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# Role of the Bone Morphogenetic Proteins pathway in leukemic stem cell regulation and resistance in acute myeloid leukemia

Mario Flores Violante

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**Role of the Bone Morphogenetic  
Proteins pathway in leukemic stem  
cell regulation and resistance in  
acute myeloid leukemia**

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## ABSTRACT

Acute myeloid leukemias (AML) are heterogeneous hematological malignancies characterized by a clonal proliferation of myeloid blasts which infiltrate the bone marrow, blood and other organs. Identified as the most common type of acute leukemia in adults with 80% of cases, AML is associated with high relapse and poor prognosis where 70% of patients face mortality within one year after diagnosis. Leukemic stem cell (LSCs) presence has been related to resistance to chemotherapeutic agents and relapse in AML. The tumor microenvironment has been described for its key role regulating LSCs through the crosstalk of signaling pathways. Bone Morphogenetic Proteins (BMP) pathway is highly involved in hematopoietic stem cell (HSC) regulation, but has also been recognized to regulate LSCs. Here, we have identified high concentrations of BMP2 and BMP4 in bone marrow (BM) AML samples at diagnosis. Furthermore, we have identified for the first time a new signaling cascade, involving the binding of BMP4 to BMPR1A receptor, which induces the expression of  $\Delta$ Np73 and NANOG. Activation of this signaling promotes a stem-like phenotype in leukemic cells. Therefore, we hypothesized that this signaling is responsible for the resistant capacity of leukemic cells to chemotherapy. In addition, we have reported BMPR1A/ $\Delta$ Np73/NANOG as potential AML prognosis markers, due to their overexpression at diagnosis associated to an increased rate of relapse of AML patients within three years. When we analyzed AML samples at relapse, higher levels of  $\Delta$ Np73 isoform were found compared to patients at diagnosis. Moreover, we have identified high expression of the BMPR1A receptor,  $\Delta$ Np73, NANOG, SOX2 and ID1 in short-term resistant primary leukemic cells. These results correlate with what we observed in AML resistant cells, where BMPR1A,  $\Delta$ Np73, NANOG and ID1 seem to be implicated in driving the resistant capacity of AML cells to drug therapy. Therefore, modulation and targeting of the BMP pathway elements and related genes identified with our study, represent a promising approach towards the development of new and more effective therapeutic strategies against AML.

**Key words:** AML, tumor microenvironment, leukemic stem cell, BMP pathway, resistance.

## RÉSUMÉ

Les leucémies aiguës myéloïdes (LAM) sont des maladies hématologiques hétérogènes caractérisées par une prolifération clonale des blastes myéloïdes qui s'infiltrent dans la moelle osseuse (MO), le sang et d'autres organes. Identifiée comme le type le plus courant de leucémie aiguë chez l'adulte avec 80% des cas, la LAM est synonyme de rechute et de mauvais pronostic, avec 70% des patients étant confrontés à une mortalité dans l'année suivant le diagnostic. La présence des cellules souches leucémiques (CSL) a été associée à une résistance à la chimiothérapie et à une rechute dans la LAM. Le microenvironnement tumoral a été décrit pour son rôle clé dans la régulation des CSL par l'interaction des voies de signalisation. La voie des Bone Morphogenetic Proteins (BMP) est fortement impliquée dans la régulation des cellules souches hématopoïétiques (CSH), mais elle a également été reconnue pour réguler les CSL. Ici, nous avons identifié des concentrations élevées de BMP2 et BMP4 dans la MO des patients atteints de LAM au moment du diagnostic. De plus, nous avons identifié pour la première fois une nouvelle cascade de signalisation impliquant la liaison de BMP4 au récepteur BMPR1A, qui induit l'expression de  $\Delta$ Np73 et NANOG. L'activation de cette signalisation favorise un phénotype proche des cellules souches dans les cellules leucémiques. Par conséquent, nous avons émis l'hypothèse que cette voie est responsable de la capacité de résistance des cellules leucémiques à la chimiothérapie. En outre, nous avons identifié BMPR1A/ $\Delta$ Np73/NANOG comme marqueurs potentiels du pronostic dans la LAM, en raison de leurs surexpressions au moment du diagnostic associé à une rechute dans les trois ans. Lorsque nous avons analysé des échantillons de LAM lors d'une rechute, nous avons constaté des taux plus élevés de l'isoforme  $\Delta$ Np73 par rapport à ceux de patients au moment du diagnostic. D'autre part, nous avons identifié une forte expression du récepteur BMPR1A,  $\Delta$ Np73, NANOG, SOX2 et ID1 dans les cellules leucémiques primaires résistantes à court terme. Ces résultats sont en corrélation avec ce que nous avons observé dans les cellules résistantes de LAM, où BMPR1A,  $\Delta$ Np73, NANOG et ID1 semblent être impliqués dans la capacité de résistance des cellules de LAM face à la chimiothérapie. La modulation et le ciblage des éléments de la voie BMP et des gènes associés identifiés au travers de notre

étude représentent donc une approche prometteuse pour le développement de stratégies thérapeutiques innovantes et plus efficaces contre les LAM.

**Mots clé :** LAM, microenvironnement tumoral, cellule souche leucémique, voie BMP, résistance





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## LIST OF ABBREVIATIONS

<b>AGM</b>	Aorta-Gonad-Mesonephros region
<b>ALL</b>	Acute Lymphoid Leukemia
<b>AMKL</b>	Acute Megakaryoblastic Leukemia
<b>AML</b>	Acute Myeloid Leukemia
<b>ANAE</b>	$\alpha$ -Naphthyl Acetate Esterase
<b>Ang1</b>	Angiopoietin-1
<b>APL</b>	Acute Promyelocytic Leukemia
<b>ARA-C</b>	Cytosine-Arabinoside
<b>ATRA</b>	All-Trans Retinoic Acid
<b>bHLH</b>	basic Helix-Loop-Helix
<b>BM</b>	Bone Marrow
<b>BMP</b>	Bone Morphogenetic Protein
<b>BPA</b>	Bisphenol A
<b>BRAM1</b>	Bone morphogenetic protein Receptor Associated Molecule 1
<b>CAR</b>	Chimeric Antigen Receptor
<b>CBF</b>	Core Binding Factor
<b>CBF-AML</b>	Core Binding Factor-AML
<b>CD</b>	Cluster of Differentiation
<b>CH</b>	Clonal Hematopoiesis
<b>CHIP</b>	Clonal Hematopoiesis of Indeterminate Potential
<b>CLL</b>	Chronic Lymphoid Leukemia
<b>CML</b>	Chronic Myeloid Leukemia
<b>CR</b>	Complete Remission
<b>CSC</b>	Cancer Stem Cell
<b>CXCL12</b>	C-X-C-motif Chemokine Ligand 12
<b>DBD</b>	DNA-Binding-Domain
<b>DHH</b>	Desert Hedgehog
<b>DMAPT</b>	Dimethylaminoparthenolide
<b>DNA</b>	Deoxyribonucleic acid
<b>DNR</b>	Daunorubicin
<b>DOX</b>	Doxorubicin



<b>ECM</b>	Extracellular Matrix
<b>EFS</b>	Event-Free Survival
<b>EGFR</b>	Epidermal Growth Factor Receptor
<b>EMPs</b>	Erythroid-myeloid progenitors
<b>EMT</b>	Epithelial to Mesenchymal Transition
<b>EPI</b>	Epirubicin
<b>EPO</b>	Erythropoietin
<b>ERK</b>	Extracellular signal-Regulated Kinase
<b>ESC</b>	Embryonic Stem Cell
<b>FAB</b>	French-American-British classification
<b>FDA</b>	Food and Drug Administration
<b>FGF</b>	Fibroblast Growth Factor
<b>FLT3</b>	Fms-Like Tyrosine Kinase 3
<b>FLT3-ITD</b>	Fms-Like Tyrosine Kinase 3-Internal Tandem Duplication
<b>G-CSF</b>	Granulocyte colony-stimulating factor
<b>GDF</b>	Growth Differentiation Factor
<b>GDNF</b>	Glial Derived Neurotrophic Factors
<b>GLI1</b>	Zinc-Finger Glioma 1
<b>GO</b>	Gemtuzumab Ozogamicin
<b>GPI</b>	Glycosylphosphatidylinositol
<b>GS</b>	Glycine-Serine
<b>HDAC</b>	Histone Deacetylase
<b>HGF</b>	Hepatocyte Growth Factor
<b>HH</b>	Hedgehog
<b>HIF-1<math>\alpha</math></b>	Hypoxia Inducible Factor-1 $\alpha$
<b>HSC</b>	Hematopoietic stem cell
<b>HSPC</b>	Hematopoietic Stem Progenitor Cell
<b>ID</b>	Inhibitor of Differentiation
<b>IDA</b>	Idarubicin
<b>IDH</b>	Isocitrate Dehydrogenase
<b>IHC</b>	Immunohistochemistry
<b>IHH</b>	Indian Hedgehog
<b>IL</b>	Interleukin

<b>iPSC</b>	induced Pluripotent Stem Cell
<b>I-SMADS</b>	Inhibitory-SMADS
<b>IT-HSC</b>	Intermediate-term Hematopoietic Stem Cell
<b>JNK</b>	C-jun N-terminal Kinase pathway
<b>Lin</b>	Lineage
<b>LSC</b>	Leukemic Stem Cell
<b>LT-HSC</b>	Long-Term Hematopoietic Stem Cell
<b>MAPK-p38</b>	Map Kinase p38 pathway
<b>MDS</b>	Myelodysplastic Syndrome
<b>MEN1</b>	Multiple Endocrine Neoplasia 1 gene
<b>MIXL1</b>	Mesoderm Inducer in Xenopus Like 1 gene
<b>MLL</b>	Mixed-Lineage Leukemia
<b>MMAE</b>	Monomethyl Auristatin E
<b>mPB</b>	mobilized Peripheral Blood
<b>MPP</b>	Multi-Potent Progenitor
<b>MPO</b>	Myeloperoxidase
<b>MRD</b>	Minimal Residual Disease
<b>MSC</b>	Mesenchymal Stromal Cell
<b>NF-<math>\kappa</math>B</b>	Nuclear factor kappa-light-chain enhancer of activated B-cell
<b>NK</b>	Natural Killer cells
<b>NPM</b>	Nucleophosmin
<b>NSCLC</b>	Non-Small Cell Lung Carcinoma
<b>OB</b>	Osteoblast
<b>OCL</b>	Osteoclast
<b>OD</b>	Oligomerization Domain
<b>OS</b>	Overall Survival
<b>OSM</b>	Oncostatin M
<b>PB</b>	Peripheral Blood
<b>PC</b>	Prostate Cancer
<b>PGE2</b>	Prostaglandin E2
<b>PI3K</b>	Phosphatidylinositol 3-kinase
<b>P-Sp</b>	Para-aortic Splanchnopleura
<b>PPAR<math>\gamma</math></b>	Peroxisome Proliferator-Activated Receptor Gamma

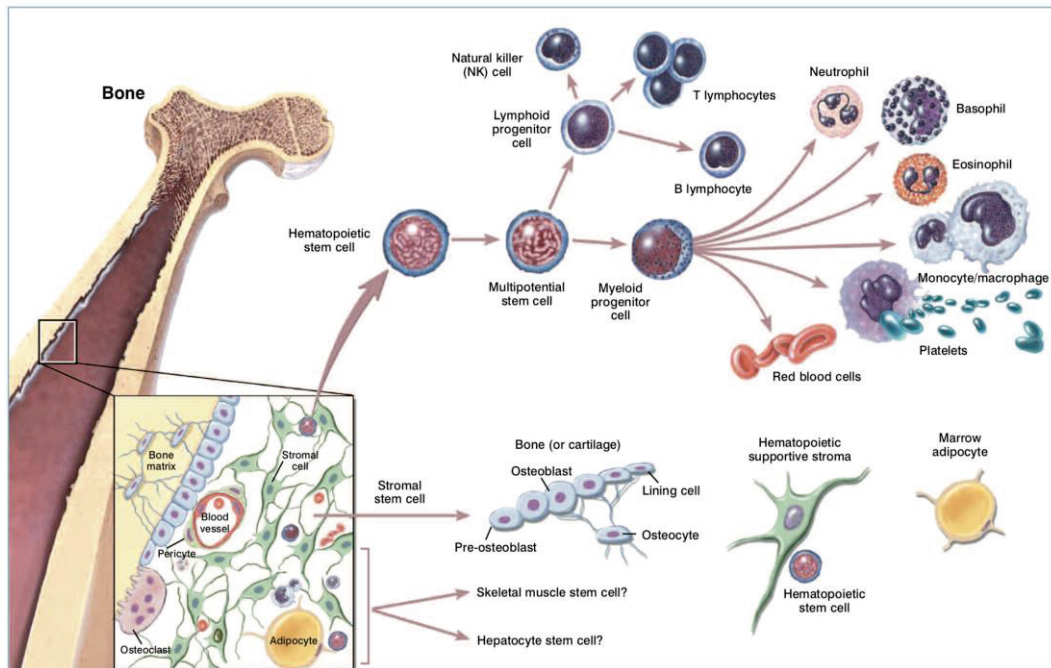
<b>PTCH</b>	Patch receptor
<b>RGM</b>	Repulsive Guidance Molecule
<b>RNA</b>	Ribonucleic Acid
<b>ROS</b>	Reactive Oxygen Species
<b>RTK</b>	Receptor Tyrosine Kinase
<b>SC</b>	Stem Cell
<b>SCF</b>	Stem Cell Factor
<b>SEER</b>	Surveillance Epidemiology and End Result program
<b>SF</b>	Scatter Factor
<b>SHH</b>	Sonic Hedgehog signaling pathway
<b>SLAM</b>	Signaling Lymphocyte Activation Molecule
<b>SL-IC</b>	SCID Leukemia Initiating-Cell
<b>SMO</b>	Smoothed receptor
<b>ST-HSC</b>	Short-Term Hematopoietic Stem Cell
<b>TA</b>	Transactivation domain
<b>TAB1</b>	TAK1 binding protein
<b>TAK1</b>	TGF- $\beta$ Activated Kinase I
<b>TAp73</b>	Transcriptionally Active p73
<b>TCGA</b>	The Cancer Genome Atlas
<b>TGF-<math>\beta</math></b>	Transforming Growth Factor- $\beta$
<b>TP73</b>	Tumor Protein 73
<b>THPO</b>	Thrombopoietin
<b>VCAM-1</b>	Vascular Cell Adhesion Molecule 1
<b>VEGF</b>	Vascular Endothelial Growth Factor
<b>WHO</b>	World Health Organization
<b>XIAP</b>	X-linked Inhibitor of Apoptosis Protein
<b>YS</b>	Yolk sac
<b><math>\Delta</math>Np73</b>	Dominant-Negative p73

# **I. INTRODUCTION**

# 1 Hematopoiesis and Hematopoietic Stem Cell (HSC)

## 1.1 Generalities of the hematopoietic system

Blood is a tissue in constant regeneration with approximately one trillion cells produced every day in a normal human adult bone marrow (BM). The hematopoietic system is formed by the blood in which it is possible to identify several types of cell lineages holding specific functions in the organism (Figure 1). Many of the different blood cells present a short lifespan, this is the reason why a constant regeneration is carried out daily in the medullary cavity of certain bones in adult mammals. The permanent blood cell regeneration rest on a key player. This is the hematopoietic stem cell (HSC); described in 1909 by Alexander Maximow, as the head of a hierarchy and main common precursor of all different multilineages progenitors giving rise to more differentiated blood cells. The HSC is capable to produce two main progenitor populations (**Figure 1**). The myeloid progenitor cells can differentiate into red blood cells on charge of oxygen transportation along the tissues, platelets able to stop the bleedings through clot formation or granulocytes and macrophages with the task of fighting infections. On the other hand, the HSC can give rise to the lymphoid progenitor cells holding the ability to differentiate into B-lymphocytes that can produce antibodies, T-lymphocytes able to kill directly agents recognized as strangers for the body. And also, natural killer (NK) cells are a product from the lymphoid progeny (**Figure 1**)<sup>1,2,3</sup>.



**Figure 1. Hematopoietic and stromal differentiation<sup>1</sup>.** The hematopoietic stem cell (HSC) and all the different blood lineages produced from the HSC.

## 1.2 Hematopoiesis in humans

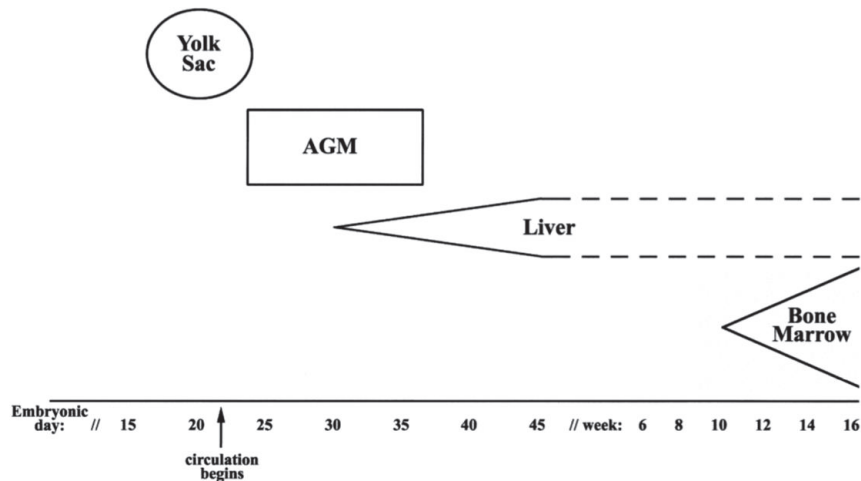
Hematopoiesis is the dynamic process of blood cell components formation, in which the multipotent hematopoietic stem cells are capable to differentiate and give rise to all different blood lineages. It is a procedure that takes place for the first time during embryonic development and continues during adulthood in order to restore the blood cell populations. Hematopoiesis has been described in vertebrates as a two-wave event, the primitive and the definitive events. The differences between these two waves are the type of cells produced and the anatomic region where it occurs. The so-called “primitive wave” happens temporarily and generates the erythroid progenitors able to differentiate into erythrocytes and macrophages. This wave takes place during the early embryonic development and its aim is essentially the production of red blood cells in order to transport the oxygen along the tissues in the embryo. This erythroid population can be seen in mammals for the first time in the extra-embryonic yolk sac. As mentioned before, this “primitive” stage gives rise only to a non-pluripotent lineage with no self-renewal capacity. The “definitive” hematopoiesis wave appears later during development and can be different depending of the

specie<sup>4,5</sup>. In the majority of species, a first “definitive” hematopoiesis wave is observed in the blood islands by erythroid-myeloid progenitors (EMPs) production. Following this first wave, the “definitive” hematopoiesis implicates the presence of hematopoietic stem cells holding the multipotent capacity and provides all blood lineages of a normal organism<sup>4</sup>.

The process of hematopoiesis in humans as the rest of the vertebrates initiates in the yolk sac (YS) during the embryo stage and evolves its location to the liver, to finally establish permanently in the thymus and bone marrow (**Figure 2**). This first stage of the hematopoiesis in the YS is the primitive wave seen after 18.5 days of development, where it is possible to identify the presence of erythroblasts and CD34+ hematopoietic cells. At day 22 the heart will start beating allowing the circulation of erythroid cells within the embryo. Once the embryo arrives to day 24 the YS mesoderm stops differentiation and the intravascular blood islands are no longer present. Following the YS, the next hematopoiesis wave occurs in the Aorta-Gonad-Mesonephros (AGM) region. Between days 24 and 34, it has been identified in the Para-aortic Splanchnopleura (P-Sp) the existence of hematopoietic cells with myeloid and lymphoid potentials. Moreover, CD34+ hematopoietic cells can be found in the mesenchyme surrounding the dorsal aorta at day 26, while these cells will also be observed between days 26-36 within the endothelium of the dorsal aorta and the ventral cell clusters<sup>4,5</sup>.

As embryo development advances, the liver appears as the next important hematopoiesis site. During day 23, liver growth starts and the hematopoietic cells begin the establishment and expansion in this region. On day 30 the first hematopoietic progenitors CD45+ CD34+ can be found and by day 42 the CD34+ population reaches a considerable number, turning the liver into the principal hematopoietic organ at this point. Mainly erythroid cell population is seen in the embryonic liver at that point, extravascularly located. The hematopoietic capacity of the liver concludes at birth. Nonetheless, some erythroid precursors can be found in newly born. Finally, as mentioned before, the bone marrow represents the last and main hematopoiesis source. Between weeks 8.5 and 9 an invasion of osteoblasts, osteoclasts and perichondral precursors takes place within the marrow cavity. In addition, it is possible to identify from week 10.5 the beginning

of hematopoiesis in the bone marrow through the recognition of CD45+ hematopoietic cells, CD15+ and CD68+ cells and in a lower amount CD34+ hematopoietic cells. The bone formation is accomplished by week 16 and the dynamic process of hematopoiesis carries on during the adulthood<sup>4,5</sup>.



**Figure 2. General representation of the chronological appearance of hematopoiesis in humans<sup>4</sup>.**

### 1.3 Hematopoietic Stem Cell (HSC)

#### 1.3.1 Defining the concept of HSC

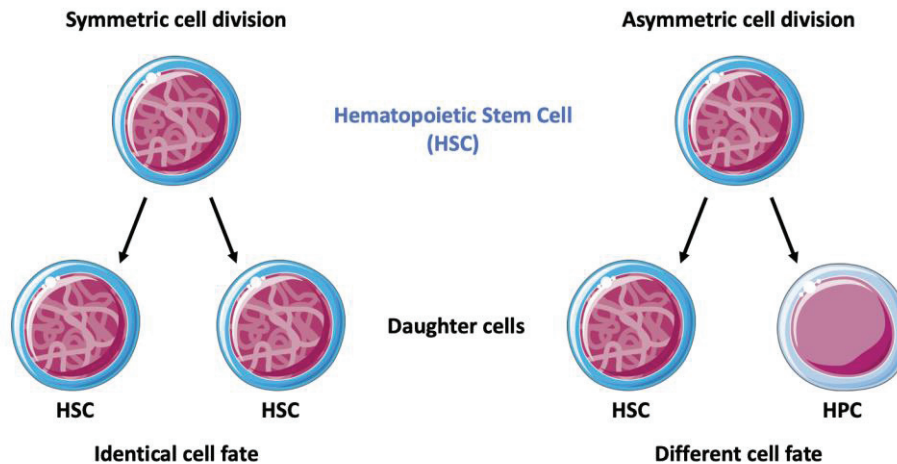
First evidence proposing the existence of the Hematopoietic Stem Cell (HSC) came right after the nuclear bombings in 1945. Individuals who did not die during the main bomb explosions were exposed to different radiation levels, depending of their location during and after the bombings. For example, people exposed to the radiation for a long period developed failures in their hematopoietic system, facing the lack of regeneration of all different blood cell types and in consequence leading to death due to an abnormal organism function. Treatment for these patients was based on transplantation of bone marrow cells from donors who were not exposed to radiation. Interestingly the patients achieved a total blood system regeneration after this procedure, demonstrating that cells present in the BM and more specifically, the HSC population was able to repopulate the BM host with the different blood lineages essential for a normal body function<sup>1</sup>.



The functional capacity of the HSC was assessed in 1961 by Till and McCulloch who actually proposed for the first time the “stem cell” concept in hematopoietic cells. Their original studies describing the HSC capacity were based on the regeneration of the blood system *in vivo*. Irradiated mice received transplantation of genetically identical and immunologically compatible bone marrow cells. Ten days after the transplant, cellular colonies corresponding to myeloid and erythroid lineages were found in the spleen of the mice. Colonies were recovered and after analyzing them, a small sub-fraction owned two major characteristics making them different from the rest of the cells found. The first characteristic was the ability of this population to give rise to myeloerythroid lineages. While the second one, was the self-replication capacity shown by these cells. Based on these two parameters it was possible to identify that the HSCs are the only cells within the bone marrow presenting the multi-potency and in parallel self-renewal to give rise to an identical HSC without suffering differentiation<sup>6,3,1</sup>.

### 1.3.2 Major characteristics

Hematopoietic stem cells are a population holding unique and specific properties that render them different from other progenitor or more mature cells. HSCs possess a special trademark and this hallmark combines different abilities. For instance, self-renewal capacity where the HSC can maintain the stemness status after cell division or follow the path of differentiation towards a mature hematopoietic cell. Division of HSCs can follow either a symmetrical division in which a generation of two daughter HSCs takes place or asymmetrical division where an HSC is generated along with a daughter cell that holds the task of differentiation to give rise to other more mature blood cell types (**Figure 3**). Moreover, migration is another important characteristic of HSCs not only during the stages of development but also in adulthood. Finally, apoptosis is another important element able to regulate the number of HSCs to maintain hemostasis<sup>7,1</sup>.



**Figure 3. HSC symmetric and asymmetric division.** HSCs are able to follow a symmetric division generating two identical HSCs carrying identical cell fate. Otherwise undergo into asymmetric division where two cell daughters are generated: one HSC and a more differentiated cell HPC (Hematopoietic progenitor cell) holding different cell fate.

Self-renewal is the capability of cells to generate a copy of themselves which presents either exactly the same or highly similar potential. This is a main characteristic of the HSCs, since other populations as hematopoietic progenitor cells can give rise to other mature blood cells during a long but limited period of time. Therefore, the presence of HSCs is crucial to sustain the long-term generation of the hematopoietic lineages. At the present time, it is not possible yet to confirm that HSCs can go through endless self-renewal and several research studies are trying to understand the mechanism driving this major characteristic. A first hint linked to self-renewal is telomerase. This enzyme also known as “terminal transferase” is a ribonucleoprotein on charge of maintaining the telomeres; regions that protect the end of the chromosomes from DNA (Deoxyribonucleic acid) damages or fusion with close chromosomes due to DNA replication<sup>8</sup>. Although telomerase has been identified as an essential factor for self-renewal, it seems to be only one of other elements driving this potential. Studies using HSCs from mice showed that lack of telomerase was related to a decreased self-renewal ability. Nonetheless, forced expression of telomerase in cells was not sufficient for HSCs to reach undefined capacity of transplantation<sup>9</sup>. Even though self-renewal is a main characteristic in HSCs, it is highly difficult to

achieve expansion of these cells in culture, while other types such as neural or embryonic stem cells are relatively easy to maintain and expand in culture conditions. In order to find elements that can help to elucidate self-renewal but also HSC culture expansion, Reya, Willert and colleagues analyzed transcriptional gene expression in purified mice LT-HSCs (Long-Term Hematopoietic Stem Cells) finding elements related to Wnt/fzd/beta-catenin signaling pathways linked to the capacity of the cells to perform self-renewal divisions<sup>10,11</sup>. Additional studies exhibit proteins such as HoxB4 and HoxA9 in HSC self-renewal. Notch and Sonic Hedgehog (SHH) signaling pathways have been studied to elucidate their potential and role in this aspect<sup>12</sup>. Moreover, genes such as *Bmi-1*, *c-Myc* or *JunB* have been identified as fundamental in stemness maintenance<sup>13,14</sup>.

Another property of HSCs is the ability of differentiation into progenitor lineages and different mature hematopoietic cell types. Growth factors, cytokines and signaling pathways are presumed to be involved in driving the direction of differentiation. Once HSCs are committed towards differentiation, this state will be maintained and self-renewal cannot be reached anymore. Here relies the importance of the bone marrow microenvironment and all the factors found in the niche in HSCs proliferation, suggesting that any deregulation could lead to malignant diseases which will be described in the following chapter<sup>1</sup>.

During early stages of embryonic development and adulthood the HSCs will be able to migrate in response to a natural or induced stimulation. For example, cytokine-induced mobilization is an important procedure used in the clinical field, in order to produce HSC migration and proliferation in patients with hematological disorders that require the use of drugs such as cytoxan. Because of the low percentage of LT-HSCs (around 8%) that undergo into cell cycle activity daily, this population is not affected by the use of cytoxan, which targets fast dividing cells. Nevertheless, myeloid and lymphoid progenitor populations are directly killed by the use of this type of drugs, due to their role in high division activity. This generates a blood system which needs the presence of more HSCs able to compensate and replace the lost populations. The use of certain factors as granulocyte colony-stimulating factor (G-CSF) and stem cell factor (SCF), will

induce migration of HSCs from the bone marrow into the blood system and these cells will be able to engraft into other hematopoietic sites different from the bone marrow, such as the liver or the spleen. This procedure allows self-renewal and differentiation of HSCs, in order to achieve a normal reconstitution of all blood lineages that were affected during the use of chemotherapeutic drugs<sup>15,16</sup>.

Despite the fact that HSCs, as described previously, represent a key element in repopulation of all different blood lineages from progenitor to mature cells, they are not exempted of the well-known mechanism of programmed cell death “apoptosis”. The necessity to regulate the number of HSCs will trigger precise signaling leading the cells to the self-destructing mechanism<sup>1</sup>.

### **1.3.3 Identification of the HSC phenotype**

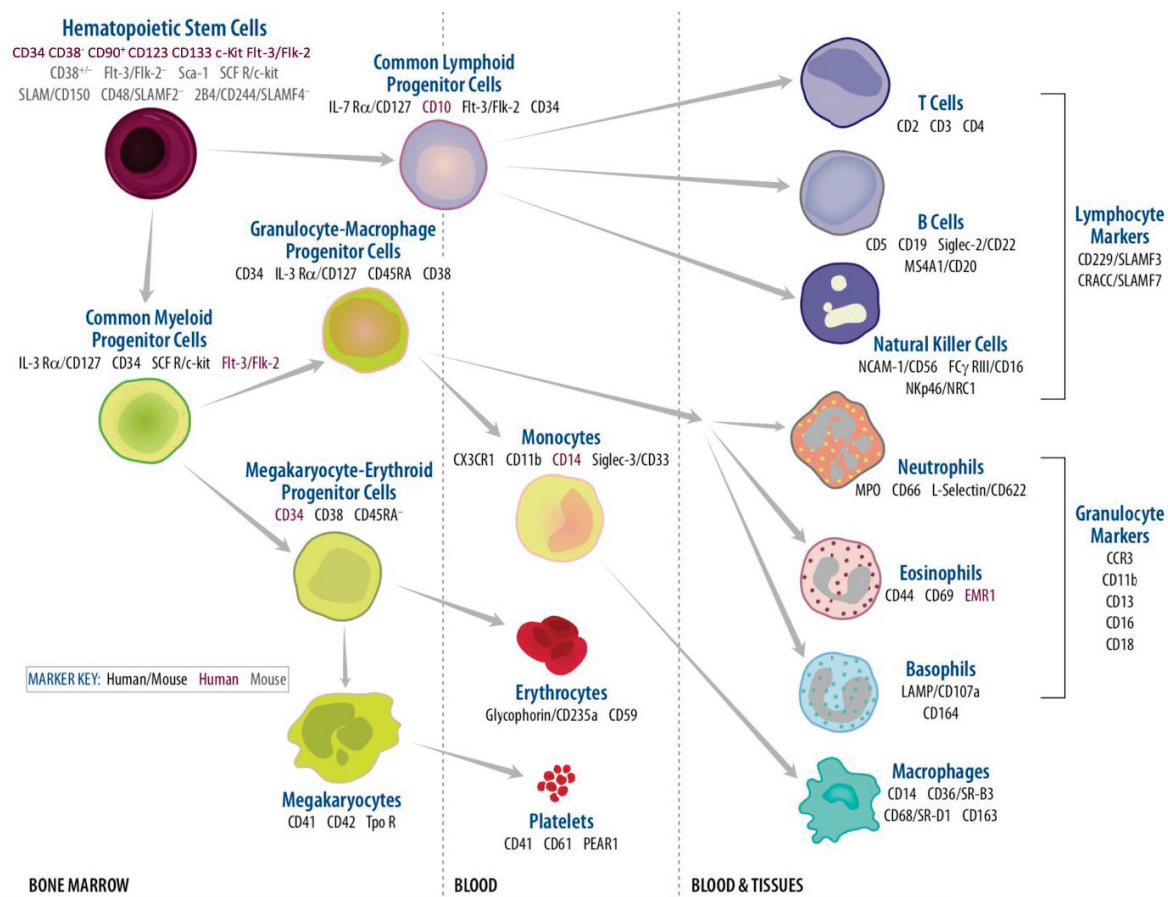
Although in 1961 the HSC was better described and its potential identified, it was not until 1981 when for the first time the hematopoietic stem cells from mouse bone marrow were isolated and purified. Using new technology based on multi-color fluorescence-activated cell sorting and monoclonal antibodies, a small fraction of approximately 0.05% BM mouse cells presenting the surface marker phenotype of Thy-1<sup>low</sup>(CD90)/Lin(Lineage-markers)-/Sca-1<sup>+</sup> were identified<sup>17</sup>. The small cell population identified was responsible for the blood system reestablishment in the lethally irradiated mice. Later on, in 1994 a research work conducted by Spangrude and colleagues showed the presence not only of a single type of multi-potency population, but three different; identified as Long-Term (LT)-HSC, Short-Term (ST)-HSC and Multi-Potent Progenitor (MPP). This last population lacking the self-renewal ability presented by the other two types<sup>17,6</sup>.

In human hematopoietic stem cells, the first cell surface marker employed to identify this minor population, was the cluster of differentiation CD34. The CD34 marker is a transmembrane phosphoglycoprotein and a ligand for L-selectin expressed only by 0.5 to 5% of the blood cells found in human fetal liver, cord blood, adult bone marrow or mobilized peripheral blood (mPB). Multi- and Oligo-potency can be observed in cells expressing this membrane marker.

Nevertheless, the cells presenting CD34 at the membrane are a heterogeneous population not completely specific to HSCs. Antibody Anti-CD34 was produced for the first time in 1984 as a monoclonal antibody, able to identify surface antigen on KG1a myeloid leukemia cell line<sup>18</sup>. Afterwards, a wider variety of cell surface protein markers were discovered for a more specific HSC targeting. Among these markers it is possible to identify CD34<sup>+</sup> CD90<sup>+</sup> Lin<sup>-</sup>. The Lin (Lineage) markers are basically surface proteins allowing to identify all the different blood lineages more differentiated as myeloerythroid population and other markers specific for lymphocytes B, T and NK cells. In contrast to this phenotype identified, cells not expressing CD90 at the membrane showed no potential to generate clones from the myeloid and lymphoid lineages. Addition of the cluster of differentiation CD38 contributed to obtain a more specific HSC phenotype. Despite the fact that between 90-99% of cells expressing CD34<sup>+</sup> also presents the CD38 expression, it was observed that the few cell population CD38<sup>-</sup> and CD90<sup>+</sup> were the cells with the multi-potent ability to give rise to multi-lineage colonies. Furthermore, the CD34 positive and CD38 negative populations have been described as the cell fraction with the highest number of colonies in the functional assay for Long-Term Culture-Initiating Cells (LTC-IC)<sup>19</sup>.

Despite the fact that CD34 expression is often related by default to human hematopoietic stem and progenitor cells (HSPCs), it was in 1997 when Goodell and colleagues described with their studies based in humans and rhesus monkeys, the existence of a population identified as primitive hematopoietic cells with a lack of CD34 expression<sup>20</sup>. Later on, in 1998 the research work conducted by Bhatia and colleagues demonstrated a human cell population with CD34 negative expression and severe combined-immunodeficiency (SCID)-repopulating cell (SRC) activity holding a phenotype Lin<sup>-</sup> CD34<sup>-</sup> CD38<sup>-</sup><sup>21</sup>. Moreover, studies conducted by Anjos-Afonso and collaborators described a rare population presenting not only self-renewal capacity but more importantly, settled at a higher position in the hematopoietic hierarchy than the HSC CD34<sup>+</sup>; this subset displayed a phenotype Lin<sup>-</sup> CD34<sup>-</sup> CD38<sup>-</sup> CD93<sup>hi</sup>. Notch signaling activation can be seen within this population with Delta4 allowing the maintenance of a primitive state, whereas quiescence is controlled through TGF- $\beta$  and Jagged1 signals<sup>22</sup>.

The existence of a CD34 negative HSC population has not only been described in humans but also in several studies performed in mice, where it has been observed for example, that knock-out CD34 mice still hold the capacity to generate hematopoiesis with multilineage progeny and self-renewal capacity as shown for CD34 positive HSCs<sup>23</sup>. Among other human HSC markers identified, it is possible to list CD123<sup>24</sup>, CD133<sup>25</sup>, c-Kit<sup>26</sup> or Flt-3(flk-2)<sup>27</sup>. **Figure 4** summarizes some of the different markers in human and mouse, employed to identify HSCs from more mature hematopoietic lineages.



**Figure 4. Principal markers of HSCs and mature hematopoietic lineages identified in humans and mice (adapted from<sup>28</sup>).** Principal markers specific or shared in humans and mice to identify HSCs and more differentiated hematopoietic lineages.

### 1.3.4 HSC heterogeneity

The hematopoietic stem cell population could be seen as a homogeneous and single group of cell type owning a flexible behavior providing the ability for self-renewal, proliferation or differentiation. Nevertheless, the HSC population is a heterogeneous group of different subpopulations, or subset of cells, holding different self-renewal capacity and differently programmed when speaking about differentiation. The distinct HSC subpopulations present different characteristics such as: variations of their cell cycle status, presence of cell surface markers or the way they react to extrinsic signaling. The idea of sorting HSCs in order to obtain an homogeneous population with an identical behavior is now questioned<sup>29,30</sup>.

One of the pioneer groups to illustrate the heterogeneity of the HSCs was Muller-Sieburg and colleagues, through the study of the kinetic of repopulation using single HSCs transplanted into either irradiated or c-Kit receptor tyrosine mutant mice. After statistical analysis, it was possible to observe 16 different kinetic repopulation arrangements, registered along 8 months in the recipient which received a clonal stem cell from whole BM. The clonal transplantation of HSCs sorted for the phenotype Lin<sup>-</sup> Rho<sup>-</sup> exhibited a selective enrichment for certain subsets from all the HSC types in BM. In addition, it was observed that HSCs daughters coming from the primary host, followed the same repopulation kinetics from the original transplanted HSC, when transplanted into a secondary host<sup>31</sup>.

Self-renewal capacity has been used to analyze the heterogeneity of HSCs, via the performance of transplantation experiments based on measurements of the lifetime of HSC clones obtained from whole BM within a period of 4 months to 2 years after transplantation. HSC clones were isolated from BM by culturing them on a stromal cell line S17, which produced small colonies of granulocytic cells. And using statistical approaches, verified experimentally, it was determined that 90% of the colonies derived from a single initiating HSC. The results derived from this study revealed that HSC clones as expected, are able to reconstitute myeloid and lymphoid lineages but the level of reconstitution for each of this lineage was different<sup>31</sup>. Therefore, HSCs seem to present an intrinsic pre-defined fate

responsible of driving the reconstitution towards a specific lineage. A second element considered as a presumed factor contributing to heterogeneity is the intrinsic cell cycle activity. For instance, Wilson and colleagues conducted a study using a two labeled-retention model in mice, with either BrdU (Bromodeoxyuridine) or histone marking with H2B-GFP which is synthesized during the active cell cycle. Interestingly, the results showed that most of the label-retaining cells were dormant HSCs<sup>32</sup>. It is known that this dormant population can be “activated” or switched into an active state by stimulation with G-CSF or in response to a bone marrow injury. Normally, active HSC population are on charge of daily hematopoiesis while the dormant HSCs not often undergo division unless there is an injury which stimulates its activation. Further analysis concluded that dormant HSCs enter cell cycle once every 149 to 193 days. On the other hand, the active population does it every 28 to 36 days<sup>33,34</sup>.

Hematopoietic stem cell heterogeneity can be observed for instance, when irradiated mice are transplanted with HSCs. This cell population holds the primary function of producing mature hematopoietic cells from the lymphoid and myeloid progeny once transplanted into the irradiated or myelodeficient mouse. Considering these studies based on HSCs transplants, a classification can be established dividing HSCs into “myeloid-biased” (lymphoid-deficient), lymphoid-biased (myeloid-deficient) and myeloid-lymphoid balanced population, having as main parameter for this categorization the tendency to favor the generation of a predominant lineage<sup>34</sup>. For example, according to Dykstra and colleagues, HSC myeloid-lymphoid balanced cells are more common in a classic animal model used as young BM from C57BL/6 mice. While in older mice (>38 weeks) the number of myeloid-biased population is increased<sup>35</sup>. Chen and colleagues identified high expression of histidine decarboxylase (Hdc) in certain HSCs and this population displaying this high expression exhibited a myeloid-biased differentiation capacity *in vivo*<sup>37</sup>. More recently, additional HSC subtypes have been defined as “platelet-biased” and “platelet-myeloid-biased”, both expressing the von Willebrand factor (vWF); a blood glycoprotein involved in hemostasis<sup>36</sup>. In addition, cell surface markers have been employed to separate the different lineage-biased HSC populations. An example of this are the signaling lymphocyte activation molecules (SLAMs), such as CD150 and CD229. High CD150



expression has been linked to myeloid-biased HSCs while low expression of this marker is related to the lymphoid-biased populations. In contrast, CD229<sup>-</sup> is a characteristic of myeloid-biased HSCs and the lymphoid-biased differentiation ability can be observed in CD229<sup>+</sup> HSCs<sup>38,39</sup>. The existence of lineage-biased HSC populations strongly highlights heterogeneity of the HSCs.

Another classification of HSCs exists, relating on the kinetics of reconstitution in a period of 24 weeks after clonal HSC transplantation into the primary recipient and 20 weeks in secondary recipients. In other words, this classification considers the *in vivo* engraftment abilities of the HSCs and the capacity to sustain the multilineage hematopoiesis for a long period and after serial transplantation. Three different repopulating HSCs are defined as; long-term (LT-HSC), intermediate-term (IT-HSC) and short-term (ST-HSC). Multilineage repopulation can be observed in the 3 types of HSCs, nonetheless, Yamamoto and collaborators demonstrated that LT-HSC displayed high donor chimerism in the five lineages they tested (neutrophil/monocyte, erythrocyte, platelet and lymphocytes T and B) and after 4 weeks of transplantation they reached a threshold and maintained it at 24 weeks in the primary and secondary recipient. In the case of IT-HSCs, this population showed lower level of chimerism and start of loss of myeloid and erythroid lineages in the secondary recipient. On the other hand, ST-HSCs are not able to provide a constant level of chimerism for any of the five lineages studied, while an increase of lymphoid population was observed in the secondary recipient<sup>40</sup>.

Studies conducted by Cho, Dykstra, Yamamoto and colleagues to establish the two different classifications of HSC subtypes, were useful to try to define a link between both classifications. In 2014, Ema and collaborators compared data related to these classifications and this allowed them to confirm that the majority of LT-HSCs in the BM correspond to myeloid-biased, the IT-HSC population correspond to a mix between balanced and lineage-biased populations and that most of the ST-HSCs are lymphoid-biased. Another important aspect, is the ability to sort these different subtypes to assess their capacities by using a pure homogeneous population. So far, only a few specific markers have been

identified such as CD150 for LT-HSCs, CD49b in IT-HSCs (integrin  $\alpha 2$ ) and platelet integrin CD41 (Itga2b) in myeloid-biased HSCs<sup>41,35,40,42</sup>.

At the functional level, the different HSC populations vary in their self-renewal capacity and lineage differentiation production<sup>43,44</sup>. Whereas, at the molecular level they share a common signature gene expression and a dissimilar chromatin architecture in the lineage-specific gene loci and present a lineage-specific priming, responsible of their cell fate<sup>45</sup>.

### **1.3.5 Aging of HSCs**

Self-renewal capacity and regenerative potential are two primary hallmarks of hematopoietic stem cells allowing an equitable blood cell regeneration during life. As HSCs age, both abilities are gradually decreased. Moreover, aging is also presumed to be a process in which the hematopoietic stem cell pool is gently decreased. However, aging represents a more complicated and contradictory process as it is thought. Actually, it has been observed that aging in individuals increases the number of HSCs in the BM. On the other hand, as mentioned before, the capacity for self-renewal and repopulation is lower than those observed in younger individuals. For example, old LT-HSC population presents a similar myeloid-biased differentiation potential to that exhibited by young HSCs, whereas the lymphoid potential is reduced<sup>46,47</sup>.

