Università degli Studi di Padova

Facoltà di Scienze MM. FF. NN.

Corso di Laurea Specialistica in Biologia Molecolare

In vitro effect of retinoic acid and epigenetic modifying drugs on mesenchymal stem cells

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Anno Accademico 2008-2009

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1 Abstract

Mesenchymal stem cells (MSC) are adult stem cells present in many tissues, such as bone marrow, adipose tissue and peripheral blood. They are able to differentiate into several mesodermal cells, such as chondrocyte, osteoblast and adipocyte. In addition, MSCs share the same microenvironment of hematopoietic stem cells (HSC) and they play a central role in the regulation of proliferation and self-renewal of HSCs. Retinoic acid (RA) is a well-known morphogen-like agent, is widely used alone or in combination with epigenetic modifying drugs, such as demethylating agents and HDAC inhibitors, in therapeutic approaches to treat acute myeloid leukemia and solid tumors. Here, we test the in vitro effects of all-trans retinoic acid, 5-azacytidine and valproic acid on the biological properties of MSCs. We show that both VPA and AZA reduce the number of CFU-Fs by increasing the number of apoptotic cells and committing them towards a non-proliferative state. In addition, we demonstrate that ATRA treatment exerts opposite effect on CFU-Fs and cultured MSCs, by decreasing colony formation of CFU-Fs and enhancing the proliferation of cultured MSCs. We demonstrate that ATRA signaling is mediated by the RARβ2 receptor in both early progenitors and MSCs, and it differentially triggers the expression of genes involved in the self-renewal and pluripotency of embryonic and adult stem cells.

2 Introduction

2.1 Mesenchymal Stem Cell

Human mesenchymal stem cells, also known as osteogenic stem cells or multipotent mesenchymal stromal cells (Friedenstein 1987, Dominici 2006), were first isolated from bone marrow in 1966 for their ability to form discrete colonies (the colony-forming unit fibroblastic, CFU-Fs) initiated by single cell when seeded at clonal density(Friedenstein 1966). Later experiments of in vivo transplantation demonstrated that single MSC generates multiple mesodermic tissue, as bone, cartilage, adipose and fibrous tissue. (Friedenstein 1990)

Further studies have led to the better characterization of MSC and the identification of three peculiar criteria that define them: their property of adherence to plastic; their phenotype, assessed by the expression of a combination of surface antigens (CD14-, CD19-, CD34-, CD45-, HLA-DR-, CD73+, CD90+, CD105+); and their capacity of in vitro differentiation into three lineages, chondrocyte, osteoblast and adipocyte. (Dominici 2006) As any other stem cell, the mesenchymal stem cell has the ability to differentiate towards different lineages and to perpetuate itself by asymmetrical division. Indeed, MSCs are able to proliferate in vitro and to maintain their differentiation potential after multiple population doublings. The use of specific in vitro condition stimulates MSC differentiation into different mesodermal cells, such as stromal cells, chondrocyte, osteoblast, adipocyte and myocite. (Figure 1)(Friedenstein 1966; Pittenger 1999, Caplan 2007). Furthermore, compelling evidence from in vitro studies demonstrate the plasticity of MSCs, inducing them to become nonmesodermal cells such as neurons, glial cells, hepatocytes, and endothelial cells (Abdallah 2008). However, this multilineage differentiation potential is still controversial, due to the lack of in vivo evidence. In addition, the gold standard assay for demonstration of stemcellness is based on the ability of cells to reconstitute in vivo a stem cell compartment with phenotypic and functional properties identical to the original cell population only acquired through in vivo transplantation in immunocompromised mice. Evidence for self renewal and maintenance of stemcellness capacity of MSCs after serial implantation has only recently started to emerge, suggesting the presence of bona fide stem cell characteristics (Sacchetti et al. 2007)

MSCs are present in different tissues in the organism, and have been isolated from tissues other than bone marrow, such as adipose tissue, peripheral blood,

dental pulp, fetal liver, amniotic fluid or umbilical cord blood. (Lazennec 2008) Traditionally, MSCs are isolated by density-gradient fractionation and selected by their ability to adhere on the plastic surface. However, they remain a heterogeneous mixture of cells with a variable differentiation and proliferation potential. For example, only around 30% of the clonal MSCs (ie CFU-F) are multipotent and able to form bone after in vivo transplantation. (Kuznetsov 1997) and no markers are available to distinguish multipotent CFU-Fs from more committed ones. Nevertheless, several investigators have tried different methods to enrich cultures for multipotent MSC. The most widely used approach employs monoclonal antibodies. One of the first antibodies shown to enrich for MSCs is the Stro-1 antibody, which identifies an as yet uncharacterized cell surface epitope expressed by MSCs and erythrocytic cells. (Gronthos 1994) Since other antigens such as CD271 (low-affinity nerve growth factor receptor), CD18 (b2 integrin) or the embryonic stem cell marker SSEA-4 have been identified (Abdallah 2008). Enrichment of multipotent MSC has also been attempted by combining Stro-1 antibody with anti-CD106 (VCAM-1) or anti-CD146 (MUC18). (Abdallah 2008) However, sorting clonogenic progenitor by surface phenotype or sorting them by plastic adherence has the same pratical result (Sacchetti 2007).

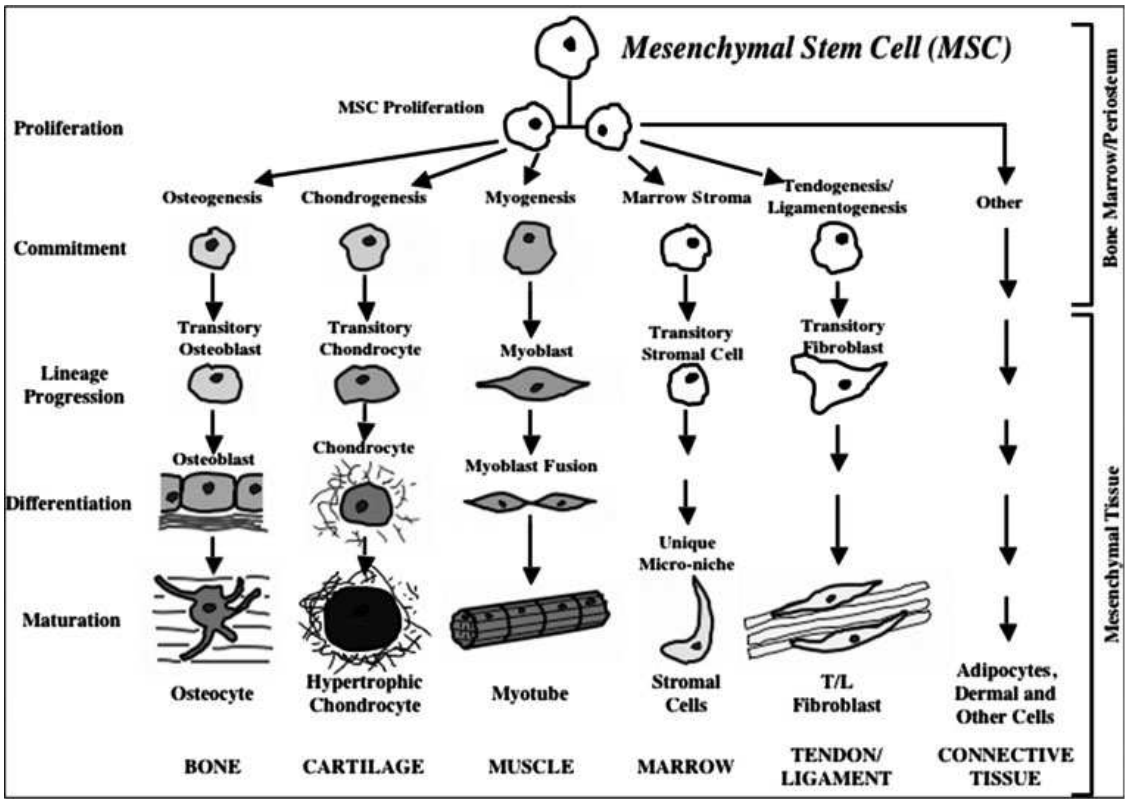


Figure 1: the diagram depicts the plasticity of mesenchymal stem cells and their capacity to differentiate into different mature cells. From Caplan, 2007.

Bone marrow MSCs exert essentially two functions: - one is the classically recognized function of providing a supporting microenvironment for hematopoiesis; - the other is related to the development and maintenance of the sinusoidal network. (Sacchetti 2007). Both HSCs and bone marrow MSCs localize to the sinusoidal walls of bones (Kiel 2006, Sacchetti 2007), and when hematopoietic development is modeled in vivo, MSCs do so prior to the establishment of hematopoiesis. (Sacchetti 2007). MSCs secrete cytokines and growth factors that sustain HSCs maintenance and differentiation. Indeed, in the HSC niche, MSCs release a number of growth factors, such as stem cell factor (SCF), interleukin(IL)-6, lymphocyte inhibitory factor and granulocyte macrophage-colony stimulating factor (GM-CSF), but also negative regulators of hematopoiesis, such as IL-8/CXCL8 and transforming growth factor (TGF-β). (Lazennec 2008) The interaction between HSCs and MSCs represents a unique, dual system of stem/progenitor cells that functionally interact in the regulation of hematopoiesis and bone physiology.

The multipotency and secretory activity of MSCs make these stem cells an attractive target for cell based therapy. Furthermore, compelling evidence has shown the immunosuppressive properties of MSCs and their low immunogenic potential, allowing the use of allogeneic MSCs in therapeutic applications. (Le Blanc 2003, Bartholomew 2002) One approach takes advantage of MSC capacity to differentiate in distinctive mesenchymal phenotype to tissue engineering, encasing cells in tissue specific scaffolds and implanting into different tissue sites. For example, MSCs have been delivered to long bone repair sites in calcium phosphate porous ceramics to produce morphologically and biomechanically superior bone. (Petite 2000) Likewise, Solchaga et al. used hyaluronan and polymeric scaffolds loaded with MSCs for cartilage repair. (Solchaga 2005) A second therapeutic strategy uses transplantation of MSC in nonskeletal sites for the repair of myocardium, brain and more (Barry 2003). Indeed, animal or human MSCs have been used in animal models to affect heart infarct ischemia, stroke ischemia and spinal cord interruption (Caplan 2007). Although evidence for the transplanted MSCs to transdifferentiate into nonmesodermal cell types has been controversial, the beneficial effects of transplantation may reside in the nursing effect conveyed by paracrine factors secreted by MSCs, that promote angiogenesis and stimulate the mitosis of tissue-intrinsic stem or progenitor cells. (Caplan 2007) Another promising area of clinical application is the use of systemic transplantation of MSCs in treatment of autoimmune diseases, involving their immuneregulatory effects. For example, systemic delivery of

MSCs has been tested to combat graft versus host disease (GVHD) during allogenic HSC transplantation and Crohn disease. (Ringden 2006, Caplan 2007). The clinical use of MSC requires a better understanding of the regulatory pathways which control their self renewal and differentiation.

2.2 Retinoic acid: a potent morphogenetic and therapeutic agent

Retinoic acid (RA) is the biologically active naturally occurring member of a family of molecules called retinoids, all of which are derived from vitamin A. RA exerts specific control in embryonic development, regulating morphogenesis and organogenesis, and adult tissue cell growth, differentiation and apoptosis. (Chambon 1996, Altucci 2007) RA signaling is mediated by the activation of two ligand-activated nuclear transcription factors, the RA receptors (RARs) and the retinoid X receptors (RXRs). In human, rat and mouse, there are three RARs (RARα, RARβ and RARγ) and three RXRs (RXRα, RXRβ and RXRγ), that originate from six distinct genes. (Chambon 1996, Germain 2006(2)) RAR and RXR proteins share a common organization of functional domains: an amino terminal A/B region containing a transcriptional activation domain (AF-1), a centrally located C region corresponding to the DNA binding domain (DBD) plus a weak dimerization domain and the E region, which includes the ligand binding domain (LBD), a strong dimerization interface and a surface allowing binding of transcriptional coregulators.(Alvarez 2007) (Figure 2) For each RAR subtype, several isoforms exist that differ from one another in their N-terminal region A. These isoforms arise from the differential usage of two promoters and alternative splicing. The downstream promoters, referred to as P2, are induced by retinoids owing to the presence of a retinoic acid response element (RARE, see below) (Germain 2006).

