulator: a first level is represented by the tumor suppressor PTEN, the phosphatase which dephosphorylates PIP3 (phosphatidylinositol 3, 4, 5 triphosphate), thus maintaining the PI3/Akt signal down, under resting conditions; it has been demonstrated that the constitutive phosphorylation of PTEN by CK2, while regulating the PTEN protein stability, has an inhibitory effect on its phosphatase activity as well, with the final effect of stimulating Akt-dependent signalling. A second level of CK2 intervention on this pathway is represented by Akt itself: beside a physical interaction between the two kinases, a direct phosphorylation

of Akt on Ser 129 by CK2 has been found, which promotes an hyper-activated state of Akt[88]. There is moreover an indirect effect of this CK2-mediated phosphorylation, since it contributes to maintain an high level of phospho Thr-308, by ensuring a stable association with the chaperone protein Hsp90, known to protect Thr308 from dephosphorylation.

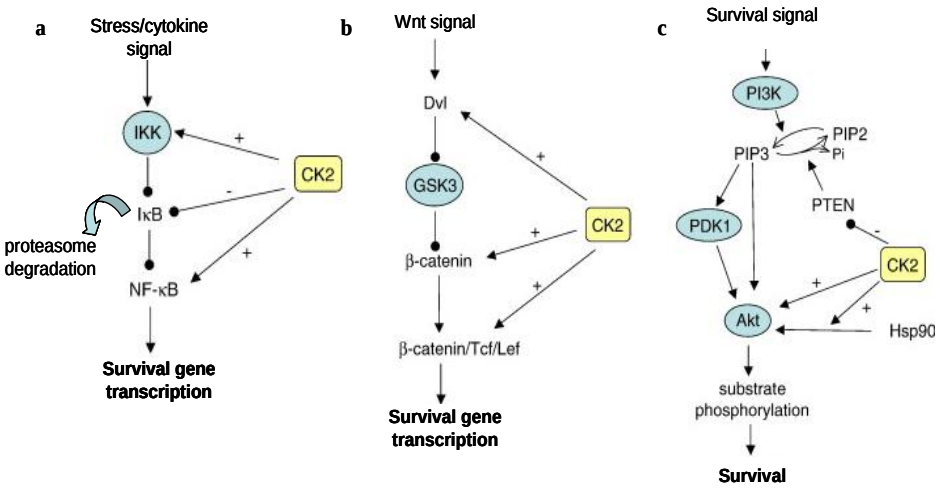

Figure 11. CK2 –dependent multisite regulation of NF-kB (a), β-catenin (b), and Akt (c) signalling.

- Jak-Stat pathway: Zheng and co-workers provided the first evidence that ser/thr kinase CK2 binds and phosphorylates Jak2 and these events are critical for the activation of Jak2-Stat signalling pathway [89]**.**
- CK2 and apoptotic signalling: the caspase inhibitor ARC is phosphorylated and activated by CK2 while survivin, a member of the inhibitor of apoptosis protein (IAP) family, is upregulated whenever CK2 expression is increased. Other CK2 targets are Bid, Max, HS1, presenilin, connexin, whose previous phosphorylation generates caspase resistant sites. Caspase 9 itself falls in this category, since its phosphorylation by CK2 protects caspase 9 from caspase 8 cleavage [90].
- PML: CK2 regulates PML protein levels by promoting its ubiquitin-mediated degradation dependent on direct phosphorylation at Ser517 [91].

- CK2 participates in the regulation of proteins that have important functions associated with cell cycle progression: topoisomerase II, p34, cdc34, $p27^{kip}$**,** MDM2, p21WAF/CIP and p53 [79].
- CK2 cooperates also with proto-oncogenes such as c-Myc, c-Myb, c-Jun, Ha-Ras and A-Raf.
- The RNA polymerase I and RNA polymerase II complexes were among the first substrates to be discovered. Then RNA polymerase III was also shown to be target of CK2. Phosphorylation by CK2 of the TATA-binding protein (TBP), a subunit of TFIIIB (the core component of the Pol III transcriptional machinery), promotes a remarkable increase in Pol III activity, favouring the synthesis of tRNA and 5SrRNA. Thus CK2 enhances rRNA and tRNA biogenesis [79]**.**
- Y. Miyata and colleagues demonstrated that the cochaperone cdc37 is a CK2 target. Cdc37 is involved in the folding process of several protein kinases in tight collaboration with Hsp90; however, cdc37 shows molecular chaperone activity *per se*. Phosphorylation of cdc37 by CK2 is essential for the proper function of the chaperone, moreover, CK2 itself operates in a cdc37-dependent manner being directly associated with the latter one. Thus CK2 may control many growth-related protein kinases simultaneously via the cdc37 phosphorylation (Fig.12) [90]**.**

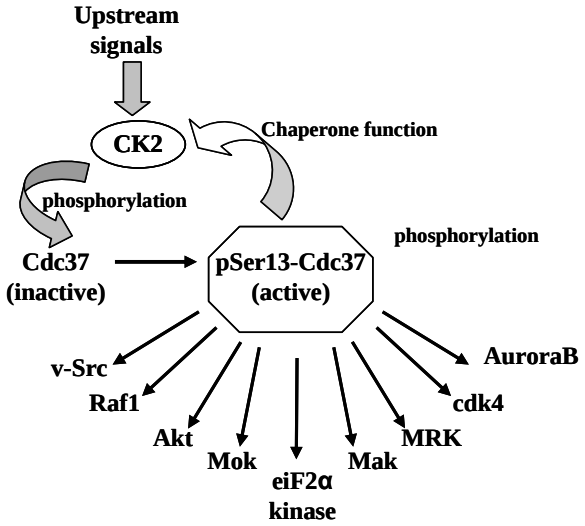

Figure 12. Cdc37 as substrate of CK2. CK2 and Cdc37 constitute a positive feedback to control a number of important kinases.

The implication of CK2 in signalling cascades is atypical with respect to the other protein kinases because it is not hierarchical. Owing to its lack of molecular mechanisms capable to readily turn on or off its activity, CK2 is exempted from the control of other kinases and it is excluded from the canonical signalling pathway "dropping vertically" into the cell from the membrane to the nucleus. It rather plays a "lateral role" impinging on many of these longitudinal pathways. CK2 can be seen as a "master regulator" of cellular functions, committed to the integration and crosswise consolidation of different pathways.

CK2 and cancer.

A number of genetic alteration can occur which bypass the physiological mode of activation of a kinase, given the rise to a constitutively active enzyme no more subjected to its physiological mechanism of control. The molecular alterations that interfere with protein kinase activities and are causative of cancer may be several, including gain/ loss of function, gene deletion, translocation with generation of fusion proteins. However CK2 does not conform to this general paradigm, merely because only active forms of it apparently exist. In addition, mutations of CK2 have never been reported, while its physiological concentration is one of the highest found, suggesting that CK2 is one of the more represented kinase. Remarkably, CK2 levels has been found to be invariably higher in malignant cells than it is in normal cell of the same type. There is also a correlation between the grading of the malignancy and the level of CK2: the higher is this latter, the worse the prognosis is [93]. It is felt now-a-days that such elevated CK2 is neither the cause nor the consequence of neoplastic transformation while it may well reflect the tendency of the tumour, regardless the genetic alterations causing it, to preferentially colonize those cells where CK2 is higher. Ruzzene and Pinna introduced the concept of "non oncogene addiction" in order to understand the role of CK2 in ensuring survival of a variety of cancer cells, where its elevated activity seems to be relied to epigenetic events. They propose a model: assuming that stochastically such high CK2 level occurs only in a little part of the cellular population, its contribution to the overall tissue phenotype will be almost null. Any oncogenic mutation occurring in these cells will be counteracted by a number of opposing mechanism in the majority of cells, where CK2 is normally represented; in contrast it will find a more favourable environment in the few cells were CK2 is abnormally elevated and which are predisposed to evade apoptosis, to stabilize the onco-kinome, to develop drug resistance

etc..(Fig.14) These cells are destined to proportionally increase in number, conferring to the tissue a malignant phenotype which will be associated and maintained by elevated CK2. At this stage it can be said that the tumour has become "addicted" to CK2**.** There is the possibility that tumour cells more strictly rely on CK2 functionality for their survival pathway than normal cells do. This concept is supported by the observation that, when primary tumour cells are treated with CK2 inhibitors, their susceptibility to cell death tends to be higher than in the case of normal precursor cells. This has been found e.g. for multiple myeloma (MM)**,** for acute myeloid leukemia[94]**,** and for T-Acute lymphoblastic leukemia (T-ALL)[95].

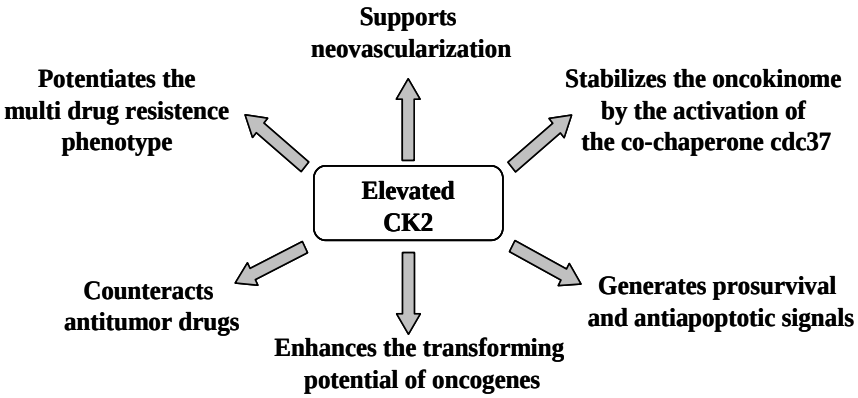

Figure 13. CK2 as a cancer driver. Effects promoted by abnormally high CK2 levels.

CK2 and hematopoiesis-related signaling molecules and pathways .

The function of CK2 in normal hematopoiesis is up to now not fully known. However, some studies demonstrated that this kinase regulates several hematopoietic transcription factors and molecules as well as influences signaling pathways involved in hematopoietic stem cell biology.

Among the transcription factors targets of CK2 we annoverate:

-c-Myb: it is phosphorylated at the amino terminal serine 11 and 12 by CK2. These sites are constitutively phosphorylated and this post-translational modification is essential for high affinity specific DNA binding activity of c-Myb**.**

-PU.1 is phosphorylated by CK2 at Ser 148. Phosphorylation at this site is required to allow interaction between PU.1 and IRF-4 and to allow the trans-activation function of PU.1 in LPS-stimulated murine macrophages**.**

-Ikaros: it is a transcription factor essential for hematopoiesis and an established tumor suppressor. Studies by Georgopoulos and colleagues have identified several amino acid that are phosphorylated by CK2. Phosphorylation of Ikaros at its terminalc-region regulates its ability to control cell cycle progression from G1 to S phase [96]. Following studies identified four novel CK2 phosphorylation sites located at the N-terminal end of Ikaros: for instance phosphorylation at 13 and 294 aminoacids causes a decrease in Ikaros DNA binding affinity and influences its subcellular localization. It was demonstrated that its phosphorylation changes during T-cell differentiation: Ikaros, indeed 13 and 294 sites undergo dephosphorylation, resulting in increased Ikaros binding to TdT regulatory gene and repression of its transcription. Moreover Ikaros contains PEST sequences (associated with increased degradation of protein following phosphorylation) that show multiple phospho-sites targeted by CK2. Thus, CK2-mediate phosphorylation might regulate Ikaros stability and turnover [96].

-HDAC1 and HDAC2: the first one can be phosphorylated by CK2, cAMP-dependent protein kinase, and protein kinase G, the second one results to be phosphorylated uniquely by CK2**.** Thus, CK2 influencing HDACs phosphorylation alter the balance of hystone deacetylase and acetyltransferase activities. HDAC1 and 2 are associated with Sp1 and Sp3 transcription factors and also these proteins are targets of CK2; indeed Sp1 is phosphorylated at the c-terminus, decreasing its DNA binding activity [97]**.**

-c-Myc and Max **:** Bousset and colleagues identified these proteins as targets of CK2. C-Myc is a proto-oncoprotein that plays a pivotal role in cell growth control. It promotes cell cycle progression into S phase and inhibits differentiation. The importance of c-Myc is further supported by the this protein is essential for murine embryonic development. To perform its functions, c-Myc heterodimerizes with Max. Max can interact also with Mad proteins that negatively regulate cell growth**.** CK2 phosphorylation of Ser2 and 11 in Max resulted in enhanced DNA binding kinetics of both Max/max homo- and Myc-Max heterodimers [98]**.**

CK2 modulates also signaling pathways involved both in normal and maligant hematopoiesis such as:

-Wnt pathway: as showed in the previous paragraph, CK2 displays several site of phosphorylation, dishevelled (Dvl), β-catenin, promoting their stabilization, and TCF/LEF, facilitating its association to partner molecules. Wnt proteins were found at sites of fetal hematopiesis and Wnt5a was involved in self-renewal and proliferation of HSC. Wnt3a also increases HSC self-renewal *in vivo* in adult mice. Human CD34+ lineage also expand under Wnt stimulation. β-catenin constitutive expression determined a depletion of HSC and a multilineage differentiation block, instead Wnt signaling appeared fundamental for HSC long-term repopulating activity and for the leukemia stem cells (LSC) repopulating activity of chronic myeloid leukemia (CML). Therefore these results underline the importanmce of Wnt/ β-catenin signaling in normal and in leukemic stem cells.

-PI3/PTEN pathway: some studies performed in mice have demonstrated that PTEN deletion in hematopoietic compartment caused the entry of HSC into the cell cycle and fast depletion of HSC pool. PTEN-/- HSC showed the lack of long-term repopulating activity, furthermore mice null for this protein developed myeloproliferative disease and ALL. Thus the presence or absence of PTEN allows the discrimination between normal HSC and leukemic LSC. As represented in the previous Fig.12, CK2 through phosphorylation inhibits PTEN and activates Akt [86]**.**

-The Hedgehog (Hh) pathway is transduced through a reception system at the plasma membrane that includes the receptor complex Patched (Ptc-Ihog) and the signal transducer Smoothened (Smo). Binding of Hh to Ptc-Ihog relieves the inhibition of Smo by Ptc, which allows Smo to activate the Cubitus interruptus (Ci)/Gli family of zing finger transcription factor (Fig.15) [99]**.** The Hh signal is essential for normal embryonic development and tissue repair. Hh has been reported to play a role in hemangioblast formation [100]; B cell, T cell, and thymocyte development [101]; erythrocyte proliferation and differentiation [102] and the HSC and progenitor cell compartment [103]. Thus, the available literature indicates that there is an important contribution of Hh signal transduction in normal hematopoietic development. Of further interest, mutations in the Hh signaling pathway lead to severe developmental abnormalities and have been associated with several types of cancers. The role of Hh signaling in hematopoiesis has been studied primarily by modulating the activity of Patched and Smoothened, but results have been conflicting. Merchant and colleagues have analyzed hematopoietic stem cell (HSC) and progenitor function in mice with a

homozygous deletion of *Gli1* (*Gli1*^{null}). *Gli1*^{null} mice have more long-term HSCs that are more quiescent and show increased engraftment after transplantation. In contrast, myeloid development is adversely affected with decreased in vitro colony formation, decreased in vivo response to granulocyte colony-stimulating factor (G-CSF), and impaired leukocyte recovery after chemotherapy. Levels of the proto-oncogene *Cyclin D1* are reduced in *Gli1*^{null} mice and may explain the loss of proliferation seen in HSCs and progenitor cells [104]. CK2 is a positive regulator of the Hh signal transduction pathway. Jia and co-workers demonstrated that Smo is phosphorylated by CK2 at multiple Ser residues in the C-terminal region. These event is necessary for Hh-induced Smo accumulation. In addition, they found that CK2 regulates the pathway also downstream of Smo; indeed, they showed that phosphorylation of Ci prevents its ubiquitination and degradation through proteasome. (Fig.15) [105]**.** There are also other kinases involved in the control of this pathway such as PKA, GSK3, CK1, which phosphorylate Smo and Ci [105].

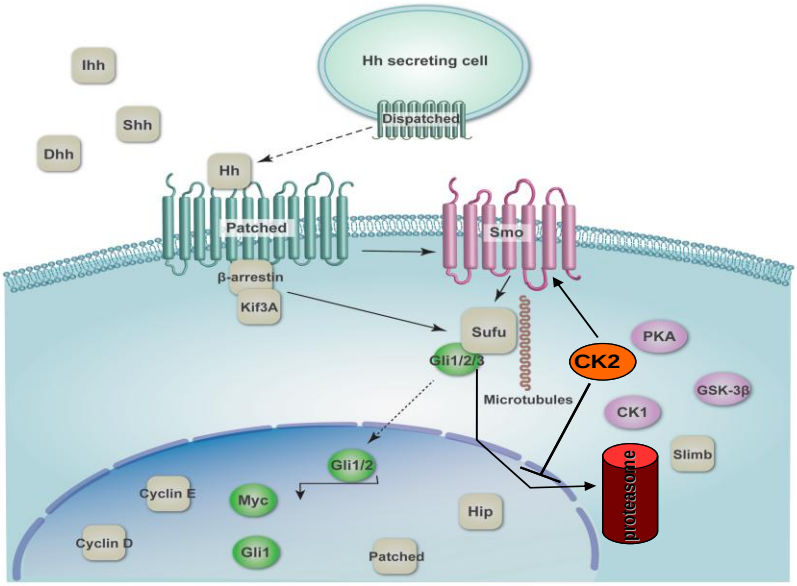

Figure 14. The Sonic Hedgehog signaling pathway.

Protein kinase CK2 inhibitors.

Interest in developing small molecule inhibitors of CK2 was heightened with the identification of adenosine -5'-triphosphate (ATP)-binding sites specific chemotypes. An increasing number of reports dealing with the development and usage of CK2 inhibitors appeared in literature. However, as with many inhibitors of other kinases, questions regarding their specificity arose immediately. This cautionary note has to be considered especially for these kind of compounds that are competitors of ATP, since ATP is the substrate for all members of protein kinase family in addition to a vast array of other cellular enzymes. Some years ago five inhibitors, all competitive with respect to ATP, the structure in complex with the catalytic subunit of maize CK2 has been solved: emodin, MNX (1,8-dihydroxy-4-nitro-xanthen-9-one), DAA (1,4-diamino5,8-dihydroxyanthraquinone), TBB (4,5,6,7-tetrabromobenzotriazole), and IQA (5-oxo-5,6-dihydroxyindolo-1,2-a-quinazolin-7-ylacetic acid) [106]. Although these inhibitors belong to different classes of chemical compounds, all are accommodated in a hydrophobic pocket which in CK2 is smaller than in the majority of protein kinases. This may well account for the selectivity of these inhibitors, which, with the partial exeption of emodin, are more effective on CK2 than they are on a panel of >30 different protein kinases. Among these inhibitors, TBB proved especially successful for in cell and *in vivo* studies (Fig.16c). While the selectivity of TBB is remarkable, its potency, though higher than that of the most other CK2 inhibitors, is not outstanding: *in vitro* it inhibits purified CK2 with IC_{50} values around 1μM, while the concentration required to induce half-maximal apoptosis of Jurkat cells is 17 μM. The relatively low potency of TBB, as well as of other CK2 inhibitors, is due to their mode of binding, which is almost exclusively based on apolar contacts with unique hydrophobic side chains, while polar interactions, which are common with potent inhibitors, are nearly absent. On the basis of these assumptions, Pagano and colleagues started to develop TBB derivates in which the tetrabromobenzene moiety, responsible for selectivity is conserved, while the triazole ring is replaced by an imidazole one derivatized with substituents that could provide interactions with polar side chains of the kinase. Some TBB analogues revealed a markedly higher potency and specificity than the previous compounds both *in vitro* and *in vivo* [107] such as K17 (4,5,6,7-tetrabromobanzinidazole; IC_{50} 0.5 μM) and K27 (4,5,6,7-tetrabromo-2-amino-benzimidazole; IC_{50} 0.25 μM) (Fig. 16a and b respectively).