Another important tool to describe aging of HSCs is linked to gene expression. Some genes have been observed to be increased in aged HSCs. These genes are related to stress response, inflammation, cell cycle, proliferation, myeloid lineage differentiation and nitric-oxide (NO)-mediated signal transduction. Contrarily, downregulation is present in those genes linked to DNA repair, chromatin remodeling or DNA replication<sup>47</sup>.

The molecular mechanisms involved in aging are essential to better understand this subject. Nonetheless, a major problem faced studying the HSC aging is the heterogeneity of the populations used along the different studies performed. The purity of a homogeneous HSC population reached in several studies is no higher

than 50%. The complication related to using not pure populations is that, it has been shown through significant data that aging increases the functional heterogeneity of HSCs. Therefore, identification of a specific phenotype for aged HSCs, such as surface markers, can be a key player for a proper functional characterization of this population that can decipher the molecular mechanism of aging<sup>46</sup>.

An important parameter which can help to conduct more efficient research studies focused on aging is to identify the specific role of cell-intrinsic and extrinsic parameters and understand if one of the two has a main role. As observed by several studies performed using mice, most of cell-intrinsic changes seem to be at least moderately influenced or initiated by extrinsic elements as the bone microenvironment. Transplantation of aged HSCs into a young recipient that holds a “young” BM microenvironment resulted in no modifications or changes of the HSCs functionality<sup>48</sup>. Nonetheless, it has been observed that young cells from bone marrow have a better capacity of engraftment when they are placed into a young environment than in an aged one. As described during this chapter, HSCs are tightly regulated by the different elements that surrounds them and for instance, hematological malignancies are an evident consequence of BM microenvironment irregularity<sup>46</sup>.

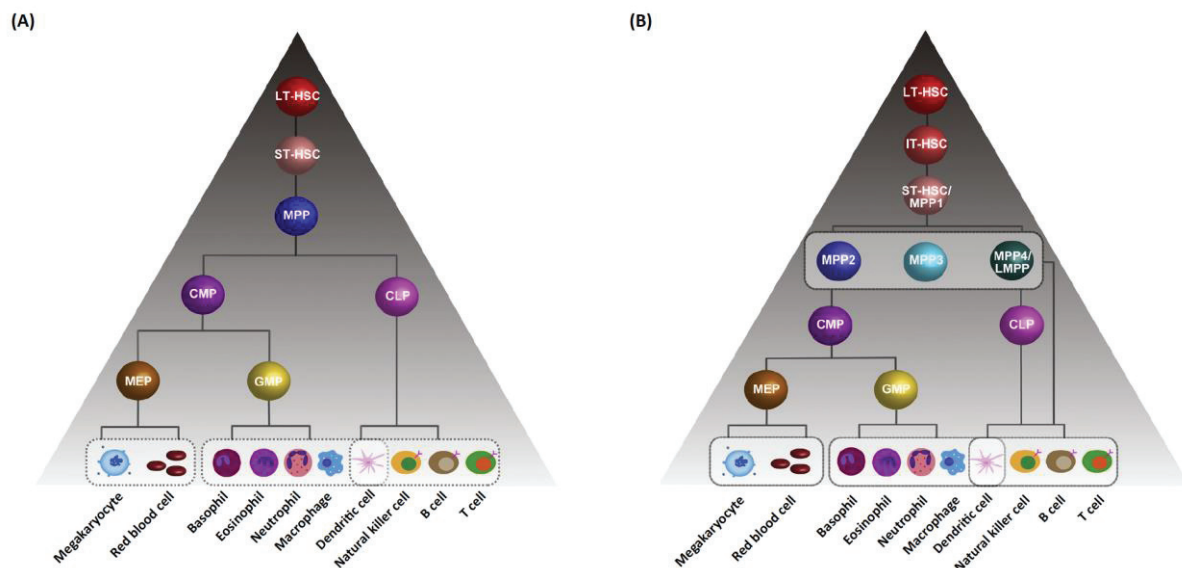
Among the different cell-intrinsic alterations in HSCs, the most common and irreversible is DNA damage accumulation. Genomic integrity is crucial to maintain HSC homeostasis. This integrity helps to diminish the chances of BM failure or transformations driving malignancies. Main causes of DNA damage are attributed to DNA replication errors, spontaneous chemical reactions and external or metabolism agents. Studies performed in mice and humans described premature aging process, by identifying mutated genes involved in encoding proteins necessary for DNA repair. This leads to decreased HSC functioning as low repopulating potential and reduction of HSC reservoir. Aside from random DNA damage, erosion of telomeres is another common disturbance observed in healthy individuals as part of the aging process. This is a particular DNA damage, where shortening of telomeres takes place producing reduction of HSC functioning<sup>49,50,51,52</sup>.

Other intrinsic factor in the list is senescence, which can be observed through a normal accumulation of cell cycle arrested cells along aging. In addition, an outstanding feature of old HSCs is the so called “increased polarity”, which is the asymmetric distribution of specific proteins and according to some studies, it can have an influence in HSC functioning. Within this category of intrinsic factors, impaired autophagy is highly present in a wide number of aged HSCs, which produces mitochondrial accumulation driving metabolic stress induction. The HCS malfunctioning derives from the presence of high levels of reactive oxygen species produced by the mitochondria. Finally, the accumulation of aberrant epigenetic marks can easily and progressively lead HSCs into loss of stem cell potential<sup>53,46</sup>.

#### **1.4 HSC and hematopoietic hierarchy**

As previously described, the HSC has been recognized as the head of a hierarchy in which relies the production of all the different hematopoietic lineages that are essential part of the blood system<sup>54</sup>. The classical road map suggested the HSC at the head of a hierarchical tree with branches representing the multistep differentiation, showing the multi-, oligo- and uni-potent progenitor populations leading to mature blood cells<sup>55</sup> (**Figure 5**). This classical road map describes the presence of the rare LT-HSCs at the head of the tree, this population is known for its quiescent state but able to enter the cell cycle when facing different stimuli<sup>56</sup>. Located one step down the hierarchy, the ST-HSCs are the next population holding the short-term capacity to rebuilt the hematopoietic system<sup>57,58</sup>. This population is able to differentiate into multipotent progenitors (MPPs) which although they do not exhibit self-renewal capacity, are able to give rise to the common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). CLPs are identified as progenitors with a lymphoid-limited differentiation capacity, while the CMPs can differentiate into megakaryocyte/erythrocyte progenitors (MEPs) and granulocyte/macrophage progenitors (GMPs)<sup>55</sup>.

Later on, an update of the classical tree-branch hierarchy has been made, in which the IT-HSCs were placed between the LT- and ST- populations due to their self-renewal capacity<sup>59</sup> (Figure 5). Moreover, within this revised hierarchy the MPPs are divided into four different populations. MPP1 are similar to ST-HSCs and can be placed at the same level, while MPP2, MPP3 and MPP4 are one step down in the hierarchy, giving rise to CMPs and CLPs<sup>60</sup>. In addition, an HSC subpopulation was identified with a high expression of the hematopoietic gene FLT3, which can be related to lymphoid lineages. This population identified as lymphoid-primed multipotent progenitors (LMPPs) presents similarities to MPP4 and can be found at the same level in the hierarchy<sup>60</sup>.



**Figure 5. Classical and revised hematopoietic hierarchy model<sup>61</sup>.** (A) Classical model including LT-HSCs and ST-HSCs as the two main populations. (B) Update version of the classical model including the IT-HSC population and the MPP1-MPP4 populations.

The recent advances studying hematopoiesis and HSC differentiation have clearly improved our knowledge regarding the classical hierarchical model, which established a multistep differentiation, proposing evident boundaries between the different cell populations involved in hematopoiesis. The use of techniques such as functional assays, non-invasive lineage tracing and single-cell analysis

confirmed that hematopoiesis is a continuous process where a lack of boundaries among the several cell populations can be observed<sup>62</sup>.

New technologies have allowed to perform individual continuous analysis of single hematopoietic cells from HSC to more differentiated lineages. For instance, the use of single-cell RNA sequencing allowed to perform single-cell transcriptome analysis leading to identify the hematopoiesis as a continuous procedure<sup>63,64</sup>. Based on this technique, Velten and colleagues described hematopoiesis as a process performed by a continuum low-primed HSPCs differentiating progressively into lineage-restricted populations, this at the cellular level<sup>64</sup>. At the molecular level, the single-cell analysis allowed to identify activation of lineage-specific genes or the suppression of cell-division related genes during the continuous differentiation process<sup>65</sup>.

Physiological conditions can also have an impact in the way hematopoiesis takes place and the populations playing the main role repopulating the blood system. Two different types of hematopoiesis occur depending on the situation. Native hematopoiesis represents the hematopoiesis produced in normal and unperturbed physiological condition (steady-state)<sup>66</sup>. While stress hematopoiesis describes the hematopoiesis occurring under stress conditions such as transplantation, cell culture and injury<sup>67</sup>.

Currently, the use of lineage-tracing techniques has been used in order to understand and better describe the hallmarks of native hematopoiesis. For instance, transposon tagging for clonal labeling in HCs has shown that hematopoietic progenitors are responsible for maintenance of differentiated progenies in this type of hematopoiesis<sup>68</sup>. Busch and collaborators used labeling of hematopoietic lineages by the fluorescence reporter system driven by the *Tie2* locus, identifying that adult hematopoiesis was maintained by ST-HSCs<sup>69</sup>. In contrast, the use of another fluorescent reporter driven by *Pdzk1ip1* which is able to label a more purified LT-HSCs, confirmed this population as a major source of the different blood lineages in native hematopoiesis<sup>70</sup>.

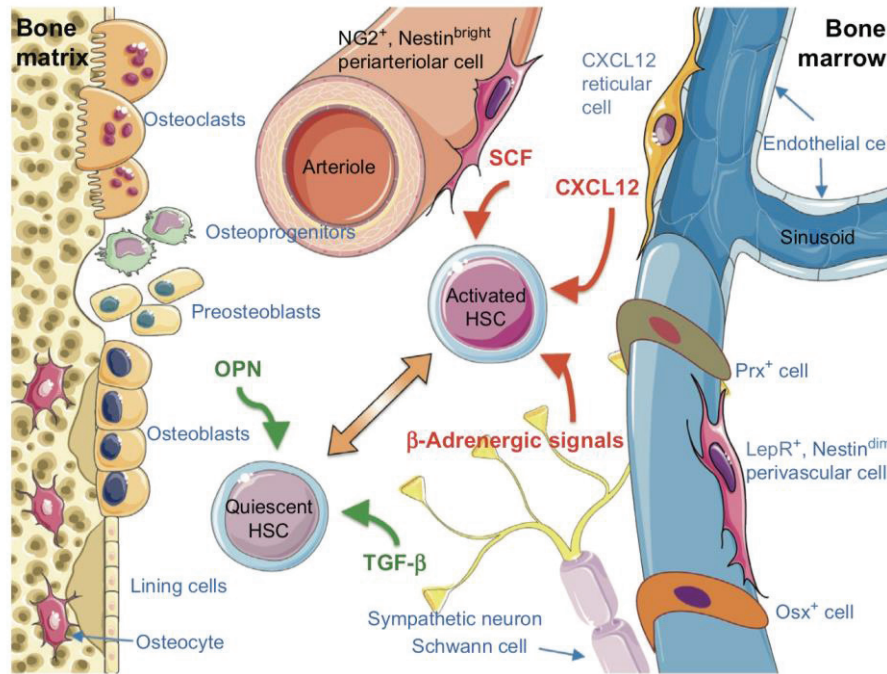
LT-HSCs are considered the main element involved in hematopoiesis maintenance at the head of the hierarchy. This idea has been confirmed by functional transplantation assays which may not represent a hematopoiesis under normal physical conditions. Studies suggest that transplanted HSCs are able to reconstitute the blood system. Nevertheless, they exhibited a differentiation trend to particular lineages at steady-state<sup>71</sup>. Transplantation-derived stress, injury-induced infection or inflammation are elements that can produce alterations in HSC differentiation in native hematopoiesis<sup>61</sup>. It has been reported that M-CSF and IFN- $\gamma$ , proinflammatory factors, are able to stimulate HSC towards myeloid cells for injury protection<sup>72</sup>. These current findings describe the native hematopoiesis as a process mainly maintained by multipotent progenitors and in lesser extent by HSCs, while in stress hematopoiesis the HSCs play the main role re-establishing the blood system<sup>61</sup>.

## **1.5 Bone marrow microenvironment**

### **1.5.1 Generalities**

The bone marrow (BM) microenvironment represents an essential niche able to provide the necessary support for hematopoietic stem cells. In 1978, the concept “niche” was used for the first time by Schofield, by describing with his studies that HSCs interaction with bone and also cell-cell contact, were both responsible for the capacity of the HSCs to proliferate in an unlimited manner and being able to inhibit the maturation of this population. Nowadays, it is well known that HSCs behavior is guided by interactions with all the components contained in this niche. The bone microenvironment consists of heterogeneous cell populations as stromal cells and several molecular components, controlling the signaling on charge of regulating three main features of HSCs; self-renewal, quiescence and differentiation. Moreover, within the BM two different niches have been described to host the HSC, endosteal and vascular niches (**Figure 6**). Both are complex structures housing not only HSCs but also an extensive range of other cell types as bone lineages (osteoblasts and osteoclasts), mesenchymal stromal cells (MSCs), endothelial and vascular cells, immune cells, among others, described

during this chapter. In addition, several molecules are present in the niches which are better described in the following subtopics of the chapter<sup>73,74,75</sup>.



**Figure 6. Bone marrow microenvironment<sup>76</sup>.** Heterogeneous cell populations and several molecular components build the BM microenvironment, regulating HSCs functions and maintaining a quiescent or activated state of this key player of hematopoiesis.

### 1.5.2 Cellular components of the niche

Different cellular elements constitute the bone marrow microenvironment and each of these elements carries out roles linked to the hosting and regulation of HSCs. In first place, osteolineage population is crucial in supporting and regulating HSCs. Osteoblasts (OBs) are cells involved in bone formation and mineralization of the bone structure. Conversely, osteoclasts (OCL) have the function of removing bone tissue by the process called “bone resorption”. HSCs have a direct and close contact with osteoblasts in the niche. Shown by *in vivo* experiments, the alteration of osteoblasts (OBs) had an impact in hematopoietic stem and progenitor population numbers<sup>77,75</sup>. Mice genetically manipulated with



*Runx2* deletion, gene essential in osteoblastic differentiation, suffered an abnormal BM hematopoiesis caused by the aberrant osteoblast differentiation<sup>78</sup>.

Another important factor in the BM microenvironment is the perivascular area, which seems to have an impact on HSC regulation and preservation. This idea stands on the hypothesis that HSCs can often be found surrounding the blood vessels. Mesenchymal stromal cells (MSCs) are localized next to the blood vessels and have the capacity to differentiate into osteolineage cells. They can also MSCs can express factors with the capacity to uphold HSCs maintenance<sup>76</sup>.

Endothelial cells are a further common element observed in the bone marrow niche, with the task of cytokine and paracrine growth factor production. In addition, endothelial cells can generate several adhesion molecules as vascular cell adhesion molecule 1 (VCAM-1), E-selectin, P-selectin or intercellular adhesion molecule 1<sup>79,80</sup>. Altogether these molecules regulate HSCs homeostasis. As an example of this, bone marrow sinusoidal endothelial cells showed the ability to enhance HSC expansion in culture by the expression of ligands from the Notch pathway<sup>81</sup>.

Within the list of cellular components, adipocytes can be found. As humans age, the different functions held by the HSCs are decreased, whereas the amount of adipocyte cells present in the bone marrow increases. This observation generated the hypothesis that adipocytes may play a role in hematopoiesis. Two different mice models were used to confirm this idea. One model, known as lipodystrophy which is unable to generate adipocytes. On the other hand, the second model was treated with peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ) to inhibit adipogenesis. After HSC engraftment, it was possible to observe how engraftment took place faster in the fatless mice, confirming the negative regulation of adipocytes in hematopoiesis<sup>82</sup>.

Monocytes and macrophages represent other types of cells identified in the BM niche playing a specific role in HSCs mobilization into the blood stream. Macrophages specifically, are capable of retaining HSCs within the marrow niche through signaling of stromal cells such as mesenchymal stromal cells. This

mechanism of HSC retention in the niche is guided particularly by CD169<sup>+</sup> macrophages. Secretion of oncostatin M (OSM) by macrophages induces Nestin<sup>+</sup> cells and MSCs among others to express *CXCL12*. Commitment between *CXCL12* and *CXCR4* receptors present on the surface of HSCs allows the retention of this population within the bone microenvironment<sup>76,75</sup>.

### 1.5.3 Molecular components of the niche

Different types of molecules can be found within the bone marrow microenvironment. Each single molecule holds a specific function in signaling and all together can help to drive homeostasis in the niche by regulating HSCs. The cellular components of the BM microenvironment, are responsible for the soluble factors secreted in the niche as growth factors or cytokines. Examples of these molecules are: stem cell factor (SCF), transforming growth factors (TGF- $\beta$ ), bone morphogenetic proteins (BMPs), C-X-C- motif chemokine ligand 12 (*CXCL12*), angiopoietin-1 (Ang1), granulocyte-colony stimulating factor (G-CSF), Wnt ligands, thrombopoietin (TPO), vascular endothelial growth factor (VEGF), prostaglandin E2 (PGE2), Notch ligands and fibroblasts growth factors (FGF)<sup>83</sup>.

As mentioned before, the importance of all these molecular elements relies on their capacity to regulate hematopoiesis but also immune responses or inflammation in the niche. For instance, *in vitro* but also *in vivo* studies, have helped to better describe the real function of cytokines in the niche that interact with surface receptors present in HSCs driving characteristics such as quiescence, self-renewal, differentiation, apoptosis and mobility<sup>84</sup>.

SCF also called as “steel factor”, is a cytokine that binds to the tyrosine kinase receptor c-Kit expressed on all HSCs. It plays an essential role in preventing HSC apoptosis. In addition, SCF combined with other factors showed potentiation of symmetric self-renewal of fetal liver HSCs in culture<sup>85</sup>. TPO is another crucial cytokine in the niche on charge of regulation of megakaryocytes and platelets development. During definitive hematopoiesis TPO supports generation and expansion of HSCs<sup>84</sup>.

Notch ligands carry out a primary role in lymphopoiesis. Nonetheless, the ligands Delta and Jagged participate in generation, antidifferentiation and expansion of HSCs<sup>86</sup>. In the same way, Wnt signaling is strongly related to generation and expansion of HSCs. Through the noncanonical Wnt5a protein, HSCs repopulation and maintenance of quiescence is possible<sup>87</sup>.

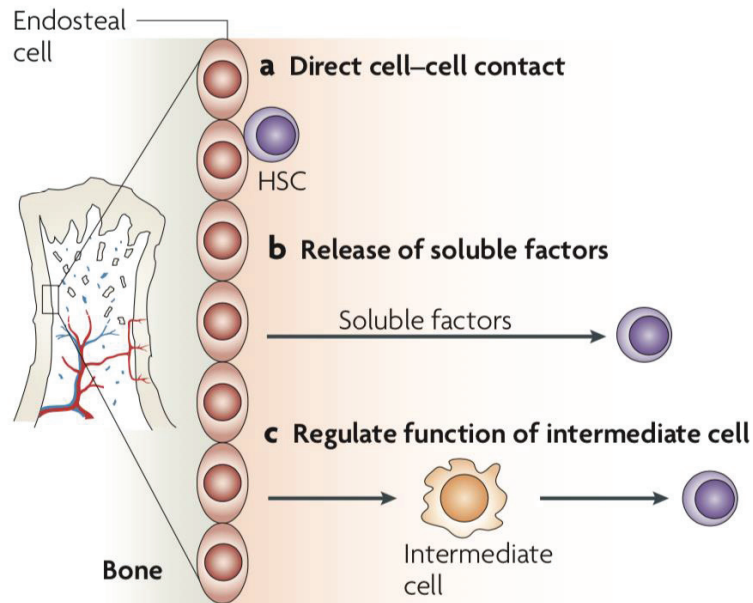
The effect of TGF- $\beta$  *in vitro* has been described as a strong inhibitor of HSC activity. On the other hand, *in vivo* studies demonstrate that deficiency in TGF- $\beta$  signaling has no proliferative effect in HSCs. Important members of this superfamily of proteins, are the Bone Morphogenetic Proteins (BMPs), which participate in specification of HSCs during development. For example, BMP4 protein upholds HSC expansion in culture and moderately has an impact on the effect of Sonic Hedgehog on cultured human HSCs<sup>88</sup>.

Other elements found in the BM microenvironment are FGF-1 and FGF-2 that hold expansion of HSCs but also hematopoietic stem cells express fibroblast growth factor receptor. Angiopoietin (Ang) growth factors can be found in the bone marrow niche as mentioned before, Tie-2/Ang1 signaling pathway plays a decisive role maintaining HSCs in a quiescent state inside the bone marrow niche<sup>84</sup>.

#### **1.5.4 Endosteal niche**

HSCs have been localized in special microenvironment areas called “bone marrow niches”. Two sites have been described as potential residencies for HSCs: endothelial and vascular niches. In the internal bone shell surface, it is possible to identify the endosteal niche (**Figure 7**). The endosteum is a vascular layer of connective tissue between the bone and the bone marrow. This space is coated with different cell types as endosteal (bone-lining) cells and osteoblastic populations, at different stages of maturation as osteoblasts and osteoclasts. Also, endothelial cells, macrophages, fibroblasts and adipocytes can be found close to the endosteum<sup>89</sup>. Different ideas suggest how the endosteal cells specifically regulate the HSC functioning: direct contact between the endosteal cells with HSCs, release of molecules involved in HSC regulation or by controlling

intermediate actors as other cells which can have an impact in HSC functions **(Figure 8)**<sup>90</sup>.



**Figure 8. Hypotheses of endosteal cells mechanism involved in HSC regulation**<sup>90</sup>. Suggestions describe three different mechanisms employed by the endosteal cells in HSC regulation. **a) Direct cell-cell contact.** HSCs are in direct contact with cell components of the endosteal niche as endosteal cells and osteoblasts. **b) Release of soluble factors.** Production of molecules able to control HSC activity. **c) Regulate function of intermediate cell.** Controlling of other cells within the endosteal niche could lead to regulation of HSCs.

Key players in the endosteal niche are the osteoblastic cells capable of synthesizing cytokines tightly involved in maintenance and regulation of HSCs. Within the endosteal microenvironment a large number of cytokines have an impact on HSC behavior. Some of these cytokines have been described already in the previous section, such as; angiopoietin (Ang-1) and thrombopoietin (THPO) showing an impact in HSC numbers and specifically THPO wield quiescence in LT-HSCs<sup>89</sup>. In addition, osteoblastic cells are responsible for retaining the HSCs inside the endosteal niche, this through the expression of membrane-bound ligands and adhesion receptors in the osteoblastic population<sup>91</sup>.

The oxygen gradient in the endosteal niche plays an important role also in HSC localization within the niche. Endosteal region is the area where HSCs are mainly located, while myeloid and lymphoid lineages together with more mature populations can be found distributed along the central marrow region, indicating that hematopoiesis takes place towards the longitudinal axis of the marrow. The reason of this specific distribution of hematopoietic stem, progenitor or more mature lineages within the endosteal niche seems to be linked to oxygen gradient found within the BM cavity. Stem cell function is highly regulated in the niche by hypoxia, reason why HSCs are localized at the lowest side of the oxygen gradient<sup>92</sup>.

Quiescence and activation of HSCs are two main functions controlled in the endosteal niche. Both actions are driven by regulatory signals produced by neighboring cells and released in the niche as soluble molecules or ligands. Moreover, physical signals are also involved such as oxygen tension. HSCs present in this niche exhibit a higher self-renewal capacity, granted by the cytokines, adhesion molecules and hypoxia<sup>91,93</sup>.

#### **1.5.5 Vascular niche**

Enough data and studies describe another type of bone marrow microenvironment involved in maintenance and regulation of HSCs as the endosteal niche. The vascular niche is able not only to support but also to regulate the self-renewal and differentiation abilities of HSCs (**Figure 7**). The importance of the vascular niche can be explained from the fact that, during fetal development, functional HSCs originate from the endothelium of the vasculature. Later on, these fetal HSCs will occupy the liver and spleen, areas where early hematopoiesis can be observed with the absence of osteoblastic cells or the endosteal niche<sup>91</sup>.

The bone marrow is densely composed of blood vessels which play a role not only as a barrier between the hematopoietic compartment and peripheral blood circulation, but also contribute to the hematopoietic process and also to stem cell mobilization and homing of this population. Blood flow and nourishment within

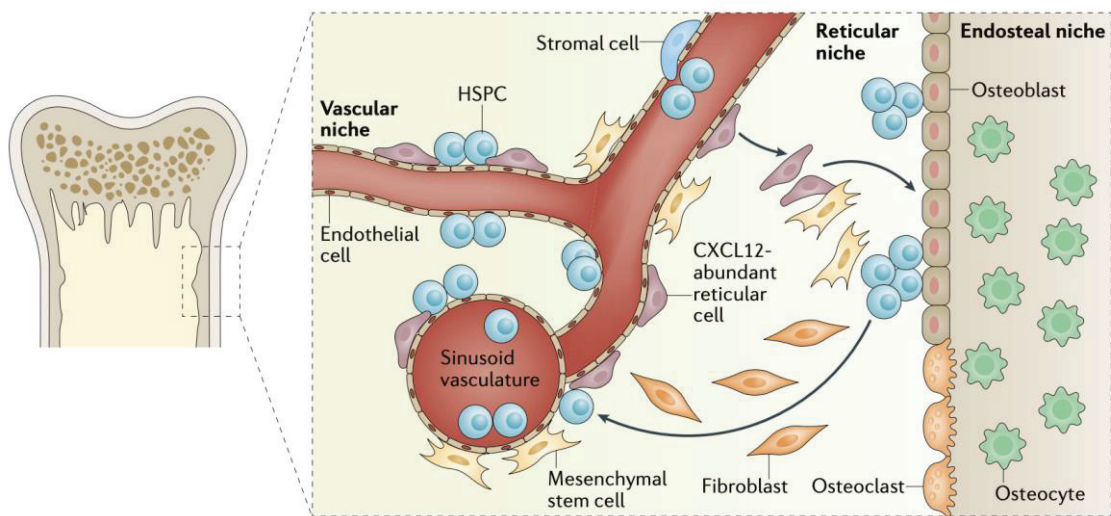
the bone marrow takes place through the medullary artery that provides the “fuel” to arterioles, capillaries and sinusoids. Sinusoids are important structures when speaking about the vascular niche. These blood vessels are specialized structures with thin walls containing the endothelial cells distributed in a very special way called “fenestrated arrangement”. In other words, the endothelial cells form a porous or window structures providing a passage for hematopoietic cells. The BM contains a large-scale system of sinusoids, and together these specialized vessels and the endothelial cells that constitutes them, are vital for HSCs in the bone marrow niche<sup>90</sup>.

The importance of endothelial cells in the vascular niche is based on several studies performed *in vitro* and *in vivo*. For instance, co-culture of HSCs together with mice endothelial cells, enhanced *in vitro* expansion of HSCs thanks to the endothelial population. *In vivo* research works conducted by Yao and colleagues demonstrated the importance of endothelial cells in hematopoiesis. By using a mice model with a conditional deletion of glycoprotein 130 (*gp130*) in both HSCs and endothelial cells, they described the importance of endothelial gp130 signaling in hematopoiesis maintenance<sup>94,95</sup>.

A large number of HSCs seem to be localized within the bone marrow just near the sinusoids. This idea has been proposed due to the fast migration of HSCs, which can take only a few minutes once the glycoprotein G-CSF is administrated. The fast mobilization of the cells suggested that a significant portion of HSCs must be located really close next to the blood vessels. Moreover, in 2005 the surface markers signaling lymphocyte activation molecule (SLAM) were identified. These markers are able to discriminate between HSC and progenitor populations in mice and include CD150, CD244 and CD48. The different marker's expression allowed to identify the HSC location within the bone marrow. The most immature HSCs presented a profile CD150<sup>Positive</sup>, CD244<sup>Negative</sup>, CD48<sup>Negative</sup> and around 60% of this population were in direct contact with sinusoidal endothelial cells. Meanwhile, the other 40% were identified at the endosteal surface<sup>96</sup>.

Another crucial cell component within the vascular niche is the reticular cells. These cells are found surrounding the sinusoid endothelium and are essential in

homing and localization of HSCs in the bone marrow. Reticular cells present a high secretion of the C-X-C motif chemokine 12 (CXCL12). For this reason, these cells are identified as CXCL12-abundant reticular (CAR) cells. Sugiyama and collaborators performed immunofluorescence staining assays in tissue from BM CXCL12-GFP knock in mice, finding interestingly that around 97% of HSCs in the bone marrow were localized next to CAR cells. In addition, all HSCs localized at the endosteum were likewise located to CAR cells. These findings confirmed how important CAR cells are in HSC homing in both endosteal and vascular niches<sup>97</sup>.



**Figure 7. Bone marrow microenvironment: endosteal and vascular niches<sup>98</sup>.** The BM microenvironment is responsible for regulation of the main characteristics of HSCs and HSPC: self-renewal, quiescence and differentiation. Two different niches possess cellular and molecular components maintaining HSC functioning. Vascular niche seems to promote self-renewal and expansion of HSCs, whereas the endosteal niche controls the number of HSCs through quiescence and storage of this population.

## 1.6 Clonal hematopoiesis and malignancies

Normal hematopoiesis is the mechanism by which all the elements of the blood system are regenerated, including all the different lineages from hematopoietic progenitor to more differentiated populations, as described along this chapter. Self-renewal, differentiation and proliferation are processes that need to be strictly regulated by the different elements, whether cell or molecular

components, within the bone marrow niche. Throughout the life of HSCs, aging is a normal stage reached by this population and allows the replacement of these cells. Somatic mutations can be found in aged HSCs caused by time and environmental exposure to mutagens. Mutations can be acquired not only by fast dividing cells but also by low rate dividing HSCs which are able to pass these mutations to the next generation of cells that they give rise to. The effect of these mutations will impact the hallmarks of stem cells as increased the self-renewal capacity, high proliferation or reduce cell death<sup>99</sup>.

Clonal hematopoiesis (CH) also known as clonal hematopoiesis of indeterminate potential (CHIP), is described as a phenomenon where mainly hematopoietic stem cells but also hematopoietic progenitors can expand to give rise to a clonal population of cells holding one or several DNA somatic mutations. Diseases related to CH are not only those linked to bone marrow malignancies but also cardiovascular diseases<sup>99</sup>. In addition, hypotheses suggest that diabetes, inflammatory syndromes and cancer also seem to be caused by CH<sup>99</sup>.

According to numerous research studies, individuals presenting CH mutations showed a raised predisposition to develop hematological malignancies. The explanation for this predisposition may be based on two different facts. In the first place, as described before, this clonal population holds mutations that allow increased self-renewal and fast proliferation. In the second place, gene mutations can have a synergic effect with secondary or tertiary gene mutations (see review<sup>99</sup>).

For instance, in adults *DNMT3A* gene is the most common mutated gene present in CH in individuals with hematological but also non-hematological malignancies. In acute myeloid leukemia (AML) (described in more detail in the next chapter) and acute lymphoid leukemia (ALL), *DNMT3* is present in about 22 to 35% of patients with these diseases. This mutation seems to be able, as mentioned before, to establish a synergic link with other mutations observed in AML patients such as; receptor tyrosine kinase *FLT3*, RAS signaling members *NRAS*, *KRAS* and *PTPN1* and mutations of the nuclear protein *NPM1*. For instance, *NRAS* mutant expression synergizes with *DNMT3* and is linked to a fast leukemia



development. Another clear example shown is the combined mutations of *DNMT3* and *FLT3* being able to trigger myeloid and lymphoid leukemias. Likewise, *TET2* mutation combined with *DNMT3* are a synergic combination involved in AML development. Although, when speaking about CH, *TET2* mutations are always found, regarding leukemias, *TET2* mutations are not always seen as the first or dominant clonal mutation<sup>99</sup>.

## 1.7 Overview of hematologic malignancies

The hematological malignancies are a type of blood cancer, arising from the blood forming tissue as the bone marrow or the cells belonging to the immune system. These malignancies comprise the different forms of leukemia, lymphoma and myeloma. The first hematological disease was described by Thomas Hodgkin in 1832, but it was more than thirty years later when Samuel Wilks baptized this specific type of lymphoma as Hodgkin's disease. Following these research works on lymphoma, new studies revealing accumulation of leukocytes in the blood introduced the major principle of leukemias. These works focused on leukemia for the first time, were conducted by John Hughes Bennett and Rudolph Virchow in 1845. Finally, myeloma was described in 1844 by William Macintyre, Thomas Watson and John Dalrymple, while the term "multiple myeloma" was coined by Von Rustizky in 1873 after the analysis of multiple tumors in marrow and bone<sup>100,101</sup>. The years between 1830 and 1950 were essential to start to better describe and classify the countless hematological malignancies discovered<sup>100</sup>.

In order to study the different hematological malignancies in a more effective and organized way, a classification is essential. Also, to classify these malignancies is crucial to adapt treatments in patients. Thus, together the European Association of Hematopathologists and the Society of Hematopathology carried out the task of establishing a classification of hematologic neoplasms for the World Health Organization (WHO). The base of this classification is mainly dividing the malignancies depending of the lineage involved into; myeloid neoplasms, lymphoid neoplasms, mast cell disorders and histiocytic neoplasms.

And inside of each of these five major categories it is possible to identify all the different diseases depending on a mixture of parameters such as morphology, immunophenotype, genetic features and clinical syndromes<sup>102</sup>.

Hematological malignancies embrace a large number of different diseases as mentioned before. Each malignancy can be related to several factors from oncogene formation generated by spontaneous mutations or DNA mutations produced by a deficient repair mechanism. In addition, external factors will also play a role in hematologic malignancies as exposure to certain chemical substances or pathogens. Genetic predisposition is another element to consider as it is also important the normal aging process. During the next chapter, a deep description of several aspects surrounding acute myeloid leukemias (AML) will be developed in order to understand this thesis work<sup>100,102</sup>.

## **2 Acute myeloid leukemia (AML) and leukemic stem cell (LSC)**

### **2.1 Overview of leukemia**

The concept “Leukemia” was established from the Greek words “*leukos*” and “*hemia*” that indicates the high amount of white blood cells in the body. Leukemia is a general term to define cancers of the blood forming tissue. These malignant disorders affect the normal production of healthy and functional blood cells involved in hemostasis. The abnormal blood cell population is called “leukemic cells”, once they accumulate within the bone marrow, they will be able to lead into a BM failure and alterations in the immune system. This because leukemic cells present a more immature state compared to a normal blood cell, holding a lack of proper functional activity. Depending of the lineage affected and the maturation state of the cell, a long list of leukemias have been described<sup>101</sup>.

In 1845, John Hughes Bennet was the first to diagnose a leukemia case and published his results in the Edinburgh Medical and Surgical Journal. Combined to this, Alfred Donné contributed by identifying blood diseases under the microscope. Later in 1877, Paul Ehrlich developed a method that helped to stain and identify blood cells which derived in the classification of leukemias based on the morphology of the leukemic cells. By the end of the 19<sup>th</sup> century, the most used classification when speaking about leukemias emerged by categorizing this disease into: acute or chronic myeloid leukemia and acute or chronic lymphocytic leukemia. And later on, in 1960 Peter Nowell and David Hungerford identified the importance of chromosomal abnormalities, and specifically missing chromosomes in a chronic myeloid leukemia patient<sup>101</sup>.

In the United States of America in 2011 around 44,600 individuals were diagnosed with leukemia, from which 21,780 included men and women faced death. Moreover, for 2019 the estimated number will increase to 61,780 new cases with an estimation of 22,840 deaths. Considering men and women, caucasian or white individuals represents the population with the highest incidence of leukemia, whereas Asian inhabitants as Chinese, Japanese and Koreans hold the lowest incidence. Furthermore, incidence in men has been observed to be almost 50% higher than in women considering all the different

racas or ethnic groups except in Vietnamese, with 18.1 men compared to 10.9 women from 100,000 new cases. Within the young adult population with an age between 30-54 years old, ethnicity seems not to have a big impact in incidence but it becomes more evident when speaking about older age individuals. Leukemia is most commonly diagnosed in the group of 65-74 years old patients with a 23.9% of all new cases. The second group are patients between 75-84 years old represents 20.5% of cases just followed after by adults between 55-64 years old with 18.1%. Moreover, leukemias in children show a higher rate within white Hispanics and non-Hispanics, Filipinos and black people<sup>103,101</sup>.

## **2.2 Acute myeloid leukemia (AML)**

### **2.2.1 Generalities**

Acute myeloid leukemia (AML) is characterized by an abnormal clonal expansion of hematopoietic myeloid progenitor cells (blasts) within the bone marrow (BM) and peripheral blood (PB), due to a myeloid blocking of differentiation giving rise to the uncontrolled proliferation<sup>104,105</sup>. Due to the heterogenicity of these hematological disorders, this malignancy has been also recognized as acute myeloid leukemias. AML is the most common type of acute leukemia in adults with around 80% cases of this form. These disorders seem to reach a peak of incidence during later adulthood but also the second population affected are individuals in early childhood. Nowadays, survival rates in the young group of patients have improved while unfortunately prognosis in 65-year and older patients remains low with 70% mortality within one year after diagnosis<sup>104,106</sup>. Therefore, development of more effective therapies in elderly AML patients represents a key challenge which can be achieved by a better understanding of these diseases and the mechanisms which can lead to a better patient outcome<sup>107</sup>.

### 2.2.2 Epidemiology

The worldwide highest incidence of AML can be observed in the United States, Australia and Western Europe. In the United States, the number of cases arises to a total between three to five for every 100,000 inhabitants. In 2015, 20,830 new AML cases were diagnosed in which only 50% of the patients reached remission while the other 50% died. The incidence of AML is highly linked to age. For instance, around 1.3 cases of AML under 65-years old were reported out of 100,000 individuals. Number of cases increases as individuals get older, finding around 12.2 cases in patients above 65 years old from 100,000 individuals<sup>104</sup>. The median age for AML is about 68 years-old and the prevalence is higher in men than women with 3 male cases for every 2 female cases<sup>107</sup>. According to the Surveillance, Epidemiology, and End Result (SEER) program from the United States of America, incidence of AML is additionally related to ethnicity. The SEER documented in their data base that children between 1-4 years old have shown an incidence of 0.8 per 100,000 people in both boys and girls. Moreover, during the first year of life the caucasian race presents a 3-times higher incidence compared to black race population. In adult patients since 2003 the prevalence of AML is slightly increased for caucasian population with 3.8 cases compared to 3.2 cases in the black inhabitants per 100,000 individuals<sup>106</sup>. Reports generated by the SEER program in a range between 2009-2015 showed that only 28.3% of AML patients faced a surviving of 5 years after diagnosis<sup>103</sup>.

For 2019 the estimated new cases of AML within the United States seem to maintain a steady number with around 21,450, which does not reflect a big change compared to those observed in 2015. Within these new cases, the number of estimated fatalities surrounds the 50% of cases with around 10,920. The median age of diagnosis of this malignancy reported by the SEER program for 2019 remains in 68-year old. In addition, the group with higher incidence contains individuals between 65 to 75 years old representing 25.1% of all the cases. While the lowest population affected are under 20-years old with only 4.5% of cases reported<sup>103</sup>. Statistics are clear and reflect the lack of more effective methods of diagnosis and treatments for AML patients, in order to be able to increase in the first place the percentage of survival cases and in the

second and more important place, decrease the mortality observed specially in the old population.

### 2.2.3 Classification

The idea of classifying AML or any other disease is based on the importance to organize the different diseases into a group sharing similar biological characteristics. In addition, it is essential to classify these malignancies to have a better understanding of the pathogenesis. Defining different subgroups into a classification is a tool which can lead to identify prognosis and the best therapeutic approach depending on the type of AML<sup>108</sup>.

In 1976, for the first time an international classification system was defined in order to be able to establish and organize the different subtypes of AML. This new system recognized as the “French-American-British (FAB)” classification, categorized AML into six different subtypes from M1 to M6, using as a reference the morphological characteristics and the cytochemistry of the leukemic cells. Discrimination between monoblasts and myeloblasts was achieved considering morphology and using  $\alpha$ -naphthyl acetate esterase (ANAE) to establish the M1-M6 AML subtypes. Later on, in 1985 the FAB group composed by Bennett and colleagues, determined a lack in the classification due to the fact that the morphological characteristics were not sufficient to discriminate between myeloblasts and lymphoblasts. Therefore, the FAB group employed myeloperoxidase (MPO) or Sudan B Black reaction to identify the positive or negative blasts in AML and ALL. Moreover, during this re-revised FAB classification, immunological markers were used to identify two new AML subtypes; acute myeloid leukemia with minimal myeloid differentiation (AML-M0) and the megakaryoblastic leukemia (AML-M7) both exhibiting a negative Sudan B Black reaction, completing the FAB classification known until these days (**Table 1) (Table 2)**<sup>109,108</sup>.

**Table 1. French-American-British (FAB) classification of Acute Myeloid Leukemia<sup>109</sup>.**

Subtype	Name
<b>M0</b>	AML with no Romanowsky or cytochemical evidence of differentiation
<b>M1</b>	Myeloblastic leukemia without maturation
<b>M2</b>	Myeloblastic leukemia with maturation
<b>M3</b>	Acute promyelocytic leukemia (APL)
<b>M4</b>	Acute myelomonocytic leukemia (AMML)
<b>M5</b>	Acute monoblastic leukemia (AMoL)
<b>M6</b>	Erythroleukemia
<b>M7</b>	Acute megakaryoblastic leukemia (AMkL)

**Table 2. French-American-British (FAB) classification of Acute Myeloid Leukemia based on morphology and cytochemistry<sup>108</sup>.**

Summary of the FAB classification of AML

FAB subtype	Definition
M0	< 3% SBB + * blasts, My + **, Ly - ***
M1	> 90% blasts
M2	30–90% blasts > 10% myeloid cells < 20% monocytoid cells
M3	majority promyelocytes hypergranular
M3V	microgranular
M4	> 20% mature myeloid cells and > 20% ANAE + **** blasts > $5 \times 10^9$ /l monocytes in the blood
M5	> 80% monocytoid cells
M6	> 50% nucleated erythroid cells > 30% blasts of myeloid cells of the non erythroid population
M7	< 3% SBB + blasts > 30% megakaryoblasts defined by CD41/CD61 or electron microscopy with platelet peroxidase

\* Sudan Black B.

\*\* Myeloid markers positive (CD13, CD33, myeloperoxidase, CDw65).

\*\*\* Lymphoid markers negative (CD3, CD5, CD10, CD19).

\*\*\*\*  $\alpha$ -Naphthyl acetate esterase.

Improvement achieved in the diagnosis and care of AML patients during the years after the establishment of the FAB classification, made necessary to integrate all this new data generated into a new system of classification proposed by the World Health Organization (WHO). In 2001 the WHO generated this new system to categorize the different types of AML and in 2008 this classification had its first update. Finally, in 2016 the new version was issued and published. Several aspects including genetic information as cellular and molecular genetics, immunophenotype, morphology and clinical data were used to determine into this classification six main categories of AML malignancies: with recurrent genetic abnormalities, with myelodysplasia-related features, therapy related AML, not otherwise specified, myeloid sarcoma and myeloid proliferation linked to Down syndrome (**Table 3**). In the category of genetic abnormalities, improvement can be observed compared to the 2008 version. Chromosomal translocations are used to describe 11 different subtypes of AML. For instance, two new types of AML with mutated NPM1 and CEBPA are also included in this revised version<sup>104,110</sup>.

