Università degli Studi di Milano
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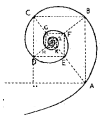

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PhD THESIS:

Regeneration-associated Wnt signaling activation in acute myeloid leukemia

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1.1 SOMMARIO

Recenti evidenze sperimentali su modelli murini indicano che la leucemia mieloide acuta (LMA) è contraddistinta dalla presenza di una popolazione cellulare definita come *Leukemia Initiating Cells* (LICs), la quale è in grado di autorigenerarsi dando inizio e sostenendo la LMA. Una questione tuttora dibattuta riguarda l'esclusiva presenza delle LICs in una ristretta popolazione cellulare definita dai marcatori CD34+CD38- e nuove evidenze sperimentali suggeriscono che una più ampia eterogeneità della componente cellulare caratterizzante le LICs sia contenuta nella suddetta popolazione. Uno dei marcatori più efficaci ai fini dell'arricchimento della popolazione staminale è l'antigene AC133, che definisce una popolazione cellulare di progenitori e cellule staminali identificate sia nella frazione cellulare $CD34^{bright}CD38-$ che nella frazione $CD34^{bright}CD38+$. L'AC133 è stato utilizzato, per la definizione della *cancer stem cell* (CSC) in grado di sostenere il tumore in altri contesti neoplastici rispetto alla LMA, quali tumori cerebrali umani e la leucemia linfoblastica acuta infantile. In recenti studi su modelli murini è emerso che il pathway WNT/β-catenine interpreta un ruolo importante nel processo patogenetico della LMA. Nonostante ciò, la relazione tra il WNT/β-catenine signaling e i programmi trascrizionali che sostengono l'autorinnovamento cellulare resta ancora poco chiara. A tal proposito è nata la necessità di identificare i programmi trascrizionali coinvolti nel mantenimento dello stato autorinnovativo delle LSCs. Considerando il ruolo di alcuni TFs nella riprogrammazione cellulare, abbiamo generato un dataset di regolatori trascrizionali (TRs) con l'obiettivo di interrogare i dati di espressione ottenuti mediante microarrays su frazioni cellulari AC133+ in una casistica di 33 pazienti con diagnosi di LMA e da 10 donatori sani. L'analisi multistep dei dati generati, ha evidenziato il coinvolgimento del pathway rigenerativo WNT-dipendente, come la funzione cellulare più significativamente deregolata nelle frazioni cellulari, arricchite per l'AC133, in pazienti affetti da LMA. L'obiettivo di questo studio è volto alla caratterizzazione dell'attivazione del Wnt pathway associato al contesto rigenerativo nella LMA. I nostri dati suggeriscono che il signaling canonico WNT/β-catenine è attivato nella frazione leucemica AC133+. Le evidenze ottenute suggeriscono l'attivazione costitutiva del Wnt signaling attraverso l'espressione delle β-catenine attive (il cui stato defosforilato

permette il loro l'accumulo nel citoplasma e la migrazione nel nucleo, promuovendo la trascrizione dei geni target) e del WNT10B, proteina associata al pathway rigenerativo nelle cellule staminali ematopoietiche. La consapevolezza che una linea cellulare primaria possa meglio mimare il contesto leucemico *in-vivo* ha portato all'allestimento di una coltura cellulare (A46) selezionata per la frazione AC133, ottenuta dal sangue midollare di un paziente affetto da LMA. La strategia per valutare *in vivo* gli effetti funzionali correlati ai segnali indotti dalle A46, è stata quella di utilizzare il modello di zebrafish come biosensore. Le cellule leucemiche AC133+, negli embrioni di zebrafish, sono in grado di sviluppare strutture ectopiche secondarie attivando i marcatori dell'organizzatore che agiscono a valle del WNT pathway. La stretta associazione tra insorgenza tumorale e danno tissutale cronico è più volte emersa in letteratura. Inoltre durante il processo rigenerativo indotto da un danno tissutale è stato evidenziato, nell'omeostasi midollare, un'aumentata attivazione del signaling del Wnt/β-catenine. In accordo con quanto riportato in letteratura, i nostri risultati forniscono risultati a sostegno di una de-regolazione nel signaling rigenerativo Wnt-dipendente in pazienti affetti da LMA.

1.2 ABSTRACT

Ample evidence exists in mouse models that acute myeloid leukemia (AML) develops through stepwise acquisition of collaborating genetic and epigenetic changes in self-renewing leukemia-initiating cells (LICs) that exhibit a committed myeloid immunophenotype. Recently, critical questions emerged regarding the characterization of LICs in the CD34+CD38- fraction and the AC133 antigen (a glycosylation-dependent epitope of CD133) seems to be one of the markers more appropriate to enrich for the LICs-containing fraction. The requirement of the Wnt/β-catenin pathway in the pathogenesis of acute myeloid leukemia (AML) has recently emerged in mouse models. However, its relationship to genetic programs promoting retention of self-renewing leukemia stem cells (LSCs) remains elusive. Hence, the need to identify transcriptional programs involved in the maintenance of a self-renewing state in LSCs. Given the predominant role of transcription factors (TFs) function in myeloid leukemia and recent progresses in reprogramming research for a critical role of TFs in the establishment and maintenance of cellular phenotypes, here we generated a specially designed transcriptional regulators (TRs) dataset to interrogate expression microarray data obtained from AC133+ cells in AML patients and healthy donors. Our data suggest that the canonical Wnt/β-catenin signaling is activated in leukemic human long-term reconstituting AC133+ restricted cells. In a series of molecular and functional studies we found that leukemic cells AC133+ synthesize and secrete WNT10B, a hematopoietic stem cells (HSCs) regenerative-associated molecule, and increase the levels of dephosphorylated β-catenin. Nevertheless, AML AC133+ cells, in zebrafish embryos, are able to develop ectopic structures by activating organizer markers that act downstream of the Wnt pathway. In light of the long-known association between cancer and chronic tissue injury, and because of the higher homeostatic range of Wnt/β-catenin signaling occurring during regeneration upon an acute injury, altogether our data provide compelling evidence that regeneration-associated Wnt signaling exceeds the homeostatic range in human AML and affects responsive cells whose renewal is promoted by Wnt pathway activity.

INDEX

INDEX OF FIGURES

LIST OF ABBREVIATIONS

LMA = Leucemia mieloide acuta
HSC = Hematopoietic Stem Cell
LIC = Leukemia Initiating Cell
CSC = Cancer stem cell
AML = Acute Myeloid Leukemia
TR = Transcription regulators
Wnt = wingless-type MMTV integration site family
Shh = Sonic Hedgehog
Hoxb4 = Homeobox B4
Bmi-1 = BMI1 polycomb ring finger oncogene
Lnk = Lymphocyte-specific adapter protein
FGF-1 = Fibroblast growth factor-1
CFU-S = Colony forming units-spleen
BM = Bone marrow
CMP = Common myeloid progenitor
CLP = Common lymphoid progenitor
LMPP = Lymphoid-primed multipotent progenitor
LT-HSC = Long term hematopoietic stem cell
ST- HSC = Short term hematopoietic stem cell
MPP = Multipotent progeniors
GMP = Granulocyte-monocyte progenitor
MEP = Megakaryocyte-erytrocyte progenitor
SNO = N-cadherin-expressing osteoblasts
CXCL-12 = CXC-chemokine ligand 12
CAR = CXCL-12-abundant reticular
ECM = Extracellular matrix
BMPRIA = Bone morphogenetic protein receptor type IA
PTH = Parathyroid hormone
BMCE = Bone-marrow sinusoidal endothelial cells
VCAM1 = Vascular cell adhesion molecule 1
LSC = Leukemia Stem Cell
LS-ICs = SCID leukemia-initiating cells
NOD/SCID = Nonobese diabetic/ several compromise immunodeficient.
WT1 = Wilms tumor 1
AML1-ETO = Acute myeloid leukemia 1 protein - Eight twenty one protein
BCR-ABL = Breackpoint cluster region – Abelson oncogene
PML-RARα = Promyelocytic leukemia – Retinoic Acid Receptor α
MLL = myeloid/lymphoid or mixed-lineage leukemia
MAb = Monoclonal antibody
TIC = Tumor Initiating cell
ALL = Acute lymphoblastic leukemia
ITD = Internal tandem duplication
CML = Chronic Myeloid Leukemia
hnRNPE2 = heterogeneous nuclear ribonucleoprotein E2
IL-3Rα = Interleukin-3 receptor alpha
C/EBPα = CCAAT/enhancer binding protein
RUNX1 = runt-related transcription factor 1
CBFβ-MYH11= Core binding factor beta - myosin, heavy chain 11

FAB = French-American-British classification
SPI1 = SFFV proviral integration
iPS = Induced pluripotent stem
Oct4 = Octamer-binding protein 4
Sox2 = SRY-related HMG-box
Klf4 = Kruppel-like factor 4
c-Myc = myelocytomatosis viral oncogene homolog
Hip = huntingtin interacting protein 1
Ptch = Patched
Smo = Smoothened
Sufu = Suppressor of fused
Gli = glioma-associated oncogene family zinc finger
BCC = basal-cell carcinoma
HH = Hedgheog
SHH = Sonic/Hedgheog
BMP4 = bone morphogenetic protein 4
TGF-β = transforming growth factor beta
AF9 = ALL1-fused gene from chromosome 9 protein
APC = adenomatous polyposis coli
Tcf/LEF = Transciption factor / lymphoid enhancer-binding factor 1
Fz = Frizzled
LRP5/6 = low density lipoprotein receptor-related protein
GSK3β = glycogen synthase kinase 3
Notch1 = Neurogenic locus notch homolog protein 1
PPARδ = peroxisome proliferator-activated receptor delta
PI3K/AKT = phosphoinositide-3-kinase / v-akt murine thymoma viral oncogene homolog
FLT3 = fms-related tyrosine kinase 3
SMYD3 = SET and MYND domain containing 3
DKK = dickkopf homolog
PROP-1 = prophet of Pit1, paired-like homeodomain transcription factor
PYGO2 = Pygopus 2
KLHL12 = kelch-like 12
E2F1 = E2F transcription factor 1
DACT 1= dapper, antagonist of beta-catenin, homolog 1
HBP1 = HMG-box transcription factor 1
STAT3 = signal transducer and activator of transcription 3
ABCC = ATP-binding cassette, sub-family C
DLX3 = distal-less homeobox 3
MARK4 = MAP/microtubule affinity-regulating kinase 4
TNTG = Tris-NaCl-Triton-Glycerol
BSA = Bovin Serum Albumin
ABC = Active β catenin
CM = Conditioned Medium
WISH = wholemount in-situ hybridization
DAPI = 4',6-diamidino-2-phenylindole
IQR = Interquantile range
MNC = Mononuclear cells
hpf = hour post fertilization
boz = bozozok
sqt = squint
gsc = goosecoid
Cnh = chordoneural hinge
n = notochord
ntl = no tail
pax2a = paired box 2
OV = Otic vesicles
OS = Optic Stalk
e = eye
MHB = midbrain-hindbrain boundary

2. INTRODUCTION

2.1 Stem cells and their hallmark

Stem cells are defined by their ability to self-renew, which means they are replenished by leaving at least one identical copy of themselves behind after each cell division, and to differentiate to all the mature cells of the organ or tissue to which they belong[1]. Both characteristics need to be full-filled. Self-renewal without differentiation is what bacteria and other unicellular organisms do, and differentiation without self-renewal is the hallmark of cells in developmental transit between stem cells and mature cells. Thus, the ultimate test for a candidate stem cell is that it, as a single cell, should be able to completely recreate the tissue from which it is derived. This criterion, in its strictest sense, is to date only fulfilled by murine hematopoietic stem cells (HSCs)[2], and might not be applicable to all kinds of stem cells.

Stem cells are classified by the diversity of their progeny into:

- TOTIPOTENT STEM CELLS are one of the most important stem cell types because they have the potential to develop to any cell found in the human body. Moreover they have the ability to replicate in unlimited numbers without lose their total potency thus they can create an entire organism.

- PLURIPOTENT STEM CELLS are self-replicating cells that have the potential to develop into cells and tissues of the three primary germ layers (ectoderm, mesoderm, endoderm).

- MULTIPOTENT STEM CELLS are unspecialized cells that have the ability to self-renew for long periods of time and differentiate into more specialized cells with specific function. Multipotent stem cells are essentially committed to produce specific cell types that give rise to a specific tissue.

Recently a number of papers have been described an unexpected plasticity of adult tissue stem cells blood which could be turned into heart[3-5], pancreas[6-7], muscle[8-10], brain[11-12], liver[13-14], skeletal[15-16] or more tissues, and stem cells from other tissues could be turned into blood[17,18,19]. Several models have proposed different possible mechanisms responsible of this plasticity, such as **transdifferentiation** of tissue specific stem cells into other tissue lineages; **de-differentiation** to a more primitive and multipotent stage with subsequent re-differentiation along a different lineage; the simultaneous occurrence of many different tissue restricted stem cells residing in the same organ; the existence of rare pluripotent stem cells with the capacity to generate progeny of all germ layers; or fusion of a stem cell (or any cell) with a cell of a different lineage. A stem cell can self renew by two different types of cell divisions either by a **symmetrical division**, where both daughter cells are stem cells, and which therefore expands the stem cell pool, or by an **asymmetrical division**, where one daughter is a stem cell and the other enters differentiation, leading to a steady state with regard to the stem cell compartment. In a third, non self-renewing type of division is the stem cell symmetrically producing two differentiated daughters, which depletes the stem cell pool. Much has been made of the idea that asymmetric division is a characteristic trait of stem cells. However, it seems obvious that most, if not all, stem cells at times divides mainly (or perhaps even exclusively) symmetrically to expand themselves, as for example during embryonic development, or during wound healing or regeneration; and therefore this trait cannot be necessary for the stem cell identity[20]. Very little data exist on the mode of division of mammalian stem cells *in vivo*[20, 21], and it is theoretically conceivable (even if unlikely) that stem cells upholds their activity mainly, or even exclusively, by symmetrical divisions, producing either more stem cells or more differentiated progeny, depending on the needs of the organism. The scarcity and quiescence of most stem cells *in vivo* makes this question technically very challenging to approach. Regardless of the mode of cell divisions, stem cells can perpetuate themselves and serve their host during a life time and beyond, and recent years have seen a dramatic progress in identifying signalling pathways influencing HSC self renewal, such as Wnt[22,23], Notch, sonic hedgehog (Shh)[24], Hoxb4 [25-28] , Bmi-1, Lnk , and thrombopoietin [29]. The use of extrinsic signalling to amplify HSCs, like cytokines and growth factors,

has shown at best very limited expansion[30-35], with perhaps recent reports of purified Wnt3a protein, fibroblast growth factor-1 (FGF-1) [36] and angiopoietin-like proteins[37] expanding murine HSCs as notable exceptions. The quest for promoting HSC self renewal continues, however, since success could clinically benefit HSC transplantations where donor cells are limited in numbers (such as adult patients receiving cord blood transplants) or where large doses of HSCs are desired to overcome immunological barriers (such as for haploidentical transplantations). Mastering HSC self-renewal can also enhance gene delivery to HSCs for gene therapy[38]. The most striking feature of stem cells is that they can self renew: i.e. produce exact copies of themselves. It is often stated that stem cells can perform three kinds of cell divisions: symmetrical divisions producing two new stem cells, or asymmetrical divisions producing one stem cell and one daughter cell which commits to differentiation, or symmetrical divisions in which two committed daughter cells are produced. It is unclear what influences the HSC to choose either self-renewal or differentiation, but most believe that the decision is largely stochastic, or random – which would imply that a true HSC inherently, at the same time, contain both choices, as yet unrealized. However, once the differentiation choice is realized in cell division, the HSC status of the preceding cell collapses and considering that we do not know to what extent a cell can differentiate without dividing suggests further complexity.

2.2 HEMATOPOIETIC STEM CELLS AND HEMATOPOIESIS

2.2.1 Hematopoietic stem cells

HSCs are the best characterised of all adult stem cells for several reasons. First, they represent a minor subpopulation of dissociated cells within a floating organ, which is easily accessible in relatively large quantities. Second, they are vitally important providers of cells in an organ with a very high cell turn-over which may make both *in vitro* and *in vivo* assays faster and more yielding. A third reason is that the discovery of HSCs now dates almost half a century back, during which time instrumental methodological developments and insights have been made,

which have aided in the enormous progress in the field that we witness today. The first steps towards identifying HSCs was taken 1961 by Till and McCulloch, as they identified the **colony forming unit – spleen (CFU-S)**[39]. In their work on radiation sensitivity of tissues they stumbled upon the observation that bone marrow (BM) cells intravenously injected into lethally irradiated mice gave rise to macroscopically visible colonies of proliferating cells in the spleens of these animals. The colonies contained hematopoietic cells of all myeloid lineages at variable maturational stages, and were derived of recipient BM cells only, as irradiated but not transplanted mice never displayed such colonies. These colonies were termed colony forming units (CFUs). In order to prove that these colonies were derived from single cells, recipient mice received a sublethal irradiation dose, followed by injection of donor BM cells and subsequent further irradiation of the recipient up to a lethal dose, which exposed the donor BM cells *in vivo* to a significant irradiation dose, enough to confer recognizable chromosomal abnormalities in a proportion of the cells without killing them, and at the same time ablating the host HSCs. In some of the resulting colonies abnormal karyotypes were found by cytogenetic analysis in all investigated cells, and as chromosome breakage by irradiation is a random process, the clonality of the colonies were proven. In a third landmark paper they defined a stem cell as a cell that should have the capacity to give rise to differentiated progeny and have an extensive proliferative potential, two features they already had been able to assay in the spleen colonies, but a stem cell should also posses the capacity for self-renewal, such that an organ like the BM throughout the life-time of an organism always has stem cells that can continuously give rise to both differentiated progeny and retain a sufficient stem cell pool. In order to test the CFU-S for self-renewal capacity they injected dissected and resuspended CFU-S into secondary hosts, and found that primary spleen colonies often, or possibly always, contained cells capable of forming more spleen colonies. Thereby they proved that their CFU-S assay detected cells with the capacities for differentiation, extensive proliferation and self-renewal, and cautiously suggested that they might be HSCs.

