UNIVERSITY OF TURIN

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XXX CICLO

Characterization of Mesenchymal Stem Cells Isolated from Bone Marrow, Placenta and Amniotic Fluid: immunophenotype, immunomodulant properties and epigenetic aspects for their clinical use.

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Subject: Molecular Oncology

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THESIS STRUCTURE AND DECLARATION

The abstract summarise the different aims and the results of the work performed during the PhD course and is followed by a comprehensive introduction about Mesenchymal Stem Cells with the different aspects that I investigated in this field (Chapter 1).

There are 3 aims explaining in the Chapter 2 and a paper session that collects the 5 manuscripts published in these years with the results obtaining to reach my aims. In particular. In Chapter 3 the manuscripts reported the results obtained for the aim 1.

In Chapter 4, 2 manuscripts (4.1 and 4.2) are inserted to describe the aim 2 and in the chapter 5 other 2 manuscripts are described, 1 published and 1 submitted to explain the aim 3.

In the Chapter 6 I have inserted some data obtained from my work *in progress* in this moment. The paragraph 6.1 describe the experiments performed using the same study design described in the 3.1 to investigate if MSCs isolated and expanded in Good Manufacturing Practice (GMP) preserve the immunomodulant properties described in the aim 1.

The paragraph 6.2 describe some preliminary data obtained analysing the epigenetic profile of MSCs always expanded in GMP conditions.

Finally, in Chapter 7, I have summarized and discussed the results presented in this thesis, and I have also debated the future perspective on the use of MSCs for clinical use.

In the Appendix, I have listed all the manuscripts published during my PhD work, where I am a co-author.

I, Katia Mareschi, declare that I have not obtained a previous qualification from the University of Turin or elsewhere based upon any of the work contained in this thesis. I conducted the experiments in the laboratory Stem Cell Transplantation and Cellular Therapy at the Pediatric Onco-Hematology Division, University Hospital City of Science and Health of Turin, Regina Margherita Children's Hospital, and in the Department of Public Health and Pediatrics, University of Turin, Turin, Italy.

I presented and wrote the thesis under the supervision of the Professor Franca Fagioli.

Data presented in this thesis are yet in part published. The experiments evaluating the epigenetic aspects were performed in collaboration with an international group headed by Fernandez AF, and at the Department of Clinical and Biological Sciences, University of Turin, Orbassano, Turin, Italy.

ABSTRACT

Mesenchymal stem cells (MSCs) are a promising tool for cell therapies for their multipotent, bystander and immunomodulant properties. Therefore, MSCs are used for a very wide range of therapeutic applications and the possibility to have a cell factory laboratory has allowed us to setup up a method to expand them from bone marrow (BM) in Good Manufacturing Practice (GMP) conditions for clinical use. Although BM represents the main source of MSCs, the need remains to identify a stem cell source that is safe, easily accessible, providing high cell yield and for which cell procurement does not provoke ethical debate.

My experience has been to isolate MSCs from BM from healthy paediatric and adult donors, to characterize them and to validate a method to expand them in large scale in GMP conditions for clinical use and to isolate MSCs from foetal origin tissues (cord blood, placenta, amniotic fluid) to use as alternative sources of BM-MSCs.

My PhD project focused on 3 major aims:

1. to evaluate immunophenotype, differentiative potential, embryonic markers and immunomodulant properties of MSCs isolated from amniotic fluid (AF-MSCs) and placenta (PL-MSCs) and compare them with BM-MSCs.
2. to understand the role of MSCs infused in bone marrow grafts and to evaluate the mesenchymal stromal cell engraftment after allogeneic HSCT in paediatric patients
3. to investigate new stemness markers, their expression and their epigenetic regulation on BM-MSCs routinely isolated from BM of healthy donors during their ex-vivo expansion

Aim 1:

In this study, AF-MSCs and placenta PL-MSCs were compared with BM-MSCs. Their immunomodulant properties were studied on total activated T-cells with phytoemoagglutin (PHA-PBMCs). In particular, an *in vitro* co-culture system was performed to study: 1) the effect on T Lymphocyte (Ly) proliferation; 2) the presence of T regulatory Ly (Treg); 3) the immunophenotype of various T subsets

(Th1 and Th2 naïve, memory, effector Ly); 4) the cytokine release and master gene expression to verify Th1, Th2 and Th17 polarization; 5) the IDO production. In all the co-culture conditions with PHA-PBMCs and MSCs (independently from the tissue origin) data showed: 1) T proliferation inhibition; 2) Naïve T Increase and Memory T decrease; 3) Treg increase; 4) strong Th2 polarization associated to increase IL-10 and IL-4; Th1 inhibition (IL-2, TNF-a, IFN-g and IL-12 significantly decrease) and Th17 induction (production of high concentrations of IL-6 and IL-17); 5) IDO mRNA induction in MSCs co-cultured with PHA-PBMCs. AF-MSCs showed a more potent immunomodulant effect on T-cells than BM-MSCs, only slightly higher than PL-MSCs. This study shows that MSCs isolated from foetal tissues may be considered a good alternative to BM-MSCs for clinical applications.

Work in progress:

Using the same experiment design described above, we compared three different culture condition. The standard condition usually described in the literature containing foetal bovine serum (FBS) (Alpha-Mem + FBS 10%)] was compared with cell cultures considered in GMP conditions (without animal serum and containing human platelet lysate (HPL and inactivated HPL underwent to a pathogen inactivation procedure). We firstly analysed the immunophenotype, differentiative potential and embryonic markers, than deepen the MSC immunomodulant properties in the three experimental conditions. We performed *in vitro* co-culture system to study the inhibitory effects of MSCs on total PHA-PBMC. We observed:

1) T proliferation inhibition; 2) Naïve T Increase and Memory T decrease; 3) Treg increase; 4) strong Th2 polarization associated to increase IL-10 and IL-4; Th1 inhibition (IL-2, TNF-α, IFN-γ and IL-12 significantly decrease) and Th17 induction confirmed from the production of high concentration of IL-6 and IL-17; 5) IDO mRNA induction in MSCs co-cultured with PHA-PBMC. No significant differences emerged from co-culture with FBS-MSCs and iHPL-MSCs. This study shows that MSCs exerted a strong immunomodulant effects on T-cells. Their immunomodulant properties were maintained even when MSCs were cultured in iHPL. For this reason, we considered iHPL a good alternative to FBS even safer

than HPL to expand, in GMP condition, BM-MSCs for clinical applications.

Aim 2:

MSCs are multipotent stem-cells able to differentiate into mesenchymal origin tissue and support-the growth of hematopoietic stem cells. In order to understand-the role of MSCs infused in bone marrow grafts, 53 consecutive-patients were analyzed for engraftment, acute and chronic graft versus-host disease (GvHD), transplant-related mortality (TRM), relapse incidence, and overall survival. The MSC content was-measured as MSC expansion at the second passage. When *in vitro* expanded MSC (cumulative population doubling at second passage,-cPDp2) values were stratified according to the median value-(2.2-fold increase), the univariate analysis showed a significant difference-in TRM (23% vs. 3.8%, P=0.05.) and in acute GvHD III-IV-incidence (12% vs. 4%, P= 0.04), while the multivariate analysis-did not confirm its independent role. No clinical parameters in-donors and recipients were identified as predictors of cPDp2-expansion. Our study suggests a role for short-term *ex vivo*-expanded MSCs in reduced aGVHD III-IV incidence and TRM in univariate analysis. A multicenter, larger study is warranted to confirm these data.

Moreover, as the MSC role after allogeneic haematopoietic stem cell transplantation (HSCT) is still matter of debate, and MSC engraftment in recipient bone marrow is unclear, we investigated the mesenchymal cell chimerism following allogeneic HSCT. A total of 46 patients were analyzed for MSC and haemopoietic stem cell engraftment after HSCT. The majority of patients had bone marrow as stem cell source and acute leukemia was the main indication for HSCT. Chimerism analysis was carried out through specific polymorphic tandemly repeated regions (STRs) on whole BM and MSCs isolated and expanded in vitro until the 2nd passage from BM after hematopoietic engraftment. We observed that All patients reached complete donor engraftment but no evidence of donor derived mesenchymal stem cell engraftment was noted. Our data indicate that MSCs after HSCT remain of recipient origin despite: i) myeloablative conditioning, ii) the stem-cell source, iii) the interval from HSCT to BM analysis (3, 4), iv) the underlying disease before HSCT, v) the patients’ or the donors’ age at HSCT.

Aim 3:

In differentiated cells, aging is associated with hypermethylation of DNA regions enriched in repressive histone posttranslational modifications. However, the chromatin marks associated with changes in DNA methylation in adult stem cells during lifetime are still largely unknown. In collaboration with an international group, we analysed DNA methylation profiling of BM-MSCs obtained from individuals aged 2 to 92. We identified 18735 hypermethylated and 45407 hypomethylated CpG sites associated with aging. As in differentiated cells, hypermethylated sequences were enriched in chromatin repressive marks. Most importantly, hypomethylated CpG sites were strongly enriched in the active chromatin mark H3K4me1 in stem and differentiated cells, suggesting this is a cell type independent chromatin signature of DNA hypomethylation during aging. Our results indicate that the dynamics of DNA methylation during aging depend on a complex mixture of factors that include the DNA sequence, cell type and chromatin context involved, and that, depending on the locus, the changes could be modulated by genetic and/or external factors.

Other studies about epigenetic regulation were performed in our laboratory to investigate the expression analysis of human endogenous retroviruses (HERV) in correlation with the stemness markers and the epigenetic profile associated with these stemness markers in MSC culture during the expansion.

The "HERV" are endogenous retroviruses that are inserted into the germ cell DNA of primate over 30 million years ago and their core transcription factors are involved in pluripotency, including POU class 5 homeobox 1 (OCT-4), sex determining region Y-box 2 (SOX-2), and NANOG homeobox (NANOG) representing approximately 80% of the LTRs and regulating the expression of the 50 most highly expressed HERV-H proviruses.

Our aim was to evaluate pol gene expression of HERV-K and HERV-H in MSCs that are adult multipotent stem cells during their expansion.

MSCs were isolated from BM of healthy donors and expanded until the 5th passage in alpha-MEM with 10% FBS and HPL. HERV-K, -H pol gene, NANOG, OCT-4, SOX-2 and GAPDH expression was quantified by real-time PCR in MSCs during

the expansion.

HERV-K and HERV-H expression was always higher at p1 compared to other passages and this difference reached a high statistical significance when passage p1 was compared with passage 3. In addition, NANOG, OCT-4 and SOX-2 expression at p1 was significantly higher than the expression at p3. Spearman's test demonstrated a strong correlation between the expression of HERV-K and HERV-H and expression of NANOG, OCT-4 and SOX-2

Our findings showed that HERV-K and -H were concurrently expressed with pluripotency biomarkers NANOG, OCT-4 and SOX-2. These findings might suggest that the HERVs pol genes play an important role in the differentiation of the MSCs and should be considered as new markers of stemness or differentiation for MSCs.

Work in progress: To investigate the epigenetic profile associated with the expression of stemness markers during MSC expansion, we performed Chromatin Immunoprecipitation (Chip) assay against histone modifications marks. It was used for evaluation of H3K4me3, active gene mark, and H3K27me3, silence gene mark, on promoter for OCT4, SOX2 and Nanog transcription factors. We analysed these aspects on MSCs cultivated in FBS (FBS-MSCs) and in iHPL (HPL-MSCs) as described above.

Although the HPL-MSC showed a higher proliferative potential, we did not observe significant difference about the molecular expression of the stemness markers and their epigenetic profile.

In particular, we observed a specific epigenetic profile for each analysed sample. For example, in one MSC batch, H3K4me3, active gene mark, enrichment was present on Nanog and OCT-4 promoter in early stages and H3K27me3, silence gene mark, increased in late stages. However H3K27me3 enriched on Nanog and SOX2 promoter in all stages. Altogether, these results suggest that epigenetic marks describe mesenchymal stem cell status as pluripotency state and proliferative conditions, however these cells lost active epigenetic marks on some stemness regulatory regions, such as Nanog and SOX2 promoter, showing a possible switching versus differentiated cells. These preliminary data suggest

us to identify other specific regulatory regions for this cellular type using an integrated analysis of the data deriving from ChIP-seq experiments.

CONCLUDING REMARKS FROM MY PhD ACTIVITY

Regenerative medicine is of growing interest in biomedical research and in this context, MSCs are a promising tool for cell therapies for their multipotent, bystander and immunomodulant properties. For these reasons, MSCs are used for a very wide range of therapeutic applications, the majority of which are in Phase I, Phase II, or a mixture of PhaseI/II studies (see www.clinicaltrial.gov). Most MSCs used in these clinical trials are isolated from BM and are considered safe and efficacious for their multipotent and immunomodulant properties. However, the clinical application of BM-derived cells is limited for the relatively invasive procedure for sample collection, the difficulties of obtaining a sufficient number of MSCs to appropriately perform studies, and a marked reduction in cell number, proliferation, and differentiation capacity with age. During the activity of my PhD, we obtained useful data to clarify some mechanisms of action at the cellular, molecular and epigenetic level of MSCs isolated from BM, AF and PL to use for clinical use.

We showed that:

- MSCs isolated from foetal tissues may be considered a good alternative to BM-MSCs for clinical application, because AF and PL-MSC were considered multipotent stem cells with the immunophenotypic characteristics and differentiative potential established by guidelines by the International Society of Cellular Therapy , a greater proliferative potential associated with the presence of embryonic markers and great immunomodulant properties.
- The immunomodulant properties of MSCs is maintained when FBS is substituted from inactivated HPL to have a method safer and more advantageous for large scale expansion in GMP conditions
- BM-MSCs have an important role in reduced aGVHD III-IV incidence and TRM and remain of recipient origin after HSCT despite: i) myeloablative conditioning, ii) the stem-cell source, iii) the interval from HSCT to BM analysis (3,4), iv) the underlying disease before HSCT, v) the patients' or the donors' age at HSCT)

- MSCs are a population of cells extremely heterogonous and have an interindividual variability of DNA methylation. The dynamics of DNA methylation during aging depend on a complex mixture of factors that include the DNA sequence, cell type and chromatin context involved, and that, depending on the locus, their changes can be modulated by genetic and/or external factors.
- Endogenous retroviruses such us HERV-K and -H should be considered as new markers of stemness or differentiation for MSCs
- The preliminary data obtained from ChIP-seq experiments showed that the activator histone mark, H3K4me3 and the repressive histone mark, H3K27me3 cannot be considered good markers to analyze the stemness maintenance during the expansion of MSCs in vitro and suggest us to identify specific regulatory regions different from NANOG, OCT-4 and SOX-2 to identify stemness stage.