**Table 3. WHO classification of AML and related neoplasms**<sup>104,110</sup>.

<i>Types</i>	<i>Genetic abnormalities</i>
<b>AML with recurrent genetic abnormalities</b>	AML with t(8:21)(q22;q22); RUNX1-RUNX1T1 AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFβ-MYH11 APL with PML-RARA AML with t(9;11)(p21.3;q23.3); MLLT3-KMT2A AML with t(6;9)(p23;q34.1); DEK-NUP214 AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM AML (megakaryoblastic) with t(1;22)(p13.3;q13.3); RBM15-MKL1 AML with BCR-ABL1 (provisional entity) AML with mutated NPM1 AML with biallelic mutations of CEBPA AML with mutated RUNX1 (provisional entity)
<b>AML with myelodysplasia-related changes</b>	---
<b>Therapy-related myeloid neoplasms</b>	---



<b>AML otherwise not specified (NOS)</b>	AML with minimal differentiation AML without maturation AML with maturation Acute myelomonocytic leukemia Acute monoblastic/monocytic leukemia Acute erythroid leukemia Pure erythroid leukemia Acute megakaryoblastic leukemia Acute basophilic leukemia Acute panmyelosis with myelofibrosis
<b>Myeloid sarcoma</b>	---
<b>Myeloid proliferations related to Down Syndrome</b>	Transient abnormal myelopoiesis (TAM) Myeloid leukemia associated with Down Syndrome

#### 2.2.4 Etiology

AML development has been associated with several risk factors that include genetic abnormalities, age, previous hematological diseases, exposure to chemicals, radiation or viruses, chemotherapy and hazardous employments (**Table 4**). These different risk agents seem to be responsible for some AML cases, in which the HSCs must be vulnerable to any of these factors that trigger the leukemogenesis. Nonetheless, the biggest part of AML cases emerges as *de novo* diseases without a specific and clear leukemogenic exposure<sup>111,112</sup>.

Genetics plays an important role in AML. For instance, in children the genetic defects are an essential factor in AML development, examples of this is the increased rate of acquiring AML in patients with Down syndrome, Klinefelter or Li Fraumeni syndrome<sup>113,114</sup>. Risk of AML development can also increase by exposure to several environmental and chemical agents. Ionizing radiation is related to AML as observed in individuals exposed to radiation from the atomic bombs dropped over Hiroshima and Nagasaki<sup>1</sup>. But also, therapeutic radiation is considered as a risk factor that can promote a secondary AML. In this context, secondary AML can also be triggered by exposure to chemotherapeutic agents as alkylating or topoisomerase II inhibitors<sup>106</sup>.

Few studies have studied the relationship between exposure to pesticides or solvents and AML development<sup>115</sup>. In addition, tobacco smoke has been linked to AML but it can be considered so far as a weaker factor compared to others, until more studies can show a stronger link to AML. Moreover, benzene is a chemical that has been on the spotlight due to data suggesting that exposure to high concentrations can drive AML development<sup>116</sup>. For instance, a follow-up study of 2-17 years was conducted in 44 pancytopenic patients exposed between 4 months and 15 years to high concentrations of benzene (150-650 ppm). These patients had worked in the shoe industry or as laborers involved in pliofilm production. AML development occurred in 6 patients out of the 44 and was linked to chronic exposure to benzene<sup>116</sup>. Smith and colleagues performed experiments based on *in vitro* exposure of CD34+ cells or lymphocytes to benzene which induced aneuploid cytogenetic abnormalities. Otherwise, there are not reliable studies that relate individuals exposed to benzene as automobile users, employees from gas stations or vehicle mechanics with increased risk of AML development<sup>117</sup>.

Although viruses and specially RNA retroviruses exhibited the capacity to cause different neoplasms including leukemia in studies using experimental animals, it has not been identified or reported so far, any virus as a main cause for AML development. Recently new research works started to study the link between Parvovirus B19 and its role in AML but specially in children<sup>118</sup>.

Another important cause of AML development has been described as “secondary AML”, which basically arises often in patients with a clinical history of myelodysplastic syndromes (MDS), myeloproliferative malignancies or as mentioned before, individuals under chemotherapeutic agents. It has been observed that AML emerged in between 10 to 20% of patients who were treated with chemotherapy for other malignancies. Furthermore, the incidence of acquiring AML increases 100 times in patients under a high and intensive dose of chemotherapy<sup>106</sup>.

Finally, within the life style factors, diet is another element that has been considered in AML development. Nonetheless, conducted studies have not been able to demonstrated the daily diet as a risk factor in AML<sup>106</sup>

**Table 4. Selected risk factors associated with Acute Myeloid Leukemia<sup>106</sup>.**

<b>Genetic disorders</b>	Down syndrome Klinefelter syndrome Patau syndrome Ataxia telangiectasia Schwachman syndrome Kostman syndrome Neurofibromatosis Fanconi anemia Li-Fraumeni syndrome
<b>Physical and chemical exposures</b>	Benzene Pesticides Cigarette smoking Embalming fluids Herbicides
<b>Radiation exposure</b>	Nontherapeutic, therapeutic radiation
<b>Chemotherapy</b>	Alkylating agents Topoisomerase-II inhibitors Anthracyclines Taxanes Drugs such as Vercyte (pipobroman)

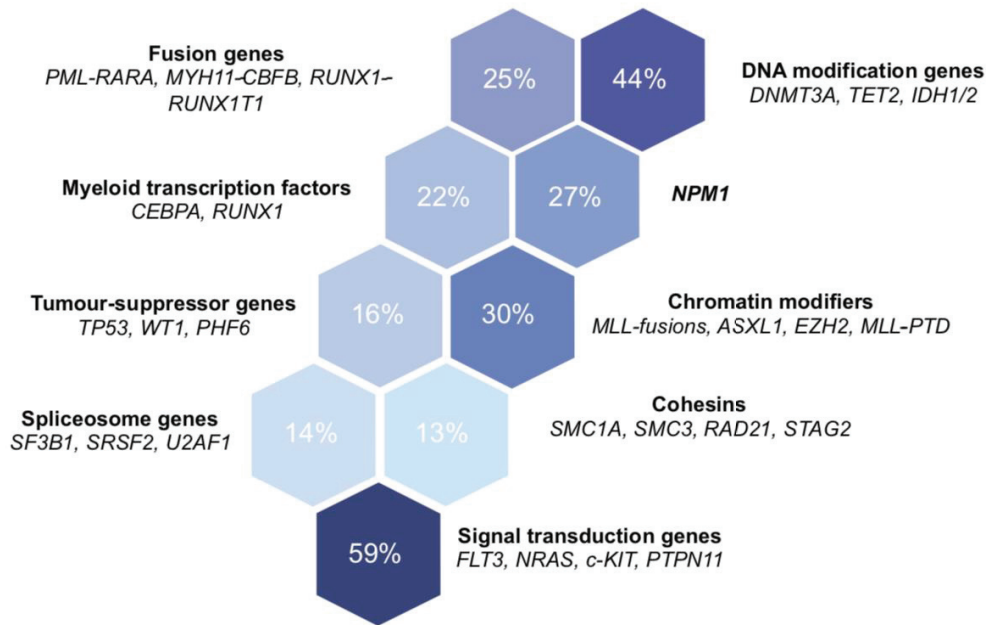
### 2.2.5 Pathogenesis

Previously described, the causes leading to AML development are several and the risk factors among them differ. Some of them have been described in several research studies while others remain as hypothesis needing a more exhaustive research. Nevertheless, most of the AML cases are considered as *de novo* malignancies that arise in individuals seemingly healthy. Independently of the causes leading to AML, this malignancy is based on the concept of abnormal differentiation and proliferation of a specific clonal population; the myeloid stem cells<sup>104</sup>.

Specific chromosomal translocations have been identified in several AML subtypes, example of this is the translocation t(8:21) in core-binding factor AML

(CBF-AML) which leads to the formation of chimeric protein RUNX1-RUNX1T1. Also in acute promyelocytic leukemia (APL) is possible to identify t(15:17) with PML-RARA protein formation. Not only chromosomal rearrangements have been described in AML development, but involvement of molecular changes is also an important factor in AML and genetic mutations are found in around 97% of AML cases. Despite the fact of the common presence of genetic mutations in AML, this malignancy presents the lowest number of mutations per case compared to other adult types of cancer<sup>119,120</sup>.

Abnormal progenitor blasts in AML are generated from normal blasts affected by two-type class genetic alterations. These transformations described by Gilliland and Griffin are called the “two-hit model”, in which both lesions are necessary to trigger AML, since each lesion by its own is not able to cause the disease. This model proposes a class I mutation involving the activation of cell-surface receptors such as FLT3 (Fms-like tyrosine kinase 3)-ITD (internal tandem duplication), N-RAS and c-KIT; activation of different downstream signaling pathways allow the capacity of proliferation and survival of the involved hematopoietic progenitor population by diminishing apoptosis, with no influence in differentiation. While class II mutations, are able to block the hematological differentiation and by consequence the apoptosis, orchestrated by fusion genes (*PML/RAR $\alpha$*  and *MLL*). Type class II mutations seem to be the initiating lesions in AML while class I mutations are posterior events<sup>105</sup>. Moreover, a type class III mutations has been identified recently, involving a modification in genes linked to epigenetic regulation. Interestingly, these class III mutations have a downstream effect in cellular differentiation and also proliferation. Among these mutations it is possible to include DNA-methylation related genes as *DNMT3A*, *TET2*, *IDH-1* (isocitrate dehydrogenase-1) and *IDH-2* (isocitrate dehydrogenase-2), identified in 40% of AML events<sup>104</sup>. During the last years new mutations have been identified (**Figure 9**), some seem to not fit precisely in any of the class type mutations described, though the synergic effect they produce can be compared to the one defined by the two-hit model. The most relevant are mutations affecting the tumor suppression genes as TP53, WT1 and PHF6<sup>121,105,104</sup>.

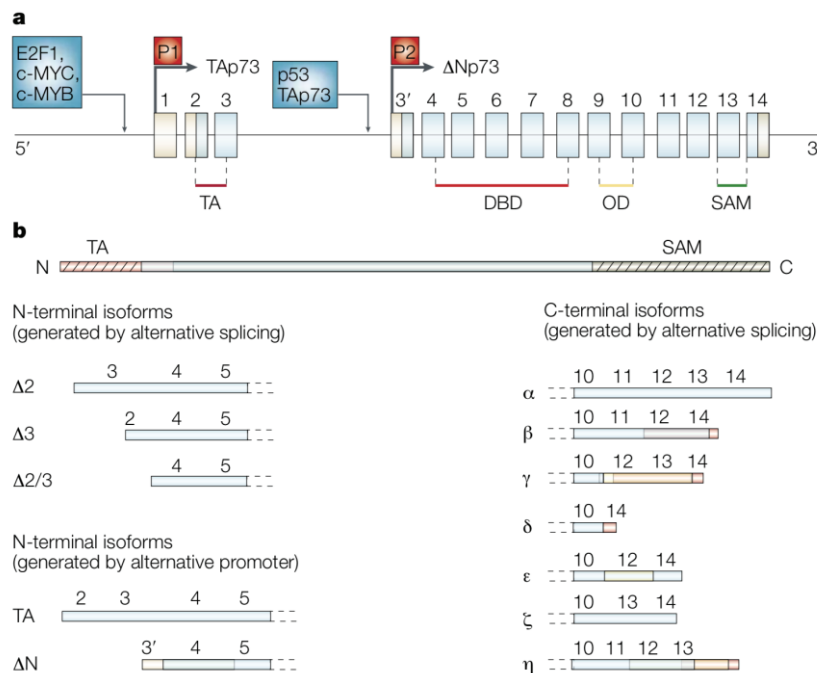


**Figure 9. Frequent mutation groups identified in *de novo* AML cases<sup>105</sup>.** Genes regularly mutated in AML and classified in different groups and pathways. Data obtained from The Cancer Genome Atlas (TCGA) Research Network, 2013.

Tumor protein p53 (TP53 or p53) is an essential tumor suppressor involved in prevention of cancer formation. p53 has been reported to be inactivated in more than 50% of solid tumors through a mutation in the *TP53* gene<sup>122</sup>. Poor survival, refractory disease and chemoresistance have been linked to inactivation of p53 in cancer. Despite the fact that TP53 mutations have been reported only in 5% of AML patients holding 17p monosomy, this mutation has been identified as a negative factor to chemotherapy response and poor prognosis<sup>123,124</sup>.

During the end of the 20<sup>th</sup> century, the p73 (TP73) proteins were identified and classified as members of the p53 family<sup>125</sup>. p73 contains three different functional domains: the amino-terminal transactivation domain (TA), the DNA-binding domain (DBD) and the carboxy-terminal oligomerization domain (OD)<sup>126</sup>. Genes regulated by p73 transcription factors are involved in cell-cycle arrest induction and apoptosis<sup>127</sup>. p73 presents several isoforms carrying out different roles in cancer development. A classification of two main groups has been established to categorize the different p73 isoforms, the first is the group of transcriptionally

active p73 isoforms (TAp73) that possess the capacity to induce apoptosis and activate transcription of cell cycle regulators. The second group is the N-terminally truncated or Dominant Negative variants (DNp73 or  $\Delta$ Np73), these isoforms present a lack of the N-terminal p73 (**Figure 10**)<sup>127</sup>.



**Figure 10. p73 isoforms**<sup>127</sup>. **a.** Regulation of the two different promoters P1 (extrinsic) and P2 (intrinsic) generates both isoforms TAp73 and  $\Delta$ Np73 respectively. First 3 exons encode for the TA (transactivation) domain and codifies TAp73. Alternative promoter P2 is located in the intron 3 and transcription of  $\Delta$ Np73 proceeds from exon 3', leading to a N-terminal end since AA are translated from the included exon 3'. **b.** Generation of amino-terminal isoforms occurs by alternative splicing or alternative promoter use. Alternative splicing of the exons 11, 12 and 13 encoding for the SAM (Sterile Alpha Domain) domain, produces the seven different carboxy-terminal isoforms.

TAp73 isoform is able to activate genes regulated by p53. Example of these genes are *CDNK1A*, *p53R2*, *PUMA*, *NOXA*, *PERP* and *BAX* which are responsible for growth arrest and apoptosis<sup>125,128</sup>.  $\Delta$ Np73 lacks the TA domain; in consequence this isoform is not capable of inducing gene expression directly and does not present the capacity for growth arrest and apoptosis as observed in TAp73.

The specific role of p73 in cancer has been widely discussed. Mutations of this protein are unusual in cancer with around 0.5% in tumors. This is a very low percentage when compared to its family homologue p53, which mutations are present in over 50% of cancers. An important parameter to understand the role of p73 is the fact that TP73 locus encodes the two p73 isoforms. TAp73 is identified by its role as a tumor suppressor, whereas  $\Delta$ Np73 is described as an anti-apoptotic agent and a presumed oncogene<sup>129</sup>.

Both p73 isoforms, TAp73 and  $\Delta$ Np73, have started to be described in the cancer context. For instance, the first studies about this subject reported the oncogenic potential of  $\Delta$ Np73 by *in vitro* and *in vivo* transformation assays. p53 and TAp73 functions are inhibited in cancer cell lines overexpressing the anti-apoptotic isoform. In lung cancer the anti-apoptotic isoform was identified in the cytoplasm of 77 patients out of a 132 cohort. Patients with a positive expression of  $\Delta$ Np73 were linked to poor prognosis<sup>130</sup>. The role of  $\Delta$ Np73 in AML has been described in certain studies. For example, Rizzo and colleagues identified high levels of the isoform at the transcript and protein levels in 96.4% of a cohort of 71 AML patient samples at diagnosis, which included the subtypes M0, M1, M2, M4, M5 and M6. In contrast, only 31.7% of the 41 AML-M3 patient samples analyzed at diagnosis presented high levels of  $\Delta$ Np73<sup>131</sup>. Nevertheless, Lucena-Araujo and collaborators identified later on that a high  $\Delta$ Np73 / TAp73 mRNA expression ratio in AML-M3 patient samples at diagnosis, was linked to low survival and higher risk of relapse, suggesting the use of these isoforms as potential prognosis markers<sup>132</sup>.

Among the different types of AML, it is possible to identify several numbers of mutations driving to the development of the disease. According to data from the The Cancer Genome Atlas (TCGA) Research Network, the study conducted in the entire genome/exome of 200 AML showed a range between 0 and 51 mutations with a mean of 13 belonging to the tier 1 group mutations<sup>133</sup>.

New discoveries in the genomic field using high-throughput technologies allowed to reach a better comprehension of the molecular pathogenesis in AML. Cytogenetic aberrations identified in myeloid neoplasms had brought advances

in diagnostic and prognosis during the last decades for AML patients. Cytogenetic abnormalities are considered important prognosis predictors for short and long-term patient outcome<sup>106</sup>.

### **2.2.5.1 Type class I mutations**

Previously described, leukemogenesis in AML is defined by the clonal proliferation and escape from apoptosis of an immature progenitor population named blasts, in which blocking of differentiation can be observed. The activation of growth factor receptor signaling pathways seems to contribute to this process. Analysis of AML bone marrow samples displayed that around 50% of these primary specimens presented aberrant and constitutive activation of signal transduction molecules. Within the most common surface receptor tyrosine kinase (RTK) linked to AML it is possible to identify FLT3, N-RAS / K-RAS and Kit<sup>134</sup>.

Since 1996, the role of the protein FLT3 has been on the spotlight as an important factor in AML development, progression and also considered as a potential therapeutic target. This protein is a member of the RTK class III and can be identified also as Flk-2 or STK-1. Mainly expressed in hematopoietic tissue, activation of this protein in a normal hematopoietic context drives increased proliferation and survival. Within a malignant context, FLT3 is highly expressed in around 60 to 92% of AML cases<sup>135</sup>. Studies conducted by Müller-Tidow and collaborators showed higher FLT3 expression in AML blasts compared to normal hematopoietic progenitors. Frequency of FLT3-ITD in AML is more often seen in APL, whereas AML-M2 and AML-M6 subtypes present a lower frequency of this mutation<sup>136</sup>.

RAS mutations represent another essential element to analyze in AML pathogenesis. Mainly N-RAS and K-RAS mutations can be identified in around 10-27% and 5-10% of AML cases. Prognosis of patients presenting RAS mutations is inconsistent, while two studies related the presence of N-RAS to low blast count in patient BM and a favorable outcome. Other studies associated RAS mutations to a worse patient outcome. This type of mutation seems not to be an



initiating leukemia event but more a progression factor. As observed in several studies, RAS mutations depend a lot of other mutations present in the microenvironment to lead into AML<sup>135</sup>.

Another member of the receptor tyrosine kinase (RTK) of class III involved in AML development is the transmembrane protein Kit. Studies focused on this protein demonstrated the presence of two regions in the receptor, which is susceptible to acquire function mutations. This area is the juxtamembrane region that acts as a negative regulatory region and activation loop. Although in some subtypes of AML this mutation seems to be present in isolated cases, in CBF-AML 6 out of 17 cases presented the activation loop<sup>137</sup>.

#### **2.2.5.2 Type class II mutations**

Hematopoiesis is based on the principle that only a proper balance between general and specific lineage transcription factors can drive the appropriate gene expression profile, which will be responsible for differentiation of hematopoietic progenitor populations into a more specific and differentiated lineage. AML is a perfect example in which this balanced is disrupted and the transcription factors are targeted by the chromosomal translocations and mutations. In AML, balanced translocations are observed giving as a result the fusion of two genes. Around 50% of this kind of abnormalities is reported in new diagnosed AML events<sup>138</sup>.

Translocations such as t(8;21), t(15;17) or inv(16) are the cause for fusion proteins identified commonly in AML<sup>139</sup>. For instance, in CBF-leukemias the core binding factor (CBF) complex is affected by the translocations t(8;21), inv(16) and t(16;16). In this case, the function of the transcription factor CBF is affected, which is formed by the subunits; RUNX1 (AML1), CBP $\alpha$ 2 and PEBP2 $\alpha$ B. Both subunits will function as a heterodimeric transcriptional activator where AML1 (RUNX1) holds the DNA binding ability<sup>140,141</sup>. The translocation t(8;21) is present in 10-15% of adult AML cases. And it generates the fusion protein AML1-ETO (CBFA2-CBFA2T1), through a replacement of the C-terminus of the transcriptional activator AML1 with the ETO repressor. Blocking of differentiation is thought to be induced by this fusion protein(see review<sup>142</sup>).

Acute promyelocytic leukemia (APL) is another example where fusion proteins play an essential role. Classified as an AML-M3 subtype by the FAB, it represents 10% of AML cases. The affected gene in this malignancy is *RAR $\alpha$* . The translocation t(15;17) is responsible in 99% of the cases for the fusion of the PML coding sequence to *RAR $\alpha$* . Normal feedback of *RAR $\alpha$*  to retinoic acid is disrupted by the PML- *RAR $\alpha$*  fusion protein responsible for blocking of differentiation<sup>143</sup>.

Among the different factors described in the type class II mutations, the mixed-lineage leukemia (MLL) protein is another frequent factor in AML pathogenesis. A histone methyltransferase activity can be identified in MLL. Although the function of MLL in normal hematopoiesis has not been properly identified, this gene can be found in aggressive forms of AML and also ALL malignancies<sup>135</sup>.

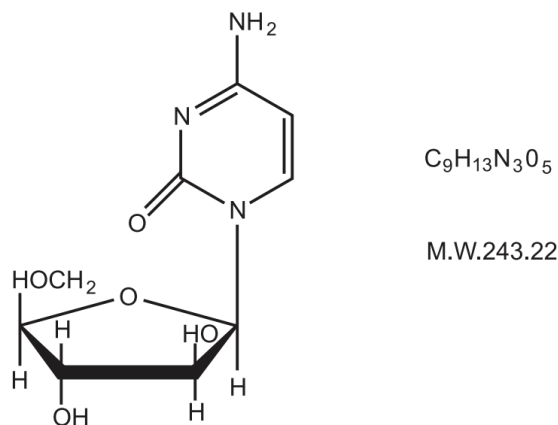
### **2.2.6 Treatments**

General treatment of AML patients has remained nearly the same during the last 30 years. The strategy followed related to the use of chemotherapeutic agents continues to be almost the same<sup>144</sup>. Initially, patients need to be evaluated in order to determine the best option for treatment. Prognostic factors are used to determine the best treatment option. Among the different factors employed to evaluate the AML patient, the Zubrod scale is a scoring given to the patient from 0 to 5, where 0 represents perfect health and autonomy and 5 death. Age of the patient is one of the main parameters and together with the physical shape, it is possible to establish the best strategy by clinicians. Parameters from clinical analysis also used as prognosis factors are levels of serum albumin, bilirubin and creatinine among others. Moreover, it is crucial to consider resistance to treatment of AML patients. The best way to identify the tendency to resistance in patients, is by analyzing at diagnosis the cytogenetic and molecular genetic profile of AML blasts. Using this profile, AML patients can be classified into favourable, intermediate and adverse cases, which can help to better adapt the therapeutic approach<sup>107</sup>.

### 2.2.6.1 Standard chemotherapy

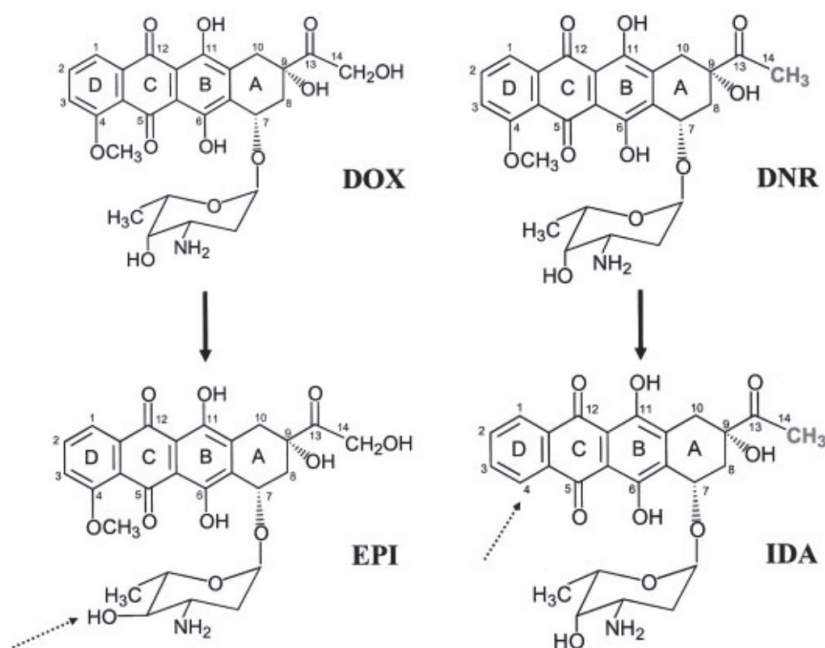
Combination of chemotherapeutic agents as anthracycline (daunorubicin or idarubicin) and cytarabine (ARA-C (Cytosine-Arabinoside)), is the standard therapeutic approach for AML patients. Treatment is composed of two phases; the first has the aim to achieve a complete remission with a bone marrow containing less than 5% blasts, neutrophil counting above 1000 and platelet number higher than 100,000<sup>145</sup>. In the second phase, once the complete remission has been reached it is necessary to prolong this status. AML patients are usually treated with medium or high doses of ARA-C and additionally to this, depending of the favourable/intermediate/adverse classification of the patient, allogenic or autologous transplants are employed. Normally, AML cases under a remission of 3 years, have a 10% of chances or less to relapse<sup>146</sup>.

Cytarabine also recognized as cytosine arabinoside is a molecule (**Figure 11**) which was synthesized for the first time in 1958 and has been used in AML treatment since 1969 after the approval of the Food and Drug Administration (FDA). Oral administration of ARA-C is ineffective due to the fast metabolism of the drug by the liver and intestines. Thus, ARA-C is applied by intravenous injection. ARA-C presents cell phase specificity by killing the cells undergoing DNA synthesis (S-Phase) and also by blocking the transition of cells from phase G<sub>1</sub> to S-phase. This compound presents a high inhibition of DNA synthesis by inhibiting DNA polymerase. The drug can also cause marked chromosomal aberrations as chromatid breaks and extensive fragmentation. A limited amount of the drug can incorporate into DNA and RNA<sup>147</sup>.



**Figure 11. Chemical structure of cytarabine (ARA-C)<sup>148</sup>.**

Anthracyclines (**Figure 12**) are the other main therapeutic agent together with cytarabine. These chemotherapeutic drugs were isolated in the 1960's from *Streptomyces peucetius*. The first two molecules obtained were daunorubicin (DNR) and doxorubicin (DOX). Later on, epirubicin (EPI) and idarubicin (IDA) were developed and approved for AML treatment. The most described cytotoxic mechanism of action of these compounds are the following: they act as DNA intercalators; by insertion into the double helix of DNA they achieve to block DNA/RNA/protein synthesis, cause fragmentation of DNA and inhibit its repair. In addition, anthracyclines have the ability to bind to cell membrane and cause functional alterations. Finally, reactive oxygen species (ROS) are produced by anthracyclines through its reduction to a semiquinone free radical. Excessive ROS will produce oxidative stress, DNA damage and lipid peroxidation leading to apoptosis<sup>149</sup>.



**Figure 12. Chemical structure of anthracycline molecules**<sup>150</sup>. Doxorubicin (DOX), Daunorubicin (DNR), Epirubicin (EPI) and Idarubicin (IDA). Main anthracyclines employed in treatment of acute myeloid leukemias.

### 2.2.6.1.1 Induction phase

As mentioned before, the first phase of AML treatment consists in the “Induction phase”. The aim is to eliminate as fast as possible the highest number of leukemic cells in the newly diagnosed patient. Usually young adults under 60 years old are candidates for this treatment and the level of intensity might variate depending of the health of the patient. The so called “3+7 combination” is the most common therapy strategy used in AML patients, in which they receive intravenously during 3 days 45 mg/m<sup>2</sup> or 60 mg/m<sup>2</sup> of an anthracycline as daunorubicin. Daunorubicin dose can be increased in patients with mutations considered of poor prognosis as DNMT3 and KMT2A<sup>104</sup>. These patients also receive 100 mg/m<sup>2</sup> of cytarabine intravenously during 7 days. This combination has shown a complete remission in 65-70% of patients between 18 and 60 years old. In addition, reports generated by the Australian Leukemia Study Group demonstrated a complete remission in 80% AML cases with high dose therapy of cytarabine combined with idarubicin and etoposide<sup>107</sup>. In older patients above 60-years old, the remission rate is lower

(between 40-60%). These patients often present unfavorable cytogenetics abnormalities and conventional chemotherapy results in a low benefit. Therefore, some of these AML cases are eligible for investigational novel therapies<sup>151</sup>.

#### **2.2.6.1.2 Consolidation phase**

Consolidation phase, also called post-remission therapy, is focused on eliminating any remaining leukemic cell and prevent relapse. The strategies used involves chemotherapy and allogenic or autologous hematopoietic stem cell transplantation. Chemotherapy is usually the first option specially in favourable patients. In young adults (18-60 years old), chemotherapy is applied in 2 to 4 cycles of cytarabine in either one of the following concentrations; high-dose (3 g/m<sup>2</sup>), intermediate-dose (400 mg/m<sup>2</sup>) or standard-dose (100 mg/m<sup>2</sup>)<sup>152,107,104</sup>.

Patients from the intermediate or high-risk groups, normally are the candidates for a treatment combining high chemotherapy doses with either allogenic (donor) or autologous (patient) hematopoietic stem cell transplantation. Allogenic transplantations present a higher benefit with a better relapse-free survival than the autologous transplant. The reason for this advantage in the allogenic procedure, relies on the graft-versus-leukemia effect which is not observed in the autologous engraftment<sup>107</sup>. Nonetheless, allogenic transplantations do not have the same effect in every patient. For instance, AML patients presenting *NPM1* mutations and a lack of FLT3 alteration and with a survival around 60% through standard chemotherapy consolidation, probably an allogenic transplant will not upgrade their outcome. Unfortunately, high-risk AML cases face a poor outcome often lower than 10% relapse-free survival after high-dose cytarabine treatment combined with autologous transplantation<sup>153,107</sup>.

#### **2.2.6.2 Novel therapeutic agents**

During the last years, development of new therapeutic agents against AML malignancy is on the center of attention. The poor prognosis and high relapse rates make essential to establish novel therapeutic strategies.

Inhibitors of the tyrosine kinase receptor (TKR) have been widely used in several solid and hematological cancers. In AML, FLT3 mutation plays an important role and for this reason, the inhibition of this TKR has been identified as a new potential therapeutic target. Within the first generation of this type of inhibitors, Sorafenib showed high complete remission (CR) in phase I and II trials, but 50% of patients faced relapse within 9 months<sup>154</sup>. Midostaurin, another first-generation inhibitor, has been tested. Patients presented resistance to this inhibitor as for sorafenib. Nonetheless, midostaurin combined with other chemotherapy drugs as azacitidine exhibited a better response in patients<sup>104</sup>.

Second-generation inhibitors of TKR targeting specifically FLT3 have been developed. Quizatinab is an example of these inhibitors, in which off-target effects are diminished thanks to its selectivity. In phase I, oral administration of Quizatinab to 76 AML relapsed/refractory patients reported 30% of response and 13% patients achieved CR<sup>155</sup>. Crenolanib is another agent being tested, which targets FLT3-ITD and FLT3-TKD mutants. A cohort of 38 AML patients from a phase II study carrying the FLT3 mutation, received 200 mg/m<sup>2</sup> of Crenolanib, achieving event-free survival (EFS) for 8 weeks and overall survival (OS) of 19 weeks<sup>156</sup>.

Another new important target in therapeutic development is the STAT signaling pathway. Around 50% of AML cases present STAT3 tyrosine phosphorylation which is linked to poor prognosis<sup>157</sup>. Different STAT3 inhibitors are being tested such as molecules C188-9 or MM-206 with good apoptosis induction in AML cell lines. In addition, the molecule OPB-31121, a STAT3 and STAT5 inhibitor showed good results in solid tumors and growth inhibition in AML cell lines<sup>158</sup>.

The use of monoclonal antibodies represents another important new therapy not only in AML but in a high number of types of cancer. The mechanism of these antibodies relies on their capacity for a direct antibody-dependent toxicity or through the combination with cytotoxic agents leading to a direct impact of the drug against the neoplastic cells. For instance, gemtuzumab ozogamicin (GO) is a human recombinant antibody that targets a protein present on myeloid cells which is the cluster of differentiation CD33. GO is an antibody conjugated with

the DNA-cleaving cytotoxic drug calicheamicin<sup>159</sup>. The fatal toxicity presented by this antibody stopped its use in 2009 but, in 2014 new studies showed a survival benefit in patients from intermediate and high-risk groups<sup>160</sup>. Another molecule tested is the AGS67E which is a human antibody against CD37. This cluster of differentiation is a transmembrane protein highly expressed in non-Hodgkin lymphoma and CLL. AGS67E is conjugated to monomethyl auristatin E (MMAE), known as a microtubule-disrupting compound. Apoptosis of malignant cells is driven through the delivery of the drug by the antibody targeting CD37+ cells. *In vivo* tests using a murine AML model exhibited a decreased capacity for tumor engraftment<sup>104</sup>.

Development of novel inhibitors against the mutant enzymes IDH1 and IDH2 represents a promising therapy showing encouraging results<sup>161</sup>. For instance, 153 AML patients newly diagnosed presenting IDH1 and IDH2 mutations were treated with standard chemotherapy combined with the new oral IDH inhibitors ivosidenib and enasidenib. Response rate of ivosidenib/chemotherapy treatment was 71% and 56% in patients treated with enasidenib/chemotherapy. While remission and better overall survival within the first year were achieved in 75% patients from the high-risk population (>65-years old)<sup>162</sup>. Moreover, a study based on treatment with only ivosidenib, reported an overall response of 57.6% patients achieved in 2.8 months previous to therapy. This study in phase I included 34 patients, where seven patients presented *de novo* AML and 27 had secondary AML. Corresponding to intermediate and poor-risk cytogenetic features<sup>163</sup>.

## **2.3 Leukemic stem cell (LSC)**

### **2.3.1 Overview of the cancer stem cell (CSC)**

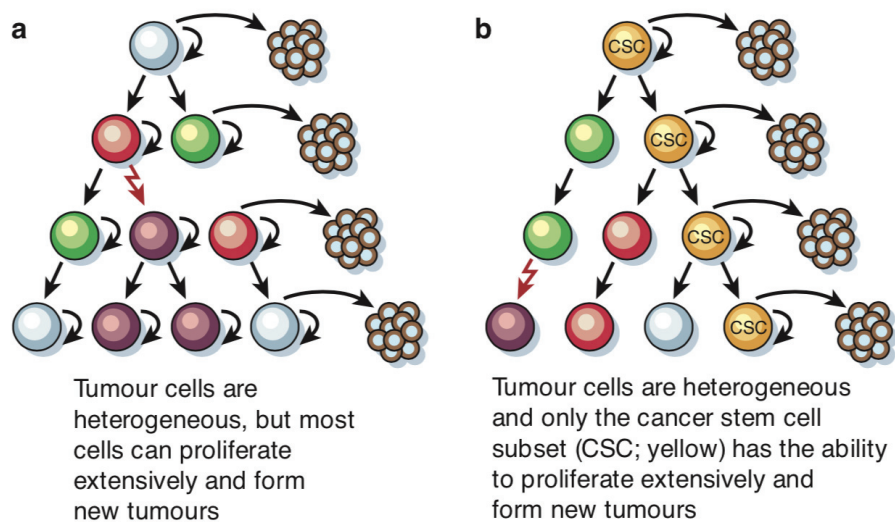
Understanding the molecular mechanisms and the signaling pathways involved in tumorigenesis as well as the malignant transformations, have been a result achieved thanks to the identification of genetic abnormalities driving cancer development. A lot of the research made during the last decades was focused on the molecular biology of cancer, whereas the cellular biology was in some way not a main priority. Although new discoveries have contributed to understand at



least partially the effect of mutations on proliferation or survival, there is a lack of understanding the direct effect of such mutations on the actual cells involved in cancer<sup>54</sup>.

Normal stem cells (SCs) are involved in development of tissues in the body. They present as described before in the HSCs, the abilities of self-renewal, proliferation and differentiation. When speaking about cancer, any type of tumor can be seen as an altered organ in the body which has been produced by a tumorigenic cancer cell. Therefore, by using the principle of normal SCs biology, it is possible to present a tumor as an “aberrant organ” that arises from a modified stem cell which has been identified as a cancer stem cell (CSC). These CSCs must present as their normal counterparts, self-renewal capacity, potential of proliferation and differentiation. Heterogeneity is observed in normal SCs and in CSCs. This heterogeneity can be produced either by the continuous mutations in the tumorigenic cancer cell or from the aberrant differentiations in cancer cells<sup>164,54</sup>.

An important aspect of the CSCs is to understand if only this population holds the capacity to proliferate and give rise to other cancer cells, or if also other cancer cell populations possess this ability. As showed in leukemia and multiple myeloma, a small bulk of cancer cells were identified to present an extensive proliferation capacity. By using myeloma cells from mice, only 1 in 10,000 to 1 in 100 cancer cells, were able to produce colonies in an *in vitro* colony-forming assay<sup>165</sup>. From these results, two different hypotheses (**Figure 13**) are considered; either only a defined small population of “special leukemic cells” have the capacity for extensive proliferation or all leukemic cells present this ability<sup>54</sup>.



**Figure 13. The two different scenarios of heterogeneity in cancer<sup>54</sup>.** **a.** All cancer cells present the ability of extensive proliferation but when placed *in vitro*, they present a low capacity in clonogenicity or tumorigenicity assays. **b.** Only a defined small subset of cancer cells has unlimited proliferative potential observed in clonogenic assays.

Both hypotheses remain in discussion for solid tumor cancers, while in AML the research work conducted by John Dick and colleagues, confirmed the presence of a small portion of leukemic cells holding the abilities of a normal SC. In addition, only these specific leukemic cells had the capacity to transfer AML into mice and proliferate extensively<sup>54</sup>.

### 2.3.2 The concept of LSC in AML

The evidence showing the existence of CSCs was described for the first time in AML. During the decade of the 90's, John Dick and colleagues published their research investigation focused in the identification of a small and rare bulk of leukemic cells. This population presented a phenotype of cell surface markers CD34<sup>+</sup>CD38<sup>-</sup> and was able to initiate AML when transferred into non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID). Identified at that time as SCID leukemia-initiating cell (SL-IC), this population owned self-renewal capacity together with extensive proliferation and differentiation abilities. Once isolated, these SL-ICs showed *in vivo* to be able to differentiate into leukemic blasts, providing the evidence of hierarchy organization

of the leukemic clones<sup>166,167</sup>. Also Identified later on as leukemia-initiating cells or leukemic stem cells (LSCs), this population became a major target to better understand not only AML but all the different hematological malignancies and other types of solid cancers. As seen in hematopoietic stem cells, based on different studies the LSCs are found at the top of a hierarchy and are responsible for the generation of poorly differentiated blasts which are able to maintain AML<sup>168</sup>.

Analysis of the LSCs have showed how similar they are to their normal stem cell counterpart. Both HSCs and LSCs share several functional characteristics such as: quiescence cell cycle status, capacity to outflow drugs and the other aspects described before as self-renewal properties and the fact of being rare in number<sup>169</sup>.

### **2.3.3 LSC phenotype**

Identification of a specific LSC phenotype is a fundamental key to understand the role and mechanisms played by these cells in cancer. In addition, recognition of the LSC bulk with specific surface markers is the tool needed to target in a more efficient way these cells responsible for AML maintenance.

The first step was given by Dick and collaborators by identifying the leukemic stem cells (LSCs) as a rare population presenting the cell surface markers CD34<sup>+</sup>CD38<sup>-</sup>. Nonetheless, the HSCs share the same immunophenotype than the LSCs<sup>167</sup>. This is the reason why cancer treatments cannot be based only considering this immunophenotype. Cell surface markers in LSCs seem to be more complex than thought decades ago. For instance, the analysis of AML patient samples with NPM (nucleophosmin) mutation demonstrated the presence of LCSs displaying the immunophenotype CD34<sup>-</sup> / CD38<sup>-</sup> / Lin<sup>-</sup> and not the typical CD34<sup>+</sup>/CD38<sup>-</sup>/Lin<sup>-</sup> profile observed in LSCs<sup>170</sup>. In addition, other studies have confirmed the fact that LSCs are a truly rare population holding different potential of self-renewal. Other surface markers of LCSs also present in HSCs are CD71<sup>-</sup> /HLA-DR<sup>-</sup>. Moreover, normal committed progenitor cells likewise share with LSCs the profile Lin<sup>+</sup>CD38<sup>+</sup>CD45RA<sup>+</sup>. These findings give strength to the theories

describing the origin of LSCs, originated not only from HSCs but also from a more differentiated population as committed progenitor lineage<sup>171</sup>.

More recently, the presence or lack of new surface markers have been identified, in order to establish a more precise and specific immunophenotype of the LSCs. For example, Blair and Sutherland identified the deficiencies of CD90 and CD117 (c-kit) expression in AML LSCs<sup>172</sup>. Contrarily, several studies reported high expression of CD123 (IL-3 receptor  $\alpha$  chain), TIM3 (T-cell Ig mucin3), CD47, CD96, CLL-1 and the IL-1 receptor (IL1RAP) by this population<sup>171</sup>. Moreover, additional surface markers such as were CD32 and CD25 have been reported to be highly expressed in LSCs as compared to normal HSCs<sup>173</sup>.

#### **2.3.4 Role of the LSC in AML resistance**

As previously described in the section “Treatments”, the standard therapy for AML patients is based on the use of cytotoxic agents, which usually leads to a complete remission (CR) of around 70% in young adults (18-60 years old) and between 40-60% in patients above 60-years old. Nonetheless, around 70% of AML patients face relapse and poor prognosis despite the different treatments employed. Resistance of AML patients in AML can be explained by different hypotheses. Drug efflux capacity of the leukemic cells, the presence of enzymes on charge of detoxification of therapy agents or the low availability of the drug within the tumor microenvironment are some of the factors considered that lead to resistance<sup>174</sup>. However, the most common and studied theory of resistance in AML, relies on the idea that the presence of a population of malignant cells is responsible for this: the LSCs. These LSCs seem to resist to the chemotherapy treatments and are able to re-establish AML in the patient. This event is known as “minimal residual disease” (MRD) which eventually drives to resistance<sup>175</sup>.

Two different studies have shown that LSCs presenting the immunophenotype profile CD34<sup>+</sup> CD38<sup>low/-</sup> CD123<sup>+</sup>, where highly found in AML patients at diagnosis. More interestingly, these patients faced poor response to treatment and poor survival. Therefore, these results suggest that MRD is related to the presence of

quiescent LSCs which are able to drive resistance in AML and in consequence patient relapse<sup>176,177</sup>.

### **2.3.5 Targeting the LSC in AML treatment**

Current therapies are focused on eradication of the leukemic blast population in AML patients. The chemotherapy drugs target the fast dividing cells with the aim of eliminate the high presence of blasts in the patient. Unfortunately, as previously described, LSCs are able to remain within the tumor microenvironment either as an active or quiescent population with the capacity to re-establish AML even after a remission of the patient. Nowadays, the obvious need for a therapy targeting the LSC has become a primary urgency<sup>178</sup>.

Identifying the specific phenotype of LSCs is crucial to develop new therapeutic approaches in AML. For instance, the high CD123 expression in CD34<sup>+</sup> CD38<sup>-</sup> LSCs compared to HSCs has been used to generate a monoclonal antibody anti-CD123 named "7G3". A significant decreased engraftment was observed in a xenograft model by using this antibody<sup>179</sup>. Investigation based on other specific surface markers of LSCs are being tested, as antibodies targeting CD47, CD96, TIM3 and CLL-1, which are being tested for elimination of primary AML LSCs<sup>171</sup>. Another type of therapies that has been studied recently and developed apart from the monoclonal antibodies, is the design of bi-specific and tri-specific antibody fragments. Immunotoxins, CAR (chimeric antigen receptor) modified T-cells and nanoparticles carrying surface markers-targeted medication are being developed. For instance, DT388IL3 (SL-401) is in phase I/II trials. This novel agent is a recombinant immunotoxin developed through the fusion of diphtheria toxin and the ligand targeting IL-3 receptor<sup>180,171</sup>.

