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MicroRNA function in monocyte differentiation

Candidate: Dott.ssa Fabiana Mammoli

Supervisor: Prof. Sergio Ferrari

Direttore della scuola di dottorato: Prof. Rossella Tupler

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ABSTRACT

MicroRNAs (miRNA) are a small noncoding family of 19- to 22-nt RNAs that regulate gene expression by targeting mRNAs in a sequence specific manner.

Currently, about 1500 different human miRNAs are listed in the miRBase registry.

They are involved in the regulation of gene expression during development, cell proliferation, apoptosis and cancer.

Recent studies demonstrate a significant role of miRNAs also in normal and malignant hematopoiesis.

Hematopoiesis is a highly regulated process controlled by complex molecular events that simultaneously regulate commitment, differentiation, proliferation, and apoptosis of hematopoietic stem cells (HSC). The activation or inhibition of a network of transcription factors (TFs) is required to initiate the commitment of HSC to different lineages precursors. TFs and miRNAs act in concert to regulate gene expression during hematopoietic differentiation: TFs regulate the expression of miRNA genes, whereas TFs are miRNA targets.

miRNA misexpression may contribute to the development of hematopoietic malignancies such as acute myeloid leukemia (AML).

In a recent study, a microRNA expression analysis has been performed in AML samples, focusing on the differentially expressed microRNAs between M1 and M5 subtypes. The results of this comparison highlighted that miR-146ab, miR-181abd, miR-130a, miR-663 and miR-135b are overexpressed in M1 samples. Interestingly, the targets of these miRNAs are key transcription factors involved in monocyte/macrophage differentiation, i.e. KLF4, MAFB, HOXA10 and IRF8.

The aim of this study is to analyse the role of miRNA-130a that is upregulated in AML and probably involved in the differentiation block characterizing myelogenous leukemia.

The expression profile of miR-130a was evaluated in myeloid differentiation (HSCs, myeloblasts, monoblasts, granulocytes and monocytes) and in HSCs cells stimulated with Vitamin D3. The data obtained show a miR-130a gene expression reduction in monocitopoyesis.

In order to investigate the functional role of miR-130a, we have performed gain- and loss-of-function experiments (by means of transfection of pre-Mir or anti-miR molecules) in CD34+ cells. The differentiation capacity of treated cells was monitored by qRT-PCR, Western blot, immuno-phenotypic and functional assays.

The samples transfected with pre-miR-130 shown a slowdown of monocyte differentiation, documented by CFU assay, which shows a significant higher percentage of colonies CFU-GM and a significant smaller percentage of colonies CFU-M, and confirmed by gene expression analysis of differentiation markers (CD14, CD163 and MRC1), inflammatory cytokines (IL-1, IL-6, IL-8, IL10RB, CCL2 e TNFa) and transcription factors (CEBPβ) involved in the monocyte/macrophage differentiation that result all down regulated.

We have performed the same monocyte differentiation analysis in CD34+ cells transfected with anti-miR-130a; accordingly with what expected, in this case the data obtained show an increase of monocyte differentiation.

This study highlights the role of miR-130a in the modulation of monocyte differentiation and confirmed the hypothesis that its overexpression can contribute to the differentiation block of AML M1.

These data suggest the use of antimiR strategy to restore a correct gene expression, in order to allow the normal differentiation of myeloid progenitors.

For this reason, we have decided to use peptide nucleic acids (PNA) as an approach to silence the expression of miR-130a.

The evaluation of the monocyte differentiation in HSCs and in leukemic cell lines transfected with anti-miR130a PNA, documents an higher increase of monocytopoiesis when compared with the sample transfected with anti-miR molecules, demonstrating that the biological effect of PNAs in the modulating miRNA expression is strong and very stable.

The data obtained in this study highlight that the modulation of miR-130a expression is necessary to a correct myeloid differentiation and they form the basis for possible future use of R-pep-PNA-a130a as therapeutic agent to restore a correct gene expression in hematological malignancies like AML.

INTRODUCTION

MicroRNA

MicroRNAs (miRNA) are evolutionally conserved, small non-coding RNA of 19-22 nucleotides (nt) long. They modulate gene expression by repressing their targets translation or inducing mRNA degradation through binding to the complementary sequence in target messenger RNA .

The first miRNA was discovered in the Ambros and Ruvkun labs in 1993 when a gene, lin-4, crucial for the nematode *Caernohabditis elegans* development, was found to not encode a protein but to give rise to a small 22-nucleotide RNA. The RNA itself was responsible for silencing the lin-14 gene, via antisense complementarity to its 3' untranslated region (UTR) [1].

The second important miRNA, let-7, was identified also in the nematode *Caernohabditis elegans* in 2000 [2]. Let-7 was identified soon both in humans and in animals [3] [4] .

Subsequently, hundreds of miRNAs and their biological functions have been identified, and thus far (June 2014) 28,645 miRNAs have been registered in the miRBase database (http://microrna.sanger.ac.uk). This makes miRNAs one of the most abundant regulators of gene expression in humans.

MicroRNA genes represent 1 to 3% of the currently known genes in the human genome[5]; their genes are located in either introns (a larger number) or exons of protein-coding genes[6].

The miRNA genes are first transcribed by RNA polymerase II into primary transcripts (pri-miRNA) in the nucleus; these pri-miRNAs are long about hundreds to thousands of nt and contain a characteristic hairpin stem-loop structure. Then, a micro-

processing complex including an RNase III enzyme, Drosha, and a double-stranded RNA binding protein DiGeorge syndrome critical region gene 8 (DGCR8), cuts the hairpin stem-loop structure and process the species into precursor miRNA (pre-miRNA).

The 70-100 nt pre-miRNA is then actively exported into cytoplasm via Exportin 5, where it is cleaved by another RNase III enzyme, Dicer, resulting in a miRNA:miRNA* duplex. Then, the mature miRNA is selected and assembled into a ribonucleoprotein complex, called miRNA-induced silencing complex (RISC), which contains an Argonaute family member, that directs the identification of target mRNA [6]. The miRNA target recognition is mediated through the sequence complementary between the 2-8 nt at the 5' end of miRNA (seed sequence) and the 3'-untranslated region (UTR) of target mRNA. The mechanisms of miRNA post-transcription regulation on its targets include blocking its translation and/or inducing mRNA degradation by deadenylation and decapping. No matter which mechanism predominates, the overall output is the reduction of amount of protein encoded by the target messenger [7](Fig.1).

Nevertheless, recent advances in miRNA studies have indicated that, besides the above mentioned "canonical" mechanisms of gene regulations by miRNAs, other alternative mechanisms of miRNA regulation exist. Several studies suggested that miRNAs could use multiple mechanisms to cause the inhibition of target translation through "non-slicer" mechanism, including de-adenylation, repression of translation initiation and elongation stages, or nascent polypeptides degradation [8-13].

When targeted for silencing by miRNAs, mRNAs can be concentrated, sequestered from translational machinery, degraded or stored for subsequent use in large macroscopic cytoplasmic foci, named processing bodies (P-bodies). The P-bodies

contain a wide range of enzymes involved in RNA turnover, including de-capping enzymes, de-adenylases and exonucleases [14].

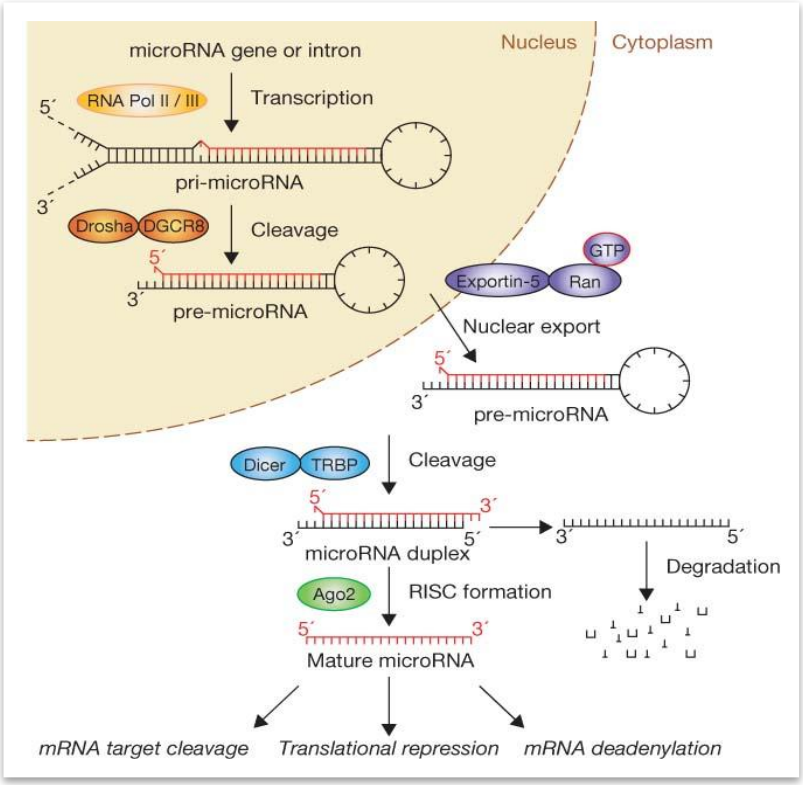

Figure 1.The 'linear' canonical pathway of microRNA processing [15]

Recent evidences indicated that miRNAs can also bind to 5'-UTR of mRNAs and that the up-regulation of the translation can, in specific conditions, result from their binding to mRNA [16]

In addition, miRNAs with a specific nuclear localization sequence can also be re-localized into the nucleus [17]. In the nucleus, miRNAs can interact with, and regulate, the expression of transcribed ultraconserved regions encoding particular sets of ncRNAs (non-coding RNA), whose expression is altered in human cancer [18].

Different sets of cells can release RNA and miRNA directly to the body fluids (as peripheral blood, plasma, saliva, and breast milk) or associated to exosome through

the exosomal pathway. Exosomes are small (50-100nm) membrane-bound vesicles of endocytic origin released by a variety of cells and able to mediate cell-to-cell communication. Usually they contain different kind of molecules as protein, mRNA and at least several RISC components including Ago2 and miRNAs [19].

MicroRNA Function in gene regulation

miRNAs represent one of the most exciting areas of modern medical sciences as they possess unique ability to modulate an immense and complex regulatory network of gene expression [20] in a broad spectrum of developmental and cellular processes including tissue development [21, 22], cell proliferation [23, 24], cell division [24, 25], cell differentiation [26], metabolism [27], stem cell proper

ties [28], apoptosis [29].

It is becoming clear that they have a big impact on shaping transcriptomes and proteomes of eukaryotes [30]. Aberration or perturbation in their expression levels has significant correlation with serious clinical consequences, including disease of divergent origin and malignancy [29, 31]. Certainly, disease-miRNAs associated represents a substantial class of targets for the miRNA-based novel therapeutic or diagnostic/prognostic biomarkers [32, 33].

In the postgenomic era, the accepted notion is that a single miRNAs can regulate hundreds of targets, even if only to a mild degree, but, conversely, several miRNAs can bind to their target mRNA and cooperatively provide fine-tuning of a single mRNA target expression [34]. Although a steeply growing computational analysis has identified a range of potential targets for miRNAs, to date, only a small number of them have been validated by experimental approaches [35, 36].

Many miRNAs have a function in biological processes and are involved in important regulatory networks.

For example, miR-34a is involved in the feedback loop with p53. It regulates p53 protein levels but it is also regulated by p53. In other cases, miRNAs have been shown to be important for cell fate decisions. In the megakaryocyte-erythrocyte progenitor, miR-150 expression drives the cell differentiation toward megakaryocytes at the cost of erythrocytes, by regulating the MYB transcription factor [37].

The miRNAs miR-1 and miR-133 are specifically expressed in skeletal and cardiac muscle tissues. This tissue specificity is conserved between species. In the genome, both miR-1 and miR-133 are located next to each other and transcribed from a single primary miRNA transcript. Both contribute to muscle differentiation but in a different manner. Expression of miR-1 in myoblast cells induces myogenesis. However, expression of miR-133 promotes the proliferation, by regulating the levels of histone deacytelase 4 (HDAC4) and serum response factor (SRF)[24].

miRNAs are also important for the self-renewal capacity of hematopoietic stem cells. One example is miR-125a that is highly expressed in long-term hematopoietic stem cells. Increased expression of miR-125a causes an increased number of hematopoietic stem cells [38].

Another example of miRNA function is their ability to generate induced pluripotent stem cells (iPSc). Anokye-Danso et al reported an efficient induction of pluripotent stem cells by the introduction of the miR-307/367 cluster, which is partially due to induction of octamer binding transcription factor 4 (OCT4) by miR-367 [39].

Since miRNAs participate in a vast array of normal functions, it seems logical that they would also be associated with abnormalities in disease states.

Deregulation of miRNA expression may have many undesired effects and contribute to the development of disease. Indeed, investigations based on this idea have

revealed that alterations in miRNA genes have a significant involvement in cancer initiation and progression [40, 41]. miRNA expression is influenced by genomic abnormalities such as chromosomal amplifications or deletions, mutations, and rearrangements, which cause aberrant gene expression when compared to normal tissues. Calin et al. [42] found that over 50% of miRNAs are overrepresented at fragile chromosomal regions prone to breakage or rearrangement, and are frequently altered in cancer. For example, two known tumour suppressor miRNAs that are clustered together, miR-15a and miR-16a, are down-regulated in over 70% of human chronic lymphocytic leukemia (CLL) and their genes are situated at the 13q14.3 loci, which is the region of deletion in human CLL [40] .

Abnormal miRNA expression is also caused by impairments of the miRNA processing machinery. Drosha upregulation is apparent in approximately half of all cases of cervical squamous cell carcinoma (SCC), likely caused by an amplification mutation at chromosome 5p where the Drosha gene is located [43]. Additionally, Melo et al. found that mutations in TRPB led to Dicer destabilization in colorectal and endometrial cell lines, resulting in the global downregulation of miRNAs [44]. This widespread miRNA downregulation has also been determined in other studies. Lu et al. [45] did a systematic analysis on 217 different miRNAs in various tumours samples such as brain, breast, lung, colon, stomach, pancreas, kidney, and found that 129 of them were downregulated in the tumours compared to normal tissues.

Other miRNA profiling studies such as that performed by Volinia et al. [45], which looked at characterization of six different tumour types (prostate, lung, breast, colon, pancreas, and stomach) have revealed that an abundance of miRNAs are both upregulated and downregulated in cancer cells compared to normal tissue [41]. Moreover, many studies have been performed such as that by Liu et al. on human

and mouse RNA profiling, which have revealed a specific pattern of miRNA expression in numerous tumour types [46, 47].

When aberrantly expressed in cancer, miRNAs can act as tumour suppressors that downregulate oncogenic mRNAs, or as oncogenes that downregulate tumour suppressor genes, both ultimately leading to deregulation of targets involved in cell survival, differentiation, growth, and angiogenesis[42, 48] (Fig.2).

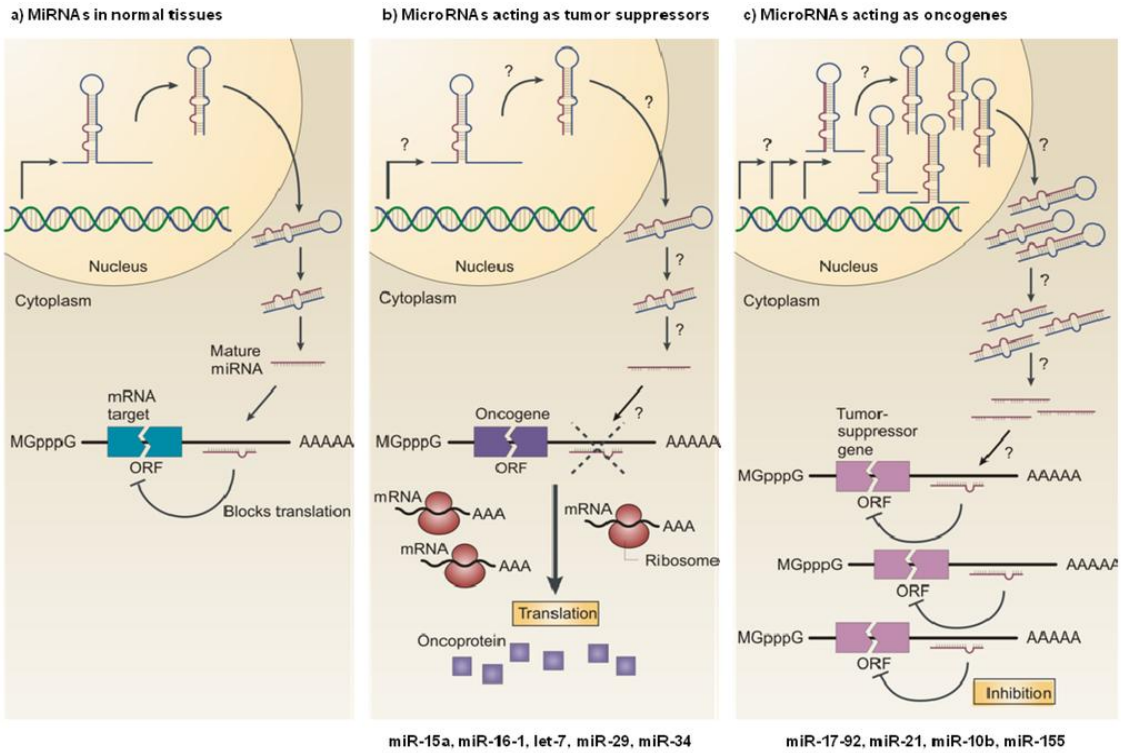

Figura 2. MicroRNAs as tumor suppressors and oncogenes. *(**a**) In normal cells, miRNA transcription, processing and binding to complementary sequences in the target mRNA lead to the repression of their target genes, by either mRNA translation inhibition or mRNA degradation. (**b**) The reduced expression of a miRNA that acts as a tumor suppressor, as a result of chromosomal deletion or defects at any stage of miRNA biogenesis (indicated by question marks) leads to the increased synthesis of the miRNA-target oncoprotein (purple squares), and ultimately to the development of an oncogenic phenotype. (**c**) The increased expression of a miRNA that acts as an oncogene, as a result of (among others) amplification of the miRNA gene or constitutive promoter activation (indicated by question marks), leads to the repression of a miRNA-target tumor-suppressor gene (pink), which favors the development of an oncogenic phenotype. ORF: open reading frame; mGpppG: 7-methylguanosine [48].*

For instance, Trang et al. [49] used a transgenic mouse model to demonstrate that the let-7 family of miRNAs act as tumour suppressors in lung cancer and negatively

regulate the expression of the Ras oncogene that contributes frequently to uncontrolled growth in tumorigenesis. Furthermore, overexpression of let-7 led to reduced tumour growth and development, while loss of the let-7 family resulted in constitutive overexpression of Ras [50].

In contrast, it has been found that miR-21 promotes cell survival and inhibits apoptosis by acting as an oncogene in cholangiocarcinoma cells and targeting PTEN (phosphatase and tensin homolog), a tumour suppressor known to inhibit cell growth and invasion [51].

miRNAs can function differently depending on the cell or tissue type and that the same miRNA can act both as a tumour suppressor and as an oncogene. For example, miR-205 is downregulated in prostate and breast cancers, but is upregulated in lung, bladder, and pancreatic cancers [52]. Additionally, miR-221 and miR-222 act as tumour suppressors in erythroblastic leukemia by inhibiting the oncogene KIT, but also act as oncogenes in CLL and hepatocellular carcinoma by targeting tumour suppressors PTEN and TIMP3 (tissue inhibitor of metalloproteinases 3) [42].

Hematopoiesis

Hematopoiesis is a hierarchical process by which multipotent stem cells (HSC) gradually reduce their differentiation potential, generating unipotent progenitors and precursors from which they originate terminally differentiated blood cells [53] (Fig.3).

At embryonic level this process takes place first in the yolk sac, then in the liver and spleen, while in adult it is located in the bone marrow, mainly in the bone of the vertebrae, pelvic bones, sternum and ribs.

The two fundamental characteristics of HSCs are the ability to give rise to cells equal to themselves, property called "self-renewal" that has the purpose to maintain a constant pool of stem cells in bone marrow and the ability to differentiate into all cell types present in the blood.

The microenvironment surrounding the HSCs within the bone marrow, is usually called “the stem cell niche” and has a crucial role for HSC function [54].

The mechanisms that induce a stem cell to self-renew or to differentiate towards well-defined hematopoietic lineages, and then to completely maturate, are thought to be finely tuned by marrow microenvironment. Different cell types reside in the bone marrow; together with the differentiated cells, they support the production of various growth factors and cytokines, which carry out their action through binding to specific receptors on the surface of the progenitors [55].

The interaction between receptor and cytokines has the dual effect to lead the increased expression of the receptor itself and to activate a specific route of signal transduction that brings to a specific differentiation.

Some of these cytokines, such as Stem Cell Factor (SCF), FLT3-ligand (FLT3-l), Interleukin 11 (IL-11), interleukin 6 (IL-6) and interleukin 3 (IL-3), are primarily responsible for proliferation of precursors cells and their commitment.

Others, such as Thrombopoietin (TPO), erythropoietin (EPO), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), Granulocyte Colony Stimulating Factor (G-CSF) and Macrophage Colony Stimulating Factor (M-CSF), are involved in precursor proliferation and expansion.