Recently, a new selective CK2 inhibitor has been developed: CX-4945 (5-(3-chlorophenylamino)benzo[c][2,6]naphthy ridine-8-carboxylic acidis) the first orally bioavailable small molecule inhibitor of CK2 to advance into human clinical trials, thereby paving the way for an entirely new class of targeted treatment for cancer (Fig.15d). It was designated by Cylene Pharmaceuticals and entered Phase I clinical trials for advanced solid tumors and multiple myeloma. [108]

The crystal structure of human CK2α in complex with CX-4945 shows two direct protein-inhibitor hydrogen bonds with CK2α. Two well ordered water molecules mediate additional protein-inhibitor contacts between the carboxylate group of CX-4945 and CK2α**.** Collectively, this extensive combination of direct and water-mediated hydrogen bonds and van der Waals contacts observed between CX-4945 and CK2α establishes the structural basis for the high affinity binding for the small molecule inhibitor [108]**.**

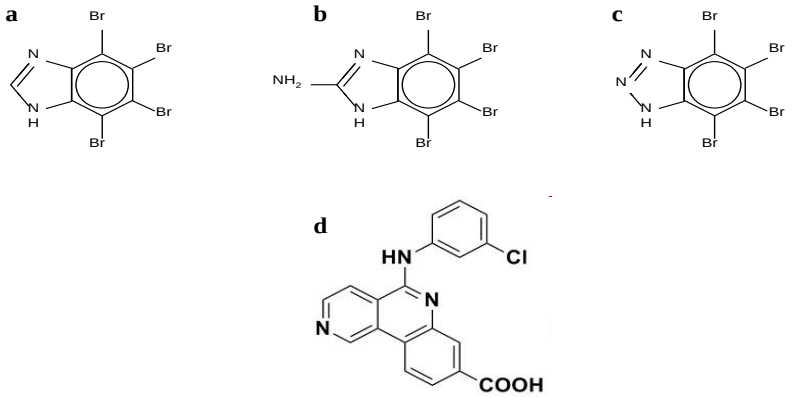

Figure 15. The most specific inhibitors of CK2. (a) K17; (b) K27; (c) TBB; (d) CX-4945

1.5 GENERATION OF *KNOCKOUT* MICE MODEL

Conditional mutagenesis is a critical tool to study pleiotropic genes, that exert their functions in several organs and tissues during embryogenesis and adult life.

For this reason, several mice models have been generated in order to obtain the conditional activation of gene expression in one or more cell types (spatial control) or in specific developmental stages (temporal control). One of the principal strategies used for these purposes, combines the homologous recombination with the properties of the Cre recombinase.

Cre Recombinase is a tyrosine recombinase enzyme derived from the P1 Bacteriophage. The enzyme uses a topoisomerase I like mechanism to carry out site specific recombination events. The enzyme (38kDa) is a member of the Integrase family of site specific recombinase and it is known to catalyse the site specific recombination event between two DNA recognition sites (loxP sites). This 34 base pair (bp) loxP recognition site consists of two 13 bp palindromic sequences which flank an 8bp spacer region. The products of Cre-mediated recombination at loxP sites are dependent upon the location and relative orientation of the loxP sites. Two separate DNA species both containing loxP sites can undergo fusion as the result of Cre mediated recombination. DNA sequences found between two loxP sites are said to be "floxed". In this case the products of Cre mediated recombination depends upon the orientation of the loxP sites. DNA found between two loxP sites oriented in the same direction will be excised as a circular loop of DNA whilst intervening DNA between two loxP sites that are opposingly orientated will be inverted. The enzyme requires no additional cofactors (such as ATP) or accessory proteins for its function. Figure 16A and 16B

The enzyme plays important roles in the life cycle of the P1 Bacteriophage such as cyclization of the linear genome and resolution of dimeric chromosomes that form after DNA replication.

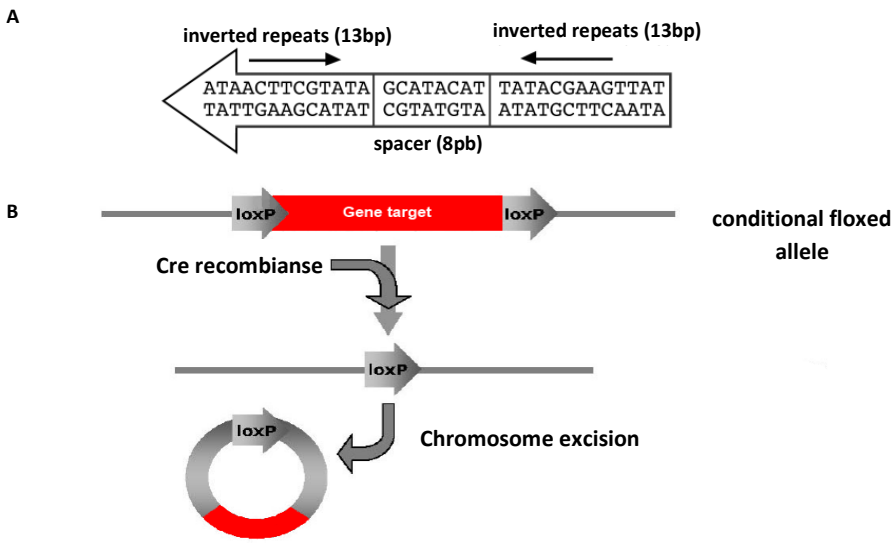

Figure 16: A) Canonic loxP sequence (Modified by Araki K *et al*., 2010); **B) Conditional genetic inactivation through the loxP system** (Modified by Kim JE, 2006).

The combination of gene targeting and site-specific recombination techniques, it is possible to generate *knockout* mice in a desired specific tissue or cell line. The rational consist in using a target vector to insert two loxP sites flanking the gene of interest or a sequence included within the open reading frame (ORF), maintaining its correct expression. The embryo or the homozygous mice that possess both alleles flanked by the loxP sites, and transgenic for the expression of Cre recombinase will be characterized by a normal gene function, all but for the tissue or the cell type of interest [109].

With the term gene targeting we identify the manipulation of the murine genome based on the homologous recombination. Linear DNA molecules represent the preferred substrate for the homologous recombination, which is more frequent during the S phase of cell cycle.

The targeting vector is studied to introduce loxP sites in the desired genomic site in embryonic stem cells (ES). The selection of the recombined ES cells is carried out through a marker gene (generally a gene that confers a drug resistance) insert in the targeting vector. For example, the gene that codifies the neomycin phosphotransferase (*neo*): only the cells that have correctly made the recombination are able to survive when in their culture medium is present G418, as this drug is lethal for cells that don't bear the neo cassette. The negative selection eliminates ES cells that have incorporated the vector through a mechanism of non-homologous recombination. A marker gene is usually insert externally to the vector region included in the genome. The timidine kinase, isolated from the herpes virus (HSV-tk) constitute an example: if the vector incorporation happens through the linearized far ends, HSV-tk gene is inserted together with *neo* gene. Adding the 2'-fluoro-2'-deoxy-1beta-D-arabinofuranosyl-5-iodouracil (*FIAU*) occurs the negative selection of HSV-tk^+ cells. After the enrichment of ES cells that bear the correct modified genetic locus, the next step is to generate chimeric mice, that are able to pass on the offspring the mutant gene. For this reason, ES mutant cells are injected in a host blastocyst that originates the chimeric mice after being transplanted in a foster mother. In order to make easier the isolation of mice bearing the

mutant gene, ES cells and the host blastocyst derive from mice that express distinguishable pigmentation alleles [110].

Finally, to obtain mice with conditional deletion of the target gene, it is necessary to cross mice that bears the floxed gene with mice that express the Cre recombinase under the control of a tissue or cell type specific promoter (figure 17).

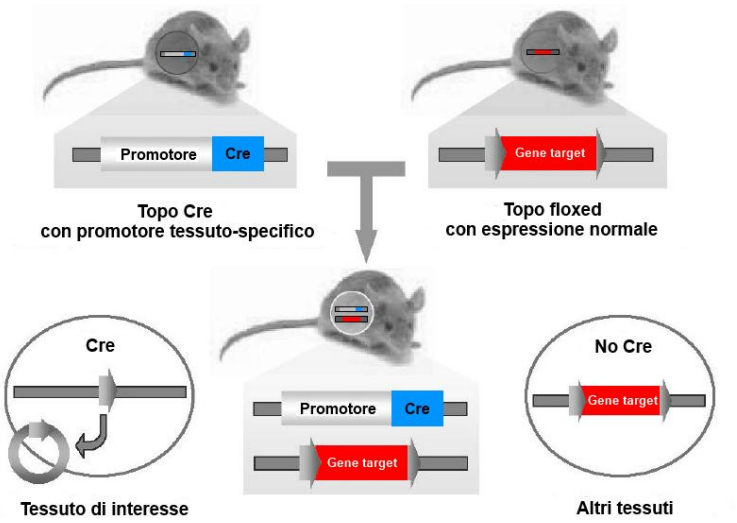

Figure 17: Tissue specific conditional mutagenesis. Target gene is excised by Cre recombinase, selectively expressed in floxed mice. Arrows indicate the orientation of the loxP sites. (Modified by Kim JE, 2006).

The offspring will have the gene deleted only in the cells that express the inegrase. Another accurate control of recombination is possible if this mice are transgenic also for a reporter gene, the expression of which is activated by Cre recombinase. A common example is the β galactosidase gene (LacZ), included in ROSA26 locus, that is active only after the excision of a floxed STOP codon. The efficiency of recombination can be site-dependent, for this reason the recombination pattern obtained is not necessary comparable for different genes. Another important parameter is the control of potential phenotypes generated by the only presence of the Cre transgene (Feil R, 2007).

2. AIM OF THE STUDY

The ser/thr kinase CK2 was reported to regulate ~~also~~ molecules involved in hematopoiesis. In particular CK2-dependent phosphorylation modulates transcriptional factors such as PU.1, c-Myb, c-Myc and Max, Ikaros, HDAC1 and HDAC2, protein kinases such as AKT/PBK, as well as components of the Wnt and NF-kB pathways.

While very low in normal granulocytes, CK2 levels are markedly increased in highly proliferating myeloblasts from patients with acute myeloid leukemia (AML) or with chronic myelogenous leukemia (CML) in blast crisis.

Thus, it could be hypothesized that CK2 is involved in hematopoietic maturation and in the pathogenesis of acute myeloid leukemias.

Thus, in this work we aimed to investigate a potential novel role of CK2 in normal hematopoiesis, through the study of a conditional KO mouse model for CK2β in the hematopoietic compartment and the role of CK2 in the biology of LSC collecting samples from AML patients and using as model Kasumi-1 AML cell line.

3.MATERIALS AND METHODS

3.1 Generation of conditional CK2β KO mice in hematopoietic compartment

CK2β KO mice in hematopoietic cells were generated through several sequential crossing.

The first cross made was between C57BL6 mice homozygous for the *Csnk2b floxed* ($CK2\beta^{Fl/Fl}$) allele (C. Cochet, Grenoble), with C57BL26 mice hemizygous for the transgene *Vav1-Cre* ($Vav^{+/Cre}$) (Jackson laboratories). This cross originates mice with the genotype $CK2\beta^{+/Fl}$ $Vav^{+/Cre}$ (heterozygous mice). To obtain knockout (KO) mice, these heterozygous mice were then crossed with $CK2\beta^{Fl/Fl}$ mice. The genotype of our KO mouse model possesses the following genotype $CK2\beta^{Fl/Fl}$ and $Vav^{+/Cre}$.

Figure 18 shows the $CK2\beta^{Fl}$ and Vav^{Cre} alleles.

All the experimental procedures performed and described hereafter have been approved by the Institutional Review Board for animal Experimentation of the University of Padua.

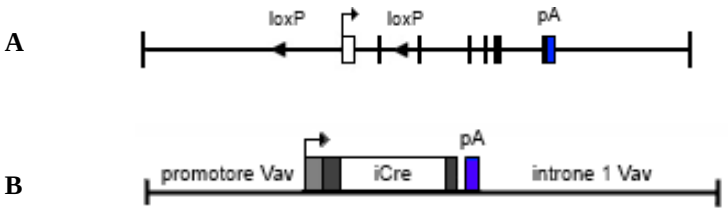

Figure 18: A) $CK2\beta^{Fl}$ allele. Rectangles represents the exons of the *Csnk2b* gene. the first exon is indicated by the white rectangle, with an arrow that indicates the transcription initiation site. loxP sites are indicated by black arowheads. The promotes (not highlighted in the picture) is located between the first loxP site and the first exon. The blue rectangle, contained in the last exon represents the polyadenilation site (pA); **B) *Vav1-Cre* transgene.** *Vav1-Cre* trangene expresses the Cre recombianse enzyme. The gene contains also the promoter and the first intron of the Vav gene. The arrow indicates the transcription initiation site. The light gray rectangle indicates an untraslated part of the exone 1 of the Vav gene. The dark rectangles are splicing sites of SV40 virus. The blue rectangle, contained in the last exon represents the polyadenilation site (Adapted by de Boer *et al.*, 2003).

3.2 Isolation of genomic DNA from mice tales and embryos

The far ends of tails of adult mice and of fetuses were digested for 3 to 4 hours at 55°C, after being resuspended in 500µl of a suitable lysis buffer, the composition of which is described below:

- 250 µL of *mouse tail* 2X lysis buffer,
- 25 µL di Proteinase K 10 mg/mL (Ambion, Life Technologies, Carlsbad, USA),
- 25 µL of SDS 10% w/v,
- H_2O Milli-Q to 500 µL.

mouse tail 2X lysis buffer contains:

- Urea 8M,
- EDTA (pH 8.0) 20 mM,
- SDS 1% w/v,
- Tris-HCl (pH 8.0) 1M,
- NaCl 5M.

Each sample was subsequently centrifuge at 13,000 rpm for 10 minutes at 4°C and the supernatant was collect and transferred to a new eppendorf containing 1ml of absolute ethanol and mix vigorously.

This leads to the formation of a white precipitate that correspond to genomic DNA.

Samples were centrifuge at 13,000 rpm for 30 minutes at 4°C, the supernatant was discard and the pellet dry up at room temperature for 10 minute.

DNA was then rehydrate with 500µl of H_2O and store at 4°C till the usage

3.3 Protocol for mouse genotyping

Determination of $CK2\beta^{Fl}$ allele

The presence of $CK2\beta^{Fl}$ allele was determined through PCR using the following primers (F = Forward; R = Reverse):

- F (BB3): 5´-CTAGCTCGAGATGAGTAGCTCTGAGGAGGTG-3´
  [Tm= 61.4°C]

- R(BB4): 5´-GGATAGCAAACTCTCTGAG-3’ [Tm= 47.2 °C]

The reaction mix for one sample was composed by:

- 12.5 μL of REDTaq® ReadyMix™ (Sigma-Aldrich, Steinheim, Germania), containing Tris-HCl (20mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), gelatine (0.002%), dNTPs and DNA *Taq* polimerase (0.006 Units/μL),
- 1.0 μL of each primer (20 pmol/μL),
- 8.5 μL of H_2O, included in the commercial kit,
- 2.0 μL of purified genomic DNA.

The thermal protocol of the PCR reaction is listed below

Amplification protocol		
94°C	5min	Initial denaturation
94°C	30sec	denaturation
55°C	30sec	annealing of primers-genomic DNA
72°C	2min	extension
72°C	7min	Final extention
4°C	endless	Hold
denaturation, annealing and extension: 40 repeats		

The amplification products were separated by gel electrophoresis on a 1.5% agarose gel. The size expected for the amplification products are comprises between 400 and 600 bp.

Determination *Vav1-Cre* of transgene

Vav1-Cre transgene is composed by the promoter of the murine Vav gene, the Cre recombianse gene, and by a polyadenilation site isolated by SV40 virus.

PCR reaction is carried out using the following primers

(F = Forward; R= Reverse):

- Internal positive control:
    - F: 5’-CTAGGCCACAGAATTGAAAGA-3’ [Tm= 52.4°C]
    - R: 5’-GTAGGTGGAAATTCTAGCATC-3’ [Tm= 50.6°C]
- Transgene:
    - F: 5’-AGATGCCAGGACATCAGGAA-3’ [Tm= 55.9°C]
    - R: 5’-ATCAGCCACACCAGACACAGA-3’ [Tm= 58.5°C]

The internal positive control corrispond to the interleukin 2 (IL-2) gene.
The reaction mix for one sample was composed by:

- 12.5 μL of REDTaq® ReadyMix™ (Sigma-Aldrich, Steinheim, Germany), comtaining Tris-HCl (20mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), gelatine (0.002%), dNTPs and DNA *Taq* polimerase (0.006 Units/μL),
- 1.0 μL of each primer (20 pmol/μL),
- 6.5 μL di H_2O, included in the commercial inclusa kit,
- 2.0 μL of genomic DNA.

The thermal protocol of the PCR reaction is listed below:

Amplification Protocol		
95°C	1min50sec	Initial denaturation
94°C	30sec	Denaturation
64°C	45sec	annealing primers-genomic DNA
72°C	45sec	extesion
72°C	2min	Final extension
10°C	endless	Hold
denaturation, annealing and extension: 35 repeats		

This assay will NOT distinguish $Vav^{+/Cre}$ hemizygous from $Vav^{Cre/Cre}$ homozygous transgenic animals. The size of the expected amplification products are :236 bp for the transgene and 324 bp for the internal positive control (IL-2).
The amplification products were separated by gel electrophoresis on a 1.5% agarose gel.

Determination of *ROSA26^{LacZ}* allele

$ROSA26^{LacZ}$ allele codifies the β galactosidase gene (LacZ). The enzyme is expressed only after the removal of a floxed STOP codon inserted in the promoter. So the transcript is present only when Cre recombinase enzyme is present.
The PCR reaction to determine the presence of $ROSA26^{LacZ}$ allele is carried with the primers listed below:

- R1295: 5'-GCGAAGAGTTTGTCCTCA-3' [Tm= 49.7°C]
- R523: 5'-GGAGCGGGAGAAATGGAT-3' [Tm= 51.9°C]
- R26F2: 5'-AAAGTCGCTCTGAGTTGT-3' [Tm= 48.7°C]

The reaction mix for one sample was composed by:

- 12.5 µL of REDTaq® ReadyMix™ (Sigma-Aldrich, Steinheim, Germany), containing Tris-HCl (20mM, pH 8.3), KCl (100 mM), $MgCl_2$ (3 mM), gelatine (0.002%), dNTPs and DNA *Taq* polimerase (0.006 Units/µL),
- 1.0 µL of each primer (20 pmol/µL),
- 7.5 µL of H_2O, included in the commercial kit,
- 2.0 µL of genomic.