Activity and function of LSCs are affected by transcriptions factors. Therefore, as surface cell markers, transcription factors have been identified as possible key elements in development of novel therapies. For instance, p-53 inactivation seems to be involved in survival and evolution of LSCs. Moreover, the activity of p53 appears to be regulated by histone deacetylases (HDACs). This is the reason why protein modulators of HDACs could be used to boost p53 activity and render

LSCs sensitive to therapy. Another target might be a low oxygen regulator identified in AML; hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), which plays a key role in self-renewal capacity of LSCs<sup>173</sup>. In 2015 Bonnet and Colleagues described HIF-1 $\alpha$  and HIF-2 $\alpha$  fundamental in LSC survival<sup>181</sup>.

Moreover, the nuclear factor kappa-light-chain enhancer of activated B-cells (NF- $\kappa$ B) is crucial in survival, proliferation and differentiation. NF- $\kappa$ B has been reported as highly expressed in LSCs, whereas low in HSCs<sup>182</sup>. The idea suggested for therapy, is the employment of dimethylaminoparthenolide (DMAPT), which is an inhibitor of NF- $\kappa$ B. Clinical trials testing this inhibitor have started to treat AML but also acute lymphocytic leukemia (ALL) and chronic myeloid leukemia (CML)<sup>183</sup>.

Lastly, another promising therapy is the use of inhibitors of the  $\beta$ -catenin molecule. The role of  $\beta$ -catenin is basic in Wnt/ $\beta$ -catenin signaling for self-renewal, development, recurrence and drug resistance of LSCs. This is the reason why, inhibition of this molecule could drive LSC eradication<sup>173</sup>.

Development of specific and effective therapeutic agents against LSCs could finally lead to a total eradication of AML in patients through a complete remission without any relapse and increase the overall survival.

## **2.4 Signaling pathway alterations in AML**

Hematopoiesis is the key player involved in maintaining homeostasis in every tissue in the body. A normal control of self-renewal, proliferation, differentiation or survival of HSCs is essential to maintain this normal hematopoiesis. In addition, quiescence and mobility are also regulated in HSCs by different elements within the BM microenvironment. Several signaling pathways are involved in driving the necessary stimuli that control HSCs. Nonetheless, aberrations in the signaling transductions are able to modify the hallmarks of HSCs, as survival or proliferation which can easily lead to the different hematological malignancies. A complex network of cell-extrinsic and intrinsic

signaling pathways are involved in HSC regulation. In consequence, alterations of these signals are able to transform and generate the initiating leukemic stem cells<sup>184</sup>. In this subtheme of the present chapter, some of these pathways involved in AML will be briefly described.

### **PI3K/AKT/mTOR pathway**

Activation of this pathway has been found in the entire AML leukemic populations. Works developed by Park and colleagues identified activation of PI3K (phosphatidylinositol 3-kinase) in 50% of AML samples analyzed, while mTORC1 was reported to be activated in all of them. In addition, they found the activation of PI3K in leukemic cells to be linked to expression of the isoform p110 $\delta$  of class IA PI3K. There are three different classes of PI3K/AKT/mTOR pathways. Being class IA PI3K the most described pathway in cancer. Studies developed in AML patients, reported around 50-80% of cases where protein kinase B (AKT) phosphorylation can be seen on serine 473 (Ser473). Blast cell proliferation and the clonogenicity of the leukemic progenitor population seem to be controlled by this pathway<sup>185</sup>.

### **NOTCH signaling pathway**

The NOTCH signaling pathway is an essential element when speaking about cell fate decisions in development, self-renewal capacity in stem cells or in differentiation in hematopoiesis. Dysregulation of the pathway has been related according to several research studies, to solid cancers but also to hematological malignancies. Interestingly, the specific role of the pathway either as an oncogenic factor or as a tumor suppressor is not completely clear and understood. For instance, Tohda and Nara performed studies in eight AML cell lines and 15 AML samples, where they identified the high expression of two NOTCH ligands; NOTCH1 and JAGGED1. But later on, they observed that NOTCH1 and NOTCH2 expression is not necessarily linked to AML growth. This was confirmed through a knockdown of both ligands with no growth effect in AML cell lines<sup>186</sup>. In contrast, the tumor-suppressor role of the NOTCH pathway has been reported in research works using patient samples, where the pathway was identified to be silenced. Once the pathway was re-activated, cycle arrest and apoptosis were observed. Therefore, at the present time the specific role of

the pathway is not completely elucidated and more studies are necessary to better understand its real duty in AML<sup>187,186</sup>.

### **Wnt/ $\beta$ -catenin signaling pathway**

As the previous pathways, the Wnt signaling belongs to the regulators of normal hematopoiesis. Nevertheless, alterations of the pathway have been described in several studies, not only in AML but also in other malignancies. Moreover, the pathway represents an important signaling in leukemic stem cell maintenance. Abnormal expression of  $\beta$ -catenin was reported in AML<sup>188,189</sup> and Ysabaert and colleagues identified the overexpression of  $\beta$ -catenin as a poor prognosis factor in patients<sup>190</sup>. In addition, it has been observed the relation between the hypermethylation of Wnt inhibitors and the genetic aberrations of class II mutations in AML as AML1/RUNX1, MLL/PTD and PML/RAR $\alpha$ <sup>191</sup>.

### **Hedgehog signaling pathway**

Hedgehog (HH) signaling has been related to several types of cancer. During the recent years, the pathway has been on the spotlight due to its role in leukemic stem cell regulation and AML drug resistance leading to poor prognosis of patients<sup>192</sup>. For this reason, targeting the pathway for new therapeutic approaches represents an interesting subject in research. Within the canonical pathway, three types of Hh ligands are identified; sonic hedgehog (SHH), Indian hedgehog (IHH) and desert hedgehog (DHH). And two receptors, a positive regulator Smoothed (SMO) and a negative Patched (PTCH). Kobune and colleagues, showed expression of the ligand IHH and the receptor SMO in CD34<sup>+</sup> cells from AML patients. Once the IHH ligand was counterbalanced the CD34<sup>+</sup> cells underwent apoptosis and exhibited higher sensitivity to cytarabine<sup>193,194</sup>. In addition, the transcription factor zinc-finger glioma (GLI1) has been reported to be overexpressed in AML cell lines and in patients in relapse. Suggesting a crucial role of GLI1 in drug resistance. Moreover, inhibition of the positive regulator SMO receptor resulted in decreased resistance effect in AML patients<sup>194</sup>.



### **Transforming Growth Factor- $\beta$ (TGF- $\beta$ ) signaling pathway**

In normal hematopoiesis, the TGF- $\beta$  pathway plays a crucial role in negative regulation of proliferation and triggers in parallel differentiation and apoptosis. The role of this pathway has been described in several hematological malignancies. The mechanisms leading to dysregulation of the pathway involve mutations or deletions of members of the signaling or the presence of oncoproteins with an impact in pathway alteration. Decreased expression of TGF- $\beta$  receptors or repression of the pathway are two of the main alterations identified in hematological malignancies. For instance, blocking of the pathway has been reported in AML subtype M2; the fusion protein AML1/ETO binds to Smad3 in the cytoplasm and blocks the signaling. In addition, AML patients exhibited expression of the receptor T $\beta$ RI presenting a higher frequency of polymorphism in the sequence T $\beta$ RI(6A) compared to healthy individuals. This alteration affected the antiproliferative capacity of the pathway<sup>195</sup>.

### **BMP signaling pathway**

The BMP (bone morphogenetic proteins) pathway is a major signaling that plays an essential role in normal hematopoiesis. As the previous pathways described, the BMP signaling and its elements are not only involved in normal hematopoiesis, but also its dysregulation has been reported in several types of solid cancers and hematological malignancies<sup>196</sup>. The specific role of BMP signaling in AML will be further described during the following chapter, representing the central axis of the present doctoral dissertation.

### **3 BMP signaling pathway in acute myeloid leukemia (AML)**

#### **3.1 Overview of the BMP pathway**

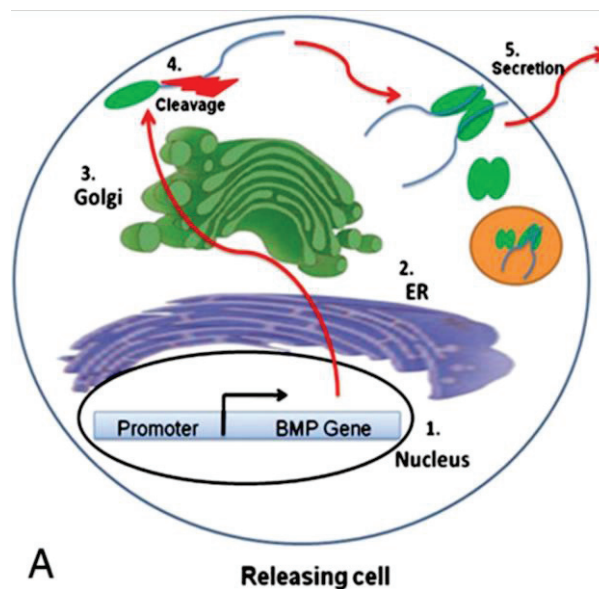
It was in 1965, when Urist described for the first time the capacity of the bone morphogenetic proteins (BMPs), to induce ectopic bone formation and cartilage generation<sup>197</sup>. And later on, at the end of the 1980's Wozney and collaborators conducted research works that allowed the characterization, purification and cloning of the first BMPs, leading to the study of the biochemical aspects of these protein<sup>198</sup>. After identifying their role in bone and cartilage formation, the BMPs are nowadays known to be cytokines present in both vertebrates and invertebrates<sup>199</sup>.

The bone morphogenetic proteins (BMPs) belong to the super family of Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) proteins. Inside this category of proteins, it is possible to identify TGF- $\beta$ s, activins, inhibins, Growth Differentiation Factors (GDFs), Glial Derived Neurotrophic Factors (GDNFs), Nodal, Lefty and the anti-Müllerian hormone<sup>200</sup>. BMPs have been identified by their key role not only in bone formation, but also in embryogenesis development and adult tissue homeostasis. Among the several tasks that BMPs perform and regulate, it is possible to identify their importance in cellular lineage commitment, morphogenesis, cell growth, differentiation and apoptosis in a wide range of cells all over the body<sup>201</sup>. When speaking about adult tissue, joint integrity, fracture repair or vascular remodeling are just some of the roles played by the BMPs. Due to their capacity to regulate several processes in all organ systems, the BMPs hold another “unofficial name”: the Body Morphogenetic Proteins. Alterations of the functioning or deficiency of these essential proteins have been related to an enormous number of pathologies<sup>200</sup>.

During the present chapter, the BMP signaling pathway will be described, including its ligands and receptors involved in both canonical and non-canonical pathways. In addition, the dysregulation of the pathway will be defined in hematological malignancies and more specifically in AML, which remains as the central topic of this dissertation.

### 3.2 BMP signaling pathway

BMP precursors are generated within the cytoplasm as dimeric pro-protein complexes of 400-500 amino acids, presenting association with pro-protein convertases allowing the production of the N- and C-terminal fragments. Therefore, during their synthesis (**Figure 14**), the BMPs are seen as precursor proteins containing a prodomain (folding and secretion) and the mature domain (C-terminal). This C-terminal mature fragment drives the capacity to bind to its receptor<sup>200</sup>. The BMP active form possesses between 50 to 100 amino acids and is composed by seven cysteines.



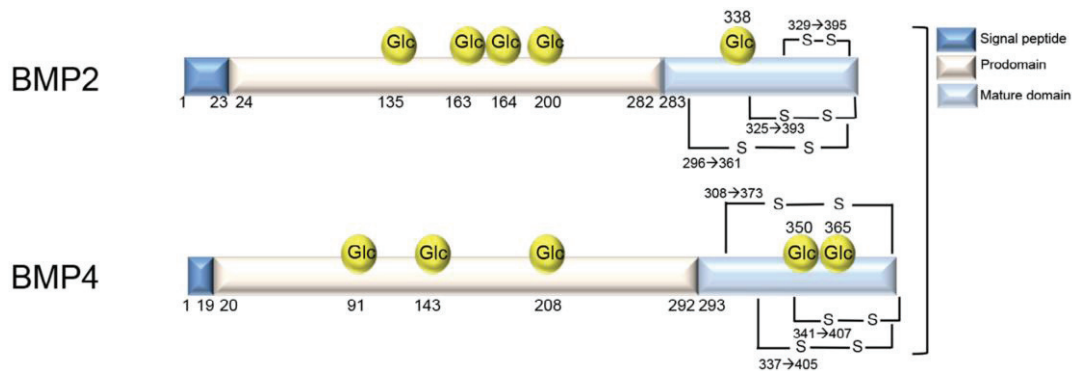
**Figure 14. BMPs synthesis and secretion**<sup>202</sup>. A. Synthesis of BMP in the cell and secretion into the extracellular microenvironment. **1.** Within the nucleus, BMP transcription starts. **2.** Translation takes place in the endoplasmic reticulum (ER). **3.** Post-translational modifications are carried in the Golgi. **4.** Proteolytic cleavage of prodomain and dimerization of monomers. **5.** BMPs are secreted from the cell either as a dimeric form carrying or not the prodomain or packed in vesicles.

According to phylogenetic trees, BMPs can be clustered into groups: BMP2/4, BMP5/6/7/8, BMP9/10 and GDF 5/6/7 (**Table 5**). For instance, BMP2 and BMP4, which present structural homologies (**Figure 15**), are secreted as precursor molecules and then cleaved by extracellular proteins such as Furin to obtain a mature protein (**Figure 14**). These active proteins form homo- or hetero-

complexes which bind to receptors complexes and induce cell transduction (Table 5)<sup>203</sup>.

**Table 5. BMP family ligands and receptors<sup>203</sup>.**

BMP Ligand	Associated Type I Receptor	Associated Type II Receptor
BMP2, BMP4	BMPR1a, BMPR1b	BMPR2, ActRIIa
BMP5, BMP6, BMP7	BMPR1a, BMPR1b, ALK1/2	BMPR2, ActRIIa, ActRIIb
BMP9, BMP10	BMPR1a, BMPR-1b, ALK1	BMPR2, ActRIIa, ActRIIb
GDF5, GDF6, GDF7	BMPR2, ActRIIa,	BMPR1a, BMPR1b



**Figure 15. Structure and protein domains of soluble BMP2 and BMP4<sup>203</sup>.** The full-length BMP structure is formed by a signal peptide (N-terminal: hydrophobic region), a prodomain (Propeptide: folding, dimerization and regulation) and a mature domain (C-terminal: monomer). Mature BMP protein is secreted as a homodimer linked by disulfide bonds<sup>204</sup>.

### 3.2.1 BMP receptors

Ligands members of the TGF- $\beta$  family are able to interact with two types of receptors in order to trigger the signaling transduction of the pathway. Although type I and II receptors are necessary for signal transduction, BMPs can bind to

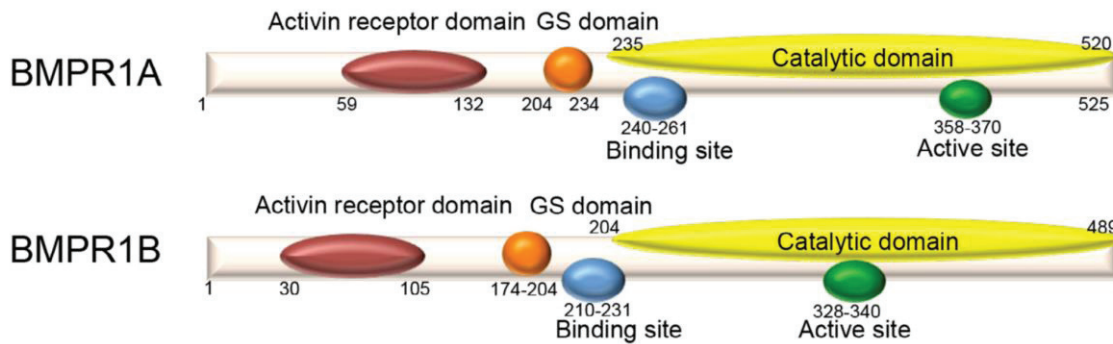
the type I receptors if lack of type II. Nonetheless, presence of both receptors boost the binding affinity<sup>205</sup>.

Type I and type II receptors are composed of three different parts: a short extracellular domain (ligand's binding site), a single transmembrane domain and a domain localized in the intracellular region which presents the serine / threonine kinase activity. The BMP type I receptors, also known as activin receptor-like kinase I, are composed of seven different subtypes (ALK-1 to ALK-7)<sup>205</sup>. These receptors can be divided in three different groups considering as a main parameter their analogies of function and structure (**Table 6**).

**Table 6. Classification of type I receptors.**

BMPR-I	ALK-I	TβR-I
BMPR-1A (ALK-3)	ALK-1	ALK-4 (ActR-IB)
BMPR-1B (ALK-6)	ALK-2	ALK-5 (TβR-I)
		ALK-7

The specificity of the ligand-receptor binding depends directly on the BMP ligand and type of receptor involved. Certain ligands present higher affinity for specific receptors. For instance, BMP-2 and BMP-4 tend to bind to BMPR-1A and BMPR-1B receptors (**Figure 16**). While it has been reported that BMP-6 and BMP-7 are able to bind to ALK-2 more than to BMPR-1B<sup>206</sup>.



**Figure 16. Structure and protein domain of the BMPR-I type receptors: BMPR1A and BMPR1B<sup>203</sup>.** BMPR1A (ALK3) and BMPR1B (ALK6) receptors formed by the activin receptor domain, GS domain and the catalytic domain.

BMP Type II receptors are divided in BMPR-II, ActR-II and ActR-IIB. The first two receptors are specific for BMP ligands. ActR-IIB is a receptor not only for BMPs, but also activins and myostatin are able to interact with this receptor<sup>205</sup>.

As described before, activation of the intracellular BMP signaling is achieved through the binding of the ligand and its receptor. In addition, it has been identified the presence of co-receptors, which can enhance the BMP signaling by regulating the ligand-receptor binding process. For instance, glycosylphosphatidylinositol (GPI)-anchored proteins of the repulsive guidance molecule (RGM) family, are co-receptors described by Babitt and colleagues for BMP2 and BMP4 (in particular RGMa which can enhance the signaling)<sup>207</sup>. Also, RGMb (DRAGON) and RGMc (hemojuvelin) are co-receptors described in the literature. They interact with both type I and type II receptors and are able to bind to BMP2 / BMP4, whereas no interaction has been observed with BMP7 or TGF- $\beta$ 1<sup>208,209</sup>.

### 3.2.2 Regulation of the BMP pathway

BMP signaling is directly controlled at extracellular, membrane and intracellular levels. Extracellular soluble antagonists are able to control the bioavailability of the BMPs within the cell microenvironment. Chordin, Follistatin, FLRG, Noggin, Gremlin, Cerberus and Tolloid are antagonists that bind BMPs and prevent their

binding to the receptor, thus modifying the local concentration of the available ligand<sup>210,211,212,213,214,215</sup>.

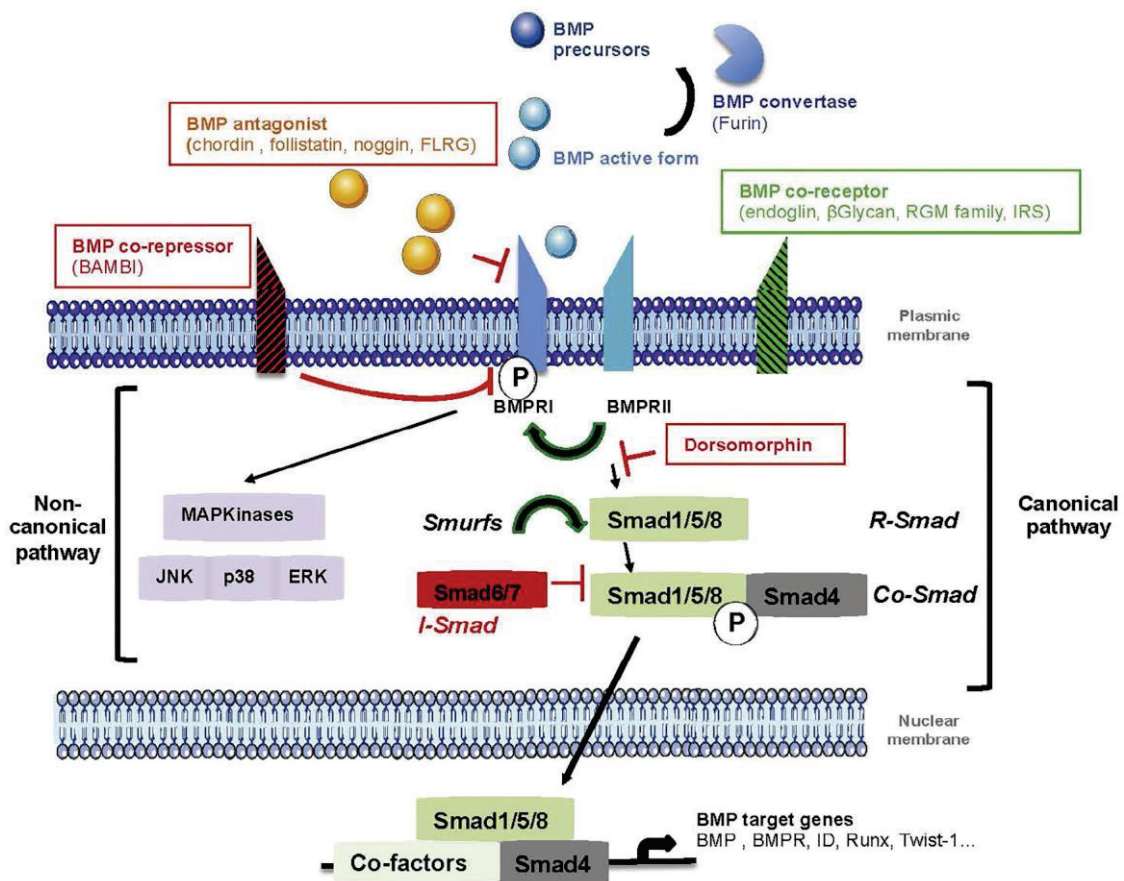
Other BMP regulators act at the membrane level to either activate (Endoglin, Endofin and RGM-Repulsive guidance family members) or inhibit (BAMBI: BMP and Activin membrane-bound inhibitor) the BMP pathway, according to the context, through direct binding with BMP (RGM) or both BMP and BMP receptor (Endoglin, BAMBI)<sup>216</sup>. BAMBI binds with type II receptors to prevent the formation of the molecular complex that transduces the signal. Finally, cytoplasmic SMAD6/7 molecules inhibit SMAD1/5/8 phosphorylation and therefore, preclude complex formation with the SMAD4 co-factor; they also recruit SMAD ubiquitin ligases (SMURF1/2) to mediate their degradation<sup>217</sup>.

### 3.2.3 BMP activation: canonical / non-canonical pathway

BMPs are able to signal through two different ways, the canonical and non-canonical pathways (**Figure 17**). In the canonical pathway, the BMP signaling cascade is triggered by the binding of the ligands to cell surface receptors, in order to form the heterotetrameric complex constituted by two dimers of type I and type II serine / threonine kinase receptors. Once the heterotetrameric complex has been formed, the type II receptor transphosphorylates the type I receptor at the GS (glycine-serine) rich domain. Activation of type I receptor drives phosphorylation of the substrate proteins recognized as receptor-regulated Smads (R-Smads), producing intracellular activation of the canonical pathway started by the SMAD1/5/8 signaling. The following step, after the Smads activation consist in the binding of the co-factor SMAD4 with the complex SMAD1/5/8. The formed complex, translocates into the nucleus of the cell and plays a crucial role as a transcription factor together with co-activators and co-repressors being capable to regulate expression of the target genes of the BMP pathway. The complex SMAD1/5/8-SMAD4 can be inhibited through inhibitory SMADS (I-SMADS); specifically, SMAD6 and SMAD7 identified as inhibitors of the signaling pathway<sup>200</sup>.

Activation of the BMP signaling can be driven also through the SMAD-independent pathways, also named non-canonical pathways (Figure 16). In this case the signaling is initiated by the activation of the BMP type I receptors which are able to start downstream pathways independent from the SMAD1/5/8. Examples of these pathways are the extracellular signal-regulated kinase (ERK), map kinase p38 (MAPK), C-jun N-terminal kinase (JNK) and nuclear factor kappa beta (NFkB). The way that the non-canonical pathways are activated has been described in several articles, that suggest interaction between the molecules BRAM1 (Bone morphogenetic protein Receptor Associated Molecule 1) or XIAP (X-linked inhibitor of apoptosis protein), and downstream molecules TAK1 (TGF- $\beta$  activated kinase I) and TAB1 (TAK1 binding protein) with the receptor type IA. For instance, JNK, p38 or NFkB pathways are activated by the downstream once TAK1 is active. Activation of TAK1 occurs through the interaction of XIAB and TAB1. Nevertheless, the mechanism of activation of other non-canonical pathways remains unclear and more studies are needed to understand their signal transduction<sup>202</sup>.





**Figure 17. Schematic representation of the BMP signaling pathway<sup>203</sup>.** BMPs are able to activate the canonical or non-canonical pathway through the binding of BMP ligand to cell surface receptors. In the canonical signaling, BMP type II receptor transphosphorylates the type I receptor, leading to phosphorylation and activation of the R-SMADS (SMAD1/5/8). SMAD4 (Co-SMAD) associates with R-SMADS and this complex translocates into the nucleus to trigger transcription of target genes of the pathway. In the non-canonical pathway, BMPs cytokines bind to type I receptors and this association starts downstream pathways independent from the SMAD1/5/8 signaling. Examples of these pathways are MAPKases JNK, p38 or ERK<sup>200</sup>.

### 3.3 Transcriptional factors and target genes of the pathway

Transcriptional regulation of certain target genes of the BMP canonical pathway occurs through the binding of SMADS with DNA sequences, but also by interaction with several DNA-binding proteins and recruiting transcriptional co-activators or co-expressors<sup>218</sup>.

For instance, RUNX1 is a member of a family of transcription factors identified to interact directly with BMP-specific R-SMADS (BR-SMADS). These factors have been described in regulation of hematopoiesis, bone formation, and homeostasis of gastric mucosa. So far RUNX1, RUNX2 and RUNX3 forms have been identified in mammals, showing interaction with R-SMADS<sup>218,219</sup>. Interestingly, RUNX1-3 are also target genes of the BMP pathway which indicates that there is a regulation loop<sup>220,219</sup>. *RUNX2* expression has been reported in different studies due to its importance in osteoblasts maturation and osteogenesis. The autosomal dominant bone disease “cleidocranial dysplasia” has been linked to the haploinsufficiency of RUNX2. Moreover, the role of RUNX3 as a main tumor suppressor in gastric cancer was described by Li and colleagues<sup>221</sup>. In addition, the importance of *RUNX1* expression is crucial in hematopoiesis and its lack of function is related to alteration of HSCs generation<sup>140</sup>. Due to the role of RUNX1 in myeloid precursors differentiation, loss-of-function mutations of this form can be found in around 30% of all human leukemias. And it is a common target for chromosome translocations in acute leukemias. These results are able to define RUNX1 as a tumor-suppressor gene<sup>219</sup>.

Another transcription factor recognized for its interaction with BR-SMADS and RUNX2 is MENIN. Generated from *MEN1* (multiple endocrine neoplasia 1) gene, MENIN seems to be necessary for mesenchymal stem cell differentiation towards osteoblasts lineage. A double role of MENIN is described, by the interaction of this factor with SMAD3, acting as a negative regulator of BR-SMAD/RUNX2 in the maturation phase of osteoblast differentiation<sup>222</sup>. Moreover, the YY1 (Ying Yang 1) transcription factor has been studied considering its importance in positive and negative gene regulation. Genes involved in TGF- $\beta$ - and BMP-early response (regulated within few hours after activation of the pathway) such as *PAI-1* and *ID1*, were observed to be repressed by YY1. Inhibition of the transcriptional activities of SMADS have been found, in which YY1 can interact with SMAD4, SMAD1 AND SMAD2/3, and restricts their binding to DNA-binding elements<sup>223</sup>.

One of the main target genes of the BMP signaling is ID (inhibitor of differentiation or inhibitor of DNA binding) protein. Four ID proteins have been identified (ID1-ID4) and hold two main capacities; one is the duty as a negative regulator in cell

differentiation and the second is their ability to act as a positive regulator in cell proliferation<sup>199,205</sup>. ID genes represent early elements regulated in the BMP signaling. An increase in the expression of ID proteins can be also stimulated by the effect of other growth factors but BMPs represent a major element in synthesis of ID1, ID2 and ID3<sup>224</sup>. Furthermore, studies focused on the analysis of the ID1 promoter confirmed that its activity in cells was higher after BMP treatment, whereas no response to TGF- $\beta$  was observed. And interestingly, the activation of the ID1 promoter was linked to the presence of SMAD1 or SMAD5 and SMAD4<sup>225,226,227</sup>. Interaction of ID proteins with bHLH (basic helix-loop-helix) transcription factors have been reported. ID proteins are not able to bind DNA. In consequence, they drive antagonism of the transcription induced by basic HLH (helix-loop-helix) transcription factors such as MyoD and myogenin in muscle and NeuroD, Mash1 and neurogenin in neurons. BMP signaling is able to control differentiation in different lineages through ID gene expression. Ying and colleagues confirmed this idea, when they identified that BMP pathway induced ID1 expression, which was able to block embryonic stem (ES) cell differentiation and promote self-renewal<sup>218,228</sup>.

New technologies as DNA microarray have allowed the identification of other BMP target genes. Using the osteoprogenitor cell line C2C12, it was possible to identify target genes of the pathway related to osteoprogenitor differentiation. Around 184 BMP-early response genes were found. The group of “immediate early genes” includes: *ID1-ID3*, *SMAD6*, *SMAD7*, *OASIS*, *Prx2*, *TIEG* and *Snai1*<sup>229</sup>.

### **3.4 Role of BMP in hematopoiesis**

BMP molecules have been described in hematopoiesis regulation, for being involved in various levels of SC differentiation<sup>88,230,231,232,233</sup>. BMPs exhibit a highly important role in the formation of hematopoietic and endothelial precursors emerging from the ventral mesoderm<sup>234</sup>. Indeed, BMP2 and BMP4, alone or in combination with Activin A, have been shown to be involved in the regulation of erythropoiesis in various models. In particular, in *Xenopus laevis* embryos,

ectopic expression of BMP4 results in the induction of several specific hematopoietic genes, such as transcription factors and the erythroid-specific globin<sup>234,235,236,237,238,239</sup>. Moreover, in human CD34<sup>+</sup> progenitor population, BMP4 exhibited the capacity to induce differentiation towards megakaryocytes through all stages<sup>240</sup>.

BMP2, BMP4, BMP7, BMPR1A, and BMPR1B knock-out mice therefore have a lethal phenotype due to a lack of formation of hematopoietic tissues or a defect in the development of mesoderm or endoderm<sup>200</sup>. In vitro, BMP4 plays an important role in the induction of hematopoietic differentiation in human and murine embryonic stem cell models<sup>235,241</sup>. In mice, BMP co-receptor expressions have been detected during hematopoietic system development in early immature cells that display hematopoietic and endothelial potential<sup>242</sup>. Indeed, the induction of endothelin-dependent hematopoiesis requires response to BMP2 and BMP4 modulation in the yolk sac. Moreover, a mouse knock-out model of SMAD5 invalidation leads to a delayed lethal phenotype during development due to a yolk sac circulatory defect associated with an inhibition of progenitor expansion<sup>243,244</sup>. Homozygous BMP4-deleted mice display a lethal phenotype characterized by reduced extraembryonic mesoderm including blood islands<sup>245</sup>. More recently, it has been demonstrated that BMP pathway activation occurs in all hematopoietic cells during the hematopoiesis-emerging phase in aorta gonad mesonephros<sup>246</sup>. Interestingly, in the following steps of the hematopoietic process, these HSCs could distinguish themselves as being BMP-positive or BMP-negative and generate a majority of cells no longer activated by this pathway. These findings suggest a role for the BMP signaling axis in the regulation of HSC heterogeneity and lineage output. The role of BMPR-1A in regulating adult HSC development has been studied *in vivo* by analyzing mutant mice with conditional inactivation. These mice have an increased number of spindle-shaped N-cadherin CD45.2 osteoblastic (SNO) cells, associated with an increased HSC number. This study showed that the BMPR-1A signaling pathway allows long-term HSC adhesion to SNO cells by the molecules N-cadherin and  $\beta$ -catenin and regulates the niche size<sup>247</sup>.

In human adult cells, phenotypic analysis revealed the expression of BMP receptors such as BMPR-1A and BMPR-1B and the expression of the effector elements of the canonical pathway, SMAD1/5/8, in CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup> cells<sup>248</sup>. Treatment of CD34<sup>+</sup>CD38<sup>-</sup> human cells with high concentrations of BMP2 and BMP7 blocks their proliferation while maintaining their immature phenotype. Conversely, treatment of the same subpopulation of cells with high concentrations of BMP4 maintains their ability to reconstitute immunodeficient mice. Conversely, low doses of BMP4 enhance cell proliferation rather than differentiation. In the same manner, CD34<sup>+</sup> cells are expanded when treated with low doses of BMP7 without affecting their engraftment capacity in immunodeficient mice<sup>249</sup>. In addition, BMP4 regulates homing of murine and human hematopoietic stem/progenitor cells through the direct regulation of Integrin- $\alpha$ 4 expression in SMAD-independent p38 MAPK-mediated signaling<sup>250</sup>. BMP2 or Activin A treatment alone decreases the expression of GATA-2 and increases the expression of EPO-R on CD34<sup>+</sup> cells. SMAD6 overexpression blocks erythropoiesis in vitro by transcriptional regulation of *KLF1* and *GATA-2* and indirectly participates in HSC maintenance by blocking the differentiation process<sup>251</sup>. Conversely, only simultaneous treatment of BMP4 with Activin A is able to modulate erythropoiesis in a Follistatin- and FLRG-mediated way<sup>212</sup>. In addition, both Follistatin family members were described in human hematopoiesis regulation. Adhesiveness of human hematopoietic cells to fibronectin was controlled by Follistatin and FLRG<sup>252</sup>. Fibronectin plays an essential role in adhesion of human hematopoietic progenitors to the bone marrow. In consequence, this adhesion represents a crucial element mediating migration, retention, self-renewal, proliferation and differentiation of this population.

In the murine system, BMP4 is able to induce erythrocyte differentiation of CD34<sup>+</sup> cells in the presence of stem cell factor (SCF) and erythropoietin (EPO)<sup>253</sup>. Comparatively, in human cells, BMP4 in combination with various hematopoietic cytokines, including EPO, is able to induce several blood lineages from other human tissues. This unexpected cell fate has been demonstrated using human muscle and neural gestational tissue suspension cultured in a serum-free

medium with interleukin (IL) 3, IL6, SCF, and FLT-3 ligand with addition of EPO and BMP4<sup>231</sup>. Conversely, BMP4 alone is able to induce human megakaryopoiesis from CD34+ cells through the JAK/ STAT and mammalian target of rapamycin pathways. These data clearly showed that, unlike the murine system and despite their very high protein sequence homology and similar use of BMPR signaling, BMP2- and BMP4-soluble molecules exhibit very distinct effects on human HSCs. Interestingly similar observations were made for BMP2 and BMP4 on the control of other human adult tissue stem cells such as mammary epithelial SCs<sup>254</sup>.

### **3.5 Cancer stem cells and the BMP pathway**

BMPs represent an important element in stem cell (SC) regulation, holding a wide range of essential tasks in embryonic development and tissue regeneration<sup>255</sup>. In hematopoiesis, BMP signaling maintains regulation of the HSCs controlling their maintenance and differentiation at distinct levels. In contrast, the pathway has been reported to be essential not only in normal SC but also in maintenance and differentiation of cancer stem cells (CSCs). CSCs are a malignant population holding the hallmarks of SCs, able to trigger cancer initiation and growth, and relapse according to several studies<sup>256</sup>.

Signaling involved in stem cell regulation is a complex network of pathways with cross-talk signals. Although the precise role played by the BMPs in CSC regulation is still not completely understood, several studies have reported the involvement of elements of the pathway in this context. A study demonstrated the effect of BMP4 in epithelial to mesenchymal transition (EMT) but more importantly in the induction of a stem-like phenotype in oral squamous cell carcinoma<sup>257</sup>. Also, BMP2 cytokine was reported to increase motility and invasiveness capacity of colon cancer cells<sup>258</sup>.

Crosstalk of the ligand BMP4 and HH signaling was reported in ovarian cancer. Where a population of human carcinoma-associated mesenchymal stem cells had the capacity to increase the number of CSCs and enhance resistance to

chemotherapy by activation of the crosstalk<sup>259</sup>. Another crosstalk interaction identified was described in an aggressive phenotype of colorectal cancer. *Notch-1* expression was observed together with other elements such as EMT/stemness-associated molecules CD44, Slug and SMAD3<sup>260</sup>.

Involvement of the BMP signaling in a specific CSC population, identified in hematological malignancies has been studied. The leukemic stem cells (LSCs) described for the first time in AML, present a narrow relationship with the BMPs. In CML, BMP alterations were reported in TKI-resistant patients. High expression of the receptor BMPR-1B and BMP4 were identified in LSCs from resistant patients<sup>261</sup>.

Conversely, the BMP pathway has been already described for its role reducing the number of CSCs in certain cancers. For instance, BMP4 enhances differentiation, apoptosis and self-renewal capacity of CSCs, but also the cytokine renders CSCs more sensitive to chemotherapy<sup>262</sup>. BMPR-1B was described in glioblastoma, where its forced expression in CSCs enhances the differentiation of this population and loss of tumorigenicity<sup>263</sup>. In addition, Piccirillo and colleagues reported that BMP4 treatment of neural CSCs reduced their proliferation capacity and cells were pushed towards differentiation into neural precursors and their capacity for tumor formation was also diminished<sup>264</sup>. BMP2 cytokine was able to repress the expansion and migration of renal cancer cells presenting ALDH<sup>+</sup> activity. And also, the cytokine had an impact in downregulation of embryonic stem cell markers in these ALDH<sup>+</sup> CSCs<sup>265</sup>. Moreover, in breast cancer, the heterodimer BMP2 / BMP7 presented a reducing effect of the number of CSCs exhibiting the phenotype ALDH<sup>hi</sup>/CD44<sup>hi</sup>/CD24<sup>low</sup>. Furthermore, this heterodimer diminished tumor invasion by inhibiting activation of TGF- $\beta$  driven SMAD signaling<sup>266</sup>.

### **3.6 BMP signaling in solid cancer**

As mentioned during this chapter, the BMP signaling plays a major role from embryogenesis to adult tissue homeostasis in an enormous number of tissues all

over the body. Nevertheless, the BMP pathway also has been studied due to its importance in carcinogenesis, where it is able to control regulation of cancer cells and the tumor microenvironment<sup>267</sup>. Molecules from the TGF- $\beta$  pathway are well-known by their double function in cancer, at early stages acting as a tumor suppressor and as a tumor promoter during late stages of the disease<sup>268</sup>. Several research studies describe the BMP signaling as a contradictory pathway, which can play a double role either as a suppressor or as a promoter in cancer. And this double function of the BMPs seems to be linked to the type of cancer<sup>199</sup>.

Genetic mutations of the different BMP pathway elements have been described in several studies. For instance, mutations of the genes encoding the receptor BMPR-1A and co-factor SMAD4, were identified in polyposis syndrome which presents a high risk factor in cancer development<sup>269,270</sup>. Dysregulation of the BMP signaling is also present in colorectal cancer (CRC), where mutations of BMPR-1A and SMAD4, combined to lack of phosphorylated SMAD1/5/8 have been identified<sup>271,272</sup>. In the context of CRC, the BMP pathway is also able to regulate the initiation and evolution of this cancer depending of the SMAD4 and p53 status, by regulating the Wnt pathway. Initiation, metastasis and chemosensitivity in CRC are linked to the activation of the Wnt signaling. Voorneveld and colleagues described inhibition of the Wnt pathway through activation of the BMP signaling. This was only achieved when SMAD4 was present and p53 exhibited no mutations<sup>273</sup>.

Prostate cancer (PC) progression has been reported to be related to an increased BMP expression. This type of cancer is commonly characterized by osteosclerotic bone metastasis. Significant preclinical data correlates the osteoblastic promotion in prostate cancer to be driven by BMP6<sup>256</sup>. In addition, invasion of the bone microenvironment by the prostate cancer cells is enhanced thanks to the BMPs. Overexpression of BMP7 has been described in PC as a marker for clinical progression but also in vivo and in vitro studies linked its upregulation with hepatocyte growth factor (HGF) and scatter factor (SF)<sup>274</sup>. On the other hand, BMP10 presented an effect of suppression in growth and aggressiveness of PC cells inducing their apoptosis through activation of the SMAD-independent pathway<sup>274</sup>.



Moreover, breast cancer is another malignancy in which the BMP signaling has been largely described. BMP2 and BMP4 have been defined as proteins involved in regulation of fate and differentiation of human mammary epithelial stem cells. BMP2 promotes luminal progenitor commitment and expansion while BMP4 avoid lineage differentiation. Interestingly, external factors such as pollutants can impact the BMP signaling. For example, bisphenol A (BPA) modified localization of type 1 receptors and pre-activated the BMP signaling in a mammary cell line (MCF10A). In this study, BMP4 drove cell differentiation to a myoepithelial phenotype due to the effect of BPA. Also, BPA avoided the BMP2 effect in the luminal commitment and expansion, which produces the maintenance of stem-like properties<sup>275</sup>. In addition, interaction of BMP2 ligand and BMPR-1B has been reported as an initiator of epithelial stem cell transformation into a luminal tumor-like phenotype<sup>276</sup>.

Studies based on pancreatic cancer demonstrated that BMP2 highly contributes to tumor growth, confirmed in both a tumor cell line and a xenograft mice model. In this case, activation of the SMAD-signaling was observed through immunohistochemistry, where phosphorylated SMAD1/5/8 was found. The use of the molecule Spp24 (secreted phosphoprotein 24 kD) produced blocking of BMP2 and tumor reduction<sup>277</sup>. Also, BMP2 and BMP4 are greatly cited due to their role in lung cancer. BMP2 overexpression has been found in non-small cell lung carcinoma (NSCLC) and also enhanced tumor growth in mice after injection of the lung cancer cell line A549<sup>278</sup>.

Cancer progression is characterized by modification of the cancer cells in different aspects such as morphology, adhesive capacity to other cells or to the extracellular matrix (ECM). Invasion and distant metastasis are a result of those changes in cancer cells; these cells employ normal mechanisms as wound healing, epithelial to mesenchymal transition (EMT), tissue specific morphogenesis and cellular motility to achieve cancer sustainment and metastasis. The BMP pathway seems to be one of the elements that grants migratory and invasive properties to cancer cells<sup>268</sup>. Activation of the pathway has been identified as an element inducing EMT<sup>279</sup>. For instance, in prostate cancer cell lines, BMP2 and BMP4 increased their motility and invasiveness capacity<sup>280</sup>.

BMP2 was also reported to promote invasion and tumor growth in breast cancer<sup>281</sup>. Increased adhesion, motility and invasion of cancer cells were also reported in colon cancer and gastric cancer. In colon cancer, the BMP4 overexpressing cells displayed these features, while gastric cancer cells exhibited these characteristics due to activation of the PI3K pathway stimulated by BMP2<sup>282, 283</sup>.

### **3.7 BMP signaling in leukemias**

BMP pathway is a major actor in hematopoiesis as described previously during this chapter. In contrast, BMPs have been identified for playing an important role not only in solid cancers as presented during this chapter, but also diverse studies have shown dysregulation of elements of the pathway in hematological malignancies. BMP signaling presents the double role showed in solid cancers, acting either as a tumor suppressor or as tumor promoter<sup>199</sup>.