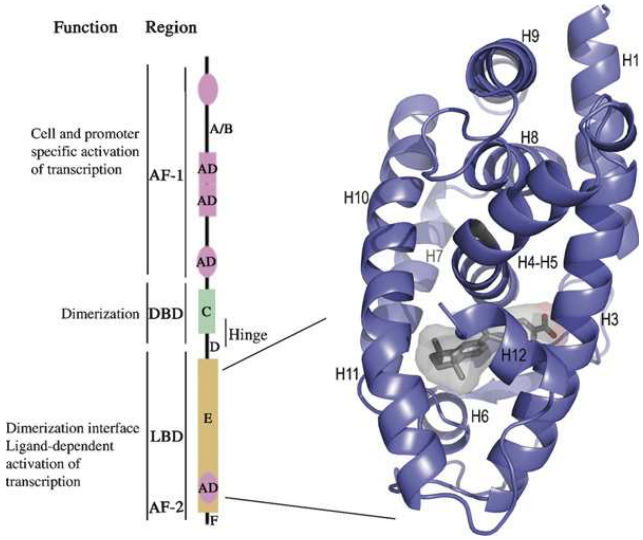


Figure 2: Structural organization and functional description of nuclear receptors commonly divided in modules A/B, C (DBD), D, E (LBD) and F. AD: activation domain. The structure of the ligand-binding domain of hRARβ bound to TTNPB is depicted. From Alvarez et al, 2007.

The RARs and RXRs act as heterodimers (for example, RXRα–RARβ), and they recognize consensus sequences known as RAREs in the control elements of RA-responsive genes. In the absence of ligand, the RXR-RAR heterodimer recruits the corepressor proteins NCoR or SMRT and associated cofactors such as histone deacetylases (HDACs) or DNA-methyl transferases, that may lead to an inactive chromatin structure, preventing transcription. (Nagy 1997, Privalsky 2001) Binding of RA to the RAR ligand binding pocket induces a conformational change of the LBD that creates a surface allowing the association of co-activators and the release of co-repressors. The co-activators (e.g. TIF2 and SRC-1 of the p160 co-activator family) subsequently mediate histone acetylation resulting in decondensation of the chromatin and activation of target gene expression (Perissi 2005) (Figure 3).

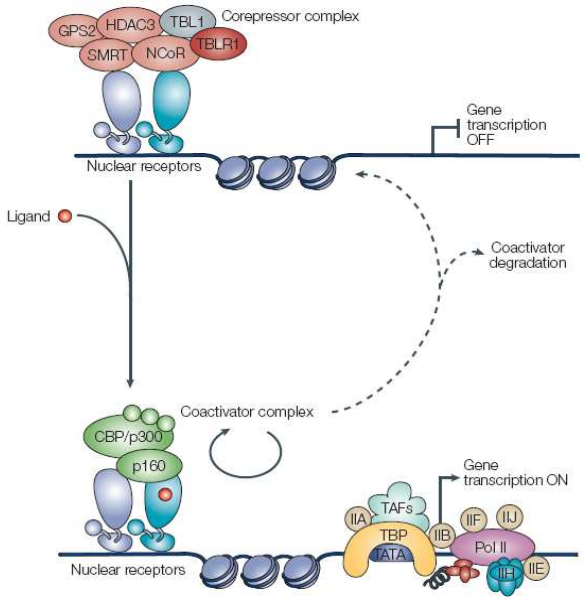


Figure 3: mechanism of RAR-RXR heterodimer activation. In the absence of the ligand, RAR-RXR heterodimer is associated with NCoR or SMRT corepressor complexes, that mediate chromatin condensation and transcription repression. The binding of retinoic acid to RAR binding pocket allows the exchange of corepressors for coactivators, such as CBP/p300 complex, which permits transcription activation by mediating histone acetylation. Modified from Perissi and Rosenfeld, 2005.

The different RAR subtypes exert diverse functions among various cell types. For instance, RARγ is involved in maintenance of a balance between hematopoietic stem cell self-renewal and differentiation (Purton 2006). On the other hand, RARα is involved in the terminal differentiation of promyelocytes, and the altered RARα, PML-RARα fusion protein, contributes to malignant transformation in acute promyelocitic leukemia (APL) by mediating an inappropriate repression of genes necessary for myeloid differentiation. Indeed, the PML fusion with RARα results in an enhanced recruitment of co-repressor HDAC complexes that epigenetically silence gene programmes. Supraphysiological ATRA levels reestablish the normal signaling in malignant cells, inducing RARα expression (Chomienne 1991) activating the differentiation program and tumour-selective apoptosis. (Chomienne 1989, Altucci 2007) However, several other genes can form fusion protein with RARα, giving rise to ATRA-insensitive APL variants. The best known example is the PML zinc finger (PLZF)-RARα fusion protein: PLZF itself recruits the co-repressor HDAC complex, such that ligand-induced dissociation from the RARα moiety is insufficient for derepression and differentiation (Guidez 1994). The combination of ATRA with HDAC inhibitor like VPA can restore ATRA sensitivity in ATRA-resistant APL variants. (Grignani 1998) In addition, the combined therapy with HDAC inhibitor

and demethylating agents, such as 5-azacytidine (5-AZA), have been proposed to sensitize AML cells to the effects of ATRA. (Ferrara 2001, Soriano 2007) This therapeutic effect is probably due to the induction and re-expression of the *RARβ2* gene (Di Croce 2002) The RARβ2 isoform derives from the use of the ATRA-responsive promoter of *RARβ* gene, acting as a tumour suppressor. Indeed, RARβ2 is frequently lost or epigenetically silenced in various cancers and, moreover, its expression correlates inversely with tumor grade. (Xu 2007) Furthermore, restoration of *RARβ2* expression reactivates RA-dependent growth control. (Sirchia 2002) In contrast, RARβ4, another RA-inducible isoform of *RARβ* gene, seems to have oncogenic effects. In fact, RARβ4 was found to be increased in esophageal cancer tissue and the increase was associated with reduced expression of RARβ2. (Xu 2005) RARβ4 is generating from the same RARβ2 primary transcripts by alternative splicing, producing a much shorter A region – only 4 amino acid long. Because RARβ4 protein retains the ability to heterodimerize with RXR and to interact with transcription cofactors but lack the DNA-binding capacity to regulate gene expression, it may act as a dominant negative form of RARβ2. (Nagpal 1992)

Explained by its crucial role in embryogenesis, it is expected that retinoic acid plays a central role also in pluripotent embryonic stem (ES) cell commitment and differentiation. It is largely used alone or in combination with other differentiating factors to induce the terminal commitment of ES toward a specific cell lineage. For instance, treatment of ES cell-derived embryoid bodies with RA from day 0 to either day 2 or day 5 following embryoid body formation results in differentiation of ES cells to neurons and glial cells (Soprano 2007) Indeed, several lines of evidence demonstrate the role of RA in the perturbation of the genetic and epigenetic network that controls ES pluripotency and self-renewal. For example, Oct 4 expression, a POU homeodomain transcription factor essential for ES self-renewal and pluripotency, is repressed by RA-induced differentiation by both a deactivation of a distal upstream stem cell-specific enhancer and the silencing of its promoter (Okazawa 1991, Schoorlemmer 1993) Moreover, microRNAs (miRNA), a class of short RNAs mediating sequence-specific post-transcriptional repression of target transcript, targeting Oct4, Nanog and Sox2 mRNA are upregulated during RA treatment of mouse ES cells, modulating their differentiation (Tay 2008) Indeed, Oct-4, Sox2 and Nanog proteins are essential transcription factors that operate coordinately to maintain ES pluripotency by both activation of downstream self-renewal genes and repression of differentiation-promoting genes. (Boyer 2005, Loh 2006) In fact, they establish a complex genetic network by a reciprocal regulation of each

other's expression and the induction of downstream genes important for ES maintenance and self-renewal, such as Tcl1 and Sall4. (Loh 2008) In addition, the overexpression of a defined group of transcription factors (e.g. Oct4, Sox2, Klf4 and c-Myc) is sufficient to reestablish a pluripotent state in mouse embryonic fibroblast. (Yamanaka 2006) This genetic network is also interconnected with epigenetic regulation of chromatin structure. (Figure 4) In fact, it has been shown that Oct4, Sox2 and Nanog co-regulates certain genes encoding components of chromatin remodeling and histone modifying complexes (Boyer 2005), and they can interact directly or indirectly with them (Wang 2006). In addition, Polycomb group proteins (PcG), a well-known group of factors involved in the silencing of developmental regulators, co-occupy a significant fraction of Oct4, Sox2 and Nanog regulated genes, raising the possibility that these transcription factors recruit PcG proteins to their target sites. (Lee 2006)

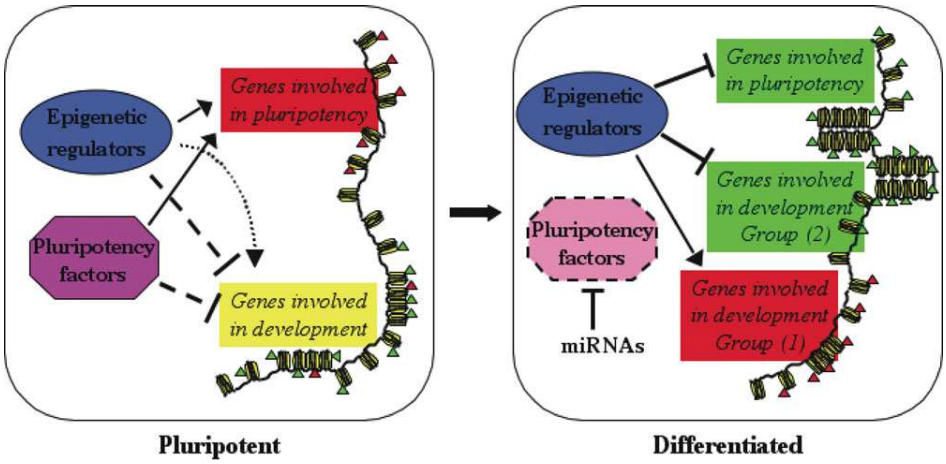


Figure 4: interplay between genetic and epigenetic factors in regulation of pluripotency in ES cells. The pluripotency transcription factors (e.g. Oct4, Sox2 and Nanog) act both in the activation of downstream genes involved in stemcellness maintenance and self-renewal and in the repression of developmental genes. Epigenetic regulators, such as PcG proteins and histone modifying factors, cooperate with genetic factors to the regulation of the gene expression programmes. Differentiating stimuli, such as retinoic acid treatment, downregulate the expression level of pluripotency factors by different mechanism (e.g., miRNA) and induce the expression of developmental genes. From Chen and Daley, 2008.

2.3 Aim of the project

The aim of this project is to study the effects of *in vitro* treatment of epigenetic and differentiation modifying drugs (*all-trans* retinoic acid (ATRA) alone or in combination with 5-azacytidine and valproic acid) in mesenchymal stem cells derived from healthy donors and leukemic patients. We first evaluated the effects on the biological properties of MSCs, such as proliferation, apoptosis, senescence and differentiation potential. We then focused on the RA signaling pathways in MSC cells by, assessing the level of expression of the three RARs

and RXRs after ATRA treatment. Finally, we investigated the effect of ATRA on the expression of genes known to be involved in embryonic and adult stem cell self-renewal and multipotency, such as Oct4, Sox2 and Nanog.

The long term goal of the project is to evaluate the effect of ATRA, AZA and VPA either alone or in combination in MSCs, due to their potential clinical application in regenerative medicine and their intimate role in regulating HSC self-renewal and differentiation. This could lead to a better understanding of the mechanism of action and side effects of these three drugs in the cure of hematological malignancies.

3 Materials and Methods

3.1 MSC isolation and culture

MSCs were isolated from bone marrow samples obtained from 7 healthy donor and 2 AML patients after informed consent was obtained. Bone marrow samples were diluted with one third vol/vol of PBS+EDTA (2mM EDTA) and posed in a layer of Ficoll of density of 1.073 g/mL (Invitrogen). The cells were centrifuged at 1.100 RPM for 30 minutes at 4 ^{o}C. Mononuclear cells were collected from Ficoll layer and washed twice with PBS+EDTA. Cell pellet was suspended in stem cell medium consisting of α-modified Eagle's Medium (αMEM, Invitrogen) containing 10% of Fetal Calf Serum (FCS, Hyclone), 1 ng/mL basic FGF (R&D), 1% (vol/vol) GlutaMAX (Invitrogen), and a combination of antibiotics/antimycotics. Cells were plated at a density of 5x10^3 cells/cm^2. After 24-48 hours at 37 ^{o}C with 5% of CO_2 pression, non-adherent cells were discarded and the culture medium was changed. Medium was changed every 2-3 days until cells reached confluence: they were detached with trypsin and seeded at a density of 5000 cells/cm^2 for propagation.