The thermal protocol of the PCR reaction is listed below:

Amplification protocol		
94°C	2min	Initial denaturation
94°C	45sec	Denaturation
59°C	45sec	Annealing of primers-genomic DNA
72°C	1min	Extension
72°C	7min	Final extension
4°C	endless	Hold
Denaturation, annealing and extension: 30 repeats		

The size of the expected amplification products are: 625 bp for the *wild type* allele and e 325 bp for *ROSA26^{LacZ} allele*. In heterozygous mice are present both amplification products.
The amplification products were separated by gel electrophoresis on a 1.5% agarose gel.

3.4 Fetal livers isolation

Fetal livers were isolated from fetuses at 12.5, 14.5 and 15.5 dpc. After having sacrificed the pregnant female with CO_2, the uterus was extracted and put in PBS 1X.
All the following phases of work are made under sterile hood.

Fetal livers were extracted by the fetuses and put in a dish containing PBS.
Then livers were disrtupting through pipetting and the solution obtained was filtered with a cell strainer of 70 μm (Becton Dickinson, Milan, Italy) posed on the topo of a 50ml Falcon tube.
The cell strainer were washed several time with RPMI 1640 supplemented with 1% v/v (100 U/μl) antibiotics (penicillin/streptomycin, Euroclone, Italy) and 10% v/v of fetal bovine serum (Euroclone, Italy).
After this passage, the tube was centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant is discard and the cells resuspended in PBS 1X without Calcium and Magnesium.
The cells were counted after staining with trypan-blue dye

3.5 May Grümwald- Giemsa staining

May Grüwald-Giemsa staining was performed in order to distinguish and analyze the morphology of blood cells: this staining combines the acidity of eosin with the alkaline methylene blue.
Nuclei are visible in different violet shades. The cytoplasm staining ranges from blue to pink shades.
The staining protocol is listed below:
- the slide is completed immerse in pure May Grüwald dye for 3 minute;
- Then, the slide is put in diluted May Grüwald dye (the dilution is made 1:1 with milliQ water) for other 3 minutes;
- After, the silde is immerse in a solution of Giemsa dye, previously dilute 1:20 with milliQ water for 25 minutes;
- In the end, the slide is washed in clean milliQ water and it is leaved to dry up before the vision at the microscope.

3.6 *Touch Preparation*

Touch Preparation protocol allows the investigation of cells without needing cytologic preparation or the disruption of the tissue of interest.
This protocol consist in touching the surface of a defined area of the slide for microscopy with the entire fetal liver. After few seconds the organ is removed and it is possible to proceed with the May Grüwald- Giemsa staining.

3.7 Cytologic analysis of fetal liver

The histologic analysis of fetal livers was made on sections of 4-5 μm, fixed with formalin, paraffin embedded and stianed with hematoxylin and eosin. Hematoxylin stains chromatin, thus highlighting the nuclei with violet/blue. Eosin stains cytoplasm in pink and acts also as a contrast for the nuclear staining.

Fetuses $CK2\beta^{+/+}$ $Vav^{+/Cre}$ (CTRL) e $CK2\beta^{Fl/Fl}$ $Vav^{+/Cre}$ (KO) isolated at 15.5 dpc were analyzed.

3.8 Colony Forming Unit assay

The colony forming unit assay (CFU assay) was developed in order to easily quantify mouse hematopoietic progenitors.

The CFU-units protocol is described below:

- Thaw tubes of MethoCult® medium (StemCell technologies, Vancouver, Canada) overnight under refrigeration or at room temperature.
- Vortex tubes to ensure all components are thoroughly mixed.
- Prepare cells at 10X the final concentration required.

Example: To achieve 1 x 105 cells per dish, a cell suspension of 1x106 cells per mL is prepared.

- Add 0.3 mL of cells to 3 mL of MethoCult® medium for duplicate cultures or 0.4 mL of cells to 4 mL of MethoCult® medium for triplicate cultures.
- Vortex tubes to ensure all cells and components are thoroughly mixed.
- Let tube stand for 5 minutes to allow bubbles to dissipate.
- To dispense MethoCult® medium into culture dishes, attach 16 gauge blunt-end needle to a 3 cc syringe.
- To expel most of the air from the syringe, place needle below surface of solution and draw up approximately 1 mL. Gently depress the plunger and expel medium completely. Repeat until no air space is visible.
- Draw up methylcellulose medium into syringe. Dispense 1.1 mL per 35 mm dish.
- Distribute methylcellulose medium evenly by gently tilting and rotating each dish.
- Place the two dishes into a 100 mm petri dish. Add a third, uncovered 35 mm dish containing 3 mL of sterile water. Replace lid of 100 mm petri dish (The use of a 100

mm petri dish and water dish helps maintain humidity and minimize contamination during culture and handling).

- Place cultures in an incubator maintained at 37°C, 5% CO2 in air and ≥95% humidity.
- Colonies were counted after 3, 7 and 10 days of cultures

The classes of mouse hematopoietic progenitors detected using MethoCult® media include:

CFU-E: Colony-forming unit-erythroid. These are mature erythroid progenitors that form 1-2 clusters of maturing erythroblasts in the presence of erythropoietin (EPO). CFU-E are counted after 2-3 days of culture. **Description:** These colonies are very tiny as seen under 40-50X magnification. One cluster contains at least 8 (~8-32) erythroblast cells. Erythroblast cells within the cluster are irregular in shape and appear fused together.

Mature BFU-E: Mature burst-forming unit-erythroid. The mature BFU-E form small colonies containing 3 or more clusters of erythroid cells or single larger colonies in the presence of EPO only. Mature BFU-E are generally counted after 3-4 days of culture.

BFU-E: Burst-forming unit-erythroid. BFU-E require EPO and cytokines with burst-promoting activity such as Interleukin-3 (IL-3) and Stem Cell Factor (SCF) for optimal growth. BFU-E are enumerated after 7-14 days of culture. BFU-E are made up of erythroid clusters and minimum of 30 cells. Each individual cluster contains a group of cells that are tiny, irregular in shape and difficult to distinguish. The cells appear fused together. BFU-E do not usually have a dense core and the clusters are relatively scattered. However, it is best to confirm BFU-E by looking at the individual clusters within each colony.

CFU-GM: This classification includes CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), and CFU-granulocyte macrophage (CFU-GM). The colonies contain 30 to thousands of granulocytes (CFU-G), macrophages (CFU-M) or both cell types (CFU-GM). CFU-GM are made up of at least 30 cells per colony. CFU-GM colonies often contain multiple cell clusters (dense core surrounded by cells). The monocytic lineage cells are large cells with an oval to round shape and appear to have a grainy or grey centre. The granulocytic lineage cells are round, bright, and are much smaller and more uniform in size than macrophage cells. It is easy to see individual cells of a CFU-GM colony, especially in the periphery of the colony.

CFU-GEMM: CFU-granulocyte, erythroid, macrophage, megakaryocyte. CFU-GEMM are multi-potential progenitors that require EPO and two or more cytokines to support the growth and differentiation of lineage-committed daughter cells within the forming colony. Because of their primitive nature, CFU-GEMM tends to produce large colonies of >500 cells containing erythroblasts and recognizable cells of at least two other lineages. CFU-GEMM colonies are generally large (>500 cells per colony) and have a highly dense core with an indistinct border between the core and peripheral cells. Erythroblast clusters should be visible along the periphery of the CFU-GEMM colony. Monocytic and granulocytic cells (see CFU-GM) should be easily identifiable and clusters of large megakaryocytic cells are usually seen.

CFU pre-B: A subset of B-lymphoid progenitors can be detected in the presence of Interleukin-7 (IL-7). Made up of at least 30 cells. Although CFU pre-B colonies vary in size and morphology, individual cells appear tiny and irregular to oval in shape. Some CFU pre-B colonies are very dense with very few cells in the periphery and some have a smaller core with more cells in the periphery.

CFU-Mk: CFU-megakaryocyte. Although megakaryocytic progenitors can be cultured in methylcellulose-based medium, it can be difficult to distinguish CFU-Mk based on colony morphology.

3.9 X-gal staining

Fetal liver cells from fetuses homozygous for *ROSA26*^{LacZ} allele were assayed for the β-galactosidase activity through the X-gal staining.

This assay is based on the ability of the β- galactosidase enzyme to hydrolyze the colorless X-gal substrate (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). X-gal, when cleaved by β-galactosidase, yields galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter then spontaneously dimerizes and is oxidized into 5,5'-dibromo-4,4'-dichloro-indigo, an intensely blue product which is insoluble. So only cells that possess an active β-galactosidase (so only cells where the Cre recombinase is present) will be blue stained.

The protocol for this technique is described below:

- add 50,000 to 60,000 cells on each polylisinated well of an immunofluorescence slide;
- Allow the fixation incubating for 15 minute at 37°C;

- Wash cells twice with PBS 1X, after having checked at the microscope that the cells are fixed on the slide;
- Add 50µL of *Fixation Solution* (included in β-Galactosidase Reporter Gene Staining™ kit- Sigma-Aldrich, Steinheim, Germany) diluited 1:10 in sterile H_2O. Incubate for 10 minutes at room temperature.
- Prepare the *Staining Solution* using the following reagents (supplied in the kit):
  - 0.5 µL Reagent A,
  - 0.5 µL Reagent B,
  - 0.5 µL Reagent C,
  - 2.5 µL *X-Gal Solution* (20 mg/mL),
  - 46.5 µL PBS 1X
- Wash cells twice with PBS 1X;
- Add the *Staining Solution* on the wells and incubate at 37°C for 30 minutes to 2 hours or more, until the cells stain blue.
- Observe the cells under the microscope. Count the cells and calculate the percent of cells expressing b-galactosidase.

3.10 **Cells**

Cell line

In the present study was used the human cell line KASUMI-1.
KASUMI-1 was established from the peripheral blood of a 7-year-old Japanese boy with acute myeloid leukemia (AML FAB M2) (in 2nd relapse after bone marrow transplantation) in 1989; these cells carry the t(8;21) leading to RUNX1-RUNX1T1 (AML1-ETO) fusion gene and were described to carry the KIT mutation N822.
This cell line was buy from DSMZ (*Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH*) cell line bank (Leibniz, Germany).

Patient samples

Patients were referred to the Hematology Division of Padua University Hospital and they were grouped in three categories of factor risk according the European Leukemia Network guideline (Table 4).

Peripheral blood from 10 AML patients was enriched by Ficoll-Hypaque density gradient, centrifugation and washed in Sodium Cloride 0.9% solution in order to purify PBMC.

The material used in the present study from healthy donors was all buy from StemCell tecnology (Vancouver, Canada).

Table 4 . Correlation between cytogenetic and molecular alteration and risk factor (assigned following the European Leukemia Network guidelines).

PATIENT CODE	KARYOTYPE	MOLECULAR ANALYSIS	RISK FACTOR
1	NORMAL	NORMAL	INTERMEDIATE I
2	NORMAL	MUT.FLT3, MUT.NPM1	INTERMEDIATE I
3	NORMAL	NORMAL	INTERMEDIATE I
4	NORMAL	MUT.FLT3, MUT.NPM1	INTERMEDIATE I
5	NORMAL	NORMAL	INTERMEDIATE I
6	NORMAL	NORMAL	INTERMEDIATE II
7	NORMAL	NORMAL	INTERMEDIATE II
8	T(9,22)	NOT DONE	ADVERSE*
9	COMPLEX	NOT DONE	ADVERSE
10	COMPLEX	NORMAL	ADVERSE
*Patient affected by chronic myeloid leukemia in blast crisis			

Cell culture

Kasumi-1 were cultured in 25cm^2 flasks (Falcon) at a final concentration of 0,5-10^6 cells×ml in a final volume of 8ml. They were maintained in incubator at 37°C in a modified atmosphere with 5% of CO_2.

The culture medium used was RPMI 1640 (EuroClone, Italy) with HEPES and L-Glutamin and supplemented with 20% v/v of decomplemented fetal bovine serum (FBS) (EuroClone, Italy) and 1% v/v of antibiotics (penicillin and streptomycin) (EuroClone, Italy).

All the procedure of handling of the cells were done under sterile hood.

Cell treatments.

Cells were counted with trypan blue (Sigma-Aldrich, Steinheim Germany) in Neubeuer chamber, centrifuged at 800rpm for 5' and plated. They were treated with different compounds:

-CK2 synthetic inhibitor:

- CX-4945 (Activate Scientific GmbH);
- Daunorubicin (Pfizer, Italy).

Cell were incubated with this compounds of 18 hours

3.11 Flow citometry

Flow cytometric analysis of hematopoietic progenitors purified from fetal liver

To investigate the effect of the CK2β KO on hematopoietic progenitors, at least 500,000 cells obtained from fetal liver at 12.5 dpc were stained with the following cocktail of antibodies: anti-CD3, anti-CD19, anti-B220, anti-NK1.1, anti- GR1(Ly6G-Ly6C), anti-Mac1, anti- Ter119, anti- CD5 (lineage markers, after indicated with the abbreviation Lin), cKIT (CD117), ska1 (ly6A/E), IL7Ra (CD127), CD34, CD16/32 (BD biosciences, NY, USA).
After the staining cells were acquired through FACSAria III (BD biosciences, NY, USA).
The analysis of the dot plot obtained was carried out with FlowJo software (TreeStar).

Analysis of different stages of erythroid maturation through flow cytometry

To deepened our analysis, we have investigated the different staged of erythroid maturation through flow cytometry.
At least 500,000 cells obtained from fetal livers at 14.5 dpc were stained with the following antibodies: anti-Ter119 and anti-CD44 (BD biosciences, NY, USA).

After the staining procedure, cells were acquired through FACSCanto II (BD biosciences, NY, USA).
The analysis of the dot plot obtained was carried out with FlowJo software (TreeStar).

Cell sorting of leukemia stem cells (LSC) and CD34+/CD38+ cells

The present study aimed at investigating the role of CK2 in LSC and in CD34+/CD38+ cells of patients affected by acute myeloid leukemia, both using samples isolated from patients and both purifying this populations from the AML cell line KASUMI-1.

The purification of this population was performed through FACS sorting using a FASCAriaIII cell sorter (BD Biosciences,NJ USA).
The protocol is described below:

- Count cells with trypan blue (Sigma-Aldrich, Steinheim, Germany) in Neubeuer chamber.
- Collect almost $100x10^6$ cells and divide them in three equal aliquots. Wash with PBS 1X and centrifuge at 800 rpm for 5 minutes.
- Discard the supernatant and gently resuspend cells.
- Proceed with the staining using the following cocktail of antibody: anti-CD2, anti-CD3, anti-CD14, anti-CD15, anti-CD16, anti-CD19, anti-CD20, anti-Glycophorin A, anti-CD34, anti-CD38, anti-CD90, anti-CD123 (BD Biosciences).
- Incubate cell for 20 minute at room temperature in dark, mixing every 10 minutes.
- Wash with PBS 1X and centrifuge at 800 rpm for 5 minutes.
- Discard the supernatant and reunite all the aliquots and filter the sample with cell filter of 40μm.
- The sample is ready to being sorted.

Proliferation and cell cycle analysis

The thymidine analog, 5-bromo-2-deoxyuridine (BrdU),is a common reagent used for cell proliferation assays and for the detection of apoptotic cells. BrdU is a uridine derivative and a structural analog of thymidine, and it can be incorporated into DNA during the synthesis-phase of the cell cycle as a substitute for thymidine, thereby

serving as a marker for proliferation. Cells marked by BrdU incorporation may be detected by multiple detection methods using fluorescently-labeled or enzyme-linked anti-BrdU antibodies. Below it is described the protocols for the incorporation of the BrdU for cytometric analysis:

- Plate $2x10^6$ cells for each condition to be tested;
- Add BrdU at the final concentration of 10μM and incubate for 1 hour at 37°C in modified atmosphere with 5% CO_2.
- Collect cells (prepare also a tube to be stained with the secondary antibody only) and wash them with cold PBS; centrifuge at 1200 rpm for 5 minutes at 4°C.
- Discard the supernatant and resuspend the pellet
- Fix cells with cold ethanol 70% mixing well to avoid the formation of cell clumping.
- Store at -20°C if the samples are not stained immediatly.
- Wash cell with PBS 1X and centrifuge at 1200 rpm for 5 minutes.
- Add 2ml HCl 2N and incubate for 10 minute
- Wash cell with PBS 1X and centrifuge at 1200 rpm for 5 minutes.
- Discard well the supernatant, resuspend cell and add 2ml of $Na_2B_4O_7$ pH 8.5 and incubate for 10 minutes.
- Wash cell with PBS 1X and centrifuge at 1200 rpm for 5 minutes.
- Discard the supernatant and add 2ml of PBS+BSA 0.5% w/v + Tween20 0.1% v/v and incubate for 10 mimutes.
- Wash cell with PBS+BSA 0.5% w/v + Tween20 0.1% v/v and centrifuge at 1200 rpm for 5 minutes.
- Discard the supernatant, resuspend cells in the drop left and add anti-BrdU antibody (BD biosciences) and incubate for 45 minutes at room temperature.
- Wash cell with PBS+BSA 0.5% w/v + Tween20 0.1% v/v and centrifuge at 1200 rpm for 5 minutes.
- Discard the supernatant and stained with the secondary conjugated antibody (goat anti- mouse FITC, DAKO) and incubate for 30 minute in the dark.
- Wash cell with PBS+BSA 0.5% w/v + Tween20 0.1% v/v and centrifuge at 1200 rpm for 5 minutes.

- Discard the supernatant and add 300µl of the solution containing PBS 1X supplemented with propidium iodide 10µg/ml+ RNAse 50µg/ml and incubate for 30 minute in the dark.
- The sample is ready to be analyzed at the cytometer.

In figure 19 is shown an example of the outcome of BrdU and of cell cycle staining.

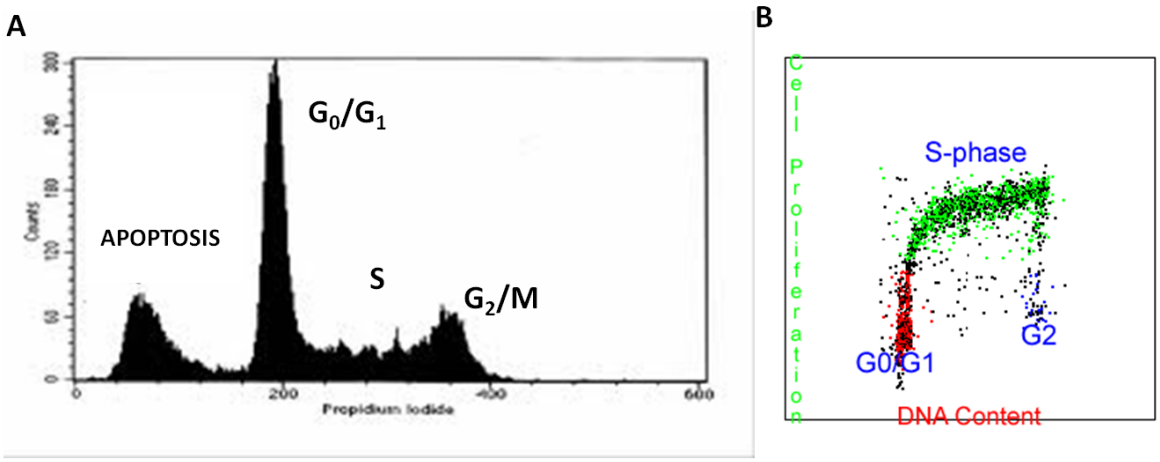

Figure 19: A) Cell cycle phases analyzed through propidium iodide staining; **B)** Cell cycle analysis through BrdU staining.