#### **3.7.1 Lymphoid leukemias**

Various research groups have started to investigate the role of the BMP pathway in lymphoid leukemias. Using different patient cohorts, BMP2 overexpression has been identified in pre-B acute lymphoblastic leukemia (ALL) compared with CD34+ healthy cells, but not in T-cell ALL. This BMP2 overexpression was associated with alteration of other genes involved in the dialogue of immature leukemic cells with their bone marrow microenvironment<sup>284</sup>. In addition, overexpression of BMPR1A/BMPR1B and BMPR2 receptors was detected at the cell membrane of B-cell chronic lymphoid leukemia (CLL) leukemic cells. Moreover, disease progression seemed to be accompanied by a more pronounced overexpression of BMPR1A and BMPR1B, as measured in samples from patients in more advanced stages<sup>285</sup>. These findings suggest that overexpression of the receptor could favor the proliferative process. Finally, analysis of gene expression of the BMP signaling pathway in a cohort of 160 CLL patient samples showed alterations of *SMAD1/8* that appeared overexpressed and correlated with a decrease in *SMAD4* expression compared with control samples. High levels of *SMAD1/8* transcripts expression were associated with

poor prognosis<sup>286</sup>. Interestingly, gene expression analysis of two independent datasets showed that the levels of inhibitory Smads varied across different B-cell lymphomas<sup>287</sup>. In this context, the BMP pathway acts as a negative factor with exogenous BMPs, especially BMP7, that inhibit DNA synthesis of lymphoma cells. SMAD7 overexpression in cancer cells is sufficient to escape the negative effects of BMPs by inhibiting SMAD 1/5/8 signaling<sup>203</sup>.

### **3.7.2 Myeloid leukemias**

#### **3.7.2.1 Acute myeloid leukemia**

BMP pathway alterations have started to be studied in AML. Nevertheless, few evidences have been found and described regarding the role and mechanism of dysregulation of the pathway in these heterogeneous hematological malignancies. In this sense, BMP4 has been associated with AML in adults by the direct induction of *MIXL1* (Mesoderm Inducer in Xenopus Like 1) gene through the binding of the cytokine and the BMP type I receptor, involving the activation of Smad signaling (SMAD5). *MIXL1* factor grants the AML cells an anti-apoptotic function which can explain the drug resistance of AML cells<sup>288</sup>. In addition, activation of the BMP signaling has been demonstrated in pediatric acute megakaryoblastic leukemia (AMKL), where the fusion protein CBFA2T3-GLIS2 is responsible of *BMP2 / BMP4* and *ID1* overexpression linked to an increased self-renewal capacity in hematopoietic progenitors<sup>289</sup>.

Acute promyelocytic leukemia (APL) has also been part of several studies. Though APL represents a type of AML with a high rate of complete remission in patients treated by all-trans retinoic acid (ATRA), resistance to treatment can be seen in some patients. The mechanism of resistance seems to involve *BMP4 / BMP6* gene expression driving in consequence the expression of genes involved in blocking of differentiation as *ID1* and *ID2*. This has been correlated to PML/RAR $\alpha$  fusion oncogene which is able to block differentiation of abnormal promyelocytes and induce resistance<sup>290</sup>.

### 3.7.2.2 Chronic myeloid leukemia

Studies performed by our team revealed both intrinsic and extrinsic deregulation of the BMP pathway in CML as early as at the time of diagnosis in chronic phase<sup>291</sup>. We demonstrated higher expression levels of the BMPR-IB receptor ALK6 in CD34+ cells, together with the existence of several molecular and functional alterations of the BMP pathway in chronic-phase CML. We also detected high concentrations of the soluble cytokines BMP2 and BMP4 produced exclusively by the tumor microenvironment. These alterations participate in the leukemic phenotype through their involvement in the survival of LSCs and in the expansion of leukemic myeloid progenitors. These data were further confirmed by other groups showing that BMP2/BMP4 and BMP7, together with their receptors and SMAD proteins, contribute to myeloid cell alteration, giving rise to CML LSCs<sup>292,293</sup>. Our study demonstrated for the first time that very early transforming events initiate intrinsic deregulation of the BMP signaling pathway in stem cells. Indeed, introduction of the BCR-ABL oncogene results in an increase in BMPR1b cell membrane expression<sup>291</sup>. This deregulation is then specifically amplified in transformed cells by exposure to exogenous ligands such as BMP2/BMP4 provided by the SC microenvironment<sup>254</sup>.

Later during disease progression, the CML leukemic bone marrow niche that produces high levels of BMPs can be suspected to be at the origin of secondary myelofibrosis. Indeed, this alteration, often reported upon CML evolution, represents an overproduction of extracellular matrix that has been previously attributed to excessive BMP cytokines within the BM niche<sup>294</sup>.

## **II. AIM OF THE STUDY**

## 1 Research problem

Acute myeloid leukemias are heterogeneous malignancies, defined by abnormal differentiation and clonal proliferation of the myeloid progenitor population (blasts) in bone marrow and peripheral blood<sup>105</sup>. This hematological disorder represents 80% of acute leukemias in adults. A high rate of relapse combined to a poor prognosis specially for patients above 65 years old is a common outlook, where 70% of these patients face mortality within one year subsequent to diagnosis<sup>104</sup>. The presence of leukemic stem cells (LSCs), described for the first time in this type of leukemia<sup>167</sup>, has been recognized to be at least partially responsible for relapse, resistance and poor prognosis in AML patients. Standard chemotherapy, allogenic and autologous transplants have failed to eradicate LSCs. Development of more specific and effective therapies targeting the LSCs represents a promising approach in AML treatment.

In order to generate new therapies, it is imperative to understand the mechanisms that govern the regulation of LSCs. Several research studies have shown the importance of signaling pathways in AML development and LSC maintenance. External signals within the tumor microenvironment are transduced into the leukemic cells which activate the intracellular signaling<sup>295,296</sup>. The bone morphogenetic proteins (BMP) pathway represents a key player in hematopoiesis and HSC regulation. Conversely, in a malignant context, BMPs have been studied for their role in LSC regulation<sup>203</sup>.

In chronic myeloid leukemia (CML), our research team has identified alterations of the pathway in the tumor niche. High concentrations of BMP2 and BMP4 within the tumor microenvironment were observed<sup>291</sup>. Moreover, LSCs from TKI resistant patients displayed high expressions of BMP4 and BMPR1B receptor<sup>261</sup>.

The role of the BMP pathway has not been widely studied in AML and few evidences have been reported. Nevertheless, BMP4 ligand and BMP type I receptor have been described as inducers of MIXL1 gene, which grants anti-apoptotic functions to leukemic cells in AML. In addition, activation of the SMAD signaling (SMAD5) was also identified<sup>288</sup>. Furthermore, research work conducted

in acute megakaryoblastic leukemia (AMKL) reported overexpression of BMP2/BMP4 and of the target gene ID1 in hematopoietic progenitors related to an increased capacity of self-renewal<sup>289</sup>.

Based on the context described above, for the present thesis research project, identification of alterations of the extrinsic and intrinsic elements of the BMP pathway in acute myeloid leukemia became an essential goal. Understanding the BMP signaling dysregulation represents the path to decipher the key role played by the pathway in LSC regulation and resistance in acute myeloid leukemias.

## **2 Aim of the project**

The aim of the present thesis research project is to decipher the role played by the BMP signaling pathway in leukemic stem cell regulation and resistance in acute myeloid leukemia.

### **2.1 Objectives**

In order to achieve the aim of the present research project, the following objectives have been established:

- I. Obtain a large cohort of bone marrow (BM) and peripheral blood (PB) AML patient samples at diagnosis and healthy samples used as control.
- II. Analyze the tumor and healthy microenvironments, by measuring the concentration of soluble cytokines BMP2 and BMP4 in the plasma of AML and healthy samples.
- III. Isolate the mononuclear cells (MNCs) from AML and healthy samples, in order to analyze at the transcriptional and protein levels the expression of intrinsic and extrinsic elements of the BMP pathway (ligands-receptors-target genes of the pathway).
- IV. Analyze membrane expression of BMP type I receptors, using MNCs obtained from BM and PB AML and healthy individuals.

- V. Evaluate the impact of soluble BMP cytokine in the immaturity phenotype of MNCs from AML and healthy samples.
- VI. Perform functional (CFC/LTC-IC) assays and ALDH activity to identify modifications in the immature phenotype of the leukemic cells.
- VII. Verify and compare the results obtained in AML and healthy individual specimens, using in parallel an AML cell line model (KG1A).
- VIII. Analyze the correlation between BMP pathway elements and AML patient outcome.
- IX. Obtain AML samples from relapse patients to evaluate the transcript and protein levels of BMP signaling extrinsic and intrinsic elements.
- X. Employ AML resistant cell lines as models that reproduce the resistance mechanisms of AML primary cells, to evaluate the status of intrinsic and extrinsic elements of the BMP pathway.



# **III. RESULTS**

# 1 Article 1: A new signaling cascade linking BMP4, BMPR1A, ΔNp73 and NANOG impacts on stem-like human cell properties and patient outcome

## Introduction

Bone marrow (BM) microenvironment represents the niche where functional activities of hematopoietic stem cells (HSCs) are regulated<sup>297</sup>. In the context of cancer, studies have revealed the importance of the tumor niche in cancer stem cell (CSC) maintenance and protection<sup>298</sup>. Leukemic stem cells (LSCs), described for the first time in acute myeloid leukemia (AML)<sup>167</sup>, have been proposed as main actors in relapse, resistance and poor prognosis<sup>299</sup>. LSC regulation and protection seems to be granted by the tumor niche and crosstalk between signaling pathways and LSCs<sup>300</sup>.

The BMP pathway is well-known for its key role in stem cell (SC) regulation. Several studies have highlighted the involvement of the pathway in solid cancer and leukemias. For instance, BMP2 and BMP4 have been identified as crucial elements in regulation of normal and cancer SC<sup>248</sup>. Our research team has described in chronic myeloid leukemia (CML), alterations of the pathway involving BMP2/BMP4 and the receptor BMPR1B. This dysregulation holds an impact in LSC resistance and poor prognosis<sup>291,261</sup>. Furthermore, some studies have started to elucidate the role of the pathway in AML. BMP4 and BMP type I receptors have been identified as inducers of *MIXL1* expression, which is linked to AML initiation<sup>288</sup>. In addition, in acute megakaryoblastic leukemia (AMKL) the fusion protein CBFA2T3-GLIS2 was reported to be a factor leading to *BMP2/BMP4* and *ID1* overexpression in leukemic cells, which is linked to acquisition of immature properties<sup>289</sup>.

The aim of this research work was to elucidate the extrinsic and intrinsic alterations of the BMP pathway in the tumor niche and the leukemic cells in AML, by identifying the crosstalk of this signaling with other related genes involved in promotion of a more immature phenotype in the leukemic cells.

ARTICLE

Open Access

# A new signaling cascade linking BMP4, BMPR1A, $\Delta$ Np73 and NANOG impacts on stem-like human cell properties and patient outcome

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## Abstract

In a significant number of cases cancer therapy is followed by a resurgence of more aggressive tumors derived from immature cells. One example is acute myeloid leukemia (AML), where an accumulation of immature cells is responsible for relapse following treatment. We previously demonstrated in chronic myeloid leukemia that the bone morphogenetic proteins (BMP) pathway is involved in stem cell fate and contributes to transformation, expansion, and persistence of leukemic stem cells. Here, we have identified intrinsic and extrinsic dysregulations of the BMP pathway in AML patients at diagnosis. BMP2 and BMP4 protein concentrations are elevated within patients' bone marrow with a BMP4-dominant availability. This overproduction likely depends on the bone marrow microenvironment, since MNCs do not overexpress BMP4 transcripts. Intrinsically, the receptor BMPR1A transcript is increased in leukemic samples with more cells presenting this receptor at the membrane. This high expression of *BMPR1A* is further increased upon BMP4 exposure, specifically in AML cells. Downstream analysis demonstrated that BMP4 controls the expression of the survival factor  $\Delta$ Np73 through its binding to BMPR1A. At the functional level, this results in the direct induction of NANOG expression and an increase of stem-like features in leukemic cells, as shown by ALDH and functional assays. In addition, we identified for the first time a strong correlation between  $\Delta$ Np73, BMPR1A and NANOG expression with patient outcome. These results highlight a new signaling cascade initiated by tumor environment alterations leading to stem-cell features and poor patients' outcome.

## Introduction

The current paradigm on the initiation of leukemogenesis implies a multistep process involving different types of genetic alterations, with no obvious hierarchy and understanding of the sequential clonal selection<sup>1</sup>. Nevertheless, crosstalk between leukemic stem cells and the associated bone marrow (BM) stroma appears to be essential for leukemic progression and response to

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therapy<sup>2,3</sup>. More globally, understanding interactions between tumor stem cells (SCs) and their microenvironment is a challenge to develop strategies to avoid relapses after therapy. Among the main elements implicated in the crosstalk between the microenvironment and both normal and tumor SCs, we have investigated the role of bone morphogenetic proteins (BMPs), because they govern SC regulation including hematopoietic<sup>4,5</sup>, neural and epithelial systems<sup>6</sup> by directly and indirectly affecting their niche<sup>7-9</sup>. Alterations of the BMP signaling pathway have been observed in numerous cancers, in some cases closely associated with cancer stem cells (CSC) properties<sup>10</sup>. According to the context, BMPs could participate in initial tumor suppression or favor CSC maintenance and metastasis<sup>8</sup>. Within the BMP family, BMP2 and BMP4 have emerged as key regulators of normal and cancer SCs<sup>11-13</sup>. We have previously demonstrated that alterations in the BMP pathway at intrinsic (BMP receptors and downstream partners) and extrinsic (BMP extracellular ligands) levels constitute major events in transformation, expansion and persistence of immature cells in chronic phase chronic myeloid leukemia (CML) and breast cancer, by diverting their normal functions<sup>11,12,14,15</sup>.

Acute myeloid leukemia (AML), the first tumor where CSCs were described<sup>16</sup>, is a heterogeneous disease, in which the accumulation of genetic aberrations results in the uncontrolled growth of malignant undifferentiated cells. Relapse in the first years following complete remission is prevalent and may reflect the survival of resistant immature-like tumor cells able to regenerate the entire tumor<sup>17</sup>. The BMP pathway has been implicated in adult AML. For example, the overexpression of the transcription factor *MIXL1*, which is sufficient to initiate AML, can be induced by BMP4. Consistent with this induction, AML cells that express *MIXL1* are sensitive to type BMP type 1 receptors (BMPRI) inhibitors<sup>18</sup>. In addition, in acute megakaryoblastic leukemia, the appearance of a specific fusion protein CBFA2T3-GLIS2 leads to the overexpression of BMP2 and BMP4 by leukemic cells and is associated with colony-forming capacities, a property ascribed to immature cells<sup>19</sup>.

Here we have identified alterations of the BMP pathway and revealed their importance in immature properties exhibited by AML cells. Initially focusing on the analysis of AML patient samples collected at diagnosis and subsequently experimentally deregulating the BMP pathway, we have identified alterations in BMP ligands, receptors and target genes. Our data highlight a new signaling cascade likely involved in the cell survival and features of immature AML cells in their microenvironment.

## Materials and methods

### Protein quantification

Bone marrow plasma obtained from allogeneic BM healthy donors and AML patients was harvested and

cleared. BMPs concentration was determined using the human BMP2-ELISA or BMP4-ELISA kits (RayBiotech) following the manufacturer's instructions.

### Primary cells, cell lines culture conditions, and treatments

Patient samples were obtained after informed consent in accordance with the Declaration of Helsinki in the hematology departments involved in this study. Mononuclear cells (MNCs) from 54 blood and BM samples were obtained from AML, excluding acute promyelocytic leukemia, patients at diagnosis. AML characteristics are presented in Table S1. Normal samples correspond to steady-state peripheral blood and BM samples from healthy donors for allogeneic BM transplantation, collected after informed consent. When necessary, primary cells were maintained in IMDM culture medium containing 10% fetal calf serum (FCS). KG1A myeloid leukemia cells were cultured in RPMI-1640 medium containing 10% FCS. BMP4 and LDN-193189 (20 nM) (Sigma-Aldrich) were added in serum-free medium as indicated<sup>18,20</sup>. Normal goat IgG control (AB-108-C) and anti-hBMPRI1A (AF346) (R&D Systems) were used at 4 µg/mL.

### Functional assays

Colony-forming cell (CFC) and long-term culture-initiating cell (LTC-IC) assays were performed as described<sup>20</sup>. LTC-IC number was expressed as W5-CFC/10,000 initial cells.

### Expression vectors, transfections and luciferase assay

KG1A cells were transfected with pBabe-ΔNp73<sup>21</sup>, pMXS-NANOG or empty vector (control) (Addgene) using a Neon (ThermoFisher Scientific) electroporation device according to the manufacturer's instructions. For luciferase assays, the pNANOG-Luc reporter vector was cotransfected with empty pCDNA3 or pCDNA3-ΔNp73 in KG1A cells. Luciferase assays were performed using Dual-Glo® Luciferase Assay System (Promega) following the manufacturer's instructions. Relative activation as compared with cells transfected with the empty vector<sup>22</sup>.

### RNA isolation and analysis

Quantitative RT-PCR was performed using standard protocols<sup>20</sup>. MNCs were isolated by a Ficoll gradient and total RNA was purified using TRI REAGENT™ (Sigma) and the mini-RNA extraction kit (Qiagen, Valencia, CA). For RT-qPCR, cDNA was produced using Superscript II (Invitrogen) and amplified using Sybr-green (Quantifast, Qiagen) and the Real-Time PCR system (Roche). TBP (TATA-binding protein) and HPRT (hypoxanthine-guanine phosphoribosyl transferase) genes were used for normalization. Arbitrary unit (AU) corresponds to the ratio of expression between samples and a single normal

sample used as a reference in each PCR. Primer sequences are presented in the Table S2.

#### Flow cytometry analysis

Cells were incubated with antibodies specifically recognizing CD34, CD38 (Becton Dickinson) and/or BMPR1A (R&D System), or an irrelevant isotype-matched control antibody. MNCs separated by Ficoll gradient were subjected to sorting using BMPR1A antibody and an FACSARIA III cell sorter (Becton Dickinson). ALDH (aldehyde dehydrogenase) activity was determined using an Aldefluor kit according to the manufacturer's recommendations (StemCell Technologies).

#### Western blot analysis

MNCs from AML samples were treated with BMP4 (20 ng/ml) during 24 h, then proteins were extracted. Per lane, 40 µg of proteins were loaded on SDS-PAGE and transferred onto polyvinylidene difluoride membrane (Bio-Rad). Membranes were incubated with monoclonal antibodies anti-ΔNp73 (Abcam, ab13649) or anti-βactin (Abcam, ab8226) from mouse, or anti-NANOG (Abcam, ab109250) from rabbit. Then membranes were incubated with their relevant HRP-coupled secondary antibodies (Jackson ImmunoResearch).

#### Datasets

Normalized Reads Per Kilobase Million (RPKM) Illumina GA-IIX RNA-seq profiles were downloaded from the TCGA AML data portal. One hundred and sixty-one patients in the TCGA AML cohort had both RNA-seq and clinical data. RPKM values were log-transformed to the base two after adding a value of 1. Expression levels were extracted from the log-transformed data. A representative probe set with the highest average intensity was selected for each gene in each dataset. The median of expression of each gene was used for patients' stratification (high vs. low score).

#### Statistical analysis

Unless otherwise specified, statistical analysis was performed using the Mann–Whitney *U* test. Except for overall survival, the statistical analyses and graphs were performed with Graphpad prism (version 6). Significant *P* values are given in the text or symbolized by asterisks (\**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001; \*\*\*\**P* < 0.0001). Overall survival (OS) curves from the date of sampling onwards are illustrated using the Kaplan–Meier method. Log-rank tests were conducted to evaluate the effect of markers on survival. Best cut-off values for expression of each gene of interest were determined with the Youden index computed on Cumulative/Dynamic time-dependent ROC curve using Inverse Probability of Censoring Weighting (IPCW) estimation between 12 and 36 months of

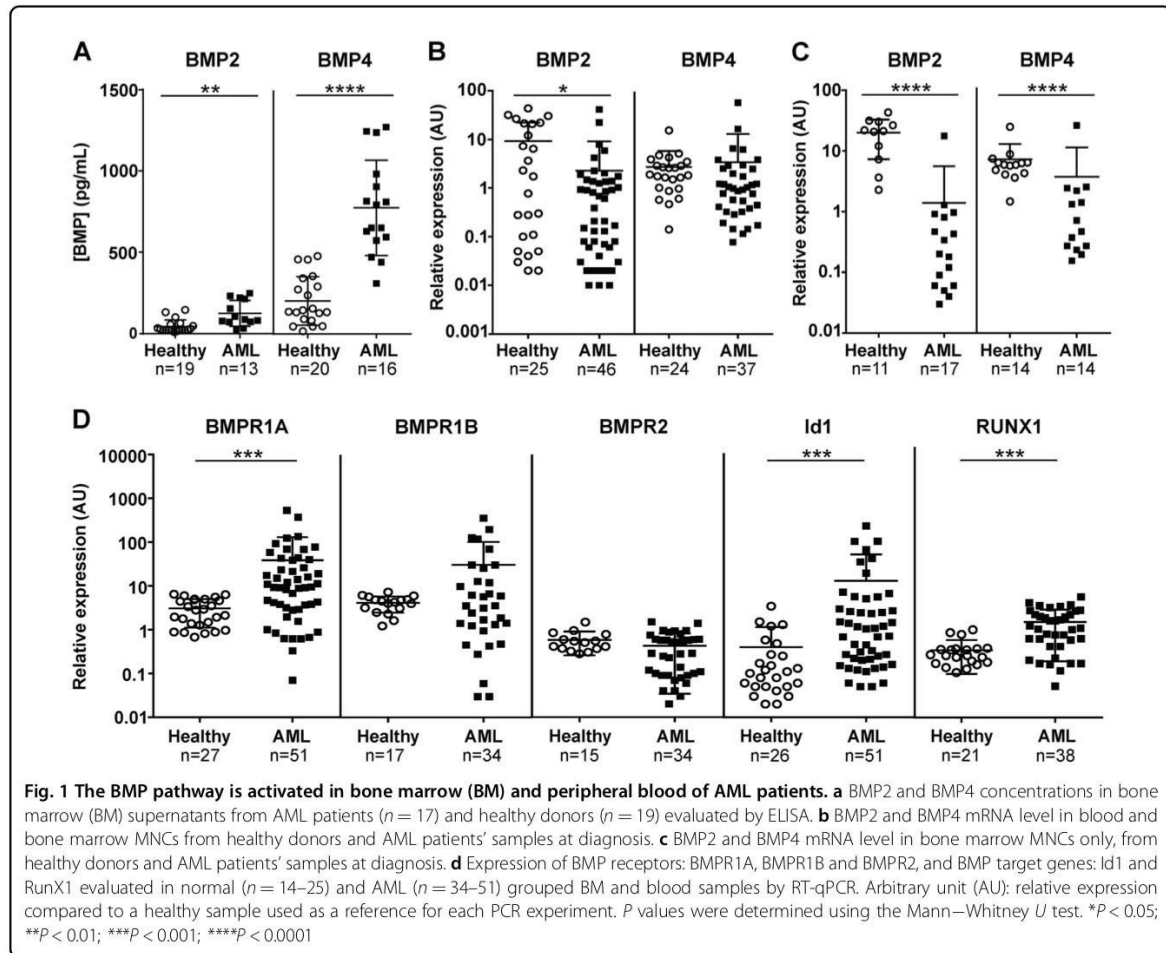
follow-up. The best cut-off value for NANOG expression that discriminates the most death events during time was 13 at 24 months of follow-up (AUC = 0.77, sensitivity = 0.61/specificity = 0.85). The level of significance was set at 5%. OS analysis and graph were performed with the R program (version 3.2.3) using the “survival” and “ggplot2” packages.

## Results

### Identification of intrinsic and extrinsic alterations in the BMP pathway in AML

Elements of the BMP pathway were analyzed in AML samples (characteristics in Table S1) in both leukemic cells and their BM environment (Fig. S1a). We first measured the levels of soluble BMP2 and BMP4 in BM plasma from healthy donors and AML patients at diagnosis (Fig. S1b for experimental design). The levels of soluble BMP2 and BMP4 were significantly increased in AML patients compared to healthy donors by 2.9-fold (*P* = 0.0100) and 3.6-fold (*P* < 0.0001), respectively (Fig. 1a). Thus, in the leukemic microenvironment, cells are exposed to elevated concentrations of BMP2 and BMP4, the latter being almost sixfold more abundant than BMP2. In order to determine whether BM leukemic cells themselves produced these high BMP levels, the expression of the BMP2 and BMP4 genes was monitored in MNCs from both peripheral blood and BM of AML or healthy donors (Fig. 1b). BMP2 expression appeared reduced in leukemic cells compared to normal samples (*P* = 0.0421), while BMP4 expression was the same. Interestingly, when we analyzed BMPs transcripts in BM samples, both BMP2 and BMP4 mRNA levels were reduced in AML samples (Fig. 1c), while no significant changes were observed in circulating peripheral blood cells (Fig. S2a). These data suggest that the higher concentration of BMP2 and BMP4 in BM is not related to its autologous production by leukemic cells themselves, but is rather provided by other cells in the microenvironment.

We next analyzed gene expression profiles of intrinsic BMP pathway components in AML MNCs, with a focus on BMP membrane receptors and target genes. We observed significantly (*P* = 0.0005) higher levels of *BMPR1A* transcripts, but not of *BMPR1B* and *BMPR2*, as compared to MNCs from healthy donors (Fig. 1d). With respect to the BMP-target genes, *Id1* and *RUNX1* expression was significantly upregulated compared to the levels found in samples from healthy donors (*P* < 0.0001 and *P* = 0.0001; respectively) in leukemic cells (Fig. 1d), whereas *Id3* and *RUNX2* expression remained unchanged (Fig. S2b). The observed increases in expression were detected both in BM and peripheral blood MNCs from AML patients (Fig. S2c). These results identify intrinsic dysregulations of the BMP pathway at different levels of



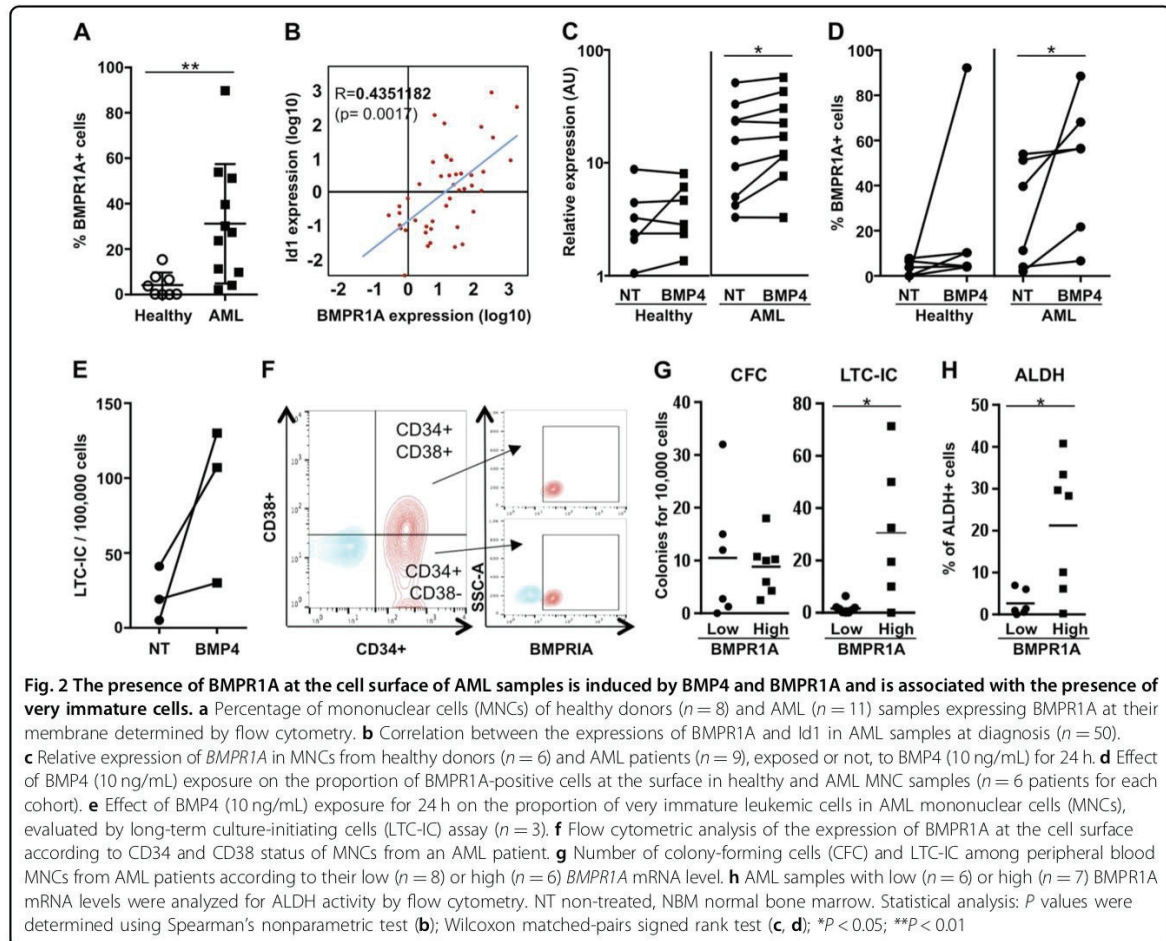
the signaling cascade in addition to extrinsic alterations in human primary adult AML samples at diagnosis.

#### BMP4 and BMPR1A increases are associated with stemness features

In order to identify BMP-responsive cells in BM, we assessed whether a correlation existed between deregulated BMP elements and the presence of BMPR1A at the cell surface. By flow cytometry analysis, we found a significantly higher number of primary MNCs over-expressing membrane-BMPR1A (BMPR1A<sup>+</sup>;  $P = 0.0024$ ) in AML than in normal samples (Fig. 2a). We also observed that the expression of BMPR1A was significantly correlated with that of *Id1* in AML samples ( $P = 0.0017$ ) (Fig. 2b) and that after sorting MNCs according to their membrane-BMPR1A content (Fig. S3a), we observed a higher *Id1* expression in BMPR1A<sup>+</sup> cells (Fig. S3b). Furthermore, when we exposed healthy and AML MNCs to BMP4 for 24 h, we could observe an increase in

BMPR1A expression at the transcript level in only AML MNCs ( $P = 0.0195$ ; Fig. 2c). This was confirmed at the protein level by the increase of BMPR1A-expressing cell numbers in AML samples following BMP4 treatment ( $P = 0.0313$ ; Fig. 2d and illustrated in Fig. S3c).

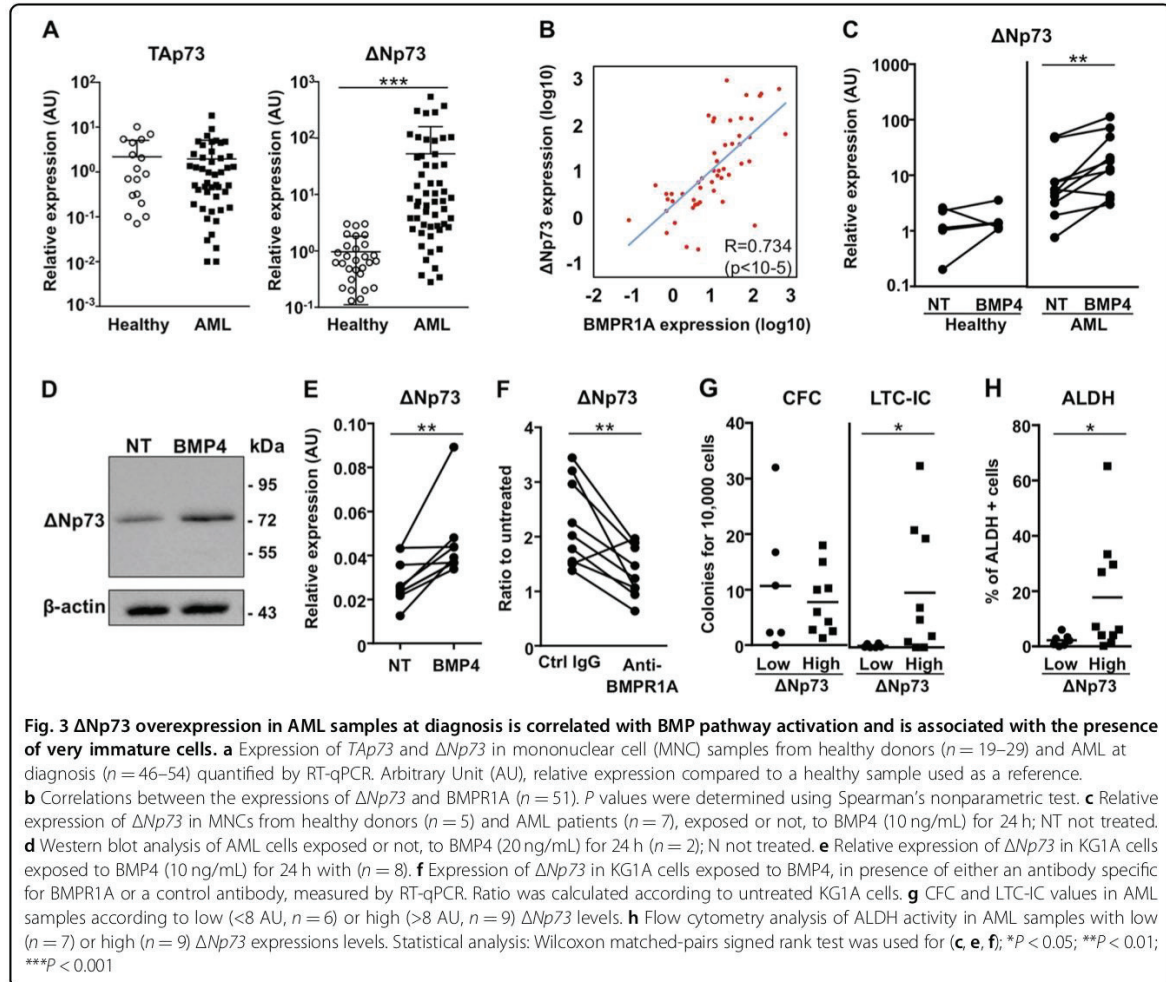
Since BMP4 is linked to the regulation of SCs<sup>10-12,20,23</sup>, we evaluated its involvement in immature features observed in leukemic cells. We first analyzed the effect of BMP4 exposure of AML cells on the number of long-term culture-initiating cells (LTC-IC) as a read-out of SCs properties maintenance through time (8 weeks assay). As previously reported for normal hematopoietic cells<sup>20,24</sup>, BMP4 treatment of AML MNCs induced a reproducible increase in the number of LTC-IC (Fig. 2e). This indicates that exposure to BMP4 favors the survival of very immature AML cells. Through cytometry analysis, we observed that BMPR1A<sup>+</sup> AML cells were specifically detected in the CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> BM fractions, respectively enriched in stem and



progenitor cells (Fig. 2f). We then divided at-diagnosis AML MNC samples into two groups according to their *BMPR1A* transcript expression. The “*BMPR1A*-low” group represented AML samples that expressed *BMPR1A* mRNA levels similar to healthy donors, while the “*BMPR1A*-high” group displayed *BMPR1A* levels exceeding the maximum value measured in healthy donors' cells. Functional assays confirmed that AML samples expressing high levels of *BMPR1A* contain 15-fold more immature cells able to generate LTC-IC ( $P = 0.0426$ ) but as many more mature progenitors identified as CFCs as *BMPR1A*<sup>low</sup> AML samples (Fig. 2g). This was confirmed by the observation that such samples are also enriched in ALDH-positive cells ( $P = 0.0394$ ), recognized as an SC marker in several cancer types including AML (Fig. 2h)<sup>25,26</sup>. Taken together, these findings indicate that leukemic cells harboring high levels of membrane *BMPR1A* receptors preferentially display immature features, this latter being increased after *BMP4* exogenous exposure.

#### **BMP4 induces $\Delta$ Np73, a p53 family member associated with stemness features**

*BMP4*, *BMPR1A*, and *Id1* are commonly associated with cell survival probably by controlling cell proliferation and differentiation, as reported in normal and leukemic contexts<sup>4,12,20</sup>. In order to identify new *BMP4*/*BMPR1A* downstream partners, we evaluated the expression of other genes involved in cell survival, such as the p53 family members<sup>27</sup>. We focused on  $\Delta$ Np73, the anti-apoptotic isoform produced by the *TP73* gene<sup>27,28</sup>, which has been found to be overexpressed in AML patients<sup>29,30</sup>. We confirmed this finding in our samples where a highly significant ( $P < 0.0001$ ) overexpression of  $\Delta$ Np73 (Fig. 3a, right panel) at diagnosis, but not of the pro-apoptotic TAp73 isoform (Fig. 3a, left panel). Interestingly,  $\Delta$ Np73 expression was strongly correlated with that of *BMPR1A* ( $P < 10^{-5}$ ) (Fig. 3b). This correlation was further confirmed by cell sorting MNCs from AML BM samples with an increase in  $\Delta$ Np73 expression within the *BMPR1A*<sup>+</sup> cells subfraction being found (Fig. S4a).



Furthermore, treatment of AML or normal primary MNCs with exogenous soluble BMP4 for 24 h resulted in a significant increase in  $\Delta Np73$  transcripts only in AML samples ( $P = 0.0059$ ; Fig. 3c). This increase was also observed at the protein level (Fig. 3d). A similar  $\Delta Np73$  upregulation after exposure to BMP4 was observed in the KG1A cell line, a model of immature AML<sup>31</sup> ( $P = 0.0078$ ; Fig. 3e). To confirm the direct link between BMP4, *BMPR1A* and the increase of  $\Delta Np73$  expression, KG1A cells were treated with BMP4 in the presence of either an anti-*BMPR1A* blocking antibody or the chemical *BMPR1* inhibitor, LDN-193189. BMP4-mediated induction of  $\Delta Np73$  was abrogated by both the *BMPR1A* blocking antibody (Fig. 3f) and the LDN 193189 (Fig. S4b). Quantification of CFC and LTC-IC in two groups of samples based on their  $\Delta Np73$  expression revealed a dramatic and significant increase in LTC-IC number in the  $\Delta Np73$ -high group ( $P = 0.0356$ ) (Fig. 3g, right panel),

whereas CFC remained equal (Fig. 3g, left panel). Next, we determined the number of ALDH<sup>+</sup> cells in  $\Delta Np73$ -low and -high expression groups and observed a significant correlation between  $\Delta Np73$  expression and ALDH positivity ( $P = 0.0350$ ; Fig. 3h). These data show that deregulation of the BMP pathway contributes to the overexpression of the survival factor  $\Delta Np73$  in AML cells through the binding of BMP4 to *BMPR1A*-overexpressing leukemic cells. As this upregulation is associated with immature cell features, we next investigated potential mechanism through which  $\Delta Np73$  could participate in SC regulation.

#### $\Delta Np73$ activates *NANOG* expression in BMP4-responsive cells

Since  $\Delta Np73$  has been described as a regulator of *NANOG*<sup>32,33</sup>, we evaluated the potential link between  $\Delta Np73$  and genes known to drive SC features in AML



samples. For this, we analyzed the expression of the three self-renewal regulators *NANOG*, *SOX2*, and *OCT4*<sup>33–35</sup>. Although no difference was observed as compared with normal MNCs for *OCT4* ( $P = 0.5500$ ), a significantly higher expression of *NANOG* ( $P = 0.0025$ ) and *SOX2* ( $P = 0.0359$ ) was measured in AML MNCs (Fig. 4a). In addition, the expression of  $\Delta Np73$  and *NANOG* and  $\Delta Np73$  and *SOX2* were strongly correlated (Fig. 4b). Using KG1A cells, we observed that ectopic  $\Delta Np73$  induced *NANOG* expression ( $P = 0.0286$ ; Fig. 4c), whereas ectopic *NANOG* did not affect  $\Delta Np73$  expression (Fig. 4c). In addition, the reduction of  $\Delta Np73$  expression by RNA interference consistently decreased *NANOG* expression ( $P = 0.0286$ ; Fig. 4d). Finally, using a luciferase reporter assay, we showed that  $\Delta Np73$  is able to activate the *NANOG* promoter (Fig. 4e), as previously reported in a reprogramming iPS fibroblast model<sup>32</sup>.

A significant correlation between *BMPR1A* and *NANOG* expression in AML samples was also observed ( $P < 10^{-5}$ ; Fig. 4f). This link was further confirmed in BM cells isolated from three different AML samples in which we detected higher levels of *NANOG* transcripts in total MNC cells sorted only for the *BMPR1A* cell membrane expression (Fig. 4g). Furthermore and in agreement with the robust correlation between *BMPR1A*,  $\Delta Np73$ , and *NANOG* expression, we observed the induction of the *NANOG* protein after BMP4 treatment (Fig. 4h). Lastly, treating primary AML cells by BMP4 allows correlation in the same cells of the BMP4-increased expression of *BMPR1A*,  $\Delta Np73$ , and *NANOG* with the amplification of cells with stemness features identified by the LTC-IC assay (Supplemental Table S4). Altogether, our data indicate that BMP4 induces the expression of  $\Delta Np73$ , which in turn, activates *NANOG* transcription.

#### The combined *BMPR1A*/ $\Delta Np73$ /*NANOG* overexpression at diagnosis identifies AML patients with a higher risk of early relapse

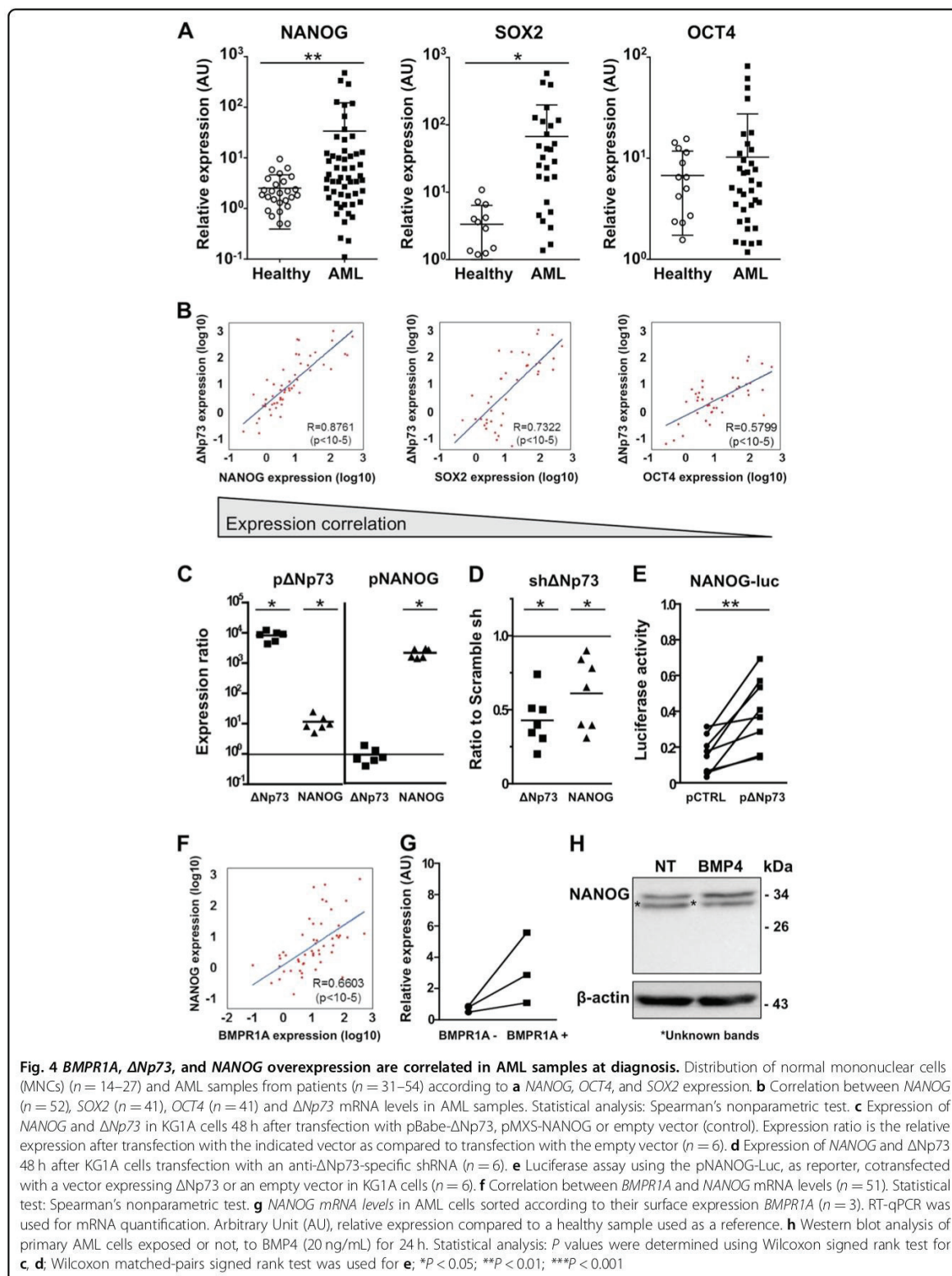
Finally, we analyzed the significance of *BMPR1A*,  $\Delta Np73$ , and *NANOG* transcript levels in AML patients at diagnosis with respect to clinical outcome at 3 years post-diagnosis. We observed that levels of the three markers at diagnosis did not predict the initial complete remission status (Table S3). Conversely, high expression of either *BMPR1A*,  $\Delta Np73$  or *NANOG* at diagnosis was associated with an increased rate of relapse within 3 years (Fig. 5a). Combining the three markers led to an increase in the clinical predictability for AML patient outcome at diagnosis, by identifying patients with a higher risk of relapse (from 33 to 86% risk of relapse; Fig. 5b). In order to evaluate the importance of *NANOG*, we performed a Kaplan–Meyer analysis on a larger number of patients and observed that high *NANOG* transcript levels are associated with poor survival (Fig. 5c). We performed a

multivariate parameter analysis and, as expected, identified a significant correlation only between *NANOG* and  $\Delta Np73$  or Sox2 or Oct4 expression. No correlation with any other parameters appeared significant, as indicated in Supplemental Table S5. Lastly, a similar analysis using the independent TCGA dataset<sup>36</sup>, this time restricted to patients within the intermediate risk group ( $n = 92$ ), showed again the association between high expression level of *NANOG* and shorter survival ( $P = 0,054$ ). Altogether, it suggests that *NANOG* could be considered as an independent risk factor and of high interest for patient within the intermediate risk group.