2.2.2 Hematopoietic hierarchy

Over the past four decades, much has been learned regarding the hematopoietic hierarchy that ultimately produces all mature blood-cell types from rare hematopoietic stem cells. This has been done to a greater detail in mice than in humans. The multipotent hematopoietic compartment is heterogeneous and consists of cells with decreasing levels of self-renewal capacity but increasing mitotic activity[40].

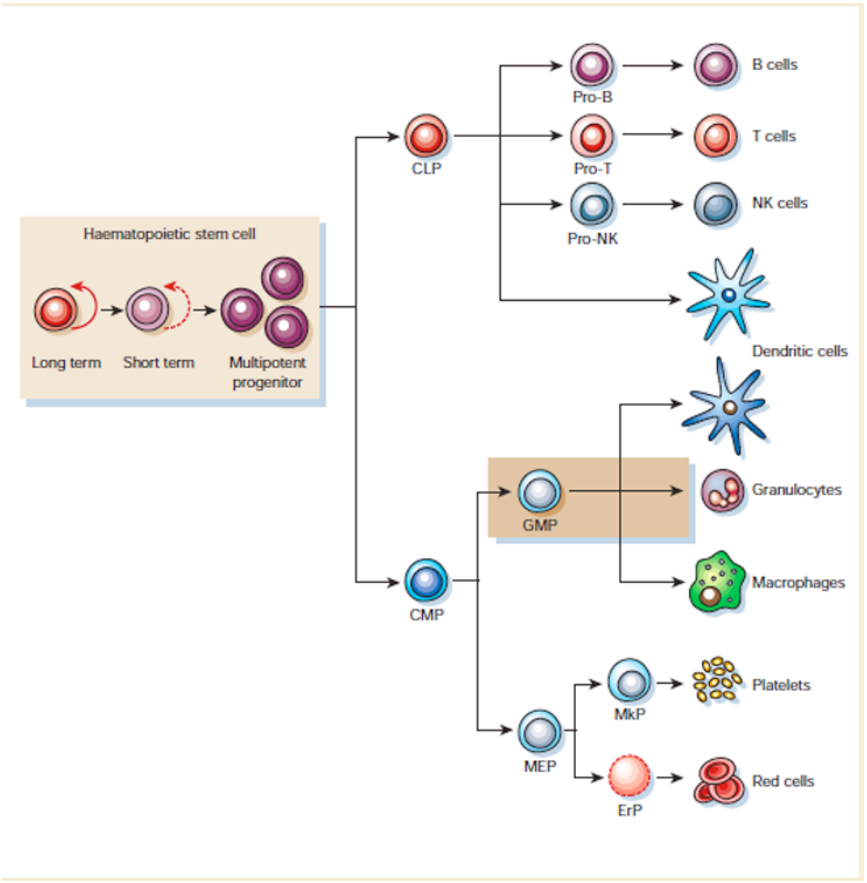

Figure 1. Cellular hierarchy of the hematopoietic system. HSCs can be subdivided into long-term self-renewing HSCs, short-term self-renewing HSCs and multipotent progenitors (red arrows indicate self-renewal).They give rise to common lymphoid progenitors (CLPs; the precursors of all lymphoid cells) and common myeloid progenitors (CMPs; the precursors of all myeloid cells). Both CMPs/GMPs (granulocyte macrophage precursors) and CLPs can give rise to all known mouse dendritic cells. (from *Reya et al. Nature, 2001*)

This compartment in mice can be phenotypically subdivided and prospectively purified into **long-term HSCs** (**LT-HSCs**, sustaining hematopoiesis for life), **short term HSCs** (**ST-HSCs**, sustaining hematopoiesis for up to 6 weeks) and **multipotent progenitors** (**MPPs**, without any self renewal capacity). A similar hierarchy is evident in humans, although it has not been phenotypically characterised[41,42]. For a long time, the first lineage branching point has been thought to be between myeloid and lymphoid cells. Common progenitors for these two cell populations (**common myeloid progenitor**, **CMP**, and **common lymphoid progenitor**, **CLP**) have been identified in both mice[43,44] and humans[45-47]. Recently, however, an alternative model has been proposed, where the first branching point is represented by a division between a common progenitor for red blood cells and megakaryocytes (the precursor for platelets) on the one hand and a progenitor with potential for all other lineages, called **lymphoid-primed multipotent progenitor** (**LMPP**), on the other[48]. An alternative way of interpreting the data has been offered though[49]. The CLP gives rise to committed precursors differentiating into the effectors cells of the lymphoid system; B-, T- and NK cells, and the CMP differentiates into **granulocyte-monocyte progenitor** (**GMP**), the common precursor for granulocytes and monocytes/macrophages, and the **megakaryocyte-erytrocyte progenitor** (**MEP**)[43,47], although it is somewhat unclear if this last progenitor is a direct descendant from a MPP or from a CMP. None of these oligoclonal progenitors have any measurable self-renewal capacity[40].

2.2.3 HSC and their nice

Although during homeostasis, a small minority of HSCs are present in the circulation, the vast majority of HSCs are found in the bone marrow and they are thought to be localized in specific microenvironments called stem cell niches. A stem cell niche can be defined as a three-dimensional spatial structure in which one or several stem cells are housed. The function of a stem cell niche is

maintenance of stem cell numbers by allowing self-renewal in the absence of differentiation[50]. This type of niche may be thought of as a "self-renewing" or "homeostatic" niche[51]. Such a self-renewing stem cell niche would be the essential unit that maintains normal tissue homeostasis and would be located close to the border separating the niche from the non-niche microenvironment, which could provide signals that would induce differentiation and/or cell division. The regulation of the balance between HSC self-renewal and differentiation is dependent on the interaction between HSCs and the cells comprising the niches. Moreover, the maintenance of long-term dormant HSCs, which may serve as a reserve pool of stem cells to be activated in response to injury signals, is thought to occur in "dormant" niche[51-52].

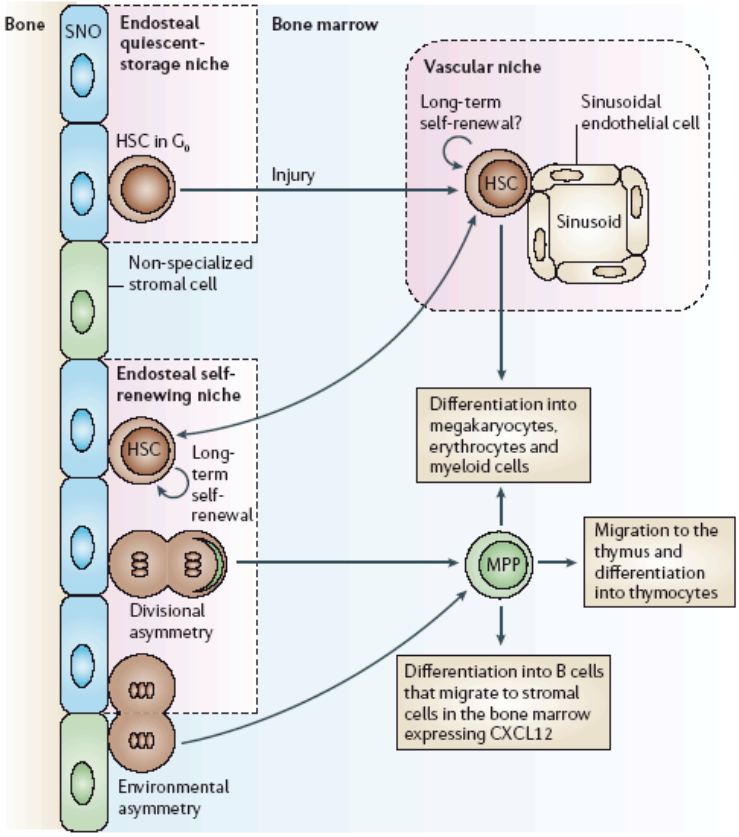

Figure 2. Endosteal bone surfaces are lined with stromal cells. Spindle-shaped N-cadherin-expressing osteoblasts (SNOs) serve as niche cells to maintain quiescence and prevent differentiation of attached haematopoietic stem cells

(HSCs). The quiescent endosteal niche would maintain dormant HSCs longterm. In response to injury, quiescent HSCs might be activated and recruited to the vascular niche. The self-renewing niche would contain quiescent HSCs intermingled with dividing HSCs. Self-renewing HSCs produce multipotential progenitors (MPPs) either by divisional or environmental asymmetry. More HSCs can be generated by symmetrical divisions which might provide the vascular niche with new HSCs. Whether HSCs long-term self-renew in the vascular niche remains to be determined, and it is probable that influx of HSCs from endosteal niches is necessary to ensure prolonged haematopoietic-cell production at the vascular niche. HSCs in the vascular niche promote differentiation and expansion along megakaryocytic and other myeloid-cell lineages, particularly in response to injury. MPPs can give rise to all haematopoietic lineages, including B-cell precursors attached to randomly distributed CXC-chemokine ligand 12 (CXCL12)-expressing stromal cells that constitute a B-cell niche. Unidentified T-cell precursors migrate to the thymus where they enter a microenvironment, promoting T-cell maturation. (from *Wilson A. and Trumpp A. Nat Rev Immunol. 2006*.)

The localization and cellular composition of niches harbouring HSCs is still poorly understood. The main reason for this paucity of information is that unlike most other stem cells, HSCs are not spatially embedded in a solid tissue structure, but are migratory and hidden inside bones. Morphological and functional studies have indicated that HSCs and immature colony-forming progenitors reside in relation to the bone surface, away from the central cavity[53-55]. In the mouse BM two types of niches have been identified.

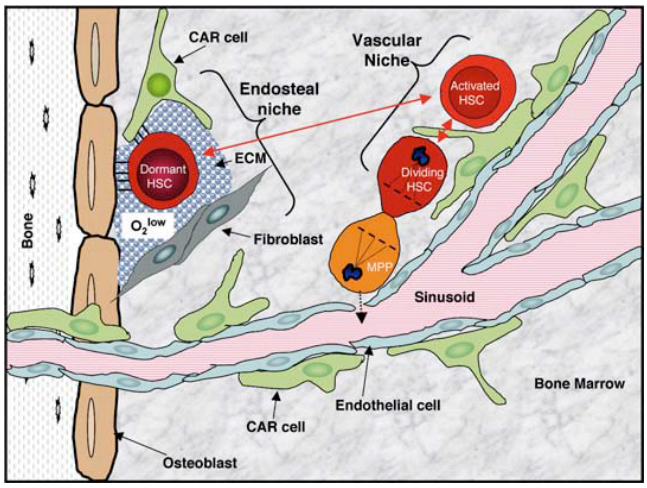

Figure 3. The two different locations of hematopoietic stem cells (HSCs) in the mouse bone marrow. Dormant HSCs are in direct contact with specialized osteoblasts lining the endosteal surface of the bone. These cells are also in contact

with CXCL12-abundant reticular (CAR) cells that are found at the endosteum, but are more frequently associated with sinusoids. In the **"**endosteal niche**"** or **"**dormant niche,**"** CAR cells and osteoblasts together with stromal fibroblasts and potentially other cell types such as osteoclasts (not shown) generate a hypoxic environment with a dense extracellular matrix (ECM) that retains the dormancy of HSCs. Dormant HSCs are activated stochastically or in response to injury signals. Activated HSCs are located adjacent to perivascular CAR cells near sinusoids forming the vascular niche. On a self-renewing division, asymmetry is generated, which leads to the generation of two daughter cells: a multipotent progenitor (MPP) and an activated HSC. MPPs and/or their progeny enter the circulation through the fenestrated endothelium of sinusoidal microvasculature. (from *Wilson A. et all.Ann N Y Acad Sci. 2007*).

First the endosteal niche located at the endosteum of trabecular bone and comprising specialized osteoblasts, CXCL12-abundant reticular (CAR) cells osteoclasts and stromal fibroblasts. It has been observed that the bone tissue, and particularly the bone-forming **osteoblast**, by itself can support HSCs in cocultures[56-58]. More recently, several mutant mice in which hematopoiesis is defective as a consequence of primary defects in bone development or remodelling, have implicated osteoblasts and/or osteoclasts in the formation and function of the bone marrow HSC environment or niche[59-61]. In two different works, genetically engineered mouse models where used to convincingly show the osteoblast dependency of HSCs[62,63]. In the first, Zhang *et al* observed that in mice, in which the bone morphogenetic protein receptor type IA (BMPRIA) were conditionally inactivated, an increase in HSCs correlated with an increase in trabecular bone lining within the BM. Histological analysis revealed that long-term HSCs attached only to immature, spindle-shaped osteoblasts expressing N-cadherin, and that N-cadherin adhered specifically to β-catenin on HSCs. The number of the spindle shaped osteoblasts correlated to a high degree with the increased number of HSCs, suggesting that the rise in HSC numbers where due to an expanded niche. In the second paper, Calvi *et al.* utilized the fact that osteoblasts are activated by parathyroid hormone (PTH), and constitutively expressed the PTH receptor under the control of a osteoblast-specific promoter (α1-collagen promoter) in mice. They observed, similar to Zhang *et al.*, a correlation between increased osteoblasts and an increase in HSC numbers and function. In both papers, the increase in hematopoietic cells was restricted to HSCs. A third group reported the complementary finding, that induced osteoblastic

deficiency was followed by reduction of HSC numbers, and that restoration of osteoblast function normalized hematopoiesis[64]. The presence of a second specialized niche, the vascular niche has been recently postulated where HSCs are found next to CAR cells adjacent to BM sinusoids, which are low pressure vessels with a fenestrated. A close interaction between HSCs and endothelial cells is not unexpected because both lineages arise from a common embryonic precursor, the haemangioblast[65]. Bone-marrow sinusoidal endothelial cells (BMECs) are functionally and phenotypically distinct from microvasculature endothelial cells of other organs 66. Indeed BMECs constitutively express cytokines such as CXC-chemokine ligand 12 (CXCL12) and adhesion molecules such as endothelial-cell (E)-selectin and vascular cell adhesion molecule 1 (VCAM1) that are important for HSC mobilization, homing and engraftment[67-69]. A vascular bone-marrow HSC niche has previously been predicted to form during HSC mobilization after myeloablation. Quiescent HSCs detach from the endosteal niche and migrate towards the centre of the bone marrow to the vascular zone from where they re-establish haematopoiesis[66,68,70]. The finding that CD150+ HSCs are attached to the sinusoidal endothelium now raises the possibility that a vascular bone marrow HSC niche might also exist during homeostasis[71]. It is probable that the pool of HSCs located in the vascular and self-renewing endosteal niches are freely exchanged to maintain homeostasis in a constantly changing haematopoietic environment. In addition, HSCs that are located in the self-renewing endosteal niche produce multipotential progenitors (MPPs) by divisional and/or environmental asymmetry. These cells give rise to myeloid-cell lineages as well as lymphocyte precursors. B-cell progenitors are uniformly distributed throughout the bone marrow attached to CXCL12-expressing fibroblasts (in the B-cell niche)[72,73]. Because deletion of osteoblasts results in extramedullary hematopoiesis[74], the vascular bone marrow HSC niche alone might not be sufficient to maintain long-term hematopoiesis. This indicates that in the bone marrow the vascular niche might be a secondary niche, requiring an influx of HSCs from the primary endosteal niches. Collectively, the vascular and endosteal niches strongly cooperate to control HSC quiescence and self-renewing activity (and therefore HSC number), as well as the production of early progenitors to maintain homeostasis or re-establish it after injury.

2.3 LEUKEMIA INITIATING CELL (LIC)

2.3.1 Definition and origin of leukemia initiating cell in acute myeloid leukemia

The idea that only a minor subpopulation of so called “cancer stem cells” is responsible for maintenance of the neoplasm emerged about 50 years ago, with the best evidence with the discovery that the large majority of AML blasts do not proliferate and only a minority of them (≈ 1%) is capable of forming colonies when plated in a semisolid medium: the colonies generated under these conditions were called AML-CFU (colony-forming units)[75]. However, there are many doubts that AML-CFUs may represent leukemia stem cells (LSCs). The first demonstration of the existence of LSCs comes from the identification of a very rare population of human termed SCID leukemia-initiating cells (SL-ICs) that were capable of propagating acute myeloid leukemia in a xenograft transplant using NOD/SCID immunodeficient mice. The leukemias that developed in the secondary recipients closely resembled the human cancer, demonstrating that SL-ICs have long-term self-renewal capabilities and also determine the stage of the differentiation block during leukaemogenesis[75]. A given leukemia can be viewed as a newly formed aberrant hematopoietic tissue initiated by tumorigenic leukemic cells that have kept or reacquired the capacity for indefinitive proliferation through accumulated mutations. This concept suggests that leukemias are produced by a few leukemia initiating cells (LICs) that undergo an aberrant and poorly regulated process of organogenesis analogous to that of normal HSCs. Because normal stem cells and LICs share the ability to self-renew, as well as various developmental pathways, it has been postulated that newly arising cancer cells appropriate the machinery for self-renewing cell division that is normally expressed in stem cells and become leukemic as the result of accumulated mutations. Because normal stem cells and LICs share the ability to self-renew, it is possible that LICs are HSCs which have accumulated mutations and have become leukemic. There are two reasons to think that this may be the case. First, because stem cells have the machinery for self-renewal already activated, maintaining this activation may be simpler than turning it on *de novo* in a more differentiated cell; that is, fewer mutations may be required to maintain self-renewal than to activate it ectopically. Second, by self-renewing, stem

cells often persist for long periods of time, instead of dying after short periods of time like many mature cells in highly proliferative tissues. This means that there is a much greater opportunity for mutations to accumulate in individual stem cells than in most mature cell types.