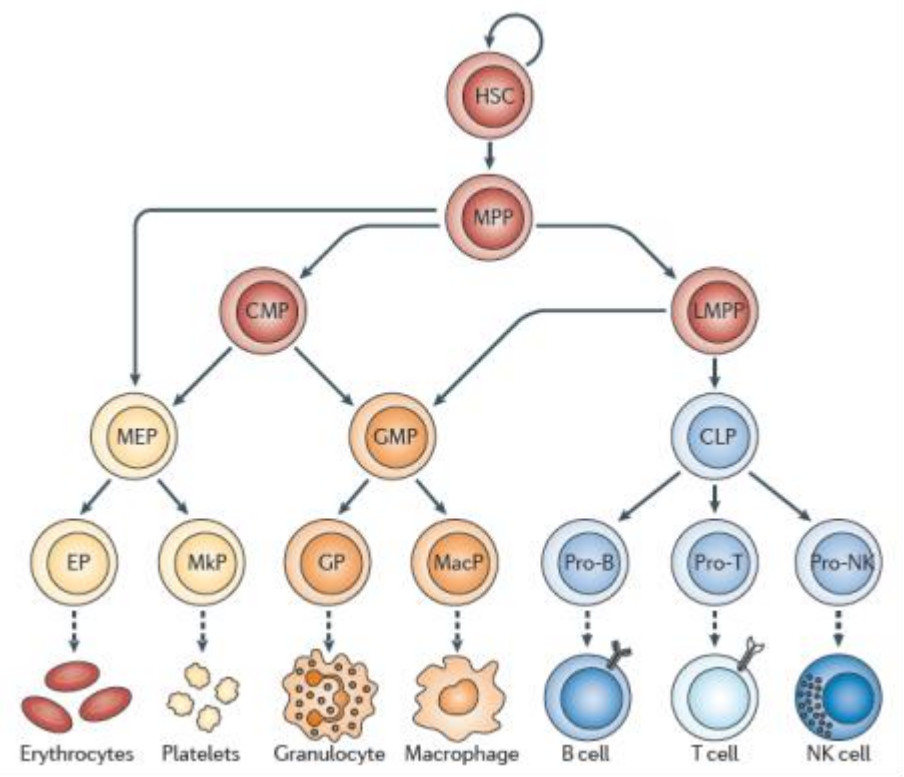

Figure 3. Hierarchy of differentiation in the haematopoietic system. *The hematopoietic stem cell (HSC) differentiates in a stepwise manner. Over the course of the differentiation process, progenitor cells choose a specific branch of the hematopoietic system and lose the potential to differentiate into other lineages until the cells reach the stage of committed progenitors, which can give rise to only one lineage. The differentiation of a committed progenitor to a mature cell is a multistage process, represented here as a dashed arrow. CLP, common lymphoid progenitor; CMP,common myeloid progenitor; EP, erythrocyte progenitor; GMP, granulocyte–macrophage progenitor; GP, granulocyte progenitor; LMPP, lymphoid primed multipotent progenitor;MacP, macrophage progenitor; MEP, megakaryocyte–erythrocyte progenitor; MkP, megakaryocyte progenitor; MPP, multipotent progenitor; NK, natural killer [56].*

At the molecular level, during the early steps of hematopoietic differentiation from the HSC, the chromatin progressively becomes more accessible at genes poised for expression, rapidly followed by an increased expression of lineage-associated genes with concomitant repression of alternative lineage genes, resulting in differentiation. These events are established by the coordinated action of microenvironmental

stimuli, transcription factors (TFs), chromatin remodeling factors and microRNAs (miRNAs) [57].

The transcription factors are able to activate specific genes that generate a cascade of gene expression response for each different lineages.

Considering the transcription factors activated by cytokines, it is possible a further distinction between factors involved in the commitment (which is a specific lineage choice) and “master regulators” (able alone to promote the commitment of undifferentiated stem cells or to cause the phenotypic switch of committed progenitors) [53].

Once HSCs divide and generate more differentiated daughter cells, within 10-15 divisions the genetic programs of the descendent cells become fixed toward a single lineage. The first decision concerns the choice to generate progeny myeloid or lymphoid. The first cells generated following such decision have been termed common lymphoid precursors (CLPs) and common myeloid precursors (CMPs).

CLP forms the whole lymphoid lineage: B lymphocytes and T lymphocytes.

CMP, also called CFU-GEMM (Colony Forming Unit Granulocyte, Eryhtroid, Myeloid, Megacaryocyte), is a cell capable of giving rise to four separate lines: granulocytic, monocytic, megakaryocytic and red cell line [58].

Myeloid differentiation

In the myeloid compartment, as in the hematopoietic system in general, the lineage-specific gene expression is controlled by a combined action of transcription factors.

The activation of different programs of differentiation occurs through the action of the “master regulators”, which lead to the induction or the inhibition of specific pathways .

To try to understand the process of myeloid differentiation, it is important to identify

and characterize the “master regulators” that selectively activate important genes for myeloid lineage.

The expression of the transcription factor PU.1 is one of the first events that lead to the commitment of HSCs to the myeloid lineage.

CMPs can differentiate into either common precursor for granulocytic and monocytic lineages (GMPs) or common precursors for both erythroid and megakaryocytic lineages (EMPs); a separate pathway leads to eosinophils (Fig.4).

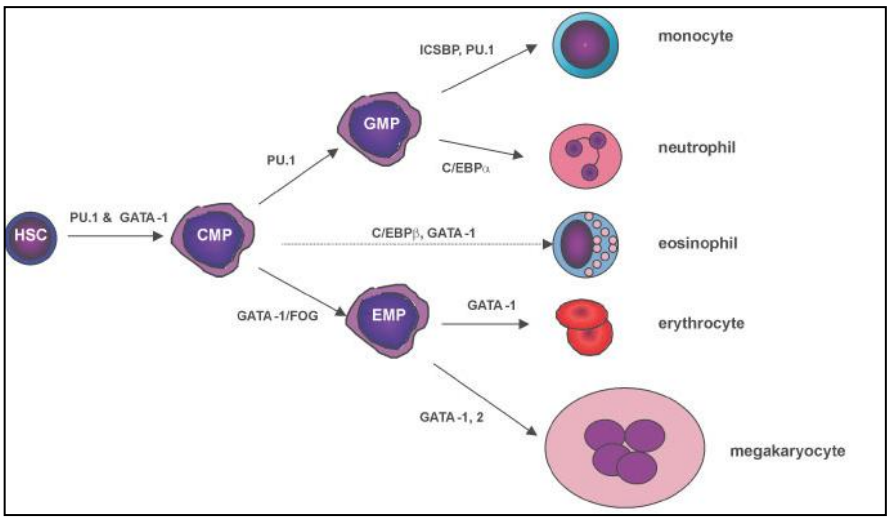

Figure 4.Transcriptional regulation of common myeloid precursor (CMP) commitment [59].

GATA and PU.1 are co-expressed in common myeloid progenitors; subsequently the expression of one or another, leads to the choice between the granulocytic/monocytic or megakaryocytic/erythroid differentiation.

The expression of GATA-1 decreases in granulocytic/monocytic differentiation while PU.1 decreases with differentiation to either megakaryocytic or erythroid direction. Then, the ratio of GATA-1/PU.1 in common myeloid precursors determines the commitment towards GMP or EMP. PU.1 was found to inhibit the transcriptional activity of GATA-1 upon its target genes, and viceversa [60].

The over-expression of PU.1 inhibits DNA binding of GATA-1 to its consensus element on target gene promoters and this inhibiting effect could be relieved by the over-expression of GATA-1.

The initial ratio of GATA-1/PU.1 protein at an early, critical time point in the CMP determines the subsequent lineage restricted fate of the cell [61, 62]. The execution of lineage-committing function of PU.1 might at least partly be mediated through the extrinsic M-CSF/M-CSFR and GM-CSF/GM-CSFR signaling pathways [60].

Co-expression of GATA-1 and FOG, instead of GATA-1 alone, destines the precursor cells to erythroid and megakaryocytic development [63]. The differential expression of FOG in eosinophils or megakaryocytic/erythroid cells directly influences the cell fate choice, and so FOG may act as either a co-activator or a co-repressor to GATA-1 in different cellular and promoter contexts [60].

In addition to these well-documented roles of PU.1, GATA-1 and M-CSFR in the commitment of GMPs and EMPs, other transcription factors and cytokines seem also to be implicated in these processes. TGF-β1 might be a potent cytokine that biases the commitment of erythroid at the expense of granulocytic and monocytic development [64].

The differential expression of C/EBPα appears to influence the lineage choice of bi-potent G/M precursors; it was found preferentially expressed and required for granulocytic lineage, but not for monocytic lineage.

The retinoid receptor RARs/RXRs might act as a differentiation-checkpoint switch at the promyelocytic stage of granulopoiesis. The C/EBPα-induced terminal granulocytic differentiation is preceded by an inhibited G1/S transition in cell cycling [60].

Several transcription factors have been shown to be implicated in the commitment of monocytes from bi-potent precursor GMPs; the interferon consensus sequence binding protein (ICSBP/IRF-8), a putative transcriptional regulator of PU.1 [65], was

recently found to be essential for monocytic development while inhibiting the production of neutrophils. ICSBP may be very crucial for maintaining normal myelopoiesis over and above its role in monocyte commitment [60]. Cell cycle arrest might be one prerequisite for the terminal differentiation of monocytic cells. ICSBP-induced macrophage differentiation coincides with cell growth arrest [66].

In erythroid commitment, different transcription factors are involved: GATA-1, LMO2, TAL1, LDB1 and E2A/E47, these lead to transcription of genes important for erythroid differentiation.

In megakaryocytic commitment the transcription factors GATA-1, GATA-2, FOG and RUNX1 are involved [67, 68].

Monocyte-Macrophage Differentiation

The mononuclear phagocyte system (MPS) is composed of monocytes, macrophages and dendritic cells (DCs) and has crucial roles in maintaining organismal homeostasis. The diverse activities of the MPS are relevant in inflammation, autoimmunity, infection, cancer and organ transplantation. The commitment to the mononuclear phagocyte lineage is determined at the stage of the macrophage and DC progenitor (MDP), when erythroid, megakaryocyte, lymphoid and granulocyte fates have been precluded (Fig.5). MDPs give rise to monocytes and common DC progenitors (CDPs) [69, 70].

While monocytes can directly participate in effector immune responses or differentiate into macrophages or DCs, the differentiation potential of CDPs is restricted to the DC lineage. CDPs give rise to plasmacytoid DCs and pre-DCs, which subsequently give rise to DCs [71, 72].

Monocytes are a conserved population of leukocytes that are present in all vertebrates. They are defined by their location, phenotype and morphology, as well as by characteristic gene and microRNA expression signatures [73-75].

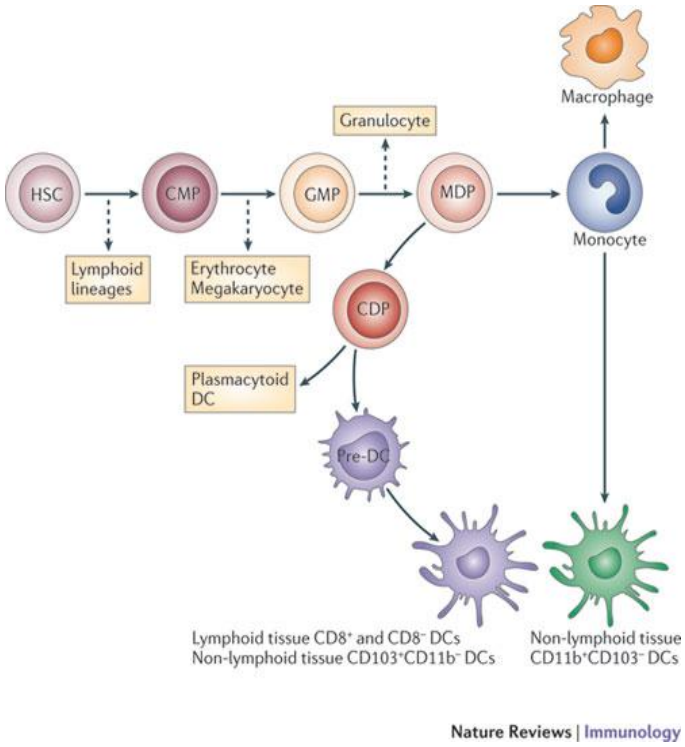

Figure 5. Lineage of mononuclear phagocytes*.Commitment to differentiation into a monocyte, macrophage or dendritic cell (DC) occurs at the stage of the macrophage and DC progenitor (MDP). MDPs can give rise to common DC progenitors (CDPs) or monocytes. CDPs are committed to the DC lineage and give rise to both CD8+ and CD8− DCs in lymphoid tissues and CD103+CD11b− DCs in peripheral non-lymphoid tissues. In addition, monocytes can give rise to some CD11b+CD103− DCs or to macrophages. CMP, common myeloid progenitor; GMP, granulocyte and macrophage progenitor; HSC, haematopoietic stem cell [76].*

In mice and humans, monocytes represent the 4% and the 10%, respectively, of the nucleated cells in the blood, with considerable marginal pools in the spleen and lungs that can be mobilized on demand [74].

Monocyte development and survival in mice completely depend on colony-stimulating factor 1 (CSF1; also known as M-CSF). Mice that are deficient in this growth factor or its receptor CSF1R (also known as CD115 and M-CSFR) exhibit severe monocytopenia [77-79]

Monocytes arise from myeloid precursor cells in primary lymphoid organs, including the fetal liver and bone marrow, during both embryonic and adult haematopoiesis.

Monocytes migrate from the bone marrow in peripheral blood as non-proliferating cells. Their half-life in peripheral blood is 1-3 days, and it is assumed that monocytes at least partially mature in circulation [80, 81] During their lifetime in circulation, they perform surveillance of the organism and comprise the first line of defence to invading pathogens and tissue injuries. They can reach any part of the body, and they are the principle source for repopulation of tissue macrophages and DCs [82-85]. Depending on the tissue environment, monocytes differentiate into macrophages that acquire tissue specificity, like splenic macrophages, alveolar macrophages in the lung, Kupffer cells in the liver, microglia cells in the brain, or osteoclasts in the bone. Monocytes might also differentiate into mDC under inflammatory conditions.

Thus, the plasticity of monocytes to differentiate into various cell types exhibits their potential to take part in a broad range of cellular processes . Since, the main function of macrophages is the phagocytosis and the central function of mDC is to process and present antigens to T-cells, monocytes are considered as the cell type that is on the cross-road between the innate and adaptive immunity.

One phenotypical characteristic of monocytes is the expression of CD14 molecule on their surface. CD14 is considered as monocyte-specific linage marker and its principal role is to cooperate with TLR4 and MD-2 in binding the LPS, which is the component of the outer membrane of Gram-negative bacteria [82].

Monocytes also express the receptors that mediate phagocytosis such as CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI) [84]. Furthemore, they express molecules indispensable for the antigen presentation and activation of Tcells, including MHC class II molecules, CD80 and CD86.

Based on the expression of surface markers CD14 and CD16, the whole population of peripheral blood monocytes in humans is divided into two subsets: the “classical or resident” monocytes, which highly express CD14 molecules and weakly CD16, (CD14hiCD16low), and the “inflammatory” monocytes that express weakly CD14 but strongly CD16 (CD14lowCD16hi) [81].

The main role of resident monocytes is to renew tissue macrophages and DCs, but also to patrol blood vessels, to scavenge oxidized lipids, dead cells and potential pathogens. The crucial role of inflammatory monocytes is to produce the pro-inflammatory mediators [83].

Coordinated regulation of gene expression by transcription factors is essential for the differentiation of immune cells[86]. Gene knockout studies have shown several transcription factors to be essential for monocyte and macrophage differentiation. PU.1, a member of the ETS family transcription factors, is necessary for the earliest steps of myeloid and lymphoid lineage commitment, and its deficiency results in loss of monocytes, granulocytes, and B cells [87, 88]. Mice transplanted with Krüppel-like factor-4 (KLF4)-deficient fetal liver cells (***Klf4***$^{-/-}$ chimera mice) have fewer monocytes [89].Others studies documentate that ectopic expression of the transcription factors MafB, c-Maf, Egr1, ICSBP/IRF8, C/EBPβ,Tf3 and HoxA10 drive monocyte/macrophage differentiation.

Below it is illustrated the role of some transcription factors involved in monocyte differentiation:

PU.1

PU.1 is one of the most important Ets transcription factors, a transcription factors family which play a key role in the growth, survival, differentiation, and activation of

hematopoietic cells and it is characterized by an 85 amino acid [90], winged helix-turn-helix DNA-binding domain.

PU.1 expression is limited to hematopoietic cells, including primitive $CD34^+$ cells, macrophages, B lymphocytes, neutrophils, mast cells, and early erythroblasts [62]. PU.1 is thus crucial in directing many facets of haematopoiesis and concordant with this, its expression fluctuates dynamically in the various haematopoietic differentiation pathways (Fig.6).

Importantly, the regulation of differentiation by PU.1 is not merely via a “presence or absence of expression” mechanism but by a dose-dependent effect. For instance, the expression of PU.1 is low in long-term reconstituting (LT)-HSCs but rises as these progenitors become more lineage restricted and form precursor cells common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). Upon further lineage differentiation and maturation, PU.1 is expressed at varied levels in mature blood cells, with higher levels found in macrophages than B cells and low levels in mature erythroid cells, megakaryocytes, and T cells [91, 92]. Moreover, not only is PU.1 differentially expressed in the various haematopoietic cells, but also lineage specification is sensitive to, and directed by, the varied dosage of PU.1 in differentiating progenitor cells. In addition, inappropriate expression of PU.1 in specific haematopoietic cells can result in leukaemic transformation, as in the case of T-cell lymphomas and, as mentioned previously, erythroleukaemias [93].

It regulates a large number of genes of the myeloid and lymphoid lineages. In myeloid lineages, the putative PU.1 target genes include G-CSF receptor, GM- CSF receptor and M-CSF receptor[94, 95]. Besides, PU.1 also transactivates other genes involved in the acquisition of a mature monocyte phenotype.

This allows monocytes/macrophages to carry out their immunological functions following an activation signal. For instance, PU.1 controls the expression of genes

encoding FcRI [96] and FcRIIIA [97], the high- and low-affinity receptors, respectively, for the constant region of immunoglobulin G.

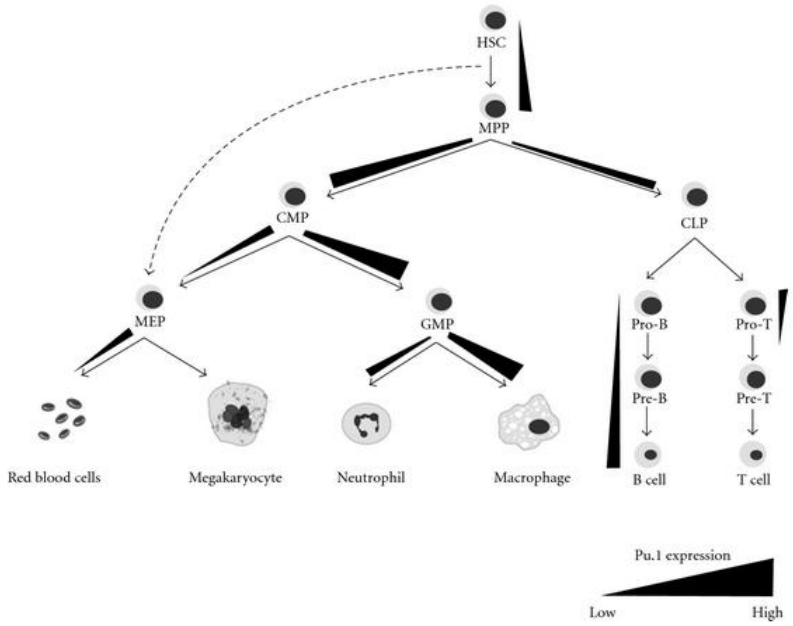

Figure 6. Schematic showing the changing expression of PU.1 during haematopoiesis*. PU.1 levels, where known, are represented by gradient bars. Gradient bars are not drawn to scale. Differentiation pathways are denoted by arrows. Abbreviations: HSC, haematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythroid progenitor; GMP, granulocyte-macrophage [98].*

Once expressed on the cell surface, these molecules allow the macrophage to recognize and phagocytose IgG-opsonized bacteria. PU.1 also induces the expression of the adhesion molecules CD11b [99], CD18 [100]and CD14 [95] during myeloid differentiation. CD18 is the β chain of integrins and, when associated with CD11b, they both constitute the membrane glycoprotein Mac-1, which mediates the adhesion of monocytes to endothelium and subsequent diapedesis, and the phagocytosis of complement-opsonized particles. CD14 is a membrane glycoprotein that specifically binds to lipopolysaccharide, a structural component of the bacterial wall. This recognition is a crucial step in triggering the microbactericidal function of the macrophage. Likewise, PU.1 mediates the expression of Scavenger receptors type I and II, which are maximally expressed during the terminal differentiation of

monocytes to macrophages and are involved in the capture and subsequent degradation of proteins that have been chemically modified at inflammation sites [101].

C/EBPβ

CCAAT/enhancer-binding protein β (C/EBPβ) is a member of the C/ EBP family of transcription factors currently consisting of 6 members: C/EBPα, C/EBPβ (also known as NF-IL6, IL6-DBP, CRP2, NF-M, AGP/ EBP, ApC/EBP, or TCF5), C/EBPγ, C/EBPδ, C/EBPε, and C/EBPξ [102, 103]

Transcription of the C/EBPβ gene results in the expression of a single mRNA product yielding the formation of three different C/EBPβ isoforms by alternative translation [104]. The two larger isoforms (liver-enriched activating protein* (LAP*; ***i.e.*** full-length C/EBPβ) and its slightly shorter version LAP) contain a dimerization and a DNA binding domain, a negative regulatory region, and several transactivation domains, whereas the considerably shorter variant, liver-enriched inhibiting protein (LIP), does not contain these transactivation domains [102]. In addition to other tissues (liver, lung, spleen, kidney, etc.), C/EBPβ is highly expressed in myelomonocytic cells and macrophages [105-108].

A variety of extracellular signals such as differentiation- or proliferation-inducing agents, hormones, cytokines and inflammatory substances as well as bacterial and other microbial products can act as activators or inhibitors of C/EBPβ via distinct signal transduction pathways [102]. The expression and/or activation of C/ EBPβ is regulated in a complex way, e.g. by transcriptional mechanisms, mammalian target of rapamycin (mTOR)-mediated alternative translation, post-translational modifications, and protein–protein interactions, and have been extensively reviewed [109-111].

Following activation, several classes of genes are induced or repressed by C/ EBPβ: cytokines, chemokines, their respective receptors, and other proinflammatory genes, proproliferative or differentiation-related markers as well as metabolic enzymes [102]. Consequently, C/EBPβ is ivolved in relevant cellular functions such as proliferation [108] , differentiation [108, 112], metabolic regulation [113, 114]. C/EBPβ KO mice exhibit a deregulated proliferative potential in the hematopoietic compartment[115] . The mice develop lymphadenopathy and splenomegaly and are more susceptible to pathogenic infections [116]. C/EBPβ KO mice are characterized by enhanced susceptibility to Candida albicans, Listeria monocytogenes, and Salmonella typhimurium infections [108,116].