Apoptosis analysis.

After treatment of KASUMI-1 cell line with CK2 inhibitor and Daunorubicin , apoptosis was evaluated using the Apoptosis Detection Kit (Immunostep, Italy).

AnnexinV is a member of a highly conserved protein family that binds acidic phospholipids in a calcium-dependent manner. The protein has been shown to possess a high affinity for phosphatidylserine. Depending on the nature of the stimuli, phosphatidylserine is translocated from the inner side of the plasma membrane to the outer layer. When cells undergo death by apoptosis or cell necrosis, exposed phosphatidylserine serves as one of several signals by which cell, that are undergoing apoptosis, are recognized by phagocytes. If AnnexinV binds to the cell surface, this indicates that cell death is imminent. In order to differentiate apoptosis from necrosis, a dye exclusion test with propidium iodide is performed to establish whether membrane integrity has been conserved or whether membranes have become leaky. A combination test measuring Annexin-5 binding and dye exclusion thus allows discrimination between intact cells, apoptotic cells, and necrotic cells.

$2X10^5$ cells were washed in PBS to remove medium and resuspended in 100µl of binding buffer; 3µl of AnnexinV-Fitc were added and cells were incubated for 10 minutes at room temperature in the dark. 100µl of binding buffer were further added to the cell suspension and DNA was stained with 10µl of propidium iodide, added immediately before proceeding with flow cytometry analysis.

3.12 Protein extraction

Whole cell protein extraction.

All steps were performed at 4°C. Cells ($1\text{-}5x10^6$) were collected and washed in PBS. Pellets were resuspended with 30-50µl of lysis buffer composed of:

-Triton	0,5% v/v
-Okadaic acid	1µM (Sigma)
-phosphatase inhibitors cocktail(100X)	1X (Thermo scientific)
-DTT	1mM (Sigma)
-PMSF	1mM (Sigma)
-Proteases inhibitor (100X)	1X (Sigma)

-Buffer made up of TRIS (pH7.5) 20mM, NaCl 150mM, EDTA 2mM, EGTA 2mM to final volume.

Samples were incubated for 30', vortexed every 5' and centrifuged for 10' at 13000 rpm. Supernatants were collected and stored at -20°C.

Protein quantification

To measure the concentration of protein in solution we used the Bradford (Sigma) protein assay . It is based on an absorbance shift of the dye Comassie Brilliant Blue G-250 in which under acidic conditions the red form of the dye is converted into its bluer form to bind to the protein being assayed. The bound form of the dye has an absorption spectrum maximum at 595 nm. The binding of the dye to the protein stabilizes the blue anionic form. The increase of absorbance at 595nm is proportional to the amount of bound dye, and thus to the amount (concentration) of protein present in the sample.

Bradford was diluted 1:2 in distilled water and 1-2µl of cell lysate was added; the solution was mixed and incubated 3' in the dark, and absorbance was performed using a spectrophotometer (Ultrospec 1100pro; Amersham).
Concentration values were obtained applying Lambert-Beer formula:

$A=\varepsilon x\ c$ ε=molar extinction coefficient

Molar extinction coefficient was calculated from a calibration curve, obtained using rising concentrations of BSA

Electrophoresis SDS-PAGE

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis, is a method that allows to separate proteins according to their size, and no other physical feature.
SDS is a detergent that can dissolve hydrophobic molecules but also has a negative charge (sulfate) attached to it. SDS can disrupt hydrophobic areas and coat proteins with many negative charges which overwhelm any positive charges the protein had. The resulting protein is denatured by SDS (reduced to its primary structure) and as a result is linearized. Moreover all proteins, having a large negative charge, will all migrate towards the positve pole when placed in an electric field.
When polyacrylamide, which is a polymer of acrylamide monomers, undergoes the process of polymerization, it turns into a gel and we will use electricity to pull the proteins through it. The acrylamide concentration of the gel can be varied, generally in the range from 5% to 25%. Lower percentage gels are better for resolving very high molecular weight proteins, while much higher percentages are needed to resolve smaller proteins. Polyacrylamide gel is not solid but is made of a laberynth of tunnels through a meshwork of fibers. Small molecules can move through the polyacrylamide mesh faster than big molecules.
Sample preparation:
20-50µg of protein lysate are mixed with sample buffer (1:4 v/v) composed by SDS 20%p/v, Tris (pH6.8) 1,5M, bromophenol blu 0,05% v/v, DTT 6% v/v, and β-mercaptoethanol 1:20 v/v. The samples are heated at 100°C for 3' to favour denaturation.
Preparing acrylamide gel:

This gel is composed of two different phases: the upper phase called stacking gel (pH6.8) and lower phase called separating gel. The first one allows the protein to compact and makes them enter the separating gel simultaneously. The last one allows the real separation of proteins according to their size. In this work we used both fixed concentration of acrylamide (10%v/v for sepatating gel; 5% for stacking gel) or precast gradient gels with a concentration of acrylamide varing from 4% to 20% (Thermo scientific) for the separating gel. Gradient sds page is best suited for showing high and low molecular weight proteins in the same gel; it still helps resolution in a couple of ways: first, by getting more and more restrictive as the protein moves down the gel, it helps maintain stacking. Sharper bands will not overlap as much. Second, by engineering the gradient properly one can enhance the separation of closely moving bands.

Protein samples and a standard sample, as molecular weight reference (Seeblue Plus2 Prestained Standard 1X-Invitrogen), are loaded into the gel and the electric field applied was 25mA. We used Amersham electrophoretic chambers and a specific saline running buffer (pH 8.3) (25 mM Tris, 192 mM glycine, 0.1% SDS).

Western blot.

Following electrophoresis, the protein must be transferred from the electrophoresis gel to a membrane. The transfer method that is most commonly used is electrophoretic transfer: this method involves placing a protein-containing polyacrylamide gel in direct contact with a piece of PVDF or other suitable, protein-binding support and "sandwiching" this between two electrodes submerged in a conducting solution. The sandwich is composed into a grid in the following manner: sponge, watman paper, PVDF, gel, paper, sponge. When an electric field is applied, the proteins move out of the polyacrylamide gel onto the surface of the membrane, where the proteins become tightly attached. The result is a membrane with a copy of the protein pattern that was originally in the polyacrylamide gel. The transfer was done in specific saline buffer containing (Tris 250mM, glycine 1.92M, deionized water and methanol 20%v/v).

After the transfer it is important to block the remaining surface of the membrane to prevent unspecific binding of the detection antibodies during subsequent steps. Saturation is performed for 1 hour in a solution composed of non fatty milk 5% v/v (Ristora) and TBS (tris saline buffer) supplemented with tween20 0,05%.

Saturation is followed by washing steps in TBS plus Tween-20 0,05% v/v (Sigma) in order to remove unbound reagents and reduce background.

The membrane is incubated for 1 hour with a primary antibody that recognizes a specific protein or epitope on a group of proteins. The primary antibody is not directly detectable. Therefore, tagged secondary antibodies are used as the means of ultimately detecting the target antigen (indirect detection). Our secondary antibodies were enzymatically labelled with Horseradish peroxidase (HRP), which is conjugated to the antibodies. After a final series of washes, antibodies on the membranes are ready to be detected. An appropriate chemiluminescent substrate, which produces light as a byproduct, is then added to the membrane. The light output can be captured using ImageQuant LAS500 machine (GE Healhcare Life Sciences). The intensity of the signal should correlate with the abundance of the antigen on the membrane. We used different chemiluminescent substrates:

-Pierce ECL western blotting substrate (Thermo Scientific);

-LiteAblot PLUS Enhanced Chemioluminescent Substrate (EuroClone);

-LiteAblot EXTEND Long Lasting Chemiluminescent Substrate (EuroClone).

-LiteAblot Turbo Extra Sensitive Chemioluminescent Substrate (EuroClone).

In order to detect more antibodies with the same specificity and similar molecular weight it is necessary to strip the membrane. Stripping buffer reagent (Thermo scientific) allows the cleaning and the efficient removal of primary and secondary antibodies from immunoblots without removing or damaging the immobilized antigen. This allows blots to be re-probed with new antibodies. Membranes are covered with this buffer and incubated for 10'-15' at room temperature; some washes in TBS are performed and finally the membrane can be saturated again with milk.

Antibodies

We used antibodies directed against the following human proteins:

Western blot antibodies:

Primary antibodies: anti-rabbit CK2α provided by Dr. S. Sarno, University of Padova, Italy; anti-mouse CK2β (BD Biosciences, USA); anti-Cyclin D1(SantaCruz, California, USA); anti-Caspase9 (Alexis Biochemical, Switzerland),; anti-Caspase3 (Alexis Biochemical, Switzerland), anti-PARP (Cell Signaling Technology, Beverly, MA, USA); anti-Mcl1 (Cell Signaling Technology, Beverly, MA, USA), anti-FoxO3 (Cell Signaling Technology, Beverly, MA, USA); anti-phospho-FoxO3 Ser253 (Cell

Signaling Technology, Beverly, MA, USA); anti- β Catenin (Cell Signaling Technology, Beverly, MA, USA); anti- phospho- β Catenin ser33/37 thr41 (Cell Signaling Technology, Beverly, MA, USA); anti- GSK3α/β (SantaCruz, Califormia, USA), anti-phosho GSK3α/β Ser, anti GSK3α/β tyr, anti-p65 (Abcam), anti-phospho-p65 ser259 (SantaCruz, California, USA), anti-AKT(Cell Signaling Technology, Beverly, MA, USA); anti- phospho-AKT ser 437(Cell Signaling Technology, Beverly, MA, USA); anti-phospho-AKT ser 129(Cell Signaling Technology, Beverly, MA, USA); anti- GAPDH (Ambion); anti- ACTIN (Sigma-Aldrich, Germany).

Secondary antibodies: anti-rabbit IgG HRP-linked antibody (Cell Signaling Technology, Beverly, MA, USA); HRP labeled goat anti-mouse IgG (KPL, Gaithersburg, MD, USA).

3.13 RNA purification

RNA was purified using RNeasy mini kit (Quiagen). This procedure represents a well-established technology that combines the selective binding properties of a silica-based membrane with the speed of microspin technology. A specialized high-salt buffer system allows up to 100ug RNA longer that 200 bases to bind to the RNeasy silica membrane. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions, and sample is then applied to an RNeasy Mini spin column, where the total RNA binds to the membrane and contaminants are efficiently washed away. RNA is then eluted in water. The procedure provides enrichment for mRNA since most RNAs <200 nucleotides are excluded.

Protocol:

Cells are collected and wash, removing the medium; then the appropriate volume of RLT lysis buffer, that contains guanidine-thiocyanate, is added (350 µl for $<5x10^6$ cells, 600 µl for $5\text{-}9x10^6$ cells). RLT is supplemented with β-mercaptoethanol 1:100 v/v, which inhibits RNases further. Samples are homogenized by vortexing and then 70% ethanol is added. After pipetting, lysed samples are transferred to RNeasy spin columns and centrifuged at 11000rpm for 1', discarding the flow-through. RNA bound to the silica membrane is washed with buffer RW1 and centrifuged at 1000rpm for 1'; a mix of DNase and buffer RDD (10µl and 70 µl respectively) are added directly on the

membrane and keep in incubation for 15'-30', in order to remove contaminant DNA. Afterwards a series of washes are performed, first of all with buffer RW1 (700µl) and then with buffer RPE (500µl) (containing ethanol). Samples are centrifuged at12800rpm for 2' paying attention that the membrane is dry from ethanol residues. At the end RNA is eluted using 30µl of RNase free water.
RNA was quantified by means of Nanodrop 1000 (Thermo Scientific).

RNA reverse transcription.

Reverse transcription is a reaction that exploits a RNA-dependent polymerase that is capable to synthesize a complementary strand of DNA, called cDNA, using a RNA strand as template.
RNA was reverse transcribed to cDNA by means of Promega system (USA). AMV, namely *Avian myeloblastoma virus,* is the reverse transcriptase enzyme used. AMV sythesizes single stranded cDNA from total or poly(A) isolated RNA; it shows polymerase activity from 5' to 3' versus, and RnaseH activity from 3' to 5', degrading the RNA strand when the hybrid cDNA/RNA is formed. The reaction takes place in 20µl of final volume:

-$MgCl_2$ (25mM) 4µl
-reverse transcription 10X buffer 2µl
-dNTPs mix (10mM) 2µl
-Oligo dT primer (0,5mg/ml) 1µl
-RNasin RNase inhibitor 0,5µl
-AMV Reverse Transcriptase 0,75µl
-RNase free H_20 to final volume of 20µl

Then samples undergo the following thermal protocol:
42°C for 15'
95°C for 5'
4°C maintenance

3.14 Real-time PCR

The real-time PCR is a method to quantify nucleic acid characterized by an high sensibility and specificity. This is called “real-time PCR” because it allows the scientist to actually view the increase in the amount of DNA as it is amplified. This is possible because it detects and quantify fluorescent molecules: these compounds bind the amplified DNA and emit a signal that increases in a proportional way with the rise of the amplified products. We obtain an amplification curve where the cycle numbers are found in abscissa and the fluorescence normalized on internal fluorophore is in ordinate. At the beginning of the reaction there are only little changes in fluorescence and this is called baseline region; the increasing of fluorescence above this threshold underline amplified product formation. From this point the reaction performs an exponential course that degenerate in plateau at the end of the reaction.

In the midway cycles the curve has a linear course: it represents the most important phase since in this stage the amount of amplified DNA is correlated with the amount of cDNA expressed at the beginning in the sample. In this linear region a threshold of fluorescence is chosen: from this value it is possible to obtain the Ct (threshold cycles), namely the cycle that are necessary, for the sample, to reach that threshold of emission (Figure 20). If the amount of cDNA present at the beginning in the sample is high, the curve will rise earlier and Ct values will be smaller.

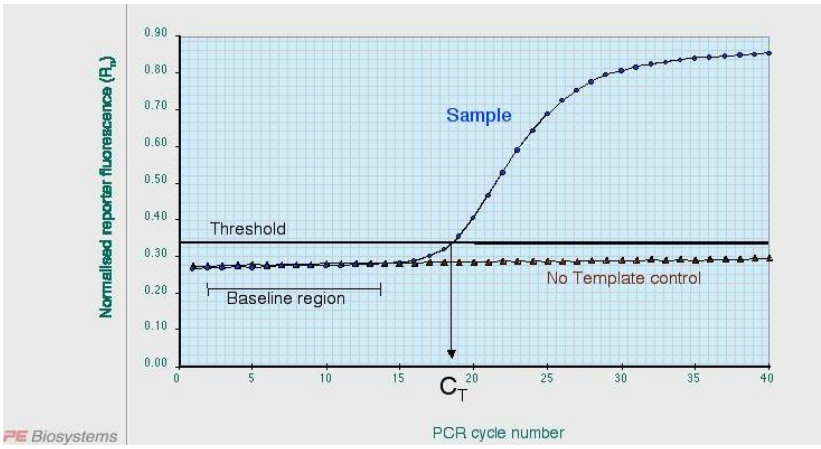

Figure 20. Amplification curve. Cycle numbers are found in abscissa, the fluorescence normalized on internal fluorophore is found in ordinate.

As detector we used SYBR GREEN: its molecules emit low levels of fluorescence if present in solution, on the contrary the signal becomes stronger if the dye binds to double strand DNA. However SYBR GREEN is not a selective dye and binds to all

DNA, even to primer dimers. For this reason it is recommended the introduction of a further step after amplification, that is the dissociation protocol: temperature rises gradually until all the DNA double strands are denaturated; this method allows the identification of contaminants or unspecific amplification products since they shows different melting temperatures. There is also a second dye called ROX; it works as an internal reference used by the instrument to normalize the SYBR GREEN fluorescence.
For the evaluation of gene expression we chose a relative quantification method, using the ΔΔ Ct formula:

1) ΔCt= Ct (target gene)- Ct (reference gene)
2) ΔΔ Ct= ΔCt (of treated sample)- ΔCt (of untreated sample, the internal calibrator)
3) $2^{\wedge -\Delta\Delta C}$

The "2"value in the last formula represents the higher efficiency for reaction that means a doubling of the product at every cycle of amplification.
The thermalcycler used is the Sequence Detection System 7000 (Applied Biosystem) and the software is ABI PRISM 7000.
The reagents of the following reaction mix are provided by Invitrogen:

-Platinum SYBR GREEN supermix	6,25µl
-ROX	0,25µl
-Forward primer (10pmol/ µl)	1µl
-Reverse primer (10pmol/ µl)	1µl
-H_2O	4,7µl
-$MgCl_2$	0,3µl
cDNA	1,5µl
Final volume	20µl

Platinum Sybr Green supermix contains:
-SYBR GREEN fluorochrome;
-the Uracil DNA Glycosylase enzyme (UDG), that allows the removal of contaminant DNA, called carryover, coming from previous amplifications. Indeed UDG recognizes deossiuridine residues, that commonly substitute timidine residues in dNTPs mix, and

degrade this carryover DNA, preserving cDNA. UDG is activated at 50°C and this step precede the amplification protocol; then it is inactivated at 95°C.

-Platinum Taq DNA Polymerase is an hot start polymerase.

Amplification protocol

-UDG activation 50°C 2’

-Polymerase activation 95°C 10’ ⎫

-Denaturation 95°C 30’ ⎬ for 40 cycles

-Annealing and amplification 60°C 60’’ ⎭

-Dissociation protocol: increasing temperature from 60°C to 95°C.

The sequences of primers, used in real-time PCR, were found using Primer Express program (Applied Biosystem) (Table 5).

Table 5. Real-time primer sequences.