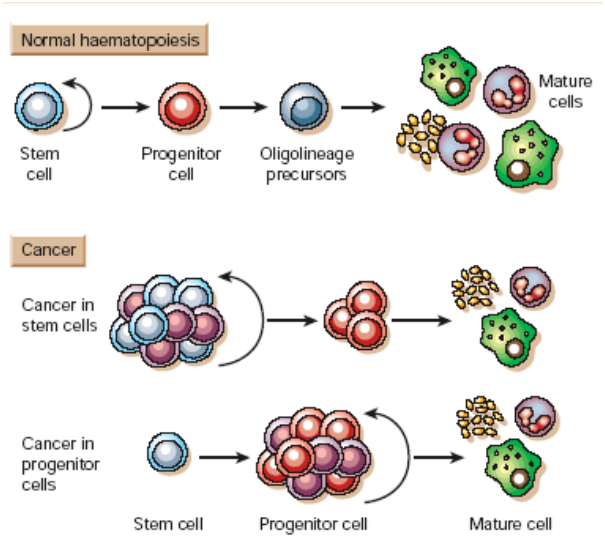

Figure 4. Comparison of self-renewal during haematopoietic stem cell development and leukaemic transformation. Unlike physiologic hematopoiesis, during leukemic transformation self-renewal pathways resulted impared, thus the leukemic stem cell become an easy target for mutations. If the mutational event arises from the progenitors compartment, it is necessary that the cells hit mantain a great self-renewal potential. (from *Reya T. et all. Nature, 2001)*

Even restricted progenitor cells are less likely than stem cells to undergo neoplastic transformation because they proliferate for a much shorter period of time before terminally differentiating. Restricted hematopoietic progenitors of the lymphoid[40] and myeloid lineages all fail to self-renew detectably on transplantation. Thus, restricted progenitors would first need to acquire the extensive self-renewal potential of stem cells to have the opportunity to experience additional mutations that would lead to transformation. Nonetheless, restricted progenitors could potentially be transformed either by acquiring mutations that cause them to self-renew like stem cells, or by inheriting existing mutations from stem cells such that

only a single mutation is required in the progenitors to cause transformation. Reya et al.[40] and Passeguè et al.[76] showed the necessity to highlight the cell that gives rise to the malignant transformation. Bonnet et al.[77] have demonstrated that the cells capable of initiating human acute myeloid leukaemia (AML) in NOD/SCID (non-obese diabetic/severe combined immunodeficiency) mice have a CD34+CD38– phenotype in most AML subtypes, and thus have a phenotype similar to normal HSCs. Conversely, $CD34^{-}CD38^{+}$ leukemia cells cannot transfer disease to mice in the vast majority of cases, despite the fact that they exhibit a leukaemic blast phenotype. This suggests that normal HSCs rather than committed progenitors are the target for leukemic transformation[78]. Several studies have analyzed in depth the characteristic AML mutational events that can lead to the leukemogenic process, such as one of the most frequent chromosomal abnormalities in AML involve the 8;21 translocation, which results in AML1–ETO chimaeric transcripts in leukaemic cells. In human HSCs from patients in remission, AML1–ETO transcripts were found in a fraction of normal HSCs in the marrow[79]. These prospectively isolated HSCs and their progeny were not leukemic, and could differentiate to normal myeloerythroid cells *in vitro*. This indicates that the translocation occurred originally in normal HSCs and those additional mutations in a subset of these HSCs or their progeny were necessary for the leukaemia development. Miyamoto et al.[79] suggested that the normal HSCs were CD34+CD38–Thy-1+, whereas the leukemic blasts were CD34+CD38–Thy-1–. Committed progenitors or even differentiated cells may also be the targets of malignant transformation. Leukemic fusion proteins such as BCR-ABL, PML-RARα can be transforming in myeloid progenitors and be able to induce leukemia. In *de novo* human leukemia, stem cells likely accumulate mutations necessary for the neoplastic proliferation even if the effects are detectable only in restricted progenitors. Thus, mutations accumulate in stem cells may lead to neoplastic proliferation of primitive progenitors. A final hypothesis of the malignant clones origin is the cell fusion between a HSC and a progenitor cell, in which one is normal and the other is partially transformed. In this case the cell fusion contributes to the full malignant potential. There are, however, any data supporting this sequence of events[80-81].

2.3.2 CD34+CD38- as population enriched for the LICs: is that the only one?

LICs can be prospectively isolated based on surface-marker expression in the BM and peripheral blood of AML patients. Dick and Bonnet demonstrated that the leukemia initiating cells are identified by the immunophenotypic marker Lin- CD34+ CD38-. Their experiments showed that the capacity to transfer human AML to recipient mice resided exclusively within the CD34+ CD38- fraction. This cell fraction represents from 0.1 to 1% of the AML cell population contained all SL-ICs, whereas only clonogenic leukemia progenitors were found in other fractions. Upon transplantation of CD34+ CD38- cells, the entire cellular diversity was recapitulated, conclusively establishing that leukemia is a hierarchy sustained by rare LSCs, closely resembling normal development[75]. The mechanism that controls the hierarchical structure of the LICs poll derives from a heterogeneity in the self-renewal potential of individual LICs. However recently Taussig et al.[82] have shown that using anti-CD38 antibodies there was a profound inhibitory effect on the engraftment of both normal and leukemic repopulating cells. Thus by treating mice with immunosuppressive antibodies they have demonstrated that the SL-ICs from some AML samples were CD34+ CD38+, indicating a greater heterogeneity in the leukemic stem cell compartment. Moreover they observed from 7 AML patients that the SL-ICs was found also in the CD34+ CD38- and it is possible that these cells might have self-renewal potential. This appear to conflict with previous studies[83, 77] suggesting that SL-ICs are restricted to the CD34+ CD38-. The likely explanation for this apparent discrepancy lies in the heterogeneity of AML. The similarity of AML SL-ICs and normal repopulating cells (ie, CD34+CD38-) led some authors to hypothesize that AML is derived from an HSC. Others argue that the features of myeloid differentiation that define AML point to a progenitor origin. In support of this, it has been demonstrated that mouse myeloid progenitors can be transformed by the MLL oncogene[84]. It suggests that SL-ICs derived from a normal progenitor would have phenotypic features of progenitors with expression of CD34 and CD38. Notably, it has been demonstrated that one AML patient, which contained the translocation t(11;19)(q23;p13.1) involving the MLL oncogene showed SL-ICs detectable only within the CD34+CD38+ fraction[82]. Recent data, though, suggest a slightly different point of view: Barabe et al.[85] used the MLL oncogene to transform

human cord blood cells into acute leukemia. This work highlighted that leukemic progenitors acquire self-renewal capacity and become a second generation of SL-ICs (the first generation comprising SL-ICs derived from HSCs). In patients, similar events may occur; this is a possible explanation for the presence of 2 types of SL-ICs in some samples: one with a primitive (CD34+CD38-) phenotype and one with a progenitor (CD34+CD38+) phenotype. Barabe et al reported that the second-generation SL-ICs came to predominate as the leukemia evolved. The same thing may happen in some patients. By the time of diagnosis, second-generation SL-ICs with a progenitor phenotype (CD34+CD38+) may predominate and SL-ICs with a primitive phenotype (CD34+CD38-) may no longer be detectable. This explanation is an alternative to the direct transformation of normal myeloid progenitors. In conclusion, the hierarchical level at which the LICs are recruited in AML is still mostly elusive. The critical questions emerged regarding the characterization of LICs in the CD34+CD38- fraction suggests that other markers could be more appropriate to enrich for the LICs-containing fraction. For example CD44 was shown to be important for homing and engraftment of BCR-ABL positive CML and AML stem cells[87]. In a NOD/SCID transplantation model, antibody blockade by an anti-CD44 mAb led to a failed engraftment of leukemic cells, although the capacity to initiate leukemia when directly injected into bone marrow was maintained. The interleukin-3 receptor alpha (IL-3Rα) chain (CD123) was widely reported to be overexpressed on AML cells and on leukemic stem cells, but not on normal hematopoietic stem cells[86]. A neutralizing monoclonal antibody against CD123 inhibited the IL-3- mediated survival of leukemic stem cells in vitro as well as homing, engraftment, expansion, and serial transplantation of AML cells in immunodeficient mice, with lower effects on normal hematopoietic cells[87]. Recent studies have shown that CD47 is upregulated on AML leukemic stem cells in comparison to normal hematopoietic stem cells and its level of expression on AML blasts is associated with poor prognosis. CD96 was described as a tumor marker for T-ALL and AML, characteristic for the AML stem cell in particular. Particularly, it was shown that the majority of CD34+CD38− AML cells in the majority of cases (19/29) abundantly express CD96, while the expression of this antigen is limited only to a minority of CD34+CD38− cells[88]. CD34+CD38−CD96+ cells are able to engraft immunodeficient animals[88]. CD96 is particularly expressed on AMLs with

mutations of the Wilms tumor 1 (WT1) gene and is associated with a poor prognosis.

2.3.3 AC133: The molecule of the moment

Between the other markers that could be more appropriately used to enrich for the LICs-containing fraction, a specific mention must be given to the epitope CD133 (prominin-1). It was the first identified member of the prominin family of pentaspan membrane proteins. The specific functions and ligands of the prominins are still relatively unclear, but they are distinct in their restricted expression within plasma membrane protrusions, such as epithelial microvilli and epididymal ductal epithelial sterocilia. In 1997, Yin et al.[89] produced a novel monoclonal antibody (MAb) that recognized the AC133 antigen, a glycosylation-dependent epitope of CD133, whose restricted expression in CD34+ progenitor populations from adult blood and bone marrow and fetal liver cells implied its function as a marker of haematopoietic progenitor cells. AC133+ cells were capable of long-term repopulation in xenografted animals and were believed to be more primitive than CD34+ cells, as AC133 was absent from endothelial cells or fibroblasts. Due to its expression by hematopoietic progenitors, interest has been directed towards the potential of CD133 as a cell surface marker of adult stem cells. In hematopoietic lineages in man, consistent with AC133 expression, CD133 antigen expression is restricted to CD34+ cells, although CD133 transcripts have been found in many human cell lines and differentiated cells[90]. Furthermore, AC133+/CD34+ cells have a higher clonogenic capacity and engraftment rate than AC133−/CD34+ cells[91-92]. Therefore, as AC133 is expressed only on stem and progenitor cells, whereas CD133 is also expressed on differentiated cells, the AC133 epitope is the most promising stem cell marker. Gallacher et al.[93] showed that AC133+ cells were the only subset amongst a CD34−CD38−lineage− population from human cord blood that could form CD34+ cells in culture, with an engraftment capability in the bone marrow of NOD/SCID mice 400-fold greater than the AC133− subset. Lang et al. showed the benefit of CD133+ cells for human allogeneic transplantation; injection of either CD133+ or CD34+ cells from both matched unrelated and mismatched-

related donors into ten patients produced successful reconstitution, but with a reduced T-cell depletion by the CD133+ selection[94]. Expanding evidence highlights the role of CD133 as a marker of tumor initiating cells (TICs) in various human tumours. CD133 was expressed in combination with CD44+ and $\alpha 2\beta 1$hi in approximately 0.1% of cells within a large series of prostate tumours, irrespective of their grade or metastatic state. AC133+ cells undergo multi-lineage differentiation to neurons, astrocytes, and oligodendrocytes *in vitro*, and can recapitulate the original tumour phenotype *in vivo*, unlike their CD133− counterparts. An interesting study has been performed on childhood acute lymphoblastic leukemia (ALL). ALL cells capable of long-term proliferation *in vitro* and *in vivo* were derived from the CD133+/CD19- subfraction. Moreover, these CD133+/CD19- cells could self-renew to engraft serial nonobese diabetic–severe combined immunodeficient recipients and differentiate in vivo to produce leukemias with similar immunophenotypes and karyotypes to the diagnostic samples. Similar results were obtained using cells sorted for CD133 and CD38, with only the CD133+/CD38- subfraction demonstrating xenograft repopulating capacity. These findings suggest that leukemia-initiating cells in childhood B-ALL have a primitive CD133+/CD19- and CD38- phenotype. Therefore, growing evidence supports the importance of CD133/prominin-1 as a central defining factor in the TICs phenotype, highlighting it as a target for successful cancer therapy.

2.4 EMERGING TRANSCRIPTIONAL PROGRAMS IN AML PATHOGENESIS

2.4.1 Transcriptional factors are pivotal for AML development

Studies of human acute myeloid leukaemia led to the concept that cancer-associated differentiation arrest is caused by mutated or dysregulated transcription factors.

Transcription factor	Mutations and effects	Frequency in AML	FAB system subtype
RUNX1–ETO (t(8;21))	RUNX1 DNA-binding domain fused to the transcriptional corepressor ETO; downregulates expression or activity of PU.1, C/EBPα and RUNX1	12–15%	M2
CBFβ–MYH11 (inv16)	Inversion of breaks in chromosome 16; joins CBFβ with the myosin gene *MYH11*	8–10%	$M4_{Eo}$
PML–RARα (t(15;17))	*PML* gene fused to *RARA*; blocks myeloid transcription factors (such as C/EBPα and PU.1)	6–7%	M3
MLL fusions (t11q23)	*MLL* gene fused with one of 30 distinct genes encoding partner proteins; believed to dysregulate HOX genes	4–7%	Diverse pattern of myeloid and lymphoid leukaemias
C/EBPα	Amino-terminal dominant negative; carboxy-terminal loss of DNA binding	7–9%	M1, M2 (most), M4 (rare)
GATA1	Amino-terminal dominant negative	Nearly 100% in AMKL associated with Down's syndrome	M7 with Down's syndrome
PU.1	Mutations decrease heterodimer formation and DNA binding*; PU.1 activity downregulated by RUNX1–ETO, PML–RARα and FLT3–ITD	<7%	M0, M4, M5, M6
RUNX1	Missense, nonsense or frameshift mutations (often biallelic); clustered within the runt domain	9%	M0 (most)

Table 1. Examples of transcription factor mutations in patients with AML. (From Rosembauer F. et Tennen D., Nat Rev Immun, 2007)

The involvement of aberrant transcription factor activity in human AML was first observed from common somatically acquired chromosomal translocations that result in oncogenic fusion products such as RUNX1–ETO (t(8;21)), CBFβ–MYH11 (core-binding factor-β–myosin heavy chain 11; inv16), fusion proteins involving MLL (mixed lineage leukaemia; t11q23), and PML–RARα (promyelocytic leukaemia–retinoic acid receptor-α; t(15;17)). More recently, small recurring mutations in defined regions of transcription factor gene coding sequences have been identified in patients with AML. In the granulocytic FAB system M2 subtype of AML, C/EBPα is the most frequently mutated transcription factor. This blocks granulocyte differentiation by reducing C/EBPα function through a dominant-negative effect of the transcriptionally inactive 30kDa isoform on the active 42kDa protein. Similarly, FLT3 with internal tandem duplication (ITD) mutations has been reported to decrease C/EBPα expression and to increase C/EBPα protein inactivation following phosphorylation of serine 21 of C/EBPα. The myeloid differentiation arrest in patients with chronic myeloid leukemia (CML) with blast crisis is also associated with reduced C/EBPα activity, through a mechanism that involves induced inhibition of *CEBPA* mRNA translation by the heterogeneous nuclear ribonucleoprotein E2 (hnRNPE2)[95]. Collectively, C/EBPα disruption seems to be a central event in human malignancies of the granulocytic lineage, an idea

that is supported by mouse models. C/EBPα-deficient mice showed blocked granulocyte development, and accumulation of myeloid precursors similar to patients with AML[96-97].Other myeloid transcription factors are also affected in myeloid cancer. *RUNX1* is mutated in 9% of cases of AML, most frequently in the immature FAB system M0 subtype, and *Runx1–/–* mice develop a myeloproliferative syndrome[98]. Although no mutations have been reported in the *IRF8* gene so far, its frequently shown the downregulation in patients with both AML and CML. In the case of PU.1, mutations in the *SPI1* gene were identified in 7% of patients with AML in one study, mainly in patients with the FAB system monocytic M4 and M5 subtypes. These mutations resulted in the decreased ability of PU.1 to synergize with interacting proteins, such as RUNX1 or JUN, in the activation of target genes. In contrast to the clear link between transcription factors coordinating early myeloid differentiation steps and leukaemia, it is less clear how relevant late-acting factors, such as C/EBPα, are for the transformation process. A plausible hypothesis is that they might act too late for their loss of function to contribute to the accumulation of proliferating progenitors[99]. How might altered transcription factors function lead to myeloid leukaemia? An attractive hypothesis is that progenitors at intermediate stages might be inherently pre-leukemic, this would explain why they are designed to proliferate rapidly for a short time to enlarge the progenitor pool, but after that, they immediately need to undergo differentiation.