3.2 MSC characterization and differentiation test

3.2.1 Analysis of cell-surface mesenchymal marker epitopes by Fluorescence-Activated Cell Sorting (FACS)

MSCs were detached with trypsin and resuspended in PBS. 1x10^5 cells were incubated at 4 ^{o}C for 15 minutes in presence of a set of different antibodies diluted 1 to 20. Antibodies used are coupled with -Fluorescein isothiocyanate (FITC) or –Phycoerythrin (PE). Cells were then washed with PBS and analyzed by FACS. Isotypic antibodies coupled with the appropriated fluorochrome were used as negative controls.

3.2.2 MSC treatment

Cells were plated in stem cell medium at a density of 5.000 cells/cm^2. After 24-48 hours the medium was removed and changed with medium contained different concentration of *all-trans* retinoic acid (ATRA, Sigma), 5-azacytidine (AZA, Sigma), valproic acid (VPA, Sigma), RARβ2 isoform specific agonist AC55649 (Tocris Bioscience) (Piu 2005) and/or RARβ subtype specific

antagonist LE135 (Tocris Bioscience) (Li 1999). Stock solutions of ATRA, LE135 and AC55649 was made by addition of DMSO (1 mM for LE135 and AC55649, 0,1 mM for ATRA). AZA was diluted with PBS at a concentration of 1 mM, instead VPA was resuspended in distilled water at a concentration of 1 M. All stock solutions were stored at -80^{o}C. The medium was changed every 2-3 days and cells were collected at the specified time. All experiments were carried out on cells from passage 2 to 5.

3.2.4 Adipogenic differentiation

Before inducing adipogenic differentiation, cultured cells were seeded in 175 cm² flask (BD) at a density of 5000 cells/cm² in basal medium added with 1 µM ATRA, AC55649, LE135 or both ATRA and LE135. After one week cells were detached and plated in 6-well plates at 70-80% of confluence. After 24 hours medium was replaced with adipogenic medium consisting of Dulbecco's modified Eagles Medium (DMEM, Invitrogen) supplemented with 1 g/L of glucose, 20% of FBS, 0.5 mM 3-isobuthyl-1-methylxantine (Sigma), 60 µM indomethacine (Sigma), 1 µM dexamethasone (Merk) and 5 µg/mL insulin in presence of ATRA and RARβ agonist or antagonist. The medium was replaced every 3-4 days, and after 14 days of culture the presence of cells containing lipidic vesicles was observed and quantified by Nile red staining. Cells were discarded with trypsin and washed twice with PBS. They were then suspended in PBS in a total volume of 400 µL and fixed by the addition of 40 µL of 4% paraformaldehyde. After washing with PBS, cells were stained with the addition of 500 µL of a solution containing 1 µg/mL of Nile red dye on ice for 30 minutes. The samples were then analyzed with a flow cytometer. Nile red fluorescence emission was measured on the FL2 emission channel through a 585±21 nm band pass filter, after excitation with an argon ion laser source at 488 nm.

3.2.5 Osteogenic differentiation

MSCs were first cultivated in 175 cm² flask (BD) at a density of $5x10^3$ cells/cm^2 in stem cell medium supplemented with 1 µM ATRA, AC55649, LE135 or both ATRA and LE135. After 7 days of culture cells were detached and seeded in 6-well plates at 70-80% of confluence. Osteogenic differentiation was induced by the addition of osteogenic medium composed of DMEM (Invitrogen) supplied with 4.5 g/L glucose, 10% of FBS, 10^{-7} M dexamethasone, 50 µg/mL ascorbic acid and 3 mM inorganic phosphate in presence of 1 µM ATRA, RARβ agonist or antagonist. The medium was changed twice per week and after 14 days of

treatment osteogenesis was quantified by alkaline phosphatase activity and after 21 days of treatment the mineralization of extracellular matrix was evaluated by the coloration with Alizarin Red.

For the quantification of ALP activity cells were lysed with a HNT (Hepes/NaCl/Triton) tampon solution and centrifuged at 300 g at 4 ^{o}C for 10 minutes. The supernatant was placed in a 96-well plate with an equal volume of reaction solution (Biorad Alkaline Phosphatase substrate kit, Biorad) and incubated at 37 ^{o}C. The reaction was stopped when a yellow coloration appeared by adding a solution of 0.4 M NaOH and optical density was read at 405 nm using a 96-well plate reader. Absorption values were then normalized to nmol of produced nitrophenol/minutes of incubation/total protein content for standardization. The experiment was carried out in duplicate for each condition tested.

The coloration with Alizarin Red evaluated the mineralization of the extracellular matrix produced by osteoblasts. Cells were fixed with 10% formaldehyde at room temperature for 15 minutes. Then cells were rinsed three times with distilled water and stained with 1 mL of staining solution containing 40 mM Alizarin Red at room temperature for 20 minutes with shaking. The excess of dye was removed and cells were washed four times with distilled water. We added 800 µL of 10% acetic acid to each well and incubated for 30 minutes with shaking. Cellular monolayer was then scrapped with the aid of a cell scraper and cells and acetic acid were transferred to a 1.5 mL microcentrifuge tube. The solution was heated at 85 ^{o}C for 10 minutes and cooled on ice for 5 minutes. We then centrifuged the solution at 20.000 g for 15 minutes and the supernatant was transferred in a new tube. 200 µL of 10% ammonium hydroxide was added to neutralize the pH and read the absorbance at 405 nm using a 96-well plate reader. The quantification of Alizarin Red staining was performed using a calibration curve of known concentrations of the dye. The experiment was carried out in duplicate for each condition tested.

3.3 Analysis of biological properties of MSC after drugs treatment

3.3.1 Colony Forming Unit-Fibroblast (CFU-F) Assay

Mononuclear cells isolated from Ficoll layer were washed twice with PBS and resuspended in stem cell medium. Cells were plated in 6-well plate at 3 different densities in duplicate: 40.000 cells/cm^2, 20.000 cells/cm^2 and 10.000 cells/cm^2. After 24-48 hours non-adherent cells were discarded and medium was

changed with fresh medium containing 1 µM ATRA, 1 µM AZA, 1 mM VPA, either alone or in combination. The medium was changed every 3-4 days and at day 14 cells were collected for colony staining with Giemsa (Biolyon, Oxoid). Cells were fixed with methanol (Merck Eurolab) for 10 minutes at 25 ^{o}C and rinsed twice with PBS. Fixed cells were incubated with Giemsa staining solution for 5 minutes and then washed twice with PBS to remove additional staining. We counted fibroblastic colonies under light microscope.

3.3.2 Detection of apoptotic cells

Cells were seeded in 10 cm^2 plates at 5x10^3 cells per cm^2 density in stem cell medium. After 24 hours the medium were replaced with the fresh medium containing 1 µM ATRA, 1 µM AZA, 1mM of VPA, 1 µM AC55649, 1 µM LE135 or combinations of drugs at the concentrations previously indicated. The medium was changed after 48 hours, and cells were collected at 5 days after treatment. Cells were detached with trypsin and washed twice with PBS by centrifugation. Apoptotic cells were detected with FITC Annexin V Apoptosis Detection Kit II (BD Pharmigen), according to the manufacturer's protocol. Briefly, PBS was removed and the cell pellet was resuspended with 100 µL of Annexin V Binding Buffer, 20 µL of 7-Amino-Actinomycin (7-AAD) and 5 µL of FITC Annexin V solution. Cells were incubated for 10 minutes at 4 ^{o}C in the dark, and then we added 400 µL of binding solution. The analysis of apoptotic cells were performed by flow cytometry.

3.3.3 Cell cycle analysis

We plated 30.000 cells per wells in 6-wells plate in culture medium to permit cells to adhere. After 24 hours the medium were replaced with fresh medium containing the concentration of drugs indicated before. At day 2 the medium was changed and MSCs were treated with 10µM bromodeoxyuridine (BrdU) for 24 h and assayed according to the protocol of BrdU flow kits (BD Pharmingen, San Diego, CA,) using flow cytometry. Briefly, cells were first fixed and permeabilized with BD Cytofix/Cytoperm Buffer, treated with DNase to expose incorporated BrdU (30µg of DNase to for each sample) and stained with FITC-conjugated anti-BrdU antibodies and 7-AAD. Stained cells were then analyzed with a flow cytometer.

3.3.4 Proliferation test

For the proliferation test we plated 500 cells/well in 96-well plate in stem cells medium. After 24-48 hours the medium was replaced with fresh medium containing serial dilutions of ATRA, AZA, VPA, AC55649 and LE135 alone (0.5, 1, 2 μM for ATRA and AZA, 0.5, 1 and 2 mM for VPA, 1 μM for AC55649, 1 μM for LE135) or combination of drugs (1 μM for ATRA, AZA and LE135, 1 mM for VPA). The medium was changed at day 2 and 5 after starting the treatment, and viable cell number was determined at day 2, 5 and 8. The determination of cell number was performed by using CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega), following the manufacturer's instructions. The CellTiter Assay is based on the cellular conversion of a tetrazolium salt into a formazan product that is detected using a 96-well plate reader, providing an indirect measure of viable cell number by measuring metabolic activity of cellular enzymes. At day of testing culture medium was removed and cells were washed with PBS. Cells were incubated with 100 μL of PBS and 20 μL of Dye solution at 37 ^{o}C. The absorbance was recorded at 570 nm after 2 hours of incubation using a 96-well plate reader.

3.3.5 Senescence-associated β-galactosidase assay

MSCs were seeded in 12-well plate at a density of 5.000 cells/cm^2 in culture medium. After 24-48 hours the medium was replaced with fresh medium supplemented with 1 μM ATRA, 1 μM AZA, 1 mM VPA or combination of the three drugs as previously described. Medium was changed after 2 days of treatment and senescence assay was performed at day 5. The detection of senescent cells were carried out using the Senescence β-Galactosidase Staining Kit (Cell Signaling Technology), which histochemically detects β-galactosidase activity at ph 6, according to the manufacturer's instructions. Briefly, cells were washed with PBS and fixed with Fixative solution for 15 minutes at room temperature. Next we added 1 mL of Staining Solution mix (containing 1 μg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopiranoside) to the plate and incubated overnight at 37 ^{o}C. Plates were observed under light microscope to recognize the development of blue color in senescent cells.

3.4 Analysis of gene expression

3.4.1 MSC treatment

Cultured MSCs were plated in 25 cm^2 flasks at a density of 8.000 cells/cm^2 in basal medium. After 3-4 days of culture we started the treatment by adding 1

µM ATRA to stem cell medium. Cells were collected at 0.5, 1, 2, 4, 8, 24, 48, 72 and 120 hours after the beginning of the treatment, depending on experimental design. For CFU-F RNA extraction, mononuclear cells isolated from Ficoll were seeded in 75 cm² flasks at a density of 5000 cells/cm² in basal medium. After 24 hours medium was replaced with fresh ones with or without 1 µM ATRA. Medium was changed every 2-3 days and cells were collected after 14 days of treatment. Cell pellets was stored at -20 ^{o}C.

3.4.2 RNA isolation and Reverse Transcription

Total RNA was extracted by cell pellet using RNeasy Mini Kit (Qiagen), following the manufacturer's instructions. The content of RNA of each sample was quantified using Nanodrop 2000 spectrophotometer (Thermo Scientific). First-strand cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystem), following the manufacturer's instructions. Total RNA (500 ng) was reverse-transcribed in a 20 µL reaction volume containing $MgCl_2$ (5 mM), PCR BufferII (1X), RNase inhibitor (1U/µL), Multiscribe Reverse Transcriptase (50 U), random primers (2.5 µM) and dNTPs (1 mM each). The tubes were placed in the thermal cycler at 25 ^{o}C for 10 minutes, 37 ^{o}C for 2 hours and 85 ^{o}C for 5 seconds. We added 60 µL of RNase-free water to each tube at the end of the reverse transcription reaction.