Table 5A: Wnt target gene

Wnt target gene		
HUMAN PRIMER	**FOWARD**	**REVERSE**
Lef1	5’- AATGAGAGCGAATGTCTGTGC-3’	5’- GCTGTCTTTCTTTCCGTGCTA-3’

Table 5B: PI3K/AKT target genes

PI3K/AKT target genes		
HUMAN PRIMER	**FOWARD**	**REVERSE**
Foxo1	5’-CGGAATGACCTCATGGATGGA-3’	5’-TAAGTGTAACCTGCTCACTAACCC-3’
Foxo3	5’-GGAACTTCACTGGTGCTAAG-3’	5’- ACTGTCCACTTGCTGAGA-3’
Ciclina D1	5’-GCAAATGGAGCTGCTCCTG-3’	5’-GCGTGTTTGCGGATGATCTG-3’

Table 5C: Sonic/Hedgehog target genes

Sonic/Hedgehog target genes		
HUMAN PRIMER	**FOWARD**	**REVERSE**

Ptch1	5’-CTGCAGCTCAATGACTTCCACCTT-3’	5’-TTCTCACAACCCTCGGAACCC-3’
BMI-1	5’-ATTGATGTCATGTATGAGGAGGAAC-3’	5’-TAGGCAAACAAGAAGAGGTGGAGGG-3’
Smo	5’-ATCTCCACAGGAGAGACTGGTTCGG-3’	5’-AAAGTGGGGCCTTGGGAACATG-3’
Gli1	5’-GCGATCTGTGATGGATGAGATTCCC-3’	5’-TGCCTTGTACCCTCCTCCCGAA-3’
Gli2	5’-GGGTCAACCAGGTGTCCAGCACTG-3’	5’-GATGGAGGGGCAGGGGTCAAGAG-3’
Gli3	5’-ACTGCAACCACAGCCCCTTGCTTTGC-3’	5’-TGACGTCCACCCCAGAGAGGTCGTC-3’

Table 5D: Primers for CK2α and CK2β subunits

CK2 primers		
HUMAN PRIMER	**FOWARD**	**REVERSE**
CK2α	5’-TCATGAGCACAGAAAGTCACGA-3’	5’-AATGGCTCCTTCCGAAAGATC-3’
Ck2β	5’-CCCATTGGCCTTTCAGACAT-3’	5’-CCGTGTGATGGTGTCTTGATG-3’

Table 5E: Primers to normalize the reaction

Genes used to normalized the real time PCR reaction		
HUMAN PRIMER	**FOWARD**	**REVERSE**
β ACTIN	5’-CCAGCTCACCATGGATGATG-3’	5’-ATGCCGGAGCCGTTGTC-3’
GAPDH	5’-AATGGAAATCCCATCACCATCT-3’	5’-CGCCCCACTTGATTTTGG-3’

Table 5F: Mouse primers

Primers used to detect mouse mRNA		
HUMAN PRIMER	**FOWARD**	**REVERSE**
CK2β	5’-AGAGCTGGAAGACAACCCCAA-3’	5’-CCAACATTTGTGCGATGCC-3’
GAPDH	5’-CACCATCTTCCAGGAGCGAG-3’	5’-CCTTCTCCATGGTGGTGAAGAC-3’

3.15 Statistical analysis

Experiments were repeated at least three times. We used t-test to analyze data as appropriate. We considered p values of 0,05 or 0,01 as significant. Analyses were performed by means of Excel (Microsoft Office) or Origene 7.0 software.

4. RESULTS

4.1 The conditional *knock out* of CK2β in the hematopoietic compartment is lethal

Until now, there are few works published dealing with the investigation of the role of CK2β subunit in mouse model.

The first purpose of this study was to generate a conditional knock out mouse model for CK2β (codified by the Csnk2b gene), in order to investigate the role of this protein in the hematopoietic compartment.

For this reason, $CK2\beta^{Fl/Fl}$ mice were crossed with $Vav1^{+/Cre}$ mice in order to obtain $CK2\beta^{+/Fl}$ $Vav1^{+/Cre}$ mice (heterozygous mice) that were than inter- crossed to obtain $CK2\beta^{Fl/Fl}$ $Vav1^{+/Cre\ mice}$ (knock out mice). The Vav1-Cre transgene expresses the Cre-recombinase starting from 9.5 dpc.

As control we have used mice with the following genotype: $CK2\beta^{+/+}$ $Vav^{+/Cre}$ (after indicated as CNTR). These mice do not bear loxP sites flanking the first two exons of the CK2β gene, but they express the Cre recombianse as they possess a single copy of the transgene Vav1-Cre. It is not possible to obtain $Vav1^{Cre/Cre}$ mice, because the presence of two copies of the transgene is embrionically lethal.

To confirm the Cre recombinase activity, we have generated mice that are also homozygous for the LacZ transgene, that is present in the ROSA26 locus.

The crossing of heterozygous mice ($CK2\beta^{+/Fl}$ $Vav1^{+/Cre}$) has generated 192 mice, on a total amount of 16 cross made. From these crossing we have obtained only 4 KO mice: all of them were found dead, and they appear pale. Probably this slow number of animals probably can be due also to cannibalism.

The evident discrepancy with the number expected has led us to think that the KO phenotype is lethal. However, we have excluded an early-stage lethality, because in some cases the gestation was completed. For this reason, all the experiments that we have performed after this result were based on the analysis of fetuses extracted at different stages of embryonic development.

4.2 Isolated fetuses have an evident phenotype

Figure 20 shows pictures of heterozygous and KO fetuses extracted at 12.5 and 14.5 dpc. All the KO fetuses that we have analyzed possess an evident phenotype.

KO fetuses (CK2β$^{Fl/Fl}$ Vav1$^{+/Cre}$) are very pale as compared to CTRL (CK2β$^{+/+}$ Vav1$^{+/Cre}$) or heterozygous mice (CK2β$^{+/Fl}$ Vav1$^{+/Cre}$), as previously observed in the KO mice born and found dead, described before.

In particular, if we observed the right side of the fetuses, it is evident that the paleness is present also in the fetal liver, that has also reduced dimension in KO mice as compare to control mice, thus indicating a possible hypoplasia. Some KO fetuses isolated at 14.5 dpc are also hydropic (Figure 20.B).

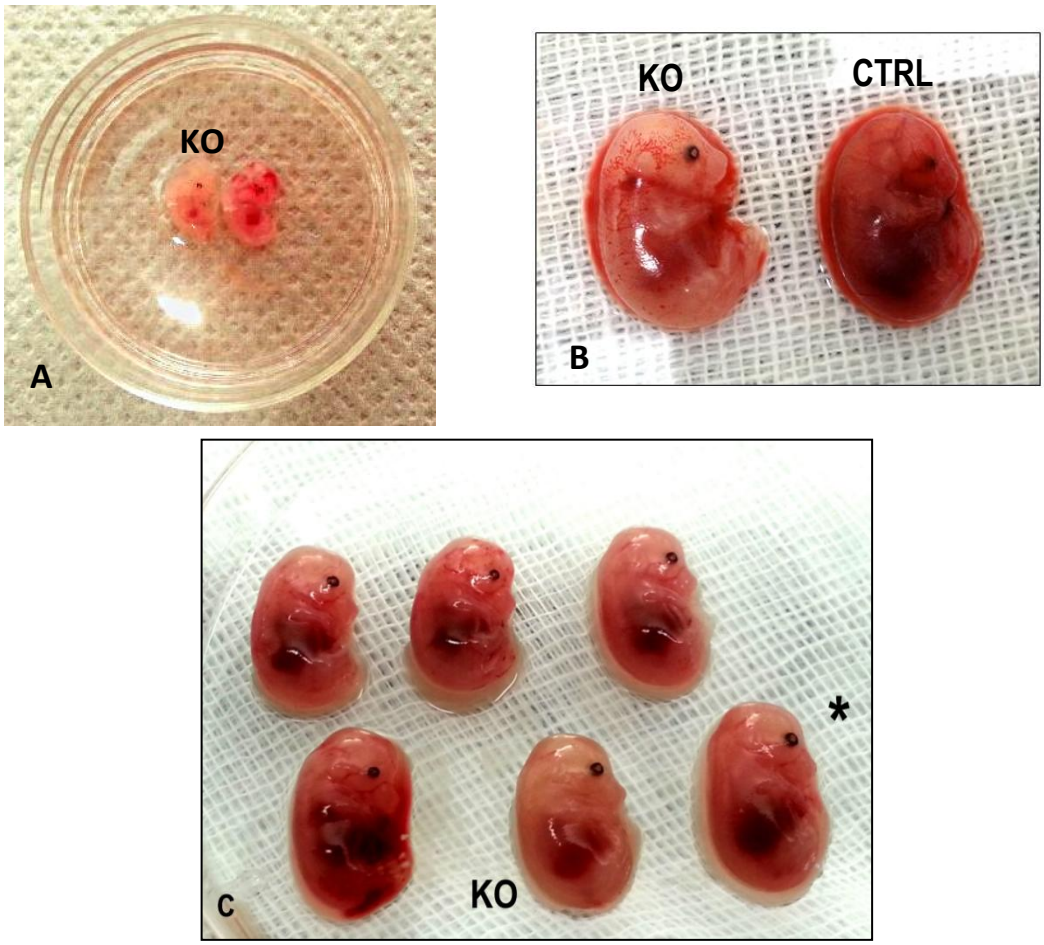

Figure 20: A) Fetuses from the some mother isolated at 12.5 dpc, generated through a cross between CK2β$^{+/Fl}$ Vav$^{+/Cre}$ ROSA26$^{+/LacZ}$ X CK2β$^{Fl/Fl}$ Vav$^{+/+}$ ROSA26$^{LacZ/LacZ}$. In the picture is indicated the KO fetus; the other fetus is an heterozygouse. **B) Fetuses isolated from the some mother at 14.5dpc,** and obtained with the some cross described before. The KO fetuses appears pale and hydropic as compared to the control (CTRL). **C) Fetuses isolated from the some mother at 14.5dpc,** and obtained with the some cross described before**.** The KO mouse is indicated with the symbol *. All the other fetuses are heterozygous mice.

4.3 X-gal staining proves the Cre recombinase activity

The X-gal staining is a technique that allows the investigator to verify the activity of the reporter gene LacZ in cells or tissues.

In the present study this technique was used to test the excision of the sequence that include the exons 1 and 2 of *Csnk2b* gene (that are flanked by two loxP sites) and that is carried on by Cre recombinase.

Locus *ROSA26*^{LacZ} is constitutively expressed , and it is constituted by the endogenous promoter *ROSA26*, by a floxed cassette with a STOP codon (that contains also a polyadenylation site) and by the LacZ gene, followed by a polyadenylation site. The trascription of the β galactosidase mRNA is not possible when the STOP codon is present (Figure 21).

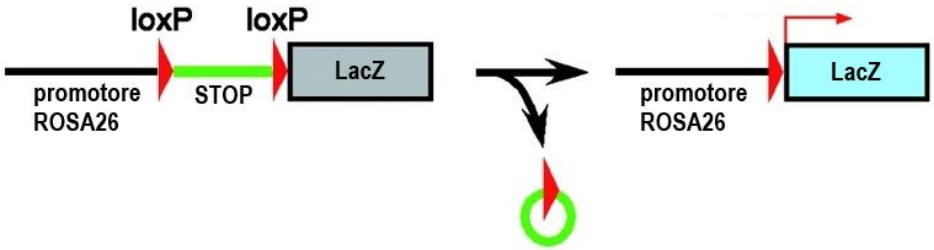

Figure 21: ROSA26^{LacZ} locus. In the left part of the picture is represented the inactive state of the allele. When Cre recombianse is present, the STOP cassette (represented by the green line) is excised at loxP sites level (loxP sites are represented by the red arrowhead) and the LacZ gene can be transcribed (right part of the figure).

The expression of the Cre recombianase, that is under the control of the Vav1 promoter in our case, causes the excision of the STOP codon, thus allowing the expression of the LacZ gene. In the meanwhile, Cre recombinase cuts also the floxed sequence of *Csnk2b*, when present, that leads to the inactivation of the gene.

To confirm the activity of the Cre recombinase in our KO mouse model, we have collected fetuses at 12.5 dpc with the following genotypes: CK2β$^{Fl/\ Fl}$ Vav1$^{+/+}$ that do not express the Cre recombianse, and CK2β$^{Fl/Fl}$ Vav1$^{+/Cre}$ (KO). All the fetuses analyzed were homozygous for the LacZ transgene. The X-gal assay was carried out on cells fetal liver cells.

Observing the pictures collected at the microscope, after having performed the staining, we can notice that fetuses CK2β$^{Fl/\ Fl}$ Vav1$^{+/+}$ (that do not express Cre recombinase) the

staining is completely absent, whereas the KO fetus the cells are positive for the staining as they appear blue colored (Figure 22).

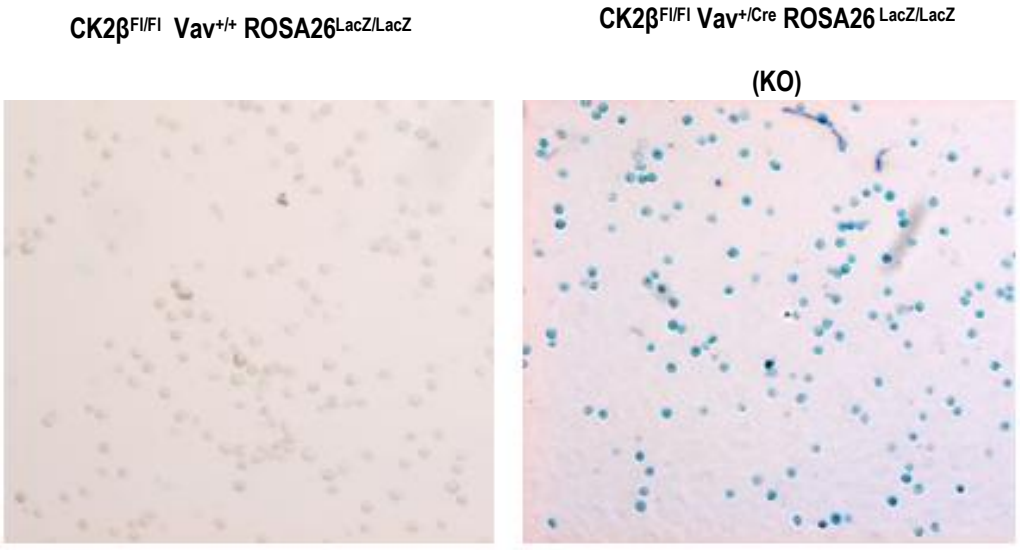

Figure 22: X-gal staining of cells isolated from fetal livers (12.5dpc). Cells were fixed with poly-lysine onto a glass slide and were stained with β-galoctosidase Reporter Kit (Sigma). 10X magnification.

4.4 *Csnk2b* KO is confirmed by mRNA and protein levels

In order to verify if the excision of the floxed sequence *Csnk2b* in the KO fetuses determines a reduction of mRNA and protein levels of CK2β, we have performed Real Time PCR and western blot experiments (Figure 23).

To this purpose, total proteins and mRNA were extracted from cells collected from KO, heterozygous and control fetuses of 12.5 dpc (Figure 23A) and 14.5 dpc (23B).

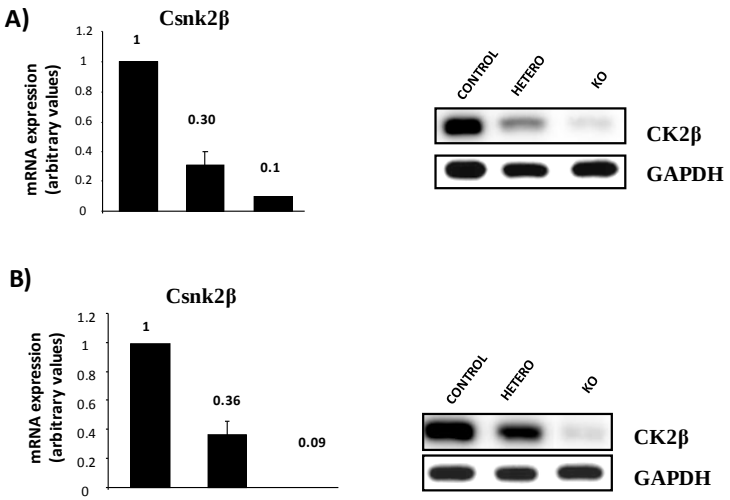

Figure 23: Quantification of mRNA of *Csnk2b* and protein level of CK2β in fetal livers: A) results of the analysis conducted in 12.5 dpc fetuses; **B)** results of the analysis conducted in 12.5 dpc fetuses.

As shown in figure 23, mRNA levels correlate with protein quantity, showing an almost complete disappearance of CK2β in the KO and, to a intermediate extent in the heterozygous.
Thus, this analysis demonstrate the our knock out system is effective.

4.5 Histological and cytological analysis of fetal liver reveals a marked depletion of hematopoietic cells

The development of the hematopoietic architecture in mouse fetal liver proceeds through specific stages, that can be identified through hematoxylin-eosin staining of tissue sections.
In fetal liver hematopoiesis four stages can be identified: Stage I that correspond to the onset of hematopoiesis at 10 dpc; in Stage II there is the expansion of the volume of the hematopoietic compartment at 11 and 12 dpc; Stage III correspond to the peak in the volume of the hematopoietic compartment and 13 and 14 dpc; and Stave IV is the involution of hematopoiesis after 15 dpc. During stages I-II, hematopoietic stem cells appeared to move from the sinusoidal lumina into primitive hepatic cell cords through the sinusoidal endothelium to give rise to colonies among hepatoblasts. At stage III, the hematopoietic colonies formed ellipsoidal foci as a structural unit of hematopoiesis. At stage IV, these cord-shape hematopoietic foci become disrupted, and round solitary foci including macrophages appeared within the hepatic cell cords on meandering sinusoids.
In order to analyze the cytological architecture of the hematopoietic compartment, fetal livers from CTRL and KO fetuses at 15.5 dpc were fixed with formalin, included in paraffin, and stained with hematoxylin-eosin (Figure 24A), or touch preparation were colored with May-Grunvald-Giemsa (Figure 24B) (in collaboration with the Institute of Pathological Anatomy Institute of the Padua University).
The hematoxylin-eosin staining highlights the hepatic parenchyma separeted by sinusoids, formed by cells with a big central nucleus, that aggregate in cords. Hematopoietic cells are the round little elements displayed with a more intense violet color, that possess a reduced cytoplasmatic volume around the nucleus. However, with this staining is not possible to distinguish the sub-cytoplasmatic structures.
May-Grunwald-Giemsa staining is suitable for this purpose and allows to discriminate different cell types according to their morphology, dimensions and intensity of the staining of the nuclei and cytoplasm.

The analysis conducted through hematoxylin-eosin staining in our CTRL and KO mice revealed a marked difference between the two type of mice; in particular, sections from CTRL mice contain numerous hematopoietic elements, whereas sections from KO fetuses revealed a dramatic depletion of hematopoietic cells (Figure 24A). The May-Grunwald-Giemsa staining confirms the depletion of the hematopoietic compartment in the KO fetuses (Figure 24B).