In immortalized macrophage-like cell lines derived from C/EBPβ KO mice, the induction of differentiation/activation markers [112] as well as the development of a typical macrophage morphology is impaired [108].

C/EBPβ-LAP* and -LAP have been shown to be considerably connected to monocytic differentiation. The differentiation of monoblast cells is characterized by significant upregulation of C/EBPβ, especially LAP* and LAP [112] [108, 117, 118]. This is also reflected by an increasing LAP/LIP ratio [22].

C/EBPβ is involved in the regulation of the expression of a variety of differentiation-associated-genes including CD14 [118-121], macrophage-2 antigen(Mac-2), Fcγ receptor II (FcγRII) [112], monocyte-specific esterase [80], 1α-hydroxylase [96], and the cytoplasmic proline-rich tyrosine kinase 2 (Pyk2) [113].

The expression of other genes such as MD-2 and chitotriosidase (CHIT1) is also supported by C/EBPβ by acting as a cofactor of transcription factor PU.1 thus mobilizing its entire transcriptional capacity [112].

The treatment of primary AML cells with deltanoids [81], U937 leukemia cells with Vit-D3 [122] or ATRA [123] or HL60 leukemia cells with 1,25(OH2)D3 [119] also induced

an increase in C/EBPβ protein as well as the differentiation of these cells into monocytes. Consequently, treatment with Vit-D3 derivates and/or analogs may represent a promising alternative therapeutical approach for leukemia patients who do not respond to a classical chemotherapy via the induction of C/EBPβ-supported differentiation [124].

IRF8

IRF8 (also known as interferon consensus sequence binding protein, ICSBP) is a member of the IRF transcription factors family. It comprises an N-terminal DNA binding domain (which is conserved among family members) and a C-terminal regulatory domain that enables it to interact with other transcriptional regulators and act as an activator or a repressor of transcription [125, 126]

IRF8 is predominantly expressed in hematopoietic cells (both in progenitors and differentiated cells), where it cooperates with other transcription factors to regulate cell fate choice and differentiation, as well as cell survival and immune function. It can heterodimerize with other IRF family members or form homodimers to bind interferon (IFN)-stimulated response elements.

In cells of the myeloid lineage, IRF8 expression is controlled by the myeloid master regulator PU.1 [119, 126]. IRF8 can be constitutively expressed or induced by cytokines such as IFN-g [127], and it can also be autoregulated (it can control its own transcription) [128, 129].

Early studies of IRF8-deficient mice reported a dramatic elevation in neutrophils and a distinct monocyte deficiency (Ly6C-expressing inflammatory monocytes in particular)[130, 131]. IRF8 regulates myeloid cell production in humans in a similar manner. A patient with a K108E mutation was shown to have an elevated neutrophil count but to lack circulating monocytes and dendritic cells, whereas an individual with

a T80A mutation was found to be selectively deficient in CD11c CD1c circulating dendritic cells [132]. Moreover, decreased IRF8 expression has been reported in both acute and chronic myelogenous leukemia patients, and IRF8- deficient mice develop chronic myelogenous leukemia (CML)-like disease [130, 131, 133-135]. Thus in both mice and humans, IRF8 deficiency compromises immune defense and underlies the development of myeloid leukemia.

IRF8 regulates myelopoiesis in at least two ways: it controls lineage choice in the commitment phase (neutrophil versus monocyte/dendritic cell production), and it restricts progenitor survival to prevent leukemia.

These conclusions have been drawn from observations of IRF8 expression and from evaluation of myeloid progenitor and differentiated myeloid cell subpopulations.

Two recent studies from Tamura and colleagues have revealed molecular mechanistic insight the regulation of neutrophil versus monocyte cell fate by IRF8 at the level of target gene induction [89, 136]. The first study demonstrates how IRF8 promotes monocyte differentiation [89] and the second shows how it suppresses neutrophil production [89].

In the first study, Tamura and colleagues employed an IRF8-deficient myeloid progenitor cell line (Tot2 cells, originally isolated from IRF8- deficient mice at the blast crisis stage), which they previously showed comprises progenitors that can be induced to differentiate to yield neutrophils using granulocyte-colony stimulating factor or rescued to yield monocytes upon retroviral transduction of the IRF8 gene [30]. They investigated how IRF8 regulates gene expression by comparing IRF8-transduced and control vector-transduced Tot2 cells [89]. With chromatin-immunoprecipitation sequencing they located regulatory regions bound by IRF8 while with gene expression analysis (microarray) they identified genes induced upon IRF8 transduction.

Integrated analysis of the two datasets revealed candidate genes that were either induced upon direct binding of IRF8 to their regulatory elements (primary response), or indirectly regulated by IRF8 through the action of other factors induced by IRF8 (secondary response) (Fig. 7 a-b).

Using this approach they identified Kruppel-like factor-4(KLF4) as a direct target of IRF8 that subsequently regulates the induction of other genes, and showed that KLF4 transduction into Tot2 cells partially rescues monocyte differentiation.

Thus many of the downstream effects of IRF8 are attributed to KLF4 induction. They also showed that IRF8 acts primarily as a transcriptional activator during monocytopoiesis, and that although IRF8 can bind promoter-proximal regions, the majority of IRF8 binding sites are in distal regions identified as enhancers (histone 3 lysine 4 monomethylated) [89]. At both proximal and distal sites, IRF8 predominantly binds composite IRF/Ets elements (EICE and IECS) that are also occupied by PU.1, which is consistent with the ability of IRF8 and PU.1 to act as heterodimers [66, 137].

In the second study, they demonstrated that IRF8 blocks neutrophil differentiation by disrupting the ability of c/EBPa to stimulate neutrophil gene expression [136]. They showed that IRF8 interacts with c/EBPa and prevents it from binding chromatin in MDPs and cMoPs, thereby blocking its ability to induce neutrophil differentiation. They demonstrated that IRF8 binding does not disrupt the formation of c/EBPa homodimers, but likely interacts with them to restrict their ability to bind to their targets (Fig.7c)

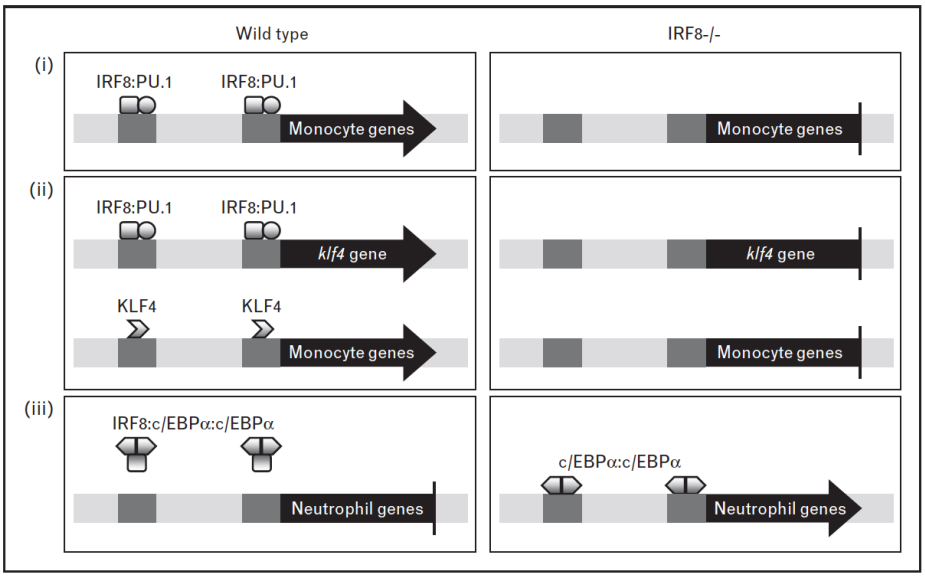

FIGURE 7 . Model of monocyte and neutrophil gene regulation by IRF8. *IRF8 favors monocyte production by regulating the expression of both monocyte and neutrophil genes. In wild type progenitors (such as MDPs, cMoPs and MPs), IRF8 binds to promoter and enhancer elements in collaboration with other transcription factors to favor monocyte differentiation (left panels). (i) Expression of some monocyte genes is instructed by binding of IRF8 to Ets/IRF composite elements and IRF/Ets composite sequence sites with PU.1 (primary response). (ii) Other monocyte genes are induced indirectly following IRF8-induced expression of primary targets such as Kruppel-like factor 4 (secondary response). (iii) IRF8 blocks c/EBPa-induced expression of neutrophil genes by interacting with c/EBPa dimers to prevent them from binding their target sequences. Deletion of IRF8 (IRF8–/–) favors neutrophil differentiation (right panels), by limiting monocyte gene expression (both direct and indirect mechanisms) and relieving the block in c/EBPa-induced expression of neutrophil genes. IRF8, interferon regulatory factor 8 [138].*

KLF4

Several members of the Kruppel-like factor (KLF) family of transcription factors play important roles in differentiation, survival and trafficking of blood and immune cell types [139-142].

Feinberg et al. [143] founded that among myeloid cells, KLF4 is expressed principally in monocytes and is induced in a stage-specific manner during myelopoiesis.

Overexpression of KLF4 in promyelocytic HL-60 cells or in primary CMPs or HSCs from bone marrow restricts these cells along the monocyte/macrophage pathway at the expense of other myeloid lineages and confers the morphologic, genetic, and functional characteristics of a mature monocyte. We further show that KLF4 is a

downstream target gene of PU.1 and is capable of binding to the monocyte-specific CD14 promoter.

Disruption of KLF4 at either the HSC or CMP stage reduced the number of monocytes with a shift toward increased granulocytes, whereas overexpression of KLF4 induced only monocyte differentiation at both of these stages. Because the relative levels and order of transcription factor expression can have profound effects in cell lineage fate, dysregulation of KLF4 may be important in leukemogenesis.

Mice with a genetic deletion of the PU.1 URE (PU.1-knockdown mice), develop acute myeloid leukemia as a result of an ~80% reduction in PU.1 expression and absence of M-CSFR and GM-CSFR expression [144].

Taken together, these data support an important role for KLF4 as a key transcriptional regulator of monocyte differentiation.

HOXA 10

The Homeobox (Hox) transcription factors originally identified as key regulators of embryonic development also play a fundamental role in normal and leukemic hemopoiesis, controlling both self-renewal and commitment-differentiation processes [145, 146]. HoxA10 is a member of this family. Its expression is higher during myeloid cell differentiation but also appears to enhance the proliferation of progenitor cells in mice. It has both anti- and pro-differentiation properties, depending on the cell and temporal context of its expression. These dual effects might reflect the intrinsic balance of proliferation and differentiation taking place during hematopoiesis. Infact, overexpression experiments performed in subsequent years, gave rise to contradictory results since they evidenced, in some cases, a role in stem cell maintenance [147] and myeloid progenitor expansion [148] whereas in others, a remarkable differentiation activity [149, 150]. HoxA10 is overexpressed in a poor

prognosis subset of human acute myeloid leukemia (AML) and *in vivo* overexpression of HoxA10 in murine bone marrow induces myeloid leukemia. Several reports indicate an involvement of the *Hox-A10* gene in the regulation of monocyte commitment that is fundamentally based on three experimental observations.

First, Hox-A10 expression is restricted to the $CD34^+$ progenitor and myeloid precursor phase of hemopoiesis [151] secondly, this gene is a primary response gene of the VD monocyte differentiation inducer [149, 152]; finally, retroviral vector-mediated expression of its cDNA induces the monocyte differentiation of U937 [149] and $CD34^+$ hemopoietic progenitors inhibiting, at the same time, the commitment to lymphoid, erythroid, and granulocyte lineages [150].

A recent report, based on a transgenic mouse model in which transcription of the transgene is controlled through an inducible system, indicated Hox-A10 expression levels as the crucial parameter able to determine different biological responses [153]. In this study, a low/intermediate expression of the transgene was associated with increased self-renewal activity of HSCs, whereas a higher expression resulted in a remarkable inhibition of erythroid and megakaryocyte differentiation.

V. Carrie Bromleigh demonstrated that stable, regulable overexpression of HOXA10 in U937 cells leads to arrest in G_1 and subsequent differentiation to the monocytic cell type. This effect closely correlates to elevated levels of p21 that follow HOXA10 induction. Remarkably, they found that HOXA10 protein can bind directly to a region of the p21 promoter and, together with its trimeric partners PBX1 and MEIS1, activate p21 transcription. This link between a transcription factor and a cell cycle regulator is a key component of the complex circuitry that leads to myeloid cell differentiation [154].

Gemelli et al, demonstrate the existence of a precise transactivation cascade in which stimulation of the VDR-dependent pathway leads to the induction of *Hox-A10* gene which in turn up-regulate MafB expression. They conclude that the vitamin D_3/Hox-A10 pathway cooperates MafB function in the regulation of monocyte commitment. This conclusion is also supported by the observation that all these inducers (VD, Hox-A10, MafB) comparably stimulated the monocyte-macrophage differentiation of $CD34^+$ cells and that treatment of the same cells with VD resulted in the sequential induction of Hox-A10 and MafB expression. This data demonstrated the important role of HOXA 10 in monocyte differentiation [155].

MAFB

The Maf family of transcription factors is characterized by a typical bZip structure; these transcription factors act as important regulators of the development and differentiation of many organs and tissues, including the kidney. The Maf family consists of two subgroups that are characterized according to their structure: large Maf transcription factors and small Maf transcription factors. The large Maf subgroup consists of four proteins, designated as MAFA, MAFB, c-MAF and and neural retinal-specific leucine zipper. Maf family shows binding activity to the Maf responsive element (MARE) and to the 5′AT-rich half-MARE , contained in the promoter regions of target genes [156] . In addition, MafB supports either transcription activation or repression depending on the interacting dimerization partner [157]. This TF is evolutionary conserved [158-160] and several reports indicate that, in human hematopoiesis, its expression is monocyte specific.

In fact, data obtained in our laboratory demonstrated that: 1) MafB is highly expressed in monoblasts, monocytes and, accordingly, in monoblastic cell lines (THP1, U937 and Kasumi-1) [161] and [162]; 2) virally mediated MafB transduction of

human hematopoietic progenitors determines a massive induction of monocyte–macrophage differentiation, coupled to a strong inhibition of erythroid commitment [5; and [161] 3) MafB is a direct target of two monocyte–macrophage related TFs, i.e. Hox-A10 [155] and TFE3 [163].

Others experiments based on MafB inactivation and over-expression, support the existence of a signaling cascade by which stimulation of macrophages with the IL-10 cytokine determines a sequential activation of STAT3 and MafB transcription factors, in turn leading to an up-regulated expression of *MMP9* and *IL-7r* genes [164].

In addition, data reported in literature underlined that high levels of MafB expression are able to activate the macrophage maturation program at the expense of dendritic cell (DC) differentiation and that its down-regulation is also required for osteoclast differentiation of myeloid cells [165].

VDR

In monocyte differentiation, Vitamin D receptor (VDR) has an important role. VDR is a member of the nuclear hormone receptor super family, and 1,25(OH)2D3 (the activated form of Vitamin D3) acts similarly to the other steroid hormones.

The active form of vitamin D3 regulates calcium and bone homeostasis, immunity, and cellular growth and differentiation through direct binding to the vitamin D receptor (VDR), and has been demonstrated to inhibit the proliferation and to induce the differentiation of various types of malignant cells, including breast, prostate and colon cancers as well as myeloid leukemia cells [166].

The effects of 1α,25-(OH)2D3 on the immune system were ascribed to its action on lymphocytes and monocytes/macrophages [167-169]. When added to mitogen-stimulated human peripheral blood lymphocytes in vitro, 1α,25-(OH)2D3 inhibits their

proliferation, Ig synthesis, and accumulation of transcripts for IL-1, IL-2, IL-6, TNF-α, and -β and IFN-γ [168, 170]. Of interest is that 1α,25-(OH)2D3 induces promyelocytes to differentiate into monocytes [171]; in addition, 1α,25-(OH)2D3 differentiates myeloid leukemia cells to nonproliferating monocyte/macrophage-like cells in both humans and mice [172] and promotes the differentiation of myeloid stem cells and normal peripheral blood monocytes toward a macrophage phenotype. Physiological levels of 1α, 25 dihydroxyvitamin D3 induce the monocytic commitment of CD34+ hematopoietic progenitors [173].

Koeffler's group, [174] and later Kelsey et al, [175] reported that oral administration of $1,25D_3$ to patients with myelodysplasia produced a partial but sustained hematological response, in terms of increases in neutrophil and platelet counts, in approximately half of the treated patients. However, hypercalcemia occurred in some patients before concentrations necessary for antileukemic activity could be achieved. Animal studies have also highlighted that the major limitation for the clinical use of the hormone is its potent calcemic activity, which results in hypercalcemia, tissue calcifications and hypercalciuria [176].

miRNA and Hematopoiesis

The self-renewal and cell fate determination of HSCs in the BM is an outcome of complex, multidimensional regulatory mechanisms that include micro-environmental factors as well as epigenetic and transcriptional regulation of key transcriptional factors (TFs) [177, 178].

Extrinsic signals such as growth factors and micro-environmental conditions are conveyed via intracellular signaling cascades that induce changes in key TFs, regulating self-renewal of HSCs during in vivo reconstitution or in vitro expansion culture [55, 177-179]. Accordingly, characteristic shifts in transcriptome profiles and some key TFs have been observed during self-renewal and differentiation into various lineage-specific cells, indicating the significance of transcriptomic changes in cell fate determination [180-182].

In addition to the changes in TFs, epigenetic modification of chromatin can induce changes in transcription profiles, thereby regulating HSC fate [183-185]. However, the mechanisms involved in the dynamic coordination of gene expression under various physiological stimuli for regeneration are largely unknown. Recently, miRNAs have been shown to function as an additional regulatory mechanism in HSCs by influencing transcription profiles and transcript stability [57].

TFs and miRNAs act in concert to regulate gene expression during hematopoietic differentiation; TFs regulate the expression of miRNA genes, whereas TFs are key miRNA targets.

Thus, miRNAs represent another axis of regulation in HSCs, thereby controlling the self-renewal and the fine-tuning of cell fate during the lineage-specification process.

Experimental evidence regarding a functional role for specific miRNAs in the control of mammalian hematopoietic lineage differentiation was first demonstrated by Chen

et al. [186]. Subsequently, numerous studies have identified more than 100 different miRNAs specifically expressed during hematopoiesis in mice and humans [187]. Recently, two studies have shown impaired hematopoiesis after the conditional deletion of Dicer and Argonaute2, demonstrating the functional impact of miRNAs on normal hematopoietic process [187-189]. Subsequently, miRNAs involved in hematopoietic regulation has been identified, thus placing miRNAs at the forefront of studies on hematopoietic regulation (Fig.8).

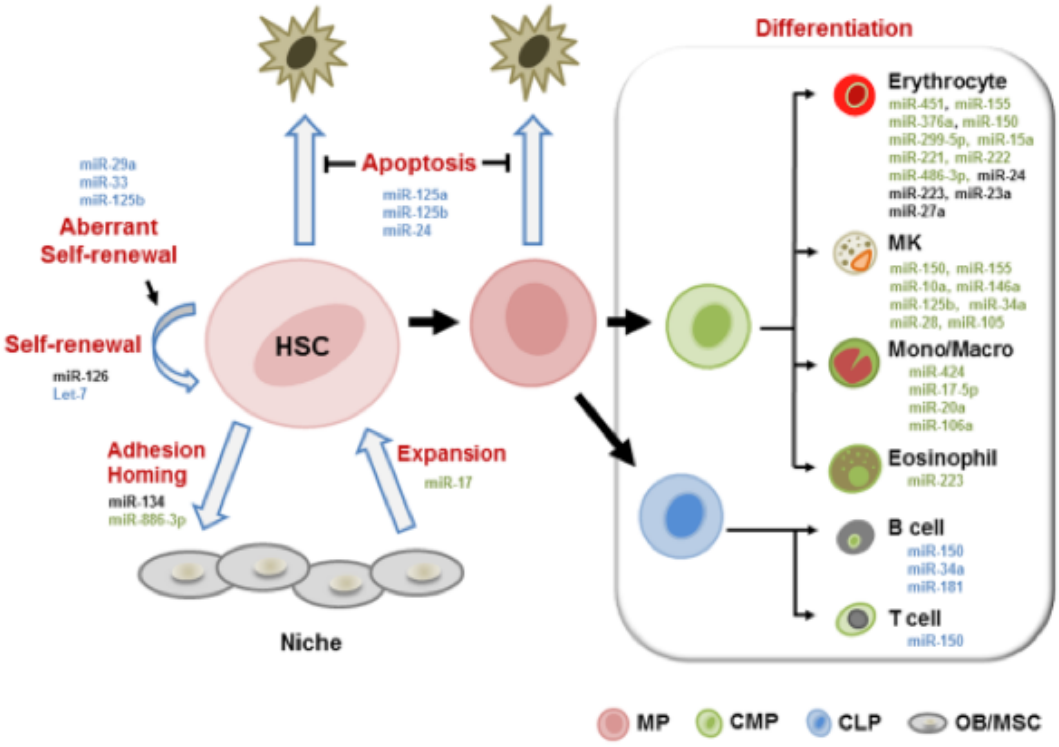

Figure 8. Involvement of miRNAs in the regulation of hematopoietic systems*. A schematic illustration representing miRNAs that regulate self-renewal and differentiation of hematopoietic stem cells (HSCs)/hematopoietic progenitor cells and intercellular crosstalk between HSCs and stem cell niches in bone marrow. The role of these miRNAs was revealed by gain- or loss-of-functional studies in murine (indicated in blue), human (indicated in green), or both murine and human (indicated in black) hematopoietic systems. Abbreviations: CLP, common lymphoid progenitor; CMP, common myeloid progenitor; HSC, hematopoietic stem cell; MK, megakaryocytes; MP, multipotent hematopoietic progenitor; MSC, multipotent stromal cell; OB, osteoblast; Mono/Macro, monocytes/macrophages [177].*

miRNAs have been found to play a role in controlling the lineage determination of HPCs toward granulocytic, erythroid, and megakaryocytes lineages.