All these data support the iHPL represents a good, GMP-compliant alternative to FBS for MSC clinical production which is more advantageous in terms of cellular growth and stemness and preserve the immunomodulant proprieties and stemness marker. Moreover, our data support the current hypothesis that MSCs could act by secreting paracrine factors in a “hit-and-run” scenario including the secretion of not only cytokines and other soluble factors but also extracellular vesicles that can contain cargos that include peptides, proteins, metabolites, microRNAs, and even mitochondria. Our future perspectives focus on the study of secretome obtained by MSCs expanded in GMP conditions to obtain a pharmaceutic product safer and extremely effective and efficient for multiple clinical use.

PREFACE

From different years I have been working in the “Stem Cell Transplantation and Cellular Therapy Laboratory” at the Regina Margherita Hospital (directed by Dr Franca Fagioli), on MSCs studying their immunophenotipic, molecular and multipotent characteristics to use them for clinical use (Mareschi K et al. 2001; Mareschi K et al. 2006; Mareschi K et al. 2006; Mareschi K. et al. 2012a, 2009a; Mazzini L et al. 2003; Ferrero et al. 2008). During my work, we isolated autologous BM-MSCs from patients with amyotrophic lateral sclerosis, a neuro-degenerative disease that selectively delays the moto neurons causing paralysis and death after 4-5 years from the diagnosis et al. 2009a; Mazzini et al. 2012a) These cells expanded in laboratory were implanted in the spinal cord in a small cohort of patients in a controlled I phase study to verify the safe and feasibility of the procedure (Mazzini et al. 2009; Mazzin et al. 2009b; Mazzini et al. 2012b; Mazzini et al. 2010; Mazzini et al. 2003). Although the procedure was considered safe and well tolerated by ALS patients, a new regulation on advanced therapy medicinal products (ATMPs) came into effect during 2007 (1394/2007/EC initiating Directive 2009/120/EC amending 2001/83/EC) and the MSCs were considered Advance Therapy Medicinal Products (ATMPs) that need to be produced in a Good Manufaturing Practice (GMP) accredited cell factory and approved by the Italian Regulatory Authority AIFA (Agenzia Italiana del Farmaco). A Cell factory was built inside the lab and meanwhile new studies were performed: to set up a method to isolate and expand MSCs in GMP conditions from bone marrow (BM) for large scale without animal component to use them for clinical application (Mareschi et al. 2012; Castiglia et al. 2014).

At that time, it was assumed that MSCs could engraft and differentiate into multiple tissue to replace damaged cells. We demonstrated in vitro that MSCs could differentiate in neuronal cells with active electro physiologically K and sodium channels. However, there were few techniques available for definitively assaying engraftment of MSCs, particularly if they acquired new phenotypes after engraftment. As the technologies for assaying engraftment of MSCs improved, the answer became clear: the assumption was wrong (Prockop et al.

2010; Keating 2012). Except for a few unusual situations, systemically administered MSCs did not engraft in significant numbers. They did not survive for long periods of time in vivo, and they showed limited tendencies to differentiate in vivo. But the cells had surprising effects in vivo. Literally hundreds of reports continued to demonstrate that MSCs produced dramatic therapeutic benefits in multiple animal models for human diseases and in a few patients (Prockop et al. 2010; Keating 2012) even though they disappeared with half-lives as short as 24 hours (R. H. Lee et al. 2009). Hence the current hypothesis that MSCs could act by secreting paracrine factors in a "hit-and-run" scenario has been established. The hypothesis has been broaden to include secretion not only of cytokines and other soluble factors but also extracellular vesicles that can contain cargos that include peptides, proteins, metabolites, microRNAs, and even mitochondria (Islam et al. 2012, 2012; Vallabhaneni et al. 2015; Phinney et al. 2015; Katsuda e Ochiya 2015). But in spite of the best efforts of thousands of scientists, we are still at a loss to explain most of the therapeutic benefits of MSCs. In effect, we have to work backward from the positive results obtained in vivo to defining their mechanisms of action at the cellular and molecular level.

GENERAL INTRODUCTION AND AIMS

1 INTRODUCTION

1.1 MESENCHYMAL STEM CELLS (MSCS)

Mesenchymal stem cell (MSCs) were identified about 40 years ago by Friedenstein et al.as the stromal cells of the bone marrow (BM) microenvironment that support hematopoiesis (Friedenstein et al. 1968). Therapeutic approaches were later achieved by using these cells in bone disorders (Luria et al., 1987).

Years later, Caplan and co-workers named these cells as MSCs (A I Caplan 1994) and in 1999, Pittenger et al demonstrated that these cells are multipotent SCs with potential to differentiate into other cells from mesenchymal tissues (Pittenger et al. 1999).

Over the next 20 or so years, Friedenstein and a large number of other investigators demonstrated that MSCs had several attractive features, including rapid expansion in culture, an ability to generate single-cell derived colonies, and ready differentiation to mineralized cells, chondrocytes, and adipocytes both in culture and in capsules in vivo.

To date MSC or MSC-like cells have also been expanded from numerous other compartments, including skeletal muscle, adipose tissue, umbilical cord, synovium, dental pulp, amniotic fluid, human embryonic stem cells, and numerous other sources and have been widely studied especially for its clinical application.

MSCs are adult multipotent stem cells able to self-renew and to differentiate into various mesodermal cell lineages. Multipotent MSCs can be isolated and efficiently expanded from almost every single body tissue and MSCs have the ability to repair diverse damaged tissues and have potent immunomodulant properties. MSCs can provide effective treatments for a wide range of diseases and possess several applications in regenerative medicine assuming a role in regenerative medicine and also in inflammatory/autoimmune disease. Relevant data have been generated in animal models of human diseases and different clinical trials have being started (www.clinicaltrials.gov). However, little is known about how MSCs can repair damaged tissues.

Once it was seen that several organs and tissue have MSCs, the name needed to be standardised. In 2005, the International Society of Stem Cell Research (ISSCR) termed

these cells as multipotent mesenchymal stromal cells for fibroblast-like plastic adherent cells isolated from any organ (Horwitz et al. 2005). And if this cell follows the minimal criteria of stem cells, then they can be called mesenchymal stem cells (MSCs).

There are three main criteria in order to determine the identity of MSCs (Dominici et al. 2006) as described inTable 1-1.

<table>
<tr><td>1</td><td colspan="3">Adherence to plastic in standard culture conditions</td></tr>
<tr><td rowspan="6">2</td><td rowspan="6">Phenotype</td><td>Positive (≥95% +)</td><td>Negative (≤2% +)</td></tr>
<tr><td>CD105</td><td>CD45</td></tr>
<tr><td>CD73</td><td>CD34</td></tr>
<tr><td>CD90</td><td>CD14 or CD11b</td></tr>
<tr><td></td><td>CD79α or CD19</td></tr>
<tr><td></td><td>HLA-DR</td></tr>
<tr><td>3</td><td colspan="3">In vitro differentiation: osteoblasts, adipocytes, chondroblasts (demonstrated by staining of in vitro cell culture)</td></tr>
</table>

Table 1-1 - Summary of criteria to identify MSC

1.2 MSC CHARACTERISTICS AND MECHANISMS OF ACTION

MSCs are adult SCs with a reduced differentiation capacity and a high plasticity capacity.

It is well known that mesenchymal cells can shift from one differentiation pathway to another under modified external conditions and can shift from quiescence to a proliferative state or that MSC differentiation can be reversed at least up to a certain stage (Das, Sundell, and Koka 2013).

Clonogenic MSCs are a heterogeneous mix of progenitors, in which a subset population is capable of differentiating into cells of mesodermal (adipocytes, osteoblasts, chondrocytes, tenocytes, skeletal myocytes and visceral stromal cells), ectodermal (neurons, astrocytes) and endodermal (hepatocytes) origin .

Figure 1-1-1 shows the ability of MSCs in the BM cavity to self-renew (curved arrow) and to differentiate (straight, solid arrows) towards the mesodermal lineage. The reported ability to transdifferentiate into cells of other lineages (ectoderm and

endoderm) is shown by dashed arrows, as transdifferentiation is still controversial.
Different techniques have been used for MSC differentiation: use of biological and pharmacological reagents (Scintu et al. 2006; Mareschi et al. 2006; Alhadlaq et al. 2004; Jørgensen et al. 2004; Sun et al. 2007); mechanical cues (McBeath et al. 2004; Engler et al. 2006) and external mechanical and electrical forces (Altman et al. 2002; Yoshikawa et al. 1997). Both mechanical and electrical stimulation have been applied separately and combined with soluble factors to facilitate MSC differentiation (Wu et al. 2008). The current MSC manufacturing platforms are reviewed with special attention regarding the use of bioreactors for the production of GMP-compliant clinically relevant cell numbers (Dwarshuis et al. 2017; Mizukami e Swiech 2018).

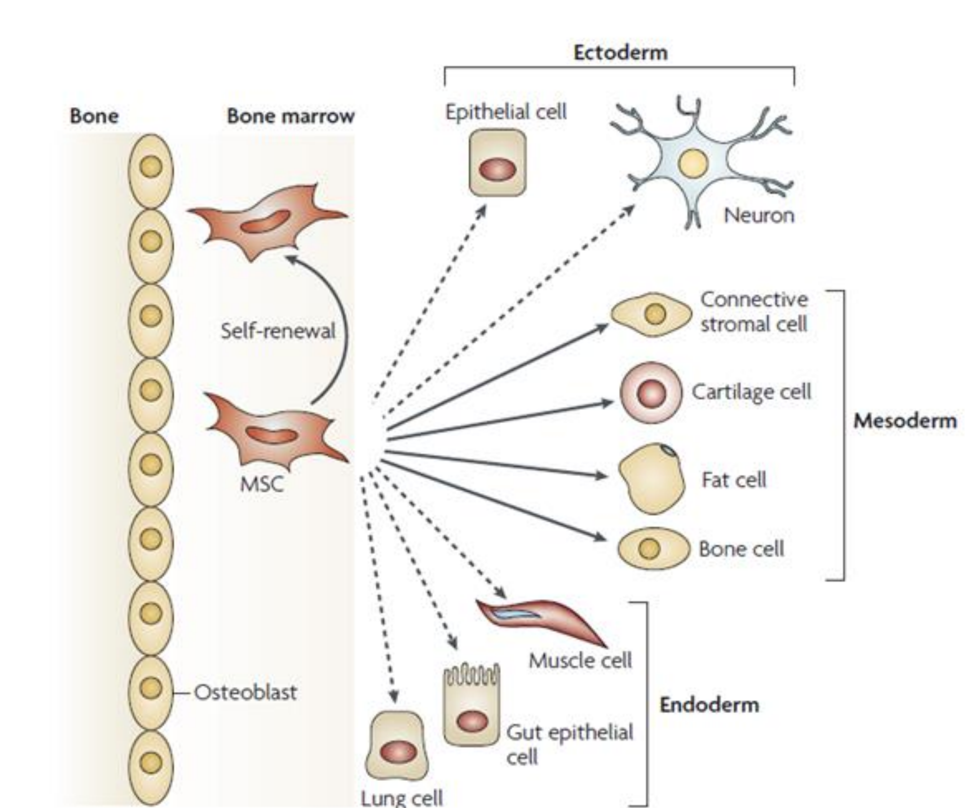


Figure 1-1-1 -The multipotentiality of MSCs (from Uccelli et al, 2008).

The criteria for differentiation need to be rigorously defined. It appears difficult to ascertain the differentiation process. However, while some markers are specific for certain cells, they do not have a functional relation. Furthermore, these markers are not the only ones that can characterize a specific cell. Delorme et al have found that MSCs express cytoskeletal proteins usually expressed in neural SCs (nestin), hepatocytes (cytokeratin-8 and -18), biliary cells (cytokeratin-19), and sarcomeric

muscle (troponins, a-C-actin), without the expression of proneural or neuronal, prohepatocytic, or myogenic key transcription factors (Delorme et al. 2009). Some of the observed differentiations may also result from reprogramming. Dezawa and colleagues have shown that rodent and human bone marrow MSCs can be reprogrammed into cells with skeletal muscle potential after specific treatment comprising first cytokines and then gene transfer of the notch intracellular domain (Dezawa et al. 2005).

MSCs obtained from different tissue sources show some differences regarding differentiation potential and gene expression profiles. Thus, it has become clear that the microenviroment in which MSCs are transplanted, growth factors and local cellular interactions, play a pivotal role in determining both MSC biology (survival, proliferation, and specific differentiation) and eventually a clinical measurable improvement.

The basis for the ability to differentiate is based on the identification or the originating cellular source of the MSCs. MSCs are thought to be derived from pericytes (Arnold I Caplan 2008). Pericytes are perivascular cells with multifunctional activities which are only now being elucidated. The functional interaction of pericytes with the endothelial cells is thought to be the source for differentiation and definition of MSC differentiation (Feng, Mantesso, e Sharpe 2010). Injury results in vascular changes even at the micro-vascular level. These changes stimulate pericyte differentiation into targeted MSCs that home to the injury and repair perturbed tissue as well as modulate the surrounding *in vivo* environment. The environment of culture *in vivo* ultimately defines the process of differentiation and end-point tissue. Variations of the tissue differentiation will depend on the quality and the quantity of specific inducers of the differentiation process.