These results demonstrate the importance of measuring levels of *BMPR1A*,  $\Delta Np73$  and even more so of *NANOG*, as they could contribute to predicting at diagnosis the risk of relapse of AML patients.

#### Discussion

Resistance to treatment is often associated with the persistence of cancer SCs within their microenvironment. In AML, owing to the poor clinical efficacy of current treatments, deciphering mechanisms through which the microenvironment can sustain and promote the survival of immature-like cancer cells remains an important issue for patient outcome. Among the mechanisms through which the microenvironment can affect leukemic SCs' survival, cytokine production by stromal cells is of major importance. We focused on the BMP pathway as BMP2 and BMP4 regulate SC fate, maintenance and differentiation processes<sup>20,24,37</sup> and contribute to cancer SC emergence<sup>11,14</sup>, maintenance and expansion<sup>15</sup>. Here, we found an abnormally elevated concentration of BMP4, and to a lesser extent of BMP2 in the BM plasma of AML patients. These cytokines are likely to be produced by the BM microenvironment of leukemic cells, since we did not observe any increase in BMP2 and BMP4 transcripts levels in primitive leukemic cells themselves as compared to normal hematopoietic cells. This appears to be a general mechanism as we have found similar profiles in CML at the time of diagnosis<sup>12</sup> and also in luminal breast cancer<sup>11</sup>. Conversely, we observed an increased expression of intrinsic actors of the BMP pathway in MNCs of AML patients. In particular, we identified a highly significant increase in the expression of *BMPR1A* both at the mRNA and protein levels, in agreement with the important role of BMPR1 in the early steps of the AML transforming process, as previously described<sup>18</sup>. Interestingly, unlike the chronic phase of CML, in which we identified BMP2 and BMPR1B as driving the deregulation of the BMP pathway and SC/progenitor maintenance and expansion<sup>12</sup>, in AML BM cells, BMP4 and *BMPR1A* alterations were already detected at diagnosis. Interestingly, with CML progression towards more advanced phases and the acquisition of resistance to treatment, the



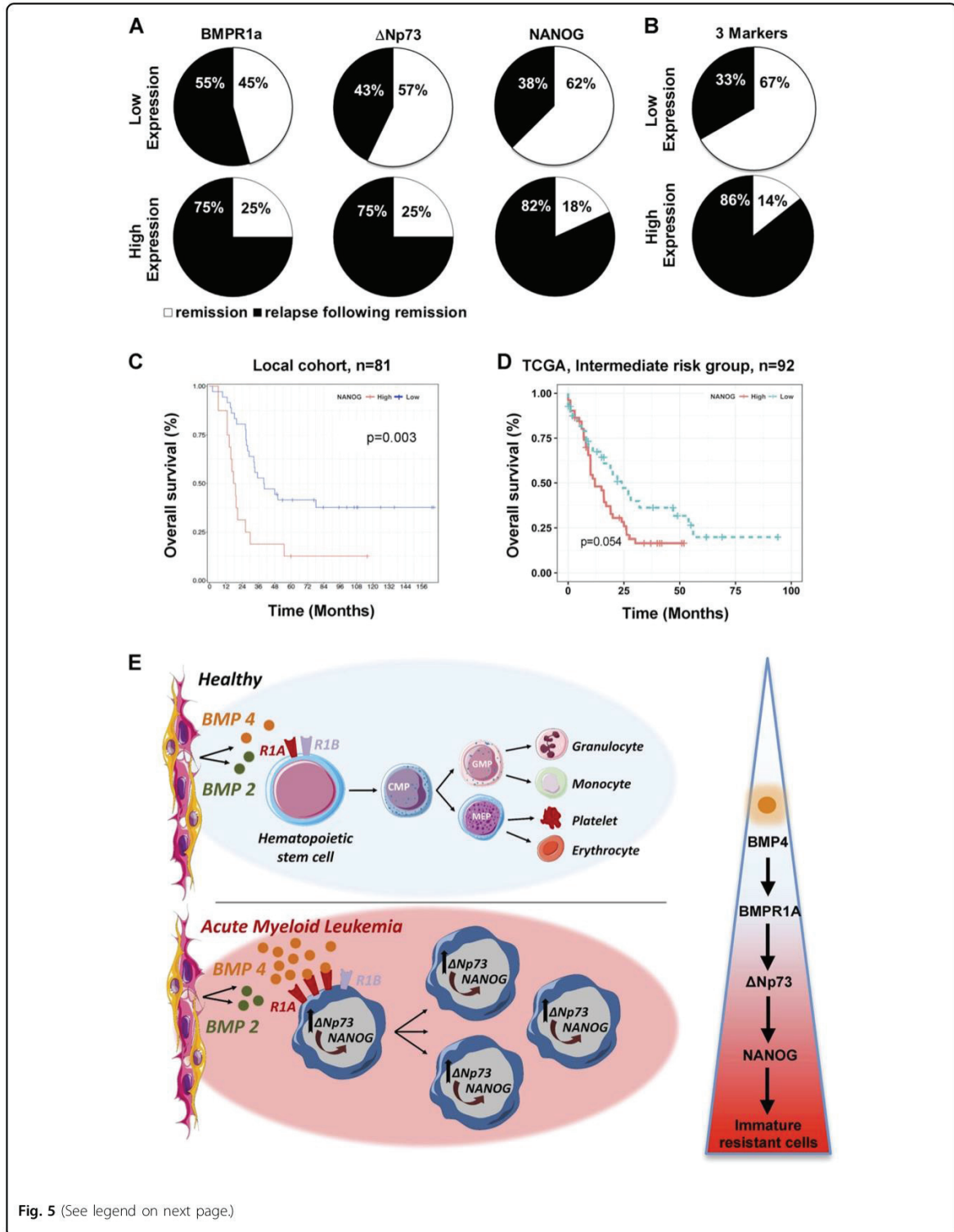


Fig. 5 (See legend on next page.)

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**Fig. 5** *BMPR1A*,  $\Delta Np73$ , and *NANOG* expression at diagnosis are correlated with patient outcome. Percentage of AML patients in remission or relapse 3 years after diagnosis, according to *BMPR1A*,  $\Delta Np73$  or *NANOG* expression levels separately ( $n = 19$ ) (a) or in combination ( $n = 13$ ) (b). c Overall survival (OS) curve of AML patients from the date of sampling onwards, by using the Kaplan–Meier method. The level of significance was determined by the log-rank test ( $n = 52$ ; 23 high and 29 low *NANOG* level). d Overall survival (OS) curve of AML patients from the date of sampling onwards, by using the Kaplan–Meier method. TCGA dataset from AML patients of the intermediate group. e Schematic representation of the role played by BMPs on homeostasis under healthy conditions (top) and the cascade of dysregulations associated with increased concentration of BMP4 in the microenvironment of leukemic immature cells (bottom)

BMP4 signal becomes predominant<sup>38</sup>. This suggests that the BMP4 signal is related to more aggressive or advanced disease as identified in cancers of other origins such as liver (HCC)<sup>39</sup>, thyroid (PTC)<sup>40</sup> and bladder<sup>41</sup>. In malignant glioma, while BMP4 appears to induce differentiation of glioma cancer stem-like cells<sup>42,43</sup>, an epigenetic repression of the BMPRIB receptors contributes to the CSC phenotype in these tumors<sup>10</sup>.

We identify here that the compartment of cells expressing surface BMPR1A is enriched in very immature AML cells as demonstrated by functional and ALDH assays. In addition, an increase in LTC-IC after exposure to BMP4 confirmed the involvement of this cytokine provided by the tumor niche and of its receptor in promoting AML stem-like cells. The BM-niche was previously reported to promote survival of leukemic cells in AML by activating different cytokine-related pathways, such as SDF1/CXCR4, Wnt/ $\beta$ -catenin, integrins<sup>2</sup>. Here, the elevated concentrations of both BMP2 and BMP4 in BM of AML patients at diagnosis also support their involvement in leukemic cells survival, as described in CML<sup>12</sup>. These results, obtained in the context of AML, highlight the importance under pathological conditions of this pathway already found to be clearly involved in SC regulation in neural<sup>44</sup>, epithelial<sup>11,14</sup> and hematopoietic systems<sup>20,24</sup> under physiological conditions.

We investigated the interplay between BMP4/BMPR1A signaling and the  $\Delta Np73$  isoform, as this member of the p53 family has been shown to be overexpressed in AML and to regulate CSCs<sup>23,30</sup>. Crosstalk between the p53 family and the BMP pathway has already been reported during breast<sup>45</sup> and epidermis<sup>46</sup> development, as well as in tumorigenesis<sup>47</sup>, though no evidence for a link between BMPs and the  $\Delta Np73$  isoform has so far been reported. Here, we clearly demonstrate a functional link between BMP4 and  $\Delta Np73$ . First, we identified a specific  $\Delta Np73$  upregulation already present at the time of diagnosis in MNCs of AML patients, while no such effect is seen for TAp73. Subsequently, we showed that this expression was higher within the BMPR1A<sup>+</sup> subpopulation and after exposure to BMP4. Finally, we established the link between both the BMP pathway and  $\Delta Np73$  with immature cell features. Indeed, AML MNCs expressing high levels of BMPR1A and  $\Delta Np73$  were mainly ALDH

positive, contained more LTC-IC and, after BMP4 treatment, exhibited an increased capacity to form colonies in an in vitro human functional SC assay.

Our results suggest that extrinsic (BMP4) and intrinsic (BMPR1A) alterations of the BMP pathway, associated with the induction of  $\Delta Np73$  expression, contribute to the increase in the number of immature cells in AML BM. The molecular mechanisms underlying such a phenomenon were evaluated focusing on the expression of some genes associated with immaturity, in particular *NANOG*, a known  $\Delta Np73$ -target gene<sup>32,33</sup>. We observed a significant correlation between the expression of  $\Delta Np73$  and *NANOG* and also of *BMPR1A* and *NANOG* in AML patients. These results highlight for the first time a cascade of events in AML, initiated by the binding of BMP4 to its type 1A receptor that leads to  $\Delta Np73$  expression, which in turn induces *NANOG* by promoter transactivation. This activation is p53-independent, since it was confirmed using the KG1A AML cell line, which does not express functional p53<sup>31</sup>.

The increased expression of three genes involved in self-renewal and in cell reprogramming,  $\Delta Np73$ <sup>32</sup> *NANOG* and *SOX2*<sup>32,48</sup>, in AML patients and the induction of  $\Delta Np73$  and *NANOG* upon BMP4 treatment supports the hypothesis that BMP4 could promote the reprogramming of cells towards immature leukemic cells. This is consistent with a recent study that identified BMP4 and its signaling pathway as a driving element of adult cell reprogramming, including fibroblasts towards functional hematopoietic SCs<sup>49</sup>.

Resistance to treatment and relapse are often associated with the persistence of cancer SCs within their microenvironment. Indeed, BMP4 involvement in resistance to treatment has been demonstrated in several tumor types, including HCC<sup>50</sup> and ovarian cancer<sup>51</sup>. BMP type 1 receptors have also been associated with resistance in CML<sup>38</sup> and AML<sup>18</sup>, as has  $\Delta Np73$ <sup>52</sup>. These latter, as well as *NANOG*, are also associated with resistance and progression in other cancers<sup>53–55</sup>.

In conclusion, our study deciphers a new signaling cascade through which alterations of BMPs secretion in the microenvironment can sustain and promote resistant immature-like leukemic cells (Fig. 5e). Activation of this signaling cascade is associated with a poor prognosis for

AML patients. Thus, measuring transcript levels of the three markers *BMPRIA*, *ΔNp73*, and *NANOG* is likely to increase the ability to predict patient outcome at diagnosis. Since involvement of CSC in clinical outcome may be a common feature of relapsing cancers, it will be worthwhile to investigate the potential involvement of this novel cascade in other cancer types.

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#### Conflict of interest

The authors declare that they have no conflict of interest.

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## Conclusions

Within the present research study, we have identified high concentrations of soluble BMP2 and BMP4 in plasma from BM AML samples. This confirmed the extrinsic alteration of the pathway in the tumor niche. In addition, mononuclear cells (MNCs) isolated from AML patients at diagnosis, displayed overexpression of *BMPR1A* receptor and of the target gene of the pathway *ID1* compared to healthy samples; whereas BMP2 and BMP4 transcript levels remained similar to those from healthy individuals, suggesting that BMPs overproduction comes from another element in the niche. Moreover, transcript levels of the anti-apoptotic isoform  $\Delta Np73$  were identified to be higher in MNCs from AML patients than in normal BM. After finding strong correlation between *BMPR1A* and  $\Delta Np73$ , we have confirmed in MNCs from AML samples, that BMP4 binding to *BMPR1A* induced expression of  $\Delta Np73$ , triggering a signaling cascade described for the first time, leading to *NANOG* induction by  $\Delta Np73$ . This new signaling cascade seems to drive leukemic cells into a more immature phenotype, assessed by ALDH activity and their colony forming capacity in the LTC-IC functional assay. Finally, we have identified association of *BMPR1A*/ $\Delta Np73$ /*NANOG* overexpression at diagnosis with an increased rate of relapse. Suggesting these elements as new potential prognosis markers.

In conclusion, our research study has started to decipher the specific elements of the BMP pathway and related genes involved in the induction of a more immature phenotype or “stem-like” state of leukemic cells in AML.

## 2 Complementary data: BMP signaling in leukemic stem cell resistance in AML

### 2.1 INTRODUCTION

The tumor microenvironment has been described as having a key role regulating and protecting cancer stem cells (CSCs)<sup>298</sup>. Cellular and molecular components in the tumor niche combined with signaling crosstalk represent the elements driving the regulation of CSCs<sup>301,302</sup>. The BMP pathway has been shown to be important in tumor initiation and progression, but also represents an essential signaling in CSC regulation and resistance<sup>203</sup>.

As described in our former article, the BMP signaling pathway promotes an immature phenotype in leukemic cells from acute myeloid leukemias (AML). The presence of leukemic stem cells (LSCs) in AML seems to be at least partially responsible for relapse and poor prognosis<sup>299</sup>.

BMPs have been described in leukemias due to their role in LSC regulation. In CML, our team identified high levels of BMP2 and BMP4 in the tumor niche<sup>291</sup>, while BMP4 and BMPR1B were overexpressed in LCSs from TKI resistant patients<sup>261</sup>. BMP4 ligand was also reported to be responsible to induce *MIXL1*, a gene involved in AML initiation<sup>288</sup>. Moreover, BMP2/BMP4 and ID1 overexpression has been related to immature features of leukemic cells in acute megakaryoblastic leukemia (AMKL)<sup>289</sup>.

Our previous results, identified BMP4 as a key actor inducing stem-like features via the binding to its overexpressed receptor BMPR1A and driving induction of  $\Delta$ Np73 and NANOG expression in leukemic cells. Evaluation of the status of these elements in AML resistant and relapse models, might elucidate the mechanisms employed by the LSCs against chemotherapy.



## 2.2 MATERIAL AND METHODS

### Primary samples at diagnosis and relapse

The cDNA employed to perform RT-qPCR was obtained from AML peripheral blood (PB) samples at diagnosis (n = 36) and relapse (n = 8).

### Primary cells at diagnosis and treatments

Patient samples were obtained after informed consent in accordance with the Declaration of Helsinki in the hematology departments involved in this study (Hospices Civils de Lyon (HCL) and Centre Léon Bérard (CLB)). Mononuclear cells (MNCs) from AML 12 bone marrow (BM) samples ((M0 = 2), (M1 = 3), (M2 = 1), (M4 = 4), (M5 = 2) were obtained from patients at diagnosis, excluding acute promyelocytic leukemia (M3). Primary cells were cultured with IMDM culture medium (ThermoFisher) containing 15% BIT 9500 (Serum substitute StemCell Technologies). Primary cells were treated during 72 hours with Cytarabine (ARA-C) (Ebewe-Sandoz) at different concentrations (0.2µg/ml-0.5µg/ml). Surviving cells to ARA-C will be referred in the following study as short-term resistant MNCs and control as untreated MNCs.

### AML sensitive and resistant cell lines

AML resistant cell lines to 0.2µg/ml ARA-C were generated by Margaux Deynoux, PhD student of Dr. Frédéric Mazurier in the team of Pr. Olivier Herault's laboratory (Université de Tours: Team Niche leucémique et métabolisme oxydatif). The AML sensitive and resistant cell lines obtained correspond to different AML subtypes from the FAB classification listed below.

Cell lines	AML subtype
KG1A	M0/M1
KG1	M1
HL-60	M2
NB-4	M3
ML-2	M4
OCI-AML2	M4
OCI-AML3	M4
THP-1	M5
MV4-11	M5
U-937	M5

### **Resistant cell line generation**

KG1A myeloid leukemia cells were cultured in RPMI-1640 medium (ThermoFisher) containing 10% fetal calf serum (FCS). Chronic exposure of the cells to ARA-C gradually increasing was performed, until KG1A cell line resistant to 0.2µg/ml was obtained.

### **RNA isolation and analysis**

Quantitative RT-PCR was performed using standard protocols<sup>240</sup>. MNCs were isolated by a Ficoll gradient and total RNA was purified using TRI REAGENT™ (Sigma) and the mini-RNA extraction kit (Qiagen, Valencia, CA). For RT-qPCR, cDNA was produced using Superscript II (Invitrogen) and amplified using Sybr-green (Quantifast, Qiagen) and the Real-Time PCR system (Roche). TBP (TATA-binding protein) and HPRT (hypoxanthine- guanine phosphoribosyl transferase) genes were used for normalization. Arbitrary unit (AU) corresponds to the ratio of expression between samples and a single normal sample used as a reference in each PCR.

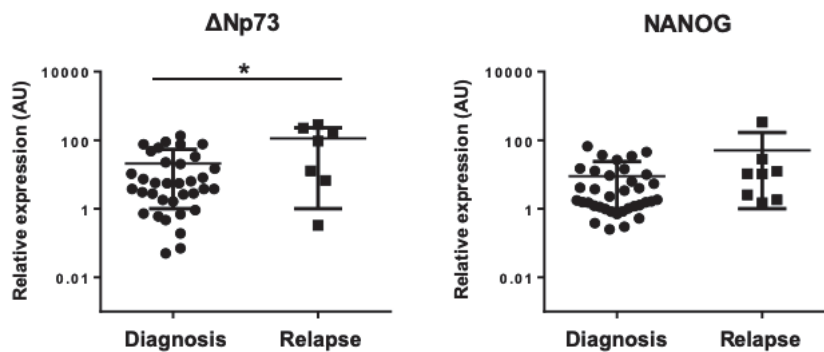
### **Flow cytometry analysis**

Cells were incubated with antibodies specifically recognizing CD34, CD38 (Becton Dickinson) and/or BMPR1A (R&D System), or an irrelevant isotype-matched control antibody.

## 2.3 RESULTS

### 2.3.1 $\Delta$ Np73 status in relapse AML patients

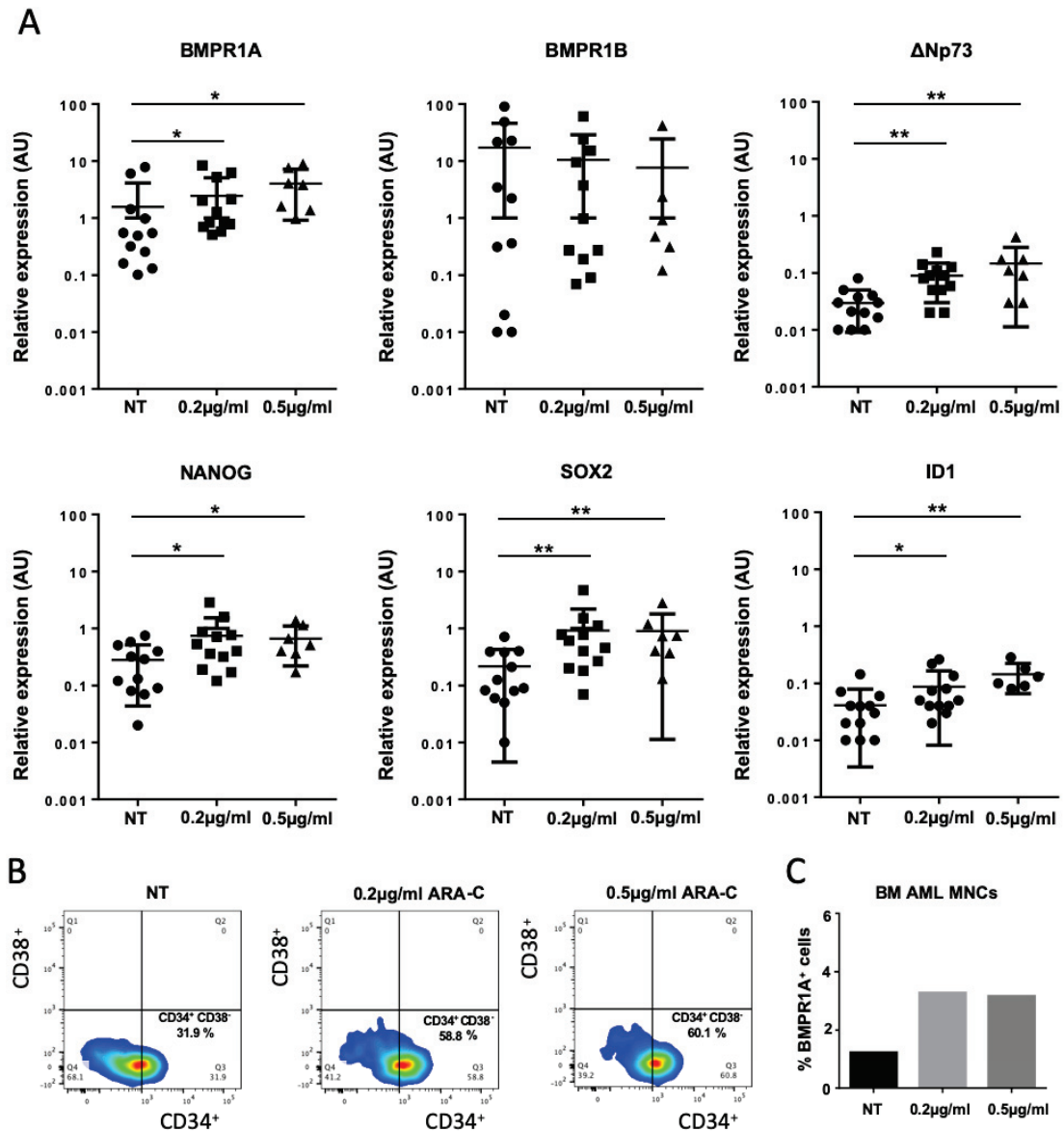
In our previous study, we identified high transcript levels of the isoform  $\Delta$ Np73 and NANOG in MNCs obtained from AML BM samples at diagnosis. Due to their role reported in by other studies in resistance and relapse, we hypothesized that expression of these two genes might be higher in AML patients at relapse compared to patients at diagnosis. In order to determine this, we used cDNA from AML PB samples at diagnosis and relapse, to measure the transcript levels of these two elements. We identified significant ( $P = 0.0284$ ) higher transcript levels of  $\Delta$ Np73 at relapse compared to samples at diagnosis. While no significant difference was observed in NANOG expression between diagnosis and relapse patients (**Figure 18**).



**Figure 18. Higher  $\Delta$ Np73 transcriptional expression is observed in AML samples at relapse compared to samples at diagnosis.** mRNA expression of  $\Delta$ Np73 and NANOG evaluated in PB AML samples at diagnosis ( $n = 33-36$ ) and relapse ( $n = 7-8$ ) by RT-qPCR. Arbitrary unit (AU): relative expression compared to a healthy sample used as a reference for each PCR experiment. Statistical analysis:  $P$  values were determined using the Mann-Whitney unpaired rank test.  $*P < 0.05$ .

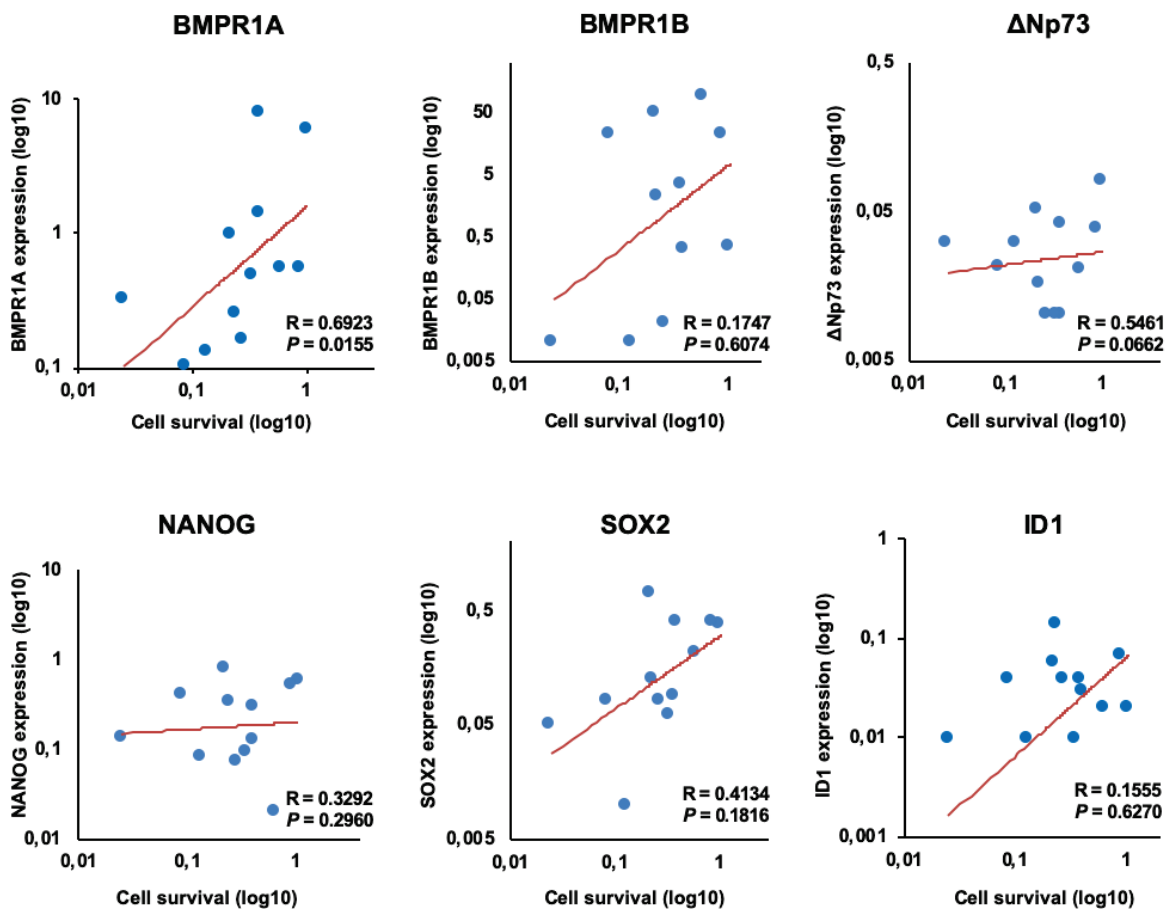
### 2.3.2 Status of BMP elements and stemness genes in short-term resistant AML leukemic cells

The high mRNA levels of  $\Delta Np73$  in relapse patients suggested that AML leukemic cells from these patients might possess a more immature phenotype leading to resistance. Although NANOG was not significantly overexpressed in AML relapse samples, the use of AML bone marrow samples could be more significant due to the idea of a higher presence of LSCs. Therefore, we obtained AML BM samples at diagnosis and isolated the mononuclear cells (MNCs). As described in the Material and Methods section, untreated and short-term resistant MNCs from AML samples were used to evaluate the transcript expression of a panel of genes, which we have identified overexpressed in AML patients at diagnosis as part of our previous study. When we analyzed the gene expression profile of untreated and resistant MNCs, we identified that short-term resistant MNCs to 0.2 $\mu$ g/ml ARA-C displayed significant higher transcript levels of BMPR1A ( $P = 0.0386$ ),  $\Delta Np73$  ( $P = 0.0014$ ), NANOG ( $P = 0.0294$ ), SOX2 ( $P = 0.0072$ ) and ID1 ( $P = 0.0333$ ) compared to untreated MNCs (**Figure 19A**). Moreover, short-term resistant MNCs to 0.5 $\mu$ g/ml ARA-C displayed significant higher transcript levels of BMPR1A ( $P = 0.0171$ ),  $\Delta Np73$  ( $P = 0.0043$ ), NANOG ( $P = 0.0283$ ), SOX2 ( $P = 0.0052$ ) and ID1 ( $P = 0.0012$ ) compared to untreated MNCs. Only the expression of BMPR1B receptor remained unchanged between resistant and untreated MNCs (**Figure 19A**). As we identified overexpression of elements linked to stemness features, we analyzed the CD34/CD38 status in untreated and resistant MNCs from one AML sample by flow cytometry. We observed that MNCs resistant to 0.2 $\mu$ g/ml and 0.5 $\mu$ g/ml ARA-C contained a more enriched population of CD34<sup>+</sup>CD38<sup>-</sup> leukemic cells compared to untreated MNCs (**Figure 19B**). In addition, using the previous AML sample where we evaluated the CD34/CD38 status, we identified a higher percentage of BMPR1A<sup>+</sup> cells in the short-term resistant MNCs compared to the untreated leukemic cells by flow cytometry (**Figure 19C**).



**Figure 19. Short-term resistant primary leukemic cells displayed stem-like features identified by its gene expression profile, CD34<sup>+</sup>CD38<sup>-</sup> phenotype and BMPR1A membrane expression. A.** mRNA expression of BMP receptors: BMPR1A and BMPR1B, the isoform ΔNp73, stemness genes: NANOG and SOX2 and the BMP target gene: ID1. Evaluated in untreated and short-term resistant MNCs from AML BM samples at diagnosis (n = 12) by RT-qPCR. Arbitrary unit (AU): relative expression compared to a healthy sample used as a reference for each PCR experiment. Statistical analysis: *P* values were determined using the Mann-Whitney unpaired rank test. \**P* < 0.05; \*\**P* < 0.01. **B.** Flow cytometry analysis identifying the percentage of CD34<sup>+</sup>CD38<sup>-</sup> population in untreated and short-term resistant MNCs from one AML BM sample at diagnosis. **C.** Percentage of MNCs untreated and short-term resistant to ARA-C, from one AML BM sample, expressing BMPR1A at the membrane determined by flow cytometry.

Identification of the gene expression profiles in untreated and short-term ARA-C resistant MNCs, allowed us to evaluate a potential correlation between the transcript level of these genes and the survival capacity of MNCs to ARA-C. We found that BMPR1A overexpression was significantly correlated with a better survival capacity of MNCs to 0.2µg/ml ARA-C treatment ( $P = 0.0155$ ). While no significant correlation was observed between the other genes analyzed and cell survival capacity (**Figure 20**).



**Figure 20. Cell survival capacity of short-term resistant MNCs to 0.2µg/ml ARA-C is correlated with a high mRNA BMPR1A expression.** Correlation between mRNA levels of BMPR1A, BMPR1B, ΔNp73, NANOG, SOX2, ID1 and cell survival capacity of short-term resistant MNCs to 0.2µg/ml ARA-C from AML BM samples ( $n = 12$ ). Statistical analysis: Spearman's nonparametric test.

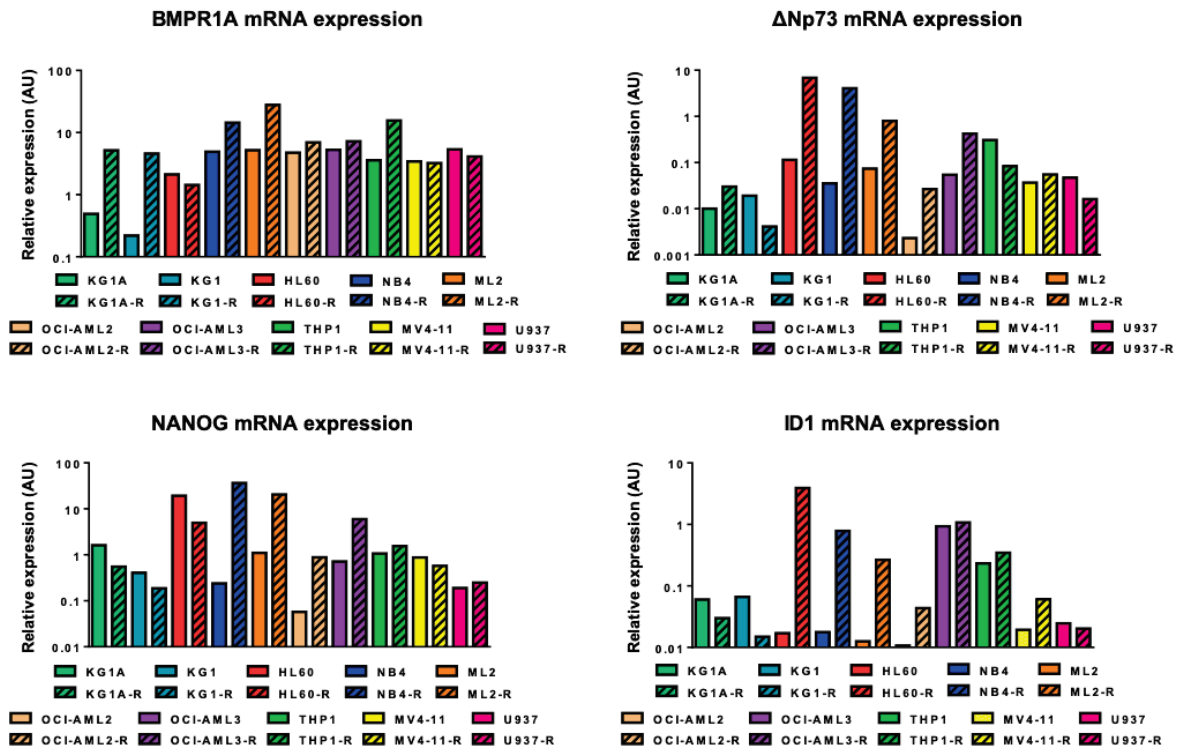
### 2.3.3 Gene expression profile linked to stem-like features in AML resistant cell lines

Short-term resistant MNCs presented overexpression of genes involved in the signaling cascade promoting immature features. Therefore, we decided to compare these results with AML sensitive and resistant cell lines. We analyzed the gene expression profile of BMPR1A,  $\Delta$ Np73, NANOG and ID1 in AML cell lines resistant to ARA-C. The selection of these genes was due to the fact that they have been linked to print an immature phenotype in leukemic cells (BMPR1A- $\Delta$ Np73-NANOG), while ID1 is also known as an inhibitor of differentiation linked to resistance.

The AML cell lines employed correspond to different AML subtypes from the FAB classification as described in the section Material and Methods. For most of the resistant cell lines, we have observed higher expression of BMPR1A compared to sensitive cells, except for HL60, MV4-11 and U937 cells.  $\Delta$ Np73 transcript levels were also higher in resistant cells compared to sensitive cells excluding KG1, THP1 and U937 cell lines. The stemness gene NANOG appeared higher in resistant cells compared to sensitive cells apart from KG1A, KG1, HL60 and MV4-11 cell lines. Finally, ID1 expression was reported higher in the resistant cell lines compared to their sensitive parental cell line, except for KG1A, KG1 and U937 cells (**Figure 21**).

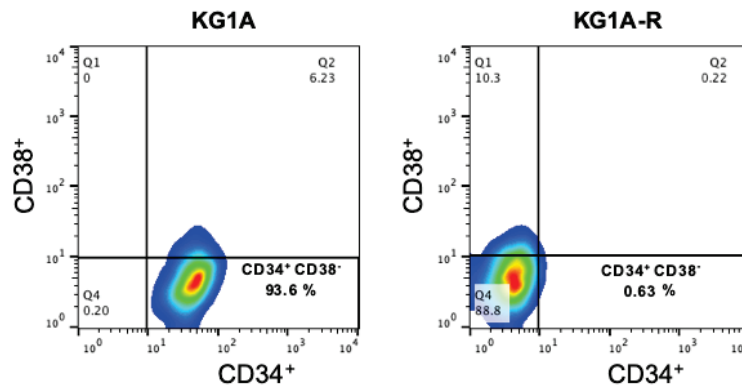
Since KG1A is the best model for immature AML M0/M1 subtype, we decided to select sensitive and resistant KG1A cell lines to analyze in more detail the status of genes involved in the signaling cascade promoting immaturity, which we evaluated in short-term resistant MNCs. Nonetheless, before developing the first experiments we assessed the immature phenotype of KG1A and KG1A-R cell lines by flow cytometry. KG1A cell line displayed a CD34<sup>+</sup>CD38<sup>-</sup> phenotype, whereas KG1A-R cells displayed an unexpected CD34<sup>-</sup>CD38<sup>-</sup> phenotype (**Figure 22**). The typical KG1A phenotype is described as positive marker CD34 and negative for CD38. For this reason, we decided to develop a new KG1A resistant cell line to ARA-C and identify if the CD34 marker is lost due to a normal process

caused by resistance or consequence of a drift of the cell line produced by long culture during development of resistance.



**Figure 21. Profile of expression of genes linked to stem-like features and blocking of differentiation in sensitive and resistant AML cell lines.** mRNA expression of BMPR1A receptor, anti-apoptotic isoform  $\Delta$ Np73, stemness gene NANOG and inhibitor of differentiation ID1 in sensitive and resistant AML cell lines by RT-qPCR. Arbitrary unit (AU): relative expression compared to a healthy sample used as a reference for each PCR experiment.



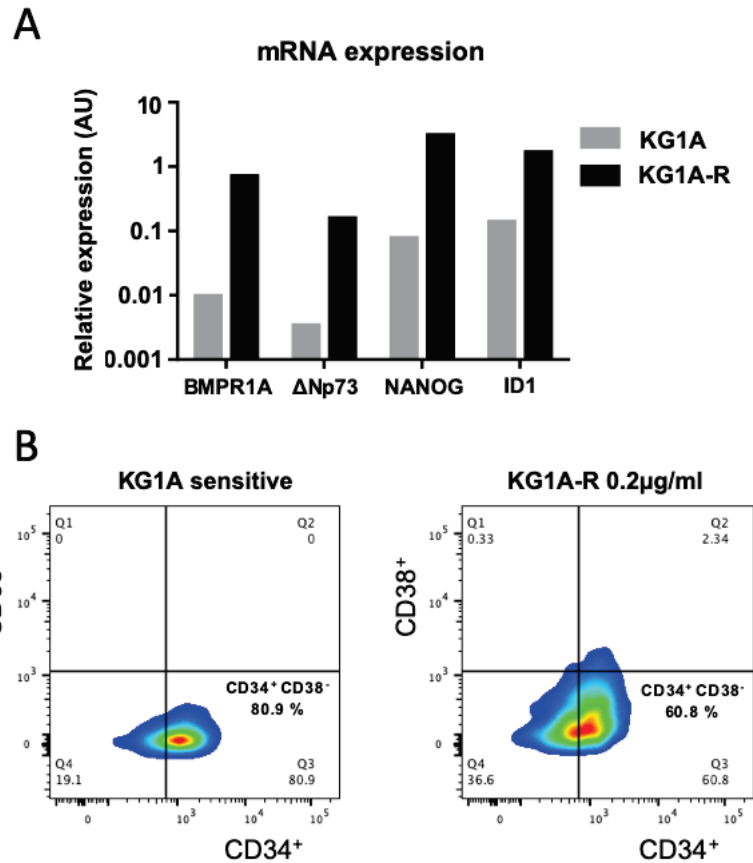


**Figure 22. CD34/CD38 phenotype of sensitive and resistant KG1A cell lines.** The status of immaturity was evaluated by analyzing the markers CD34 and CD38 in KG1A and KG1A-R cell lines by flow cytometry.

#### 2.3.4 Development of the resistant KG1A cell line and characterization

The KG1A resistant cell line developed by our team, was characterized by analyzing the expression of genes involved in promoting the immature phenotype of AML leukemic cells (BMPR1A,  $\Delta$ Np73, NANOG) and the target gene of the BMP pathway ID1 involved in blocking of differentiation and resistance. Performance of RT-qPCR reported higher mRNA levels of BMPR1A,  $\Delta$ Np73, NANOG and ID1 in KG1A resistant cells compared to sensitive cells (**Figure 23A**). This profile corresponds to the gene expression reported in cells obtained from the Pr. Olivier Herault's laboratory.

Furthermore, we analyzed the CD34/CD38 status of both cell lines, identifying that 80.9% of sensitive KG1A cells exhibited a CD34<sup>+</sup>CD38<sup>-</sup> phenotype, whereas 60.8% of resistant KG1A cells displayed the immature phenotype CD34<sup>+</sup>CD38<sup>-</sup> (**Figure 23B**).



**Figure 23. Characterization of KG1A sensitive and resistant cell lines, based on their mRNA expression of genes promoting stem-like features/blocking of differentiation and immature phenotype. A.** Transcriptional expression of BMPR1A receptor, ΔNp73, NANOG and ID1 in KG1A sensitive and resistant cell lines by RT-qPCR. Arbitrary unit (AU): relative expression compared to a healthy sample used as a reference for each PCR experiment. **B.** Evaluation of the immaturity state CD34/CD38 of KG1A sensitive and resistant cell lines by flow cytometry.

## **IV. DISCUSSION**

## 1 Tumor microenvironment: BMP dysregulation in AML

The tumor niche has been identified as an essential factor extensively studied in cancer. Composed of several cellular and molecular components, the niche represents a major element in CSC maintenance and survival<sup>303,302</sup>. Relapse and poor prognosis are key words in AML. Presence of LSCs in the tumor niche is often related to resistance, which is eventually is able to drive relapse and poor prognosis<sup>300</sup>. Understanding the mechanism by which the tumor niche regulates LSC survival represents one of the main steps in order to develop new therapeutic approaches.

The BMP pathway has been described in several studies as being involved in HSC regulation<sup>248</sup> and in a malignant context, the signaling has been described in LSC maintenance. In my research project, the identification of BMP alterations in the AML tumor niche was the first element that allowed the recognition of a signaling cascade regulating LSCs.