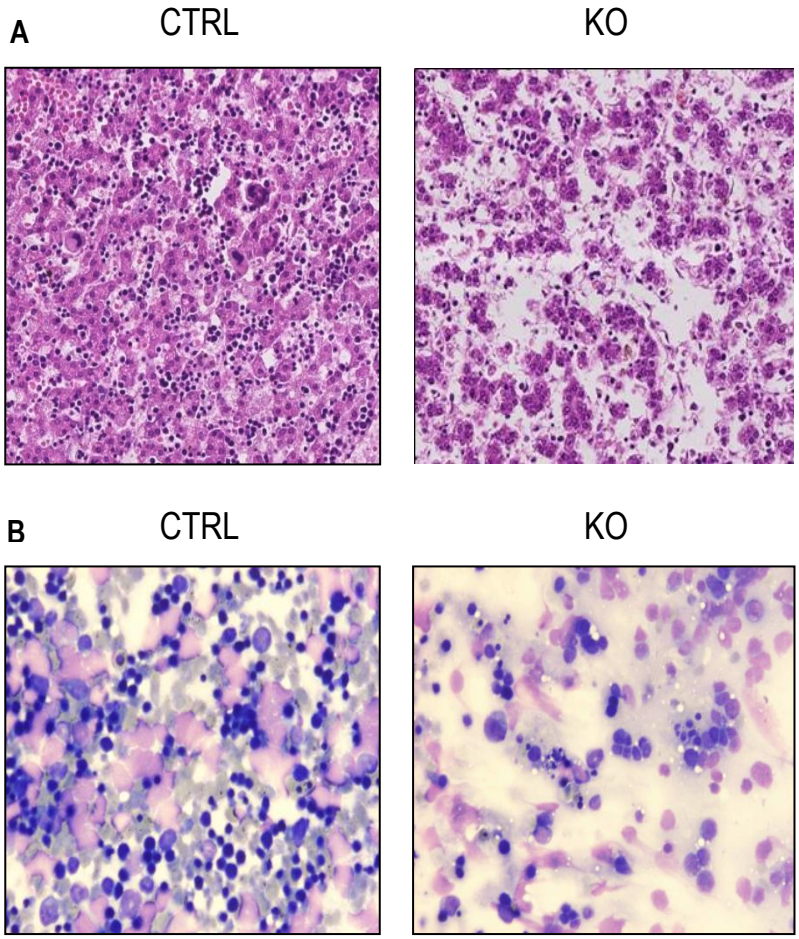

Figure 24: Histological (A) and cytological (B) analysis of fetal liver at 15.5 dpc. A) Hematoxylin-eosin staining of tissue sections of fetal liver; **B)** *Touch preparation* and May-Grunwald-Giemsa staining on fetal livers.

4.6 The colony formation assay highlights defects on erythroid, granulocyte and monocyte lineage.

To test whether or not the lack of CK2β has an influence on hematopoietic precursors, 12.5 dpc fetal liver cells were suspended in MethoCult™ (enriched with erythropoietin

and other hematopoietic growth factors). Colonies were then counted at 2, 3, 7 and 10 days.

Erythroid colonies develop earlier and have a red appearance: CFU-E (2 days), mature BFU-E (2-3 days), BFU-E (7 days). CFU-GM are counted starting from 7 days, while GEMM colonies can be seen only after 10 days. Erythroid precursors from the KO liver are able to form viable colonies but these are fewer as compared to control, both at 3 days and 7 days. At 10 days the control shows also other kinds of colonies: GM-, M-, and GEMM clusters. In contrast, in the KO sample these colonies are completely absent and the few BFU-E present seem not to be viable (Figure 25A).

All this data are summarized by the histograms shows in figure 26B. The different colonies were counted after 7 days and after 10 days of cultures. The number of colonies formed after 7 and 10 days in control mice are not significantly different, with the exception that there is a light reduction of the BFU-E number and the presence of a small amount of CFU-GEMM at 10 days of culture. It is interest to notice the dramatic reduction of BFU-E colonies in KO fetuses as compared with the CTRL mice, a difference that is even more evident after 10 days of culture.

Taken together, these results suggests that the inactivation of *Csnk2b* gene determines *in vivo* an impairment in erythropoiesis and the absence of granulocytes and monocytes.

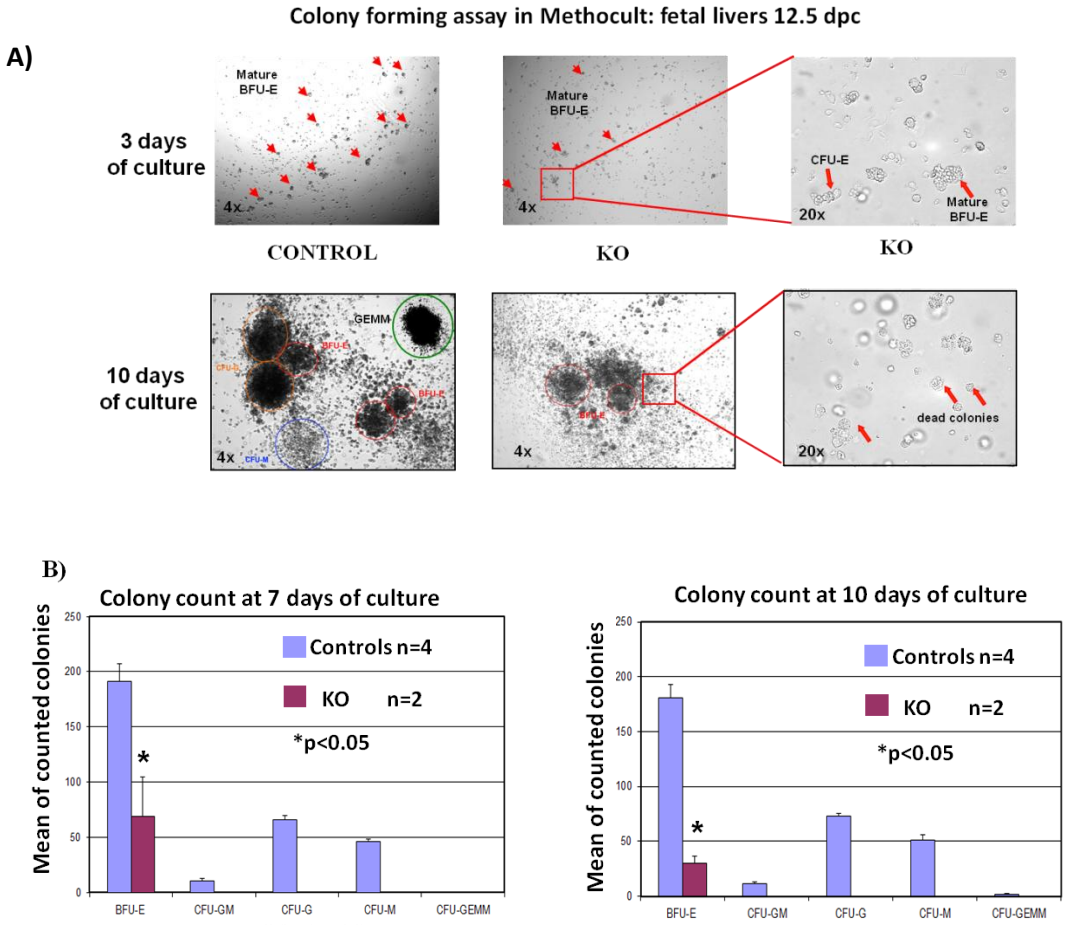

Figure 25. A) Pictures of cells plated on MethoCult^{TM} after 3 and 10 days of culture, **B)** Histograms summarize the results of colonies counted after 7 and 10 days of culture in methylcellulose. **CFU-E: colony-forming unit erythroid; BFU-E: burst forming unit erythroid CFU-GM: colony-forming unit granulocytes and macrophages; CFU-GEMM: colony-forming unit granulocyte, erythroid, macrophage, megacaryocyte.**

4.7 Cytometric analysis of fetal liver at 12.5 dpc revealed that CK2β KO affects the hematopoietic progenitors but not the HSCs

To deepen the study of precursors we used also FACS analysis. 12.5 dpc liver cells were stained with a pool of antibodies: Lin (Fitc), Sca1 (PE), CD16/32 (percpCy5.5), CD34 (APC), cKit (PECy7), IL7Rα/CD127 (V450). The subpopulations of progenitors were identified as follows: HSCs show a Lin^- IL^- $7R\alpha^-$ $Sca1^+$ $c\text{-}Kit^+$ phenotype; the CLPs are Lin^- $IL^-7R\alpha^+$ $Sca1^{low}$ $c\text{-}Kit^{low}$; CMP are Lin^- $Sca1^-$ $c\text{-}Kit^+$ $CD34^+$ $CD16/32^{low}$; GMPs are Lin^- $Sca1^-$ $c\text{-}Kit^+$ $CD34^+$ $CD16/32^+$; MEPs are Lin^- $Sca1^-$ $c\text{-}Kit^+$ $CD34^-$ $CD16/32^{low}$ [112] . As shown in figure 26, CK2β KO causes a reduction of CLP and MEP progenitors. On the other hand CMPs, GMPs and HSCs do not seem to be influenced by CK2β loss in KO mice, whereas in heterozygous mice there is a slight

increase of CMPs and GMPs and an intermediate phenotype as far as CLPs and MEP is concerned (Figure 26). The discrepancy between the colony forming assay results and the FACS aria analysis, with regard to the GMP levels, could be due to the different time points at which the two studies were done: in the first one, cells were cultured for 7 days after extraction and finally counted, in the second one cells were immediately analyzed at 12 dpc. Granulocyte and monocyte progenitors could be alive at 12.5 dpc but later they could lose the ability to form colonies and differentiate.

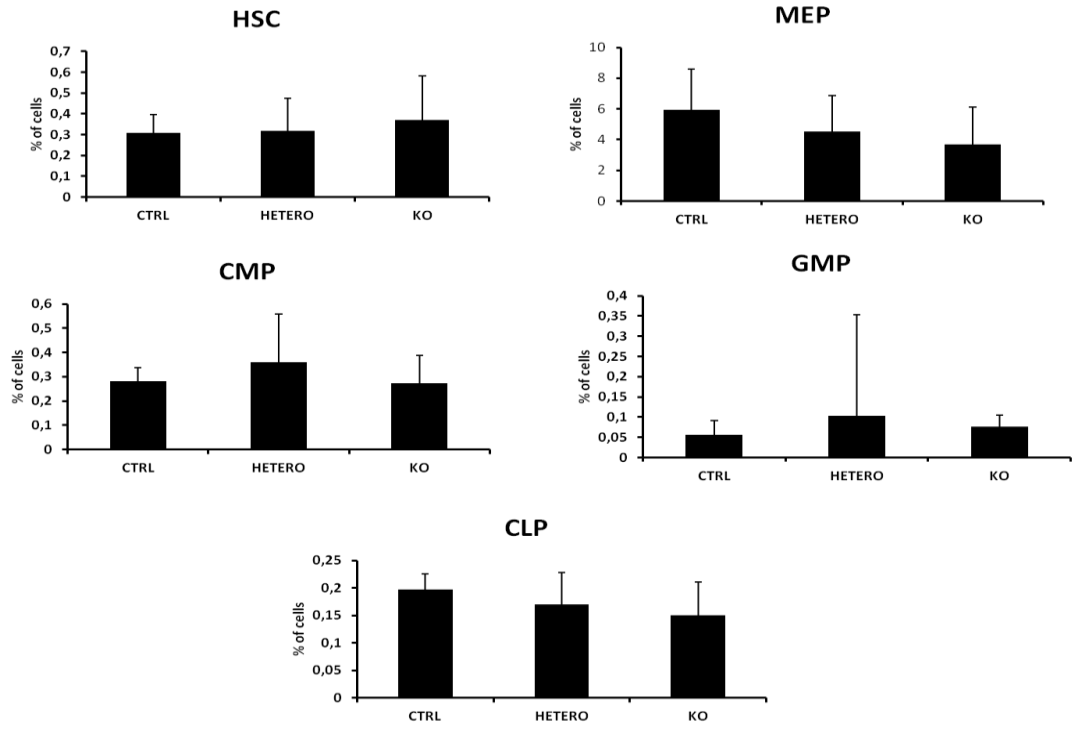

Figure 26: Histograms indicate the levels of different hematopoietic progenitors in12.5 dpc fetal liver. HSC = Hematopoietic stem cells, **MEP =** Megakaryocytes Erythroid progenitors; **CMP =** Common Myeloid Progenitors; **GMP =** Granulocytes Macrophages Progenitors; **CLP =** Common Lymphoid Progenitors. The anlysis was conducted on CTRL mice (n=4); Heterozygous mice (n=11); KO mice (n=10)

4.8 CK2β KO affects the intermediate stage of erythroid maturation.

Since erythropoiesis is the first hematopoietic event and CK2β KO caused a heavy depletion of the red compartment, we wanted to unravel which stage of erythroid differentiation was the most affected. Red blood cell maturation can be divided in 6 phases with a progressive reduction in cell size and the final enucleation (Fig.27) : proerythroblast , with large size, very high nucleus/cytoplasm ratio, basophylic

cytoplasm; basophylic erythroblasts, smaller in size with increased nuclear condensation; polychromatic erythroblasts ; orthocromatic erythroblasts ; non nucleated reticulocyte ;erythrocyte .

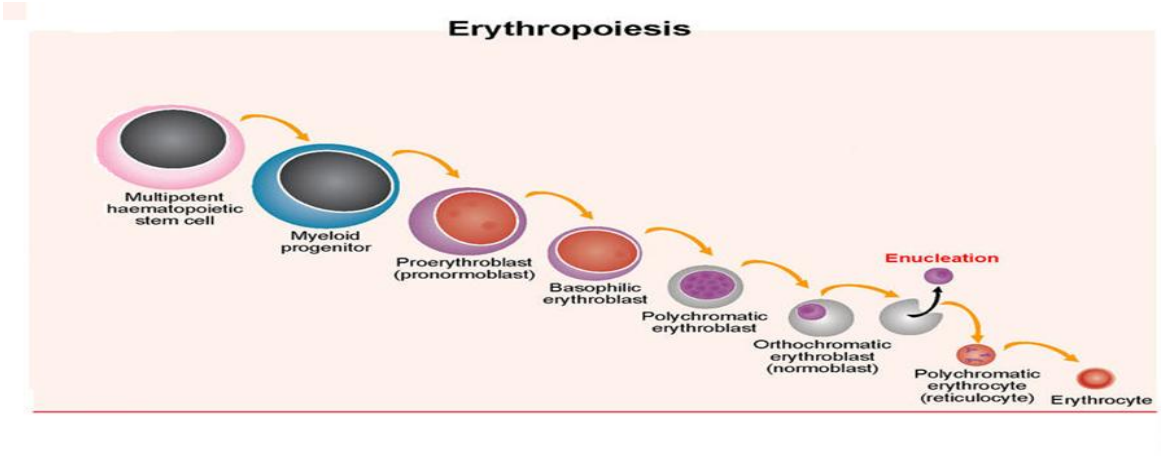

Fig. 27 Erytropoiesis; scheme of differentiation

To determine which stages of erythropoiesis is more affected by the KO of CK2β, 14.5 dpc liver cells were analysed by flow cytometry upon staining with: Ter119 (Fitc) antibody, which recognizes glycophorin A, expressed in all erythroid phases, and CD44 (PE) antibody, which identifies an adhesion molecule strongly expressed in proerythroblasts, down-regulated during maturation and that vanishes at the orthochromatic stage. CD44 was demonstrated to be a much more reliable marker for distinguishing among different stages than the common CD71 (transferrin receptor) [111]. The analysis revealded that KO fetuses possess a lower count of proerythroblast (population I) and basophilic erythroblasts (population II) when compared to the control (confirming the low count of CFU-E and BFU-E in methocult); there is also a decrease of orthocromatic erythroblasts and reticulocytes (IV) thus, less production of mature erythrocytes. It is interesting to notice that there is an accumulation of cells at othochromatic stage of differentiation (population III). We can therefore hypothesize that the few proerythroblasts in the KO enter differentiation but they are not able to proceed into the final stages, remaining blocked in the orthochromatic stage (Figure28)

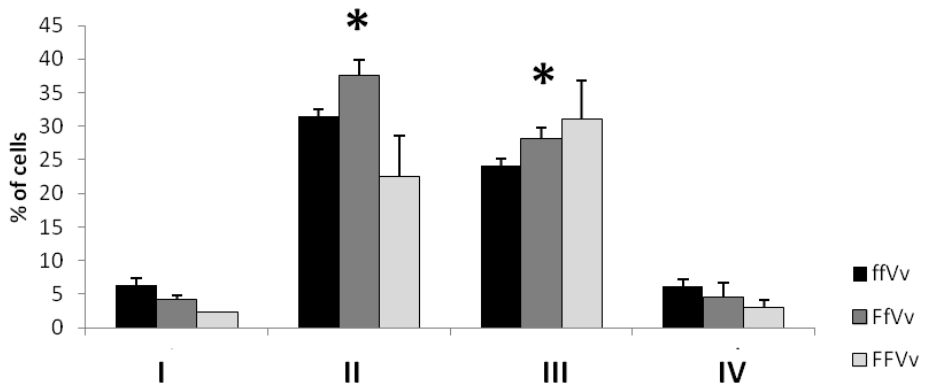

Figure 28: Flow Cytometry analysis of erythroid subpopulation: Istograms summarize the results obtained through Ter119-CD44 staining ,made on 12.5 dpc fetal livers in CTRL (ffVv), Heterozygous (FfVv) and KO (FFVv) mice. As shown in this graph, the CK2β KO (FFVv) causes a reduction of the population I (Proerythroblasts), II (Basophilic Erythroblasts) and IV (Orthochromatic erythroblasts and immature reticutocites) and an increase of the population III (Polychromatic Erythroblasts) as compared to control mice (ffVv). Heterozygous mice display a pattern that is similar to KO mice, except for the population of the basophilic erythroblasts that is increased in FfVv mice (heterozygous mice). The analysis was made on a population of 4 ffVv mice(CTRL), 11 FfVv mice (eterozygous mice) and 10 FFVv mice (KO mice).
* indicates $p<0.05$

4.9 Optimization of sorting procedure of $CD34^+/CD38^-$ subpopulation from sample obtained from AML patients

Purification of $CD34^+/CD38^-$ leukemia cells subpopulation was performed using both fresh or frozen peripheral blood or bone marrow samples from AML patients in order to determine if this procedure could be applicable to both kind of sample sources.

As far as frozen sample is concerned, after thawing, samples were resuspended in phosphate-saline buffer (PBS), marked with a specific cocktail of antibodies and then sorted with FACSAria III (BD Biosciences). Freshly isolated samples were resuspended in PBS and processed as above.

As shown in Figure 29 fresh and frozen samples did not display different CD34 expression. This result indicates that CD34 antigen is not altered by freezing procedure, allowing the possibility to study even sample stored in liquid nitrogen for a long time. This will allow to increase the number of sample available for analysis.

Fig. 29A

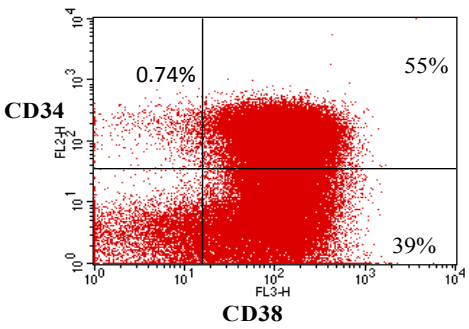

Fig. 29B

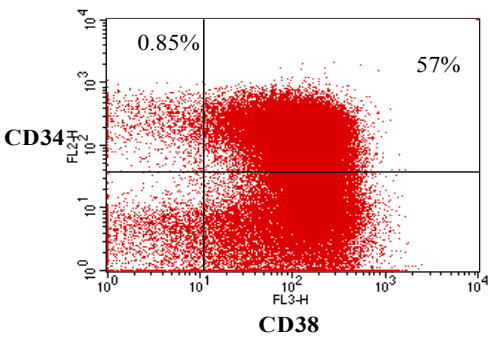

Figure 29: A:dot plot of a fresh sample; **B:** dot plot of the same sample which was frozen

4.10 Expression analysis of CK2α, CK2β in purified $CD34^{+}/CD38^{-}$ subpopulation

There are several experimental evidence that CK2 possesses an important role in tumorigenesis and in the biology of several type of cancers. In fact, CK2 was found over-expressed in several type of cancers. For this reason, in order to determines if this was true also for the leukemia stem cells compartment, expression analysis of the catalytic subunit CK2α, the regulatory subunit CK2β a was performed by quantitative real time PCR reaction on cDNA obtained from purified $CD34^{+}/CD38^{-}$ subpopulation of 10 AML patients, grouped according to the European Leukemia Network guidelines. $CD34^{+}$ from cord blood sample was used as control.