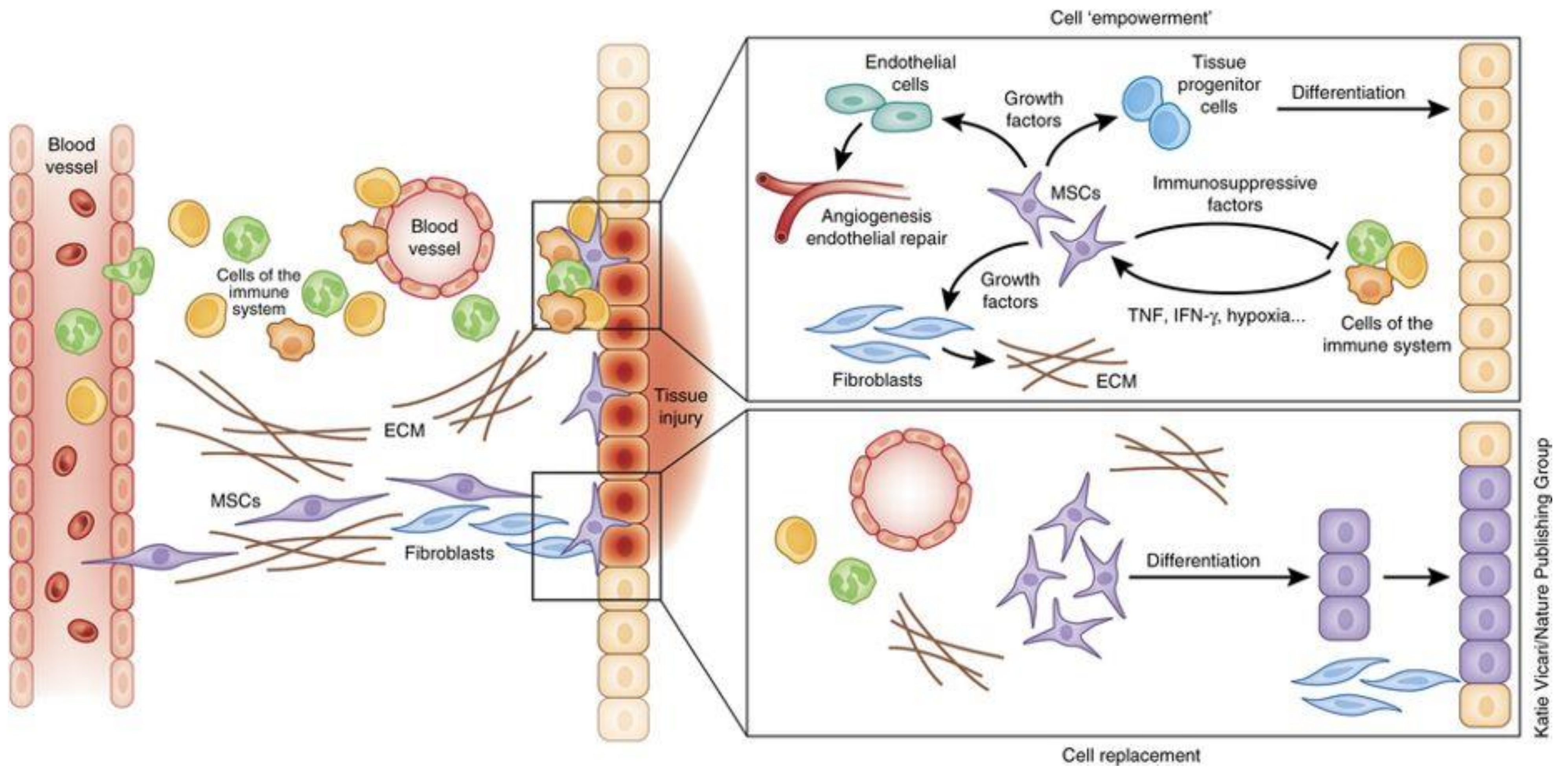


Figure 1-1-2 Modes of MSC-based therapy: cell replacement versus cell 'empowerment' from (Y. Wang et al. 2014).

Cell fusion is a process that has an important biological role in the development, physiology and disease of multicellular organisms. For example, the zygote formation and organogenesis of various tissues, such as placenta, bone and skeletal muscle. There are two types of cell fusion: homotypic and heterotypic cell fusion.

Homotypic fusion happens between cells of the same type; like fusion between myoblasts for the formation and growth of multinuclear myofibres and multinucleated cells during chronic inflammatory conditions.

Heterotypic cell fusion occurs between cells of different lineages, in specific adult SCs they can be used for clinical therapy by introducing a nuclei or functional genes in aged or degenerating cells (Singec e Snyder 2008). Recent reports show that SCs can fuse with differentiated cells in a range of tissues, including the brain, kidney, heart, lung and liver.

It is known that inflammation promotes the migration and infiltration of bone marrow-derived SCs to sites of tissue injury. Moreover, inflammation also increases the frequency of stem cell fusion. In the brain, chronic inflammation can cause an increase in spontaneous fusion events through the increase of cytokine levels, by activating immune cells or by damaging the blood brain barrier leading to increased permeability (Johansson et al. 2008).

Kemp et al demonstrated that fusion between MSCs and cerebellar neurons can occur spontaneously *in vitro* and also exhibit this potentially reparative action *in vivo*, fusing with Purkinje cells in the rodent cerebellum. These function events are also higher in the neuroinflammatory environment of experimental autoimmune encephalomyelitis (EAE), with no apparent loss in Purkinje cell numbers (K Kemp et al. 2011).

Furthermore, BM transplantation demonstrates that BM-derived cells fuse *in vivo* with hepatocytes in liver, Purkinje neurons in the brain and cardiac muscle in the heart, resulting in the formation of multinucleated cells, suggesting that genetic material derived from BM-derived cells contribute through cell fusion to the survival and function of these cells (Johansson et al. 2008; Bae et al. 2007; Alvarez-Dolado et al. 2003).

These studies demonstrate that SCs can fuse with cells of different tissues. However, additional studies in animal models will be required to determine whether this fusion

can be used in reparative cell therapy.

Although there are many ways through which SCs may ameliorate injury, the main mechanism is through paracrine and endocrine functions. Today, a wide range of cytokines and factors are known to be involved in the beneficial interaction between MSCs and other cells. Among the theories regarding the paracrine action for MSCs, by far the most explored and discussed is immunomodulation (see chapter 1.5. Immunomodulation)

Angiogenic support provided by MSCs can be considered one more supportive effect, since the re-establishment of the blood supply is fundamental to recover damaged tissues. The pro-angiogenic effect of MSCs has been demonstrated in other *in vitro* and *in vivo* studies (Hung et al. 2007; Sanz et al. 2008).

MSCs express and secrete stromal cell-derived factor 1 (SDF-1), essential for endothelial cell survival, vascular branching and pericyte recruitment, Vascular Endothelial Growth Factor (VEGF), a key component in the development of blood vessels, and other cytokines which are important for angiogenesis (Basic Fibroblast Growth Factor (bFGF); Matrix metalloproteinases (MMPs) (Bronckaers et al. 2014).

Interestingly, clinical studies have demonstrated that MSCs have the same angiogenesis property. Kim et al. demonstrated in a preliminary clinical trial that the implantation of human cord blood-derived MSC enhanced angiogenesis and collateral vessel formation in human cases with Buerger's disease (Kim et al. 2006). Kamihata and colleagues have reported that bone marrow mononuclear cells that survived engrafting can synthesize angiogenic factors such as VEGF, bFGF, and angiopoietin-1 to induce angiogenesis in the ischemic myocardium (Kamihata et al. 2001).

An important role in MSC-mediated protection is the inhibition of tissue apoptosis and augmentation of tissue turnover. Studies show that MSCs are capable of inhibiting apoptosis in kidney, liver and brain injuries (Nascimento et al. 2014; Yin et al. 2014). Recent evidence shows that this is done through increasing pro-survival factors such as Akt expression in injured cells (Morigi et al. 2004), Brain-derived Neurotrophic Factor (BDNF) and growth factors such as Insulin-like Growth Factor (IGF)-1, VEGF and Hepatocyte Growth Factor (HGF) expression which inhibit apoptosis and stimulate cell proliferation (Imberti et al. 2007). Together, this dynamic

permits high cell turn-over, renewing damaged cells and decreasing excessive cell death, thus restoring normal tissue physiology.

MSC mediate tissue repair through paracrine mechanisms. Besides mediating directly in the inflammatory process, some studies have suggested that MSCs also possess anti-oxidative characteristics. MSCs have been observed to produce a number of anti-oxidative mediators such as IGF, Platelet-derived Growth Factor (PDGF), superoxide dismutase (SOD), HGF and IL-6 (Shi et al. 2010; Kevin Kemp et al. 2010). MSC-conditioned media has also been seen to contain these anti-oxidative factors including Granulocyte-Colony stimulating Factor (G-CSF), Granulocyte-macrophage (GM)-CSF and Interleukin (IL)-12, as reviewed by Kim et al (W.-S. Kim, Park, and Sung 2009). In addition, evidence shows that MSCs and the conditioned media respectively decrease oxidative damage in culture when fibroblast cells are exposed to oxidative damage-inducing environments such as UVB (W.-S. Kim et al. 2009) or tert-butyl hydroperoxide (tbOOH) (W.-S. Kim et al. 2008).

The paracrine action of MSCs is now well accepted. In this sense, the administration of conditioned medium of stem cells in an animal model of kidney injury has been shown to improve in clinical parameter outcomes correlated with decreased apoptosis and ameliorated histological parameters. Despite the bioactive molecules secreted by MSCs, Camussi's group has described that there are also some microvesicles (MVs) inside the conditioned medium leading to an amelioration of acute and chronic experimental models of renal injury, accelerating regeneration of hepatectomized rats and activating endothelial cells (Gatti et al. 2011; Bruno et al. 2009; Bruno et al. 2009)

These MVs are circular membrane fragments that shed from the cell surface membrane carrying protein and lipids from the membranes of the cells from which they originate. Besides this, MVs may also carry mRNA and microRNA establishing a communication between one cell and another (Ratajczak et al. 2006). The shedding of MVs is a physiologic process, however some stress conditions lead to an increase in the number of MVs shed (Hugel et al. 2005).

As _previously _mentioned, _epigenetics _denotes _heritable _changes _in_gene expression _at _the _level _of _chromatin _without _changing _the highly condensed, transcriptionally inactive heterochromatin, or the less condensed, transcriptionally

active euchromatin. The dynamic balance between these 2 forms is regulated by several epigenetic mechanisms such as DNA methylation, histone modifications, microRNA and chromatin remodeling (Han and Yoon, 2012).

1.3 EPIGENETICS MECHANISMS IN GENE REGULATION

Epigenetics refers to the set of heritable changes involving the expression of genes without changes in the DNA sequence. It is known to regulate gene expression at the chromatin level as several generations of eukaryotic cells undergo mitotic and meiotic divisions (Im and Shin, 2015). The interactions between genes an associated transcriptional factors and modulators are usually modulated by DNA methylation, histone modifications, microRNAs and chromati remodeling (Eslaminejad et al., 2013). These changes regulate gene expression by modifying chromatin or nuclear architecture (Huang et al., 2015). Furthermore, epigenetics factors have a role in determining the fate of stem cells, their commitment and differentiation. In the past few years, most research investigated the effects of epigenetics on embryonic pluripotent stem cells, and it was found that epigenetic changes drive these cells to commit to a particular lineage by repressing genes associated with differentiation to alternative lineages (Herlofsen et al., 2013). On the other hand, mesenchymal stem cells (MSCs) are largely studied adult stem cells due to their promising role in experimental biology and regenerative medicine (Kobolak et al., 2016). The Figure 1-1-3 summarizes the most important mechanisms described below.

1.3.1 DNA methylation

DNA methylation refers to the addition of a methyl group preferentially at CpG islands leading to a repression of transcription (Beerman and Rossi, 2015, Fernandez-Tajes et al., 2014). Adding methyl groups is achieved by a specific class of enzymes, the DNA methyltransferase (Dnmt) such as Dnmt3b (Fasolino and Zhou, 2017). Regions dense in CpG islands are found near promoters of many human genes. In general, promoter DNA methylation is associated with repression of the corresponding gene. However, this association is not always straightforward. Genes associated with methylation-free CpG islands often remain silent while genes that correspond to methylated promoters occasionally undergo transcription (Teven et

al., 2011). The importance of DNA methylation has been widely validated as any defect can be associated with diseases involving embryogenesis and tumor formation.

1.3.2 Histone modifications

Histone modifications include post-translational acetylation, methylation, ubiquitinilation, phosphorylation, sumoylation at the N-terminal tail of histones (Moran-Salvador and Mann, 2017). Different groups lead to different effects on DNA transcription; for example, acetylation of lysine residues by histones acetyltransferases relaxes DNA coiling allowing for transcription, while removing the acetyl groups by histone deacetylases (HDACs) such as HDAC1, mediates gene silencing (Dokmanovic et al., 2007). On the other hand, histone methylation by adding up to 3 methyl groups at lysine and arginine residues within histones can either activate or repress transcription depending on the location of the methylation (Kouzarides, 2007, Tammen et al., 2013, Suganuma and Workman, 2011).

1.3.3 microRNA (miRs)

miRs are short (∼20 nucleotides) non-coding RNAs implicated in post transcriptional regulation of gene expression. MiRs pair on target messenger RNA (mRNA) via complete complementarity causing mRNA degradation, or partial complementarity resulting in the regulation of mRNA expression. MiRs were found to regulate other epigenetic mechanisms such as DNA methylation and histone deacetylation (Sato et al., 2011).

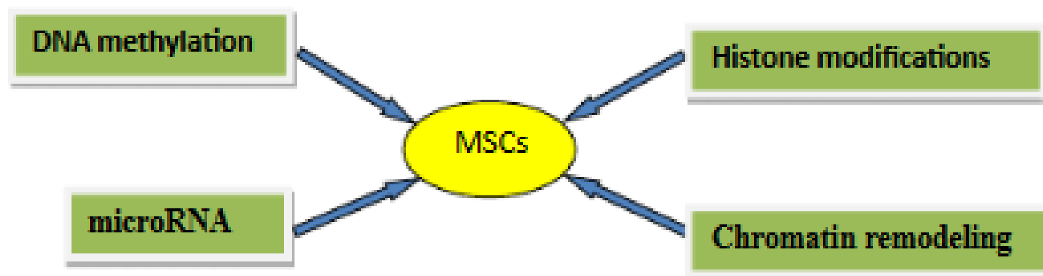
1.3.4 Chromatin remodeling

Chromatin remodeling proteins modulate rearrangements of chromatin structure increasing accessibility to DNA. The SNF2 family of chromatin remodeling proteins acts in many cellular processes such as gene expression, replication, DNA repair, and recombination. ATP is used to alter the structure of chromatin via disruption of the histone/DNA interaction. In the context of gene expression, SNF2 family members are thought to mediate transcriptional activation as well as repression depending on the SNF2 factor, and the proteins it interacts with (Geiman and Robertson, 2002). Other major chromatin remodeling proteins include the PcG repressive complex

which is known to affect stem cell differentiation (Di Croce and Helin, 2013).

1.3.5 MSCs differentiation and epigenetics

Since MSCs are emerging as a promising therapeutic tool in the management of several diseases, understanding their biology, including epigenetics interactions, in relation to their differentiation pathways is essential to maximize their usefulness and benefit. Several studies highlighted a role for epigenetics in determining MSCs senescence. The inhibition of HDAC function was found to promote apoptosis and senescence in human MSCs through the upregulation of several cyclin kinase inhibitors (Di Bernardo et al., 2009). Similarly, inhibition of DNMTs with 5-azacytidine induces the cellular senescence of human umbilical cord blood-derived multipotent



stem cells (So et al., 2011).

Figure 1-1-3 Major epigenetic factors regulating mesenchymal stem cells differentiation

1.3.6 Endogenous retroviruses

In addition to the described mechanism above, also the transcriptional and epigenetic regulation of Endogenous retroviruses (ERVs) play an important role in cell-fate commitment and establishment. ERVs are remains of past retroviral infections , are retrovirus-like elements with long terminal repeats and are widely dispersed in the euchromatic compartment in mammalian cells, comprising ~10% of the mouse genome(Mouse Genome Sequencing Consortium et al. 2002). Host organisms have accumulated these sequences into their genomes during the course of time. The expression of ERVs is repressed in pluripotent stem cells (Friedli et al. 2014; Niwa et al. 1983). Methylation of DNA restricts the ERV expression in differentiated cell type while de novo methylation of ERVs mediate ERV repression in pluripotent stem cells (Gaudet et al. 2004).