3.4.3 Real time PCR (RT-PCR)

Quantitative real-time polymerase chain reaction was performed in a 25-µL reaction consisting of 12.5 µL of TaqMan Universal PCR Master Mix (Qiagen), 2.5 µL of the RT reaction, and 600 nM primers using 7500 Fast Real-Time PCR System (Applied Biosystem). The amplification program consisted of initial denaturation at 95 ^{o}C for 10 minutes, followed by 40 cycles of denaturation at 95 ^{o}C for 15 seconds and annealing/extension at 60 ^{o}C for 1 minute, and finally 7 minutes at 72 ^{o}C. Real-time PCR assays were performed in duplicate or triplicate for each MSC tested. We measured the cycle threshold (Ct) value for each gene of interest. The Ct value of porphobilinogen deaminase (PBGD) gene was used as an internal reference for normalization. Specific primers for RAR and RXR receptors and for self renewal genes were purchased from Applied Biosystem (TaqMan Gene Expression Assays, Applied Biosystem; proprietary primers, sequence not disclosed). Primers for PBGD and RARβ2 isoform were purchased from Sigma Proligo, and primers for RARβ4 isoform were synthesized by Eurofins MWG Operon. The sequence of primers used was as follow: RARβ2 (forward, F)

5’-CTAAATACACCACGAATTCCAGTGCTGA-3’, RARβ2 (reverse, R) 5’-CAGACGTTTAGCAAACTCCACGATCTTA-3’; RARβ4 (F) 5’-TTGGAAGGAGAACTTGGGATC-3’, (R) 5’-TCAATTGCATTTTCCAGGCT-3’; PBGD (F) 5’-GGAGCCATGTCTGGTAACGGCA-3’, (R) 5’-GGTACCCACGCGAATCACTCTCA-3’.

3.4.4 Statistical analysis of the data

Comparison in the difference of all data presented between paired sample was by one-tailed paired *t* test using GraphPad Prism software (GraphPad Prism version 5.0 for Windows, GraphPad Software, San Diego, CA). In all tests, $p<0.05$ was taken as significant.

4 Results

4.1 ATRA, AZA and VPA reduce CFU-F formation

Mesenchymal stem cells were first isolated from bone marrow for their ability to form colony of fibroblastic cells (CFU-F) when plated at clonal density (Friedenstein 1966) To first investigate the effects of ATRA, AZA and VPA in MSCs, the action of the different treatments was evaluated on the formation of colonies in mononuclear cells isolated from normal bone marrow aspirates by density gradient.

Samples derived from different donors showed a variation in the number of CFU-Fs, probably due to an intrinsic variability of the number of the MSCs in bone marrow aspirates and to isolation process. Treatment of cells with 1 µM ATRA, 1 µM AZA, 1 mM VPA or a combination of AZA and VPA or of the three drugs reduced the number of colonies present in the plate (3.92 colony/10^5 mononuclear cells for untreated samples (UNT) compared to 2.62 ATRA, 2.45 AZA, 1.46 VPA, 1.15 AZA+VPA and 0.25 AVA treated samples (Fig. 5). This inhibition is statistically significant compared to untreated cells for all the treatments (p<0,05). The combination of the three drugs (AVA) showed a greater inhibitory effect on the CFU-F count, maybe due to a cooperation of drugs. In addition, the treatment with AZA and VPA alone or in combination reduced colony size and density, and changed the morphology of cells (Fig. 6 D-G). Indeed, cells appeared larger and more flattened: probably linked to their commitment toward a non-proliferative or differentiated status. In contrast, ATRA treatment showed a more variable effect on CFU-F formation: in the same well there were colonies with marked changes in cell morphology and others with no visible alteration compared to untreated cells (Fig. 6 B-C).

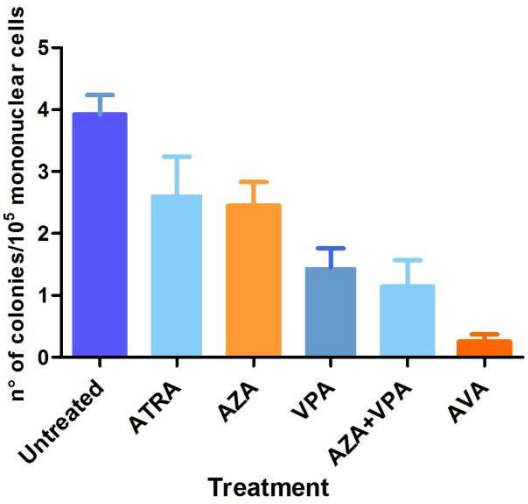


Figure 5: CFU-F number. The number of colonies is expressed as CFU-F/10^5 mononuclear cells. Data represent the mean of 6 independent experiment ±sem.

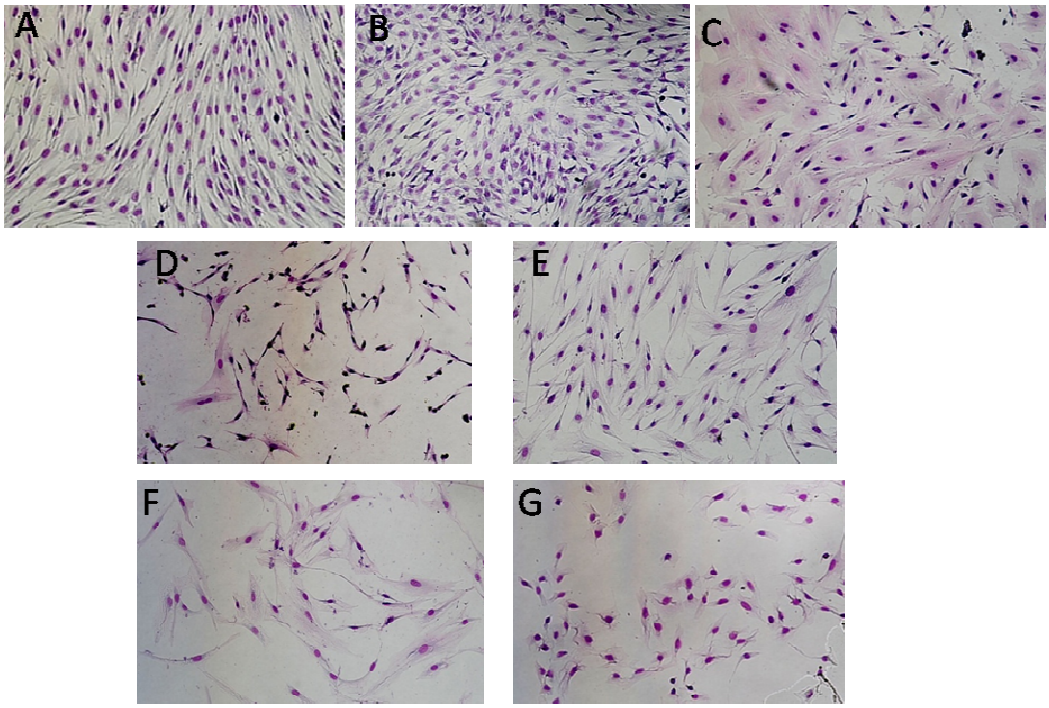
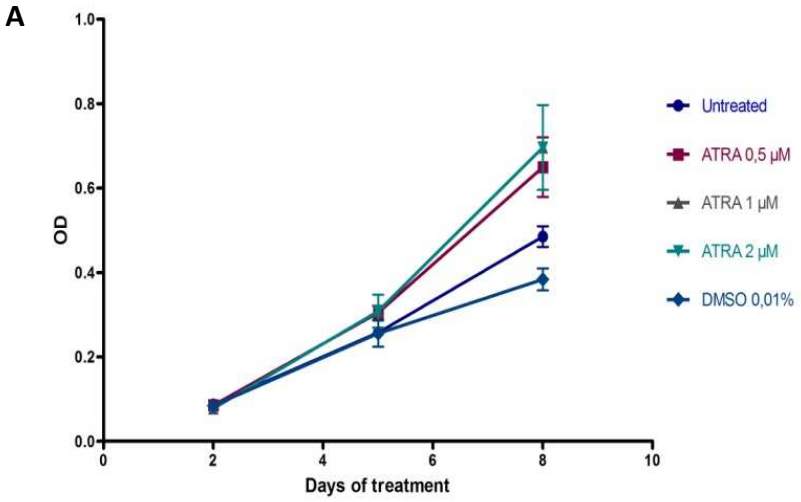


Figure 6: Microscopical aspect of untreated (A) and treated (B-H) cells. (400x). ATRA-treated CFU-Fs present both spindle-shaped (B) and flattened (C) morphology. Cells exposed to AZA (D), VPA (E), combination of AZA and VPA (F) or all the three drugs (G) undergo a drastic changing in cell shape and a reduction of colony density.

4.2 Proliferation of cultured MSC

To further characterize the effect of the three drugs on MSCs, cells were isolated by their ability to adhere to the plastic surface and then characterized by flow cytometry analysis to confirm the absence of contamination of hematopoietic and endothelial cells. The proliferation rate of cultured MSCs were then tested by an MTS assay. Viable cell number was determined after 2, 5 and 8 days of treatment with different concentrations of ATRA, AZA and VPA, either alone or in combination. For treatment with ATRA, control cells were cultured with an equal amount of DMSO.

The presence of ATRA in the culture medium enhances the proliferation of MSCs after 5 and 8 days of culture compared to cells exposed to the same amount of DMSO (p-value<0.05) and untreated cells, even if in this case the differences are not statistically significant (Fig. 7A). No concentration-dependent effect was found for ATRA treatment at the concentrations tested. In contrast, treatment of MSCs with AZA and VPA alone or in combination decreases the proliferation of MSCs compared to control cells after 5 and 8 days of treatment (p-value<0.05), as it was shown for CFU-F assay. VPA presents a concentration-dependent inhibition on proliferation 5 days after treatment and AZA after 8 days(Fig. 7 B-C). The combination of AZA and VPA has a cooperative inhibitory effect compared to AZA 1µM and VPA 1mM after 8 days(p-value<0.05) (Fig. 7B). The addition of ATRA to AZA+VPA treated cells attenuated the phenotype due to the two epigenetic drugs, confirming its role in promoting MSC proliferation (Fig. 7B).



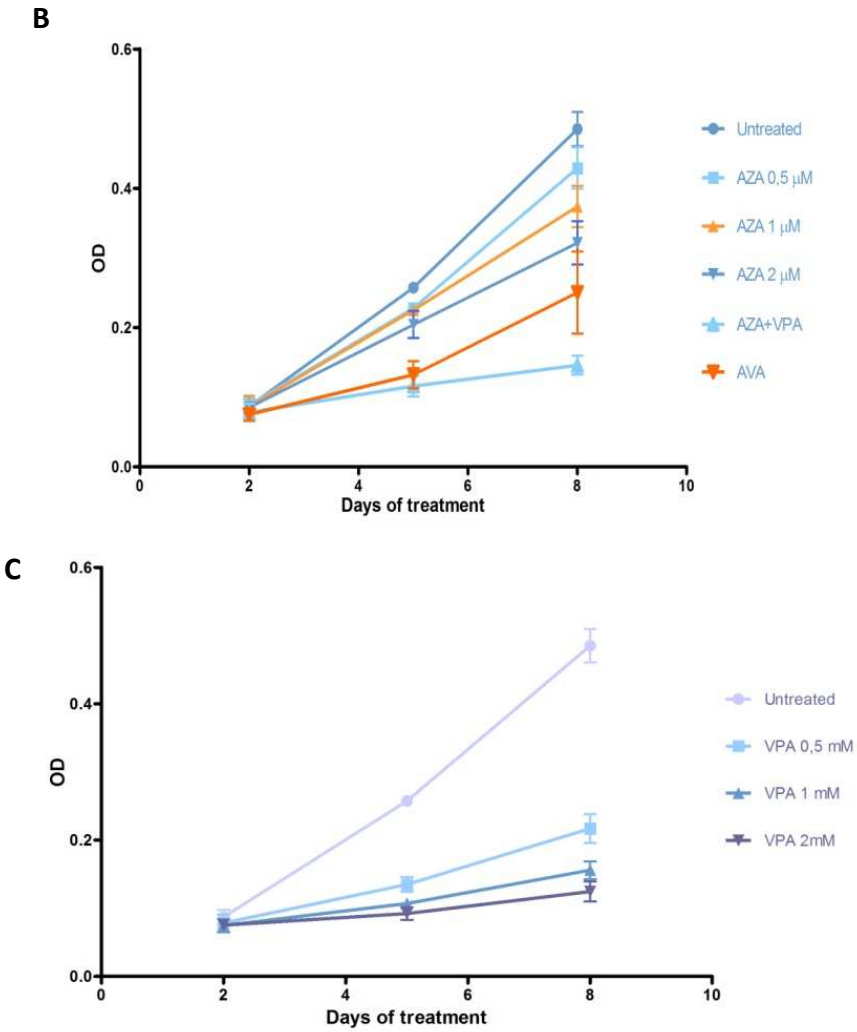


Figure 7:Results on MSC proliferation assessed by the MTS test on ATRA (A), VPA (B) and AZA (C) treated MSCs. (A) The treatment with ATRA promotes the proliferation of cultured MSCs; (B-C) in contrast, both AZA and VPA treatments show a concentration-dependent reduction of viable cells after 5 and 8 days of culture. Data represent the mean of three independent experiments ±sem. Each experiment was done in triplicate.