For example, mice lacking miR-223 exhibited a significant increase in the numbers of circulating neutrophils and granuloid hyperplasia in the BM.

The neutrophils from these mice were characterized by hypersegementation and hyperactivity in response to endotoxin challenge [190].

In this study, miR-223 was found to target Mef2c, a TF that promotes proliferation of myeloid progenitors, and the loss of Mef2c reversed the phenotype of the miR-223 mutant, indicating a role in the fine-tuning of granulocyte production.

Interestingly, a recent study revealed that the expression of miR-223 is coordinated by differential binding of TFs to the promoter of miR-223 in a lineage-specific manner, that is, miR-223 is upregulated during granulopoiesis and monocytic differentiation whereas it remains at a low level during erythropoiesis. Moreover, in vitro overexpression of miR-223 in CD341 cells increased granulocytic/monocytic colony formation, but decreased erythroid progenitors, whereas knockdown of miR-223 produced the opposite effect [191].

Together, these findings demonstrate the role of miR-223 in controlling the equilibrium from HSC to commitment to myeloid/erythroid progenitors and to terminal differentiation/maturation of granulopoiesis, thus indicating its role in fine-tuning the lineage-specification and differentiation of hematopoietic cells.

Similarly, miR-451, which was the first miRNA identified in human erythrocytes [192], was shown to be regulated by GATA-1 and its depletion in zebrafish led to impaired maturation of erythroid cells into circulating red blood cells, indicating a role for terminal differentiation of erythroid lineages [193]. Moreover, it was recently demonstrated that miR-451 is also upregulated during erythropoietic differentiation from embryonic stem cells, but reciprocally downregulated during megakaryocyte differentiation [194]. This indicates that a set of genes regulated by miR-451 is

involved in lineage determination between erythropoietic and megakaryocytic lineages.

Additionally, studies on erythroid differentiation of UCB derived CD34+ cells or K562 cells revealed that expression of miR-15b, miR-16, miR-22, and miR-185 correlate positively with the appearance of erythroid surface antigens (CD36, CD71, and CD235a), while induction of miR-28 was inversely related to the expression of these markers [195].

Recently, miR-486-3p and miR-23a have been identified as key regulators for erythroid differentiation of CD34+ HPCs by targeting BCL11A and SHP2, respectively [196, 197]. Other studies have reported the identification of miRNAs that control megakaryocyte differentiation. For example miR-150 drives megakaryocytic-erythroid progenitor (MEP) toward differentiation into megakaryocytes by targeting MYB, leading to an increase in megakaryocytes with a>60% decrease of erythrocytes in the BM [37]. Similarly, overexpression of miR-155 in CD341 HPCs impairs proliferation and differentiation of megakaryocytes by reducing Ets-1 expression [198].

Several miRNAs appear to influence the commitment of HSCs and their progenitors to monocyte (Fig.9). For instance, *miR-146a, miR-155, miR-342* and *miR-338* are upregulated by the transcription factor PU.1 [199, 200], which controls myeloid cell development. Ectopic expression of *miR-146a* is sufficient to direct HSC differentiation to the mononuclear phagocyte lineage in mouse transplantation assay [199].

miR-21 and *miR-196b* have been shown to promote monocytopoiesis and to antagonize granulopoiesis, respectively. Of note, the growth factor-independent-1-transcription repressor (GFI1), which is required for granulopoiesis, suppresses

both *miR-21* and *miR-196b*; this illustrates a cooperative role between GFI1 and *miR-21/196b* in regulating the balance between monocyto- and granulopoiesis [201].

Commitment toward the mononuclear phagocyte lineage can be instructed by the colony stimulating factor-1 (CSF-1) through its receptor, CSF-1R. Interestingly, *miR-17-5p, miR-20a* and *miR-106a* cooperate to suppress monocyte production by targeting Runt-related transcription factor-1 (RUNX1, also known as AML1), which promotes CSF-1R expression. Interestingly, transcription of these miRNAs appears to be regulated through RUNX1-mediated repression [202] or epigenetic silencing [203]. These observations suggest that a negative feedback loop, involving RUNX1 and *miR-17-5p/20a/106a*, controls monocyte output from common myeloid progenitors. Additionally, *miR-424* was shown to stimulate mononuclear phagocyte differentiation by increasing CSF-1R and decreasing NFI-A expression [204].

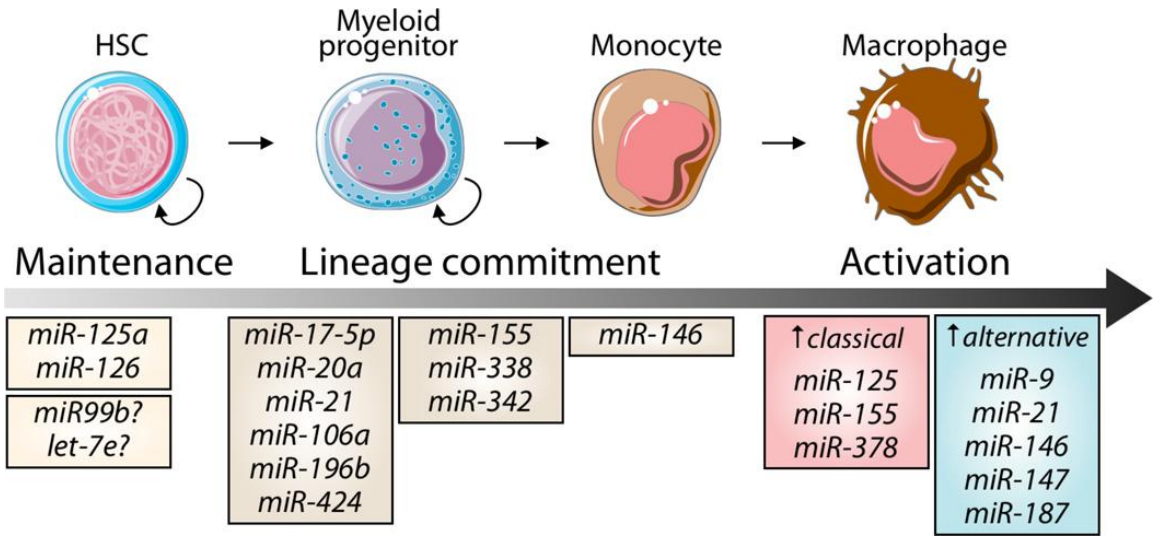

Figure 9. Overview of miRNAs implicated in macrophage development and activation [205].

Leukemogenesis: ACUTE MYELOID LEUKEMIA

Leukemogenesis is a complex process that involves multiple genetic and epigenetic events. It underlies a group of clonal malignancies of blood and bone marrow characterized by the presence of chromosomal abnormalities, such as deletions, translocations or inversions, or genetic mutations affecting the control of hematopoietic cell proliferation and differentiation. Leukemia is classified both clinically and pathologically as acute or chronic (based on differentiation state and clinical evidence) and myeloid or lymphoid (according to cell type).

AML (Acute Myeloid Leukemia) is a rapidly fatal malignancy which occurs as a consequence of the progressive accumulation of genetic aberrations in myeloid progenitor cells. These alterations lead to a block in progenitor cell differentiation and sometimes an increase in cell proliferation.

AML is morphologically, molecularly, and prognostically heterogeneous [206]. Two systems have been used to classify AML into subtypes: the French-American-British (FAB) classification and the newer World Health Organization (WHO) classification.

In the 1970s, a group of French, American and British leukemia experts divided acute myeloid leukemias into subtypes, M0 through M7, based on the types of cell from which the leukemia develop and how mature the cells are (Tab.1). This was based largely on how the leukemia cells looked under the microscope after routine staining. Subtypes M0 through M5 all start in precursors of white blood cells. M6 AML starts in very early forms of red blood cells, while M7 AML starts in early forms of cells that make platelets. Some subtypes of AML defined in the FAB system are linked with certain symptoms; bleeding or blood clotting problems are often a problem for patients with the M3 subtype of AML, also known as acute promyelocytic leukemia

(APL). Identifying APL is very important for two reasons. First of all, certain complications of APL can often be prevented by appropriate treatment.

Secondly, APL is treated differently from most other forms of AML because it responds to drugs like retinoids [207].

FAB subtype	Name
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

Table 1: *Classification French-American-British (FAB).*

The FAB classification system is useful and still commonly used to group AML into subtypes but it doesn't take into account many of the factors that are known to impact prognosis.

In 2001, the World Health Organization (WHO) published a newer system that includes some of these factors to try to help better classify cases of AML based on a patient's outlook [208].

The WHO classification system divides AML into several broad groups:

- *AML with certain genetic abnormalities:*
  - AML with a translocation between chromosomes 8 and 21;
  - AML with a translocation or inversion in chromosome 16;
  - AML with changes in chromosome 11;

- APL (M3), which usually has translocation between chromosomes 15 and 17.

- *AML with multilineage dysplasia* (more than one abnormal myeloid cell type is involved).
- *AML related to previous chemotherapy or radiation.*
- *AML not otherwise specified* (includes cases of AML that don't fall into one of the above groups; similar to the FAB classification):
  - Undifferentiated AML (M0);
  - AML with minimal maturation (M1);
  - AML with maturation (M2);
  - Acute myelomonocytic leukemia (M4);
  - Acute monocytic leukemia (M5);
  - Acute erythroid leukemia (M6);
  - Acute megakaryoblastic leukemia (M7);
  - Acute basophilic leukemia;
  - Acute panmyelosis with fibrosis;
  - Myeloid sarcoma (also known as granulocytic sarcoma or chloroma).
- *Undifferentiated or biphenotypic acute leukemias* (leukemias that have both lymphocytic and myeloid features). Sometimes called ALL with myeloid markers, AML with lymphoid markers, or mixed lineage leukemias.

Several types of chromosomal rearrangement may be found in AML cells:

- *Translocations*.
- *Deletions*
- *Inversions*
- *Addition or duplication*.

Different cases of AML can have different chromosomes changes.

In April 2004, Peter J. M. Valk and colleagues [209] have found that a molecular classification based on DNA-expression profiling offers a powerful way of distinguishing myeloid from lymphoid cancer and subclasses within these two diseases. DNA-microarray analysis has the potential to identify distinct subgroups of AML with the use of one comprehensive assay, to classify cases that currently resist categorization by means of other methods, and to identify subgroups with favorable or unfavorable prognoses within genetically defined subclasses. The studies were conducted on a sample of 285 adults with AML, taking into consideration 13000 genes. Unsupervised cluster analyses identified 16 groups of patients on the basis of strong differences in gene-expression profiles. The genes that defined these clusters have been identified and the minimal numbers of genes needed to identify prognostically important clusters with a high degree of accuracy have been determined. The clustering was driven by the presence of chromosomal rearrangment such as translocations t(8;21) and t(15;17), the inversion inv(16), particular genetic mutations (CEBPA) and abnormal oncogene expression (EVI1). In *figure 10 A* it is shown the pairwise correlations between the samples, and *figure 10B* shows all 16 clusters identified on the basis of specific correlation and their comparison to the FAB classification[209]. At the same time, Lars Bullinger and colleagues used complementary-DNA microarrays to determine the levels of gene expression in peripheral-blood or bone marrow samples from adults with AML (including a few with a normal karyotype). Their work is based on the fact that it is very difficult to make AML predictions. They have developed a program that can provide clinical-outcome predictor of disease, using an innovative algorithm and analyzing differential gene expression that was proved to be quite accurate in predicting the survival of patients, even in those who had normal karyotype [210].

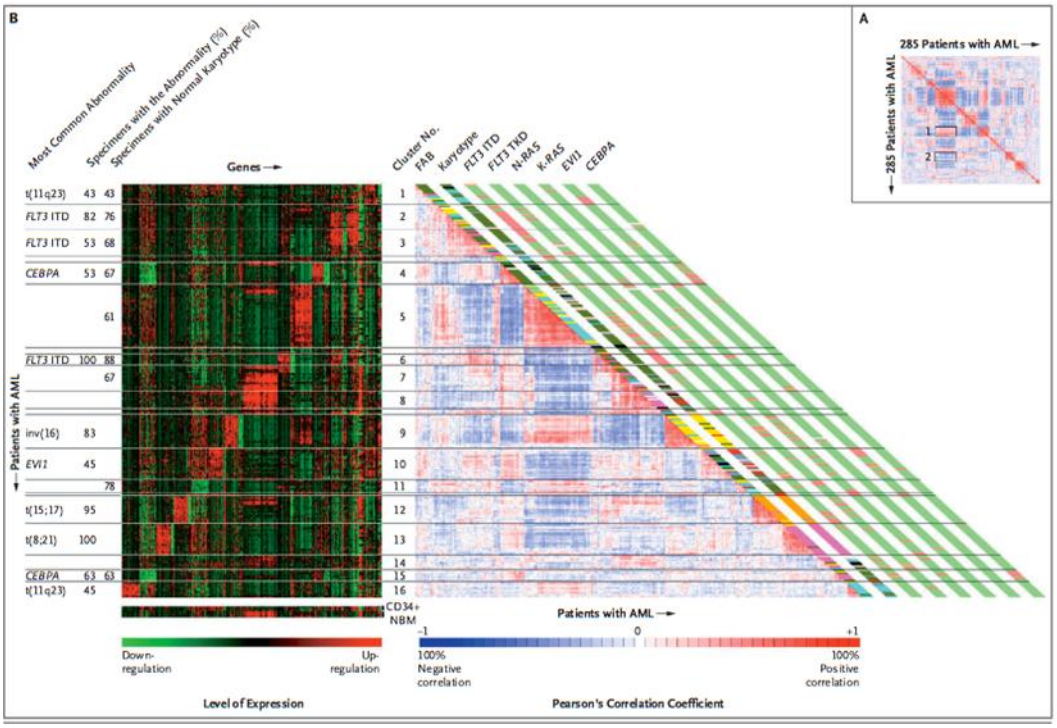

Figure 10A: *The colors of the cells relate to Pearson's correlation coefficient values, with deeper colors indicating higher positive (red) or negative (blue) correlations. One hundred percent negative correlation would indicate that genes with a high level of expression in one sample would always have a low level of expression in the other sample and vice versa. The red diagonal line displays the intraindividual comparison of results for a patient with AML (therefore 100 percent correlation).* ***10B:*** *The French-American-British (FAB) classification and karyotype based on cytogenetic analyses are depicted in the columns along the original diagonal of the figure 3A; FAB subtype M0 is indicated in black, subtype M1 in green, subtype M2 in purple, subtype M3 in orange, subtype M4 in yellow, subtype M5 in blue, and subtype M6 in gray; normal karyotypes are indicated in green, inv(16) abnormalities in yellow, t(8;21) abnormalities in purple, t(15;17) abnormalities in orange, 11q23 abnormalities in blue, 7(q) abnormalities in red, +8 aberrations in pink, complex karyotypes (those involving more than three chromosomal abnormalities) in black, and other abnormalities in gray. FLT3 internal tandem duplication (ITD) mutations, FLT3 mutations in the tyrosine kinase domain (TKD), N-RAS, K-RAS, and CEBPA mutations, and the overexpression of EVI1 are depicted in the same set of columns: red indicates the presence of a given abnormality, and green its absence. The levels of expression of the top 40 genes identified by the significance analysis of microarrays of each of the 16 clusters as well as in normal bone marrow (NBM) and CD34+ cells are shown on the left side. The scale bar indicates an increase (red) or decrease (green) in the level of expression by a factor of at least 4 relative to the geometric mean of all samples [210].*

AML e miRNA

miRNAs are located in fragile sites or common breakpoint regions in chromosome aberrations that involve oncogenes or tumor suppressor genes in cancer cells . Thus, miRNAs were implicated as drivers of leukemogenesis [211]. Starczynowski *et al.* demonstrated that, although around 70% of miRNAs are located in regions of leukemia-associated cytogenetic changes, a subset (only ~ 20%) of these miRNAs are expressed and probably relevant in myeloid malignancies [212]. In this subset, the miRNAs miR-143, miR-145, miR-146a, miR-155, miR-181, miR-221 and miR-222 are implicated in cellular processes relevant to AML . Deletion of miR-145 [213]and miR-146a results in a long-term myeloid disease in mice, and reintroduction of both miRNAs into AML cells significantly induced cell death and prevented growth *in vitro [212]*. Overexpression of miR-155 leads to fatal and aggressive myeloproliferative disorder in mice [214].

The miRNA expression profile allow the distinction of AML patients from ALL patients through the down-regulation of six miRNAs (miR-5, miR-128a, miR-128b, miR-130b, miR-151* and miR-210) and up-regulation of 21 miRNAs (for example, let-7a, -b, -c, -e, miR-21, miR-221, miR-222, miR-223 etc.); among these miRNAs, let-7b, miR-128a, miR-128b and miR-223 are the most characteristic ones [215].

Additionally, it was shown that expression profiles of miRNAs can not only be used for distinction of leukemias of different lineages, but also for differentiation of cytogenetic subtypes of adult AML. Three independent studies demonstrated that the cytogenetic AML subtypes t(8;21), t(15;17) and inv(16) offer unique miRNA expression profiles [206, 216, 217]. It was shown that miR-126 was highly overexpressed in t(8;21) and inv(16) and miR-224, miR-368 and miR-382 were exclusively overexpressed in t(15;17) in adult AML patients [217]. The overexpression of miR-24 in patients carrying translocation t(8;21) leads to an

inhibition of a mitogen-activated protein kinase (MAPK) phosphatase (MKP-7) and to an activation of downstream partners. Additionally, miR-24 blocks myeloid differentiation and speeds up cell proliferation [218, 219].

Moreover, recent reports suggested that miR-125b might act as oncogene as well as tumor suppressor, depending on the cellular context [220]. It has already been shown that this miRNA is involved in myeloid differentiation arrest in human cell lines, and that it is 6- to 90-fold overexpressed in AML patients carrying the translocation t(2;11) than in other AML subtypes or in healthy controls .

Overexpression of miR-125b was associated with the development of multiple types of leukemia, suggesting an effect of this miRNA on proliferation and inhibition of apoptosis because most miR-125b targets are involved in the p53 pathway. However, the exact role of miR-125b, as a second event in oncogenesis, has to be confirmed by further analyses [221, 222].

Furthermore, miR-223 is a known regulator of myelopoiesis with low expression in primary leukemia blasts. This expression is decreased by the interaction between the AML1-ETO fusion protein and the miRNA promoter region, which leads to miR-223 silencing [223]. On this account, it has been started the association of miRNAs with individual risk-groups of AML. For example, up-regulation of let-7b and miR-9 was detected in patients with adverse cytogenetic risk-groups, and low expression of these miRNAs was detected in patients in the favorable risk-group [216].

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Correlations of microRNA expression with marrow morphology in CN-AML

Debernardi et al were the first to demonstrate that *miR-181a* was more highly expressed in leukemic blasts with FAB M1 and M2 marrow morphology compared with FAB M4 and M5. A subsequent study confirmed these results with regard to both *miR-181a* and *miR-181b* [224].

In another study, comparing the microRNA expression profiles of FAB M1 and FAB M5 subtypes, it was found the higher expression of not only *miR-181a* and *miR-181b* but also *miR-181a**, *miR-181d*, *miR130a*, *miR-135b*, *miR-146a*, *miR-146b*, and *miR-663* in FAB M1 [225].

In FAB M5 samples, *miR-21*, *miR-193a*, and *miR-370* were overexpressed. Lutherborrow et al demonstrated significant down-regulation of *miR-181a*, *miR-181b*, *miR-181d*, *miR130a*, *miR-135b*, and *miR-146a* during induced monocytic differentiation of AML cell lines, suggesting direct involvement of these microRNAs in monocytic differentiation. They also identified key myeloid factors, such as *MAFB*, *IRF8* and *KLF4* as targets of these microRNAs [225].

miRNA Therapeutics

Recent understanding of the importance of miRNAs has attracted the interest of the biomedical research community. Researchers believe that miRNAs are the next important class of therapeutic molecules after siRNA. It has been demonstrated that the restoration of misregulated miRNAs to their normal levels can reduce or even eliminate diseases including tumors in animal models [226].

Because miRNAs are naturally occurring molecules, there are certain advantages in their application as therapeutic agents.

Morever, miRNAs have typically many targets within cellular networks, which, in turn, enable modulation of entire pathways in a disease state via therapeutic targeting of disease-associated miRNAs.

Worldwide researchers have validated the theory of "*miRNA replacement therapy*," which involves introducing synthetic miRNAs or miRNA mimetics into diseased tissues in an attempt to restore normal proliferation, apoptosis, cell cycle and other cellular functions that have been affected by the misregulation of one or more miRNAs [227].

In contrast, some researchers have utilized miRNA inhibitors in an effort to increase the endogenous levels of therapeutic proteins.

Thus, in theory, the inhibition of a specific miRNA linked to a given disease can remove the block of expression of a therapeutic protein. Mature miRNA can be inhibited using either miRNA sponges or antisense oligonucleotide, known as antimiR. A miRNA sponge uses transgenic overexpression of RNA molecules harboring complementary binding sites to a miRNA of interest to block the function of a given miRNA or a miRNA family [228]. While this approach has shown great utility as an experimental tool, antimiRs seem a greater promise from a therapeutic perspective (Fig.11).

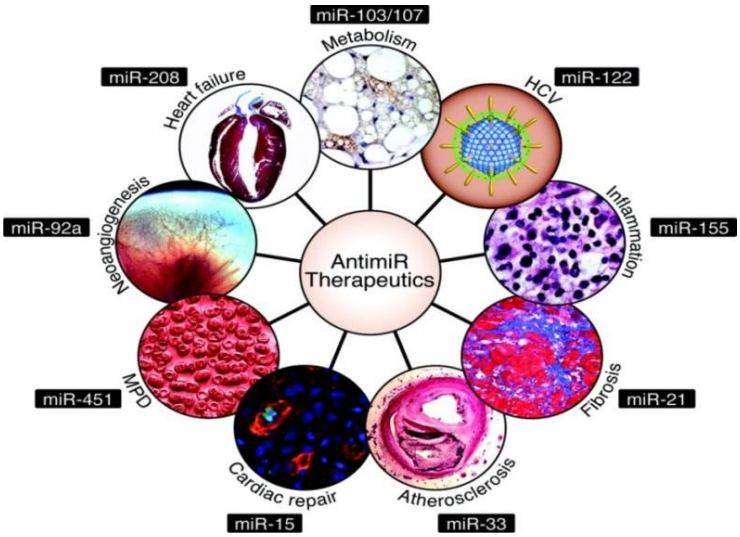

Figure 11. Specific miRNAs that are currently being pursued as clinical candidates. *A subset of the miRNAs of which inhibition has shown therapeutic promise and that are currently actively being pursued as clinical candidates for various disease indications [229]*

Several studies have demonstrated that the pharmacological modulation of miRNA represent a therapeutic benefict for the treatment of cancer, heart failure, atherosclerosis and HVC infection (Tab. 2)[230].