1.3.6.1 *ERV LTRs as regulatory of cellular genes*

More than ago 50 years ago, Barbara McClintock first discovered transposable elements in maize, which she called “controlling elements” because they altered gene expression (McClintock 1956). We now appreciate that many ERVs similarly affect cellular gene expression by contributing to the activities of nearby promoters and enhancers. The accumulating retroviral infections have induced a considerable impact on the evolution of the genome rewiring the major genetic networks in both mouse and human stem cells. This consideration derived from the many examples of ERV integration events that do not cause harmful mutations but provide additional regulatory sequences that change the regulation of genes and genetic networks in useful ways. Some of the known ways that ERVs perform useful functions are represented in the Figure 1-1-4

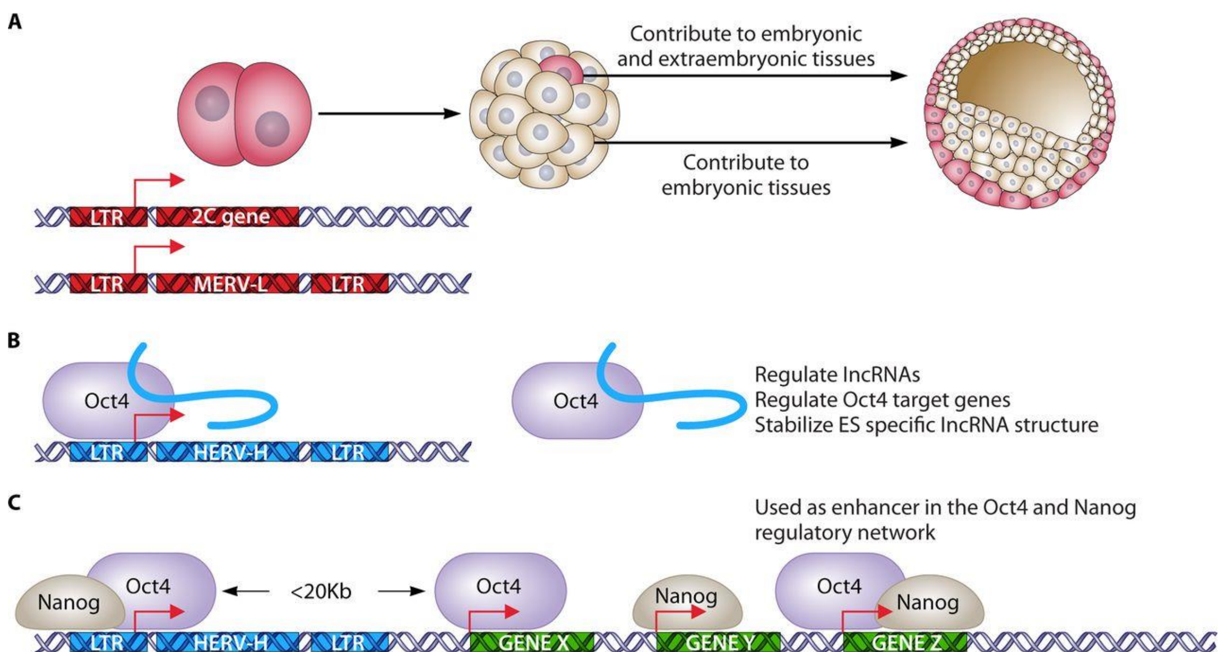


Figure 1-1-4 Examples of domestication of ERV sequences by mouse and human embryonic cells[scheme .from (Schlesinger e Goff 2015)] (A) MERV-L elements and their remnant “solo” long terminal repeats (LTRs) have been coopted to participate in gene regulatory networks by serving as primary or alternative promoters of nearby genes. A subset of mouse embryonic stem cells expresses MERV-L LTR-driven chimeric transcripts, which correlates with increased potency. (B) HERV-H interacts with Oct4 to promote the enhancer activities of LTR7 and nearby regions and to drive the expression of neighboring lncRNAs and protein-coding genes essential to hES cell identity. (C) ERV1 elements in the human and mouse genomes carry transcription factor-binding sites for Oct4 and Nanog, which can regulate genes that form the pluripotency network near insertion sites, leading to novel regulatory patterns in evolving mammals.

ERV promoters can drive the expression of tissue-specific genes, can express long

noncoding RNAs (lncRNAs) that autoregulate and transregulate cellular genes, and provide enhancer elements that are used by cellular genes. ERV sequences have been identified as part of many functional promoters in mouse and human, often functioning as alternative, tissue-specific promoters in addition to the ancestral promoters (Faulkner et al. 2009; Jern e Coffin 2008) Their contribution to the overall transcription may sometimes be small but the number of genes affected may be very large. In a comprehensive survey of the repetitive element transcriptome, up to 30% of 5'cap-selected mouse and human RNA transcripts were found to initiate within repetitive elements (Faulkner et al. 2009). More than 25% of coding genes have ERV elements in their 3' untranslated region (3'UTR), which negatively regulate their expressionan many of the ERV-initiated transcripts show high tissue specificity (Carninci et al. 2005). Thirty percent of all transcripts in human embryonic tissues were associated with repetitive elements, pointing to a clear pattern of embryonic cell specificity for some viral promoters (Fort et al. 2014).

1.3.6.2 *ERV regulatory network in pluripotent stem cells*

ERVs may play their most significant role in embryonic cells. The pluripotency of ES cells tracks closely with the expression levels of these elements. The regulators of ERV expression, such as Trim28, are also key players in development. The ERV regulatory elements are not only correlates of host gene elements but also used as host gene regulatory elements [from the review (Schlesinger e Goff 2015)]. Numerous studies reported that Kruppel associated box-Zinc Finger Proteins (KRAB-ZFPs) recognize specific DNA sequences for recruitment of TRIM28 which in association with the histone modification complexes catalyse the addition of repressive marks on the ERVs. Among them, ESET, officially named SETDB1, plays a critical role mediating repressive deposition of H3K9me3 on the ERVs (Matsui et al. 2010). SUV39H1 and SUV39H2 are the other factors which also effect the H3K9me3 modification on the intact ERVs and LINE transposons . Other factors such as YY1, KDM1A, NuRD complex, HP1, DNMT 3A/B are among the active participants of the ZFP-TRIM28-ESET axis. In most cases, they help to stabilize the complex or facilitate the recruitment of other key factors (Gautam, Yu, e Loh 2017). A regulatory network for all classes of ERVs is represented in Figure 1-1-5

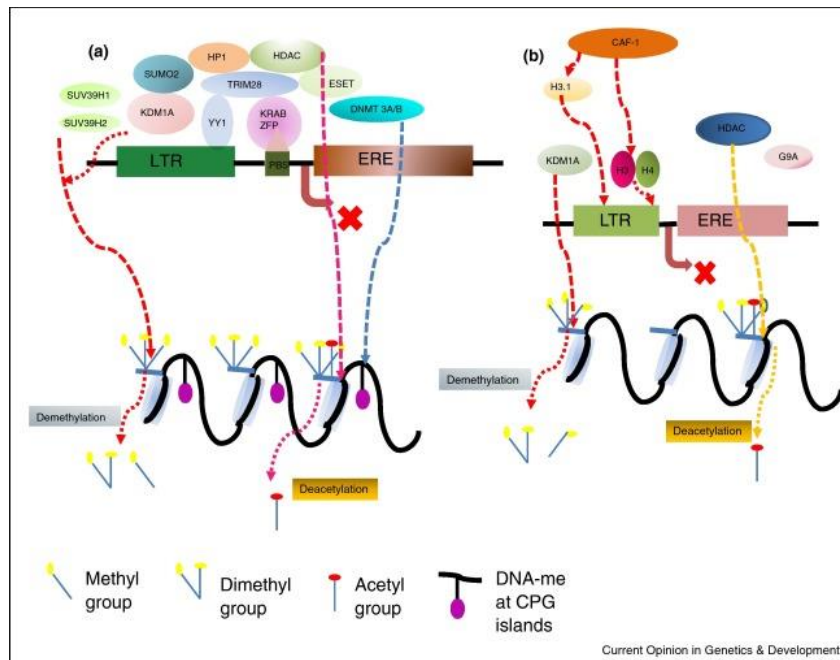


Figure 1-1-5 Regulation of ERVs (Gautam, Yu, e Loh 2017) (a) The regulatory network of class I/II ERVs. PBS sequences are recognized by KRAB-ZFP proteins which in turn recruit Trim28 and Eset . TRIM28 catalyzes the recruitment of demethylases like KDM1A, SUV39H1 and SUV39H2. HP1 reads H3K9 methylation marks on the chromatin and is closely related with Kdm1a . YY1 also interacts with TRIM28 to induce retroviral silencing. SUMO2 plays a role in retroviral silencing by sumoylation of TRIM28. The epigenetic marks deposited by TRIM28 may be used by Dnmt 3a/3b to methylate DNA at CpG islands . (b) The regulatory network of class III ERVs. CAF-1A is present on many ERVs which are independent of TRIM28-ESET-SUMO2. In such ERVs, CAF-1A interacts with KDM1A and HDAC . G9A facilitates the silencing of ERVs in TRIM28 independent manner . CAF-1A is known to deposit H3/H4 histones during DNA synthesis . Most likely H3.1 is deposited by CAF-1A onto newly formed chromatin of the ERVs to maintain the repressive state.

1.3.6.3 *ERVs and the maintenance of stemness*

Pluripotent stem cells engage the help of ERVs to reinforce stemness (Gifford, Pfaff, e Macfarlan 2013). Exemplifying this is a small subset (~1%) of mouse embryonic stem cells (ESCs) exhibiting high levels of murine ERV-L (MERVL) and a reciprocal low level of Oct4 expression. These cells resemble the transcription profile of embryos at 2-cell stage and could contribute to extraembryonic lineages in chimeric embryos, suggesting that they have retained totipotency. Remarkably, the LTRs of these MERVL act as alternative promoters for many 2-cell stage-specific genes. Collectively, the MERVL expression seems to be predominantly regulated by the histone demethylase KDM1A (Macfarlan et al. 2012; Peaston et al. 2004).

Human ESCs, also express abundant expression of H (HERVH) (Santoni, Guerra, e

Luban 2012a). Although human ESCs have been derived from preimplantation embryos, they are transcriptionally similar to cells derived from murine post implantation embryos commonly known to be in primed pluripotent state.. Wang et al. studied the functional association of HERVH expression with pluripotency and found that the element drives hESC-specific transcripts. It acts as the binding site for naive pluripotency transcription factors like LBP9 and NANOG. Surprisingly, selecting hESCs expressing high level of HERVH helps in deriving naive-like stem cells and knockdown of HERVH transcripts compromises self-renewal (J. Wang et al. 2014a). Consistent with this finding, Lu et al. down-regulated the subfamily LTR7 and observed a dramatic change in morphology to a differentiation phenotype. These ERV transcripts function as long non-coding RNAs which could interact with pluripotency factor Oct4 and assist it in maintaining pluripotency circuitry (Lu et al. 2014)

1.3.6.4 *The dynamics of ERVs during embryonic development and human somatic cell reprogramming*

The expression of ERVs during embryonic development is finely controlled and many of them exhibit a stage specific expression pattern or are associated with lineage specification(Rowe et al. 2013; Walsh, Chaillet, e Bestor 1998). Using bioinformatics approaches, Goke et al. analyzed the published RNA-seq data from different developmental stages Their study found many ERVs whose expression are tightly correlated with various stages (Göke et al. 2015a) as reported in the Figure 1-1-6. Consistently, ERV levels at each stage are accompanied by global changes in epigenetic landscapes. Using ATAC-seq in different stages of preimplantation embryos Wu et al. found out that retroelements like B1, B2 and B3 are enriched in 2-celled stages. They showed that chromatin regions harboring retroelements are more accessible in early stages of development and overlap with many cis regulatory elements . Large-scale DNA demethylation initiates during early preimplantation embryos. As such, H3K9me3 histone modifications take over the role of repressing the endogenous retroelements . Subsequently, DNA methylation is re-established during differentiation with the help from the H3K9me3 marks . These transitions of epigenetic features have drawn much attention because of their highly complex regulation and tight relationship with cell-fate determination and genomic stability

[from the review(Gautam, Yu, e Loh 2017)] .

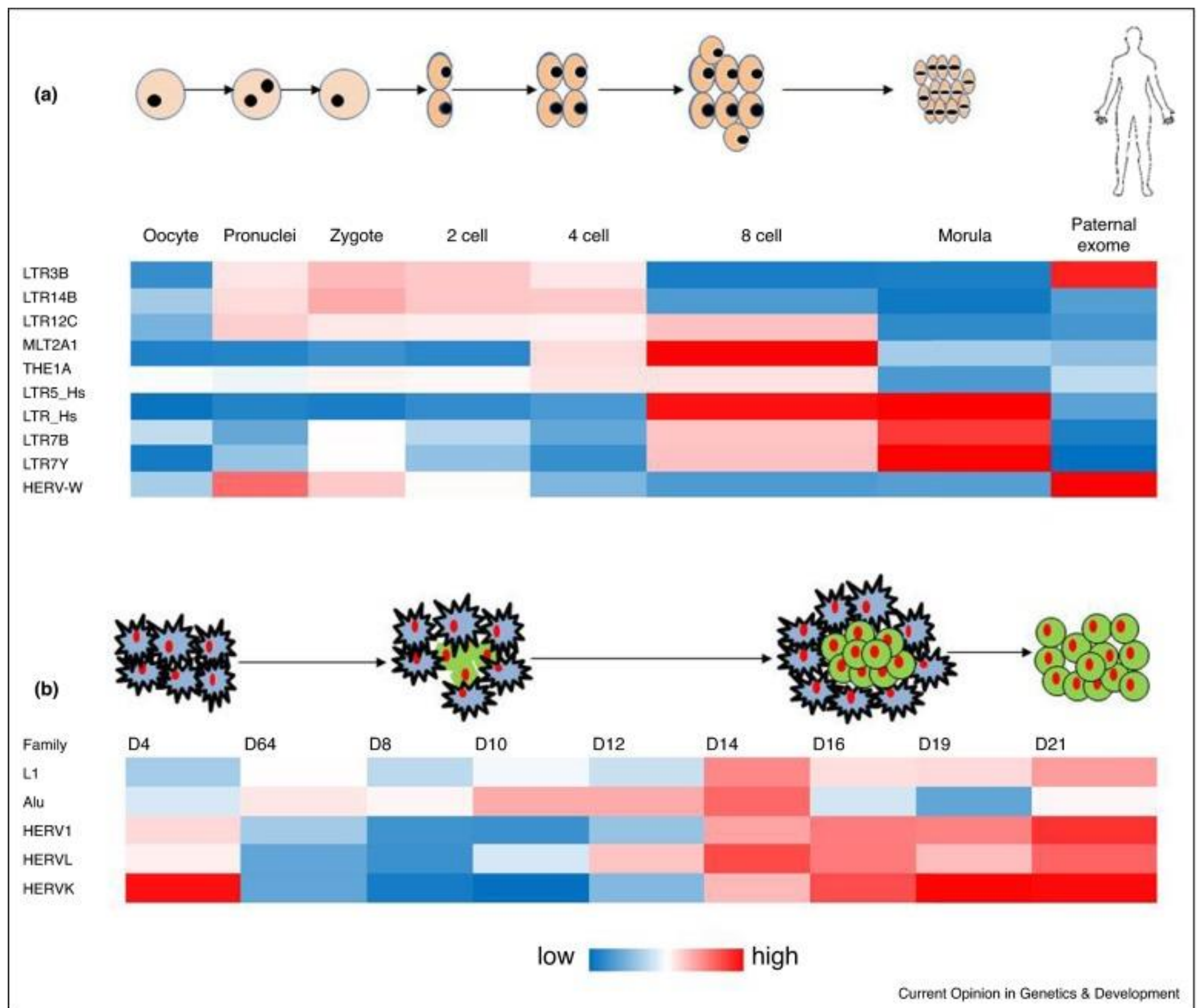


*Figure 1-1-6 Expression of ERVs during development and reprogramming. [from (Gautam, Yu, e Loh 2017)] (**a**) Relative expression of the indicated ERVs during human embryonic development. LTR33, 14 and 12C are highly enriched during the 2 cell-4 cell stage. Similarly, the high enrichment of MLT2A1 during 4-8cell stage, LTR5, 7B and 7Y during 8 cell-morula stages point to the fact that some ERVs are specifically expressed stage wise during different stages of human embryonic development. (**b**) Relative expression of ERV families during the course of human cell reprogramming. Almost all the ERV families were repressed in the early stages of reprogramming. By day 12, most of the ERVs started to show higher enrichment reaching their maximum by day 21.*