Analysis of extrinsic elements of the BMP signaling in the tumor niche, led us to identify high concentrations of BMP2 and even higher BMP4 cytokine levels in plasma AML samples compared to healthy donors. These first results correlate with previous studies from our research team, where we identified high BMP2/BMP4 concentrations in the tumor niche of luminal breast cancer and CML<sup>254,291</sup>. In order to confirm if the overproduction of BMPs was generated by the leukemic cells, MNCs from AML and healthy samples were isolated and transcriptional gene analysis performed. Expression of both ligands was slightly decreased in leukemic cells compared to healthy specimens. This excludes an autologous BMP production by the malignant cells, suggesting that another cell component within the tumor niche is responsible for this BMP secretion. A similar profile of BMP4 overproduction was reported by our team in the CML tumor niche. In this case, high transcript BMP4 expression was observed in MSCs from CML patients compared to healthy donors. In addition, MSCs derived from CP-CML patients, showed high BMP4 production compared to healthy MSCs<sup>261</sup>. An interesting perspective is to identify if MSCs derived from AML patients possess the capacity to produce high levels of BMPs as reported in CML.

Another approach to identify the cellular component responsible for this BMP overproduction would be through the obtention of AML and healthy bone marrow tissue samples. Immunohistochemistry (IHC) would allow to identify the presence of cells with higher BMP2/BMP4 expression in the niche. As shown by our team, in breast cancer a similar overproduction of BMP2 was observed in the microenvironment. IHC performed in basal tumors identified stromal and endothelial cells as the positive population expressing BMP2 responsible for the high levels of the cytokine in the tumor niche<sup>254</sup>.

MSCs have been described in AML due to their role building a leukemic tumor niche<sup>304</sup>. Huang and colleagues recently showed that MSCs derived from BM AML patients, exhibited different cytokine production compared to MSCs derived from healthy donors. Some of these cytokines were MCP-1 (Monocyte Chemoattractant Protein-1), G-CSF and IL-6. When MSCs were co-cultured with AML blasts, the cytokines produced by the MSCs enhanced growth and inhibited apoptosis<sup>305</sup>. Interestingly, these cytokines are linked to BMPs. For instance, enhanced expression of the inflammatory cytokines MCP-1 and IL-6 by BMP2 has been reported through SMAD activation<sup>306</sup>. In breast cancer, BMP2 and IL-6 have been identified for their role promoting malignant transformation of mammary epithelial cells<sup>254</sup>. While MCP-1 has been reported to be overexpressed in the aggressive type of triple-negative breast cancer. This data confirmed the importance of BMPs in the tumor niche and the interaction of these cytokines with other molecules involved in cancer development and progression.

Therefore, confirmation that BMP2/BMP4 overproduction in AML is linked to MSCs would allow to understand how the tumor niche regulates LSCs, through secretion of different cytokines or growth factors. Based on this, co-culture experiments using MSCs with AML blasts or healthy BM MNCs represent an attractive perspective. Adding standard drug chemotherapy in this co-culture experiments would help to identify if MSCs are able to grant protection and resistance to AML blasts, through cytokine secretion as BMP2 or BMP4 overproduction or a combination of other cytokines.

## **2 Intrinsic alterations of the BMP pathway in AML leukemic cells**

After identifying BMPs overproduction within the niche. MNCs were isolated from AML and healthy samples. BMP2 and BMP4 transcript levels remained decreased in BM leukemic cells compared to healthy MNCs. Conversely, we reported overexpression of BMPR1A receptor and target genes of the pathway as ID1 and RUNX1 in BM and PB MNCs from AML samples compared to healthy donors. Our results present similarities with recent publications. For instance, Raymond and colleagues identified the induction of *MIXL1* gene in AML through the interaction of BMP type I receptors and BMP4 ligand<sup>288</sup>. Interestingly, in CML it was the BMPR1B receptor overexpressed at transcriptional level<sup>261</sup>, suggesting probably two different mechanisms of BMP dysregulation when comparing AML and CML malignancies. In addition, overexpression of the inhibitor of differentiation ID1 has also been reported in acute megakaryoblastic leukemia (AMKL)<sup>289</sup>. While RUNX1 has been reported in AML due to its role promoting leukemic cell growth<sup>307</sup>.

BMP4 has been described in several aggressive types of cancer such as colorectal cancer (CRC)<sup>308</sup>, gastric adenocarcinoma<sup>309</sup>, hepatocellular carcinoma<sup>310</sup> and squamous cell carcinoma of the head and neck<sup>311</sup>. And BMPR1A receptor has been described in breast cancer as a tumor promoter<sup>312</sup> and also high expression of this receptor has been reported in liposarcoma relapse<sup>313</sup>. Although BMP4 transcript levels are not altered in AML blasts, the high levels of BMP4 within the niche and the transcript overexpression of BMPR1A, started to suggest the importance of these two elements as key factors regulating the leukemic cell behavior in AML.

## **3 BMPR1A correlation with the inhibitor of differentiation ID1 and $\Delta$ Np73 isoform**

A significant correlation was identified between the high transcript levels of BMPR1A and ID1 in AML MNCs at diagnosis. We analyzed other survival factors in the literature and focused on the isoforms TAp73 and  $\Delta$ Np73. We found

overexpression of  $\Delta Np73$  at transcript level in MNCs from AML patients compared to healthy samples, while no difference of TAp73 expression was observed. Our results are consistent with previous studies reporting high expression of the anti-apoptotic isoform in lung cancer<sup>130</sup> and in APL samples<sup>131</sup>. In these studies,  $\Delta Np73$  expression was linked to poor prognosis and high risk of relapse respectively.

As we reported for BMPR1A and Id1, we also identified a strong correlation between the overexpression of  $\Delta Np73$  and BMPR1A. In order to confirm this correlation, MNCs from AML samples were sorted according to the BMPR1A membrane expression. BMPR1A<sup>+</sup> cells displayed higher transcript levels of ID1 and  $\Delta Np73$  compared to the BMPR1A<sup>-</sup> population. The correlation between BMPR1A and ID1 can be partially explained as ID1 is known for being a downstream target gene of the pathway<sup>224</sup>. Other studies have described a link between this receptor and ID1. For example, disruption of BMPR1A receptor decreased the growth of colon cancer cell xenografts in nude mice, caused by the downregulation of ID1<sup>314</sup>. Interestingly, the correlation we have identified in our study between BMPR1A and  $\Delta Np73$  has not been previously described, it represents one of the first hints that helped us to define the new signaling cascade reported in our study.

#### **4 BMP4, BMPR1A and $\Delta Np73$ promoting an immature phenotype in AML leukemic cells**

As described in the literature the BMP pathway is an essential element in normal and leukemic SC regulation<sup>203</sup>. In addition,  $\Delta Np73$  also represents an important factor that confers immature features to leukemic cells<sup>315</sup>. Due to these observations, we performed the functional assays CFC (Colony Forming Cell assay) and LTC-IC (Long-Term Culture-Initiating Cell assay) and ALDH assay (Aldehyde Dehydrogenase), to evaluate the immaturity of the leukemic cells based on the transcript levels of BMPR1A and  $\Delta Np73$ . In addition, we evaluated the effect of soluble BMP4 in the immature phenotype of AML blasts.

In the CFC assays, which identifies the presence of progenitor populations, no significant differences were observed in MNCs with high or low expression of BMPR1A and  $\Delta Np73$ . Nevertheless, in the LTC-IC assay a higher number of colonies were identified in MNCs displaying high expression of BMPR1A and  $\Delta Np73$ . These results were confirmed with MNCs presenting high ALDH activity, which belonged to the group with high BMPR1A and  $\Delta Np73$  transcript expression. The effect of soluble BMP4 in MNCs also promoted the immature phenotype, as high number of colonies were identified in the LTC-IC assay.

Therefore, these results confirmed that AML leukemic cells are able to acquire a more immature phenotype through BMP dysregulation. High soluble BMP4 identified in the tumor niche, together with overexpression of BMPR1A receptor and  $\Delta Np73$  play a key role granting the immature phenotype to leukemic cells. Our results showing that BMP4 grants a stem-like phenotype have also been reported in oral squamous carcinoma cells (OSCC). Where BMP4 changed the morphology of Tca8113 cells (human tongue OSCC), induced nuclear OCT4 expression and upregulated EMT markers (snail, slug and vimetin)<sup>257</sup>. In addition,  $\Delta Np73$  has been described due to its capacity to increase stem-like properties in melanoma and lung cancer cells<sup>315</sup>.

An interesting perspective regarding our results, would be to modulate the expression of  $\Delta Np73$  through downregulation or knockout. And evaluate the effect of drug therapy in these leukemic cells. AML blasts from patients at diagnosis or an AML cell line would represent a good *in vitro* model. For instance, downregulation of  $\Delta Np73$  performed in human cervix carcinoma HeLa cells, played a crucial role promoting cycle arrest and apoptosis of these cells<sup>316</sup>. In addition, a study reported that cells from  $\Delta Np73$  KO mice have shown sensitivity to DNA-damaging agents<sup>317</sup>, suggesting that high levels of  $\Delta Np73$  might be linked to the resistant capacity of cancer cells to drug therapy. Thus, regulation of this isoform could lead to total eradication of LSCs in AML and malignancies where this isoform is involved.

Although TAp73 was not altered in AML MNCs, modulation of TAp73 in primary leukemic cells or AML cell lines represents an interesting aspect, for subsequent



evaluation of the sensitivity of these cells against chemotherapeutic agents. In this context, a study has reported the use of different cancer cell lines such as human gastric epithelial adenocarcinoma cells (AGS), osteosarcoma cells (Saos-2) and liver cancer cells (HepG2). Cells were infected with adenoviral TAp73, this upregulation increased the sensitivity of the cells to chemotherapy, through upregulation of death receptors (CD95, TNF-R1, TRAIL-R1 and TRAIL-R2)<sup>318</sup>.

## **5 $\Delta$ Np73 induction through the BMP4-BMPR1A binding**

BMP4, BMPR1A and  $\Delta$ Np73 alterations were identified in our study for the first time in AML. The leukemic cells in the tumor niche are in contact with high concentrations of BMP2 and BMP4 cytokines according to our results. Thus, we established the hypothesis that leukemic cells were responsive to BMPs and this cytokine was responsible for the induction of BMPR1A and  $\Delta$ Np73 expressions.

First, we identified a higher percentage of MNCs from AML samples expressing BMPR1A at the membrane compared to healthy cells. This suggested that AML MNCs were more responsive to BMP4 due to the high number of cells expressing the receptor at the membrane. We have confirmed this, by treating MNCs from both AML and normal samples with soluble BMP4. Percentage of BMPR1A<sup>+</sup> population in MNCs from AML samples increased significantly while no difference was observed in healthy samples. In addition, high expression of BMPR1A and  $\Delta$ Np73 was identified at transcript level after treatment. Thus, we have reported for the first time the activation of a new signaling cascade, in which the binding of BMP4 to BMPR1A induces  $\Delta$ Np73 expression. These three elements are responsible for driving stem-like features in AML leukemic cells as reported in our study.

Confirmation of the binding between BMP4 and BMPR1A was performed using the AML cell line KG1A. We used an antibody to target the receptor and treated the cells with soluble BMP4. Induction of  $\Delta$ Np73 was significantly reduced in cells where the receptor BMPR1A was blocked.

High expression of BMPR1A at the membrane of leukemic cells, opens the door for future research to develop therapies targeting this receptor overexpressed in AML blasts. Monoclonal antibodies in cancer treatment represent a promising field. For instance, anti-EGFR antibodies have been used in colorectal, breast, lung and head and neck cancer. The antibody binds to the EGFR receptor domain causing inhibition of the downstream targets, reporting an inhibitory effect in cancer cell cycle leading to apoptosis<sup>319</sup>. A similar mechanism could be used in AML, by blocking the BMPR1A receptor and disrupt the interaction of the receptor with the cytokine BMP4.

Crosstalk between BMPs and  $\Delta$ Np73 in AML are described for the first time with our study. And probably, this new crosstalk could be involved in other types of leukemia or solid cancers. Previously the homolog protein  $\Delta$ Np63 was identified to be regulated by BMP4 in epidermal development<sup>320</sup> and also the BMP signaling has been linked to a network involving p53,  $\Delta$ Np63 $\alpha$  and SMAD signaling in EMT and metastasis<sup>321</sup>.

Deciphering all the elements involved in this new signaling cascade that we have described, is crucial in order to understand completely the mechanism by which leukemic cells acquire the immature phenotype that renders them more resistant to therapies. A future perspective is to determine whether the canonical or non-canonical pathway is involved in  $\Delta$ Np73 induction. For instance, ID1 induction has been reported through both pathways. In hepatocellular carcinoma (HCC), SMAD1/5/8 phosphorylation has been described for its role activating ID1, causing proliferation of HCC cells and anti-apoptotic activity<sup>322</sup>. Besides, activation of the non-canonical MAPK pathway has been described in activation of ID1 in prostate cancer growth and tumorigenesis<sup>323</sup>.

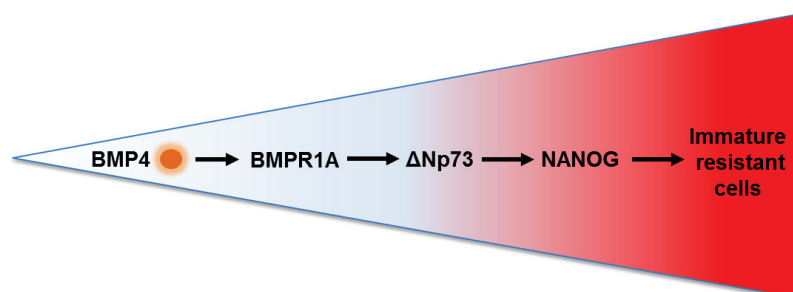
## **6 Stemness genes involved in AML**

Expression of p73 isoforms is associated with differentiation during embryogenesis<sup>324</sup> and the p53 family has been related with regulation of stemness state and reprogramming of somatic cells<sup>325</sup>.  $\Delta$ Np73 has been

described due to its role in generation of induced Pluripotent Stem Cells (iPSCs) and the capacity to induce the stem cell gene NANOG<sup>326</sup>. Based on this, as we identified the overexpression of  $\Delta Np73$  in MNCs from AML patients, we analyzed the expression of genes involved in stemness maintenance in MNCs. Using MNCs from AML at diagnosis and healthy BM samples, we detected high transcript levels of NANOG and SOX2 in AML MNCs compared to healthy cells. While no difference was observed in OCT4 transcript expression.

The overexpression of NANOG presented a high significant correlation with BMPR1A transcript high expression. mRNA levels of NANOG were higher in MNCs expressing BMPR1A at the membrane, as showed in the RT-qPCR performed in BMPR1A<sup>+</sup> and BMPR1A<sup>-</sup> populations. In addition, AML MNCs treated with soluble BMP4 increased their NANOG expression at transcript and protein levels. And we have confirmed that ectopic  $\Delta Np73$  expression is able to induce NANOG, while NANOG is not capable to induce the expression of  $\Delta Np73$ .

Therefore, these results demonstrate how BMP4 seems to act as an element reprogramming leukemic cells into a more immature phenotype, through the activation of the signaling cascade *BMP4-BMPR1A- $\Delta Np73$ -NANOG* (**Figure 24**). In our hypothesis, the stem-like features in leukemic cells would be responsible to render the cells more resistant to chemotherapy and able to cause relapse and poor prognosis in AML. As previously mentioned, the modulation of BMPR1A or  $\Delta Np73$  represents an attractive target that may drive immature leukemic cells more sensitive to chemotherapy.



**Figure 24. Signaling cascade driving the immature phenotype in AML leukemic cells<sup>327</sup>.** Soluble BMP4 binds to the BMPR1A receptor inducing the expression of the

isoform  $\Delta Np73$ , which is able to induce NANOG expression. This will print an immature phenotype in the leukemic cells and may be responsible for resistance to chemotherapy.

## **7 Potential prognosis markers in AML**

We have identified overexpression of BMPR1A/ $\Delta Np73$ /NANOG at diagnosis in AML MNCs. These three markers are part of the signaling cascade responsible for the immature phenotype of the leukemic cells. This suggests that these overexpressed elements represent key factors in leukemic stem cell resistance and may be related to prognosis. When we analyzed the clinical outcome of AML patients three years after diagnosis, we identified that patients with high transcript levels of BMPR1A,  $\Delta Np73$  or NANOG reported an increased rate of relapse within three years compared to patients with low expression. Moreover, AML patients presenting high expression of the three markers increased the clinical predictability, showing that 86% of these patients relapsed within three years.

The three markers that we have identified in AML, have been described separately in certain types of cancer as prognosis factors. For instance, reduced expression of BMPR1A receptor has been linked to poor prognosis in pancreatic cancer<sup>328</sup> contrary to what we observed in AML, whereas high expression of the receptor is observed in liposarcoma related to poor prognosis<sup>313</sup>.  $\Delta Np73$  has been described as a poor prognosis marker in lung cancer<sup>130</sup> and APL<sup>132</sup>. And high NANOG expression has been reported as a poor prognosis marker in lung<sup>329</sup>, digestive system and head and neck cancers<sup>330</sup>.

Identification of new prognosis markers in AML at diagnosis can help to improve the selection of more suitable therapeutic strategies, based on the relapse risk of the AML patients using these new markers. The combination of known molecular prognosis markers with BMPR1A/ $\Delta Np73$ /NANOG that we have described in our study, represents promising parameters which can allow in the coming years, establishment of a new classification of risk of relapse and prognosis in AML patients.

## 8 $\Delta$ Np73 overexpression in AML relapse patients

AML patients at relapse displayed higher transcript expression of  $\Delta$ Np73 compared to patient samples at diagnosis. In our previous study we already reported the role of  $\Delta$ Np73 as part of the signaling cascade promoting an immature phenotype of the leukemic cells. In our hypothesis, the stem-like state caused in part by  $\Delta$ Np73 overexpression, seems to be linked to persistence of leukemic stem cells able to escape chemotherapy leading to relapse in AML patients. As we also reported, the isoform represents a potential prognosis marker which overexpression is associated with an increased rate of relapse in AML. In concordance with our last results, high transcript levels of  $\Delta$ Np73 have been identified in cervical cancer, observed in 70.8% of patients who faced recurrence of the disease<sup>331</sup>. While in ovarian cancer high levels of  $\Delta$ Np73 were linked to patients with poor response to chemotherapy and high recurrence of the disease<sup>332</sup>. In addition, the overexpression of the isoform was reported in acute promyelocytic leukemia linked to high risk of relapse and poor survival<sup>132</sup>.

For this part of our study we had access only to data generated from peripheral blood (PB) samples from AML patients at relapse. Thus, an interesting perspective is to analyze expression of  $\Delta$ Np73 and other elements of the BMP signaling as receptors, target genes or related stemness genes, in AML samples at diagnosis and relapse obtained from bone marrow (BM). Due to the fact that Cheung and colleagues have identified that AML myeloid blasts holding a stem-like state CD34<sup>+</sup>, possessed different gene expression between those obtained from PB and BM. Chemotaxis, stress response, proliferation and apoptosis are some of the functions regulated by these genes identified to have different transcript levels, probably modulated by the tumor niche<sup>333</sup>.

Although NANOG expression was not significantly higher at relapse, a tendency of higher transcript levels at relapse compared to diagnosis was observed. Interestingly, in other types of cancer as renal cell carcinoma (RCC) NANOG expression was identified at the nucleus and the cytoplasm, linked to recurrence of the disease<sup>334</sup>. An interesting perspective is the use of a larger cohort of AML patient samples at relapse to analyze the expression of not only NANOG but

other elements involved in the BMP signaling. Since, BMPs have been linked to recurrence in certain types of cancer. For instance, overexpression of BMP4/6/7/9 have been related to recurrence and poor prognosis in hepatocellular carcinoma<sup>335</sup>.

A follow up analysis represents another interesting and significant perspective. The evaluation of intrinsic, extrinsic and associated elements of the BMP pathway at different stages of the disease as diagnosis, post-treatment and relapse, might reveal significant data linked to alteration of these factors in relapse.

Therefore, identifying the main elements involved in AML relapse might help us to decipher the mechanism of resistance employed by LSCs escaping to chemotherapy. Modulation of the factors involved in relapse, could allow us to target the LSC population and reach their complete eradication in patients.

## **9 The new described signaling cascade promoting stem-like features is linked to short-term resistant AML leukemic cells**

Resistance to chemotherapy has been mainly related to the presence of LSCs capable to endure treatment and persist within the tumor niche<sup>336</sup>. With our last data, we have identified that AML leukemic cells that survived to a short exposure of cytarabine, displayed higher transcript levels of BMPR1A,  $\Delta$ Np73, NANOG, SOX2 and ID1 compared to untreated samples. These genes were described in our previous study, as part of the activation of a signaling cascade promoting immature features in the leukemic cells. In this research study, we have reported that the expression of these genes is at least partially responsible, for granting a resistant capacity to AML blasts against ARA-C.

In concordance to the gene profile we have identified in short-resistant AML leukemic cells. High transcript levels of these genes have been reported in chemoresistance by other authors. For instance, BMPR1A receptor is able to drive chemo-resistance in colon cancer. This was confirmed after deletion of the receptor, which increased the sensitivity of cells to drug therapy<sup>337</sup>.  $\Delta$ Np73 has

been linked to radioresistance in cervical cancers<sup>338</sup>. In addition, high levels of  $\Delta$ Np73 have been reported to impact the stemness expression of pluripotency factors as CD133, NANOG and OCT4. This seems to increase chemoresistance in melanoma and lung carcinoma cell lines<sup>315</sup>. Moreover, elevated NANOG expression has been related to tumor cell radioresistance in breast cancer<sup>339</sup> and oral squamous carcinoma<sup>340</sup>. Interestingly, NANOG has been described due to its capacity to reprogram prostate cancer cells increasing their drug resistance capacity<sup>341</sup>. These findings are consistent with our results showing the importance of  $\Delta$ Np73 and NANOG induction by BMP4 and BMPR1A, promoting the reprogramming of the leukemic cells into a stem-like phenotype and enhancing their resistant capacity to chemotherapy.

Another element in our results that has been identified to be highly expressed in short-resistant AML leukemic cells is the target gene of the BMP pathway ID1. Involved in blocking of differentiation, this gene has been reported to be elevated in AML patients in almost all FAB AML subtypes (M0-M6)<sup>342</sup>. In concordance with our results, ID1 expression has been reported in AML resistant cell lines to cytarabine<sup>343</sup>. But also, this gene has been linked with resistance to cyclophosphamide in CML cell lines<sup>344</sup>. Zhang and colleagues have suggested with their study, that ID1 plays a crucial role as anti-apoptotic factor and increases resistance of cancer cells to therapy. In this study, down-regulation of ID1 in five cancer cell lines (nasopharyngeal carcinoma (CNE1), hepatocarcinoma (Huh7), cervical carcinoma (HeLa), breast cancer (MCF7) and prostate cancer (PC3)) sensitized the cells against six chemotherapy agents. Thus, these studies suggest the crucial role played by ID1 in resistance. Due to its anti-apoptotic role reported by Zhang and collaborators, another interesting perspective would be to study the correlation between ID1 and  $\Delta$ Np73, and a possible mutual regulation or synergic effect of these two elements might be involved in resistance.

## **10 AML resistant cell lines present a similar gene profile expression as short-term resistant AML MNCs**

In order to better describe the mechanisms of resistance, we obtained AML resistant cell lines corresponding to different FAB subtypes. According to preliminary data that we have generated, higher transcript levels of BMPR1A,  $\Delta$ Np73 and ID1 were identified in 7 out of 10 resistant cell lines compared to their sensitive counterpart. While NANOG was observed to be higher in 6 resistant cell lines out of 10 compared to the sensitive cell lines. These findings correlate with the results obtained in short-term resistant MNCs, where high expression of these genes has been observed.

Considering the gene profile of each AML resistant cell line and the stemness phenotype of the parental sensitive cell lines, we selected KG1A cell line to start to characterize the elements involved in resistance. Interestingly, sensitive KG1A cells presented the normal CD34<sup>+</sup>CD38<sup>-</sup> phenotype, whereas resistant cells generated by Pr. Olivier Haurault's laboratory displayed an unusual CD34<sup>-</sup>CD38<sup>-</sup> phenotype. These results raised the hypothesis that loss of CD34 expression might be linked either to a normal mechanism present in resistance or due to the drift of KG1A resistant cells into a subline caused by the long culture during its generation.

For this reason, we decided to develop a new KG1A resistant cell line, in order to evaluate if the loss of the CD34 marker was also present in this cell line and therefore, linked to a normal process in resistance.

After a long and chronic exposure to ARA-C, we obtained KG1A cells resistant to 0.2 $\mu$ g/ml ARA-C. Preliminary data generated showed that resistant KG1A cells displayed high transcript levels of BMPR1A,  $\Delta$ Np73, NANOG and ID1 as observed in the cell line obtained from Pr. Olivier Haurault's laboratory. Conversely, our KG1A resistant cells presented CD34 expression at the membrane. Further studies are necessary to completely characterize the cell line that we have generated and understand the gene expression at transcript and protein level of the elements involved in resistance. In addition, evaluation of the



expression of BMP receptors at the membrane might generate crucial data to understand the resistance mechanism to ARA-C.

Within the perspectives is the use of this cell line and the others obtained from Pr. Olivier Herault, is the modulation of the BMP pathway and target genes to evaluate significant changes in resistance. The key elements that might be identified in these cell lines will represent crucial factors that can be also modulated in primary samples, which can lead to overcome resistance in AML.

# **V. CONCLUSIONS**

## Conclusions

In this research project, we have identified extrinsic alterations of the BMP pathway within the tumor niche of AML patients at diagnosis. High concentrations of the cytokines BMP2 and mainly BMP4 were measured in BM AML plasma samples at diagnosis. The leukemic cells (LC) are not responsible for this BMPs overproduction since their transcript levels are significantly lower as compared to healthy MNCs. This suggests the involvement of other cellular components of the niche, on charge of producing the excess of cytokines.

Intrinsic alterations of the BMP signaling have also been reported in this project, by identifying high transcript expression of the BMPR1A receptor and of the target gene of the pathway ID1 in MNCs from AML samples at diagnosis. In addition, we have shown that leukemic cells have a higher BMPR1A membrane expression and in consequence they are more responsive to soluble BMP4. These findings allowed us to describe for the first time a signaling cascade triggered by BMP4-BMPR1A binding, which is able to induce  $\Delta$ Np73 and NANOG expressions, promoting a stem-like phenotype in AML leukemic cells confirmed by the functional assay LTC-IC and ALDH activity. This suggests that the immature phenotype induced in LCs seems to be linked to their resistance capacity against chemotherapy. Moreover, we have identified that high BMPR1A,  $\Delta$ Np73 and NANOG expressions in patients at diagnosis are associated with an increased rate of relapse within three years, suggesting these factors as new potential prognosis markers in AML.

Finally, by analyzing PB samples from AML patients at relapse, we have detected higher  $\Delta$ Np73 expression compared to patients at diagnosis. This data led us to evaluate the status of BMP signaling elements and related genes in resistance. Interestingly, we have identified in short-term resistant primary AML leukemic cells, higher transcript levels of the elements involved in the signaling cascade promoting immaturity (BMPR1A/ $\Delta$ Np73/NANOG) and other factors such as SOX2 and ID1 compared to sensitive MNCs. The use of AML resistant cell lines allowed us to generate preliminary data, in which we have found higher expression of BMPR1A,  $\Delta$ Np73, NANOG and ID1 in certain resistant cell lines

compared to sensitive cells. These results suggest the key role of the BMP signaling and its associated genes in the resistant capacity of AML leukemic cells, which might be related to relapse and poor prognosis in AML patients.

The involvement of elements and target genes of the BMP signaling pathway in the regulation of LSCs in AML has been described in this research study. The immature phenotype promoted by these factors in leukemic cells seems to play a key role in resistance and relapse. Modulation and targeting of these elements represent a promising approach towards the development of new and more effective therapeutic strategies against AML.

# VI. SCIENTIFIC COMMUNICATIONS

## Oral communications

- **23ème Congrès du Club Hématopoïèse et Oncogénèse 2016.**  
BMP pathway in Acute Myeloid Leukemia (AML):  $\Delta Np73$ , NANOG and Leukemic stem cells.  
12 – 15 October 2016. Presqu'île de Giens, France
- **Journée Scientifique de la SFR Santé Lyon Est 2018.**  
A new signaling cascade linking BMP4, BMPR1A,  $\Delta Np73$  and NANOG impacts on stem-like human cell properties and patient outcome.  
8 November 2018. Lyon, France.

## Posters

- **Cancer Symposium CRCL 2016.**  
BMP pathway in acute myeloid leukemia (AML):  $\Delta Np73$ , NANOG and leukemic stem cells.  
November 2016. Lyon, France. **3<sup>rd</sup> Place prize**
- **3rd International Cancer Symposium CRCL 2017.**  
Intrinsic and extrinsic BMP pathway alterations promote leukemic immature cells through  $\Delta Np73$  and NANOG induction, associated with a poor clinical outcome.  
25 – 27 September 2017. Lyon, France.
- **3ème Congrès du GDR 3697 MICRONIT «Microenvironnement des Niches Tumorales» 2018.**  
Intrinsic and extrinsic BMP pathway alterations promote leukemic immature cells through  $\Delta Np73$  and NANOG induction, associated with a poor clinical outcome.  
31 January – 2 February 2018. Marseille, France.
- **Forum de la Recherche en Cancérologie du CLARA 2018.**  
Intrinsic and extrinsic BMP pathway alterations promote leukemic immature cells through  $\Delta Np73$  and NANOG induction, associated with a poor clinical outcome.

3 – 4 April 2018. Lyon, France. **Best poster prize**

- **Scientific Days: Biologie Moléculaire Intégrative et Cellulaire (BMIC) Doctoral School 2018.**

A new signaling cascade linking BMP4, BMPR1A,  $\Delta$ Np73 and NANOG impacts on stem-like human cell properties and patient outcome.

27 – 28 November 2018. Lyon, France. **Best poster prize**

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## **viii. APPENDICES**

# 1 Review: The BMP pathway: A unique tool to decode the origin and progression of leukemia



## REVIEW

### The BMP pathway: A unique tool to decode the origin and progression of leukemia

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**The microenvironment (niche) governs the fate of stem cells (SCs) by balancing self-renewal and differentiation. Increasing evidence indicates that the tumor niche plays an active role in cancer, but its important properties for tumor initiation progression and resistance remain to be identified. Clinical data show that leukemic stem cell (LSC) survival is responsible for disease persistence and drug resistance, probably due to their sustained interactions with the tumor niche. Bone morphogenetic protein (BMP) signaling is a key pathway controlling stem cells and their niche. BMP2 and BMP4 are important in both the normal and the cancer context. Several studies have revealed profound alterations of the BMP signaling in cancer SCs, with major deregulations of the BMP receptors and their downstream signaling elements. This was illustrated in the hematopoietic system by pioneer studies in chronic myelogenous leukemia that may now be expanded to acute myeloid leukemia and lymphoid leukemia, as reviewed here. At diagnosis, cells from the leukemic microenvironment are the major providers of soluble BMPs. Conversely, LSCs display altered receptors and downstream BMP signaling elements accompanied by altered functional responses to BMPs. These studies reveal the role of BMPs in tumor initiation, in addition to their known effects in later stages of transformation and progression. They also reveal the importance of BMPs in fueling cell transformation and expansion by overamplifying a natural SC response. This mechanism may explain the survival of LSCs independently of the initial oncogenic event and therefore may be involved in resistance processes. © 2018 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.**

During embryogenesis and development, the fate of stem cells (SCs) is guided by intrinsic and environmental cues, such as surrounding cells, matrix, and soluble factors. During cancer development, the maintenance and evolution of cancer stem cells (CSCs) also depends on their microenvironment. This has been particularly demonstrated in the hematological field, where the differentiation hierarchy from hematopoietic stem cells (HSCs) is a model and in which the existence of CSCs was first shown [1–4]. Among the soluble molecules

involved in SC fate, the bone morphogenetic proteins (BMPs) have been shown to regulate self-renewal and differentiation during development [5] and are emerging as potential regulators of CSCs [6–8].

BMP proteins belong to the transforming growth factor beta (TGF $\beta$ ) superfamily of proteins, including TGF $\beta$ , activin, inhibin, growth differentiation factor (GDF), Nodal, Lefty, and anti-Müllerian hormone [9,10]. Although they belong to the same family of proteins, BMPs and TGF $\beta$  are often reported to display opposite effects on cell regulation. Moreover, even if BMP and GDF have high sequence homology [11], they also have different properties and functions [12]. BMP elements, initially described in bone formation, are now largely documented as being associated with the biology of a large

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number of organ systems, from embryonic to adult tissues, and are involved in proliferation, differentiation, and apoptosis of SCs. This major role in several biological processes and tissue homeostasis is illustrated by the fact that lack of soluble BMPs or BMP receptors is lethal or induces various severe pathologies [13].

**BMP signaling pathway**

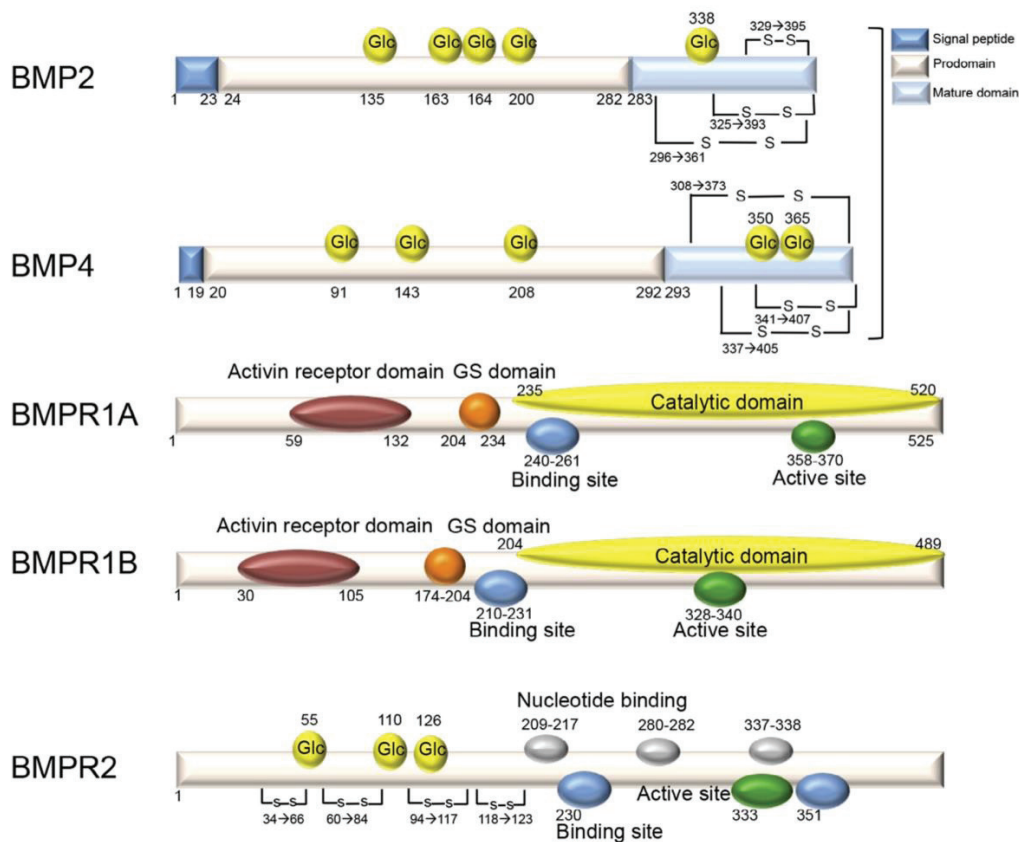
According to phylogenetic trees, BMPs can be clustered into groups: BMP2/4, BMP5/6/7/8, BMP9/10, and the GDF 5/6/7 (Table 1) [12]. BMP2 and BMP4, which present structural

homologies (Fig. 1), are secreted as precursor molecules and then cleaved by extracellular proteins such as Furin to obtain a mature protein (Fig. 2) [14–16]. These active proteins form homocomplexes or heterocomplexes, which bind to receptor complexes and induce cell transduction (Table 1) [17]. The BMP canonical pathway requires BMP type I receptor activation (BMPRIa/ALK3, BMPRIb/ALK6, or ACTRIa/ALK2), which bind and are phosphorylated by BMPR2 [10,18,19]. This phosphorylation leads to further SMAD1/5/8 phosphorylation, resulting in BMP activity as a transcription factor [20–22]. Alternatively, BMP can activate noncanonical pathways such as APK [23], p38 [24], ERK [25], JUNK [26], PI3K [27], and PKC [28].

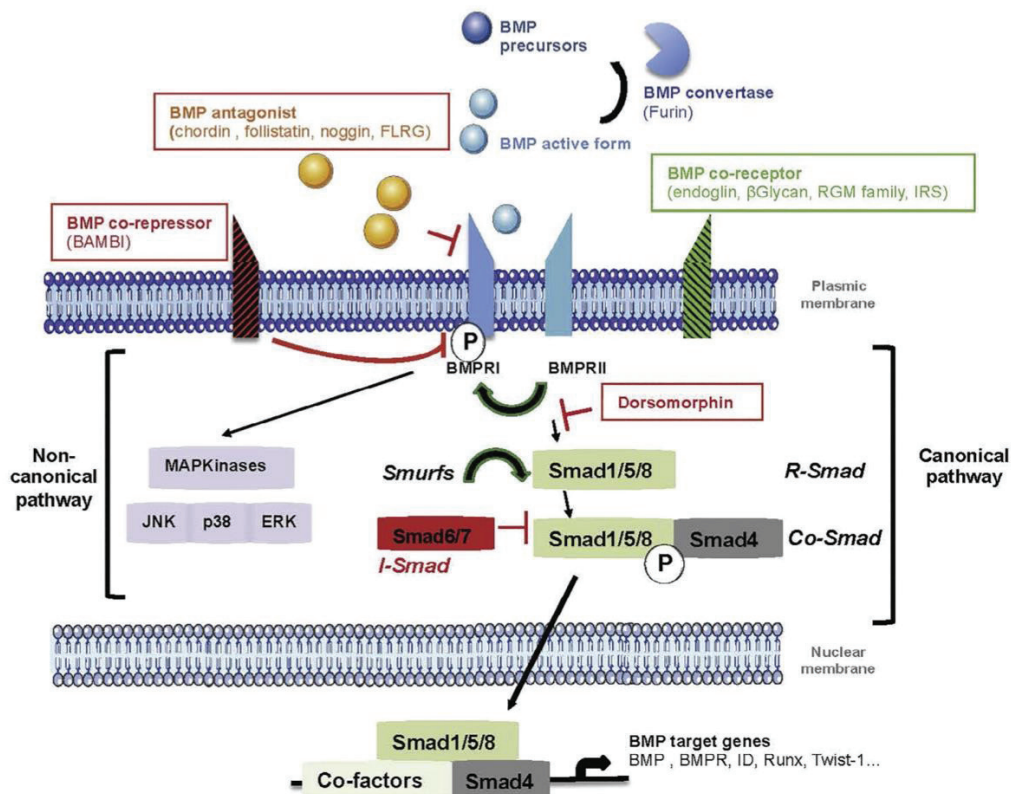
BMP signaling regulation is controlled through extracellular, membrane, and intracellular elements (Fig. 2). Extracellular soluble antagonists such as Chordin [29,30], Follistatin [31], FLRG [32], Noggin [33,34], Gremlin [35], Cerberus [36], and Tolloid [37] bind BMPs and prevent their binding to the receptor, thus modifying the local concentration of the available ligand. Other BMP regulators act at the membrane level to either activate (Endoglin, Endofin, and RGM-Repulsive guidance family members) or inhibit

**Table 1.** BMP family ligands and receptors

BMP Ligand	Associated Type I Receptor	Associated Type II Receptor
BMP2, BMP4	BMPRIa, BMPRIb	BMPR2, ActRIIa
BMP5, BMP6, BMP7	BMPRIa, BMPRIb, ALK1/2	BMPR2, ActRIIa, ActRIIb
BMP9, BMP10	BMPRIa, BMPRIb, ALK1	BMPR2, ActRIIa, ActRIIb
GDF5, GDF6, GDF7	BMPR2, ActRIIa,	BMPRIa, BMPRIb



**Figure 1.** Structure and protein domains of soluble human BMP2/4 and its associated receptors.



**Figure 2.** Schematic representation of the BMP signaling pathway.

(BAMBI: BMP and Activin membrane-bound inhibitor) the BMP pathway, according to the context, through direct binding with BMP (RGM) or both BMP and BMP receptor (Endoglin, BAMBI) [38]. BAMBI binds with type II receptors to prevent the formation of the molecular complex that transduces the signal [39]. Conversely, certain coreceptors of the BMPR (such as RGMa/b/c and DRAGON [40,41]) associate with a pre-formed complex to enhance the BMP pathway. Finally, cytoplasmic SMAD6/7 molecules inhibit SMAD1/5/8 phosphorylation and therefore preclude complex formation with the SMAD4 cofactor; they also recruit SMAD ubiquitin ligases (SMURF1/2) to mediate their degradation [42]. Importantly, the mechanisms by which the BMP pathway regulate cell fate also depend on interactions and crosstalk with other signaling pathways such as TGF $\beta$  [6], Wnt/ $\beta$ -catenin [43], Janus kinase (JAK)/signal transducer and activator of transcription (STAT) [44,45], and Ca<sup>2+</sup>/calmodulin [46].

#### Biological functions of the BMP pathway during normal hematopoiesis

BMP molecules in the regulation of hematopoiesis have been documented as being involved in various levels of SC differentiation [47–51]. BMPs exhibit a highly important role

in the formation of hematopoietic and endothelial precursors emerging from the ventral mesoderm [52]. Indeed, BMP2 and BMP4, alone or in combination with Activin A, have been shown to be involved in the regulation of erythropoiesis in various models [52–56]. In particular, in *Xenopus laevis* embryos, ectopic expression of BMP4 results in the induction of several specific hematopoietic genes, such as transcription factors and the erythroid-specific globin [52,56–58].

Bmp2, Bmp4, Bmp7, Bmpr1a, and Bmpr2 knock-out mice therefore have a lethal phenotype due to a lack of formation of hematopoietic tissues or a defect in the development of mesoderm or endoderm [13]. In vitro, BMP4 plays an important role in the induction of hematopoietic differentiation in human and murine embryonic stem cell models [53,59]. In mice, BMP coreceptor expressions have been detected during hematopoietic system development in early immature cells that display hematopoietic and endothelial potential [60]. Indeed, the induction of endothelin-dependent hematopoiesis requires response to Bmp2 and Bmp4 modulation in the yolk sac. Moreover, a mouse knock-out model of Smad5 invalidation leads to a delayed lethal phenotype during development due to a yolk sac circulatory defect associated with

an inhibition of progenitor expansion [61,62]. Homozygous *Bmp4*-deleted mice display a lethal phenotype characterized by reduced extraembryonic mesoderm including blood islands [63]. More recently, it has been demonstrated that BMP pathway activation occurs in all hematopoietic cells during the hematopoiesis-emerging phase in aorta gonad mesonephros [64]. Interestingly, in the following steps of the hematopoietic process, these HSCs could distinguish themselves as being BMP-positive or BMP-negative and generate a majority of cells no longer activated by this pathway. These findings suggest a role for the BMP signaling axis in the regulation of HSC heterogeneity and lineage output. The role of *Bmpr1a* in regulating adult HSC development has been studied *in vivo* by analyzing mutant mice with conditional inactivation. These mice have an increased number of spindle-shaped N-cadherin CD45.2 osteoblastic (SNO) cells, associated with an increased HSC number. This study showed that the *Bmpr1a* signaling pathway allows long-term HSC adhesion to SNO cells by the molecules N-cadherin and  $\beta$ -catenin and regulates the niche size [65].