Company	miRNA target	Mode of action	Indication	Status
Santaris Pharma	miR-122	antimiR	HCV	Clinical Phase II
Mirna Therapeutics	miR-34	mimic	Unresectable primary liver cancer	Clinical Phase I
	let-7	mimic	Cancer	Preclinical
Regulus Therapeutics	miR-122	antimiR	HCV	Clinical Phase I
	miR-221	antimiR	Hepatocellular carcinoma	Preclinical
	miR-10b	antimiR	Glioblastoma	Preclinical
	miR-21	antimiR	Hepatocellular carcinoma	Preclinical
	miR-21	antimiR	Kidney fibrosis	Preclinical
	miR-33	antimiR	Atherosclerosis	Preclinical
miRagen Therapeutics	miR-208	antimiR	Heart failure	Preclinical
	miR-15/195	antimiR	Post-MI remodeling	Preclinical
	miR-145	antimiR	Vascular disease	Preclinical
	miR-451	antimiR	Myeloproliferative disease	Preclinical
	miR-29	mimic	Fibrosis	Preclinical
	miR-208	antimiR	Cardiometabolic disease	Preclinical
	miR-92	antimiR	Peripheral artery disease	Preclinical

Table 2. MicroRNA-based therapeutics in development [230]

Efficient silencing of dysregulated miRNAs in vivo requires that the antimiR oligonucleotides would be chemically modified to improve their binding affinity, biostability and pharmacokinetic properties (Fig.12).

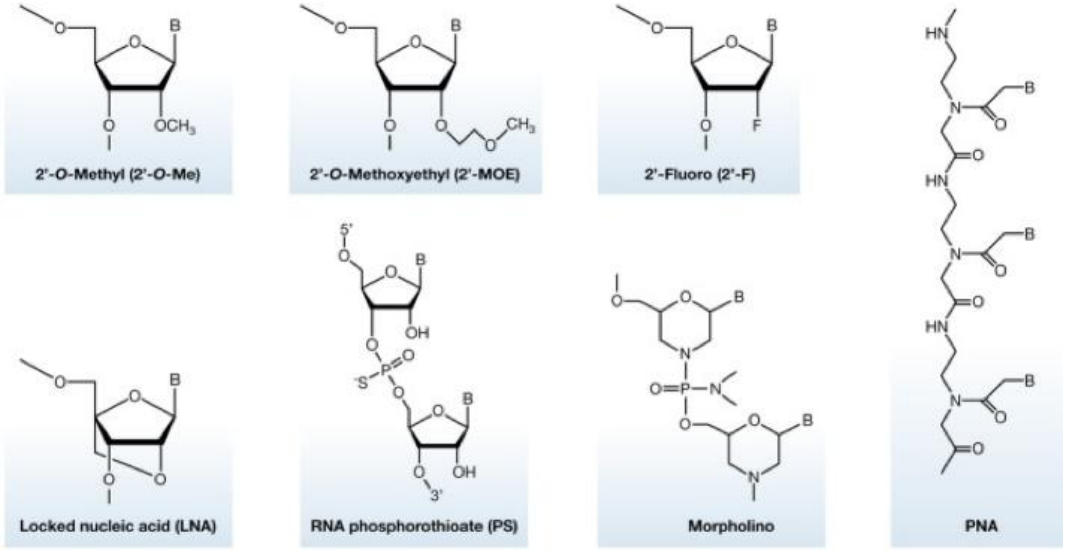

Figure 12. Design of chemically modified miRNA modulators*. Structures of chemical modifications used in miRNA modulators. A number of different sugar modifications are used to increase the duplex melting temperature (Tm) of antimiR oligonucleotides. The 20-O-methyl (20-O-Me), 20-O-methoxyethyl (20-MOE) and 20-fluoro (20-F) nucleotides are modified at the 20 position of the sugar moiety, whereas locked nucleic acid (LNA) is a bicyclic RNA analogue in which the ribose is locked in a C30-endo conformation by introduction of a 20-O,40-C methylene bridge. To increase nuclease resistance and enhance the pharmacokinetic properties, most antimiR oligonucleotides harbor phosphorothioate (PS) backbone linkages, in which sulfur replaces one of the non-bridging oxygen atoms in the phosphate group. In morpholino oligomers, a six-membered morpholine ring replaces the sugar moiety. Morpholinos are uncharged and exhibit a slight increase in binding affinity to their cognate miRNAs. PNA oligomers are uncharged oligonucleotide analogues, in which the sugar–phosphate backbone has been replaced by a peptide-like backbone consisting of N-(2-aminoethyl)-glycine units [230].*

The most commonly used sugar modifications for increasing the duplex melting temperature (Tm) and improving nuclease resistance of antimiRs include the20-O-methyl (20-O-Me), 20-O-Methoxyethyl (20-MOE) 20-fluoro and the bicyclic locked nucleic acid (LNA) modifications, respectively, (Fig 12) [231-234]. Among the different sugar modifications, LNA exhibits the highest affinity toward complementary RNA with an increase in Tm of +2–8°C per introduced LNA modification [235-237].

Increased nuclease resistance is achieved by substituting the phosphodiester (PO) backbone linkages with phosphorothioate (PS) linkages in antimiR oligonucleotides , or by using peptide nucleic acid (PNA) or morpholino oligomers, respectively, designed to target miRNAs [238-240]. Currently, there are two miRNAs that are in clinical trials: miR122 LNA which target hepatitis C virus (HCV) and has currently finished phase IIb testing, and miR34, which is currently in phase I testing.

miR-122 LNA AMO for HCV

miR-122 is a liver specific miRNA, which plays an important role in facilitation of replication of the hepatitis C virus [241]. This was shown to be through interactions with two miR-122 binding sites at the 5' noncoding region of the virus [242]. Current therapies for HCV are PEG-IFN alpha and ribavirin, which have a poor side effect profile and are often ineffective. Satnaris Pharma has developed a LNA-miR-122, known as Miravirsen, which binds to the 5' end of miR-122 and decreases HCV in nonhuman primates with no side effects [243]. In Phase II clinical trials of Miravirsen, different doses of Miravirsen 5 times a week for 29 days were given to patients with chronic HCV with a follow up to 18 weeks later. It was observed that in patients with chronic HCV there was a significant, dose dependent decrease of HCV, which was sustained after administration [244]. This trial demonstrated the safety and effectiveness of the therapy and is an important first step for the development of miRNA therapeutics.

miR-34 for Solid Cancers

The human miR-34 family contains three miRNAs; miR-34a, miR-34b and miR-34c. These miRNAs have identical seed regions and therefore, control a similar set of target mRNAs. miR-34 is transcriptionally induced by p53 [245] and has numerous

targets in many different pathways. miR-34 is decreased in many types of cancer, leading to tumurogenesis [246]. Many mouse models have shown that increased miR-34 leads to decrease of tumor size, with no side effects [247]. However, delivery of the miR has been proved to be difficult. Mirna Therapuetics It has been developed a delivery technology called NOV340, or SMARTICLES® which is a lipososme which forms particles of ~120 nm cationic at low pH and neutral or anionic at neutral or higher pH. SMARTICLEs® have efficient delivery to the liver and decreased tumor burden in mice with liver cancer [248, 249]. Recently, MRX34, miR-34 in a SMARTICLE® liposomal injection, has entered phase I clinical trial for liver cancer and metastasis from other cancers (NCT01829971).

MiRNA targeting by PEPTIDE NUCLEIC ACIDS (PNA)

PNAs are DNA analogs in which the sugarphosphate backbone is replaced by N-(2-aminoethyl) glycine units [250]. These very interesting molecules have been described for the first time by Nielsen et al. [251] and, despite a radical structural difference with respect to DNA and RNA, they are capable of sequence-specific and efficient hybridization with complementary DNA and RNA, forming Watson-Crick double helices [252, 253]. In addition, they generate triple helix structures with double-stranded DNA and perform strand invasion. Accordingly, they have been proposed for antisense and antigene therapy [250].

IMPORTANT PROPERTIES OF PNA

Thermal stability of PNA and its hybrid complexes.

PNA/DNA and PNA/RNA duplexes have an higher thermal stability compared with DNA/DNA and DNA/RNA duplexes. This stronger binding is attributed to the lack of charge repulsion between the neutral PNA strand and the DNA or RNA strand.

Stronger binding independent of salt concentration. An important consequence of the neutral backbone is that the Tm values of PNA/DNA duplexes are practically independent of salt concentration. At low ionic strength, PNAs can bind to a target sequence at temperatures at which DNA hybridisation is strongly inhibited.

Greater specificity of interaction. PNA also shows greater specificity inhibinding to complementary DNA. A PNA/DNA mismatch is more destabilising than a mismatch in a DNA/DNA duplex. A single mismatch in mixed PNA/DNA 15-mers lowers Tm by 8-20℃ (15℃ on average). In the corresponding DNA/DNA duplexes a single mismatch lowers Tm by 4 to 16 ℃ (11℃ on average).

Resistance to nucleases and proteases. PNAs with their peptide backbone bearing purine and pyrimidine bases are not molecular species that are easily recognisable by either nucleases or proteases. Therefore the lifetime of these compounds is extended both in vivo and in vitro.

Insolubitity of PNA. PNAs are charge-neutral compounds and hence have poor water solubility compared with DNA. Some recent modifications, including the incorporation of positively charged lysine residues, have shown improvements in solubility.

The first example of targeting microRNAs using PNA-based molecules is provided by miR-122. Fabani and Gait demonstrated that, using PNAs and PNA-peptide conjugates, these oligonucleotide analogs, evaluated for the first time in microRNA inhibition, are more effective than standard 2'-O-methyl oligonucleotides in binding and inhibiting microRNA actions [237].

In these experiments, PNAs were delivered by electroporation. Inhibition of miR-122 was evaluated by Northern blot and by the up-regulation effect upon both chemical and enzymatic degradation.

Interestingly, these authors showed that microRNA inhibition can be achieved without transfection or electroporation, by conjugating the PNA to the cell-penetrating peptide R6-Penetratin, or merely by linkage to four Lys residues, highlighting the potential of PNAs for future therapeutic applications as well as for studying microRNA function [237].

In a parallel work, Oh et al. described the effectiveness of miR targeting by PNA-peptide conjugates, using a series of cell penetrating peptides (CPP) as carriers, including R6 pen, Tat, a four Lys sequence, and transportan [254]. The best conditions were obtained with cationic peptides, and in particular with the Tat-modified peptide RRRQRRKKRR.

A PNA targeting miR-155 has been used in cellular systems and in mice [239]. In this study, the induction of miR-155 by bacterial lipopolysaccharide (LPS) was reduced using a PNA matching the miR target and linked to four lysine residues.

Mice challenged with sub-lethal dose of LPS were treated with 50 mg PNA/kg/day for 2 days and 24 h after the last injection (when the miR-155 expression is maximal) they were sacrified and their spleen tissue was analysed. Complete suppression of miR-155 induction was observed. Genome-wide analysis of gene expression revealed a profile of normal mice treated with LPS and then with anti-miR PNA similar to transgenic miR-155-deficient animals receiving control PBS buffer.

In a recent study Fabbri et al. evaluated the activity of a PNA targeting microRNA-210, which is firmly associated to hypoxia and is modulated during erythroid differentiation, in leukemic K562 cells . The major conclusions of this study were that a PNA against miR-210 conjugated with a polyarginine peptide (R-pep-PNA-a210) (a) is efficiently internalized within the target cells; (b) strongly inhibits miR-210 activity; (c) deeply alters the expression of raptor and -globin genes. Unlike commercially available antagomiRs, which need continuous administrations, a single

administration of R-pep-PNA-a210 was sufficient to obtain the biological effects [255].

EXPERIMENTAL DESIGN

MicroRNAs are evolutionally conserved, small non-coding RNAs 19-22 nucleotides (nt) long. They modulate gene expression by repressing their targets translation or inducing mRNA degradation through binding to the complementary sequence in target mRNA.

Moreover they are involved in the regulation of gene expression during development, cell proliferation, apoptosis and cancer. Recent studies have demonstrated a significant role of miRNAs also in normal hematopoiesis and in particular in each phase of its genetic control: self-renewal, commitment, proliferation of hemopoietic precursors, differentiation and apoptosis.

Hematopoiesis is a highly regulated process controlled by complex molecular events. The activation or inhibition of a network of transcription factors (TFs) is required to initiate the commitment of HSCs to different lineages precursors. TFs and miRNAs act in concert to regulate gene expression during hematopoietic differentiation: TFs regulate the expression of miRNA genes, whereas TFs are miRNA targets.

On the other end miRNA misexpression may contribute to the development of hematopoietic malignancies such as acute myeloid leukemia (AML). In a recent study (Lutherborrow et al., 2010) a microRNA expression analysis has been performed in AML samples, focusing on the microRNAs differentially expressed between M1 and M5 subtypes. The results of this comparison highlighted that miR-146ab, miR-181abd, miR-130a, miR-663 and mir-135b are overexpressed in M1 samples.

Interestingly, the targets of these miRNAs are key transcription factors involved in monocyte/macrophage differentiation, i.e. KLF4, MAFB, HOXA10 and IRF8; some of these have been extensively studied and characterized in our laboratory.

As in literature there isn’t any mir-130a and mir-135b functional study in HSCs that elucidate miRNAs function in differentiation of HSCs to monocyte, the aim of this study is to analyse the role of miR-130a and miR-135b in order to understand if their misregulation can have a role in the differentiation block characterizing myelogenous leukemia.

In order to investigate the functional role of miR-130a and mir-135b, we have performed gain- and loss-of-function experiments (by means of transfection of pre-Mir or anti-miR molecules) in CD34+ cells. The differentiation capacity of treated cells was monitored by qRT-PCR, Western blot, immuno-phenotypic and functional assays.

Moreoveor, once demonstrated the role of miR-130a and mir-135b in monocyte differentiation, we have analyzed the possibility to use an antimiR strategy based on PNA to restore a correct gene expression, in order to allow the normal differentiation of myeloid progenitors.

MATERIAL AND METHODS

CD34 Hematopoietic progenitor cells (CD34 HPCs)

Human CD34+ cells were purified from umbilical cord blood (CB) samples as previously described: mononuclear cells were isolated by Ficoll-Hypaque gradient separation, washed twice with PBS, and then CD34+ cells separated using a magnetic cell sorting procedure (EasySep® Human CD34 Positive Selection Kit, StemCell Technologies).

Monoblasts (CD14+ precursors) and myeloblasts (CD14- precursors) were obtained by in vitro differentiation of CB derived CD34+ cells performed as already described [56]. Briefly, CB CD34+ cells were cultured in IMDM added with 20% FCS (Bio-Whittaker, Walkersville, MD, USA), in the presence of human hematopoietic cytokines: SCF (50 ng/ml), Flt3-ligand (Flt3-l) (50 ng/ml), IL-11 (50 ng/ml), IL-6 (10 ng/ml), IL-3 (10 ng/ml) and G-CSF (10 ng/ml) (all from R&D Systems, Minneapolis, MN, USA). After 7 days of culture, hematopoietic cells were analyzed, by flow cytometry, for CD14 antigen expression.

Then monoblasts (CD14+) and myeloblasts (CD14-) cell fractions were obtained by immunomagnetic separation using the MACS technology (Miltenyi). Differentiation of CD34+ cells was monitored by by flow-cytometric analysis of CD34 and CD14 surface antigen expression.

Normal human monocytes were selected from the Ficoll separated cord blood (CB) mononuclear cells of adult samples by means of magnetic microbeads conjugated with mouse monoclonal (Mo) anti-human CD14 antibody (Ab) (Miltenyi, Auburn, CA).

Human granulocytes were initially collected from cell pellets obtained by Ficoll separation of PB samples. Erythrocytes contained in cell pellets were removed by means of osmotic lysis. Neutrophils (CD16+ fraction) were then purified using

magnetic microbeads conjugated to mouse Mo anti-human CD16 Ab (Miltenyi, Auburn, CA).

Classical monocyte activation was obtained by a 24 h treatment with a combination of 100 ng/ml LPS (Difco Laboratories) and 20 ng/ml IFNγ (Roche Diagnostics, Mannheim, Germany), alternative polarization was carried out by a 72 h stimulation with 20 ng/ml IL-4 (R&D System); macrophage de-activation was achieved by treatment with a 50 ng/ml concentration of IL-10 cytokine (Miltenyi Biotec, Auburn, CA, USA) for up to 72 h.

Cell lines culture

U937, Kasumi-1, K562, Hl60 and NB4 cell lines were cultured in RPMI 1640 supplemented with 100 U/ml penicillin and 0,1 mg/ml streptomycin (Euroclone S.p.A, Milan, Italy), 10% FBS (Sigma-Aldrich, Oakville, ON, Canada).

Vitamina D stimulation

Stimulation of CD34+ cells with VD was achieved by treatment with a 5 × 10−8 M concentration of this nuclear hormone (Hoffman-Laroche) for 7 days.

miRNA-130a expression profile

Total cellular RNA was extracted from CD34 HPCs, MYELOBLAST, MONOBLAST, MONOCYTE and GRANULOCYTES populations using the miRVana miRNA Isolation kit (Life Technologies-Ambion, Austin, TX, USA) following the manufacturer's protocol.

The quantity of each sample was quantified using Nanodrop 1000 (Thermo Fisher Scientific, Freemont, CA, USA).

miRNAs was reverse transcribed and TaqMan reactions were carried out using the TaqMan MicroRNA Assays Kit (Life Technologies-Applied Biosystems, Foster City,

CA, USA). PCR reactions were performed using TaqMan Universal PCR Master Mix without AmpErase Uracil Nglycosylase, by means of the ABI PRISM 7900 HT Sequence Detection Systems (all from Life Technologies-Applied Biosystems).

ΔΔ-CTs and RQ were calculated, for each detector using as calibrator the CD34+ sample and the TaqMan Control miRNA Assay RNU6b as endogenous control.

Pre-miR miRNA precursor molecule and Anti-miRNA inhibitor transfections

After separation, CD34HPCs were plated at the same conditions as previously described. After 2 days of culture, they were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Amaxa Biosystem), and 200μM of either the pre-miR miRNA precursor molecule-negative control # 1 (NC1) or the hsa-miR-130a-3p pre-miR miRNA precursor molecule (Ambion, Austin, TX, USA) and pulsed with the program U-008.

Pre-miR negative control 1 is a random sequence pre-miR molecule that has been extensively tested in human cell lines and tissues and validated to not produce identifiable effects on known miRNA function (http://www.ambion.com).

Anti-miRNA inhibitor transfections. After separation, CD34 HPCs were plated at the same conditions as previously described. After 2 days of culture, they were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Amaxa Biosystem), and 200μM of either the anti-NC1 or the anti-130a-3p (Ambion) and pulsed with the program U-008. Anti-miR negative control #1 is a random sequence anti-miR molecule that has been extensively tested in human cell lines and tissues and

validated to not produce identifiable effects on known miRNA function (http://www.ambion.com).

PNA synthesis

PNA were designed and synthesized by Professor Roberto Corradini (University of Parma) [255]

PNA transcfection

PNA and controls were directly added to the cellular medium at final concentration of 2μM in HSCs and AML cell lines samples.

CFC assay

After 24 h from nucleofections of the hsa-miR-130a-3p precursor, anti-miR-130a-3p and controls, or from PNA transfection, CD34+ cells were plated following the manufacturer's instructions in MethoCult GF H4434 complete methylcellulose medium for clonogenic assay (StemCell Technologies) containing a cocktail of recombinant human cytokines: SCF (50 ng/ml), granulocyte-macrophage colony-stimulating factor (10 ng/ml), interleukin-3 (10 ng/ml) and EPO (3 U/ml). After 14 days of culture at 37°C in a humidified atmosphere with 5% CO_2, the presence of colony-forming erythroid units (CFU-E), colony-forming granulocyte/macrophage units (CFU-GM), colony-forming granulocyte units (CFU-G), colony-forming monocyte units (CFU-M) and colony-forming erythroid- granulocyte-macrophage/megakaryocyte units (CFU-GEMM) were assessed.

Quantitative RT-PCR

Total cellular RNAs were extracted by means miRVana Isolation kit (Life Technologies-Ambion, Austin, TX, USA) following the manufacturer's protocol and

then analyzed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to evaluate RNA integrity and concentration.

QRT-PCR was carried out by an ABI PRISM 7900 sequence detection system (Applied Biosystems) on total RNAs (100 ng) reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. Each cDNA sample was run in triplicate for targets (MAFB, MMP9, IL-7R, IL-6, TNF-α, IL-1RA, IL8, IL10RB, CCL2, MRC1, CD163, CD14, CEBPβ) and for GAPDH endogenous control using primers and probes supplied by Applied Biosystems as pre-made solutions and the FastStart Universal Probe Master Mix (Roche Diagnostics). Quantification of QRT-PCR signals was performed using the $(2^{-\Delta\Delta Ct})$ method [25], which calculates relative changes in gene expression of the target gene normalized to the GAPDH endogenous control and relative to a calibrator sample. The values obtained were represented in terms of relative quantity of mRNA level variations.