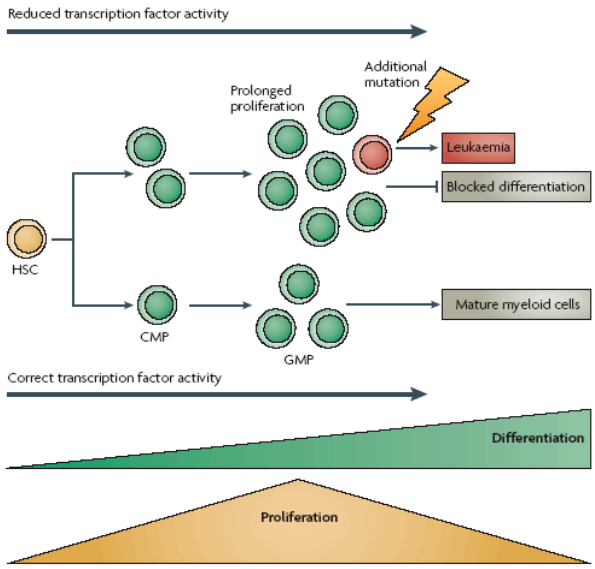

Figure 5. Model of leukemia development from block myeloid differentiation. The normally short-lived intermediate myeloid progenitors undergo rapid proliferation before they differentiate into functional immune cells. The balance between proliferation and differentiation is tightly controlled by stage-specific transcription factors. If dysregulated or mutated, these transcription factors fail to induce differentiation and therefore can cause a prolonged proliferation phase. This might place the progenitors at a higher risk of developing a true leukaemia owing to the random accumulation of additional cooperating events. CMP, common myeloid progenitor; GMP, granulocyte/monocyte progenitor; HSC, haematopoietic stem cell. *(from Rosembauer F. et Tennen D., Nat Rev Immun, 2007)*

Failure to induce differentiation might result in a prolonged proliferation phase followed by the random accumulation of additional cooperating events because of an increased rate of cell division. This places the progenitors at much higher risk to ultimately develop true leukemia, a model that is similar to those put forward to explain the development of teratocarcinomas from embryonic stem cells. Transcription factors are the key gatekeepers in this process, and their dysregulation leads to a shift from the essential differentiation process to prolonged proliferation, thereby shifting the balance towards cancer. An important breakthrough that pose the importance of the transcription factors for the cell was achieved by Yamanaka and colleagues[100] who succeeded in directly reprogramming fibroblasts into induced pluripotent stem (iPS) cells by transduction of the four transcription factors Oct4, Sox2, Klf4 and c-Myc . Another study showed that generating iPS cells from pro and pre-B cells by transduction with the reprogramming factors Oct4, Sox2, c-Myc and Klf4 and from mature B cells by the additional overexpression of C/EBPα or specific knockdown of the Pax5 transcription factor indicate that specific combinations of reprogramming factors can reset the genome of terminally differentiated cells to a pluripotent state, suggesting that dysregulating the physiological expression of transcription factors can move on the cell through an oncogenic transformation.

2.4.2 Hedgheog signaling in cancer

The Hedgehog signaling (HH) has been identified for the first time in Drosophila melanogaster, where it controls cell differentiation, the formation of organs, such as

the neural tube during embryogenesis and scientific studies, have revealed the three ligands involved, Sonic, Desert and Indian, are able to bind to transmembrane receptors Hip1, and Ptch1 Ptch2, expressed by the cells responsive to Hedgehog[101]. Shh (Sonic Hedgehog) is a glycoprotein, mainly studied in the development of the central nervous system during development as expressed by cerebellar Purkinje cells and is a major mitogen for cerebellar granule cell progenitors outside. Once secreted, binds Shh, Patched (PTCH), a receptor to twelve transmembrane domains, which inhibits another receptor with seven transmembrane passages, Smoothened (Smo), which is closely related to the family of Wnt receptors Frizzled pathway, has intrinsic catalytic activity and signaling. In the absence of the activity of Smo, the effectors of the Shh pathway, are kept in a cytoplasmic multiprotein complex that includes Fused proteins and Suppressor of Fused (Sufu). Seized in this way, Gli1 can not perform its function as a transcriptional activator, while two other members of the family, Gli2 and Gli3, are activated following a proteolytic cleavage assuming the role of transcriptional repressors. The binding of Shh to Patched activates Smo, then the complex containing Fused, and Sufu is disassembled, Gli1 is thus released while cutting Gli2 and Gli3 is locked. Among the target genes of Gli1 to note: the same Gli1, Wnt genes encoding factors, PTCH, n-myc, c-myc and cyclin D1[101].

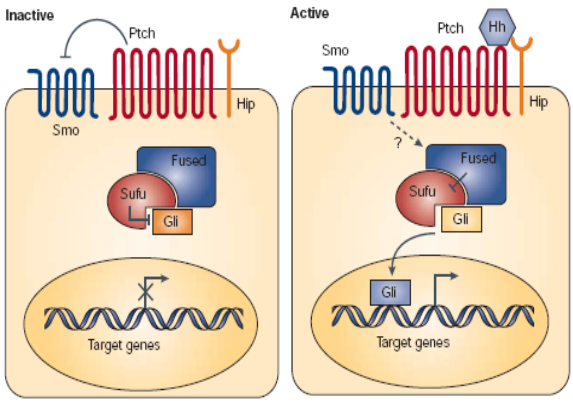

Figure 6. Hedgehog signaling pathway. On the left side is represented the Hedgehod pathway inactive. Ptch receptor inhibits Smo activity and Gli factors remain bound to the cytoplasmatic complex formed by Fused and Sufu. On the right side, the activation of the pathway is mediated by either Sonic, Desert or Indian that bind Ptch receptor. This leads to Smo activation and the translocation of

Gli in the nucleus, where targets genes transcription can start. *(from Pasca di Magliano M. et al, Nat Rev Cancer, 2003)*

The HH pathway is involved in the process of tumorigenesis, because scientific studies have shown that Shh mutations are present in squamous cell carcinoma basal-skin, and in some cases, in medulloblastoma and in breast cancer. Activating mutations in Smo have been identified in 10-20% of squamous cell cancers of the skin basal-cell carcinoma(BCC) and the amplification of the transcription factor Gli1 was originally identified in gliomas. In addition, inactivating mutations of Ptch1 and Sufu, have been identified in approximately 20% of human medulloblastomas that present preferentially but not exclusively, desmoplastic morphology. More precisely, Sufu mutations were associated with an increased risk of developing medulloblastoma in humans, while Ptch1 mutations were identified in patients with Gorlin syndrome, a disease characterized by a high incidence of BCC and medulloblastoma[102].

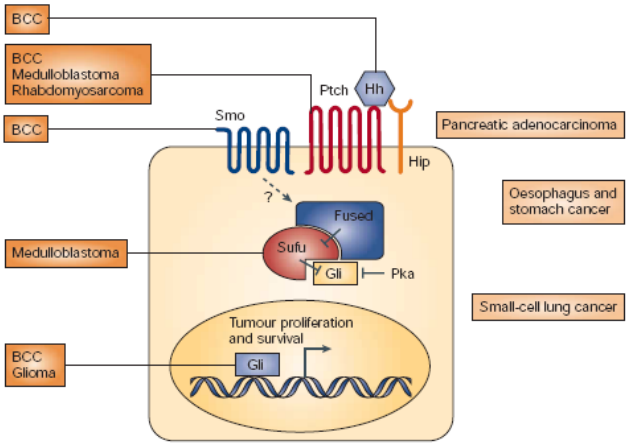

Figure 7. Hedgehog *pathway* and tumorigenesis. Mutations of HH pathway involved in different tumors. *(from Pasca di Magliano M. et al, Nat Rev Cancer, 2003)*

The deregulation of the SHH pathway has been mainly studied in solid tumors, and there is not much literature about its involvement in haematological malignancies [103]. It is interesting, however, the proposed study by Bhardwaj et al.[104], which

highlights the role of SHH in the proliferation of pluripotent hematopoietic precursor cells, the researchers point out that HH and receptors PTCH and Smo and consequently the transcription factors downstream of the cascade, such as the, are expressed by precursor cells of the hematopoietic and stromal cells from the stem cell niche. Of particular interest is the lack of expression of transcription factors of the Gli family in the cells of the hematopoietic system have already committed to a lineage differentiation, suggesting the hypothesis that the SHH pathway is important for the maintenance of cell self-renewal stem, but not essential for the differentiation process. Bhardwaj et al also highlight the importance of the niche in maintaining stem cell self-renewal, because of the activity of the cytokine BMP4 (Bone Morphogenetic Protein 4), a member of the family of TGF-β growth factors, released from the cells stromal cells, the SHH pathway is still active. Multiple studies have explored the role of the Hh pathway in development, but relatively few studies have addressed the role of Hh signaling in normal hematopoietic development and leukemogenesis To define its role it has been assessed the consequences of conditional deletion of the Smoothened (Smo) allele in an in vivo knockout mouse model[105]. Hoffman suggested that the Hedgehog pathway is not required for hematopoietic homeostasis in the adult mouse. Recently, data suggest that Hh signaling may play an important role in leukemia stem cell function in a BCR-ABL-induced model of murine hematopoietic disease[106-107]. Although Hoffman et al. observed no impact in MLL-AF9-mediated leukemogenesis in the absence of Smo, suggesting that the pathway does not play a critical role in initiation of leukemia, it remains plausible that Hh signaling could be effectively targeted in leukemias that arise *de novo*, or that there are allele-specific differences in requirement for Hedgehog signaling. Taken together, these findings demonstrate that Hh signaling is dispensable for normal hematopoietic development and hematopoietic stem cell function, indicating that targeting of Hh signaling in solid tumors is not likely to result in hematopoietic toxicity. Furthermore, the Hh pathway may not be a compelling target in certain hematopoietic malignancies.

2.4.3 WNT signaling and its role in the stem cell self-renewal

In the absence of Wnt signal, APC fosters the degradation of the oncogene β−catenin and prevents its entry into the nucleus. Wnt stimulation, loss of APC protein function, or of its associated partner Axin, all lead to the stabilization of β−catenin and to its increased concentration in the nucleus. β−catenin can then act as a transcriptional co-activator by associating with the Tcf/LEF family of transcription factors. A complex of APC with Axin and other proteins targets β-catenin for proteasomal degradation by scaffolding an association between β-catenin and kinases whose activities lead to β-catenin ubiquitinylation. This action is abrogated by the recruitment of the degradation complex to the membrane upon Wnt activation of a receptor complex that includes Frizzled (Fz), a relative of Smo, and LRP5/6. Any lesion causing β-catenin accumulation through the disruption of a degradation complex component or by mimicking complex recruitment to the receptor would be expected to promote tumour formation. Many scientific studies have highlighted the important role of the Wnt pathway in mechanisms regulating hematopoietic stem cells and more specifically, Reya et al.[22] have demonstrated *via* the inhibition of Frizzled receptor *in vitro* and *in vivo*, that the mitotic division of hematopoietic stem cells is determined by activation of Wnt signaling highlighting the role of WNT in the development of HSC. The regulation of self-renewal appears to be mediated by their own WNT proteins produced by stromal cells and the niche and in some cases from the same hematopoietic stem cells through paracrine and autocrine mechanism.

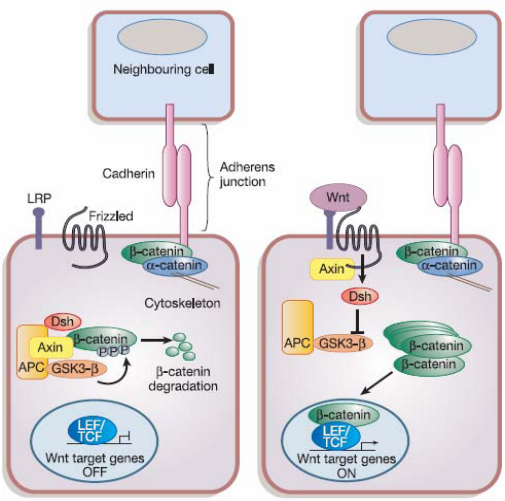

Figure 8. Canonical Wnt/β-catenin pathway. At the left, in absence of Wnt ligand, β-catenin are complexed with axin-APC-GSK3β and once phosphorilated, they are degradated. In the right, Wnt ligand binds its frizzled receptor, that induce the release of β-catenin from axin-APC-GSK3β complex and the migration of β-catenin in the nucleus where it promotes the transcription of target genes. *(from Reya et all. Nature, 2005)*

Functional studies *in vitro* have shown that the Wnt proteins work in synergy with Steel factor (also known as Stem Cell Factor), to promote cell growth, inhibiting simultaneously murine hematopoietic progenitor differentiation. It was also shown that β-catenin Wnt3A protein are able to promote self-renewal of murine stem cells *in vitro* and to reconstitute the hematopoietic system in murine irradiated models *in vivo*. The same effects detected in mouse models are present at the level of the human hematopoietic system. For example, the treatment of hematopoietic progenitors with Wnt5A in presence of stromal cells, promotes the expansion of progenitors *in vitro* and treatments with the medium conditioned by Wnt5a result in an increase of repopulating stem cells in NOD-SCID mouse models xenotransplanted. All the studies conducted *in vitro* and *in vivo*, have supported the hypothesis that Wnt signaling have a central role in the self-renewal of hematopoietic stem cells and progenitors. Reya et al.[109] have shown how each component of the Wnt signaling is necessary for self-renewal of the hematopoietic stem cell. The *in vitro* studies have revealed that the expression of activated β-

catenin allows the maintenance of the immature state of stem cells, but at the same time also the proliferation of the cell pool which appears to be increased by 20-48 times compared to the number of cells present at the start. Experiments *in vivo* demonstrated that the hematopoietic stem cell niche respond to the canonical Wnt signal through the activation of nuclear components such as Tcf/Lef determining the increase of self-renewal through the up-regulation of transcription factors that control the cell cycle, such as HoxB4 and Notch1. Reya et al. have also observed that the Wnt pathway dysregulation and possible mutations in components of the Wnt signaling pathway are present in blood diseases with special emphasis on myeloid leukemias, where it is involved in both the acute and the chronic form. Chronic myelogenous leukemia is characterized mainly by translocation t(9; 22) which determines the fusion product BCR-ABL; it has been also shown that the β-catenin play a crucial role in maintaining the pool of tumor cells which is probably necessary to generate the BCR-ABL mutation. The dyregulation of the Wnt pathway was also observed in acute myeloid leukemia, both in presence and absence of mutations that characterize the classical disease. Studies conducted by Mikesch et al. have revealed the association between AML1-ETO fusion products, PML-RARα and PLZF-RARα and activation of Wnt signaling, which are able to determine the transactivation of the TCF / LEF complex by increasing β-catenin expression, which in turn leads to target genes transcription such as cyclin D1, c-myc and PPARδ. Thus, it emerged an association between the chromosomal translocations that characterize different forms of acute myeloid leukemia and the Wnt pathway, mediated by the globin appease, co-activator complex TCF / LEF. The leukemogenic process is also determined by activating mutations of FLT3 receptor tyrosine kinase III, present in 30% of patients with AML. Analysis conducted using microarray technology in cell lines 32D/Flt3-ITD, have revealed that Wnt receptor Frizzled-4, can be induced and activated by FLT3 in myeloid progenitors, resulting in Wnt signaling activation. It is also interesting to note that the increase in β-catenin level appears to be associated with transcriptional activation of the complex TCF / LEF, but also to the induction TCF-dependent of c-myc. The observations reported by Simon et al. show that Gsk3β turns out to be inactivated by phosphorylation of Akt. The PI3K/AKT signaling cascade is in fact to be activated in several cases of AML and in particular is

activated by mutations of FLT3-ITD, suggesting that Wnt signaling in the growth of leukemic patients is partly due to inactivation of GSk3β. The results therefore suggest that the FLT3-ITD mutations may contribute to the leukemogenic process in association with the Wnt activation signaling[109]. Recent studies conducted by Jamieson et al., showed alterations of the Wnt pathway in leukemia cells, regardless of the presence of chromosomal alterations. Several cases of AML analyzed by Simon et al. show alterations of various components of the signaling cascade, such as Wnt1, Wnt2b and Lef1, as a potential mechanism of up-regulation of signaling β-catenine/TCF. β-catenin appear to be expressed at different levels in patients with AML, as affected by post-translational regulation of Wnt-mediated pathway, their expression seems to be correlated with the ability to clone proliferation of AML colony-forming cells, and then with the cell self-renewal. Studies by Duncan et al., support the hypothesis that Wnt signaling plays a role of fundamental importance in the development of leukemia, even in the absence of specific mutations, but in close correlation with other deregulated pathways, such as that of Akt and Notch[109].