4.3 Apoptosis and senescence

The decrease of viable cells by AZA and VPA may be due to an induction of apoptosis or senescence in MSC culture. To test this hypothesis, we first evaluate the rate of apoptotic cells in the presence of the three drugs. Figure 8 shows the data collected after 5 days by flow cytometry with Annexin V/propidium iodide two color staining. Surprisingly, treatment with ATRA seems to enhance the percentage of apoptotic cells compared to control cells (1.82% UNT compared to 3.07% in ATRA treated cells), even if this difference has no statistical meaning and the numbers are very low (Fig. 8 A). AZA and VPA

treatments increase the number of apoptotic cells compared to untreated cells (2.03% and 4,95% respectively), but the results are not significantly different (Fig. 8 B). The combinations AZA+VPA and AVA trigger the most striking effect on cultured MSCs viability, and this increase of apoptotic cells is statistically significant compared to untreated cells and single treatment (p-value<0.05) (Fig. 8 B). Moreover, the addition of ATRA to the combination of the two epigenetic drugs enhances apoptosis (7.96% for AZA+VPA compared to 11.39% for AVA treatment), but this increase is not statistically important.

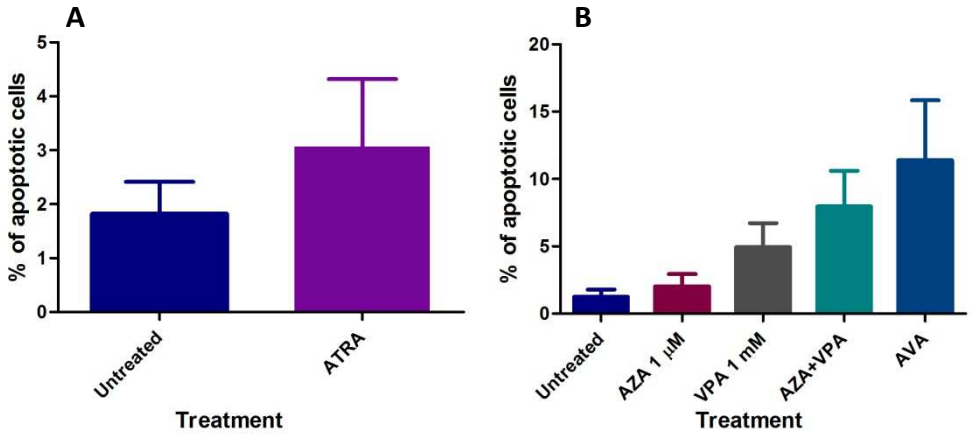


Figure 8: Effect on apoptosis. (A) The addition of ATRA in culture medium increases the number of apoptotic cells. (B) Both AZA and VPA treatments enhance apoptosis of cultured MSCS, but the differences are significant only for the combination of treatment compared to untreated and single treated cells (p-value<0.05%). Data represent the mean ±sem of four independent experiments.

The flattened morphology of cells in CFU-F is a typical indicator of the presence of suffering and non-proliferative cells in the culture. We thus evaluated the presence of senescence in control and treated cells by the recognition of β-galactosidase activity, a well-known marker of senescent cells, after 5 days of culture. As it is shown in Figure 9, no senescent cells were detected in all conditions tested. In addition, treatments with AZA and VPA either alone or in combination cause modifications of cell shape very similar to those observed for treated CFU-F (Fig. 9 C-E; Fig. 6 D-G). On the other hand, exposure to ATRA seems not to affect cell morphology of cultured MSCs (Fig. 9B) This finding is consistent with enhancement of proliferation in ATRA-treated MSCs, and it may explain the opposite effects of the drug between CFU-F and cultured MSCs.

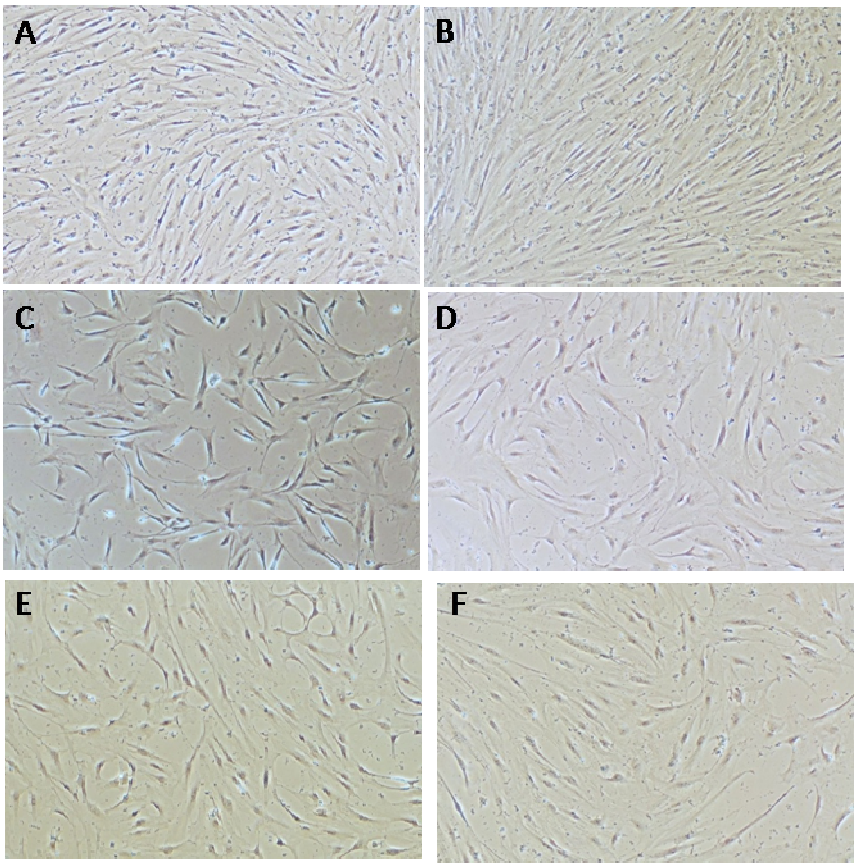


Figure 9: Detection of β-galactosidase activity after 5 days of culture. No blue cells were detected in all condition tested. Cells treated with ATRA (B) show no difference in cell morphology compared to control (A). In contrast, cells treated with AZA (C), VPA (D) or combination (E, AZA+VPA; F, AVA) appear flattened and planar. Images were taken with 400X magnification under light microscope.

Our data suggest that AZA and VPA treatments reduce the number of CFU-F and viable MSCs by the induction of apoptosis and a commitment of cells toward a less proliferative state. In contrast, ATRA seems to act in opposite ways in earlier progenitor cells (i.e., CFU-Fs) compared to cultured MSCs.

We thus focus on the treatment with ATRA to unravel the reason for the differing behavior of CFU-F and cultured MSCs.

4.4 Cell cycle analysis

To confirm the effect of ATRA treatment on MSC proliferation, the distribution along cell cycle phases was verified by the incorporation of 5-Bromodeoxyuridine (BrdU). After 3 days of culture, cells treated with ATRA show a comparable percentage of cells in sub-G_1 and G_1 phases (2.08% and 37.56% in ATRA-treated cells against 2.26% and 37.88 % of untreated cells)(Fig. 10 A-B).

Surprisingly, the presence of ATRA reduced significantly the fraction of cells in M phase of cell cycle division compared to control cells (3.20% in treated cells and 5.50% for controls, $p<0.01$), but it increases the number of cell in S phase (57.15% for ATRA and 54.36% for control cells) (Fig. 10 C-D). These data suggest that ATRA treatment does not select a subpopulation in cultured MSCs, as no significant increase of the percentage of cells in active division was seen. On the other hand, this did not explain why ATRA enhances the number of viable cells, as it seems not to influence the division rate of cultured MSCs.

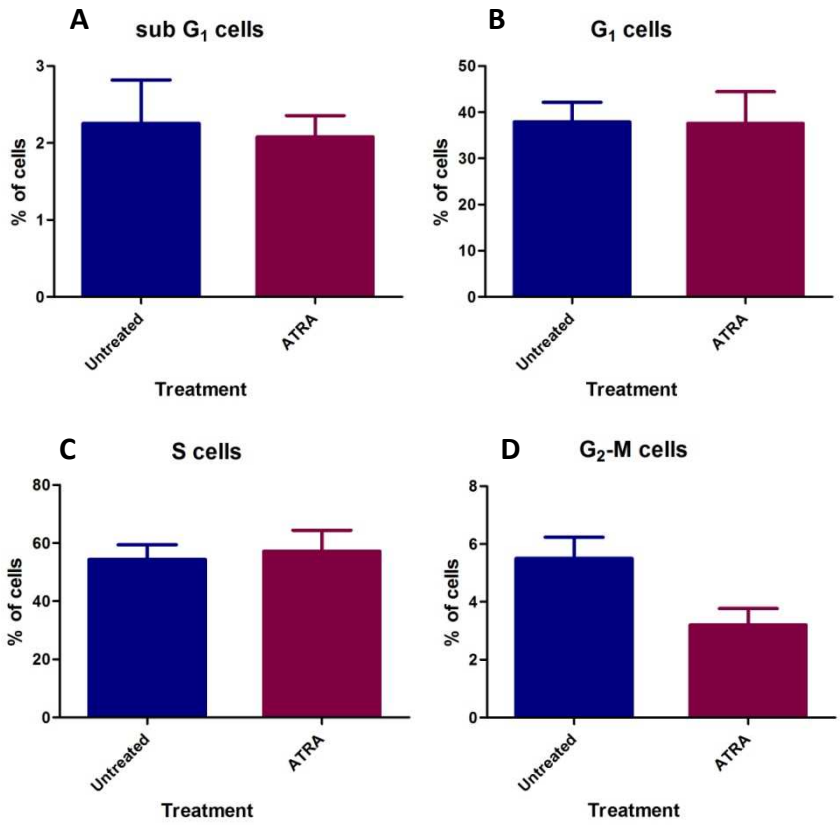


Figure 10: Analysis of distribution of cells along cell cycle phases.(A-B) No difference was found between control and treated cells in the percentage of sub-G_1 and G_1 cells after 3 days of culture. A tiny increase of s cells was seen in cell treated with ATRA (C), at which corresponds an equal reduction of G_2-M cells compared to controls (D). Data represent the mean ±sem of three independent experiments.

4.4 Expression levels of Retinoic acid receptor and retinoid X receptor

Retinoic acid signaling is mediated by the binding to and activation of RAR and RXR receptors, which are also induced by the RA treatment through the

presence of a RARE in their promoter region. To determine which receptors mediates ATRA signaling in MSCs, we assessed the basal expression level of the receptors and the variation induced by ATRA.

As it is shown in Figure 11, all RARs and RXRs are expressed in both CFU-F and MSCs, except for RXRγ. CFU-Fs express levels of RXRα and RXRβ drastically lower compared to cultured MSCs, and the differences are statistically significant (p<0.05 for RXRα, p<0.01 for RXRβ). Also RARγ expression is reduced in CFU-F compared to MSCs (0,52 and 1.30, respectively), instead RARα seems to be more expressed (3.11 in CFU-F compared to 1.76 in cultured MSCs). Differences in RAR and RXR expression level may explain the opposite effect of retinoic acid treatment in CFU-F and cultured MSCs.