Protein extract preparation and western blot analysis

Total proteins were extracted at 7 days after nucleofection in samples treated with hsa-miR-130a-3p precursor, with anti-miR-130a-3p, with PNA and controls using a small volume of lysis buffer (50 mM TRIS pH 7.8, 400 mM NaCl, 1% NP-40, 1 mM PMSF, 1 × Protease Inhibitor Cocktail), followed by incubation on ice for 30 min and centrifugation for additional 30 min at high speed. 30 µg of protein extracts was then loaded onto 10% SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose sheets. Blotted membranes were pre-blocked for 1 h at room temperature (RT) in blocking solution, composed by 5% nonfat milk (Regilait, Saint-Martin-Belle-Roche, France) in 0.05% TBST or by 3% nonfat milk and 2% BSA (Sigma Aldrich, St. Louis, MO, USA) in TBST 0.1%, according to antibodies

specificity. The following rabbit anti-human primary antibodies were used at concentrations recommended by the manufacturer's instruction: MafB polyclonal Ab (Sigma-Aldrich), Klf4 (Abcam), Irf8 (Santa Cruz Biotechnology), Hoxa 10 (Sigma Aldrich) and PU-1(Santa Cruz Biotechnology).a goat anti-rabbit IgG (Cell Signaling Technology) at 1:10000 dilutions was used as secondary antibody while a rabbit anti-human vinculin polyclonal Ab (Millipore Corporation, Billerica, MA, USA) was used to normalize analyzed protein samples. Detection of Western blot signals was carried out using the Westar EtaC enhanced chemiluminescent substrate (Cyanagen S.r.l., Bologna, Italy).

Flow cytometry analysis

Surface antigen expression was detected after 2 days of nucleofection in samples with hsa-miR-130a-3p precursor, with anti-miR-130a-3p and controls or in PNA transfected sample, using the fluorescein isothiocynate (FITC)-conjugated mouse anti-human CD14 and phycoerythrin (PE)-conjugated mouse anti-human CD163 MoAbs (Milteny). Negative controls were performed by staining cells with isotype-matched non specific antibodies (Milteny). Briefly, cell samples were incubated in the presence of the indicated MoAbs, at the proper diluition, in PBS 5%FBS and 1% Fcreceptor (FcR) blocking (Milteny) for 30 min at a 4℃. Cells were then washed twice, resuspended with PBS and analysed by a Coulter Epics XL-MCL (Coulter Electronic Inc. Hialeah, FL, USA) flowcytometry. At least 10000 events were counted for each sample to ensure statistical relevance. Analysis was performed in terms of positivity percentage.

Statistical analysis

All the statistical differences were analyzed by Student's t-test. $p < 0.05$ was considered statistically significant, and are indicated by asterisks $*p < 0.05$; $**p < 0.01$. $***p < 0.001$.

RESULTS

The differential expression of hsa-miR-130a-3p in myelopoieis

In order to clarify the role of miR-130a in monocyte differentiation, and in particular the possible involvement of miRNAs in the differentiation block characterizing the AML M1, we first investigated the expression level of miR-130a during the normal differentiation of Hematopoietic Stem Cell (HSCs) purified from umbilical cord blood (CB) samples. The evaluation of hsa-miR-130a-3p expression was monitored in HSCs (CD34+), myeloblasts (CD14-), monoblast (CD14+), monocyte and in monocyte activated (monocyte treated with LPS, IL-4 or IL-10) by means of TaqMan real-time PCR. The samples were obtained from the same cord blood. The results are illustrated in Figure 13.

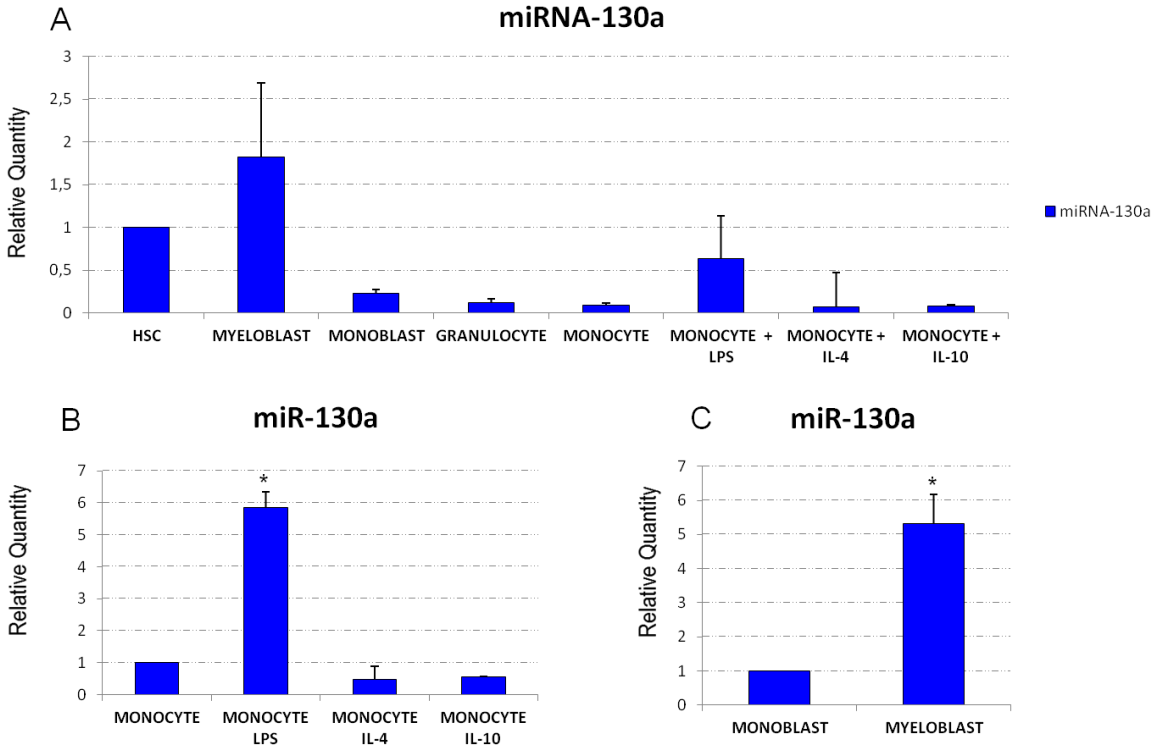

Figure 13. Evaluation of miR-130a-3p in myeloid differentiation by quantative real- time PCR*. The histogrames report on y axis the expression level and on x axis the cell type. Delta-delta-CTs and RQs were calculated for each detector using as calibrator in: A) HCS; B) Monocyte; C) Monoblast, and RNU6B as endogenous control. Mean±S.D. from three independent experiments. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001.*

Considering the HSC as a calibrator, the level of expression of mir-130a was found significantly higher in myeloblasts and in monocyte stimulated with LPS. This result can correlate with the fact that among the mRNA target of miR-130a-3p there are the transcription factors HOXA10, IRF8, KLF4 and MAFB that are considered master regulators of monocyte differentiation. Considering monoblast as calibrator, the results obtained show the differential expression of miR-130a-3p between myeloblast and monoblast. In monoblasts the miR expression is significantly lower. In this cell population the transcription factors listed above are more expressed and drive the monocyte differentiation. This can be correlated with the decrease of the miR expression. On the other end, in myeloblasts the expression level of miR-130a-3p is higher resulting in a “down-regulation” of monocyte master regulators. To verify the decrease of miR-130a expression in monocyte differentiation process, we stimulated the HSCs CD34+ cell with Vitamin D that is a strong inductor of monocyte-macrophage stimulation. After 7 day of this stimulation, we performed a real-time PCR to evaluate the miR-130a expression. The results obtained are illustrated in Figure 14

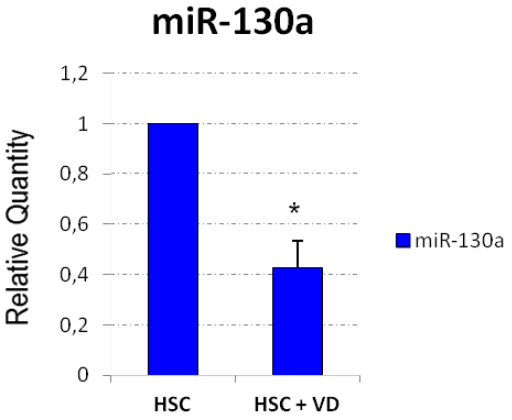

Figure 14. Evaluation of miR-130a-3p in HSC stimulated with Vitamin D*. The histogrames report on y axis the expression level and on x axis the sample. Delta-delta-CTs and RQs were calculated using as calibrator HSCs and RNU6B as endogenous control. Mean±S.D. from three independent experiments. Student’s t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001.*

The data obtained indicate that in monocyte differentiation induced by VD there is, as expected, a decrease of miR-130a expression.

Study of mIR-130A Function

In order to understand the miR-130a function in myelopoiesis and in particular in monocyte differentiation, we have performed gain and loss of function experiments.

Pre-miR miRNA precursor molecule transfections

After 2 days of culture from separation, HSCs were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Amaxa Biosystem) with 200μM of either the pre-miR miRNA precursor or molecule-negative control # 1 (NC1)(Ambion, Austin, TX, USA,) and pulsed with the program U-008. Cells were analyzed by means of real-time PCR to evaluate the over expression of miR-130a. The data obtained indicate an increase of miR-130a expression level of about 80 times. The results are illustrated in figure 1

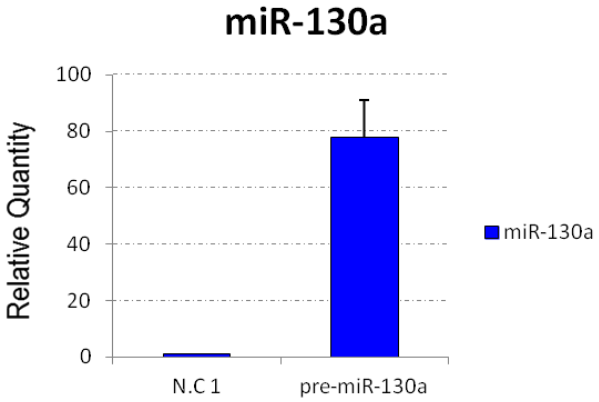

Figure 15. Evaluation of miR-130a transfection. *The histograms report the expression level of miR-130a on y axis and the sample name on x axis. NC1 represent the HSC sample transfected with miR control while Pre-miR-130a represent the sample transfected with a precursor molecule of miR-130a-3p. Delta-delta-CTs and RQs were calculated for each detector using NC1 as calibrator, and RNU6B as endogenous control. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001*

Evaluation of monocyte differentiation

The monocyte differentiation in HSCs transfected with Pre-mir-130a precursor was analyzed at different levels: morphological and immunophenotypic analysis, protein expression and gene expression.

Effect of miR-130a-3p over expression on myeloid colony formation

In order to characterize the role of miR-130a in stem cell/progenitor differentiation, 400 of CD34+ transfected with pre-miR precursor and 400 of CD34+ transfected with negative control N.C.1, were plated in methylcellulose–based medium at day 4 of liquid culture (1 day after nucleofection) in a set of three independent experiments. After 14 days of culture at 37°C in a humidified atmosphere with 5% CO2, the presence of colony-forming erythroid units (CFU-E), colony-forming granulocyte/macrophage units (CFU-GM), colony-forming granulocyte units (CFU-G), colony-forming monocyte units (CFU-M) and colony-forming erythroid- granulocyte-macrophage/megakaryocyte units (CFU-GEMM) were assessed. The result are illustrated in figure 16.

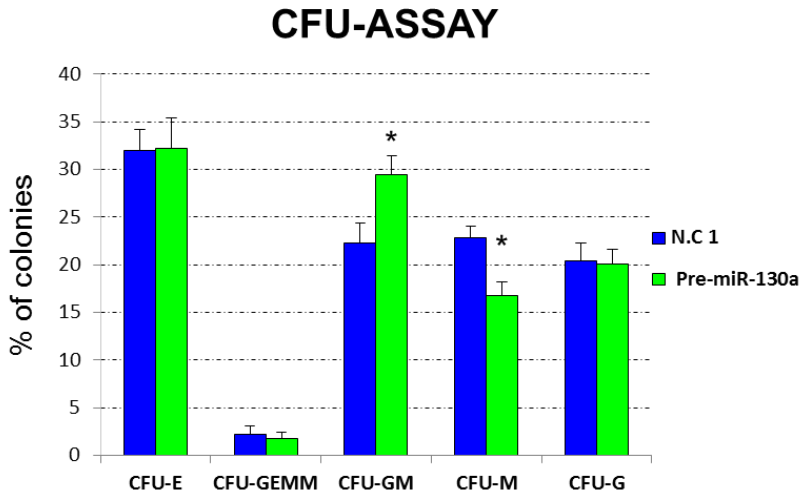

Figure 16. CFU Assay. *HSCs transfected with negative control (NC1) (blue) or with pre-miR-130a precursor (green) were plated in methylcellulose medium and scored after 14 days as CFU-E, CFU-GEMM, CFU-GM, CFU-M and CFU-G. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001.*

Clonogenic assay showed a significant increase of the percentage of colony-forming units granulocyte-macrophage (CFU-GM) from 22.3%±2 of the controls to 29.5%±1.9 of CD34+/pre-miR-130a precursor and a decrease of colony-forming units macrophage from 22.9%±1.2 of controls to 16.7%±1.4 of the CD34+/pre-miR-130a precursor. The results obtained showed a slowdown of monocyte differentiation.

Evaluation of HOXA10, IRF8, KLF4, MAFB protein expression levels.

The transcription factors Hoxa10, Irf8, Klf4 anD Mafb are master regulators of monocyte differentiation and target of miR-130a.

We analysed the protein expression level of these TFs in HSCs transfected with negative controls and with pre-miR-130a precursor molecules. Furthermore we have evaluated PU-1 that is involved in monocyte differentiation. The results are reported in figure 17.

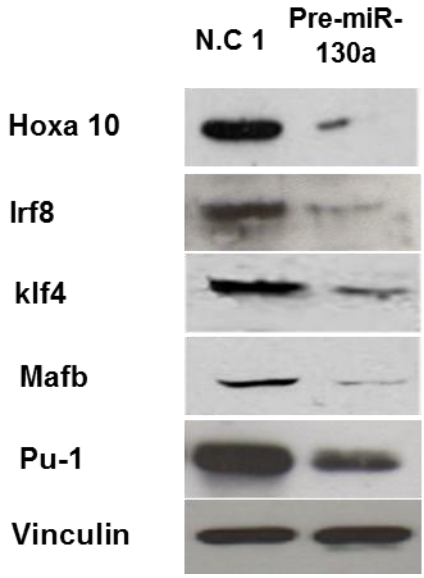

Figure 17. Evaluation of protein expression levels. *The analysis was performed in HSCs transfected with negative controls (N.C) and in HSCs transfected with pre-miR-130a-3p precursor molecules.*

The data obtained show a “down”-regulation of the transcription factorS analysed in the samples transfected with pre-miR-130a-3p precursor molecules. This result suggests that the expression level of miRNA 130a-3p have a role in the commitment of HSCs.

Evaluation of monocyte surface markers CD14 and CD163

Surface antigen CD14 and CD163 expression was detected 2 days after nucleofection of hsa-miR-130a-3p precursor and controls, using the fluorescein isothiocynate (FITC)-conjugated mouse anti-human CD14 and phycoerythrin (PE)-conjugated mouse anti-human CD163 MoAbs. Negative controls were performed staining cells with isotype-matched non specific antibodies. The results are illustrated in Figure 18.

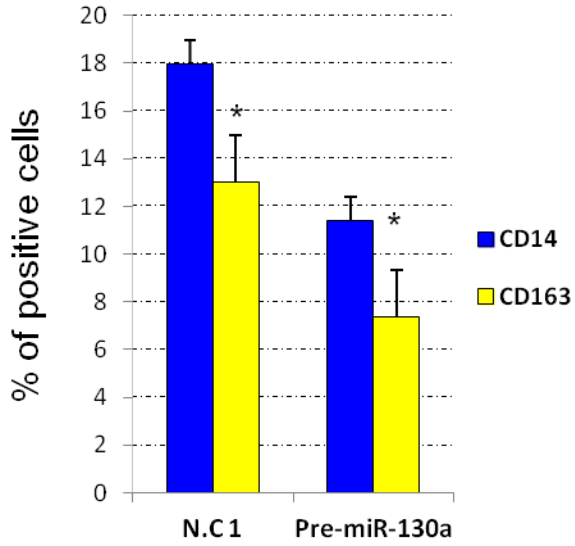

Figure 18. Evaluation of monocyte markers CD14 and CD163. *The histograms show the percentage of cell positive for CD14 and CD163 expression; the blue bars represent the HSCs transfected with negative controls (NC1) while the yellow bars the HSCs transfected with pre-miR-130a precursors. Student’s t-test $p < 0.05$ was considered statistically significant, and are indicated by asterisks *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$.*

The citofluorimetric analysis show the significant percentage decrease of monocyte differentiation markers, CD14 from 17.9%±1.8 in the control to 11.4%±1.7 in the

HSCs transfected with pre-miR-130a and CD163 from 13%±1.4 in the control to 7.35%±1.4 in HSCs transfected with pre-miR-130a.

To confirm the involvement of miR-130a in monocyte differentiation, we performed experiment in HSCs transfected with negative control or pre-miR-130a precursor molecules and we stimulated these cells with vitamin D that is a strong inductor of monocyte differentiation. Also in these experiments the expression level of CD14 results decreased in the sample transfected with pre-miR-130a-3p.

The result obtained is illustrated in figure 19 and it indicates that miRNA 130a is capable of inhibit monocyte differentiation even in presence of strong inductor of monocytopoiesis.

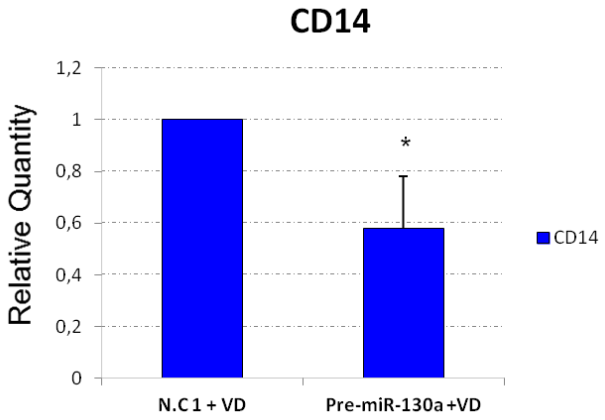

Figure 19. CD14 expression level. *The histograms report on y axis the expression level of miR-130a and on x axis the sample. NC1 rapresent the HSC sample transfected with miR control and Pre-miR-130a rapresent sample transfected with a precursor molecule of miR-130a-3p, both sample were stimulated with vitamin D. Delta-delta-CTs and RQs were calculated using as calibrator the negative controls and GAPDH as endogen control. Student's t-test $p < 0.05$ was considered statistically significant, and are indicated by asterisks *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$*

Evaluation of gene expression level of transcription factors, differentiation markers and cytokines involved in monocytopoiesis.

In order to investigate the role of mir-130a in HSCs commitment and in particular in monocyte differentiation, we performed a real-time PCR to evaluate the expression levels of the genes correlated to monocyte-macrophage differentiation.

We analysed the expression of monocyte markers CD14, CD163 and MRC1; the inflammatory cytokines IL-1,IL-6,IL-8, IL10RB, TNFα and CCL2; the transcription factosr CEBPβ; IL7r and MMP9 that are primary response genes of MAFB.

Moreover we have evaluated MPO, a granulocyte differentiation marker, and GPYa which is a marker of erythrocyte differentiation. The results are illustrated in figure 20.

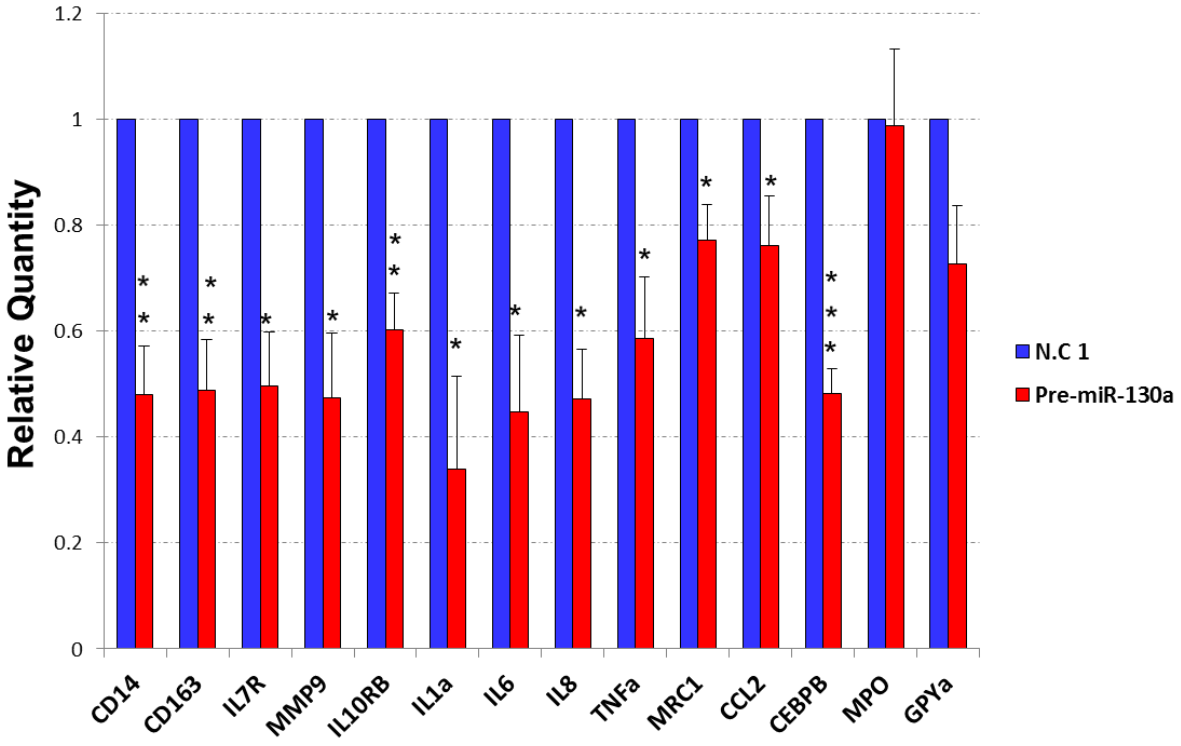

Figure 20. Evaluation of gene expression level. *The histograms report on y axis the expression level and on x axis the gene symbol. the blue bars represent the HSCs transfected with negative controls (NC1) while the red bars the HSCs transfected with pre-miR-130a precursors. Delta-delta-CTs and RQs were calculated using as calibrator the negative controls and GAPDH as endogen control. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001*

The expression of the gene involved in monocyte/macrophage differentiation result all down regulated conferming the inhibitory role of miR-130a in monocytopoiesis regulation.