1.4 Sources of MSCs

In the adult organism, reservoirs of MSCs can be found in almost all tissues where MSCs contribute to the maintenance of organ integrity. The use of these different MSCs for cell-based therapies has been extensively studied over the past years, which highlights the use of MSCs as a promising option for the treatment of various diseases including autoimmune and cardiovascular disorders. However, the proportion of MSCs contained in primary isolates of adult tissue biopsies is rather low and, thus,

vigorous ex vivo expansion is needed especially for therapies that may require extensive and repetitive cell substitution. Therefore, more easily and accessible sources of MSCs are needed.

1.4.1 Bone marrow MSCs

Bone marrow derived stem cells first described by Friedenstein et al. are still the most frequently investigated cell type and often designated as the gold standard s as described in the paragraph 1.1. Although, MSCs are effectively isolated from almost every organ such as adipose tissue, cartilage, muscle, liver, blood, and blood vessels (Steens e Klein 2018). However, there are several limitations for large scale in vitro expansion of ex vivo isolated adult MSCs: a decline of their plasticity and in vivo potency over time was reported, as well as accumulated DNA abnormalities and replicative senescence (Mimeault e Batra 2009; Ho et al. 2012). Moreover, the number of BM-MSCs declines with increasing age (Rao e Mattson 2001; Katia Mareschi, Ferrero, et al. 2006). MSCs have been isolated from a variety of tissues, including adipose tissue, umbilical cord, Wharton's jelly, placenta, and amnion. These all represent promising sources of MSCs, as they are abundant and easily obtained by non invasive procedures.

Techniques are now available to isolate and grow mesenchymal progenitors and to manipulate their growth under defined in vitro culture conditions. As a result, MSCs can be rapidly expanded to numbers that are required for clinical application. This advance has allowed the clinical testing of culture-expanded MSCs in the context of HSCT (Maria Ester Bernardo e Fibbe 2015)

MSCs are present in postnatal bone marrow and also in the bone marrow of adults, and there is evidence that the frequency declines with age (Mimeault e Batra 2009; Mareschiet al. - 2006). Recently MSCs are been isolated from a variety of tissues, including adipose tissue, umbilical cord, Wharton's jelly, placenta and amnion. These all represent promising sources of MSCs, as they are abundant and easily obtained by non-invasive procedures.

1.4.2 MSCs isolated from amniotic fluid and placenta

Extra embryonic tissues such as placenta, cord blood, amniotic fluid, discarded following birth can represent rich sources of SCs that can be used in the future for

clinical therapeutic applications. The advantage of these sources of SCs is that there are no ethical or legal considerations associated with their collection and use. To date, MSCs fitting the criteria used for defining BM-MSCs (Table 1-1) represent the best characterized subpopulation of SCs isolated from these tissues.

Our previous studies have proven that multi-potent MSCs can be isolated from amniotic fluid (AF) (Mareschi et al. 2009b) and from placenta (PL) (data not published) with MSC characteristics as defined by the International Cellular Therapy Society (Dominici et al. 2006). Interestingly, MSCs derived from these sources show greater proliferative and differentiative potentials than BM-MSCs, which are most likely due to the early embryological origin of the AF and PL-MSCs compared to BM-MSCs. These data support the importance of investigating the AF and PL–MSC properties as an essential prerequisite to allow their clinical use in cell therapy protocols for regenerative medicine.

In the Figure 1-1-7 the major sources from adult and birth associated tissues of human MSCs are represented.

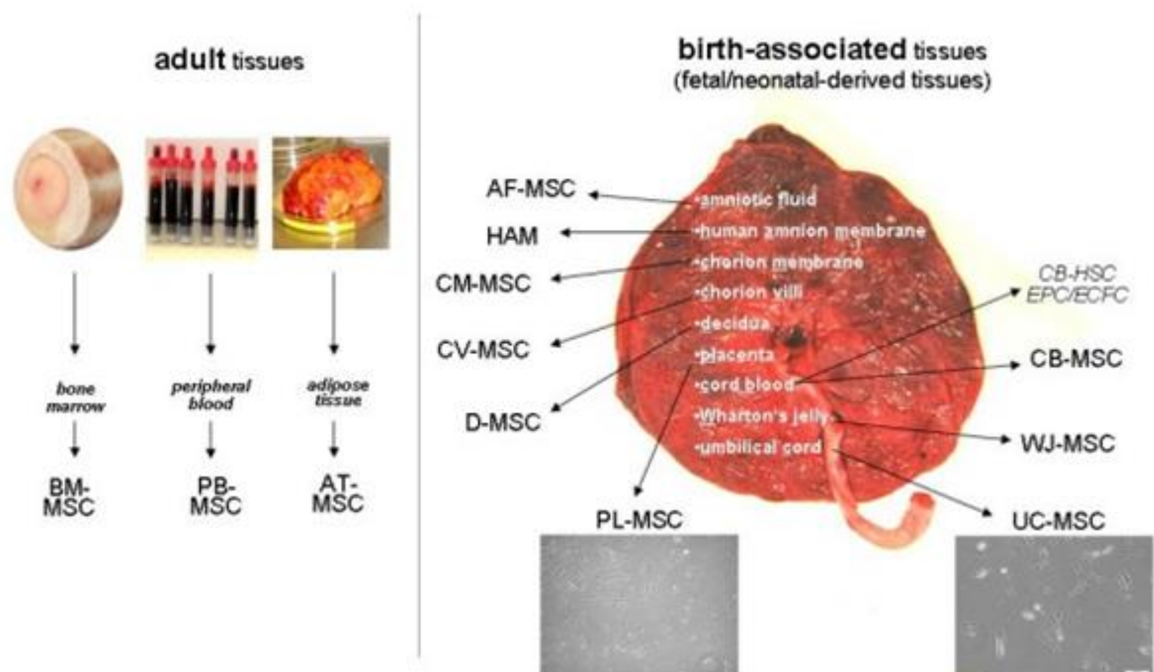


Figure 1-1-7. Major sources of MSCs [Modified from (Hass et al. 2011)]. The sources can be distinguished between adult tissues, preferably bone marrow (BM), peripheral blood (PB) and adipose tissue (AT) and neonatal birth-associated tissues including placenta (PL), umbilical cord (UC) and cord blood (CB). Besides cord blood-derived mesenchymal stem cells (CB-MSC) other stem/progenitor cell populations from cord blood also include hematopoietic stem cells CB-HSC and two endothelial populations such as endothelial progenitor cells (EPC) and endothelial colony-forming cells (ECFC).

1.4.2.1 *Placental Mesenchymal SCs (PL-MSCs)*

Numerous reports describing isolation and characterization procedures of SCs from different parts of the placenta have been published. Considering the complexity of the placenta, the region of origin and methods of isolation of cells derived from this tissue, a variable number of SCs have been described. During the first international Workshop held in Brescia, Italy in 2007 on Placenta Derived SCs (PL-MSCs), a group of researchers working in this research area, defined the minimal criteria to define PL-MSCs. These criteria are (Parolini et al. 2008):

- Adherence to plastic;
- Formation of fibroblast colony-forming units;
- A specific pattern of surface antigen expression (positivity for CD90,CD72, CD105 and negativity for CD45, CD34, CD14, HLA-DR);
- Differentiation potential toward one or more lineages, including osteogenic, adipogenic, chondrogenic, and vascular/endothelial;
- Foetal origin.

PL-MSCs can be isolated through various methods, which are generally based on the digestion of the placental pieces of interest with various concentrations and incubation times of different enzymes, including dispase, collagenase and DNase. In some cases, as for chorionic villi-derived MSCs, the isolation method may involve mechanical mincing of the chorionic tissue followed by trypsin digestion and seeding in a medium which allows colony formation and culture of fibroblast-like cells, although the explant culture method, whereby cells are outgrown from pieces of chorionic villi attached to dishes, has also been successfully applied.

PL-MSCs express several cell surface and intracellular markers typical of stem/progenitor cells, such as Stage Specific Embryonic Antigen (SSEA)-4, Tumour Rejection Antigen (TRA)1-60, TRA1-81, and octamer-binding protein (OCT)-4 (Caruso, Evangelista, and Parolini 2012).

For some placental cells, such as amniotic membrane-derived cells, the isolation protocol used may influence the levels and pattern of marker expression, and such expression patterns may also vary with passage numbers (Díaz-Prado et al. 2010). For instance, Murphy et al. have recently reported changes in the expression levels of a selection of cell surface markers in human amniotic epithelial cells (hAECs) between

P0 and P5 (Murphy and Atala 2013). Meanwhile, changes in marker expression have also been observed with varying gestational ages (Izumi et al. 2009). Interestingly, placental cells have also been reported to express several lineage-associated genes, suggesting that they could act as progenitors and differentiate into various cell types. Further details regarding the expression of such genes in hAECs and human amniotic mesenchymal stromal cells (hAMSCs) can be found in Manuelpillai et al's review (2011). (Manuelpillai et al. 2011).

As for other MSCs, PL-MSCs are able to undergo differentiation toward the osteogenic, chondrogenic, and adipogenic lineages. Furthermore, they are also able to differentiate toward several other lineages, including neurogenic, cardiomyogenic, myogenic, angiogenic; and pancreatic lineages (Caruso, Evangelista, and Parolini 2012). A recent study has also shown that Wharton's jelly-derived MSCs are able to undergo in vitro differentiation into germ-like cells (P. Huang et al. 2010).

In addition, because the placenta synthesizes various hormones, enzymes, neurotransmitters, and cytokines (Mortimer et al. 2012; Sahraravand et al. 2011), PL-MSCs may secrete active factors that make them attractive as a potential clinical therapy for inflammatory diseases.

1.4.2.2 *Amniotic Fluid Multipotent SCs (AF-MSCs)*

Amniotic Fluid Multipotent Stem Cells (AF-MSCs) can be obtained from a small amount of fluid during amniocentesis at the second trimester. Amniotic fluid can also be collected at term from routine caesarean deliveries. Kaviani et al. reported that just 2 mL of amniotic fluid contains up to 20,000 cells, 80% of which are viable (Kaviani et al. 2001). A highly multipotent subpopulation of AFSCs in the amniotic fluid and placenta can be isolated through positive selection for cells expressing the membrane receptor c-kit (CD117) (De Coppi, Bartsch, and Siddiqui 2007). C-kit is expressed on a variety of SCs including embryonic SCs (ESCs), primordial germ cells, and a number of somatic SCs. AF-MSCs exhibit typical mesenchymal marker expression, such as CD90, CD73, CD105, CD29, CD166, CD49e, CD58, and CD44, determined by flow cytometry analyses (Murphy and Atala 2013; Galende, Karakikes, and Edelmann 2010; In 't Anker, Scherjon, and Kleijburg-van der Keur 2003). Additionally, these cells expressed HLA-ABC antigens, whereas the expression of the hematopoietic markers CD34 and

CD45, the endothelial marker CD31, and the HLA-DR antigen were undetected. More importantly, the majority of cultured AF-MSCs expressed pluripotency markers such as the octamer binding protein 3/4 (Oct-3/4), the homebox transcription factor Nanog (Nanog), and the stage-specific embryonic antigen 4 (SSEA-4) (Murphy and Atala 2013).

AF-MSCs show a high self-renewal capacity and maintain a normal karyotype at late passages and display normal G1 and G2 cell cycle checkpoints. They also conserve a long telomere length in late passages due to continued telomerase activity. Like ESCs, AF-MSCs form embryoid bodies in vitro that stain positive for markers of all three germ layers. However, unlike embryonic SCs, when implanted into immunodeficient mice in vivo, AF-MSCs do not form teratomas, an essential safety characteristic for potential cell therapy. AF-MSCs have a high clonal capacity demonstrated using a technique involving retrovirally tagged cells. In this assay, a tagged single cell gave rise to a population that differentiated along six distinct lineages from all three germ layers: adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic (De Coppi, Bartsch, and Siddiqui 2007; Abdulrazzak, De Coppi, and Guillot 2013)

In the Stem Cell and Cellular Laboratory at the Regina Margherita Children's Hospital multipotent SCs were isolated from amniotic fluid but only on samples with a volume over 6 ml and it was observed that AF is an important multipotent stem cell source with a high proliferative potential able to originate potential precursors of functional neurons.(Mareschi et al. 2009)

1.5 IMMUNOMODULATION

Considering the inflammatory nature of most injuries, studies have indicated that the predominant role of MSCs in resolving tissue damage relies on toning down inflammation in specific sites of injury. The possible mechanisms of immunomodulation are described below and illustrated in Figure 1-1-8.