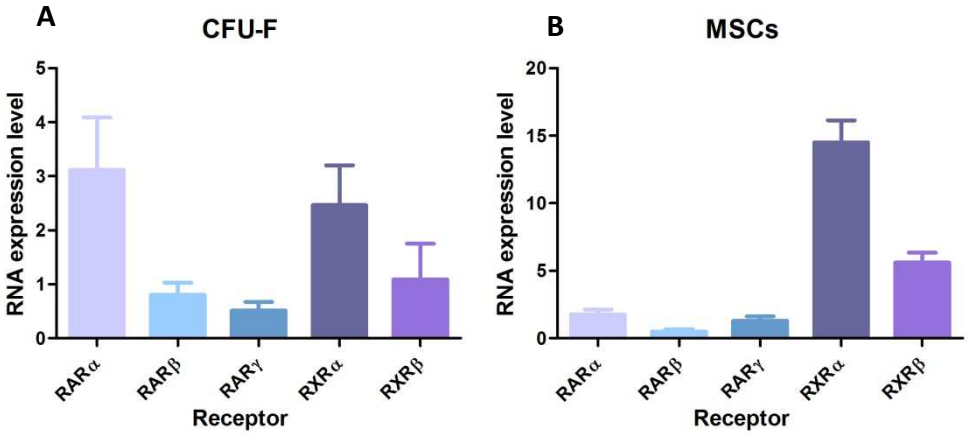


Figure 11: basal level of expression of RARs and RXRs in CFU-F (A) and cultured MSC (B). The value were normalized compared to the expression of the housekeeping gene PBGD. The data show the mean ±sem of three independent experiments.

The presence of ATRA in the medium alters in a similar way the expression profile of retinoic acid receptors in both CFU-Fs and MSCs. In fact, a remarkable increase in RARβ expression is induced by ATRA compared to control cells (Fig. 12 A-C), with no relevant variation in the expression of the other receptors (Fig 12 A-B, D-F). In cultured MSCs, the induction of RARβ starts after 2 hours of treatment, reaching a peak at 8 hours and it stabilizes 24 hours after treatment (Fig. 12 C).

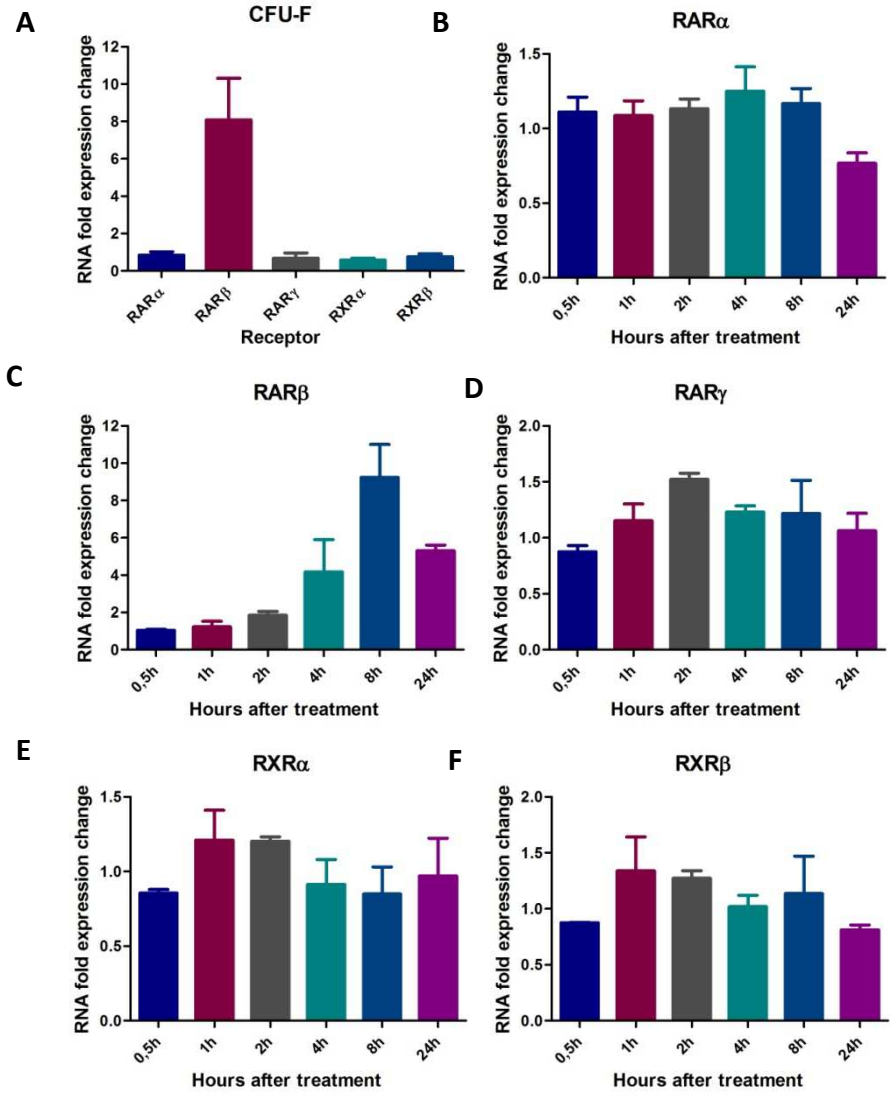


Figure 12: expression fold change of RAR and RXR receptors after addition of ATRA. mRNA levels of RARβ are notably enhance by ATRA treatment in both CFU-F (A) and cultured MSCs (C). No significant differences were seen in the expression of other retinoic acid receptors (A-B, D-F). For cultured MSCs the expression levels of the receptor were analyzed after 0.5, 1, 2, 4, 8 and 24 hours of treatment. Values are normalized to the expression of control cells collected at the same timepoint. The data shown the mean ±sem of three independent experiments.

The RARβ receptor presents two RA-induced isoforms, RARβ2 and RARβ4, that differ for their N-terminal region A, which contains the transcriptional activation domain AF-1. Indeed, the RARβ2 isoform acts as a tumor suppressor in different types of cancers and its expression correlates inversely with tumor grade (Xu 2007). In contrast, the RARβ4 isoform seems to have an oncogenic effect, because its expression is increased in esophageal cancer tissue and this correlates with reduced expression of RARβ2 isoform (Xu 2005). We evaluated

the mRNA level of the two isoform and the presence of variation after ATRA treatment in cultured MSCs. Figure 13 shows the ratio between RARβ2 and RARβ4 mRNA transcript level after 8 and 24 hours of treatment. No notable discrepancies are observed between control and untreated cells. In fact, the two isoforms arise from the same promoter, and they share the same regulation by ATRA. RARβ2 transcript is 50-60 times more expressed than the β4 isoform, suggesting that RARβ2 mediates the effect of ATRA treatment in cultured MSCs. To test this hypothesis, we verify the effect of a RARβ2 specific agonist (AC55649) (Piu 2005) and a RARβ selective antagonist (LE135) (Li 1999) in the proliferation and apoptosis of cultured MSCs.

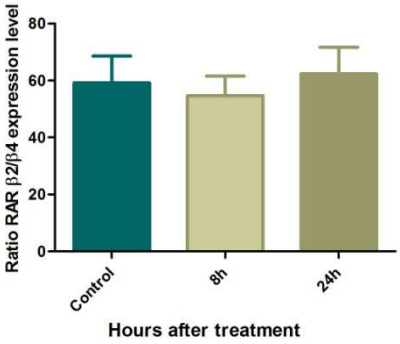


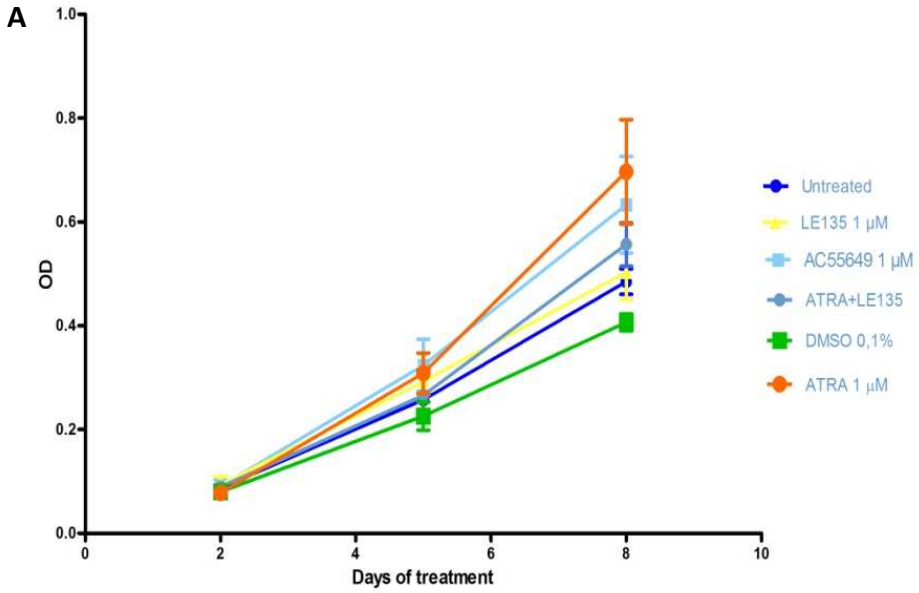
Figure 13: Ratio of RAR β2/β4 isoforms in cultured MSCs after 8 and 24 hours of treatment. No significant difference is present compared to control cells. Data show the mean ±sem of four independent experiments.

4.5 Proliferation and apoptosis in MSCs treated with RARβ selective agonist and antagonist

To control the involvement of RARβ2 isoform in mediating ATRA signaling we took advantage of a RARβ2-selective agonist, AC55649, and a RARβ-specific antagonist, LE135, at the same concentration of ATRA.

The addition of AC55649 to the medium enhances the number of viable cells compared to untreated cells and cells treated with the same amount of DMSO after 5 and 8 days of culture (Fig. 14 A), even if the differences are not statistically significant. However, with the RARβ2 agonist the stimulating effect is similar to equal concentration of ATRA (Fig. 14 A). On the other hand, LE135 mediates a tiny increase of viable cells compared with untreated cells and DMSO control cells, but its effect notably differs from ATRA (p-value<0.05). The concurrent treatment with ATRA and RARβ antagonist reduces the effect of ATRA alone, even if the differences is not statistically significant (Fig. 14 A).

The quantification of the percentage of apoptotic cells after 5 days of treatment confirms the role of RARβ2 in mediating ATRA signaling. In fact, the treatment with AG55649 increases the number of apoptotic cells compared to controls (2.21% and 1.82%, respectively), even if the difference is not statistically relevant (Fig. 14 B). Moreover, the presence of LE135 in the medium reduces significantly the number of apoptotic cells compared to untreated (p<0.01) and AC55649-treated cells (p<0.05), but this reduction is not statistical appreciable compared to ATRA treatment (1.37% for LE135 and 3.07% for ATRA) (Fig. 14 B). This protective effect of LE135 treatment might explain the small increase of viable cells compared to cells treated with the same amount of DMSO seen in proliferation test. Finally, the addition of both ATRA and RARβ antagonist in culture medium diminishes the percentage of apoptotic cells compared to ATRA alone (2.28% for ATRA+LE135) (Fig. 14 B).



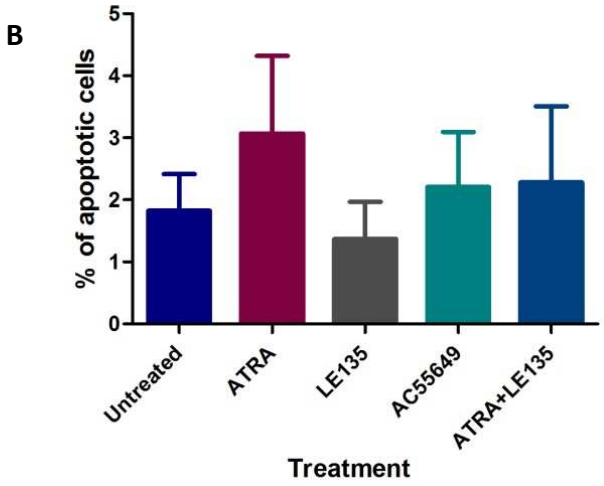


Figure 14: MTS (A) and percentage of apoptotic cells (B) in cells treated with ATRA, RARβ agonist (AC55649) and RARβ antagonist (LE135). AC55649 mediates the same effect on cell proliferation and apoptosis than ATRA, even if in a more attenuate fashion. In contrast, LE135 protects cells from apoptosis compared both to untreated and treated cells. The addition of RARβ antagonist to ATRA treatment reduces the proliferative and proapoptotic effect of ATRA. Data represents the mean ±sem of three (A) or four (B) independent experiments.