3. AIM OF THE STUDY AND STRATEGIES

The existence of a subpopulation of rare cancer stem cells (CSCs) responsible for maintenance of the neoplasm has been postulated and substantial evidence emerged in the literature in recent years[110]. The data suggesting that the leukemic stem cell (LSC) pool, like the normal hematopoietic stem cell (HSC) compartment, is organized as a hierarchy of distinct stem cell classes with decreasing self-renewal capacity[75,77], is the most compelling evidence to date of the stem cell origin of acute myelogenous leukemia (AML). Understanding the genetic and molecular regulations of the self-renewal programs and an appreciation of how perturbations in this regulation initiate proliferative diseases such as leukemia is a major challenge of the medical research. Annotation of the draft sequence of the human genome has opened up the possibility of applying novel genomic approaches to the characterization of molecular pathogenesis of human disorders. Among genomic screening methods, DNA-microarray analysis has to date provided the greatest insight into leukemogenesis.

Considering the relevant role of transcription factors in myeloid leukemogenesis, in order to investigate the common transcriptional programs in the AC133+ stem cell fraction from AML patients, we generated an *ad hoc* transcriptional regulators (TRs) dataset to interrogate expression microarray data obtained from AC133+ cells in AML patients and healthy donors.

- The main goal of the research activity performed by introducing biological ($AC133^+$ cell fraction selection) and bioinformatic (TR-dataset restricted analysis) filters is the development of a transcriptional circuitry model in which defined master “executer” genes acting to induce a stronger (possibly leukemogenic) response of the final “player” genes on self-renewal programs (**Figure 9 top**).

- To validate the *in silico* results obtained from a functional enrichment analysis, AML and healthy donors BM biopsies, AC133+ fractions and BM smear were investigated using immunofluorescence and immunoblot strategies (**Figure 9 bottom**).

- In order to investigate the circuitry model emerged from transcriptional studies a hematopoietic primary stem cell culture was established. The primary cell culture was set up using the purified $AC133^{+}CD34^{+}CD117^{+}CD38^{-}$ fraction obtained from a patient affected by AML M2 with 46XY karyotype and harbouring an Flt3 ITD mutation. Establishing of a powerful cellular model for LSC is an important goal of the project because it represents the best starting point to evaluate the deregulated pathways in AML.

- In recent years the zebrafish has emerged as a valuable organism in which to model leukaemogenesis[111]. Using the zebrafish model as biosensor allowed us to investigate the molecules released in the microenvironment by the AC133+ leukemic cells and whether these molecules interfere with the physiological development of zebrafish main tissues.

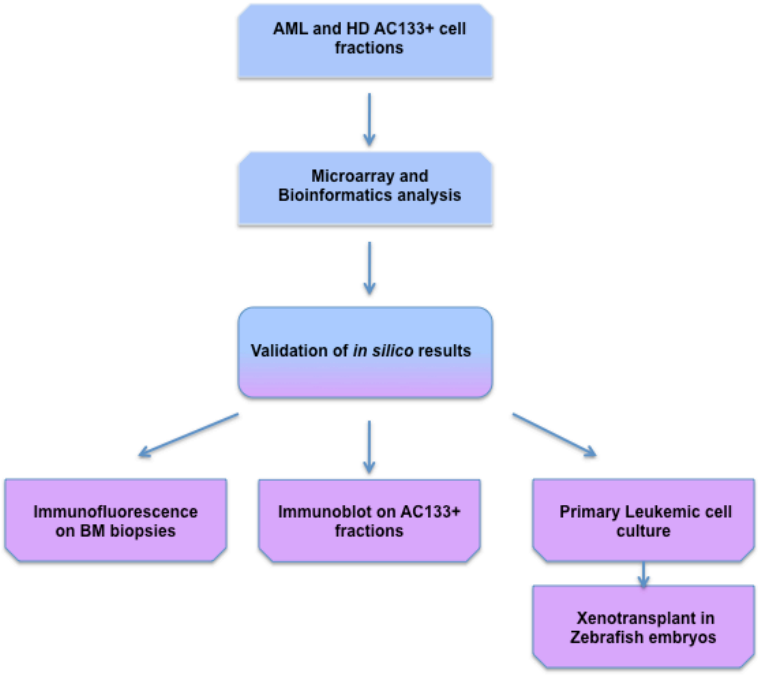

Figure 9. Strategies applied in the PhD project. AML= acute myelogenus leukemia patients; HD= healthy donors. BM= bone marrow

4. MATERIALS AND METHODS

4.1 Collection of AML patients and healthy donors sample of hematopoietic cells.

Bone marrow (BM) mononuclear cells were collected from 33 diagnosed AML patients and 10 healthy donors according to Niguarda Hospital's Ethical Board approved protocols (116_04/2010). Human adult BM cells obtained from consenting healthy donors and patients were isolated according to standard procedures using Lymphoprep (Axis-Shield PoC AS) from the BM drawn from the posterior iliac crest. (**Supplementary Table 1**)

4.2 Cell sorting and flow cytometry.

The AC133+ cell fraction was isolated by immunomagnetic separation after labelling with CD133/1 (AC133)-biotin antibody and anti-biotin MicroBeads on LS columns and Midi MACS separator (Miltenyi Biotec). Unselected BM-MNC, CD133/1-positive (AC133+), and CD133/1-negative (AC133−) cells were incubated for 20 minutes at room temperature in the dark with the appropriate monoclonal antibody (mAb) mixture, at a concentration deriving from specific titration experiments. MAbs were directly conjugated with the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC), and combined for four-color analysis. Each sample was incubated with the following mAbs panels: CD34-FITC/CD133/1-PE/CD45-PerCp/CD38-APC and CD34-FITC/CD133/2-PE/CD45-PerCp/CD38-APC. Unselected BMMNC from AML patients were also incubated with the mAbs panel CD34-FITC/CD117-PE/CD45-PerCp/CD14-APC to test for KIT (CD117) expression. At the end of incubation, red blood cells were lysed for 10 minutes at room temperature by adding 3ml of ammonium chloride. Cells were centrifuged at 800×g for 8 minutes, and the cell pellet was resuspended in 500 µl of PBS for flow cytometry analysis. All measurements were performed on a dual-laser FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and contained 10,000 to 50,000 cells, adjusted to the leukocyte subpopulations in the CD45/side scatter plot. Data acquisition was performed with the CellQUEST software, whereas both CellQUEST and Paint-a-Gate (Becton Dickinson) were used for

analysis. Multiparameter analysis including logical gates on forward scatter, side scatter, FL1, FL2, FL3, and FL4 was used to evaluate cell populations.

4.3 Microarrays expression analysis and statistical bioinformatics analysis.

4.3.1. Sample preparation and Affymetrix platform.

Total RNA for expression profiling was extracted using RNAqueous®-4PCR kit. (Ambion) from AC133 selected cells. Expression profiling has been performed on Affymetrix HGU133Plus2 arrays according to the manufacturer's procedures. The entire bioinformatics tests performed in this study were realized using the R language for statistical computing and the annotation libraries provided by the Bioconductor project. Thus, we removed, from our transcriptional regulator set, all the gene symbols lacking an annotation in the Bioconductor annotation package associated with the Affymetrix HGU133Plus2 platform. This step was realized by mean of in house written R scripts based on the Bioconductor (hgu133plus2.db package version 2.2.5). For easiness of visualization in the heatmap, in the case of genes represented by more than one probe set, the following procedure has been applied to identify a unique representative probe set for each gene: i) any probe set of type '_x_at' associated to the gene has been removed, if at least one probe sets not of type '_x_at' was also associated to the gene; ii) among the remaining probe sets, the one with the lowest P value has been chosen as representative for the gene. Microarray data have been deposited in ArrayExpress (http://www.ebi.ac.uk/arrayexpress/), with accession number E-MTAB-220.

4.3.2. A genome-wide approach: the functional enrichment analysis.

In order to identify functional groups and deregulated pathways in AC133+ fraction, a genome-wide functional enrichment analysis was performed on AML and healthy donors samples. We adopt three different functional methods to reach a more robust identification of overrepresented regulatory networks and pathways in AML cells like as GOstats, DAVID and deregulated pathway analysis. GOstats selected terms with p-value<0.01. Among them, the "positive and negative regulation of WNT receptors" terms were included in both aspects of the analysis: the genome-wide level and the TR genes restricted level. DAVID functional analysis selected

terms with p-value<0.05. In both analysis, the genome-wide level and the TR genes restricted level, the "regulation of WNT receptors signaling pathway" emerged. Using a non-parametric test like the deregulated pathways analysis, test applied by Weissman et al.[112], terms were selected at p-value<0.05. After the analysis the "WNT receptor signaling pathway regulation" came out in all the analysis performed, even using KEGG.

4.3.3. Generation of an evolutionarily conserved human transcriptional regulators dataset.

The first step in our gene expression analysis consisted in the definition of a set of genes annotated as transcriptional regulators (TRs) and conserved in a broad range of organisms, from yeast to human (**Figure 10 left**). The entry point of our selection procedure was the extraction from Pfam of all the domains involved in transcription regulation, which resulted in a set of 400 Pfam domains. Next, we used this list to query the entire set of ensemble human genes using the BioMART web interface[25]. This produced an initial set of 2,971 non-redundant genes associated to a unique HGNC symbol. We thus adopted a direct SQL access to the ensembl_compara_51 database (hosted at ensembldb.ensembl.org) in order to extract all the one-to-one orthology relationships occurring between the genes in the initial set and the complete set of genes in the following organisms: *Bos taurus, Canis familiaris, Danio rerio, Drosophila melanogaster, Equus caballus, Gallus gallus, Loxodonta africana, Mus musculus, Saccharomyces cerevisiae, Takifugu rubripes and Xenopus tropicalis*. We then compiled an evolutionarily conserved set of TRs by arbitrarily requiring the presence of at least 5 one-to-one orthology relationships disregarding the involved species. This produced a set composed of 1,989 non-redundant genes. We finally tested each of the 1,989 gene symbols obtained through the orthology filtering for the association with at least 1 probe set in the Affymetrix HGU133Plus2 array. This returned a final set of conserved putative transcriptional regulators composed of 1,919 genes. A procedure aimed to build a whole-genome set of transcription factor genes was published by Messina and colleagues[113]. The pipeline adopted by the authors resulted in the definition of a set of 1,468 human TFs. In order to explain the difference in size, we compared our pipeline with the procedure presented in Messina et al.[113]. After considering the

absence of a comparative genomics filtering in Messina et al.[113], the most relevant difference between the compared approaches is the absence in our pipeline of a filtering aimed at providing strong support for the expression of our set of TRs. We thus check our conserved set of TRs for an overlap with annotated NCBI's UniGene clusters. Again, the required annotations were obtained using the BioMART web interface and the whole human known Ensembl gene set (version 51) and limiting the extraction to a set of genes produced by the orthology 1-to-1 multi-species filter. One thousand, six hundred and twenty conserved TRs genes were found to overlap with at least 1 NCBI UniGene cluster. Therefore we removed from our set of TRs all the gene symbols lacking an annotation in the Bioconductor annotation package associated with the HGU133Plus2 array. This filtering procedure produced the reference conserved TRs set composed of 1,611 evolutionarily conserved genes (**Figure 10 right**).

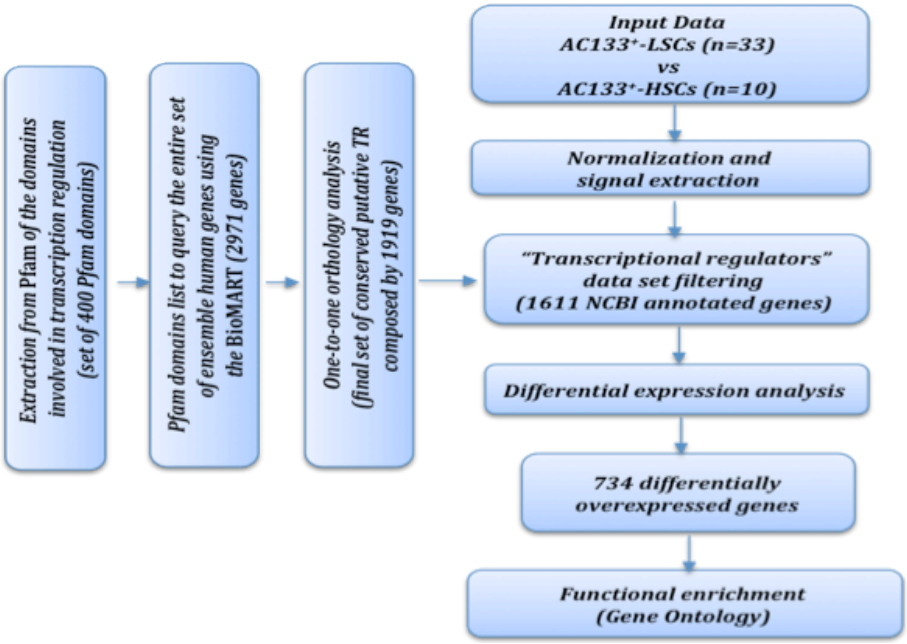

Figure 10. Bioinformatic analysis of data derived from microarrays analysis. Construction of a set of conserved human transcriptional regulators (TRs) and differential expression analysis. The 734 differentially overexpressed genes were enrolled for the functional enrichment analysis.

4.3.4. Functional enrichment using the Bioconductor GOstats package.

The genes comprised in the conserved TRs set were tested for differential expression in AC133+ AML cells. In this stage of the analysis our goal was to isolate a set of genes highly or moderately overexpressed in the pathological condition under analysis in order to identify genes suitable for ontology driven functional enrichment investigations basing on a standard hypergeometric test. A commonplace approach is to be less selective during the extraction of the differentially expressed gene sets and then to apply more stringent statistical significance cutoffs during the functional enrichment test. Following this approach we selected a set of overexpressed conserved TRs by means of Welch *t*-test (α=.05, one-tailed). This procedure identified a set of 734 conserved putative TRs overexpressed in AC133+ AML cells. The overexpressed TR genes were then tested for functional enrichment using the Bioconductor GOstats package (function: hyperGTest). As expected, all the functional terms retrieved are associated with regulatory processes because of the nature of the universe set, which is entirely composed of TRs. Among the selected functional terms the most specific one, with respect to the GO Biological Process ontology, is the term 'GO:0016055' (**Figure 11)**, associated with the "Wnt receptor signaling pathway" and composed of 38 genes, 26 of which resulted significant by the Welch *t*-test (**Supplementary Table 2**).

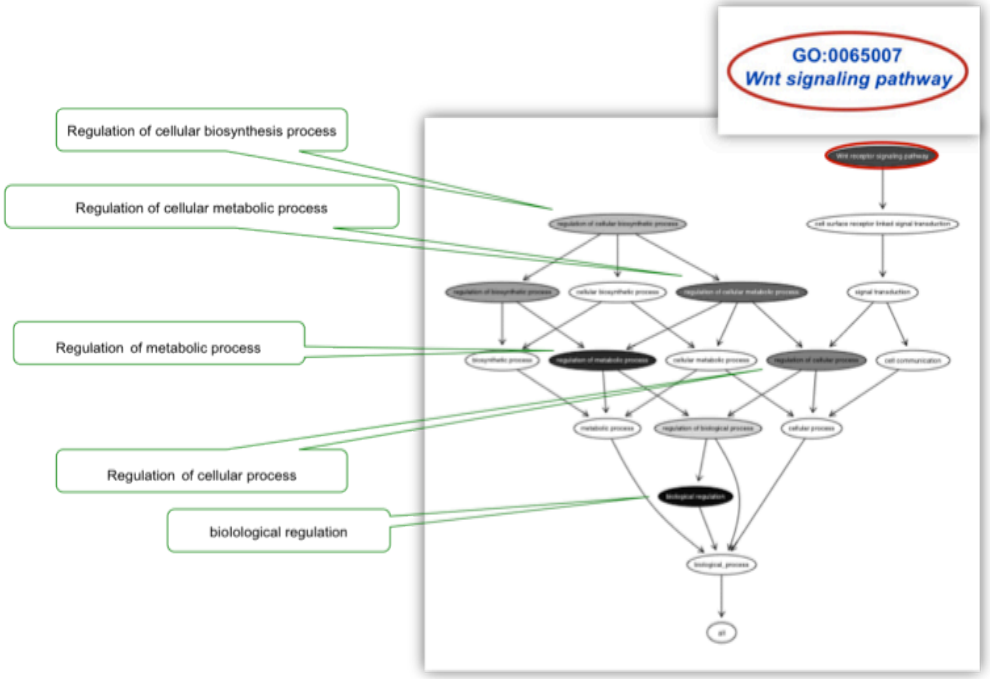

Figure 11. Gene Ontology Tree from the enrichment analysis. Enriched Gene Ontology Biological Process terms are plotted in light gray, the 'Wnt Signaling Pathway' (GO:0016055) term is highlighted in red. The P value associated to each significant term is reported along with the Gene Ontology identifier. 'GO:0016055' was the most specific one (the farthest from the root of the directed acyclic graph encoding the relationships between the depicted functional terms).

4.4 Immunoblot

Protein extraction was performed using cold TNTG buffer from AML patients and healthy donors BM cells. Protein expression was demonstrated by immunoblotting using standard procedures. Primary antibodies used included anti-active β-catenin monoclonal mouse (anti-ABC clone 8E7, Millipore) (1:5000), anti-β-catenin monoclonal rabbit (E247, Abcam) (1:5000), anti-WNT10B polyclonal rabbit (H-70, Santa Cruz) (1:1000), anti-WNT10B monoclonal mouse (5A7, Abcam) (1:1000), anti-WNT2B poluclonal rabbit (Abcam) (1:1000) anti-GAPDH polyclonal rabbit (ab97626, Abcam) (1:5000), anti-Pygopus 2 polyclonal rabbit (H-216, Santa Cruz Biotechnology, Inc.) (1:1000). Secondary antibodies used were anti-mouse-HRP,

anti-goat-HRP and anti-rabbit-HRP (Thermo Scientific) (all of them at 1:10000).