1.5.1 Immunomodulatory effects of MSC on innate immunity

Dendritic cells (DCs) have the elementary role of antigen presentation to naïve T cells upon maturation, which in turn induce the proinflammatory cytokines. Immature DC acquire the expression of co-stimulatory molecules and upregulate expression of

MHC-I and II, as well as, other cell-surface markers (CD11c and CD83). When mature DC are incubated with MSCs they have a decreased cell-surface expression of MHC class II molecules, CD11c, CD83 and co-stimulatory molecules, as well as, decreased IL-12 production, thereby impairing the antigen-presenting function of the DC (Zhang et al. 2004; Maccario et al. 2005). MSC can also decrease the pro-inflammatory potential of DC by inhibiting their production of TNF-α. Furthermore, plasmacytoid DC (pDCs), which are specialized cells for the production of high levels of type-I IFN in response to microbial stimuli, upregulate production of the anti-inflammatory cytokine IL-10 after incubation with MSC (Aggarwal e Pittenger 2005). These observations indicate a potent anti-inflammatory and immunoregulatory effects for MSCs *in vitro* and potentially *in vivo*.

Natural killer (NK) cells are key effector cells of the innate immunity in anti-viral and anti-tumor immune responses through their Granzyme B mediated cytotoxicity and the production of pro-inflammatory cytokines. NK-mediated target cell lysis results from an antigen-ligand interaction realized by activating NK-cell receptors, and associated with reduced or absent MHC-I expression by the target cell. MSCs can inhibit the cytotoxic activity of resting NK cells by down-regulating expression of NKp30 and natural-killer group 2, member D (NKG2D), which are activating receptors involved in NK-cell activation and target-cell killing (Spaggiari et al. 2006). Resting NK cells proliferate and acquire strong cytotoxic activity when cultured with IL-2 or IL-15, but when incubated with MSCs in the presence of these cytokines, resting NK-cell, as well as, pre-activated NK cell proliferation and IFN-γ production are almost completely abrogated (Spaggiari et al. 2008). It is worth noting that although the susceptibility of NK cells to MSCs mediated inhibition is potent, the pre-activated NK cells showed more resistance to the immunosuppressive effect of MSCs compared to resting NK cells. Autologous and allogeneic MSCs were susceptible to lysis by NK cells, where NK cell-mediated lysis was inversely correlated with the expression of HLA class I on MSCs. Incubation of MSCs with IFN-γ partially protected them from NK-cell-mediated cytotoxicity, through the up-regulation of expression of MHC-I molecules on MSCs (Spaggiari et al. 2006; Spaggiari et al. 2008). Taken together, a possible dynamic interaction between NK cells and MSCs *in vivo* exists, where the latter partially inhibit activated MSCs, without compromising their ability to kill MSCs,

reflecting on an interaction tightly regulated by IFN-γ concentration.

Neutrophils play a major role in innate immunity during the course of bacterial infections, where they are activated to kill foreign infectious agents and accordingly undergo a respiratory burst. MSCs have been shown to dampen the respiratory burst and to delay the spontaneous apoptosis of resting and activated neutrophils through an IL-6-dependent mechanism. MSCs had no effect on neutrophil phagocytosis, expression of adhesion molecules, and chemotaxis in response to IL-8, f-MLP, or C5a (Raffaghello et al. 2008). Stimulation with bacterial endotoxin induces chemokine receptor expression and mobility of MSCs, which secrete large amounts of inflammatory cytokines and recruit neutrophils in an IL-8 and Macrophage Migration inhibitory Factor (MIF)-dependent manner. Recruited and activated neutrophils showed a prolonged lifespan, an increased expression of inflammatory chemokines, and an enhanced responsiveness toward subsequent challenge with Lipopolysaccharide (LPS), which suggest a role for MSCs in the early phases of pathogen challenge, when classical immune cells have not been recruited yet. Furthermore, MSCs have shown the capability to mediate the preservation of resting neutrophils, a phenomenon that might be important in those anatomical sites, where large numbers of mature and functional neutrophils are stored, such as the BM and lungs (Brandau et al. 2010).

1.5.2 Immunomodulatory effects of MSCs on adaptive immunity

T-cells are major players of the adaptive immune response. After T-cell receptor (TCR) engagement, T cells proliferate and undergo numerous effector functions, including cytokine release and, in the case of CD8+ T cells (CTL), cytotoxicity. Abundant reports have shown that T-cell proliferation stimulated with polyclonal mitogens, allogeneic cells or specific antigen is inhibited by MSC (Burr, Dazzi, e Garden 2013). The observation that MSC can reduce T cell proliferation *in vitro* is mirrored by the *in vivo* finding through infusions of MSCs that control Graft versus Host disease (GVHD) following BM transplantation. Nevertheless, there is no demonstrable correlation between the measured effects of MSCs *in vitro* and their counter effect *in vivo* due to the lack of universality of methodology correlating the *in vitro* findings with the *in vivo* therapeutic potential. MSC inhibition of T-cell proliferation is not MHC

restricted, since it can be mediated by both autologous and allogeneic MSC and depends on the arrest of T-cells in the G0/G1 phase of the cell cycle . *In vitro*, MSC inhibit phytohemagglutinin (PHA) mitogen-induced and mixed lymphocyte reaction allo-antigen driven T cell proliferation. This is done by both cell-to-cell contact and humoral factors in specific, secreting IDO and Galectin-1. These SC also decrease TNF-α and IFN-γ T cell production and raise IL-10 secretion, possibly mediated by IDO, PGE2 and B7-H amongst other molecules (Cuerquis et al. 2014; Manochantr et al. 2013). MSC also induce Th2 type lymphocyte and T regulatory cell differentiation through, HLA-G5 and other molecules (Bai et al. 2009).

Regulatory T cells (Treg), a subpopulation of T cells, are vital to keep the immune system in check, help avoid immune-mediated pathology and contain unrestricted expansion of effector T-cell populations, which results in maintaining homeostasis and tolerance to self antigens. Treg are currently identified by co-expression of CD4 and CD25, expression of the transcription factor FoxP3, production of regulatory cytokines IL-10 and Transforming Growth Factor (TGF)-β, and ability to suppress proliferation of activated CD4+CD25+ T cells in co-culture experiments. MSC have been reported to induce the production of IL-10 by pDCs, which, in turn, trigger the generation of regulatory T cells (Liren Li et al. 2013; Luz-Crawford et al. 2013). In addition, after co-culture with antigen-specific T-cells, MSC can directly induce the proliferation of regulatory T-cells through release of the immunomodulatory HLA-G isoform HLA-G5 (Selmani et al. 2008). Taken together, MSC can modulate the intensity of an immune response by inhibiting antigen-specific T-cell proliferation and cytotoxicity and promoting the generation of regulatory T-cells.

Antibody producing B-cells constitute the second main cell type involved in adaptive immunity. Interactions between MSCs and B-cells have produced controversial results attributable to the inconsistent experimental conditions used. It have shown that B lymphocytes have decreased proliferation and antibody production when cultivated with MSCs regulated allogenic DC (Y. Huang et al. 2010). In addition, other studies suggest that MSCs directly inhibit B lymphocyte plasma cell differentiation through humoral factors. On the other hand, other studies indicate that MSCs induce B-cell proliferation and differentiation into plasmocytes, when stimulated with a Toll-like receptor agonist (Traggiai et al. 2008; Corcione et al. 2006). These different

results are probably due to the different stimuli used and its detailed effect on B cell outcome is still to be disovered.

1.5.3 MSC escape the immune system in vitro

Studies have shown that MSC escape the immune system, and this makes them a potential therapeutic tool for various transplantation procedures. MSCs express intermediate levels of HLA major histocompatibility complex (MHC) class I molecules while they do not express HLA class II antigens of the cell surface. However, HLA class II is readily detectable by Western blot on whole-cell lysates of unstimulated adult MSCs, thus suggesting that MSCs contain intracellular deposits of HLA class II allo-antigens (Katarina Le Blanc et al. 2003). Cell-surface expression can be induced by treatment of the cells with IFN-γ for 1 or 2 days. Unlike adult MSCs, the foetal liver–derived cells have no intracellular nor cell surface HLA class II expression (Götherström et al. 2004), but incubation with IFN-γ initiated their intracellular expression followed by surface expression.

In vitro data support the theory that MSC escape the immune system. MSCs do not express FAS ligand or costimulatory molecules, such as B7-1, B7-2, CD40, or CD40L . When costimulation is inadequate, T-cell proliferation can be induced by the addition of exogenous costimulation. However, MSCs differ from other cell types, and no T-cell proliferation can be observed when they are cultured with HLA-mismatched lymphocytes in the presence of a CD28-stimulating antibody (Tse et al. 2003). MSCs mainly fail to activate T-cells and show to be targets for $CD8^+$ T cell-cytotoxicity, although controversial. Phyto-hemagglutinin (PHA) blasts, generated to react against a specific donor, will lyse chromium-labelled mononuclear cells from that individual but it will not lyse MSC derived from the same donor. Furthermore, killer cell inhibitory receptor (KIR ligand)–mismatched natural killer cells do not lyse MSC (K Le Blanc et al. 2004). Thus, MSCs, although incompatible at the MHC, tend to escape the immune system.

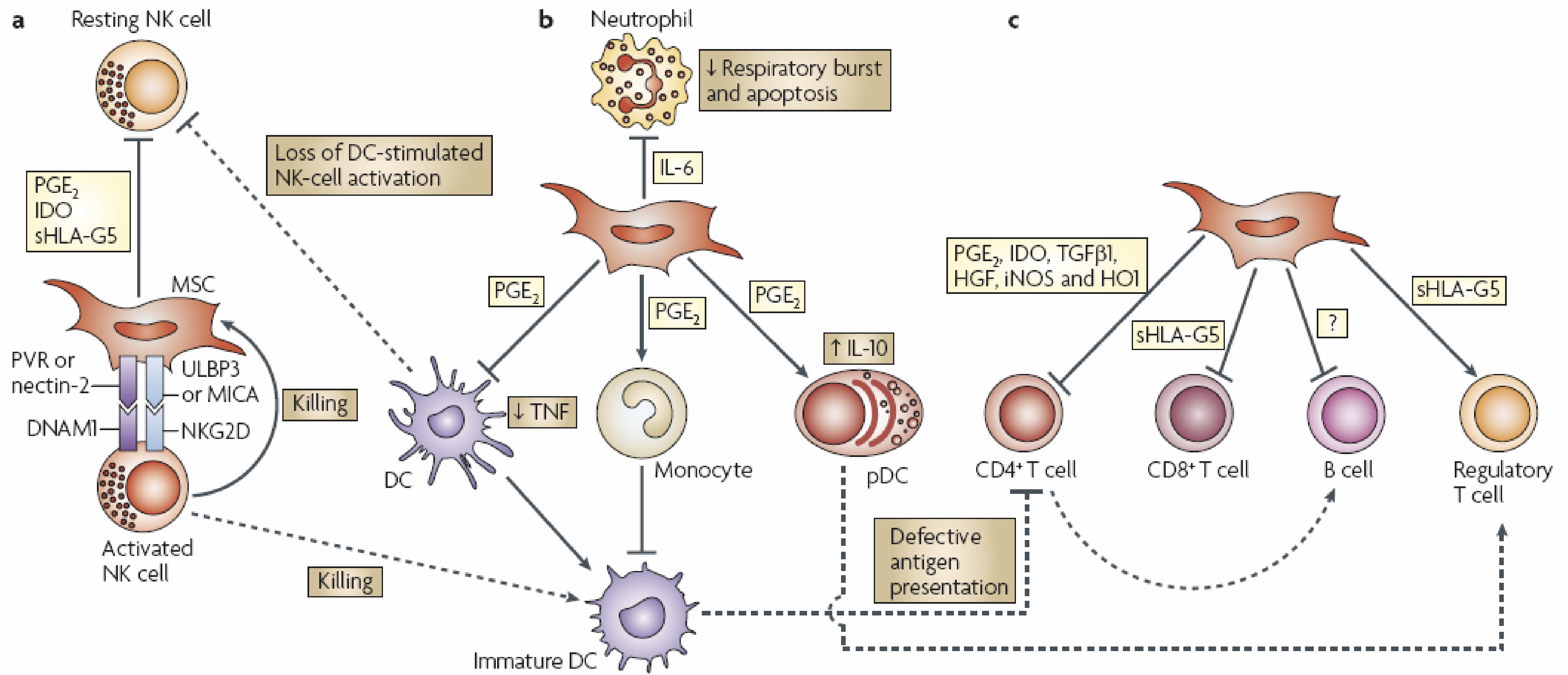


Figure 1-1-8 - Scheme of immunomodulant properties of BM-MSCs (Uccelli et. al, 2008).

1.5.4 T Cell Activation, Differentiation and T helper Subsets

T cell-mediated immunity is an adaptive process of developing antigen (Ag)-specific T lymphocytes to eliminate viral, bacterial, or parasitic infections or malignant cells. T cell-mediated immunity can also involve aberrant recognition of self-Ag, leading to autoimmune inflammatory diseases. The Ag specificity of T lymphocytes is based on recognition through the T cell receptor (TCR) of unique antigenic peptides presented by MHC molecules on Ag-presenting cells (APC). APC can take up Ag in peripheral tissues and migrate to secondary lymphoid tissues

CD4+ T helper (Th) cells are believed to play central roles in modulating immune responses. Based on the cytokine profiles and effector function, CD4+ T cells can be classified into T-helper 1 (Th1) cell, Th2 cell, Th17 cell, and CD4+CD25+ T regulatory (Treg) cell subsets . Th1 cells produce mainly interferon (IFN)-γ and promote the cell-mediated immunity , whereas Th2 cells suppress Th1-cell responses, and contribute to humoral immunity. A recent study has shown that not only Th1/Th2 imbalance but also Th17/Treg imbalance contributed to the pathogenesis of some autoimmune/inflammatory diseases, such as rheumatoid arthritis, acute coronary syndrome, and type 1 and type 2 diabetes. Th17 cells produce IL-17, TNF-α and IL-6, and induce inflammation in the pathogenesis of autoimmune diseases. Immunosuppressive Treg cells exert important effects on the maintenance of immune homeostasis and immune tolerance by producing anti-inflammatory cytokines, such as IL-10 and transforming growth factor-β (from review (Gagliani e Huber 2017) .These mechanisms are summerized in the Figure 1-1-9.