In human adult cells, phenotypic analysis revealed the expression of BMP receptors such as *BMPR1a* and *BMPR1b* and the expression of the effector elements of the canonical pathway, *SMAD1/5/8*, in *CD34<sup>+</sup>CD38<sup>-</sup>Lin<sup>-</sup>* cells [66]. Treatment of *CD34<sup>+</sup>CD38<sup>-</sup>* human cells with high concentrations of BMP2 and BMP7 blocks their proliferation while maintaining their immature phenotype. Conversely, treatment of the same subpopulation of cells with high concentrations of BMP4 maintains their ability to reconstitute immunodeficient mice. Conversely, low doses of BMP4 enhance cell proliferation rather than differentiation. In the same manner, *CD34<sup>+</sup>* cells are expanded when treated with low doses of BMP7 without affecting their engraftment capacity in immunodeficient mice [67]. In addition, BMP4 regulates homing of murine and human hematopoietic stem/progenitor cells through the direct regulation of Integrin- $\alpha$ 4 expression in SMAD-independent p38 MAPK-mediated signaling [68]. BMP2 or Activin A treatment alone decreases the expression

of GATA-2 and increases the expression of EPO-R on *CD34<sup>+</sup>* cells. *SMAD6* overexpression blocks erythropoiesis *in vitro* by transcriptional regulation of *KLF1* and *GATA-2* and indirectly participates in HSC maintenance by blocking the differentiation process [69]. Conversely, only simultaneous treatment of BMP4 with Activin A is able to modulate erythropoiesis in a Follistatin- and FLRG-mediated way [31].

In the murine system, BMP4 is able to induce erythrocyte differentiation of *CD34<sup>+</sup>* cells in the presence of stem cell factor (SCF) and erythropoietin (EPO) [70]. Comparatively, in human cells, BMP4 in combination with various hematopoietic cytokines, including EPO, is able to induce several blood lineages from other human tissues [49]. This unexpected cell fate has been demonstrated using human muscle and neural gestational tissue suspension cultured in a serum-free medium with interleukin (IL) 3, IL6, SCF, and FLT-3 ligand with addition of EPO and BMP4 [49]. Conversely, BMP4 alone is able to induce human megakaryopoiesis from *CD34<sup>+</sup>* cells through the JAK/STAT and mammalian target of rapamycin pathways [51]. These data clearly showed that, unlike the murine system and despite their very high protein sequence homology and similar use of *BMPR* signaling, BMP2- and BMP4-soluble molecules exhibit very distinct effects on human HSCs. Interestingly similar observations were made for BMP2 and BMP4 on the control of other human adult tissue stem cells such as mammary epithelial SCs [71].

#### BMP pathway in early phase of myeloid leukemia

Although BMP signaling is an important element from embryogenesis to adult tissue homeostasis, deregulation of this signaling pathway is involved in several types of cancer, mainly during late stages of solid tumor evolution [6,13,72]. Alterations in the BMP pathway have also been reported in some hematological diseases (Table 2). The existence of LSCs has been demonstrated in acute myeloid leukemia (AML), in which the deregulation of various signaling pathways in the bone marrow gives rise to an altered HSC with a strong

**Table 2.** BMP signaling pathway deregulation in hematological disorders

Hematological Disorder	Tumor Microenvironment Alteration	Soluble BMP	Signaling Element	Target Gene Modified	Reference(s)
CML	High concentration soluble BMP2/BMP4	BMP2/BMP4	high <i>BMPR1B</i> p-SMAD 1/8	<i>TWIST-1</i>	[73–75]
AML	Unknown	BMP4	BMP type I	<i>MIXL-1</i>	[76]
Acute megakaryoblastic leukemia (AMKL)	Unknown	BMP2/BMP4	BMP type I	<i>Id1</i>	[77]
Acute promyelocytic leukemia (APL)	Unknown	BMP4/BMP6	High receptor: <i>BMPR1A/BMPR2/ACTRIIB</i>	<i>Id1/Id2</i>	[78]
B-cell CLL (B-CLL)	Unknown	Unknown	High receptor: <i>BMPR1A/BMPR1B/BMPR2</i> (progressive increase with advanced stages)	Not studied	[79]
ALL	Unknown	BMP2	Unknown	Unknown	[80]
CLL	Unknown	Unknown	SMAD 1/8	Unknown	[81]

capacity for self-renewal and quiescence for tumor initiation and growth [2]. Moreover, the bone marrow niche appears to be a major factor in granting a protective microenvironment for LSCs, contributing to resistance against chemotherapy and patient relapse [1,73,82].

A unique and outstanding model to address key questions about LSCs is chronic myelogenous leukemia (CML). Indeed, this leukemia arises from the transformation of a true SC by the BCR-ABL oncogene. Without treatment, this disease evolves to an inexorable and fatal blast crisis. Our team revealed both intrinsic and extrinsic deregulation of the BMP pathway in CML as early as at the time of diagnosis in chronic phase [74]. We demonstrated higher expression levels of the BMPRIb receptor ALK6 in CD34<sup>+</sup> cells, together with the existence of several molecular and functional alterations of the BMP pathway in chronic-phase CML. We also detected high concentrations of the soluble cytokines BMP2 and BMP4 produced exclusively by the tumor microenvironment. These alterations participate in the leukemic phenotype through their involvement in the survival of LSCs and in the expansion of leukemic myeloid progenitors. These data were further confirmed by other groups showing that BMP2/BMP4 and BMP7, together with their receptors and SMAD proteins, contribute to myeloid cell alteration, giving rise to CML LSCs [73,83]. Our study demonstrated for the first time that very early transforming events initiate intrinsic deregulation of the BMP signaling pathway in stem cells. Indeed, introduction of the BCR-ABL oncogene results in an increase in BMPRIb cell membrane expression [74]. This deregulation is then specifically amplified in transformed cells by exposure to exogenous ligands such as BMP2/BMP4 provided by the SC microenvironment. Very surprisingly, we were able to decipher a similar mechanism in early phases of luminal breast cancer under the driving signals of estrogeno-mimetic pollutants [71]. We demonstrated that bisphenol or radiation are able to initiate both intrinsic [84] and extrinsic [71] deregulation of the BMP pathway in human mammary SCs and their surrounding stromal cells to initiate epithelial SC transformation [85]. These data suggest that, in addition to already documented roles in the late stages of the disease, deregulation of the BMP pathway in both SCs and their surrounding microenvironment could constitute a more general mechanism during the early stages of cancer development.

Later during disease progression, the CML leukemic bone marrow niche that produces high levels of BMPs can be suspected to be at the origin of secondary myelofibrosis. Indeed, this alteration, often reported upon CML evolution, represents an overproduction of extracellular matrix that has been previously attributed to excessive BMP cytokines within the BM niche [86].

#### **BMP pathway and resistance to treatment of LSCs**

Recently, two independent studies highlighted the important role played by the tumor niche in LSC resistance in CML. Using coculture experiments of stromal and CML cells, the

impact of cell–cell interactions in resistance and in the regulation of cytokine production was identified [87]. In this context, activation of the BMP-restricted phospho-SMAD 1/8 was identified in CML cells resistant to the highly specific BCR-ABL tyrosine kinase inhibitor. Moreover, our team revealed that alterations in the BMP pathway persist in the cells of chronic-phase CML patients under treatment, especially in the LSC compartment. Indeed, despite the effects of long-term specific BCR-ABL tyrosine kinase inhibitors, the BMP pathway remained deregulated at both the intrinsic and extrinsic leukemic levels, especially in resistant cells [75]. In addition, we showed that alterations in both LSCs and their surrounding stromal cells continue to evolve upon treatment and fuel LSC survival and treatment escape. In particular, continuous treatment pressure forced the development of a growth factor autocrine loop within the highly plastic LSC compartment. We showed that a fraction of bone marrow surviving LSCs then rely on the installation of this BMP4/BMPRIb autocrine loop that involves downstream TWIST-1 overexpression to escape treatment killing. Interestingly, we previously identified the embryonic basic helix-loop-helix transcription factor TWIST-1 to be overexpressed in immature compartments and to represent at diagnosis a predictive factor of CML treatment resistance [88]. TWIST-1 overexpression promotes cell growth, tumor-initiating properties, and drug resistance and increases clonogenic capacities in other myeloid leukemias [89,90]. Interestingly, TWIST-1 can be a regulator or a target of the BMP pathway in different cell models [91,92]. In the context of CML resistance during the chronic phase, our data clearly identified TWIST-1 as a downstream target of the BMP4/BMPRIb autocrine loop [75]. This situation likely reflects evolution with time and upon treatment adaptation, in particular of the leukemic niche, as suggested in other cancers [93]. It also argues that alteration of the BMP signaling pathway contributes to treatment survival of a subfraction of LSCs independently of the BCR-ABL oncogene because we showed that it remained efficiently targeted within these cells.

While monitoring the evolution of the BMP pathway in a CML-resistant patient over almost a decade, we identified a progressive overproduction of the BMP4-soluble signal that dominated BMP2 in both LSCs and their altered bone marrow mesenchymal stem cells. This correlates with patient evolution toward the advanced transformed stage of CML [75]. Interestingly, BMP4 has been associated with the more aggressive AML [76]. Indeed, BMP4 has been involved in the induction of the *MIXL1* gene upon binding to its type 1 receptor, BMPRI, and through the activation of SMAD signaling (SMAD5). The *MIXL1* factor exerts an anti-apoptotic function that can participate in the drug resistance of AML cells. In addition, activation of BMP signaling has been demonstrated in pediatric acute megakaryoblastic leukemia, where the fusion protein CBFA2T3-GLIS2 is responsible for BMP2/BMP4 and *ID1* overexpression and linked to an increase in self-renewal capacity for the hematopoietic progenitors [77].



Conversely, in the context of acute promyelocytic leukemia, a type of AML with a high rate of complete remission in patients treated by all-*trans* retinoic acid, resistance to treatment can be observed in some patients. In this particular subtype of adult AML, the mechanism of resistance seems to involve *BMP4/BMP6* gene expression that could control target genes involved in a differentiation block such as *Id1* and *Id2*. Interestingly, this mechanism has been correlated with the PML/RAR $\alpha$  fusion oncogene, which prevents differentiation of abnormal promyelocytes and induces resistance [78]. Last, an important role of BMPs has been identified in MSCs of the leukemic niche to support AML cell growth [94]. These combined data clearly point toward the BMP pathway as one of the major signaling pathways involved in all steps of myeloid malignancies.

#### BMP pathway and lymphoid malignancies

Various research groups have started to investigate the role of the BMP pathway in lymphoid leukemia. Using different patient cohorts, BMP2 overexpression has been identified in pre-B acute lymphoblastic leukemia (ALL) compared with CD34<sup>+</sup> healthy cells, but not in T-cell ALL. This BMP2 overexpression was associated with alteration of other genes involved in the dialogue of immature leukemic cells with their bone marrow microenvironment [80]. In addition, overexpression of BMPR1a/BMPR1b and BMPR2 receptors was detected at the cell membrane of B-cell chronic lymphoid leukemia (CLL) leukemic cells. Moreover, disease progression seemed to be accompanied by a more pronounced overexpression of BMPR1a and BMPR1b, as measured in samples from patients in more advanced stages [95]. These findings suggest that overexpression of the receptor could favor the proliferative process. Finally, analysis of gene expression of the BMP signaling pathway in a cohort of 160 CLL patient samples showed alterations of *SMAD1/8* that appeared overexpressed and correlated with a decrease in *SMAD4* expression compared with control samples. High levels of *SMAD1/8* transcripts expression were associated with poor prognosis [81]. Interestingly, gene expression analysis of two independent datasets showed that the levels of inhibitory Smads varied across different B-cell lymphomas [79]. In this context, the BMP pathway acts as a negative factor with exogenous BMPs, especially BMP7, that inhibit DNA synthesis of lymphoma cells. SMAD7 overexpression in cancer cells is sufficient to escape the negative effects of BMPs by inhibiting SMAD 1/5/8 signaling.

The studies discussed here suggest that, in addition to the context of myeloid cells and solid tumors, alterations of the BMP signaling pathway may also be involved at different stages of the transformation process of lymphoid cells. However, these findings will require further investigation because they are still in their early stages.

#### Conclusion: BMP pathway as a new therapeutic avenue

The use of chemotherapy combined with inhibitory molecules against essential elements of the deregulated signaling

pathways implicated in LSC regulation is emerging as a new and promising therapeutic choice. This could be applied to the BMP pathway in CML because we showed that BMPR1b overexpression is responsible for overexpansion of LSCs and resistance [75], suggesting the use of a combined conventional therapy with an effective BMPR1b inhibitory molecule. Depending on cancer type, BMP signaling needs to be either activated or inhibited. In several types of leukemia, BMP seems to be the key for better patient outcome [96]. This inhibition of the pathway can be achieved, for instance, by trapping the extracellular BMP ligands from the microenvironment using antagonist molecules such as Noggin, Chordin, or Gremlin to prevent ligand–receptor interaction. For example, inhibitors of type I BMP receptor such as Dorsomorphin or LDN-193189 molecules avoid the binding of the ligand to its receptor [96]. The use of several inhibitory molecules against BMP type I receptors has indeed been initiated in the context of adult AML using LDN-193189 to restore chemotherapy sensitivity [76]. Targeting the intrinsic and extrinsic elements of the BMP pathway for novel therapeutic approaches promises to be an important tool for effective long-term cancer SC eradication according to our recent findings and to the recurrent alterations of this pathway in hematological malignancies and solid tumors [72,96,97]. However, elucidating the functional importance of BMP receptor localization within different cellular compartments remains a major unresolved issue. Therefore, the development of more specific antagonist or inhibitory molecules will be necessary to target the relevant element without affecting other ligands and to preserve the normal function of this very pleiotropic and fundamental signaling pathway. Nevertheless, modulation of the BMP signaling pathway is a different and unique way to develop innovative patient treatments that could simultaneously take into account the important involvement of this pathway in the dynamic evolution of the tumor ecosystem.

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#### Conflict of interest statement

The authors declare no competing financial interests.

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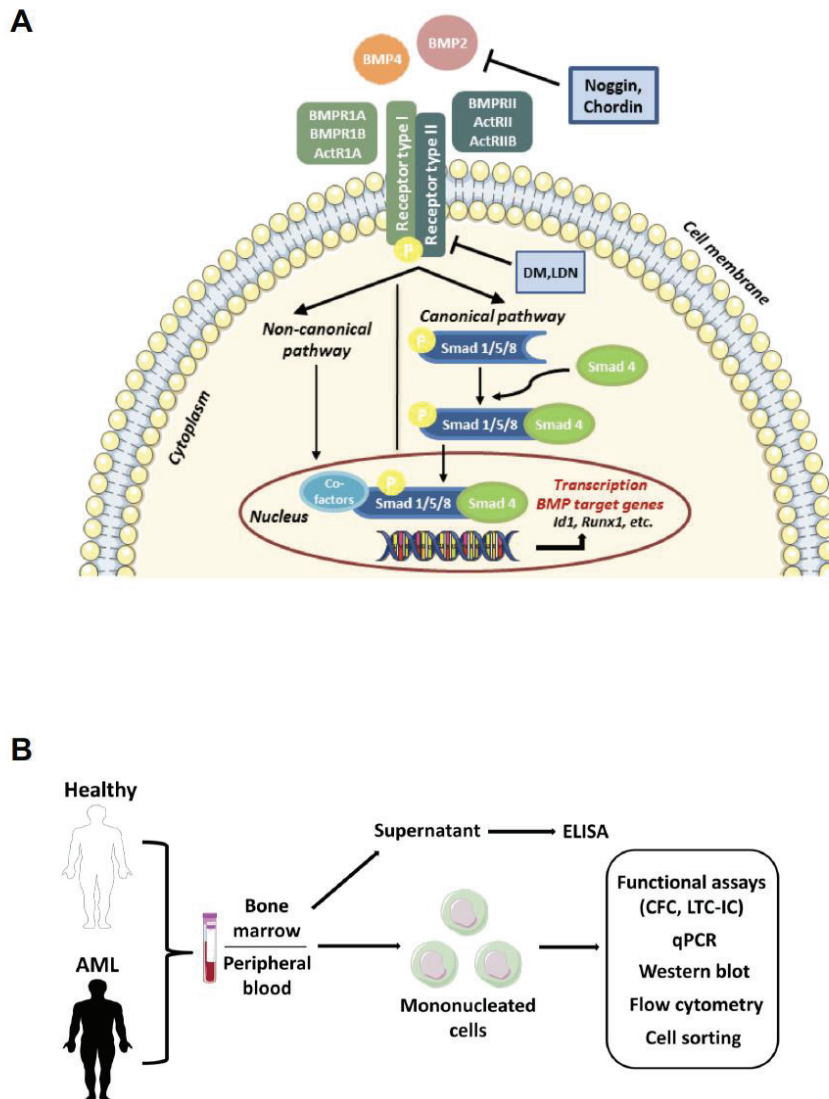
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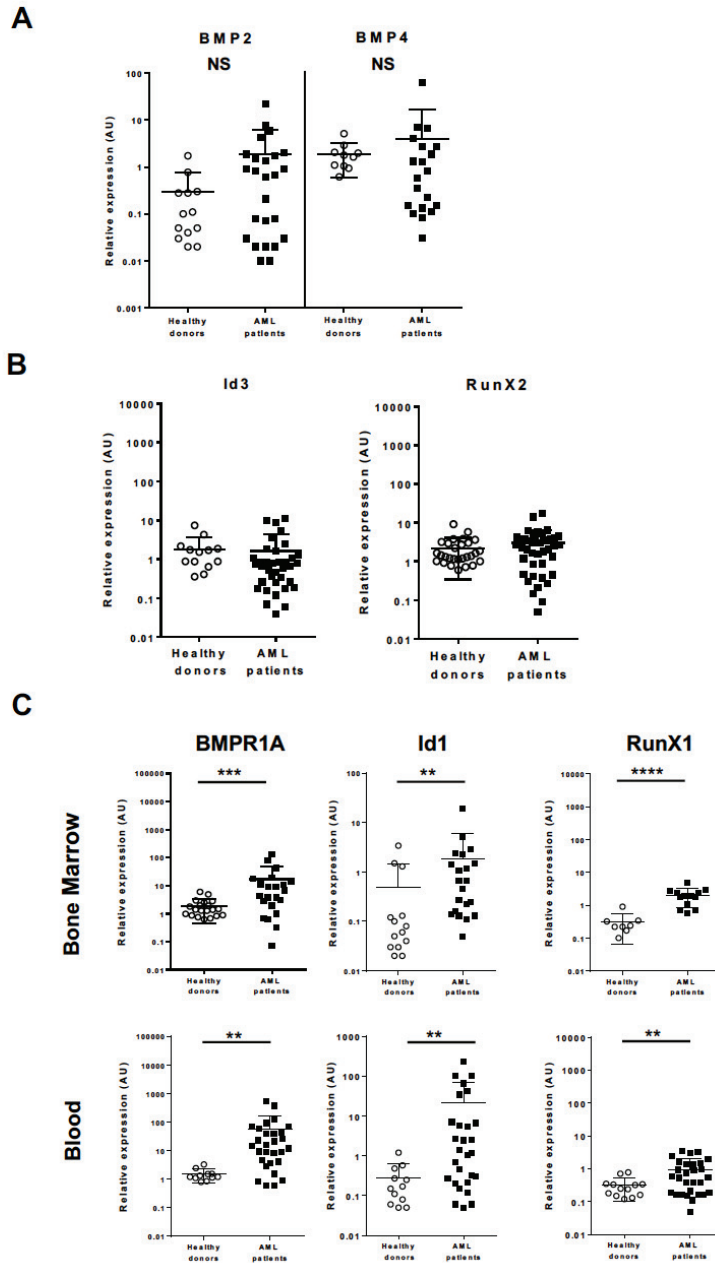
## 2 Article 1: Complementary figures

Voeltzel et al. Fig. S1

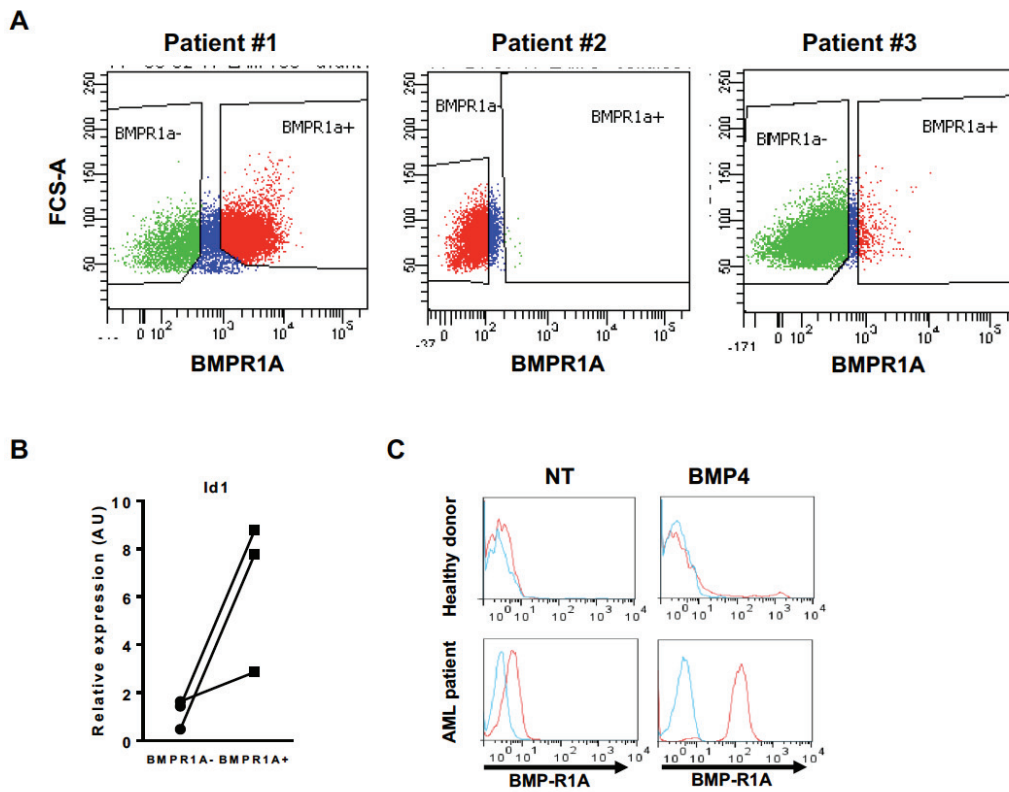
**Figure S1:** (A) Schematic diagram of the bone morphogenetic proteins (BMP) pathway. (B) Patient sample collection protocol and experiment design.



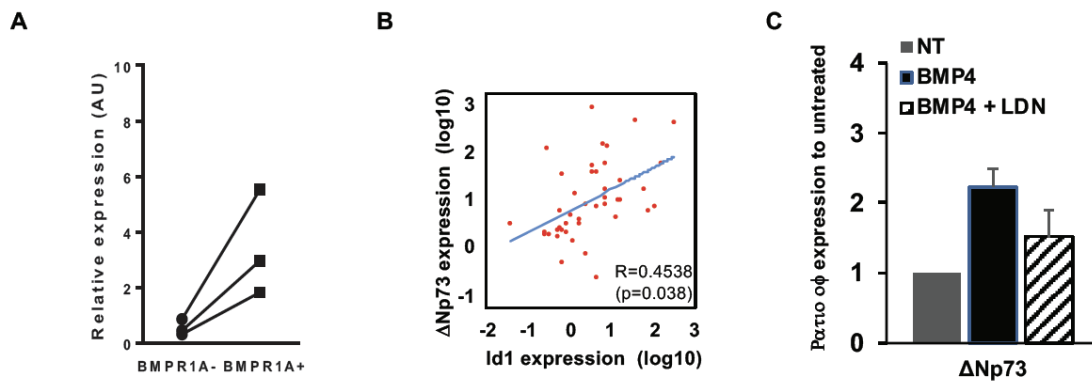
**Figure S2: Expression of BMP2 and BMP4 and some members of the BMP pathway in healthy and AML samples. (A)** BMP2 and BMP4 mRNA level in peripheral blood MNCs from healthy donors and AML patients samples at diagnosis. **(B)** Id3 and RunX2 mRNA level in MNCs of grouped blood and BM samples. **(C)** BMPR1A, Id1 and RunX1 mRNA level in BM and blood MNCs from healthy donors and AML patients samples at diagnosis. Expression was evaluated by RT-qPCR. . P values are symbolized by stars: \*P<0.05; \*\*P<0.01; \*\*\*P<0.001; \*\*\*\*P<0.0001.



**Figure S3: Expression of BMPR1A and Id1 in AML samples. (A)** FACS sorting of MNC from three AML patients samples according to the presence of BMPR1A at their surface. **(B)** Expression of Id1 evaluated by RT-qPCR in AML MNCs sorted according to their surface expression of BMPR1A (n=3). **(C)** Effect of BMP4 (10ng/mL) exposure on the proportion of BMPR1A-positive cells at the surface in healthy and AML MNC samples. Representative sample of one healthy donor.



**Figure S4: Relationship between BMP pathway activation and DNp73 expression. (A)** Expression of  $\Delta Np73$  evaluated by RT-qPCR in AML MNCs sorted according to their surface expression of *BMPR1A* (n=3). **(B)** Correlations between the expressions of  $\Delta Np73$  and *Id1* (n=50) in AML samples at diagnosis. *P*-values were determined using Spearman's non-parametric test. **(C)** Relative expression of  $\Delta Np73$  in KG1A cells exposed to BMP4 (10ng/mL) for 24 hours with (n=8) or without (n=5) the BMPR1 inhibitor LDN-193189 (20nM). Expression was measured by RT-qPCR and compared to untreated (NT) KG1A cells.





### 3 Article 1: Supplementary information

Patient	Sex	Age	AML subtype	MDS sign	Secondary AML	Karyotype	Karyotype group	Prognosis group	Sampling date	Sample type	Sample at diagnosis	% of Blasts	Treatment before diagnosis	Treatment after diagnosis	Detailed treatment	Complete remission	Remission date	Follow-up treatment	Relapse	Relapse date	Date of latest news	State to the latest news	BMPA-IA	Δnp/3	Nanog
LAM146	1	1	5	0	0	normal	3	3	28/1/02	2	1	70	1=cytoreductive 2=chemotherapy 3=allograft 4=hydroxyurea	0= no 1=cytoreductive 2=chemotherapy 3=allograft 4=hydroxyurea	ALFA800	1	02/04/02	0	1	17/15/03	11/09/04	1	19/01	542.34	17.26
LAM138	1	2	4	NE	NE	46,XY,t(21)	NE	NE	5/1/04	2	NE	7	2	2	LYM1418.95	1	01/15/05	0	1	3/17/05	6/20/05	1	371.09	372.59	476.63
LAM170	2	1	1	0	0	echec	NE	NE	8/2/05	2	NE	90	NE	NE	3+7	0	9/13/05	1	1	9/13/05	9/13/05	1	58.11	286.78	12.33
LAM205	1	1	NE	1	NE	46,XX	2	NE	30/10/06	1	0	NE	NE	NE	ALFA802	0	02/07/05	1	1	08/13/05	12/19/04	1	124.15	276.28	119.43
LAM152	2	1	5	0	0	46,XX	2	1	28/12/04	2	1	70	0	0	ALFA802	0	27/06/2007	1	1	9/13/05	27/06/2007	1	6.80	116.87	36.73
LAM169	2	1	1	0	0	echec	2	NE	15/2/04	2	1	95	0	0	ALFA802	0	06/08/04	1	1	9/13/05	9/13/05	1	42.76	103.89	5.94
LAM157	1	1	1	0	0	46,XY	2	2	14/1/05	2	1	70	0	0	ALFA802	0	01/19/04	1	1	9/13/05	6/10/04	1	97.34	100.59	11.94
LAM137	2	1	1	0	0	normal	2	2	15/7/05	2	0	NE	NE	NE	ALFA802	1	02/18/02	1	0	12/15/03	11/04/04	4	23.37	94.03	337.79
LAM130	1	1	1	0	0	normal	2	2	16/12/03	1	0	NE	NE	NE	ALFA802	1	07/18/03	1	0	12/15/03	11/04/04	4	8.82	69.88	26.72
LAM154	1	1	5	0	0	normal	2	1	17/6/03	1	1	50	NE	NE	ALFA802	1	11/12/06	1	0	10/15/08	2/15/08	1	530.27	51.80	13.74
LAM205	1	1	5	0	0	46,XY	2	NE	30/10/06	1	1	90	NE	NE	ALFA802	1	04/08/05	1	0	9/14/05	9/14/05	1	38.87	48.00	66.26
LAM135	1	2	4	0	0	46,XY	2	2	29/4/04	1	1	80	0	0	Daunorubicine Anzelyne	1	06/08/04	1	1	3/10/05	9/11/05	1	69.46	45.27	22.81
LAM133	2	1	5	0	0	normal	2	NE	19/2/04	1	1	50	0	0	Daunorubicine Anzelyne	1	03/10/05	2	0	6/29/15	6/29/15	4	25.91	33.92	11.04
LAM159	2	1	1	0	0	46,XY,t(20)	2	3	17/6/05	1	1	66	0	0	Daunorubicine Anzelyne	1	06/06/05	1	0	11/17/05	3/15/06	1	16.01	11.28	3.15
LAM230	2	1	0	0	0	normal	2	NE	6/2/17	1	1	31	0	0	ALFA802	1	08/03/05	0	1	11/17/05	3/15/06	1	31.3	10.45	8.65
LAM132	2	1	1	0	0	46,XY	2	NE	3/7/05	1	1	86	NE	NE	ALFA802	1	4/10/05	1	1	4/10/05	3/8/10	1	17.1	33.21	26.17
LAM239	1	1	5	0	0	normal	2	1	17/6/03	2	1	90	0	0	ALFA802	1	07/11/03	1	0	6/10/08	6/10/08	1	17.60	32.13	26.17
LAM166	1	1	5	0	0	normal	2	3	25/1/08	3	0	90	0	0	ALFA802	1	03/17/08	1	1	10/7/08	4/12/10	1	9.42	22.59	9.33
LAM251	2	1	5	0	0	normal	2	2	6/2/17	2	1	63	0	0	Idarubicine Anzelyne	1	12/27/16	1	0	3/28/08	3/28/08	1	10.91	15.87	1.33
LAM208	2	1	4	0	0	46,XY	2	NE	21/2/08	1	1	75	NE	NE	ALFA801	0	12/19/03	1	1	1/6/08	1/6/08	3	68.50	15.73	10.70
LAM237	1	2	NE	0	0	ECHec	NE	NE	25/11/03	1	1	25	0	0	ALFA801	1	1/6/08	1	1	1/6/08	1/6/08	3	43.81	14.29	6.36
LAM250	2	1	5	0	0	normal	2	2	6/2/17	1	1	63	0	0	Idarubicine Anzelyne	1	08/03/05	0	1	11/17/05	3/15/06	1	16.01	11.28	3.15
LAM138	2	1	1	0	0	46,XY,t(7)	3	NE	17/6/05	1	1	86	NE	NE	ALFA802	1	08/03/05	0	1	11/17/05	3/15/06	1	31.3	10.45	8.65
LAM230	2	1	0	0	0	normal	2	NE	6/2/17	1	1	31	0	0	ALFA802	1	08/03/05	0	1	11/17/05	3/15/06	1	16.01	11.28	3.15
LAM236	1	1	5	0	0	normal	2	1	13/12/16	3	0	90	NE	NE	No.	0	8/2/08	1	1	4/10/09	12/15/16	1	21.45	8.54	6.14
LAM252	2	1	5	0	0	NE	NE	NE	4/2/17	2	1	NE	NE	NE	NE	NE	NE	0	0	12/27/16	2/13/17	4	14.03	7.65	4.70
LAM243	2	2	5	0	0	46,XX	2	1	25/11/16	2	1	17	NE	NE	Idarubicine Anzelyne	1	12/27/16	1	0	3/28/08	3/28/08	1	4.69	7.49	3.43
LAM209	1	2	2	1	NE	46,XX	2	NE	13/3/08	1	1	NE	NE	NE	Idarubicine Anzelyne	0	11/29/06	1	1	1/4/08	2/15/08	1	8.21	6.59	4.40
LAM204	1	3	1	0	0	NE	47,XY,t(13)	NE	18/10/06	1	1	93	NE	NE	AML12	1	10/25/03	1	1	10/25/03	6/24/06	1	8.21	6.59	4.40
LAM204	1	3	1	0	0	NE	46,XY,t(25)	NE	13/2/03	1	1	31	0	0	AML12	1	10/25/03	1	1	10/25/03	6/24/06	1	8.21	6.59	4.40
LAM250	2	1	2	0	0	46,XY	2	2	4/17/16	2	1	63	0	0	Daunorubicine Anzelyne	1	07/17/13	1	0	9/6/12	05/11/12	4	3.82	5.62	0.54
LAM250	2	1	2	0	0	46,XX	2	2	17/11/16	2	1	83	0	0	Daunorubicine Anzelyne	1	07/17/13	1	0	9/6/12	05/11/12	4	3.82	5.62	0.54
LAM242	2	1	4	0	0	46,XY,t(8)	1	2	17/11/16	2	1	83	0	0	Daunorubicine Anzelyne	1	12/14/12	1	0	2/13/17	2/13/17	1	4.27	4.86	3.97
LAM229	1	1	4	0	0	46,XY	2	2	30/6/16	2	1	0.3	0	0	CTTAAABINE AMACARINE	1	08/09/16	2	0	3/6/12	05/14/12	4	9.13	4.56	3.37
LAM121	2	1	0	0	0	46,XX,t(20)	2	2	15/6/08	1	1	14	0	0	ALFA702	1	08/17/08	1	0	3/6/12	05/14/12	1	1.55	3.79	1.24
LAM186	1	1	2	1	0	del(5,-19)	2	3	7/2/07	1	1	NE	0	0	ALFA801	0	02/04/07	2	1	7/2/08	05/02/2009	1	0.84	3.79	4.01
LAM193	1	1	1	1	0	46,XY	2	2	16/11/06	1	1	19	0	0	AML12	1	02/04/07	2	1	7/2/08	05/02/2009	1	0.82	3.79	3.40
LAM195	2	1	5	0	0	46,XX	2	2	15/2/07	2	1	75	0	0	ALFA802	1	02/12/07	1	1	9/1/08	7/18/13	1	0.87	3.00	2.69
LAM222	1	1	1	0	0	46,XY,t(8)	1	1	17/11/16	1	1	61	0	0	Daunorubicine Anzelyne	1	07/17/13	1	1	08/13/06	37/06/2007	5	0.86	2.76	1.19
LAM222	1	1	5	0	0	46,XX	2	1	27/12/04	1	1	85	0	0	Daunorubicine Anzelyne	1	03/07/05	1	1	10/15/05	10/15/05	1	0.86	2.76	1.19
LAM224	2	1	2	0	0	46,XY,t(8;21)	1	1	9/6/15	1	0	37	0	0	Chemotherapy	1	11/01/15	1	1	10/16/16	02/28/17	5	15.06	2.68	3.62
LAM244	1	1	2	0	0	46,XX	2	2	25/4/16	1	1	72	0	0	MYLOFRANCE	0	12/09/16	1	0	10/16/16	12/09/16	1	4.17	2.63	1.89
LAM241	1	1	2	0	0	46,XY	2	1	4/11/16	1	1	61	0	0	Daunorubicine Anzelyne	0	12/12/16	1	0	2/7/17	2/7/17	4	3.66	2.43	2.05
LAM223	1	2	2	0	0	46,XY,t(8;21)	1	1	11/6/15	2	0	37	0	0	Daunorubicine Anzelyne	1	10/16/16	1	0	10/16/16	02/28/17	5	3.18	2.36	1.20
LAM221	1	2	2	0	0	46,XY,t(8;21)	1	1	9/6/15	2	0	37	0	0	Daunorubicine Anzelyne	1	10/16/16	1	1	10/16/16	02/28/17	5	2.81	2.07	2.46
LAM194	2	1	5	0	0	46,XX	2	2	15/10/12	1	1	17	0	0	ALFA802	1	02/12/07	1	1	9/1/08	07/18/13	1	0.82	3.00	2.69
LAM249	1	1	1	0	0	normal	2	1	2/11/17	2	1	61	0	0	daunorubicine anzelyne	1	02/09/17	1	0	7/21/08	03/21/09	1	1.00	1.01	1.76
LAM249	1	1	1	0	0	normal	2	1	2/11/17	2	1	61	0	0	daunorubicine anzelyne	1	02/09/17	1	0	7/21/08	03/21/09	1	1.00	1.01	1.76
LAM207	2	1	5	NE	NE	46,XX	2	NE	27/8/07	2	1	89	NE	NE	daunorubicine anzelyne	0	02/09/17	1	0	9/13/07	9/13/07	4	59.25	9.95	0.79
LAM244	1	1	1	0	0	t(8)(8)	3	3	5/12/16	2	1	95	1	1	Idarubicine Anzelyne	1	01/17/17	1	0	3/21/17	4	0.63	0.69	0.68	
LAM202	2	3	2	1	NE	46,XY	2	NE	9/11/04	1	1	24	NE	NE	Idarubicine Anzelyne	0	1/17/17	1	0	3					

<b>ΔNp73</b>	Forward	<b>AAG CGA AAA TGC CAA CAA AC</b>
	Reverse	<b>CAC CGA CGT ACA GCA TGG TA</b>
<b>BMP2</b>	Forward	<b>ACG CTC TTT CAA TGG ACG TG</b>
	Reverse	<b>GGA AGC AGC AAC GCT AGA AG</b>
<b>BMP4</b>	Forward	<b>CTT TAC CGG CTT CAG TCT GG</b>
	Reverse	<b>GGG ATG CTG CTG AGG TTA AA</b>
<b>BMPR1A</b>	Forward	<b>GAA AAA GTG GCG GTG AAA GT</b>
	Reverse	<b>TAG AGC TGA GTC CAG GAA CC</b>
<b>BMPR1B</b>	Forward	<b>CTG TGG TCA CTT CTG GTT GC</b>
	Reverse	<b>TTC CTT TCT GTG CAG CAT TC</b>
<b>BMPR2</b>	Forward	<b>TAG CAC CTG CTA TGG CCT TT</b>
	Reverse	<b>CTG AAT TGA GGG AGG AGT GG</b>
<b>HPRT</b>	Forward	<b>CGA GCA AGA CGT TCA GTC CT</b>
	Reverse	<b>TGA CCT TGA TTT ATT TTG CAT ACC</b>
<b>ID1</b>	Forward	<b>GGT GCG CTG TCT GTC TGA G</b>
	Reverse	<b>TGT CGT AGA GCA GCA CGT TT</b>
<b>ID3</b>	Forward	<b>AAA TCC TAC AGC GCG TCA TC</b>
	Reverse	<b>AAG CTC CTT TTG TCG TTG GA</b>
<b>NANOG</b>	Forward	<b>CAT CCC TGG TGG TAG GAA GA</b>
	Reverse	<b>CCA ACA TCC TGA ACC TCA GC</b>
<b>OCT4</b>	Forward	<b>GTG AAG TGA GGG CTC CCA TA</b>
	Reverse	<b>GAA GGA TGT GGT CCG AGT GT</b>
<b>RUNX1</b>	Forward	<b>TGG AAG AGG GAA AAG CTT CA</b>
	Reverse	<b>CCG ATG TCT TCG AGG TTC TC</b>
<b>RUNX2</b>	Forward	<b>GTG GAC GAG GCA AGA GTT TC</b>
	Reverse	<b>TTC CCG AGG TCC ATC TAC TG</b>
<b>SOX2</b>	Forward	<b>AAC CCC AAG ATG CAC AAC TC</b>
	Reverse	<b>CGG GGC CGG TAT TTA TAA T</b>
<b>TAp73</b>	Forward	<b>GCA CCA CGT TTG AGC ACC TCT</b>
	Reverse	<b>GCA GAT TGA ACT GGG CCA TGA</b>
<b>TBP</b>	Forward	<b>CAC GAA CCA CGG CAC TGA TT</b>
	Reverse	<b>TTT TCT TGC TGC CAG TCT GGA C</b>

**Table S2:** Primers used in RT-qPCR experiments

Initial Complete Remission	BMPR1A		ΔNp73		NANOG		3 Markers	
	Low	High	Low	High	Low	High	Low	High
Yes	11 (79%)	8 (67%)	7 (70%)	12 (75%)	8 (67%)	11 (79%)	6 (67%)	7 (70%)
No	3 (21%)	4 (33%)	3 (30%)	4 (25%)	4 (33%)	3 (21%)	3 (33%)	3 (30%)

**Table S3:** Number and proportion of patients that did or did not achieve complete remission after initial treatment according to expression levels at diagnosis of BMPR1A, ΔNp73, NANOG or all the 3 markers.

**Ratio of BMP4 treated/untreated**

AML sample	q RT-PCR				LTC-IC
	ΔNp73	BMPR1A	NANOG	Id1	
AML6	9.766598	1.824912	3.636595	1.666667	1.583333
AML66	4.015873	1.121495	not done	not done	6
AML136	4.79085	1.088643	1.997863	0.954357	6.666667
AML252	4.804556	1.292218	1.474508	1.127854	2.6
Mean	5.844469	1.331817	2.369655	1.249626	4.2125

**Table S4:** Correlation of molecular markers induced transcriptional expression upon BMP4 treatment of primary AML cells with functional amplification of immature cells, identified by the LTC-IC *in vitro* assay. (n=4).

Variable	Nanog < 13 (n=36)	Nanog > 13 (n=16)	p-value	Test
Age (cat), N (%)			0.334	Pearson's Chi2 Test with Monte-Carlo method
- 1	29 (80.56%)	13 (81.25%)		
- 2	3 (8.33%)	3 (18.75%)		
- 3	4 (11.11%)	0 (0.00%)		
Sex, N (%)			0.124	Pearson's Chi2 Test with Monte-Carlo method with Yates' continuity correction
- M	18 (50.00%)	12 (75.00%)		
- F	18 (50.00%)	4 (25.00%)		
AML type (cat), N (%)			0.825	Pearson's Chi2 Test with Monte-Carlo method
(n=35)				
- 0	4 (11.43%)	2 (12.50%)		
- 1	7 (20.00%)	2 (12.50%)		
- 2	5 (14.29%)	5 (31.25%)		
- 4	5 (14.29%)	2 (12.50%)		
- 5	13 (37.14%)	5 (31.25%)		
- 6	1 (2.86%)	0 (0.00%)		
2ndary AL, N (%)			0.262	Pearson's Chi2 Test with Monte-Carlo method
- No	26 (72.22%)	9 (56.25%)		
- Yes	2 (5.56%)	0 (0.00%)		
- NA	8 (22.22%)	7 (43.75%)		
Caryo group, N (%)			0.652	Pearson's Chi2 Test with Monte-Carlo method
(n=34)		(n=15)		
- 1	4 (11.76%)	1 (6.67%)		
- 2	25 (73.53%)	13 (86.67%)		
- 3	5 (14.71%)	1 (6.67%)		
Blasts				
(n=34)		(n=15)		
- mean (sd)	59.47 (28.98)*	59 (24.3)*		(Normality not assumed)
- median (min;max)	70 (4 ; 96)	60 (11 ; 90)	0.853	Mann-Whitney
TAp73				
(n=34)		(n=13)		
- mean (sd)	1.23 (1.91)*	1.54 (2.34)*		(Normality not assumed)
- median (min;max)	0.51 (0.04 ; 8.5)	0.86 (0.08 ; 9.06)	0.390	Mann-Whitney
DNP73				
- mean (sd)	9.7 (19.46)*	113.55 (110.47)*		(Normality not assumed)
- median (min;max)	2.83 (0.07 ; 100.58)	78.59 (20.25 ; 456.2)	<0.001	Mann-Whitney
SOX 2				
(n=32)				
- mean (sd)	10.02 (22.92)*	90.44 (112.66)*		(Normality not assumed)
- median (min;max)	2.8 (0.06 ; 128.22)	56.99 (3.11 ; 470.26)	<0.001	Mann-Whitney
Oct 4				
(n=33)				
- mean (sd)	4.23 (6.83)*	11.35 (5.56)*		(Normality not assumed)
- median (min;max)	2.76 (0.45 ; 40)	10.55 (1.74 ; 23.2)	<0.001	Mann-Whitney

**Table S5:** Multivariate statistical analysis.