4.5 Immunofluorescence and imaging analysis

Indirect Immunostaining was performed following standard procedures. Bone marrow biopsies of AML patients, previously embedded in paraffin blocks were cut in 5μm thick sections and mounted on slides. Slides were loaded into glass slide-holders and dewaxed as follows: twice in 100% xylene (Carlo Erba Reagents, Rodano, Italy) 15min and 10min, twice in 100% EtOH (Panreac, Quimica Sau, Castellar del Vallès, Spain) for 5min, once in 95% EtOH for 5min, once in 85% EtOH for 5min, once in 65% EtOH for 5min, once in 30% EtOH for 5min and three times in water for 5min each. Epitope retrieval was performed using the boiling method with buffer Citrate (10mM, pH6). Slides were dipped into already boiling buffer citrate for 20min and then brought to room temperature in water. The blocking procedure was performed overnight using Phosphate Buffered Saline (PBS) plus 5% Bovine Serum Albumin (BSA) (Sigma Aldrich, St. Louis, US) and 0.05% Tween 20 (Roche, Mannheim, Germany). The day after, slides were incubated with primary antibodies such as mouse anti-CD133.1 (AC133) (1:100, Milteny Biotech, Bergisch Gladbach, Germany), mouse anti-Active-β-Catenin (1:100, Millipore, Billerica, MA, US), rabbit anti-WNT10B (H70) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, US), rabbit anti-Pygopus 2 (H-216) (1:100, Santa Cruz Biotechnology), goat anti-SMYD3 (F-19) (1:100, Santa Cruz Biotechnology) for 5h and then washed three times with PBS plus 5% BSA and 0.05% Tween 20. Samples were incubated with the secondary antibodies donkey anti-mouse Alexa Fluor 488 (1:500, Life Technologies, Carlsband, CA, US), donkey anti-rabbit Alexa Fluor 568 (1:500, Life technologies) and donkey anti-goat Alexa Fluor 568 (1:500, Life technologies). Nuclei were counterstained with 100ng/ml DAPI (Sigma-Aldrich). Cells were analyzed using the upright microscope (Leica, DM 4000B). Image analysis was performed by means of a custom automatic routine and the ImageJ program (1.43s NIH, USA). Maps of ABC+/WNT10B+ cells were obtained using an automatic threshold based on moments algorithm newly implemented in the ImageJ program and mathematical morphology plug-in developed by D. Prodanov [http://rsbweb.nih.gov/ij/plugins/gray-morphology.html]. DAPI staining was used to identify nuclei. Finally, images of nuclei for positive cells were obtained

by Boolean AND operations between DAPI staining and cell maps. The resulting images were used to determine the percentage of ABC+/WNT10B+ cells both manually or by the Analyze Particles function of ImageJ.

4.6 Primary Cell Culture

Selected AC133+ cells from bone marrow at diagnosis of an AML patient (thereafter called A46) were cultured for 16 weeks. The culture was performed using synthetic medium StemSpam® H3000 (StemCell Technologies), HPGM™ (Lonza) and X-VIVO™ 15 Medium (Lonza) in absence of serum and cytokines. Due to the cell number, only half volume of the total medium was replaced each time. The cell culture did not change phenotype using the three different media. StemSpan®H3000 and HPGM™ media, conditioned by the cell culture, were collected after 12 weeks, refined with 0.2µm filter and stored at - 20°C. HEK293T cells were grown following ATCC procedures.

4.7 Wnt/β-Catenin responsive luciferase assay

HEK293T cells grown in 24-well plates at density of 1,7 x 10^5 cells per well were transfected with M50 Super 8x TOPFlash (plasmid 12456, Addgene), containing eight motifs TCF/LEF for β–Catenin binding and pRL-TK (Renilla luciferase, Promega) using jetPEI™ (Polyplus). Cells were treated for 12h with A46 conditioned medium (CM) or HEK293T cells transfected with BA-Wnt10b (plasmid 1831, Addgene) CM as positive control at different doses. Wnt10b expression in HEK293T transfected with BA-Wnt10b was evaluated by SYBR Green based real-time RT-PCR using Wnt10b FW-5'-GCTGTAACCATGACATGGAC-3', and RW-5'-CTGCCTGATGTGCCATGAC-3' specific primers. Luciferase activity measurement was performed with the Dual-Luciferase Reporter Assay System (Promega).

4.8 Zebrafish models and transplantation procedures.

Embryos were handled according to relevant national and international guidelines. Fishes of the AB strain were maintained at 28°C on a 14-hr light/10-hr dark cycle. Embryos were collected by natural spawning and staged according to Kimmel[114]. Zebrafish embryo cells transplantation was performed as previously reported[115]. Briefly, A46 cells concentrated at 1x104 cell/µl were incubated with Hoechst 33342 (5µg/ml final concentration), the incubation was carried out for 2h at 37°C. Fluorescently labeled A46 cells were resuspended in 1x PBS and injected into zebrafish at 3hpf using between 100 and 200 cells each injection. Live embryos were observed at 70% epiboly to ensure the presence of fluorescent A46 cells. Embryos have been collected and fixed in 4% paraformaldehyde/PBS at 70% epiboly and 24 hpf, dehydrated in 100% methanol and stored at -20°C until processed for wholemount in-situ hybridization (WISH). WISH was carried out according to Thisse and colleagues[116].

5. RESULTS

5.1 AC133 positivity is a widespread feature of AML blast.

AC133 antigen is restricted to a rare cell population with long-term reconstituting activity, ranging from 20% to 60% of all CD34+ cells, and resulting barely detectable in CD34-Lin- cells. To determine the anatomical distribution of AC133+cells in AML, we evaluated by immunostaining a number of bone marrow biopsy sections from five randomly selected cases of AML patients. In all of the samples, cells positive for AC133 antibody were organized in little islands with exception for some sporadic cell. AC133 positive cells were well recognizable: they were smaller and iper-chromatic, in the DAPI fluorescence, respect the surrounding microenvironment constituted mostly by negative tumor blasts (**Fig.12**).

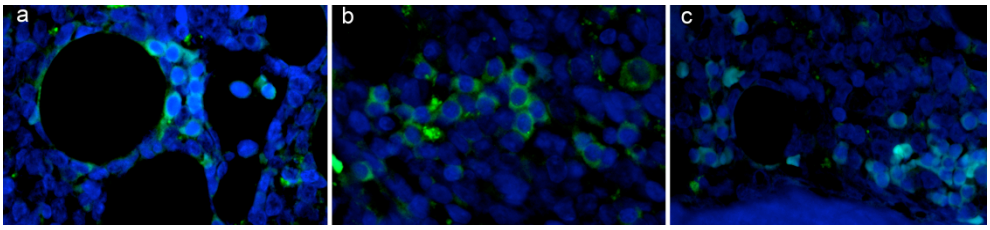

Figure 12. Immunostained AC133 positive cells localized in chromatic islands in AML BM biopsy. Representative Immunofluorescence micrographs show in **green** the cells expressing AC133 (CD133.1) and in **blue**, with the DAPI staining, the nuclei in BM section of AML#9 patient. All images were acquired with a X40 objective.

In order to establish the range of the expanded AC133+ population in AML samples, a flow cytometric quantification of CD133.1 expression was performed in single staining or in combination with the pan-hematopoietic marker CD45 on 25 primary AML bone marrow human samples and 10 age-matched healthy volunteer adult donors. (**Fig.13**)

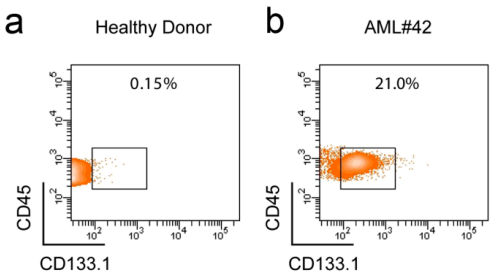

Figure 13. Flow Cytometric representation of AC133 expression in a healthy donor and AML#42. In the healthy donor AC133 positive cells constitute the 0.15% of the population while in a prototypic patient the percentage can reach the 21.0%.

CD133.1 cell fraction was dramatically expanded in all the patients, for example AML#42 shows a 200fold difference in AC133+ cells percentage respect the healthy donor . The AC133+ expansion in all the AML samples averaged about 31.5% (interquartile range (IQR): 16.5%-53.4%) compared the one in the healthy donors that did not reached the 1% of the expansion (median 0.54%, IQR: 0.17%-1.14%, *P*<0.0001) (**FIG-3**), with a increase of 300 fold respect the physiologic expression of AC133+.

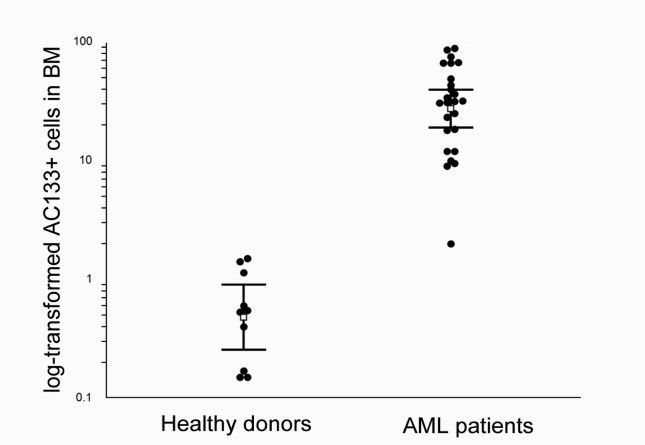

Figure 14. Distribution of AC133+ expansion in AML samples and healthy donors. Flow cytometry analysis of the CD133.1 antigen in BM MNCs of healthy donors (n=10) and AML patients (n=25).

5.2. Identification of a common human transcriptional regulators signature in AC133 positive cells of AML patients.

To obtain insight into the Wnt pathway in AC133+ leukemia cells, we performed a (re)analysis of microarray data by using all the WNT probe sets annotated to GO class 'GO: 0016055' or to any of its children GO terms. The obtained probe set list has been complemented with the probes mapping to genes in a manually selected list of WNT target genes and with probes mapping to other well-known WNT genes, not included in the previous two lists. The total list of analyzed WNT genes includes 480 probe sets, mapping to 193 different genes. To assess differential expression of WNT genes we employed Welch *t*-tests (two-tailed) on the 480 probe sets, thus identifying 103 differentially expressed genes (**Supplementary Table 3**). Taking into account the WNT ligands (WNT2B, WNT6, WNT10A, and WNT10B)[117], the Wnt/β-catenin signaling agonists (including SMYD3[118], DKK2[119], SOX4[120], PROP-1[121], PYGO2[122-123]) and antagonists (including WIF-1[124], KLHL12[125], LRP6, KREMEN1[126], E2F1[127], DACT1[128], and HBP1[129]), showing the lower *P* value as evaluated by the Bonferroni correction, we have built a network model covering the deregulated targets (including STAT3, MYCN, ABCC4[130], DLX3, MARK4, RUNX2[131], CD24[132], CD44[133]) within the canonical Wnt/β-catenin pathway (**Fig. 15**). Collectively, these data are consistent with ligand-dependent activation of the regeneration-associated Wnt pathway[112,134]. Genes that result overexpressed are promoting the WNT/β-catenin signaling while the one resulted underexpressed have an inhibitory function in the physiologic microenvironment.

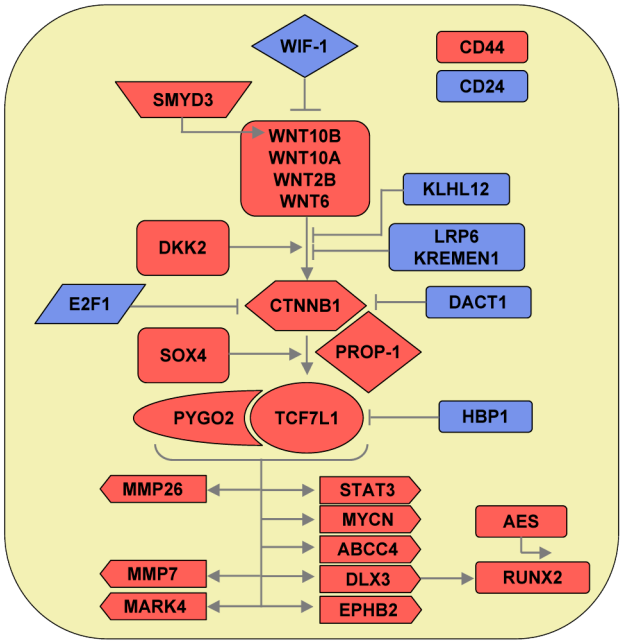

Figure 15. Model of transcriptional regulators involved in AML pathogenesis. The dysregulated genes are all insert in a model of interaction. Red color represents up-regulation, and blue color represents down-regulation in AC133+ AML cells relative to normal AC133+ LT-HSCs.

5.3. Activation of Wnt signaling resulted in diffuse expression of the hematopoietic regenerative molecule WNT10B.

Taking into consideration our findings suggesting a transcriptional activation of canonical WNTs, we focused our research on the genes that have been shown to strongly regulate stem cells functions. First of all, we wanted to investigate the key molecule of the WNT canonical pathway activation: N-terminally dephosphorylated β-catenin (active-β-catenin, ABC). Active-β-catenin were found largely expressed in AML patients in both tissues analyzed, bone marrow smear and total MNCs respectively, by immunostaining (**Fig.16**).

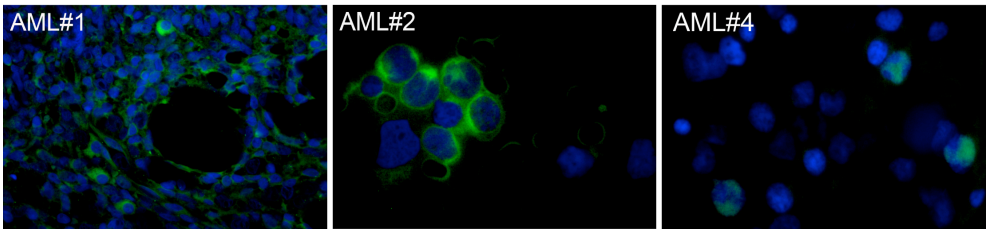

Figure 16. Expression of active β-catenin (ABC) in AML samples. The key molecules for WNT canonical pathway activation, the ABC are expressed in AMLs sample (**green**). In the BM of AML#1 only some cells does express ABC mainly in the cytoplasm. ABC are found expressed also in BM smears of AML#2 and AML#4 patients.

Our gene expression analysis disclosed that the entire set of canonical Wnt ligands activated during tissue regeneration[109], including WNT2B, WNT6, WNT10A, and WNT10B, were among the most strongly upregulated genes in AC133+ AML cells (**Fig.15**). On this purpose, we wanted to investigate the anatomical distribution of cells in which WNTs canonical pathway was activated and we choose WNT10B for their strong association to the regenerative function.(**Fig.17**)

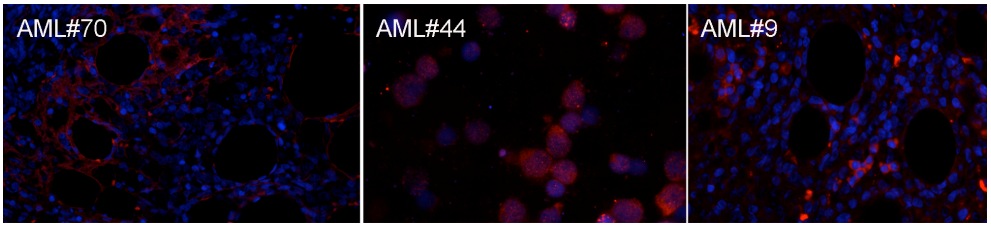

Figure 17. Expression WNT10B in AML BM smear and biopsy. WNT10B is expressed in the BM of AML patients as a ligand, well identified in AML#70 and in little island of cells, identifiable for their dimension and bright DAPI staining.

WNT10B protein was found expressed in not all the cells but in cluster of very chromatic cells. Double immunostaining for WNT10B and ABC, followed by ImageJ analysis, confirmed that WNT10B is expressed by a high proportion of leukemic cells, and its presence correlates with cytoplasmic accumulation of active-β-catenin in an estimated proportion of eight percent of cells (**Fig. 18 right**). WNT10B antibody staining was also detectable in interstitial spaces, suggesting its secretion and release in the bone marrow microenvironment. The slides revealed that WNT10B is diffusely expressed but that only a few small cells (8-10μm diameter of

the nuclei), with a *clonal* appearance and increased N:C ratio, shared the Wnt signaling activation signature represented by cytoplasmic accumulation of the active form of β-catenin[21], likely induced through an autocrine/paracrine mechanism (**Fig. 18 left**), suggesting a LIGAND-depend activation of the WNT/β-catenin pathway.