Anti-miR-130a-3p inhibition molecules transfections

To confirm the results obtained with the over expression of mir-130a-3p, we performed the same experiments in HSCs transfected with anti-mir-130a inhibitor to evaluate the effect on monocyte differentiation.

After 2 days of culture from separation, HSCs were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Amaxa Biosystem) with 200µM of either the anti-miR130a inhibitor molecules or the anti-mir-negative control # 1 (NC1)(Ambion, Austin, TX, USA,) and pulsed with the program U-008.

Evaluation of IRF8, KLF4, MAFB protein expression levels.

The protein expression level of the transcription factors Irf8, Klf4 and Mafb were analysed in HSCs transfected with anti-miR negative controls or anti-miR-130a inhibitors molecules. The results are illustrated in figure 21.

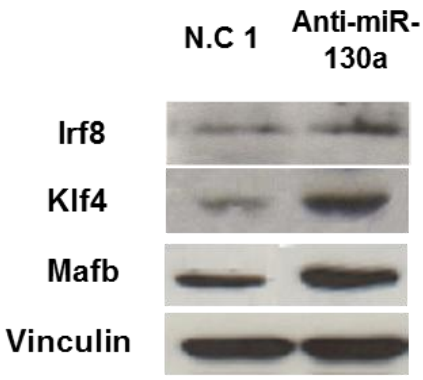

Figure 21. Evaluation of protein expression levels. *The analysis was performed in HSCs transfected with negative controls and in HSCs transfected with anti-miR-130a-3p molecules.*

The results show that monocyte master regulators protein expression level is higher in HSCs sample transfected with anti-miR-130a and confirm the role of mir-130a in monocyte TFs regulation.

Effect of miR-130a inhibition on myeloid colony formation

In order to characterize the role of miR-130a in stem cell/progenitor differentiation, 400 CD34+ transfected with anti-miR-130a inhibitor molecules and 400 of CD34+ transfected with control molecules were plated in methylcellulose –based medium at day 4 of liquid culture (1 day after nucleofection) in a set of three independent experiments. After 14 days of culture at 37°C in a humidified atmosphere with 5% CO2, plates were assessed for the presence of colony-forming erythroid units (CFU-E), colony-forming granulocyte/macrophage units (CFU-GM), colony-forming granulocyte units (CFU-G), colony-forming monocyte units (CFU-M) and coloniy-forming erythroid- granulocyte-macrophage/megakaryocyte units (CFU-GEMM). The result are illustrated in figure 22.

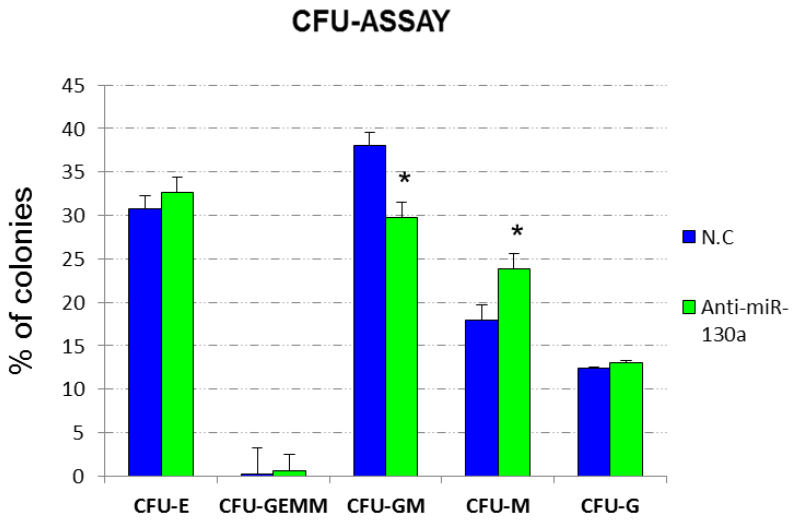

***Figure 22. CFU Assay**. HSCs transfected with negative control (NC1) (blue) or with anti-miR-130a inhibitor molecules (green) were plated in methylcellulose medium and scored after 14 days as CFU-E, CFU-GEMM, CFU-GM, CFU-M and CFU-G. Student's t-test $p < 0.05$ was considered statistically significant, and are indicated by asterisks *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$*

Clonogenic assay showed a significant decrease of the percentage of colony-forming units granulocyte-macrophage (CFU-GM) from 38.06%±3 of the controls to 29.76%±1.8 of CD34+/anti-miR-130a inhibitor sample and an increase of colony-

forming units macrophage from 17.9%±1.4 of controls to 23.8%±1.8 of the CD34+/anti-miR-130a inhibitor sample.

The results obtained show a preferential differentiation to the monocyte lineage of HSCs transfected with anti-miR-130a.

Evaluation of monocyte surface markers CD14 and CD163

Surface antigens CD14 and CD163 expression was detected 2 days after nucleofection of anti-miR-130a-3p inhibitor and control molecule, using the fluorescein isothiocynate (FITC)-conjugated mouse anti-human CD14 and phycoerythrin (PE)-conjugated mouse anti-human CD163 MoAbs. Negative controls were also performed by staining cells with isotype-matched non specific antibodies. The results are illustrated in figure 23.

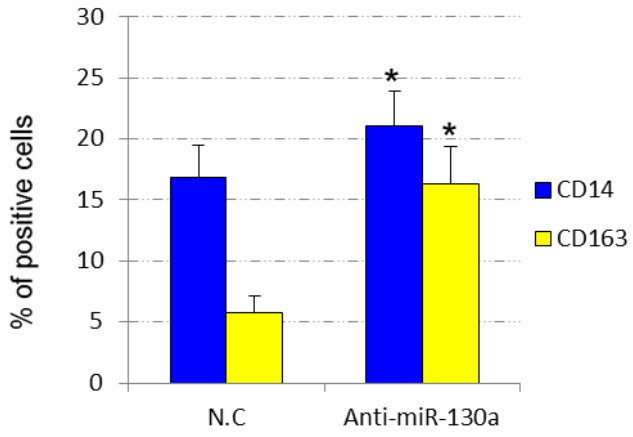

Figure 23. Evaluation of monocyte markers CD14 and CD163*. The histograms show the percentage of positive cell for CD14 and CD163 expression; the blue bars represent the HSCs transfected with negative controls (NC1) while the yellow bars the HSCs transfected with anti-miR-130a inhibitor molecules. Student's t-test $p < 0.05$ was considered statistically significant, and are indicated by asterisks $*p < 0.05$; $**p < 0.01$. $***p < 0.001$*

The citofluorimetric analysis showed the significant increase of monocyte differentiation markers, CD14 from 16.5%±1.5 in the control to 21%±2.8 in the HSCs

transfected with anti-miR-130a inhibitors and CD163 from 5.72%±1.5 in the control to 16.30%±3.51 in HSCs transfected with anti-miR-130a inhibitor.

We evaluated the gene expression level of Mafb primary response genes (IL7R and MMP9) and MRC1 which is a monocyte markers. The results are illustrated in figure 24.

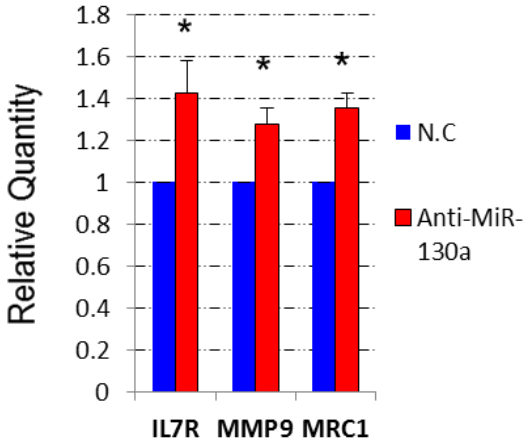

Figure 24. Evaluation of gene expression level*. The histograms report on y axis the expression level and on x axis the gene symbol. The blue bars represent the HSCs transfected with negative controls (NC1) while the red bars the HSCs transfected with anti-miR-130a. Delta-delta-CTs and RQs were calculated using as calibrator the negative controls and GAPDH as endogen control. Student's t-test $p < 0.05$ was considered statistically significant, and are indicated by asterisks *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$*

The result obtained document an increase of the gene expression level of Mafb primary response gene, that is considerated a master regulator of monocyte differentiation and an increase of monocyte marker MRC1.

The differential expression of hsa-miR-135b-5p in myelopoieis

In order to clarify the role of miR-135b in monocyte differentiation, and in particular the possible involvement of this miRNA in the differentiation block characterizing the AML M1, we investigated the expression level of miR-135b during the normal differentiation of *Hematopoietic Stem Cells* (HSCs) purified from umbilical cord blood (CB) samples . The evaluation of hsa-miR-135b-5p expression was monitored *in HSCs (CD34+)***,** myeloblasts (CD14-), monoblast (CD14+) and monocyte.

The results are illustrated in figure 25 (A and B).

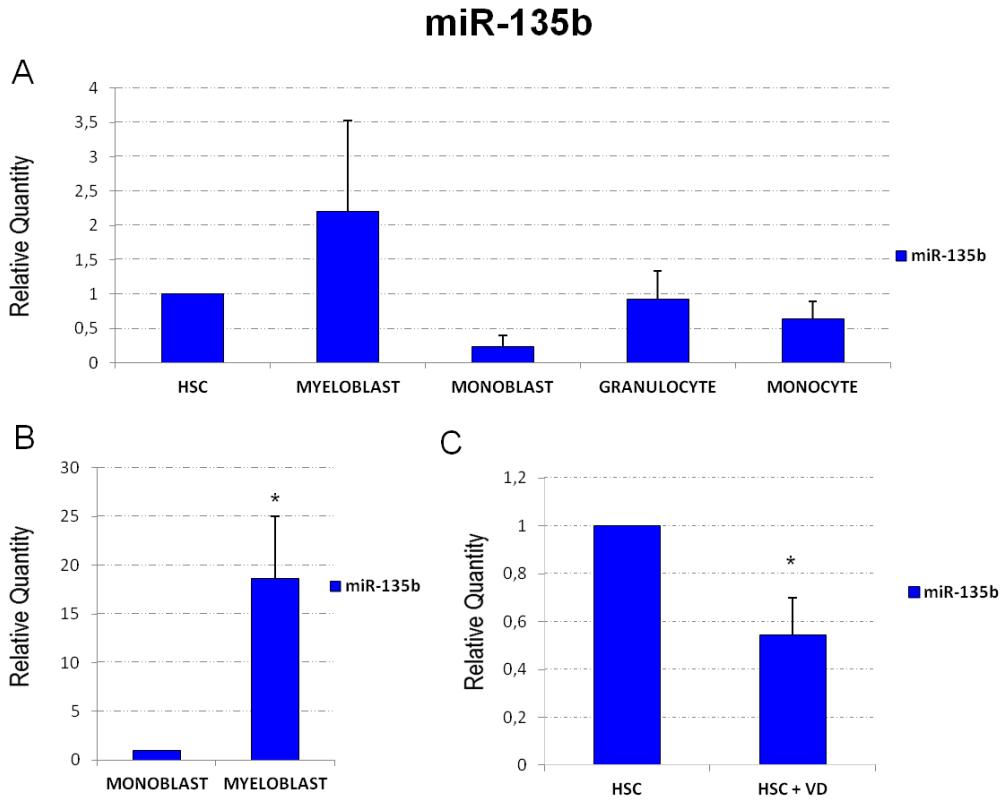

Figure 25. Evaluation of miR-135b-5p in myeloid differentiation and in HSC trattated with vitamin D by quantative real- time PCR*. The histogrames report on y axis the expression level and on x axis the cell type. Delta-delta-CTs and RQs were calculated for each detector using as calibrator in: A) HCS; B) Monoblast; C) HSC and RNU6B as endogenous control. Mean±S.D. from three independent experiments. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001*

Considering the HSC as a calibrator, the level of expression of mir-135b was found significantly higher in myeloblasts . This result can correlate with the fact that among the mRNA target of miR-135b there are the transcription factors HOXA10 and KLF4 that are considered master regulators of monocyte differentiation.

To verify the decrease of miR-135b expression in monocyte differentiation process, we stimulated the HSCs CD34+ cell with Vitamin D that is a strong inductor of monocyte-macrophage stimulation. After 7 day of this stimulation, we performed a real-time PCR to evaluate the miR-135b expression. Result is illustrated in figure 25 c.

Study of miR-135b Function

In order to understand the miR-135b function in myelopoiesis and in particular in monocyte differentiation, we have performed gain and loss of function experiments and we evaluated the HSCs differentiation by means of CFU-ASSAY.

Pre-miR miRNA precursor molecule and miR inhibitor transfections.

After 2 days of culture from separation, HSCs were transfected with the Amaxa Nucleofector Device, using the Human CD34 Cell Nucleofection Kit, according to the manufacturer's instructions (Amaxa Biosystem) with 200μM of either the pre-miR miRNA precursor or antimiR molecule and theirs molecule-negative control (Ambion, Austin, TX, USA,) and pulsed with the program U-008. Cells were analyzed by means of real-time PCR to evaluate the over expression of miR-135b. The data obtained indicate an increase of miR-135b expression level of about 65 times. The results are illustrated in figure 26.

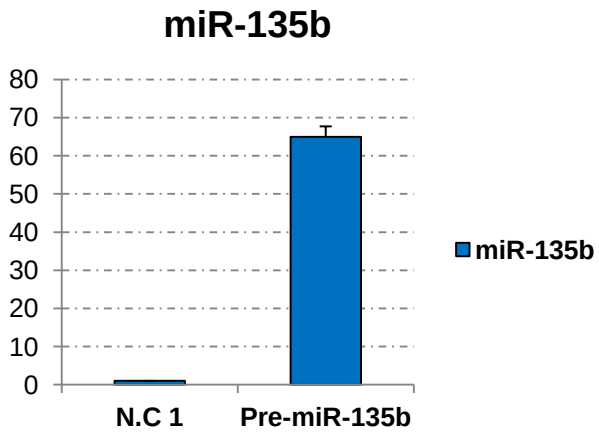

Figure 26. Evaluation of miR-135b transfection*. The histograms report the expression level of miR-135b on y axis and the sample name on x axis. N.C 1 represent the HSC sample transfected with miR control while Pre-miR-135b represent the sample transfected with a precursor molecule of miR-135b-5p. Delta-delta-CTs and RQs were calculated for each detector using NC1 as calibrator, and RNU6B as endogenous control.*

Evaluation of monocyte differentiation

The monocyte differentiation in HSCs transfected with Pre-mir-135b precursor and inhibitor was analyzed with morphological analysis.

Effect of miR-135b-5p over expression or inhibition on myeloid colony formation

In order to characterize the role of miR-135b in stem cell/progenitor differentiation, 400 of CD34+ transfected with pre-miR precursor or anti-miR and 400 of CD34+ transfected with their respective negative control N.C.1, were plated in methylcellulose–based medium at day 4 of liquid culture (1 day after nucleofection) in a set of three independent experiments. After 14 days of culture at 37°C in a humidified atmosphere with 5% CO2, the presence of colony-forming erythroid units (CFU-E), colony-forming granulocyte/macrophage units (CFU-GM), colony-forming granulocyte units (CFU-G), colony-forming monocyte units (CFU-M) and colony-forming erythroid- granulocyte-macrophage/megakaryocyte units (CFU-GEMM) were assessed. The results are illustrated in figure 27.

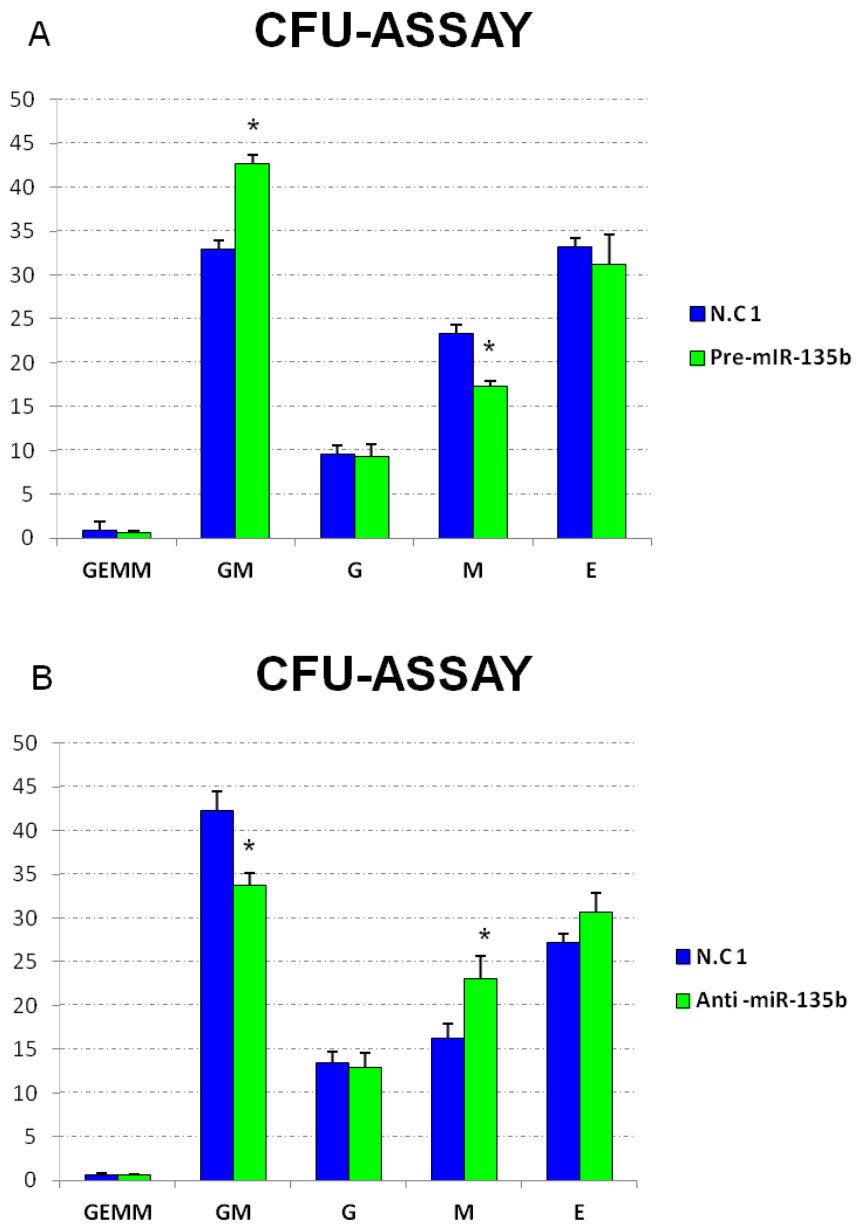

Figure 27. CFU-ASSAY*. A) HSCs transfected with negative control (N.C1) (blue) or with pre-miR-135b precursor (green) B) HSCs transfected with negative control (N.C1) (blue) or with anti-miR-135b (green) were plated in methylcellulose medium and scored after 14 days as CFU-E, CFU-GEMM, CFU-GM, CFU-M and CFU-G. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001*

Clonogenic assay showed a significant increase of the percentage of colony-forming units granulocyte-macrophage (CFU-GM) from 32.95%±1.21 of the controls to 42.61%±1.1 of CD34+/pre-miR-135b precursor sample and an decrease of colony-forming units macrophage from 23.35%±1.15 of controls to 17.38%±0.41 of the CD34+/ pre-miR-135b precursor sample.

As expected in HSCs transfected with anti-miR-135b we showed a decrease of colony-forming units granulocyte-macrophage (CFU-GM) from 42.22%±2.25 to 33.69%±1.49 and an increase of colony-forming units macrophage from 16.24%±1.72 of controls to 23.11%±2.48 of the CD34+/ anti-miR-135b sample.

The results obtained show miR-135b role in HSCs differentiation and in particular it documents that the altered expression of the miRNA can disrupt the normal differentiation.

Modulation of miRNA activity by AntimiR Peptide Nucleic Acid (PNA)

The gain and loss of function experiments highlights the role of miR-130a in the modulation of monocyte differentiation and confirmed the hypothesis that its overexpression can contribute to the differentiation block of AML M1.These data suggest the use of antimiR strategy to restore a correct gene expression, in order to allow the normal differentiation of myeloid progenitors.

For this reason, we have decided to use peptide nucleic acids (PNA) as approach to silence the expression of miR-130 as they form very stable PNA:RNA duplex which can efficiently disrupt the dsRNA duplex.

PNA synthesis

PNA were designed and synthesized by Professor Roberto Corradini (University of Parma). We used a chiral PNA conjugated with eight arginine residues on the backbone (R-pep-PNA-a130a) to allow the internalization in the absence of transfectants agents.

The sequence used are:

1. **H-R_8-CCT TTTAACATTGCA CTG-Gly-NH_2** Anti-miR-130a
2. **H-R_8-CCA TTTTACAATGCT CTG-Gly-NH_2** Degenerate sequence
3. **H-R_8-ATCTCGTATCTATCCC TGA-Gly-NH_2** Scramble PNA
4. **FI-O-R8-CCT TTT AAC ATT GCA CTG-Gly-NH_2** Fluorescent Anti-miR-130a

Uptake of chiral PNA in HSCs and AML cell lines.

Anti-miR-130a and controls were directly added to the cellular medium at final concentration of 2µM in HSCs and AML cell lines samples.

PNA conjugated with fluorescein (FI-O-R8-CCT TTT AAC ATT GCA CTG-Gly-NH_2) was used to evaluated the cellular uptake by means of cell sorting and confocal microscopy analysis. The results are illustrated in figure 28.