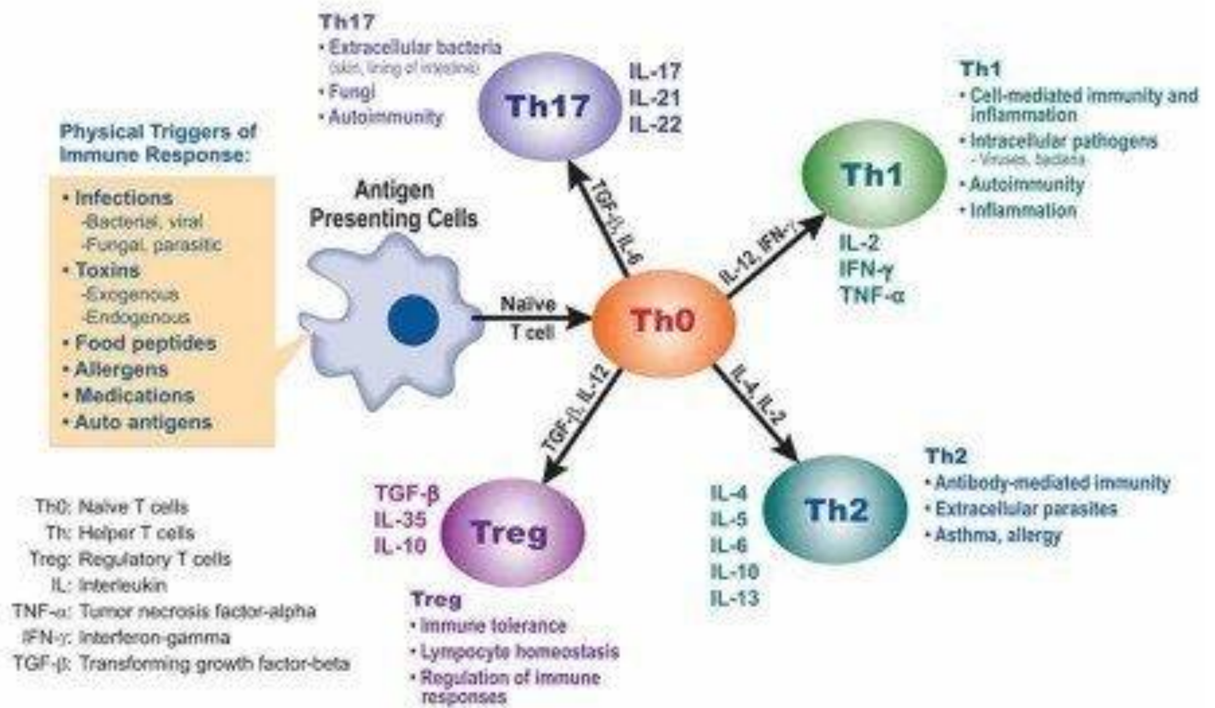


Figure 1-1-9 - Cytokine and molecular expression from different effector T subsets.[from («Are You Th1 or Th2 Dominant? Effects + Immune Response» 2014)

1.6 Mesenchymal stem and Allogeneic haemopoietic stem cells

Allogeneic hematopoietic stem cell transplantation (HSCT) is the treatment of choice for many malignant and non-malignant hematologic or immune disorders. (Sierra et al. 1997). Patients are prepared with high-dose chemoradiotherapy, followed by intravenous infusion of haemopoietic stem cells. Shortly after infusion, stem cells leave the circulation, home to extravascular spaces and repopulate the recipient's bone marrow. When all myeloid and lymphoid cells are of donor origin, this is taken as proof of engraftment and referred to as complete donor chimerism. Engraftment of stem cells depends on several factors such as:

i) the intensity of the preparative regimen; ii)the grafted cell dose; iii)the degree of histocompatibility between donor and recipient; iv) the T-cell content in the inoculum and v) the intensity of postgraft immunosuppression. Following intensive preparative regimens, such as cyclophosphamide and 1 total body irradiation, engraftment is the rule and this should lead to complete haemopoietic recovery. However, also poor haemopoietic function can be observed after HSCT. Some

patients may have slow or incomplete recovery of blood counts, while others may exhibit prompt recovery followed by decreasing counts between day +30 and day +180 after transplant. This can happen despite an intensive preparative regimen, an adequate cell dose and complete donor chimerism. Decreasing or low peripheral blood counts can be associated with events such as cytomegalovirus infections and/or graft-vs-host disease (GVHD). Maintenance of haematopoiesis depends on the self-renewal and multilineage differentiation capacity of HSCs that is thought to be regulated and controlled by the bone marrow micro-environment. The spatial organization of the stem cells in the marrow, mediated by the haematopoietic micro-environment and extracellular matrix, may be crucial for haematopoietic regeneration following HSCT [from review (Bacigalupo 2004)]

The bone marrow serves as a reservoir for different classes of stem cells. In addition to HSCs, the bone marrow comprises a population of marrow stromal cells or mesenchymal stem cells (MSCs).

1.7 MSC PRE-CLINICAL STUDIES

One important characteristic of human MSC is their ability to suppress inflammation resulting from injury, as well as, resulting from allogeneic solid organ transplants, and autoimmune disease. Consistent with in vitro studies, murine allogeneic MSCs are effective cellular therapy models in the treatment of murine models of human disease. Several studies have documented the substantial clinical improvements observed in animal models, when MSCs were systemically introduced as a therapy in mouse models of multiple sclerosis, inflammatory bowel disease, infarct, stroke, and other neurologic diseases, as well as diabetes (Uccelli, Laroni, and Freedman 2011; Tang et al. 2012; Das, Sundell, and Koka 2013). These findings strongly suggest that xenogeneic human MSCs are not immunologically recognized by various immunocompetent mouse models of disease and are able to home to sites of inflammation. However, the mechanisms behind the immunosuppressive actions at the site of inflammation and its association with the homing activity have not yet been completely elucidated. The possible mechanisms of action that leads to this improvement were described above and the main experimental models treated with MSCs are summarized in table 3.

	Experimental model	Cell(amount/way/time)	Mechanism of Action
Heart	Myocarcial infarct model	Swine MSC, $6x10^7$ cell/swine, in situ at infracted area, two weeks after artery occlusion	Engraftment at myocardium
CNS	Spinal cord injury	Human MSC, 1.5x105 cell/rat, injury in situ, at time of surgery	Immunomodulation and engraftment
	Parkinson model	Rat MSC, 1.106 cell/rat, intranasal, 3 days post surgery.	Immunomodulation, anti-apoptotic effect and engraftment
Liver	Liver Fibrosis (CCl4)	Rat MSC, 3x106 cell/rat, e.v., 42 days after CCl4 administration	Paracrine and anti-apoptotic effect
Lung	Acute Lung Injury (LPS)	Mouse MSC, $1x10^5$ cell/mouse, e.v., 2h after the first LPS inhalation	Immunomodulation
	Chronic Lung Injury (Bleomycin)	Mouse MSC, $2.5x10^5$ cell/mouse, e.v., 7 days after bleomycin administration.	Engraftment, immunomodulation
Pancreas	Diabetes Autoimmune (NOD animal)	Mouse MSC, $1x10^5$ cell/mouse, i.p. or e.v., NOD mice at 4 weeks old.	Immunomodulation

Table 3 - Some pre-clinical data on MSC therapy (Semedo et al. 2011)

1.7.1 Human trials with MSCs

Pre-clinical studies have stimulated human clinical trials. Le Blanc *et al*. were among the first to clinically administer allo-MSC to treat a none marrow transplanted boy from treatment-resistant, grade IV GvHD (Le Blanc et al. 2004) . This landmark case study provided an early glimpse of MSC' therapeutic potential. Just 8 months after the publication of Le Blanc *et al*.'s *Lancet* article, Osiris Therapeutics, an American company, began recruiting patients for the first large-scale clinical trials of allo-MSC for the treatment of acute GvHD and acute myocardial infarction

Today , numerous MSC cell preparations from academic and corporate institutions are being investigated in nearly 700 clinical trials (>80% of which are phase 1 or 2; 190 have reached their scheduled completion). Clinical trials examining the safety and efficacy of MSC have used both allogeneic and autologous cells.(www.clinicaltrials.gov at the date of 09/25/2018)

MSCs are typically manipulated by means of culture expansion, as they exist in limited quantities in situ; thus they require clinical trials to gain US Food and Drug Administration (FDA) approval and have only recently begun to reach the market. Clinical trials exploring MSC therapy have been driven predominately by companies with proprietary allogeneic MSCs (allo-MSCs) preparations. Importantly, allo-MSCs therapy has consistently been shown to be safe, enabling future trials to be conducted with improved trial design and using refined MSC-based approaches.

In 2011, FBC-Pharmicell's autologous MSC preparation, Hearticellgram-AMI, gained approval in South Korea, becoming the first culture-expanded MSC therapy to receive regulatory approval (Allison 2012). To date, one allogeneic culture-expanded MSC product has received regulatory approval: Osiris' (now Mesoblasts') Prochymal was approved in Canada in May of 2012 and shortly after in New Zealand for the treatment of steroid-refractory GVHD in children. Now, Prochymal is now available for adults and children in eight other countries including the United States for steroid refractory grade III and IV GVHD under an Expanded Access Program. Academic-investigator–driven studies without placebo controls have also shown a benefit with unmatched-allogeneic and haplo-identical MSCs in the prevention and treatment of acute GVHD. Whereas case studies and clinical studies of small groups of patients have suggested MSCs have significant clinical utility, demonstration of a beneficial effect from MSC in a large placebo-controlled trial has remained elusive (Le Blanc et al. 2008; Bernardo et al. 2011).

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2 AIMS AND OUTLINE OF THE PRESENT THESIS

In addition to stemness, MSCs have other intriguing features, which include supporting haematopoiesis, tissue remodelling/ repair properties, and angiogenesis. However, none of these properties is the main reason for the popularity of MSCs in current medicine, but it is their unique immunomodulatory property which makes them an attractive source for cell therapy.

Among several possible applications, the possibility to utilize MSCs in autoimmune, chronic inflammatory and degenerative diseases has led to notable therapeutic uses. These include bone and cartilage repair, cell types into which MSCs readily differentiate, and immune conditions such as GVHD and autoimmune conditions that utilize the MSCs' immune suppressive properties. Expectations for patient benefits are high in these therapeutic applications.

My expertise at the start of the PhD was based on good knowledge of isolation and expansion of MSCs from BM and from foetal origin tissues (cord blood, placenta and amniotic fluid), on their characterization and capacity to transdifferentiate in cells with non-mesenchymal fate and on their use in clinical Phase I studies expanded in GMP conditions. My PhD work has been inserted in a contest of yet started projects which different collaborations that have contributed to a drafting of 12 manuscripts (see Appendix) .

My PhD project focused on 3 major aims which I describe in this thesis:

1. to evaluate immunophenotype, differentiative potential, embryonic markers and immunomodulant properties of MSCs isolated from amniotic fluid (AF) and placenta (PL) and compare them with BM-MSCs.
2. to understand the role of MSCs infused in bone marrow grafts and to evaluate the Mesenchymal Stromal Cell Engraftment after Allogeneic HSCT in Paediatric Patients
3. to investigate new stemness markers, their expression and their epigenetic regulation on BM-MSCs routinely isolated from healthy donors during their ex-vivo expansion

2.1 AIM 1

Although BM represents the main source of MSCs for both experimental and clinical studies, the use of BM-MSCs is not always acceptable because of the invasive harvesting procedure. Moreover, the number of BM-MSCs has been reported to decline with increasing age (Rao e Mattson 2001; Mareschi et al. 2006).

Recent studies have indicated that MSCs can be isolated from a variety of tissues, including adipose tissue, umbilical cord, Wharton's jelly, placenta and amnion. These all represent promising sources of MSCs, as they are abundant and easily obtained by non-invasive procedures.

Previous studies performed in the "Stem Cell Transplantation and Cellular Therapy Laboratory" at the Regina Margherita Hospital (directed by Dr Franca Fagioli), have proven that multi-potent SCs can be isolated from amniotic fluid (AF) (Mareschi et al, 2009) and from placenta (data not published). Interestingly, MSCs derived from these sources show greater proliferative and differentiative potentials than BM-MSCs, and are most likely due to the early embryological origin of the AF and PL-MSCs compared to BM-MSCs. These data support the importance of investigating the AF and PL–MSCproperties as an essential prerequisite to allow their clinical use in cell therapy protocols for regenerative medicine.

For this purpose, in this study a comparison of MSCs isolated from AF and PL with BM-MSCs was made. First, the immunophenotype, differentiative potential and embryonic markers of MSC isolated from the 3 different sources were analysed. Then their immunomodulant properties also studied. In particular, we focused on the interaction of AF-MSCs, PL-MSCs and BM-MSCs with T lymphocytes (Ly). The effects of MSC isolated from different sources on Ly, by using in vitro co-culture system were analysed.

Using the same experiment design described above, we also compared three different MSC culture conditions obtained cultivating the cells in a standard condition usually described in the literature [alpha+MEM + 10% of foetal bovine serum (FBS)] and in cultures considered in GMP conditions (without animal serum and containing human platelet lysate (HPL and inactivated HPL underwent to a pathogen inactivation procedure).

2.2 AIM 2

MSCs provide to support the growth and differentiation of hematopoietic progenitor cells in bone marrow (BM) microenvironments and, in animal models, promote engraftment of hematopoietic cells. MSCs suppress proliferation of activated lymphocytes in vitro in a dose-dependent, non-Human Leukocyte Antigen (HLA)-restricted, manner. It has also been shown that stromal cells may be damaged by chemo-radiotherapy before Hematopoietic Stem Cell Transplantation (HSCT), and from a clinical point of view, data on previous studies showed how the add back of stromal cells entrapped in filters during HSCT provided an advantage in terms of reduced graft-versus-host disease (GvHD) and lower transplant-related mortality (TRM). A number of fundamental questions relating to the biology of MSCs are still unanswered, such as survival and homing capacity to populate host tissues after transplantation, if is there a relationship between immunophenotype and functions. To date, the human bone marrow fibroblast colony-forming units (CFU-F) and the adherent ratio are the easiest parameters of MSC content in the graft, but the role of MSCs to 1) expand in vivo, 2) maintain stemmess in vivo and later be able to differentiate into a committed lineage and 3) survive and engraft in the recipient is under investigation. Since the role of MSCs transplanted in the BM graft is still not fully understood, and the role of CFU-F is still under debate, we decided to study the in vitro expansion ability of MSCs and, then, to compare their role in reducing transplant toxicity, and improving survival.

In addition, because MSCs support hematopoiesis and some studies have highlighted a beneficial for graft-versus-host disease (GvHD) treatment, it is of interest to determine whether these cells are susceptible to conditioning therapy, engraft with donor hematopoietic stem cells or depend on the graft source. This information will be necessary to understand in more detail the process of engraftment and, possibly, also the immunological events after allogeneic HCT. Other aim in this contest was to determine if donor-derived MSCs could be identified after HSCT in a heterogeneous group of patients and if there are any correlations with conditioning regimen (myeloablative vs. non-myeloablative), the source of the graft (bone marrow [BM], peripheral blood stem cells [PBSC] or cord blood [CB]), the interval from HSCT, the

patient's or the donor's age at HSCT.