4.6 ATRA does not commit MSCs toward a specific tissue lineage

Retinoids are known for their effects on cell growth and differentiation in embryogenesis and adult tissues. Thus the treatment with ATRA could influence the multipotency of MSCs by priming them towards a specific cellular lineage. To address this question, we evaluated the effect of ATRA, RARβ agonist and antagonist before or during adipogenic and osteogenic differentiation of cultured MSCs.

Figure 15 shows the data for the adipogenic differentiation, revealed by the staining of lipid droplets in cytoplasm of adipocyte with Nile Red dye. All the treatments seem to enhance the percentage of differentiated cells compared to untreated cells(Fig. 15 A), even if this difference is statistically significant only for ATRA ($p<0.05$). In addition, the treatment of cells with ATRA and AC55649 before inducing differentiation doesn't prime MSCs toward the adipocytic lineage (Fig. 15 B). In contrast, LE135 pretreatment seems to decrease the number of Nile Red positive cells: indeed it has been described that this compound can drive MSCs to the chondrogenic pathway (Kafienah 2007).

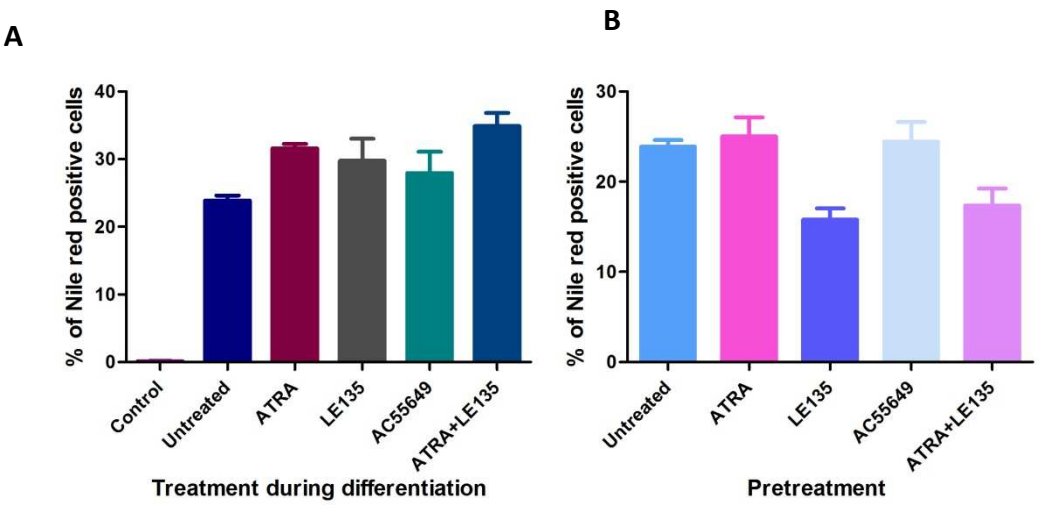


Figure 15: Nile red staining of cultured MSCs treated during (A) or before (B) inducing the differentiation. (A) All the treatments seem to enhance the percentage of differentiated cells when present in the adipogenic medium. (B) LE135 reduced the number of Nile red positive cells, instead ATRA and AC55649 don't modify the rate of differentiation. Data show the mean ±sem of two independent experiment done in duplicate.

To evaluate the osteogenic differentiation in MSCs culture we measured the activity of alkaline phosphatase, an early marker of osteogenic differentiation, and accumulation of calcium in the extracellular matrix with Alizarin red staining, an indicator of terminal differentiation into osteocyte. Treatment with ATRA during differentiation increases the level of alkaline phosphatase compared to untreated cells (Fig. 16 A), even if not significantly, but no modification of the Alizarin red quantification was seen (Fig. 16 C). On the other hand, RARβ agonist enhances alkaline phosphatase activity both during and before inducing the differentiation (Fig. 16 A-B), and in the latter case the difference is statistically significant (p-value<0.05). Surprisingly, a slight decrease of calcium deposition was observed in cells treated with AC55649 either before or during differentiation, even if the differences have no statistical significance. (Fig. 16 C-D). Treatment with LE135 during differentiation does not modify osteogenic commitment (Fig. 16 A-C), but a reduction in alizarin red staining is evident in cells pretreated with RARβ antagonist: this confirms its chondrogenic differentiation capacity (Fig. 16 D). Data from pretreated cells show that both ATRA and RARβ agonist do not mediate an osteogenic commitment of MSCs (Fig 16 B and D).

Taken together, data for adipogenic and osteogenic differentiation confirm that both ATRA and RARβ2 selective agonist do not alter multipotency of MSCs or commit them toward a specific lineage. In contrast, the reduction of cells terminally differentiated as adipocytes and osteocytes confirms the capacity

of RARβ selective antagonist LE135 to induce chondrogenic differentiation of MSCs.

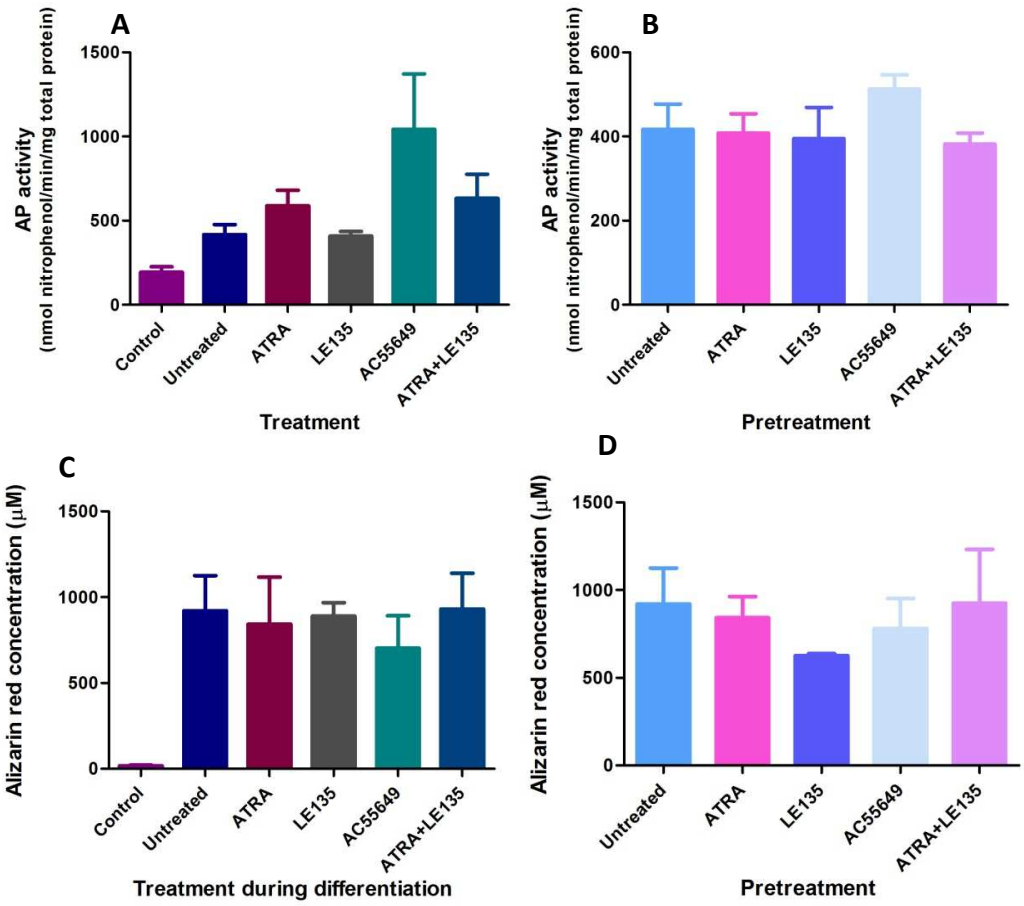


Figure 16: Alkaline phosphatase activity (A-B) and Alizarin red staining (C-D) quantification of osteogenic differentiation. Treatments were added during (A, C) or before (B, D) inducing osteogenesis. No relevant variation of the number of osteogenic cells were seen in MSCs pretreated with ATRA or RARβ agonist. LE135 diminishes the deposition of calcium in the extracellular matrix, confirming its role in the chondrogenic differentiation of MSCs. Data show mean ±sem of three independent experiment done in duplicate.

4.7 Expression of self-renewal genes in CFU-F and cultured MSCs

To further investigate the opposite behavior of CFU-F and cultured MSCs after ATRA treatment, we tested the expression level of genes involved in embryonic and adult self-renewal and multipotency (Niwa 2001, Loh 2008).

Figure 17 shows the expression fold change of all tested genes in CFU-F after ATRA treatment. No expression was found for *Zfp42/Rex1*, *Tcl1* and *GDF3*.

The treatment seems to reduce the expression of *Oct4*, *Nanog*, *Sox2*, *Sox15*, *Bmi1*, *Utf1* and *KLF4*; in contrast, *Sall4* level is greatly increased after ATRA treatment. No variation was seen for the expression of *HoxB4*. The basal expression level of *Sall4* gene is very low in untreated CFU-Fs, but it becomes comparable to the other self-renewal genes after ATRA treatment. This phenomenon might be explained by the selection of a specific population in CFU-F culture that expresses higher level of SALL4 and lower level of all the other genes.

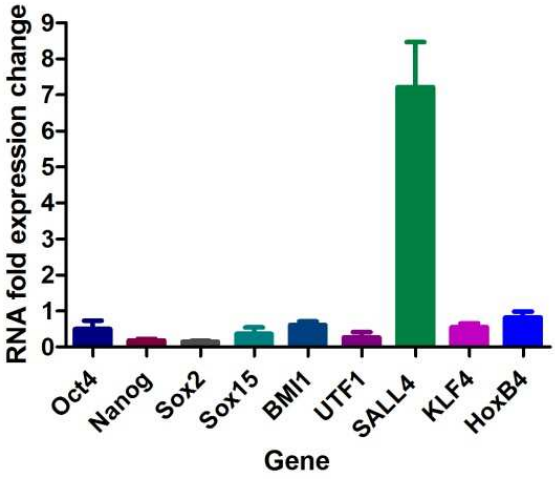


Figure 17: Expression fold increase of self-renewal genes in CFU-F after treatment with ATRA. Values represent the fold increase of mRNA transcript level in treated cells compared to controls. Data show mean ±sem of three independent experiments.

The results differ when the expression profile of cultured MSCs was determined. After 8 and 24 hours of treatment, the expression of *Oct4*, *Nanog*, *Sox2*, *Utf1* and *Sall4* increases (Fig. 18 A-E), even if important variations between MSCs derived from different donors were noted. These five genes could be induced after 72 hours, as new fresh medium was added after 2 days of culture. In contrast, the expression level of *Klf4* is reduced at all the time points, even if the lowest values were reached after 24 and 72 hours of treatment (Fig. 18 F). No expression was found for *Zfp42/Rex1*, *Tcl1* and *GDF3*, as in CFU-Fs, and no significant variation was seen for *Sox15*, *Bmi1* and *HoxB4* mRNA levels.