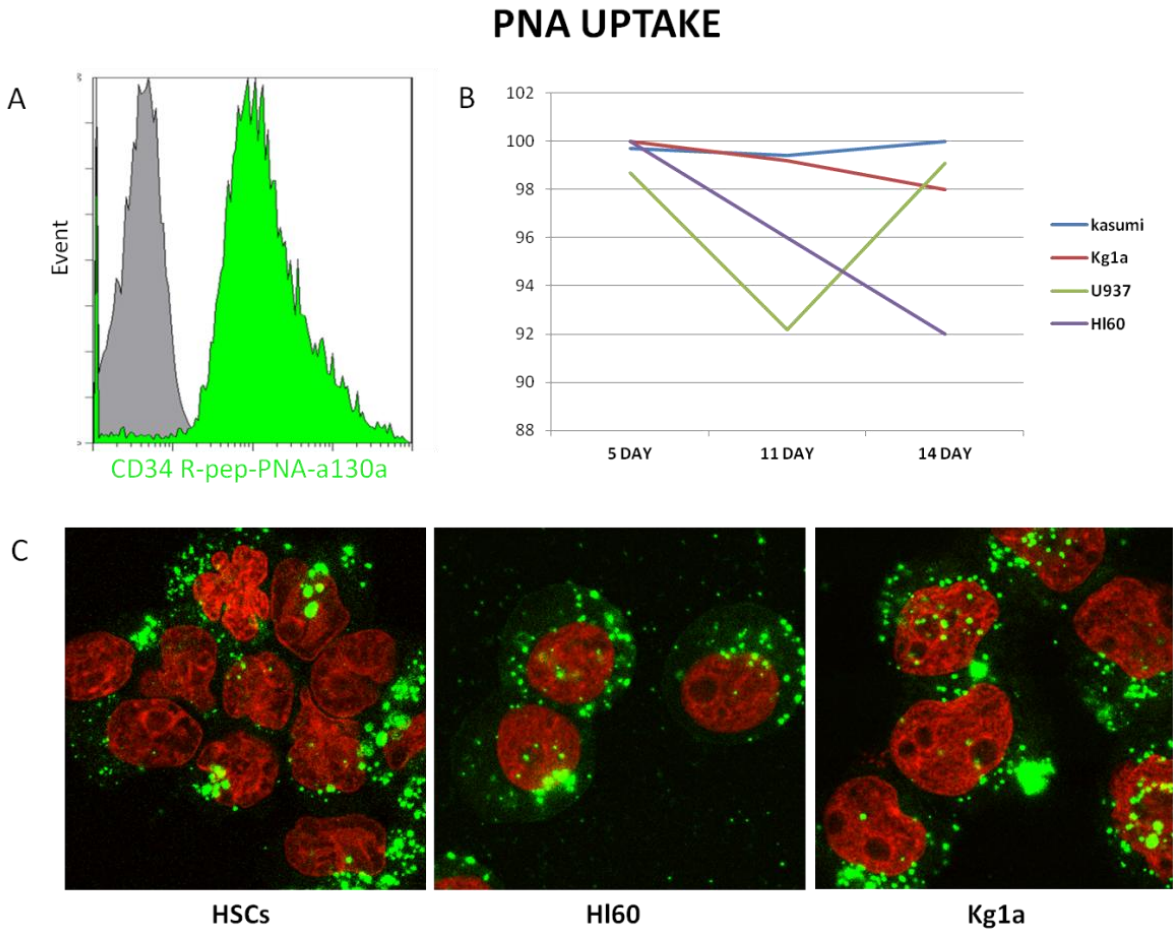

Figure 28. Evaluation of PNA uptake. *A) Cytofluotimetric analysis show HSCs uptake after incubation of fluorescein –labelled (24h) R-pep-PNA –a130a. B) The graphic represent AML cell line uptake of fluorescein –labelled R-pep-PNA –a130a at 5, 11, 14 days from transfection. C) Merged analysis of the fluorescence and the Dapi staining after 24 h of transcfection, confocal microscopy.*

The data obtained show that the uptake of HSCs and AML cell lines transfected with R-pep-PNA –a130a was very efficient, the citofluorimetric analysis documented that the 98-100% cells was fluorescent positive. AML cell lines uptake was monitored for 2 weeks. The result obtained indicate that cells are still fluorescent demonstrating the

stability of the PNs. These results were obtained in absence of transfection reagents. This element is particularly relevant for hematopoietic cells which are very hard to be transfected without nucleofection.

Evaluation of IRF8, KLF4, MAFB protein expression levels.

The protein expression levels of the transcription factors Irf8, Klf4 and Mafb were analysed in HSCs and in AML cell lines transfected with ant-imiR-130a PNA and controls. The results are illustrated in figure 29.

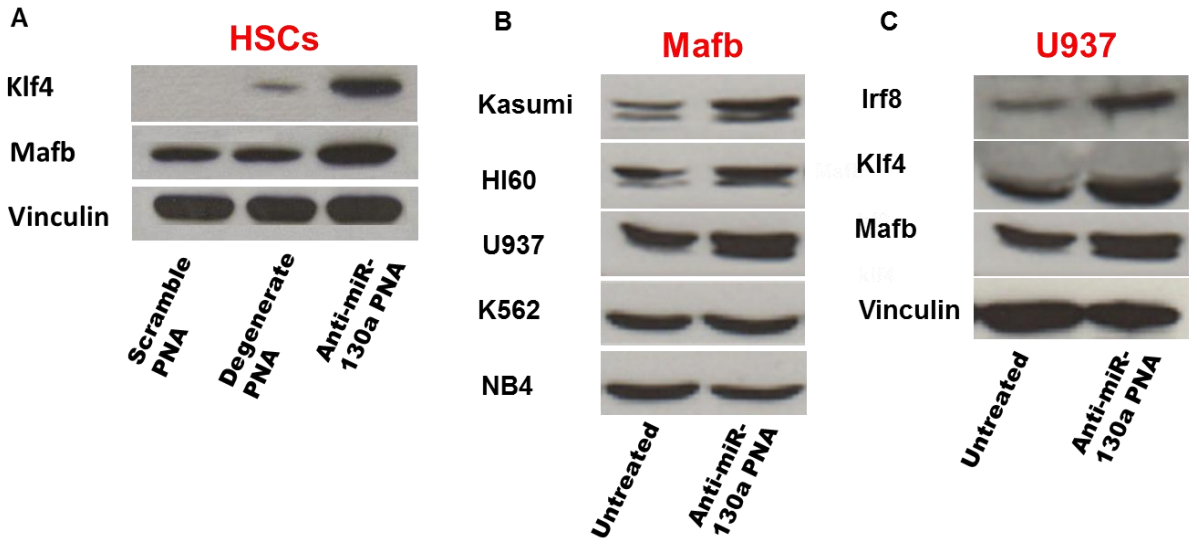

***Figure 29. Evaluation of protein expression levels**. The analysis was performed in A)HSCs transfected with Anti-miR-130a PNA and controls; B-C) AML cell lines untreated and transfected with Anti-miR-130a PNA.*

The results show that monocyte master regulators protein expression level is higher in sample transfected with anti-miR-130a PNA and the effect is stronger in respect to anti-miR molecules (Figure 21).

Evaluation of monocyte differentiation

The monocyte differentiation in HSCs transfected with Anti-miR-130a PNA was analyzed by means of morphological and immunophenotypic analysis.

Effect of anti-miR-130a PNA on monocyte marker expression

The expression level of the surface antigen CD163, a monocyte marker, was analyzed to evaluate the monocyte differentiation in sample transfected with anti-miR-130a PNA or scramble PNA, after 7 day of liquid culture.

The results are illustrated in figure 30.

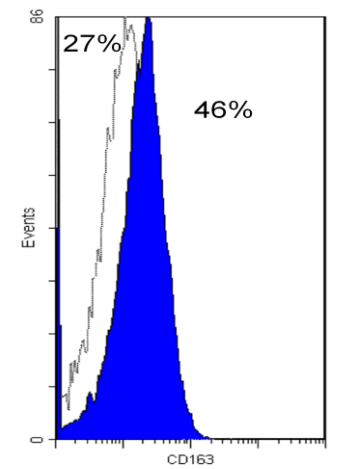

Figure 30. Evaluation of monocyte markers CD163. The histograms show the percentage of positive cell for CD163 expression; the blue signal represent the HSCs transfected with anti-miR-130a PNA while the gray signal the HSCs transfected with scramble PNA.

The citofluorimetric analysis shows an increase of the 20% of CD163 positive cells in sample transfected with anti-miR-130a PNA in respect to scramble PNA, highlighting a significant increase of monocytopoiesis.

Effect of anti-miR-130a PNA on myeloid colony formation

In order to characterize the effect of the anti-miR-130a PNA in stem cell/progenitor differentiation, 400 of CD34+ transfected with anti-miR-130a-PNA and 400 of CD34+

transfected with controls, were plated in methylcellulose–based medium at day 4 of liquid culture (1 day after transfection) in a set of three independent experiments. After 14 days of culture at 37°C in a humidified atmosphere with 5% CO2, the presence of colony-forming erythroid units (CFU-E), colony-forming granulocyte/macrophage units (CFU-GM), colony-forming granulocyte units (CFU-G), colony-forming monocyte units (CFU-M) and colony-forming erythroid- granulocyte-macrophage/megakaryocyte units (CFU-GEMM) were assessed. The results are illustrated in figure 31.

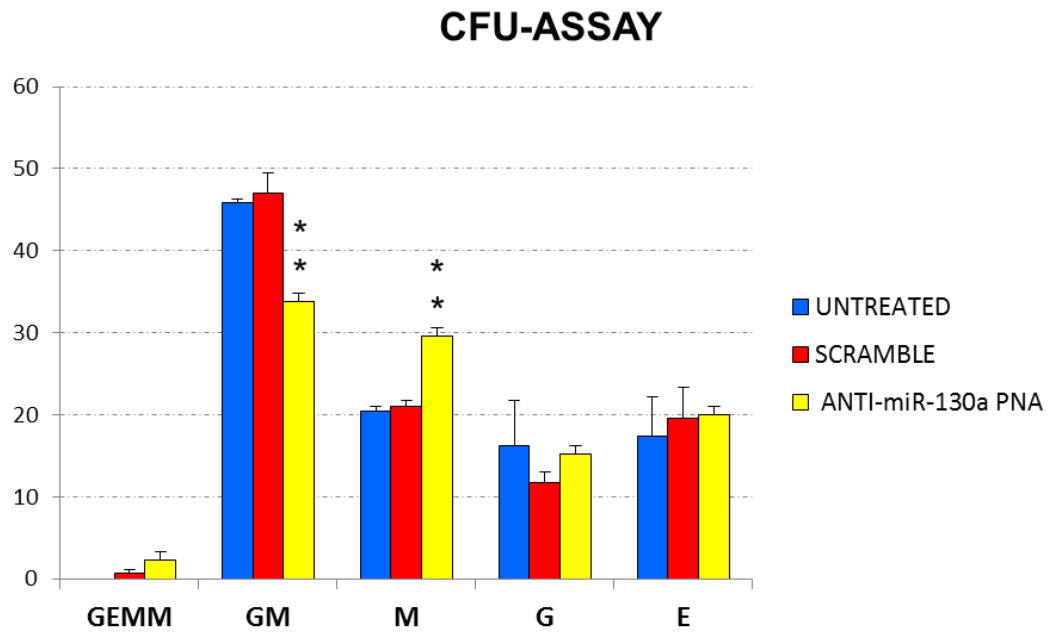

Figure 31 CFU Assay. *HSCs untransfected (blue), transfected with negative scramble PNA (red) or with anti-miR-130a PNA (yellow) were plated in methylcellulose medium and scored after 14 days as CFU-E, CFU-GEMM, CFU-GM, CFU-M and CFU-G. Student's t-test p < 0.05 was considered statistically significant, and are indicated by asterisks *p < 0.05; **p < 0.01. ***p < 0.001*

The CFU-assay data show a significant decrease of colony-forming units granulocyte-macrophage (CFU-GM) from 46.22%±2.6 of scramble PNA sample to 33.84%±2.45 of the anti-miR-130a PNA sample and an significant increase of colony-forming units macrophage (CFU-M) from 21.02%±0.74 to 29.63%±1.29.

The results obtained documents the efficiency of PNA as molecule capable to modulate the expression of the miRNA and the HSCs differentiation.

Morphological analysis of transfected HSCs

Biological effect promoted by anti-miR-130a PNA transfection was analyzed by morphological analysis of MaY-Grunwald –Giemsa stained .

The results illustrated in figure 32 document that the sample transfected with anti-miR-130a PNA is highly enriched with monocyte-macrophage lineage cells.

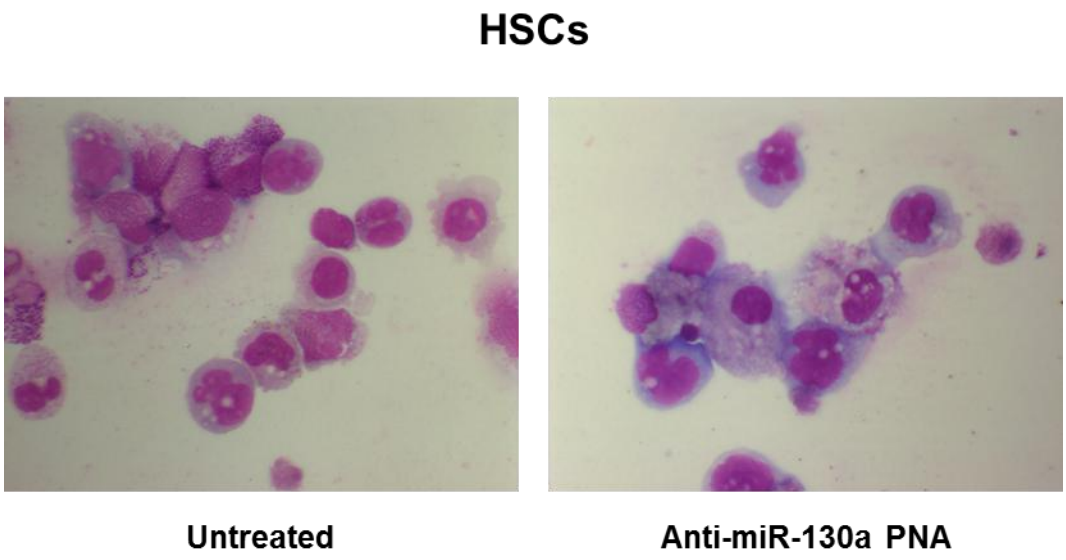

Figure 32. Morphological analysis performed at 14 day of liquid culture on HSCs transfected with anti-miR-130a PNA.

DISCUSSION

MicroRNAs (miRNAs, miRs) are a family of small (19–22 nucleotides in length) noncoding RNAs that regulate gene expression by sequence-selective targeting of mRNAs.

Since their discovery, hundreds of miRNAs and their biological functions have been identified, and thus far (June 2014) 28 645 miRNAs have been registered in the miRBase database (http://microrna.sanger.ac.uk). Considering that a single miRNA can target several mRNAs and a single mRNA might contain in the 3UTR sequence, several signals for miRNA recognition, it has been calculated that at least 30–60% of human mRNAs are a target for microRNAs.

This makes miRNAs one of the most abundant regulators of gene expression in humans and their study represents one of the most exciting areas of modern medical sciences as they possess a unique ability to modulate an immense and complex regulatory network of gene expression [6].

miRNAs regulate all aspects of a cell, including its differentiation, function, proliferation, survival, metabolism and response to changes in its environment.

Several miRNAs appear to act as master regulators of cell fate decisions in response to environmental or developmental cues by altering the expression of key regulatory genes and/or by regulating many genes in specific pathways.

Recent studies demonstrate a significant role of miRNAs also in normal and malignant hematopoiesis [218]

Hematopoiesis is a hierarchical process by which multipotent stem cells (HSCs) gradually reduce their differentiation potential, generating unipotent progenitors and precursors from which they originate terminally differentiated blood cells [53]. The differentiation process is established by the coordinated action of transcription factors

(TFs), chromatin remodeling complexes and microRNAs. The transcription factors are able to activate specific genes and to induce the expression of receptors for growth factors able to condition the differentiation towards the precursors of the different lineages.

TFs and miRNAs act in concert to regulate gene expression during hematopoietic differentiation: TFs regulate the expression of miRNA genes, whereas TFs are miRNA targets. Thus, miRNAs represent an important axis of regulation in HSCs: they play a critical role in the control of stem/progenitor, lymphoid, myeloid, erythroid and megakaryocytic biology, and in the immune function of these cell lineages [186].

In fact, in the last decade the miRNA has been describe as Key regulator of hematopoiesis and specific signature of differentially expressed miRNA have been identified as associated with each distinct phase of this process: self-renewal, commitment and differentiation of HSCs.

Similarly, individual miRNAs and miRNA signatures have been associated with specific hematological malignancies.

Acute myeloid leukemia is characterized by an accumulation of granuocytic or monocyte precursors in bone marrow and peripheral blood. miRNA patterns have been correlated with cytogenetic and molecular subtypes of AML [206].

In a recent study (Lutherborrow et al., 2011), [225] comparing the microRNA expression profiles of FAB classification M1 and FAB classification M5 subtypes, it was found the higher expression of *miR-181a/b/d*, *miR-130a*, *miR-135b*, *miR-146a/b* and *miR-663* in FAB classification type M1.

Interestingly, the targets of these miRNAs are key transcription factors involved in monocyte/macrophage differentiation, i.e. KLF4, MAFB, HOXA10 and IRF8; some of these have been extensively studied and characterized in our laboratory.

As in literature there isn't any miR-130a and miR-135b functional study in HSCs that elucidate miRNAs function in differentiation of HSCs to monocyte, the aim of this study is to analyse the role of miR-130a and miR-135b in order to understand if their misregulation can have a role in the differentiation block characterizing myelogenous leukemia.

Initially we have investigated the expression level of miR-130a and miR-135b in myelopoiesis, we use as model the in vitro differentiation of Hematopoietic Stem Cell (HSCs) purified from umbilical cord blood (CB) samples and AML cell lines.

The evaluation of hsa-miR-130a-3p and hsa-miR-135b-5p expression was monitored in HSCs (CD34+)**,** myeloblasts (CD14-), monoblast (CD14+) and monocyte. The data obtained indicate that miR-130a and miR-135b are significantly higher in myeloblasts.

This result correlates with the fact that among the mRNA target of miR-130a-3p and miR-135b-5p there are the transcription factors HOXA10, IRF8, KLF4 and MAFB that are considered master regulators of monocyte differentiation, so the lower expression of these TF is required to have a granulocytic differentiation.

To verify the decrease of miRNAs expression in monocytopoiesis, we stimulated the HSCs CD34+ cells with Vitamin D that is a strong inductor of monocyte-macrophage stimulation. As expected, a decrease of miR-130a and miR-135b expression was observed. This data represent another indication that the decrease of miRNA expression is necessary to have a monocyte differentiation.

These preliminary data show the down regulation of miR-130a and miR-135b in monocyte differentiation and suggest that their misregulation can contribute to an aberrant monocytopoiesis.

In fact, many studies documented that the expression change of a single microRNA can reinforce single lineage differentiation pathway into already committed cells or into progenitors grown in unilineage conditions.

In order to investigate the functional role and the perturbation capacity of miRNAs in mielopoyesis we have performed gain- and loss-of-function experiments by means of transfection of pre-miR and anti-miR molecules in HSCs purified from cord blood.

The differentiation capacity to monocyte lineage of treated cells was monitored by qRT-PCR, Western blot, immuno-phenotypic and functional assays.

Initially, we have studied miR-130a.

The samples transfected with pre-miR-130a show a moderate but significant slowdown of monocyte differentiation, documented by CFU-assay, which shows a significant higher percentage of colonies CFU-GM and a significant lower percentage of colonies CFU-M. This result was confirmed by gene expression analysis of differentiation markers (CD14, CD163 and MRC1), inflammatory cytokines (IL-1, IL-6, IL-8, IL10RB, CCL2 e TNFa) and transcription factors (CEBPβ, PU-1, HOXA10, KLF4, IRF8, MAFB) involved in the monocyte/macrophage differentiation that result all down regulated.

Moreover, we treated the CD34+ cell that overexpressing the miR-130a with Vitamin D that is as strong inductor of the monocyte differentiation and also in this case we observes a decrease expression of monocyte differentiation marfers.

We have performed the same monocyte differentiation analysis in CD34+ cells transfected with anti-miR-130a; accordingly with what expected, in this case the data obtained show an increase of monocyte differentiation.

At this time data on miR-135b are very preliminary, but the first results obtained are similar to miR-130a data: CFU-ASSAY indicate a significant decrease in monocyte

differentiation in sample transfected with pre-miR molecules and as aspect an increase of monocytopoiesis in HSCs transfected with anti-miR.

This study highlights the role of miR-130a and miR-135b in the modulation of monocyte differentiation and confirmed the hypothesis that their overexpression can contribute to the differentiation block of AML M1.

Moreover these data suggest the use of antimiR strategy to restore a correct gene expression, in order to allow the normal differentiation of myeloid progenitors.

For this reason, we have decided to use peptide nucleic acids (PNA) as an approach to silence the expression of miR-130a in HSCs and in leukemic cell lines.

PNAs have very important properties: thermal stability, stronger binding to RNA, capacity to form stable triple-helical complex, greater specificity of interaction, resistance to nucleases and proteases and some recent modifications, including the incorporation of positively charged lysine residues, have shown improvements in solubility.

In a recent study Fabbri et al. evaluated the activity of a PNA targeting miR-210 in K562 cells; the major conclusions of this study were that a PNA against miR-210 conjugated with a polyarginine peptide (R-pep-PNA-a210) is efficiently internalized within the target cells and strongly inhibits miR-210 activity.

Based on these experiments we decided to use the same type of PNA.

The results obtained show a strong uptake of R-pep-PNA-miR130a in absence of transfection reagent in hematopoietic stem cells and in AML cell lines that are very hard cell to be transfect. The analysis performed at day 14 of culture confirmed that PNA is very stable and that a single treatment is sufficient to have an antimiR effect.

The evaluation of the monocyte differentiation in HSCs and in leukemic cell lines treated with anti-miR130a PNA, documents an higher increase of monocytopoiesis when compared with the sample treated with anti-miR molecules, demonstrating that

the biological effect of PNAs in the modulating miRNA expression is strong and very stable.

The data obtained in this study highlight that the modulation of miR-130a expression is necessary to a correct myeloid differentiation and are the basis for possible future use of R-pep-PNA-a130a as therapeutic agent to restore a correct gene expression in hematological malignancies like AML.

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