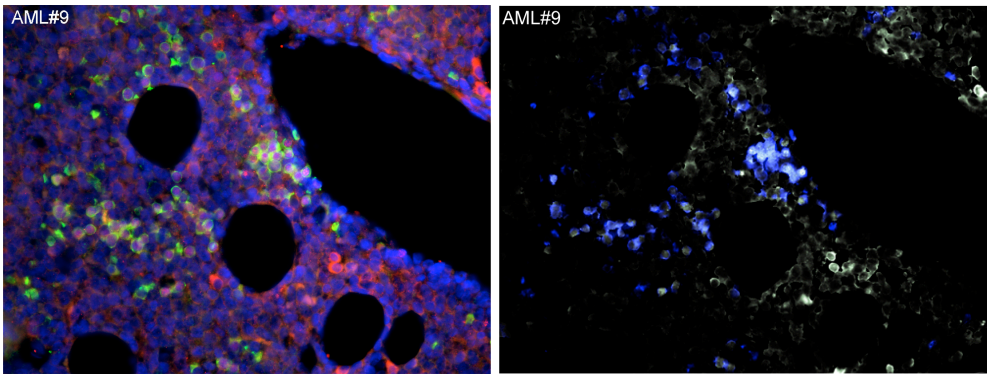

Figure 18. WNT10B/β-catenin double staining in AML patients. The double staining for WNT10B and β-Catenin in biopsy of 5 different AML patients identifies the activation of the WNT signaling induced through an autocrine/paracrine mechanism (left) and imaging analysis highlighted that in 8% of the cell there is a correspondence of WNT10B and β-Catenin signals.

The expression of WNT10B was confirmed in immunoblotting analysis. Remarkably, we found a dramatic increase in WNT10B and WNT2B expression in a subset of patients' samples, except for one, reanalyzed by immunoblot (**Fig.19**).

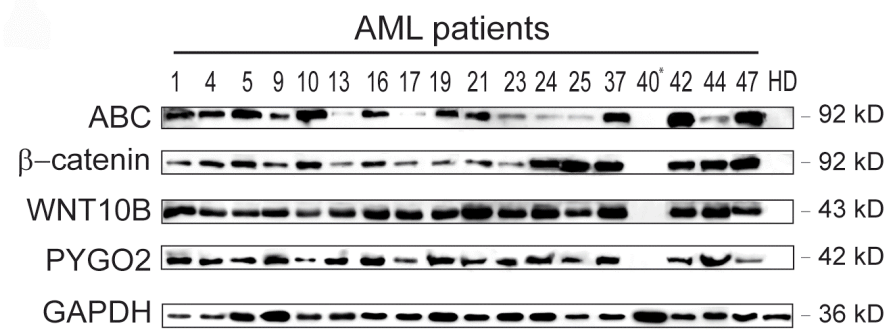

Figure 19. WNT10B/β-Catnenin pathway is activated in AML patients. Immunoblot analysis of active β-catenin (ABC), β-catenin (as detected with the N-terminal pan-β-catenin antibody), WNT10B (ab91201), Pygopus 2 proteins expression in AC133+ cells fractions from 18 patient's samples and on healthy donor introduced as control. Interestingly, AML#40 that developed an AML therapy-related does not show the activation of the WNT10B/β–catenin.

Interestingly, the only AML patient negative for the WNT10B expression (AML#40 in **Supplementary Table 1**) was affected by therapy-related AML. We then decided to investigate also a downstream actor of the WNT signaling and we decided to study PIGO2 expression. In immunostaining PYGO2 expression in associated with the Active β-catenin one suggesting that the WNT activation, marked by ABC expression, is involving also downstream proteins like PYGO2, founded expressed also with immunoblot. In order to show that this phenomenon is spread in more than one WNT signaling ligands, associate to the regeneration function, we decided to investigate by immunoblot the expression of WNT2B (**Figure 20**). The same expression pattern obtained for WNT10B was recapitulated for the WNT2B.

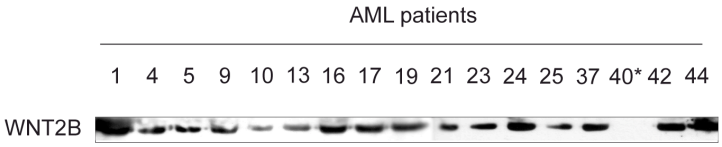

Figure 20. WNT2B is activated in AML patients. Immunoblot analysis for WNT2B protein in AML patients confirm the activation of the WNT signaling associated to the regeneration function in AML.

5.4. WNT10B expression in AC133+ leukemic primary cell culture.

Since now we could demonstrate the activation of the WNT signaling in human samples showing the expression of active β-catenin and WNTs ligands in leukemic tissues. In order to mimic the *in-vivo* state, a primary AC133+ cell culture (termed A46 hereafter) was established. A46 cells, with diploid karyotype, were selected from a 66 years old male at diagnosis of AML-M2. The immunophenotype characterization of the unselected cells revealed that there was a dominant CD133.1+CD34+CD38-CD45+CD117+ blast population (59%), that could for sure contribute to enrich the primary cell culture with a LIC population (**Fig.21**).

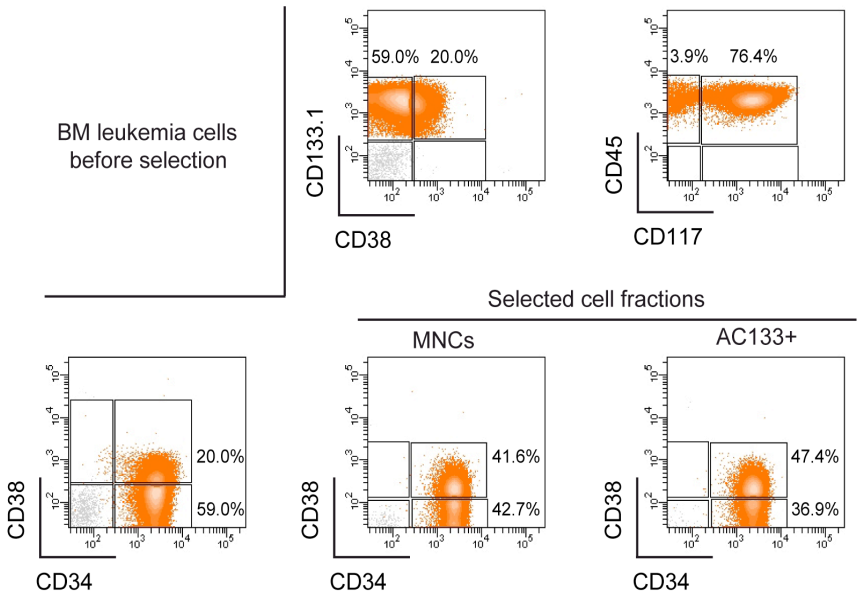

Figure 21. Dot plots of the immunophenotype analysis from AML#46 BM MNCs at diagnosis and after selection. Patterns of CD38/CD133.1 (top left), CD117/CD45 (top right), CD34/CD38 (below left) co-staining was gated on BM AML cells before selection. Representative CD34 and CD38 expression on Ficoll™-selected MNCs (below center) and AC133 sorted cells prior to culture (below right) is shown. Percentages on total cellularity are shown for gated AML populations

The cells were grown in three different media, StemSpan®H3000 (Stem Cell Technologies), HPGM (Lonza) and X15 Vivo (Lonza). All of the three cultures were kept without serum or growth factors and they were carried on for 16 weeks. The cells, once seeded, present a heterogeneous aspect but after a couple of weeks, very small, translucent and round cells constituted the only homogeneous population of cells. The A46 cells created, *in vitro*, their own microenvironment and in order to study whether A46 were secreting WNTs compounds I collected the A46 conditioned medium (A46-CM). To further investigate whether leukemia cells release WNTs in the medium and if these ligands are able to induce the β-catenin, A46-CM was used to evaluate β-catenin-mediated transcriptional activation. To this aim, HEK293T cells (H293T), transfected with Super8XTOPFlash β-catenin/TCF transcription-based reporter construct, were exposed either to pBA-Wnt10b H293T-CM as control (**Fig. 22 left**) or A46-CM. This construct could be efficiently expressed in a dose-dependent manner when transiently exposed to A46-CM for 12h (**Fig. 22 right**).

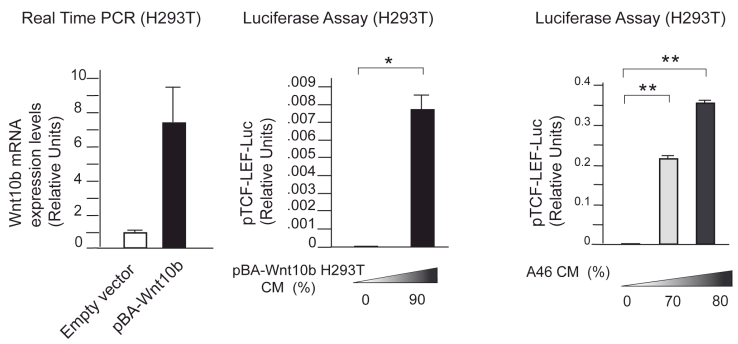

Figure 22. TOPFlash reporter assay showing luciferase expression driven by 8 TCF/LEF binding sites. (left) Positive control was obtained by conditioned medium (CM) of pBA-Wnt10b-transfected H293T cells. Expression of Wnt10b was evaluated in pBA-Wnt10b transfected H293T by real-time PCR (left). TOPFlash reporter assay shows luciferase expression induced in Super8xTOPflash-H293T cells by pBA-Wnt10b H293T-CM (centre). (right) TOPFlash reporter assay showing dose-dependent luciferase expression induced in Super8xTOPflash-H293T cells by A46-CM. Significance was evaluated by t-test (two-tailed, α=.01). Data represent the mean ± s.d. of triplicate reactions and are representative of three independent experiments.

5.5. Transplantation in zebrafish embryo of AC133+ leukemic primary cell culture induces ectopic axial structures formation by Wnt signaling activation.

At this point, we investigated the physiological response of the WNT10B expression and release effect in a developmental model; to this aim we decided to use the zebrafish as a biosensor to investigate possible tumor-derived signals after A46 transplantation. Wnt pathway is among the most evolutionarily conserved signalings, and has been strongly implicated in body axis formation. Patterning of zebrafish body plan requires the activity of the factors *bozozok (boz)* and *squint (sqt)* that trigger the expression of the organizer/mesoderm marker *goosecoid* (*gsc*), among others[48]. We hypothesized that A46 cells, transplanted into developing zebrafish embryos, can induce the formation of additional axial structures following on activation of markers that are normally dependent on the Wnt pathway. Thus, we injected Hoechst 33342 fluorescently labeled A46 and normal bone marrow-derived AC133+ cells (control) into zebrafish embryos at 3hpf stage **(Fig. 23a)**, and the effects on the expression pattern of *gsc* at the 70%

epiboly stage have been analyzed **(Fig. 23b-d)**. While normal AC133+ cells did not alter the normal expression of the gene **(Fig. 23b),** 29% of the embryos grafted with A46 cells (*n*=208) displayed both the expansion of the *gsc* endogenous domain and its ectopic activation in the dorsal and ventral aspects of the animal pole **(Fig. 23c,d)**.

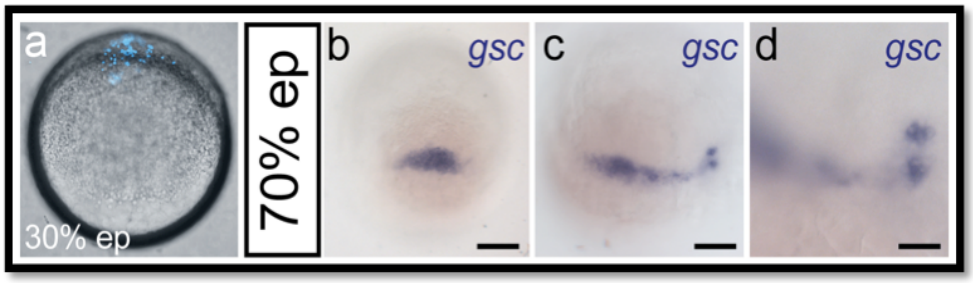

Figure 23. A46 AML cells induce ectopic gene zebrafish embryos. (a) Fluorescence microscopy of a live zebrafish embryo transplanted at 3hpf with A46 cells previously blue-stained with Hoechst 33342. (b-d) Animal pole view of 70% epiboly-stage embryos hybridized with a gsc-specific probe. Embryos have been injected at the blastula stage with (b) normal AC133+ cells as control or (c) A46 AML cells. The gsc-ectopic signals in the embryos injected with A46 are highlighted in (d).

According to the ectopic expression of this organizer marker, the embryos injected with A46 cells developed secondary structures, ranging from the formation of additional non-axial tail tissues **(Fig. 24e-g)**, to the emergence of extra axial **(Fig. 24h)** or head structures **(Fig. 24i)**. Most of the embryos displayed dramatic early development defects and lethality at 10 hours post fertilization (hpf).

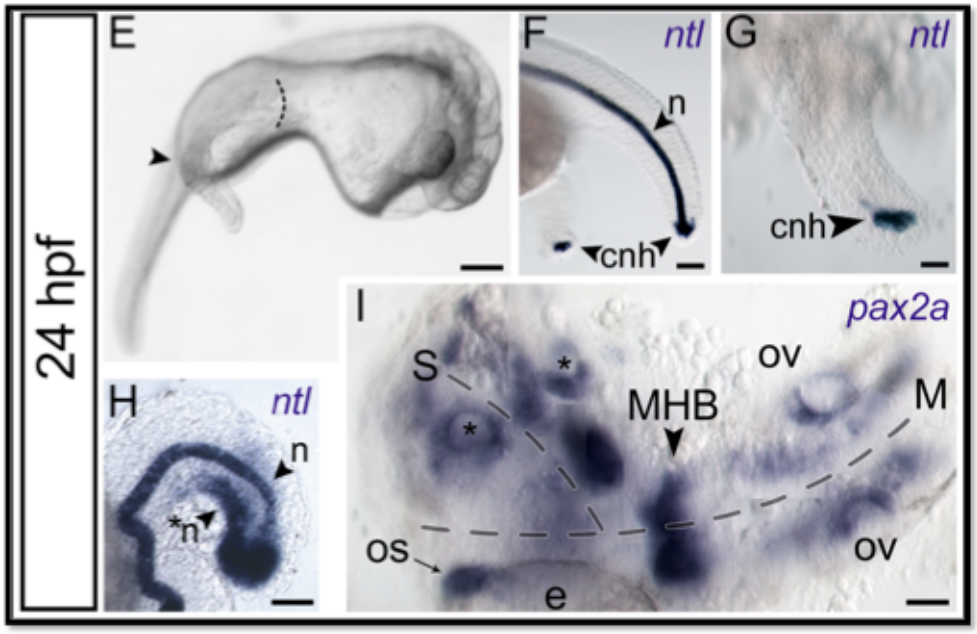

Figure 24. A46 AML cells induce secondary body axes formation in zebrafish embryos. The embryo in (E) has been hybridized with a probe specific for the mesodermal marker ntl. (F) The probe labels the notochord (n) in the endogenous trunk and the chordoneural hinge (cnh) in both tails (G, higher magnification). (H) Tail of a 24hpf embryo hybridized with ntl-specific probe. The endogenous (n) and ectopic (*n) ntl signals run parallel along the axis of the embryo, indicating the presence of additional axial structures. (I) Dorsal view of a 24hpf embryo that developed an ectopic head on the side of the endogenous one, as indicated by the expression of the brain-marker gene pax2a. The dotted lines indicate the main (M) and secondary (S) axes. The optic stalk (OS) in close vicinity to the eye (e), the midbrain-hindbrain boundary (MHB), and the otic vesicles (OV) of the embryo are stained with the pax2a riboprobe, as well as several areas of the ectopic head (the asterisks indicate the two clearly recognizable additional otic vesicles). The image is composed of different pictures corresponding to several focal planes, since the embryo is not flat, and a single focal plane cannot comprise all the labeled structures belonging to the main and secondary axes. Scale bars represent 125 mm (a,b,c,d), 150 mm (e), 40 mm (f), 15 mm (g,i), or 25 mm (h).