From our analysis emerges that, all LSCs derived from AML patients display an increased expression of both CK2α and CK2β. Thus indicating that the over expression of CK2 starts already at level of LSCs (figure 30).

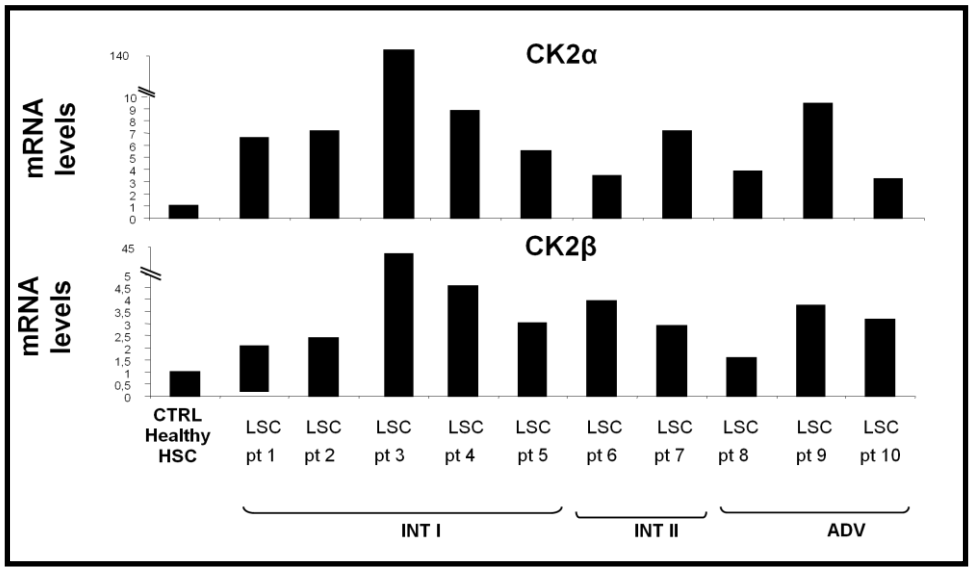

Figure 30. Real time quantitative PCR analysis of the expression of CK2α and CK2β mRNA levels in HSC and LSC taken from the patients described in table 1

4.11 Expression analysis of PI3K/AKT, Hedgehog and WNT/β-Catenin target genes in purified $CD34^+/CD38^-$ subpopulation

The involvement of the three signaling pathway of Wnt/ β catenin, Hedgehog and PI3K/AKT in the regulation of hematopoiesis is well known. For this reason we have devided to evaluate the expression pattern of some target genes of these pathways in leukemic stem cells (LSC) obtained from a cohort of 10 AML patients, grouped according to their risk factor, determined following the European Leukemia Network guideline.

As far as the PI3K/AKT pathway we have decided to analyze the expression levels of three genes: FOXO-1, FOXO-3 and CYCLIN D1.

As shown in figure 3, the expression levels of FOXO-1 and FOXO-3 seem to be related to the expression pattern displayed by the two subunits of CK2 whereas Cyclin D1 is in general down regulated highlighting the negative regulation effect of FOXO-3 on the expression of this gene as reported in literature.

As far as the HH and WNT/β catenin pathways is concerned, the expression pattern of the different target genes analyzed (GLI1, GLI2, GLI3, Smoothened and PTCH-1 for HH and LEF1 for WNT/β catenin) seems not to be related to the expression levels of the two subunit of CK2. Only BMI1, a gene with a well known role in the maintenance of stemness and that is regulated by the HH pathway, displays an expression pattern

conserved in all patients analyzed as its expression is increased in all patients studied (Figure 31).

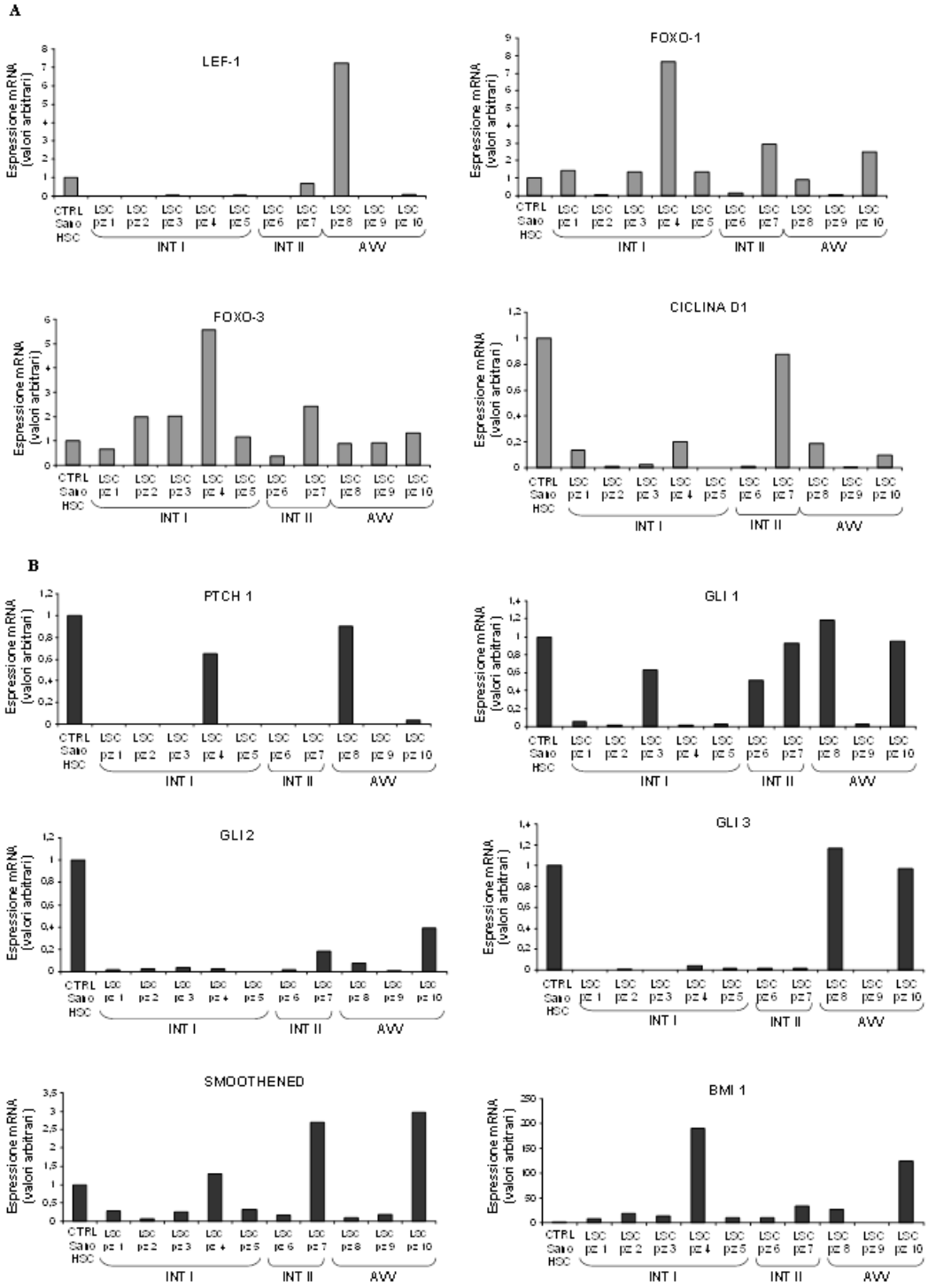

Figure 31. Representative data from Real Time quantitative PCR analysis of the expression levels of PI3K/AKT (FOXO-1, FOXO-3 and CYCLIN D1), Wnt (LEF-1) and Hh (PTCH1, GLI 1, 2, 3, SMO, BMI1) target genes in HSC and LSC taken from ten AML patients described in table 2

4.12 The inhibition of CK2 activity in sorted KASUMI-1 cell line with a specific inhibitor CX-4945 alters the cell cycle and causes apoptosis both of LSC and CD34+/CD38+ subpopulations.

To determine if CK2 is important in LSC survival, we have decided to use Kasumi-1 cell line as a model. This choice was made because this cell line posses a large amount of LSC that can be easily purified with FACS sorting as previously described in material and method section.

For this reason we have sorted the two main population of cells that composed Kasumi1 cell line (LSC and CD34+/CD38+ subpopulations) and then we have treated both of them with three increasing doses of a specific inhibitor CK2 activity, CX-4945 for 18 hours.

The two populations were then stained with Annexin V and Propidium Iodide to determine the apoptotic effect of the treatment.

As shown in figure 32 the treatment causes apoptosis in both population analyzed. In particular the apoptotic effect seems to be more pronounced in LSC as compared to the CD34+/CD38+ subpopulation.

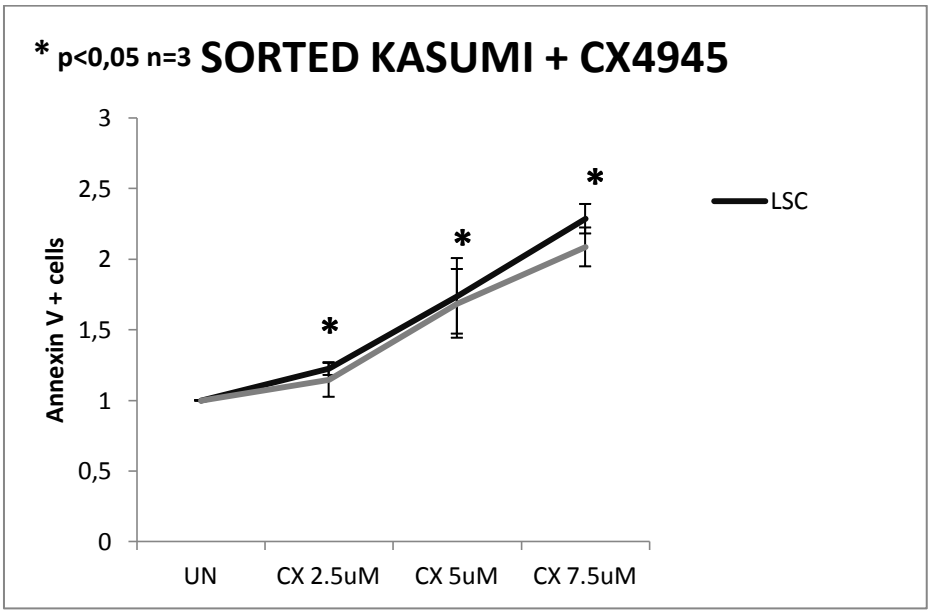

Figure 32. Inhibition of CK2 causes apoptosis both of LSC and CD34+/CD38+ subpopulations of KASUMI cell line . Two KASUMI subpopulation were sorted: LSC and CD38+/CD38+ and then exposed to three increasing doses of CK2 inhibitor CX4945 and analyzed for Annexin V expression by FACS. LSCs were fairly more sensitive to treatment as compared to CD34+/CD38+ cells. * inidates $p<0.05$ referred to UN cells.

Moreover, to deepened the analysis we have performed BrdU staining and propidiun iodide (PI) staining in order to elucidate if the inhibion of CK2 affects the cell cycle both of LSC and of CD34+/CD38+ obtained after sorting of Kasumi-1 cell line.

For this reason, after having sorted the two populations that constitute Kasumi-1 cell line, they were cultured for 18h with 5uM CX-4945.

As can be seen in figure 33A, the analysis of the cell cycle performed with PI shows that the inhibition of CK2 causes a significant increase in apoptosis (as previously seen with annexin V staining), a reduction of the G_0/G_1 phase, and a significant increase of S and G_2/M phases both in LSC and in CD34+/CD38+ subpopulation of cells. The same increase in the S phase is visible also with the analysis of the BrdU incorporation (Figure 33B).

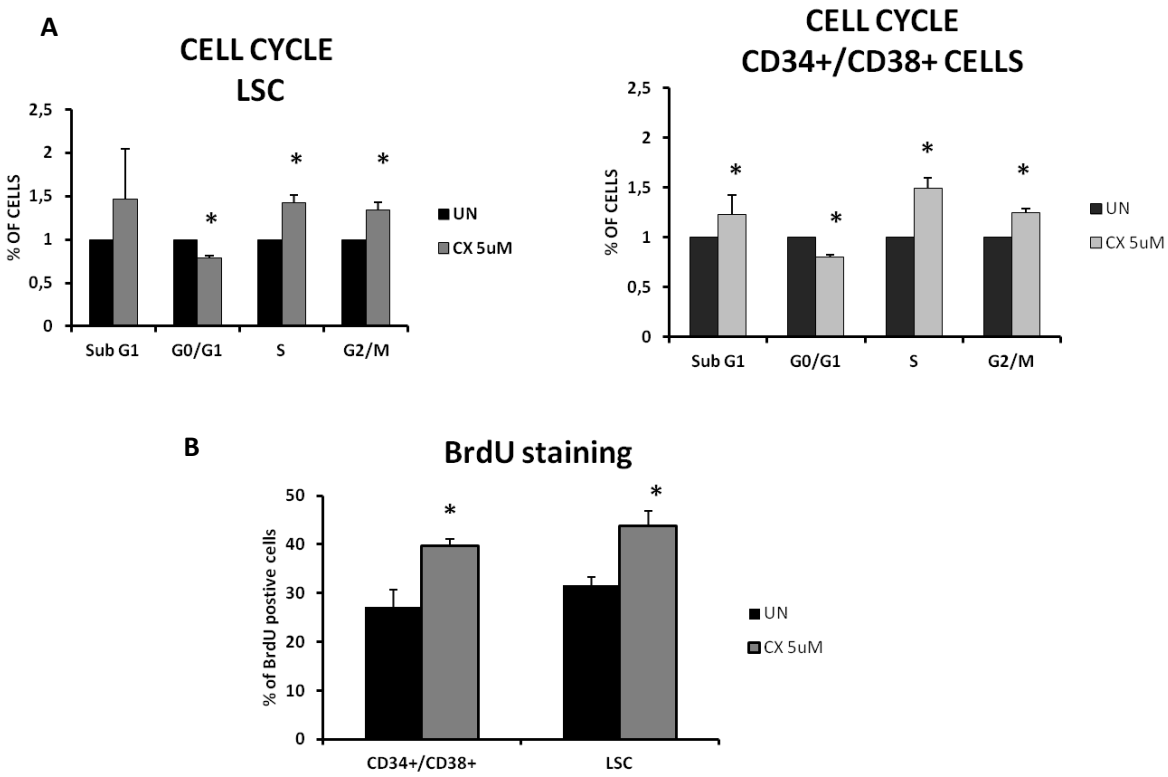

Figure 33: A) Cell cycle analysis of LSC and CD34+/CD38+ sorted from KASUMI-1 cell line and treated for 18h with CX-4945 5uM; **B)** BrdU incorporation of LSC and CD34+/CD38+ sorted from KASUMI-1 cell line and treated for 18h with CX-4945 5uM (n=6). * indicate $p<0.05$ referred to UN cells.

The apparent paradox of increased cell cycling in cells where CK2 is inhibited, can be explained by the fact that in Kasumi-1 cell line is present a p53 mutated and not active. In fact, Dixit D. and colleagues, demonstrate that CK2 inhibition can lead to cell cycle arrest with a p53 mediated mechanism; but when p53 is not functioning this mechanism is not present and cells shows an increase in proliferation rate [113].

4.13 The inhibition of CK2 with CX-4945 causes an alteration of the signaling pathway of PI3K/AKT but doesn't affect Wnt/β Catenin and of Hedgehog pathway.

Wnt/β Catenin, and of PI3K/AKT pathways are crucial for the biology of HSCs but also for LSCs. For this reason, we investigated the effect of the inhibition of CK2 in LSCs and if there was any difference between LSCs and the bulk of leukemia cells (CD34+/CD8+).

In particular, LSCs and CD34+/CD38+ subpopulations were sorted from Kasumi-1 cell line and were incubated for 18h with two increasing doses of CX-4945 (2.5uM and 5uM).

As far as the PI3K/AKT pathway is concerned, it seems to be affected by the inhibition of CK2 with CX-4945. In particular, there is a reduced phosphorylation of AKT both in ser129 and in ser 473 in CD34+/CD38+ subpopulation. Total amount of AKT seems not to vary in presence of the inhibitor in both populations.

Also AKT targets are altered in presence of the inhibitor of CK2. In particular phospho-FoxO3 Ser253 is reduced in presence of CX-4945, and the total protein FoxO3 increases in treated cells. Even Cyclin D1, which is a direct target of FoxO3 is increased in treated cells, thus confirming the results obtained with the cell cycle analysis previously described (Figure 34A).

As far as the Wnt/β Catenin signaling pathway is concerned, western blot analysis revealed that the inhibition of CK2 does not affect this pathway. In fact, none of the target analyzed seems to be altered in presence of the CK2 inhibitor CX-4945 (Figure 34B).

Phospho-p65 ser 529 was used to assess the afficacy of the inhibitor CX-4945, as the phosphorylation of p65 in Ser529 is a direct target of phosphotylation of CK2 (Figure 34C).

GAPDH is used as normalizer.