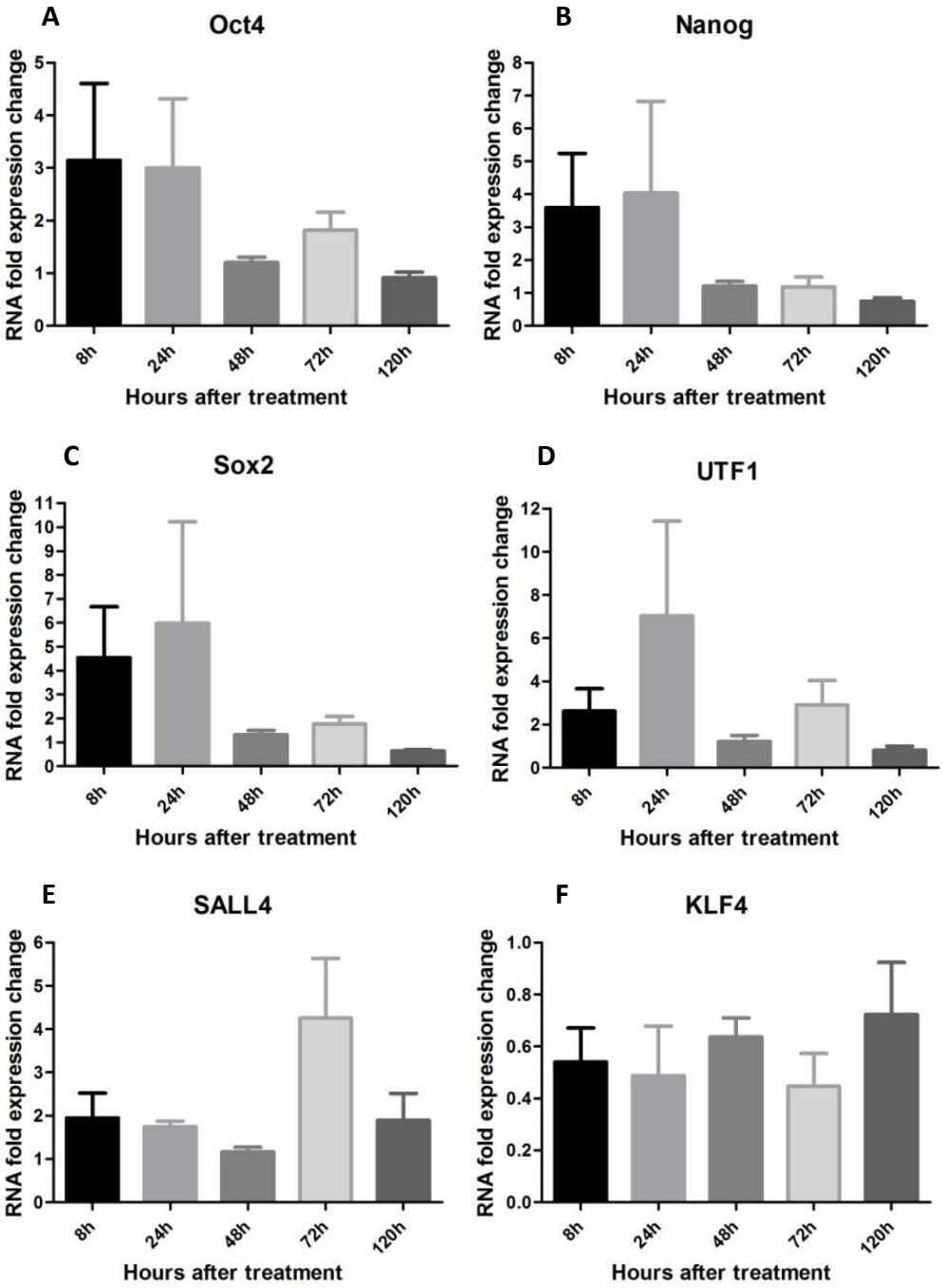


Figure 18: expression fold change of Oct4 (A), Nanog (B), Sox2 (C), Utf1 (D), Sall4(E) and Klf4 (F). Klf4 seems to be reduced by addition of ATRA to the medium, while Oct4, Nanog, Sox2, Utf1 and Sall4 transcripts augment in response to the treatment. Data show the mean ±sem of four independent experiments.

5 Discussion

Retinoic acid plays crucial role in embryogenesis and morphogenesis, and it controls cell growth, apoptosis and differentiation of several cell types. Indeed, it is used alone or in combination with chemotherapeutic agents to treat acute promyelocitic leukemia *in vivo* (Chomienne 1989, Altucci 2007). Only recently the combination of ATRA with epigenetic drugs, such demethylating agents and HDAC inhibitors, has been shown to be effective against a wider range of hematological malignancies, and clinical trials are in progress to test the therapeutic effect of combination of *all-trans* retinoic acid, 5-azacytidine and valproic acid in AML patients (Soriano 2007). Mesenchymal stem cells share the same microenvironment as hematopoietic stem cells, and they play a central role in the balance between proliferation and differentiation of these cells (Lazennec 2008). In this study, we evaluated the effects of *in vitro* treatment of ATRA, AZA and VPA in human MSC biological properties, such as proliferation, apoptosis, senescence and differentiation potential.

Our data show that treatment with AZA and VPA decreases the number of CFU-F in culture. This is consistent with the inhibition of proliferation and increase of apoptosis seen in cultured MSCs. In addition, both AZA and VPA alter the morphology of cells, that appear more flattened, suggesting the commitment towards a non-proliferative or differentiated states. Indeed, Cho *et al.* have described an inhibitory effect of VPA on MSC proliferation in culture, that associate with a stimulatory effect on osteogenic differentiation (Cho 2005). Moreover, it has recently been demonstrated that suberoyl anilide hydroxamic acid (SAHA) and MS-275, two synthetic HDAC inhibitors, induce a block in the cell cycle along with the induction of apoptotic pathway in human bone marrow-derived MSCs (Di Bernardo 2009). On the other hand, treatment with AZA alone is sufficient to promote the commitment of human MSCs in cardiomyocytes, assessed by the expression of cardiac specific markers and functional analysis (Xu 2004).

The results reported in this study show a different behavior of CFU-Fs and cultured MSCs after ATRA exposure. In fact, ATRA treatment reduces the number of colonies in CFU-F assay, whereas it enhances the proliferation rate of cultured MSCs. Oliva *et al.* have first reported a growth inhibition of human MSCs after ATRA treatment, due to an accumulation of cells in G_1 phase of the cell cycle.

This is probably linked with the increase in the protein level of the cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p16^{INK4A}$ and the consequent reduction in cdk2 activity (Oliva 2003). Cell cycle analysis by flow cytometry did not reveal significant variation in the percentage of cell in the active phases of cell cycle after ATRA treatment, because the reduction of G_2-M cells is compensated by an increase in the number of cells in S phase. In addition, no cell morphology changes were observed in cultured MSCs after ATRA treatment as previously described (Oliva 2003). The differences in the data collected from our group and Oliva's group might lay on the different methods used to isolate MSCs from bone marrow aspirates and to cultivate them.

The expression levels of the three RARs and RXRs suggest the presence of different cell populations in CFU-Fs and cultured MSCs. No expression of RXRγ was found in both early and later stem cells: in fact this RXR subtype presents a very restricted pattern of expression, mainly in the muscle and certain part of the brain (Germain 2006 (2)). RARα and RARγ have a higher expression in CFU-Fs compared to MSCs. Furthermore, the RARβ subtype is the only RAR and RXR receptor responding to ATRA treatment both in CFU-Fs and cultured MSCs. Both RARβ2 and RARβ4 transcripts are induced by the treatment, albeit with no change in the ratio between the two isoforms in treated cells compared to controls. The prevalence of RARβ2 isoform suggests that it mediates ATRA signaling in the cells: this hypothesis was validated by the employment of RARβ2 selective agonist AC55649 (Piu 2005), which exerts the same effect of ATRA on proliferation and apoptosis. The crucial role of the RARβ subtype was further confirmed by the concurrent treatment of ATRA and a RARβ specific antagonist LE135 (Li 1999), that attenuates the proliferation and apoptosis of MSCs with ATRA alone. The RARβ2 isoform is a well-known tumor suppressor. Indeed, RARβ2 is frequently lost or epigenetically silenced in various cancers and, moreover, its expression correlates inversely with tumor grade (Xu 2007). Furthermore, restoration of *RARβ2* expression reactivates RA-dependent growth control (Sirchia 2002). However, our data uncover a potential new role of this isoform in stimulating MSC proliferation and expansion in culture.

The ATRA treatment in cultured MSCs might select one of the populations present in cultured MSCs. In fact, cells in early passages are heterogeneous in morphology, rates of proliferation, and efficacy with which they differentiate (Sekiya 2002). To address this possibility, we checked the effect of ATRA treatment before or during the induction of osteogenesis and adipogenesis. No priming towards a specific lineage was found using ATRA or the RARβ2 selective agonist; on the contrary, exposure to LE135 reduces the number of adipocyte

and osteocyte after the induction of differentiation, confirming the role of this RARβ specific antagonist in the induction of chondrogenic differentiation (Kafienah 2007). The presence of ATRA and RARβ2 agonist in osteogenic medium enhances alkaline phosphatase activity, even though this is not correlated with the deposit of calcium in the extracellular matrix. The absence of concordance between alkaline phosphatase and Alizarin red staining data could be due to the fact that alkaline phosphatase is an early marker of osteocyte commitment, while calcium deposition in extracellular matrix marks later step of osteogenic differentiation. In contrast, Wan and colleagues showed that the presence of ATRA in osteogenic medium stimulate the differentiation of murine adipose-derived adult stromal cells, confirmed by an increase in both alkaline phosphatase activity and Alizarin red staining (Wan 2007). Specie-specific differences and the higher concentration of ATRA used (2.5 μM) could explain the absence of ATRA-promoting effect in our MSC culture. Finally, all the treatments enhanced the percentage of adipocytes, even if the difference is significant only between ATRA-treated and control cells. However, data suggest that this phenomenon is not mediated by ATRA or by the activation of RARβ, because the higher effect on adipocytic differentiation was seen with the combination ATRA+LE135. Further data are needed to understand the effect of these treatments in MSC adipogenesis during the induction of differentiation.

Finally, we tested the expression level of genes known to regulate embryonic and adult stem cell multipotency and self-renewal. CFU-Fs respond to the treatment with a reduction of almost all of the genes tested, except for *Sall4*. Indeed, *Sall4* mRNA level is greatly induced by ATRA exposure compared to untreated cells, even if no RARE has been described in its promoter region. The selection of a specific population in CFU-Fs culture which expresses high levels of Sall4 could explain both the reduction of colony number and the overexpression of this gene after ATRA treatment. On the other hand, the expression profile of self-renewal genes in cultured MSCs is completely different. In fact, after 8-24 hours *Oct4*, *Nanog* and *Sox2* transcript levels are enhanced by ATRA, with a resultant induction of *Utf1* and *Sall4* expression, two known target of Oct4 (Niwa 2001, Loh 2008). The levels of these 5 genes are also increased after 72 hours of treatment, maybe in response to the addition of fresh medium containing ATRA, but return to baseline levels after 5 days of treatment. Thus, ATRA seems to induce a very brief and time-restricted induction of Oct4, Sox2, Nanog, Sall4 and Utf1 transcripts, that might explain the proliferative effect on cultured MSCs. In contrast, the expression level of *Klf4* gene is reduced by ATRA at all the time intervals tested. Klf4 protein is necessary for the reprogramming of adult mouse

fibroblast (Yamanaka 2006), and it shares many common targets with Nanog, suggesting a link with the Nanog transcriptional network (Loh 2008). Moreover, no variation of the expression of *HoxB4* was found both in CFU-Fs and in cultured MSCs. This is in marked contrast with the results reported by Folberg *et al.* in mouse hindbrain segmentation, where they demonstrated the induction of HoxB4 gene by RARβ in response to RA. (Folberg 1999) In addition, RARβ and HoxB4 present a direct crossregulation for the presence of RA- and Hox-responsive enhancer in both genes (Serpente 2004). The role of Klf4 and HoxB4 in MSC maintenance and multipotency is not known, and further studies are needed to explain their expression modification in response to ATRA exposure.

6 Conclusions and future perspectives

We demonstrate the inhibitory effect of AZA and VPA treatment in both CFU-Fs and cultured MSCs, that correlates with an increase of apoptosis and a commitment towards a non-proliferative state. In addition, we show that ATRA exposure reduces the number of CFU-Fs, while it enhances the proliferation of cultured MSCs without affecting their differentiation potential. RARβ2 isoform mediates ATRA signaling both in CFU-Fs and in cultured MSCs, as it is assessed by the use of RARβ2 selective agonist and RARβ selective antagonist. Finally, we prove that ATRA treatment influence the expression of self-renewal genes in early progenitor and MSCs.

Our data suggest that ATRA selects a specific subpopulation in CFU-Fs with higher expression level of *Sall4*. To confirm this hypothesis, we will characterize CFU-Fs after ATRA treatment by surface antigens and we will evaluate the proliferation, apoptosis and differentiation potential of cultured MSCs derived from these colonies.

The combination of ATRA with epigenetic modifying drugs is now under clinical trial to evaluate its therapeutic effect in AML malignancies (Soriano 2007). Experiments to analyse whether MSCs derived from leukemic patients respond to ATRA, AZA and VPA treatment in the same way as MSCs from healthy donors will be pursued. Our preliminary data confirm that the three drugs trigger the same effect on proliferation and apoptosis of cultured leukemic patient MSCs. In addition, ATRA treatment induces the expression of the RARβ subtype, as in normal MSCs. Further studies are needed to confirm these results and to

compare the effect on the differentiation potential and expression of self-renewal genes to normal MSCs.

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