6. DISCUSSION

A fundamental challenge in cancer research is the identification of cells within a tumor that sustain the growth of the neoplastic clone. Phenotypic evidence indicates that somatic stem cells are target cells for malignant transformation[135]. The human AML-initiating cell, defined as SCID leukemia-initiating cell (SL-IC), was identified and purified by transplantation into NOD/SCID mice that recapitulates the human disease[136]. Based on these findings, it has been proposed a hierarchical organization for AML that is similar to normal hematopoiesis, in which the LSC is responsible for both self-renewal and the production of clonogenic leukemic progenitors that have proliferative capacity, but not the capacity of self-renewal. In this scenario the HSC is the most likely target for transformation into a LSC[77,137]. The concept that leukemia-initiating cell (LIC) properties occur in a self-renewing non-HSC progenitor cell population, preceded by the expansion of a preleukemic long-term hematopoietic stem cell (LT-HSC), has been recently reinforced[138-140]. However, the molecular function responsible for the preleukemic LT-HSC expansion and the acquisition of self-renewal ability in AML remain poorly defined. Human HSCs have traditionally been characterized by the expression of cell surface markers such as CD34, but not all human hematopoietic repopulating cells express CD34[141]. AC133 represents a glycosylation-dependent epitope of CD133 and its expression is restricted to a blood cell population highly enriched in HSCs CD34high, CD38low, c-Kit+[89]. This protein is thus one of the most specific markers for HSCs currently available. Furthermore, AC133+/CD34+ cells have a higher clonogenic capacity and engraftment rate than AC133-/CD34+ cells[92]. For the first time, here we show that AC133+ cells are highly expanded in AML by an average of 31.5% respect to healthy donors. In all the analyzed samples the AC133 immunostaining revealed clusters of positive cells, or single positive cells, amid a majority of negative tumor blasts. Some on the AC133+ clusters distribute in a symmetric manner, suggesting a possible clonal origin. Results achieved by a genome wide and multistep analysis of the generated data from microarray analysis defined the involvement of the distinct Wnt signaling pathway associated to the regeneration of HSCs as the master deregulated function in AC133-enriched fraction in human AML. Recently,

Wnt/β-catenin pathway requirement for LIC development in AML has emerged in a mouse model[142]. The results presented here provide direct evidence that the Wnt/β-catenin signaling is diffusely activated in human AC133+ AML cells in a ligand dependent manner, with a specific transcriptional signature involving overexpression of the Wnt pathway agonists and down-modulation of the major antagonists. Notably, WNT2B, WNT6, WNT10A, and WNT10B, known to promote tissue regeneration[117,143,144], are the WNT mediators specifically upregulated in the AC133+ AML cells. Consistent with the latter observation, we found also a dramatic increase of WNT10B and WNT2B expression in the large majority of samples from AML patients recruited to this study, with the exception of the unique therapy-related AML patient, thus suggesting an etiology-dependent deregulation. For the first time we could see the anatomic distribution of the leukemic cells in the BM of AML patients *via* the immunoblotting performed on BM biopsies. Nevertheless, exclusively we show a massive WNT10B expression in certain cells concomitantly with the diffuse release in the leukemic microenvironment of AML patients suggesting a ligand-dependent activation mode of the Wnt/β-catenin. In accordance with previous studies[145], we have not detected WNT10B gene expression in normal marrow hematopoietic cells, however WNT10B was found in the microenvironment that supports B cell differentiation[146]. In addition, there is evidence that in the hematopoietic system WNT10B and WNT10B is specifically and significantly upregulated following an injury, and that WNT10B acts to enhance the growth of HSCs[144]. It is also worth noting that grater fold expansion of murine hematopoietic stem cell progenitors was obtained after WNT10B conditioned media exposure[145]. Another ligand directly related to the regeneration function is WNT2B that we found massively expressed in AML patients except for the therapy related one, suggesting that the main “characters” of the regeneration function are implicated in leukemia initiation. Considering PYGO2 key role in promoting Wnt ligands responsiveness through its ability to interact with trimethylated K4 residues of histone H3 and its interaction with BCL9-β-catenin complexes at Wnt target genes[122-123], the shared overexpression of PYGO2 here documented in AML was not surprising. The establishment of a primary leukemic cell culture from AC133+ cells of an AML patient let us consider that this fraction was effectively enriched for the LIC since it lasted for 16 weeks without being treated with cytokines or serum.

Moreover, we are able to demonstrate that leukemia cells release WNTs ligands in the microenvironment conditioning the culture medium. Interestingly, the WNTs released in the medium are able to induce β-catenin activation in a dose dependent manner. It also is well known the pivotal role of WNTs gradients and β−catenin stabilization in establishing the dorsal organizer in zebrafish embryos[147]. Here we have demonstrated that cultured AC133+ A46 cells have the ability to cause secondary ectopic organizers marked by the Wnt/β−catenin dependent marker *gsc*. Finally, A46 cells are able to induce a secondary axis formation in zebrafish embryos through the initiation of dorsal or tail organizer activity[148] by inducing Wnt dependent markers.

7. CONCLUSIONS

A new and pivotal insight for cancer research is the identification of cells within a tumor that sustain the growth of neoplastic clone. The entire cellular diversity of AML was recapitulated establishing that leukemia is organized as a hierarchy sustained by rare leukemia stem cells (LSCs) resembling normal development, although LSCs possess higher self-renewal capacity. AC133 is one of the most specific and efficient cell surface marker available for the hematopoietic stem cell. Here we show that AC133+ population in AML patients is highly expressed compared to the healthy donor cells AC133+. In the bone marrow of AML patients, AC133+ distribute in clustered population surrounded by blasts, in a simil-symmetric disposition suggesting a clonal origin. For the first time, here we show the anatomical disposition of AC133+ cells in AML patients. The AC133+ fractions were employed to gene expression microarray analysis, gene-ontology-based functional annotations and bioinformatic tools in order to study the leukemogenesis focusing on transcriptional regulators as mediators of reprogramming. From the functional enrichment analysis, obtained using GOstats Bioconductor package, a pivotal and unique transcriptional pathway has emerged: the *wnt receptor signaling pathway*. Here we propose an interaction model in which defined master "executer" TR in synergism with chromatin re-modellers enhance the responsiveness of the system acting to induce a stronger, possibly leukemogenic, response of the final "player" genes. Thus, *in vitro* experiments performed on AML patients samples allowed us to demonstrated the massive activation of WNT/β–catenin signaling pathway in a ligand depend way, for example *via* an expression autocrine/paracrine of WNT10B, one of the principal ligand belonging to the WNT signaling that is strongly associated to the regenerative function. Also WNT2B, a second and not less important actor of the regeneration function, is highly activated in AML patients except for the only therapy-related one. Finally, AC133+ A46 leukemic primary culture is able to release in the medium WNTs that can induce β-catenin activation in a dose dependent manner. Thus AC133+A46 cell showed a regenerative potential inducing the formation of secondary axial structures in embryos zebrafish. In light of the long-known association between cancer and chronic tissue injury[107], and because of the higher homeostatic range of Wnt/β-

catenin signaling occurring during regeneration upon an acute injury[120]. Our data rise important implications for the involvement of the Wnt pathway in the origin of AML, considering its pivotal function in promoting self-renewal[22], its emerging role in myeloid leukemogenesis[137], and the effects of its constitutive activation via a stabilized form of β-catenin, a key component of canonical Wnt signalling[137], by inducing quiescent stem cells to enter the cell cycle and by arresting their differentiation.

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9. SCIENTIFIC PRODUCTS

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Supplementary Table 1. Clinical characteristics and outcome of AML patients

No	Age,y/ sex	FAB	Cytogenetics*	FLT3	NPM1	WBC, x 10^9/L	MO blast, %	EML	s-AML	Response to induction	Relapse	Outcome
#1	67/M	M2	45,XY,-7	wt	-	1,7	55	Absent	Yes°	CR	Yes	D/1st res rel
#2	45/M	M1	Complex karyotype	wt	-	1,7	60	Absent	No	PR	No	A/1st CR
#4	59/M	M4	46,XY,del(20)(q11q13)	wt	-	3,5	32	Absent	No	ref dis	No	A/1st CR
#5	47/F	M4	46,XX	ITD	-	84,6	80	Skin	No	CR	No	A/1st CR
#6	76/F	M4	47,XX,+11	wt	-	-		Absent	Yes°	Ref dis	n.a.	D/prim ref dis
#9	20/M	M0	46,XY	wt	-	3,6	80	Absent	No	CR	Yes	A/2nd CR
#10	62/F	M5a	46,XX	wt	-	69	90	Skin	No	ref dis	No	A/1st CR
#13	57/M	M1	46,XY,t(6;9)(p23;q34)	ITD	wt	39,7	89	Absent	No	ref dis	n.a.	D/prim ref dis
#14	72/F	M2	46,XX	wt	-	-		Absent	No	ref dis	n.a.	D/prim ref dis
#16	41/F	M2	46,XX	wt	wt	15,9	45	Absent	No	CR	No	A/1st CR
#17	29/M	M1	46,XY	ITD	wt	217,7	96	Absent	No	CR	Yes	D/1st res rel
#19	29/F	M1	46,XX,del(11q23)	wt	-	110,6	95	Skin	No	ref dis	Yes	D/1st res rel
#21	22/M	M1	46,XY	ITD	wt	16,7	75	Absent	No	ref dis	No	A/1st CR
#23	65/M	M1	46,XY	wt	wt	12,7	78	Absent	No	CR	Yes	A/2nd CR
#24	59/M	M1	46,XY	wt	Exon 12	20,1	75	Absent	No	CR	Yes	A/1st rel
#25	39/F	M1	46,XX,del(11q23)	wt	wt	15,7		Absent	No	ref dis	n.a.	D/prim ref dis
#30	41/M	M5a	46,XY	wt	wt	3,6	75	Absent	No	CR	Yes	A/1st rel
#32	52/F	M2	46,XX	wt	wt	2,7	55	Absent	Yes°	CR	No	D/TRM 1st CR
#34	29/M	M0	46,XY,del(11)(q13q23)	wt	wt	4	86	Absent	No	CR	Yes	D/1st res rel
#38	59/F	M1	46,XX	wt	Exon 12	1	82	Absent	No	CR	Yes	D/1st res rel
#39	51/M	n.a.	Complex karyotype	wt	wt	1,2		Absent	No	ref dis	n.a.	D/prim ref dis
#40	55/F	M2	Complex karyotype	wt	wt	0,8	20	Absent	Yes†	n.a.	n.a.	A/active dis
#41	62/F	M4	46,XX	wt	wt	17	30	Skin	Yes°	ref dis	Yes	A/1st res rel
#42	65/F	M4	46,XX	wt	wt	1,2	60	Adnexal mass	No	ref dis	n.a.	A/prim ref dis
#44	56/F	M2	46,XX,t(8;21)(q22;q22),inv(9)(p11q12)	wt	wt	8,6	65	Absent	No	PR	Yes	A/2nd rel
#46	66/M	M2	46,XY	ITD	wt	26,8	80	Absent	No	PR	n.a.	A/prim ref dis
#47	62/F	M2	47,XX,+21	wt	Exon 12	23	12	Absent	Yes°	n.a.	n.a.	A/active dis
#48	43/M	M5a	46,XY	ITD	Exon 12	63,9	85	Absent	No	CR	Yes	A/2nd CR
#49	68/F	biphen.	45,XX,-7,t(9;22)(q34;q11)	wt	wt	8,1	70	Absent	No	CR	Yes	A/2nd CR
#50	61/F	M4	Complex karyotype	wt	wt	8,8	35	Absent	No	ref dis	n.a.	D/prim ref dis
#51	15/F	M5b	46,XX	wt	wt	118	84	CNS	No	ref dis	Yes	D/1st res rel
#52	33/M	M2	46,XY	wt	Exon 12	20,9	40	Absent	No	CR	No	A/1st CR
#53	51/F	M1	46,XX	wt	Exon 12	81,1	77	Absent	No	CR	No	A/1st CR

- A, alive; CR, complete remission;PR, partially remission;D, dead; ref dis, refratory disease; res rel, resistant relapse; biphen., biphenotypic; TRM, transplant related mortality; s-AML, secondary AML; n.a., not applicable; EML, extramedullary leukemia.
- *: at diagnosis
- °: myelodisplastic-AML
- †: therapy-related AML

Supplementary Table 2: Functional enrichment analysis: selected genes associated with GO:0016055 term.

Gene ID	Gene	Gene Description
56033	BARX1	BARX homeobox 1
8945	BTRC	beta-transducin repeat containing
1499	CTNNB1	catenin (cadherin-associated protein), beta 1, 88kDa
1500	CTNND1	catenin (cadherin-associated protein), delta 1
23291	FBXW11	F-box and WD repeat domain containing 11
26959	HBP1	HMG-box transcription factor 1
51176	LEF1	lymphoid enhancer-binding factor 1
5626	PROP1	PROP paired-like homeobox 1
26108	PYGO1	pygopus homolog 1 (Drosophila)
90780	PYGO2	pygopus homolog 2 (Drosophila)
5916	RARG	retinoic acid receptor, gamma
6657	SOX2	SRY (sex determining region Y)-box 2
83439	TCF7L1	transcription factor 7-like 1 (T-cell specific, HMG-box)
7088	TLE1	transducin-like enhancer of split 1 (E(sp1) homolog, Drosophila)
7090	TLE3	transducin-like enhancer of split 3 (E(sp1) homolog, Drosophila)
7091	TLE4	transducin-like enhancer of split 4 (E(sp1) homolog, Drosophila)
8840	WISP1	WNT1 inducible signaling pathway protein 1
80326	WNT10A	wingless-type MMTV integration site family, member 10A
7480	WNT10B	wingless-type MMTV integration site family, member 10B
7482	WNT2B	wingless-type MMTV integration site family, member 2B
7473	WNT3	wingless-type MMTV integration site family, member 3
54361	WNT4	wingless-type MMTV integration site family, member 4
81029	WNT5B	wingless-type MMTV integration site family, member 5B
7475	WNT6	wingless-type MMTV integration site family, member 6
7479	WNT8B	wingless-type MMTV integration site family, member 8B
10009	ZBTB33	zinc finger and BTB domain containing 33

Supplementary Table 3: “Wnt signaling” differentially expressed genes.

a. *Differentially over-expressed genes.*

Probe	Gene	GeneID	pValue
217729_s_at	AES	166	0
217174_s_at	APC2	10297	0.011
219845_at	BARX1	56033	0.016
211518_s_at	BMP4	652	0.047
222374_at	BTRC	8945	0.041
209002_s_at	CALCOCO1	57658	0.009
208711_s_at	CCND1	595	0.034
204490_s_at	CD44	960	0
207144_s_at	CITED1	4435	0.004
202790_at	CLDN7	1366	0.014
226920_at	CSNK1A1	1452	0
229212_at	CSNK2A1	1457	0.011
201390_s_at	CSNK2B	1460	0.037
201533_at	CTNNB1	1499	0.004
1557944_s_at	CTNND1	1500	0.002
219908_at	DKK2	27123	0.009
231778_at	DLX3	1747	0.002
38707_r_at	E2F4	1874	0.004
209589_s_at	EPHB2	2048	0.005
1438_at	EPHB3	2049	0.005
209456_s_at	FBXW11	23291	0.022
214702_at	FN1	2335	0.001
207345_at	FST	10468	0.025
224337_s_at	FZD4	8322	0.004
224325_at	FZD8	8325	0.001
218468_s_at	GREM1	26585	0.016
1552338_at	GSC	145258	0.03
209945_s_at	GSK3B	2932	0.027
205718_at	ITGB7	3695	0.002
209097_s_at	JAG1	182	0.025
204584_at	L1CAM	3897	0.001
221560_at	MARK4	57787	0
1566677_at	MMP2	4313	0.004
220541_at	MMP26	56547	0.002
204259_at	MMP7	4316	0
209756_s_at	MYCN	4613	0
206022_at	NDP	4693	0.005
229481_at	NKD1	85407	0.002
232201_at	NKD2	85409	0.014
241911_at	PPP2CA	5515	0.002
211223_at	PROP1	5626	0.002
215517_at	PYGO1	26108	0.011
225370_at	PYGO2	90780	0
2041898_at	RARG	5916	0.013
1554012_at	RSPO2	340419	0.017
221283_at	RUNX2	860	0.001
223121_s_at	SFRP2	6423	0.026
204051_s_at	SFRP4	6424	0.003
218788_s_at	SMYD3	64754	0
213456_at	SOSTDC1	25928	0.003
230943_at	SOX17	64321	0.034
1567906_at	SOX4	6659	0
208992_s_at	STAT3	6774	0.007
221016_s_at	TCF7L1	83439	0.005
227130_s_at	TLE1	7088	0.013

1553813_s_at	TLE6	79816	0.001
211312_s_at	WISP1	8840	0.027
229154_at	WNT10A	80326	0.005
206213_at	WNT10B	7480	0.017
206458_s_at	WNT2B	7482	0.012
222086_s_at	WNT6	7475	0.005
214631_at	ZBTB33	10009	0.004

b. Differentially under-expressed genes.

Probe	**Gene**	**GeneID**	**pValue**
208161_s_at	ABCC3	8714	0.008
203196_at	ABCC4	10257	0
204129_at	BCL9	607	0.018
202094_at	BIRC5	332	0.017
238545_at	BRD7	29117	0.005
231288_at	CCDC88C	440193	0.019
266_s_at	CD24	100133941	0
201131_s_at	CDH1	999	0.007
202160_at	CREBBP	1387	0.011
219179_at	DACT1	51339	0
20198_at	DVL3	1857	0.001
204947_at	E2F1	1869	0
213579_s_at	EP300	2033	0.016
202894_at	EPHB4	2050	0
209189_at	FOS	2353	0
204451_at	FZD1	8321	0
219683_at	FZD3	7976	0.017
221245_s_at	FZD5	7855	0.003
203987_at	FZD6	8323	0.004
203705_s_at	FZD7	8324	0.001
236645_at	HBP1	26959	0.001
213931_at	ID2	3398	0.002
211055_at	INVS	27130	0.004
201465_s_at	JUN	3725	0.003
225068_at	KLHL12	59349	0
227250_at	KREMEN1	83999	0.014
221558_s_at	LEF1	51176	0.011
225745_at	LRP6	4040	0
211675_s_at	MDFIC	29969	0.027
203936_s_at	MMP9	4318	0
220184_at	NANOG	79923	0.042
229027_at	PPM1A	5494	0.004
203554_x_at	PTTG1	9232	0.043
204535_s_at	REST	5978	0.037
216645_at	TCF3	6929	0.002
212382_at	TCF4	6925	0.001
205255_x_at	TCF7	6932	0.035
212761_at	TCF7L2	6934	0.005
213135_at	TIAM1	7074	0
210512_s_at	VEGFA	7422	0.005
204712_at	WIF1	11197	0