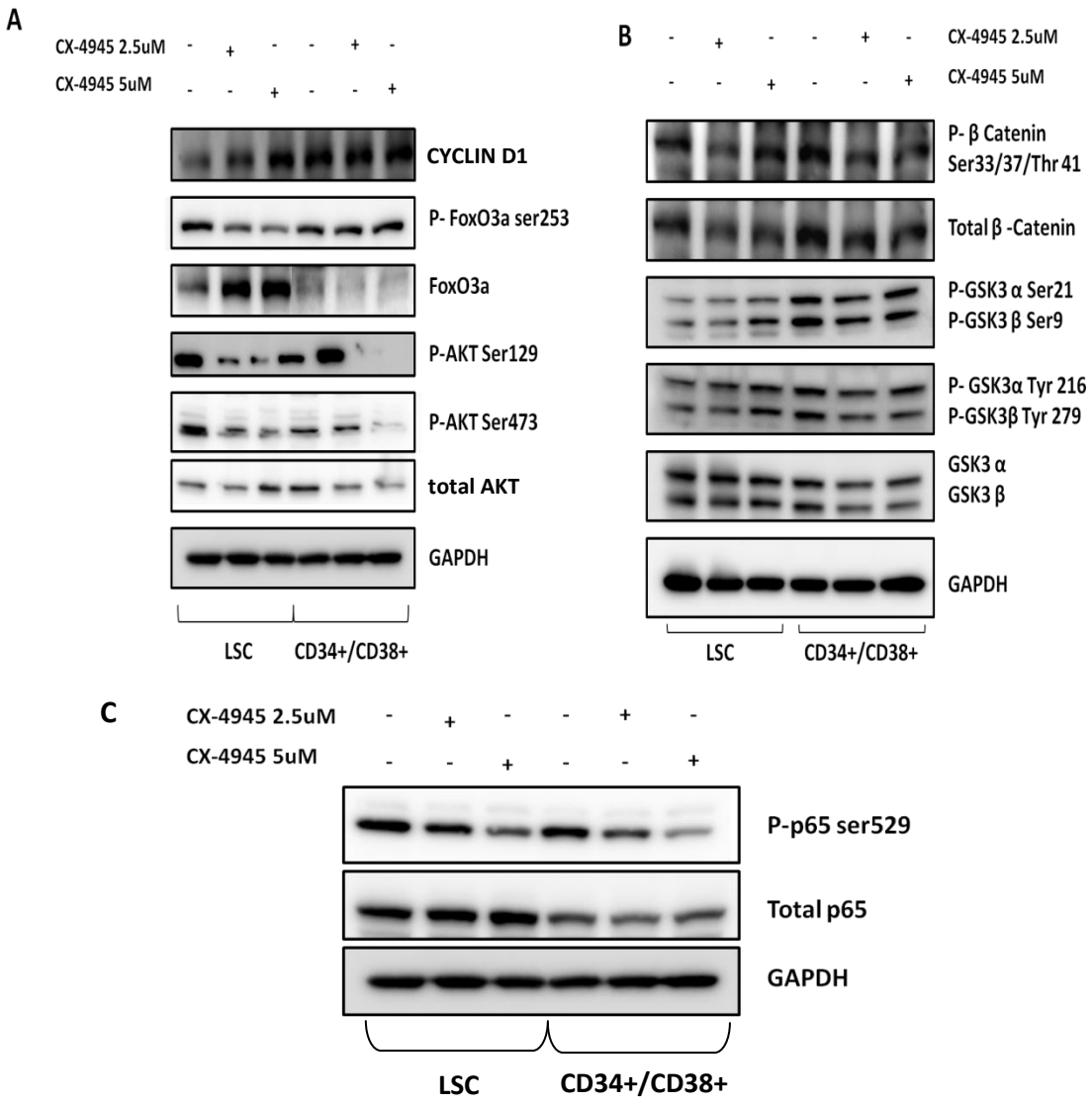

Figure 34: A) PI3K/AKT targets; B) Wnt/β Catenin targets; C) CK2 phosphorylation target

The analysis of the Hedgehog signaling pathway was performed through real time PCR on both LSCs and CD34/CD38+ Kasumi-1 subpopulations.

As shown in figure 33 the inhibition of CK2 does not affect the expression of Hedgehog target genes PTCH1 and Smoothened in LSC; whereas in CD34+/CD38+ subpopulation there is an increase in expression levels of Smoothened. Only Gli1 seems to be influenced by the inhibition of CK2 as its expression is increased in both Kasumi-1 subpopulations (Figure 35).

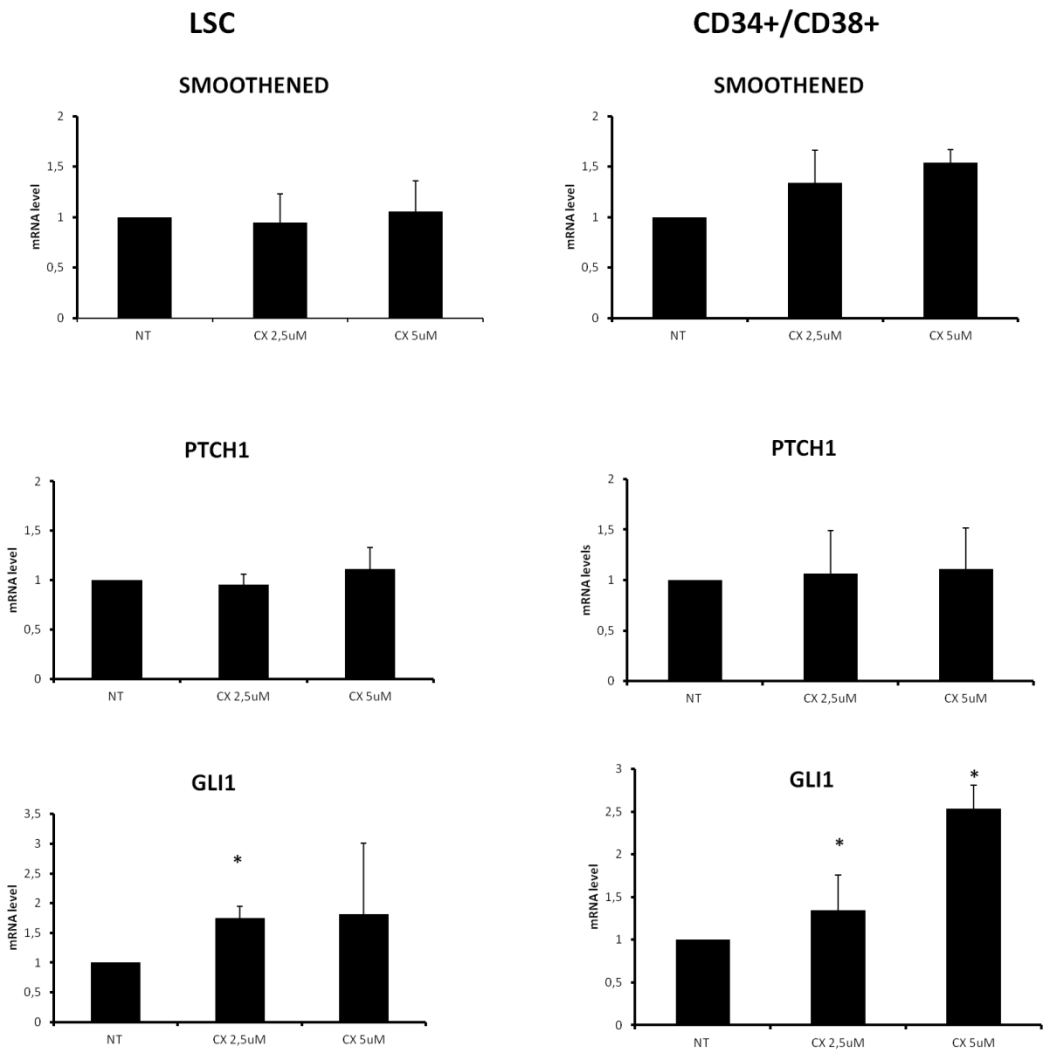

Figure 35: mRNA levels of Hedgehog target genes: Smoothened, PTCH1 and GLI1 in LSC and CD34+/CD38+ Kasumi-1 subpopulations of cells incubated 18h with CX-4945 (2.5uM and 5uM) n=3. ***** indicate $p<0.05$ referred to UN cells.

4.14 The inhibition of CK2 increases the cytotoxic effects of Daunorubicyn both in LSCs and in CD34+/CD38+ Kasumi-1 subpopulations.

It is a general concept that LSCs, as quiescent cells, are not sensitive to the chemotherapy currently used for the treatment of acute myeloid leukemia.

For this reason, in order to determined if the inhibition of CK2 could ameliorate the response of these cells to the drugs used in therapy, we have decided to treat LSCs and CD34+/CD38+ Kasumi-1 subpopulations with CX-4945 5uM and two doses of daunorubicyn (a drug that is currently used in clinic for AML treatment) for 18h.

Annexin V staining and western blot analysis were performed in order to evaluate the apoptotic effect of CX-4945 alone and in combination with Daunorubicin.

As can be seen in figure 36 Annexin V staining revealed that the cytotoxic effect exert by Daunorubicin is potentiated when is combined with CX-4945 both in LSCs and in CD34+/CD38+ cells.

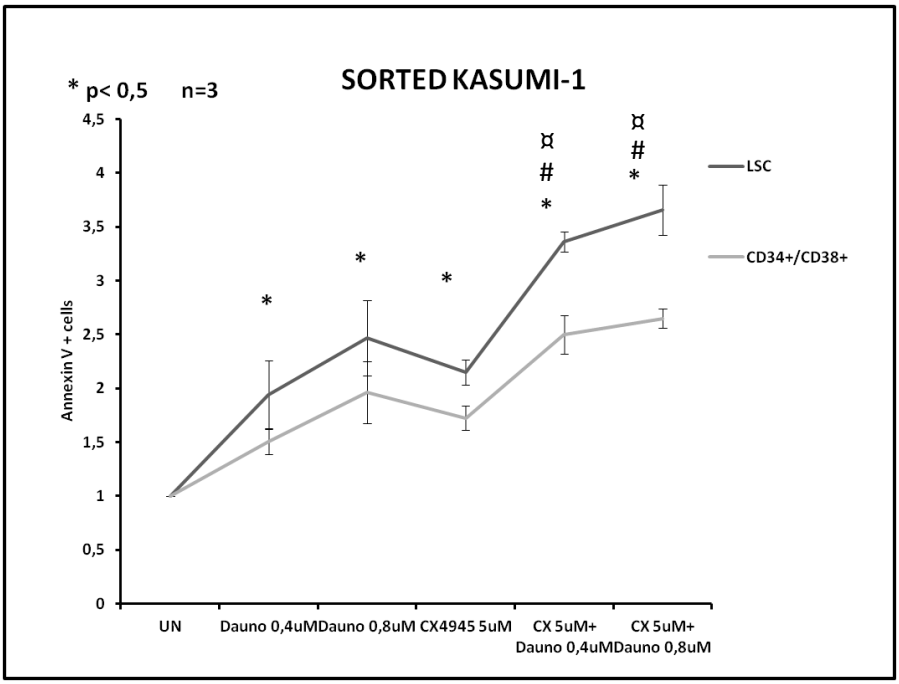

Figure 36: Annexin V staining of LSC and CD34+/CD38+ Kasumi-1 subpopulations after 18h of treatment with CX-4945 (5uM) and Daunorubicin (0.4uM and 0.8uM) alone and in combination (n=3). * indicate p<0.05 referred to UN cells. # indicates p<0.05 referred to Daunorubicin; ¤ inicate p<0.05 refereed to CX-4945 5uM.

The some result was confirmed by western blot analysis of proteins involved in the apoptotic process. In fact, as shown in figure 37 there is an increase in caspase 9 and PARP cleavage and a reduction of MCL1 protein level in the combined treatments.

Taken together, this results indicate that the combination of CK2 inhibition, can be a useful tool to potentiate the efficacy of the chemotherapeutic treatment of AML.

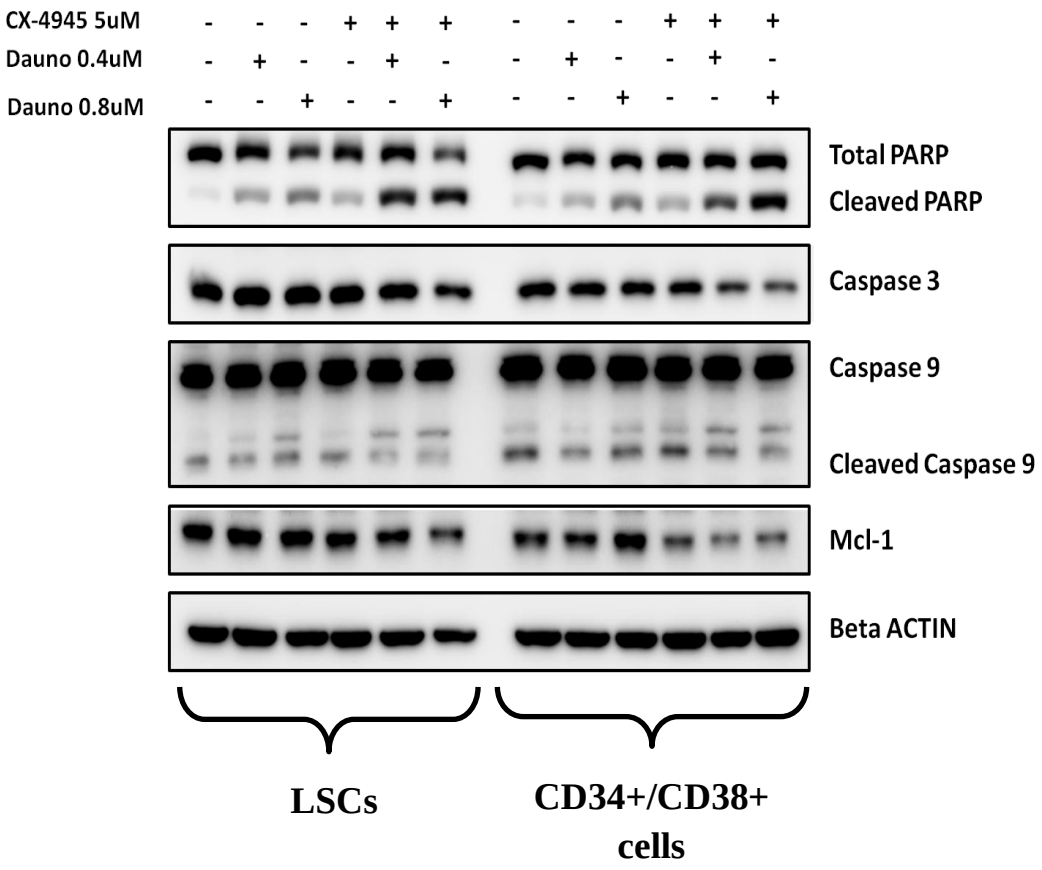

Figure 37: Western blot analysis of pro- and anti-apoptotic proteins in LSCs and CD34+/CD38+ cells treated with CX-4945 (5uM) and Daunorubicin (0.4uM and 0.8 uM) alone and in combiantion.

CONCLUSIONS

With the present work we have analyzed the role of CK2β in normal hematopoiesis using a conditional KO mouse model in the whole hematopoietic system and in leukemogenesis collecting leukemic stem cells (LSC) from patients with Acute Myeloid Leukemia (AML) and KASUMI-1 cell line.

CK2 is a pleiotropic and evolutionary conserved serin-threonin kinase, that is involved in several cellular processes. A number of studies has revealed the mechanisms through which this kinase regulates cell cycle, apoptosis, cell survival and last but not least tumorigenesis.

However, despite all this knowledge, little is known about the role that CK2 has in hematopoiesis and leukemogenesis.

As far as the hematopoiesis is concerned, we have decided to investigate the role of CK2 in normal hematopoiesis using a conditional CK2β KO mouse model in the whole hematopoietic compartment and leukemogenesis collecting sample of AML patients and using AML cell line model, KASUMI-1.

In particular, we have crossed homozygous mice for *floxed Csnkb* allele with hemizygous mice for the transgene *Vav1-Cre,* that express Cre recombinase under the control of Vav promoter, starting from 9,5 dpc. This crosses have been designed in order to generate homozygous mice for the $ROSA26^{LacZ}$, that express β-galactosidase contextually with the excision of the *Csnk2b floxed* sequence.

The first result that we have obtained, deals with the post-natal phenotype of KO mice: *i.e.* a reduced number of KO pups all dead at birth, pale and sometimes hydropic. This result has led us to hypothesize that, despite CK2β is absent starting from 9,5 dpc, the effects of its depletion are visible only after the first part of the gestation. For this reason we have moved our analysis during the fetal stages of development, focusing on fetal liver, that is the organ where hematopoiesis occurs between 12,0 and 16,0 dpc.

The phenotypical analysis of fetal livers extracted at 12,5 and 15,5 dpc revealed that KO fetuses are pale, with a smaller and faint colored liver and sometimes they appeared hydropic as compared with control mice, and these are all signs associated with a severe anemia.

Subsequent analysis with May-Grümval/Giemsa staining, MethoCult™ assay, and cytometric analysis of hematopoietic and erythroid precursors revealed a reduced

amount of cells in KO fetal livers as compare with control mice, that display an incapacity to form all the hematopoietic precursors and an impairment in erythropoiesis. A possible explanation of this phenotype can be the demonstrate role of CK2 in the positive regulation of JAK-STAT pathway, as CK2 interacts with JAK2 (*Janus kinase 2)* (8), that control STAT5 signaling. STAT5 mediates the survival and proliferation of HSCs and of lymphoid and myeloid progenitors. Moreover, JAK2 is activated by the erythropoietin receptor (EpoR), the signaling of which is important in the maturation and survival of erythrocytes.

As far as the leukemogenesis is concerned, we have collected a cohort of ten AML patients that were grouped following the European Leukemia Network (ELN) guidelines. Then we have isolated through FACS sorting LSC and from this subpopulation we have extracted total mRNA and we have performed real time PCR in order to determine the expression levels of CK2α and CK2β and of WNT/β catenin, HH, PI3K/AKT target genes, as these three pathway are involved in the biology of stem cells.

Our analysis revealed that in all patients analyzed there is an up-regulation of CK2α and CK2β mRNA levels as compared to normal HSC, with a trend toward higher levels of CK2 in Intermediate-I and Intermediate-II ELN risk subgroups.

The expression analysis of target genes of the WNT/β Catenin, HH, PI3K/AKT pathways revealed that there seem to be no correlation between the expression levels of WNT and HH target genes and CK2 in the cases analyzed. The only gene that displays a conserved pathway in all patients is BMI-1, a polycomb gene which expression is related to stemness maintenance and is controlled by HH signaling. In particular BMI-1 is up-regulated in all cases that we have analyzed, and this result probably is related to the importance of this gene in stem cell maintenance.

As far as the PI3K/AKT target genes is concerned, we focused our attention on three genes mainly: FOXO-1, FOXO-3 and Cyclin D1.

Our analysis revealed that there seemed to be a correlation in FOXO expression and CK2 levels in almost all cases analyzed and Cyclin D1 is in general down-regulated and this is probably connected to the negative regulation effect of FoxO-3.

Then we have decided to analyze the effect of the inhibition of CK2 in LSC.

For this reason, we have decided to use KASUMI-1 cell line as a model because this particular cell line possesses a 40% of LSC population.

In particular, we have sorted the LSC and the CD34+/CD38+ subpopulations of KASUMI-1 and we have treated for 18 hours with CX-4945, a specific inhibitor of CK2 that is currently in phase II clinical trial for the treatment of hematological malignancies.

Annexin-V/IP staining revealed that CX-4945 is effective in inducing apoptosis both in LSC and CD34+/CD38+ subpopulations, but is intriguing the fact that the inhibition of CK2 leads a major apoptotic effect in LSC compartment as compared to the CD34+/CD38 cells.

Moreover, the inhibition of CK2 does not cause alteration both in Wnt/β catenin and in HH signaling pathway (except for GLI1, the expression of which is slightly increased whe CK2 inhibitor CX-4945 is present). PI3K/AKT pathway, instead seems to be altered by the inhibition of CK2, as the phosphorylation state of FoxO3 and of AKT itself is altered when CK2 is inhibited.

Finally, we wanted to determine if the inhibition of CK2 can potentiate the effect on LSCs of Daunorubicin, a chemotherapeutic drug used for the treatment of AML patients.

Our results shows that the combination of CX-4945 (CK2 inhibitor) with Daunorubicin increases the cytotoxic effect of the latter one, thus indicating that the inhibition of CK2 could represent a valid approach to ameliorate the chemotherapy used for AML patients.

In conclusion, all our data indicates that CK2 possesses an important role both in hematopoiesis and leukemogenesis.

In particular our KO model indicates that CK2β is an essential regulator of hematopoiesis.

The analysis conducted in LSC derived both from AML patients and Kasumi-1 cell line indicate that CK2 is involved in control the PI3K/AKT activation pathways in AML cells, and in the survival of LSC as the inhibition of this kinase induces apoptosis in this cells and make them more sensitive to chemotherapeutic treatment.

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7. PUBLICATIONS

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