2.3 AIM 3

In the past, we isolated BM MSC from healthy adult or pediatric Caucasian donors who underwent bone marrow collection for a related patient after informed consent to characterize and to investigate their different proliferative capacity, immunophenotypic characteristics, telomere length and karyotype modifications during their cellular expansion

During that study we cryopreserved adult and paediatric BM-MSCs that was precious material to be used for a study on epigenetic evaluation to identify DNA methylation changes during MSC aging. In fact, I was contacted by a Spanish research to collaborate with him and other European and Chilean researchers to characterize the genome-wide DNA methylation status of bone marrow MSCs obtained from individuals aged between 2 and 92 yr. We participated to that project by providing 18 samples of paediatric BM-MSCs.

The possibility to have a cell-like bank of MSCs obtained from BM of healthy donors has allow us to collaborate also with other groups to investigate new stemness markers, their expression and their epigenetic regulation during their ex-vivo expansion.

In particular, in my PhD course I collaborated as well as to the international network created for the study on DNA methylation changes during MSC aging, with the Dr Bergallo's group working in my University Department to evaluate gene expression profile of endogenous retroviruses HERV-H and K in MSCs at different passages and with the Prof. DeBortoli's group at the Department of Clinical and Biological Sciences, University of Turin to investigate the expression of stemness markers during MSC expansion by Chromatin Immunoprecipitation (Chip) assay against histone modifications marks.

PAPER SECTION

3 EVALUATION OF IMMUNOPHENOTYPE, DIFFERENTIATIVE POTENTIAL, EMBRYONIC MARKERS AND IMMUNOMODULANT PROPERTIES OF MSCS ISOLATED FROM AMNIOTIC FLUID (AF) AND PLACENTA (PL) AND COMPARISON WITH BM-MSCS.

Aim 1

3.1 PAPER I :"IMMUNOREGULATORY EFFECTS ON T LYMPHOCYTES BY HUMAN MESENCHYMAL STROMAL CELLS ISOLATED FROM BONE MARROW, AMNIOTIC FLUID, AND PLACENTA"

Experimental Hematology

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Immunoregulatory effects on T lymphocytes by human mesenchymal stromal cells isolated from bone marrow, amniotic fluid, and placenta

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Abstract

Mesenchymal stromal cells (MSCs) are a promising tool for cell therapies for their multipotent, bystander and immunomodulant properties. Although bone marrow (BM) represents the main source of MSCs, a need remains to identify a stem cell source that is safe, easily accessible, providing high cell yield without ethical debate. In this study, MSCs isolated from amniotic fluid (AF) and placenta (PL) were compared with BM-MSCs. Their immunomodulant properties were studied on total activated T-cells with Phytoemoagglutin (PHA-PBMCs). In particular, an *in vitro* co-culture system was performed to study: 1) the effect on T Lymphocyte (Ly) proliferation; 2) the presence of T regulatory Ly (Treg); 3) the immunophenotype of various T subsets (Th1 and Th2 naïve, memory, effector Ly); 4) the cytokine release and master gene expression to verify Th1, Th2 and Th17 polarization; 5) the IDO production, In all co-culture conditions with PHA-PBMCs and MSC (independently from the tissue origin) data showed: 1) T proliferation inhibition; 2) Naïve T Increase and Memory T decrease; 3) Treg increase; 4) strong Th2 polarization associated to increase IL-10 and IL-4; Th1 inhibition (IL-2, TNF-α, IFN-γ and IL-12 significantly decrease) and Th17 induction (production of high concentrations of IL-6 and IL-17); 5) IDO mRNA induction in MSCs co-cultured with PHA-PBMCs. AF-MSCs showed a more potent immunomodulant effect on T-cells than BM-MSCs, only slightly higher than PL-MSCs. This study shows that MSCs isolated from fetal tissues may be considered a

good alternative to BM-MSCs for clinical applications.

INTRODUCTION

Mesenchymal stromal cells (MSCs) are adult stem cells (SCs) which maintain the capacity to self renew and to have high plasticity. Clonogenic MSCs are a heterogeneous mix of progenitors, in which a subset population is capable of differentiating not only into mesenchymal tissue cells (tenocytes, skeletal myocytes, stromal cells, adipocytes, osteoblasts, chondrocytes) but also into neurons, astrocytes and hepatocytes. However, their capability to transdifferentiate into ectodermal and endodermal cells is still controversial. In addition to stemness, MSCs have other intriguing features, which include supporting hematopoiesis, tissue remodeling/ repair properties, and angiogenesis. Although none of these properties is the main reason for the popularity of MSCs in current, medical practice, their unique immunomodulatory property, makes them an attractive source for several clinical uses [1].

Among several possible applications, the possibility to utilize MSCs in autoimmune, chronic inflammatory and degenerative diseases has led to notable therapeutic uses such as in treatment of GVHD and autoimmune conditions for [2]. These cellular therapy applications might represent new therapeutic approaches with benefits and expectations for patients.

MSCs have intermediate levels of HLA major histocompatibility complex (MHC) class I molecules while they don't have HLA class II antigens FAS ligand, and the co-stimulatory molecules [3, 4]. Several papers show that T-cell proliferation induced with specific antigen or polyclonal mitogens and allogeneic cells is inhibited by MSCs [5]. This capacity is confirmed also in vivo because MSCs are able to control Graft versus Host disease (GVHD) after bone marrow (BM) transplantation [6, 7].

The arrest of T-cells occur in the G0/G1 phase of the cell cycle and the T-cell proliferation inhibition is mediated by both autologous and allogeneic MSCs , then this mechanism is not MHC restricted . Moreover, this phenomenon seem to be caused by both cell-to-cell contact [8, 9] and humoral factors such as indoleamine-2,3-dioxygenase (IDO) ,Galectin-1 Prostaglandin E2 and B7-H [10]. These factors should be responsible to induce a decrease of tumor necrosis factor (TNF)-α and interferon (IFN)-γ and an increase of IL-10 secretion from T cells in contact with MSCs [11, 12]. MSCs also induce T helper (Th) 2 type lymphocyte and T regulatory (Treg) cell

differentiation through HLA-G5 and other molecules [13].
Moreover, MSCs, block the antigen presenting cell (APC) maturation and activation [14], modulate cytokine and growth factor production by the dendritic and T cells [15] and increase the number of Treg cells in a mixed lymphocyte reaction [16].
Although BM is the the main source of MSCs, the use of BM-MSCs is not always acceptable because of the invasive harvesting procedure. Moreover, the number of BM-MSCs decline with increasing age [17, 18] . Recently MSCs are been isolated from a variety of tissues, including adipose tissue, umbilical cord, Wharton's jelly, placenta and amnion. These all represent promising sources of MSCs, as they are abundant and easily obtained by non-invasive procedures.
Our previous studies have proven that multi-potent MSCs can be isolated from amniotic fluid (AF) [19] and from placenta (PL) (data not published) with MSC characteristics as defined by the International Cellular Therapy Society [20]. Interestingly, MSCs derived from these sources show greater proliferative and differentiative potentials than BM-MSCs, which are most likely due to the early embryological origin of the AF and PL-MSCs compared to BM-MSCs. These data support the importance of investigating the AF and PL–MSC properties as an essential prerequisite to allow their clinical use in cell therapy protocols for regenerative medicine.
For this purpose, in this study, a comparison of the effects of MSCs isolated from AF and PL with BM-MSCs on T lymphocytes (Ly) was made using an in vitro direct co-culture system.

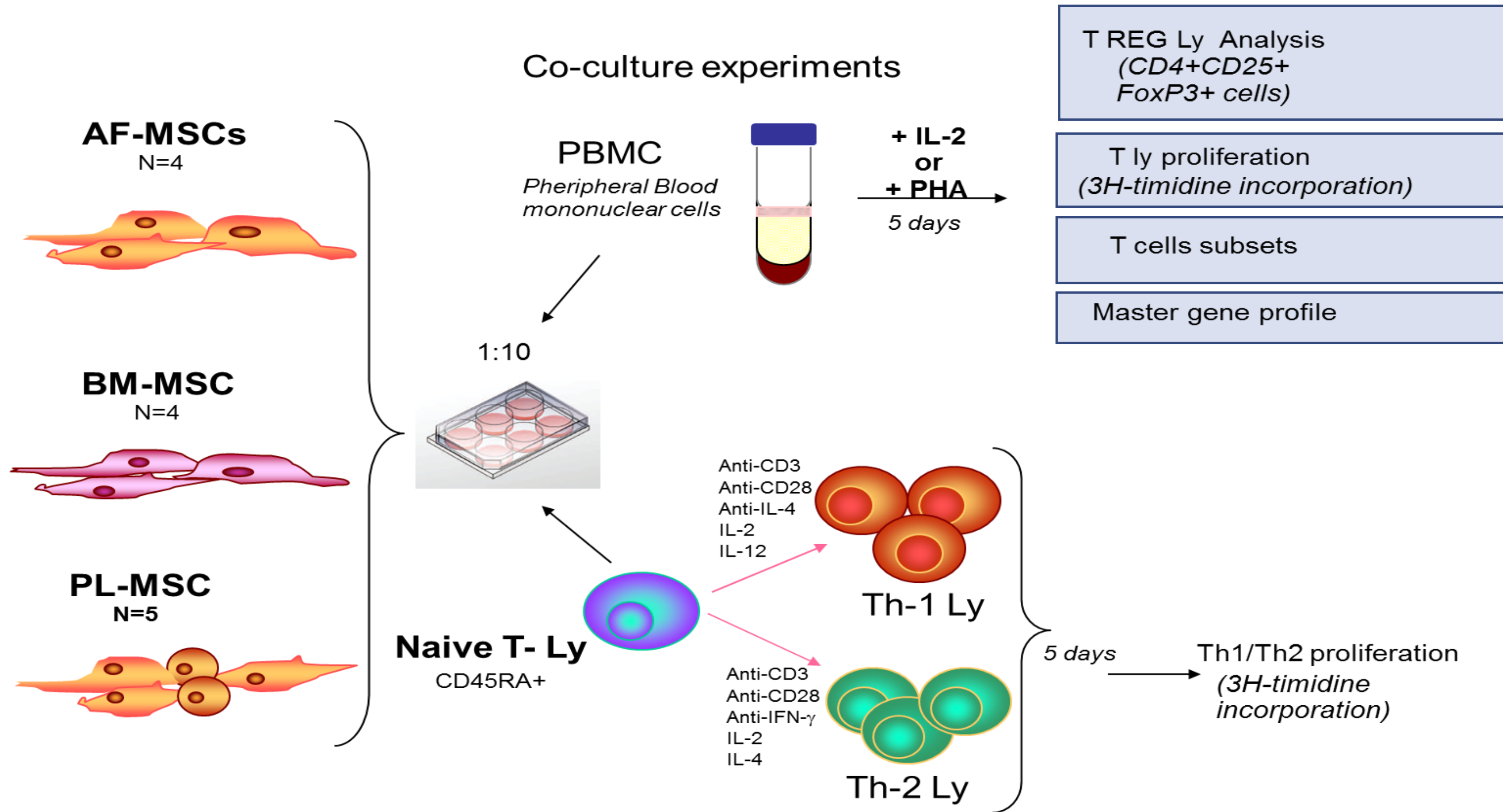

Figure 3-1- Study design for MSC/T cell co-culture

MATERIALS AND METHODS

Isolation and culture of human MSCs

All studies adhered to the Declaration of Helsinki. Human BM, AF and PL samples were collected following written consent, in accordance with the Ospedale Infantile Regina Margherita-Sant'Anna-Ordine Mauriziano hospitals' ethics committee, which approved the collection of the samples.

BM cells were harvested from the iliac crest of adult or pediatric Caucasian donors who underwent BM collection for a related patient after informed consent. When available, an unfiltered BM collection bag was also used (Baxter Healthcare Corporation, IL, USA) which was normally discarded before the BM infusion. The bag was washed 3 times with Phosphate Buffer Saline (PBS) 1X (Lonza, Versviers, Belgium) and the cells were collected and washed at 200g for 10 minutes. An aliquot of whole BM was counted and plated directly in T25- or T75-flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at $1x10^4$ cells /cm^2.

AF was harvested from women undergoing amniocentesis for routine prenatal diagnosis at 14-16 weeks of pregnancy. AF samples were centrifuged and the resulting pellets were plated in 25 cm^2 T-flasks as previously described [19].

PL was collected immediately after elective c-section (to avoid contamination with vaginal pathogens) after informed consent. Upon receiving the PL, the decidua tissue and amniotic membrane were dissected, and a piece of central PL was harvested from the basal plate. Each piece of PL was rinsed 3 times with PBS and digested mechanically and enzymatically using gentle MACS™ Dissociator (Milthenyi Biotec). After two digestion steps using trypsin (Sigma-Aldrich®, Saint Luis, MO, USA) and collagenase (Stemcelltechnologies, Vancouver, British Columbia, CA) with DNasi (Sigma-Aldrich®), the cells were plated directly in T75-flasks.

The culture medium used for all sources was α MEM (Biochrome, Berlin, Germany) supplemented with 10% FBS (Sigma-Aldrich®), 2mM L-glutamine (Sigma-Aldrich®),, penicillin/ streptomycin 1X (Euroclone, Pero, Mi, Italy). The culture was maintained at 37°C with a 5% CO^2 atmosphere. After 5-7 days, the non-adherent cells were removed and the adherent cells were re-fed every 3-4 days. In order to expand the isolated cells, the adherent semi-confluent monolayer was detached with trypsin/EDTA (Sigma-Aldrich®) for 5 minutes at 37°C and expanded for several passages until they no longer reached confluence.

Only those cells which had all the MSC criteria, as indicated by the International Cellular Society [20], and which were not senescent, were frozen as MSCs in FBS with 10% dimethyl sulfoxide (DMSO) (Euroclone, Pero, Mi, Italy) and then thawed at the moment of the experiments in this study.

MSC analysis and characterization

BM, PL and AF-MSCs used for this study, were analyzed for viability, immunophenotype, differential and proliferative potential to verify that the freezing had not altered the MSCs' characteristics.

To analyze the immunophenotype, flow cytometer analysis was performed on MSCs using the following antibodies: anti-CD90 FITC, CD73 PE, CD34 FITC, CD14 FITC; CD45 FITC (Becton Dickinson, San Jose, CA, USA); CD 105 APC, and CD146 APC (Miltenyi Biotec srl, Bologna, Italy). Details of the cytofluorimetric analysis are described below.

In order to analyze the multipotent capacity, MSCs isolated from the different sources were cultured in osteogenic, adipogenic (Stem Cell Tecnologies) and chondrogenic media (Lonza, Cologne, Germany) for 21 days, according to the manufacturer's instructions. Briefly, 5,000 and 10,000 cells, for control samples and for differentiation experiments, were seeded in a 6-well plate for osteogenesis and adipogenic culture conditions respectively. Osteogenic differentiation was demonstrated by the accumulation of crystalline hydroxapatite by Von Kossa staining, and the adipogenic differentiation, by the presence of intracellular lipid vesicles assessed by Oil Red O. MSC chondrogenic differentiation was obtained as previously described [21] and the differentiation was evaluated by Alcian Blue staining which identifies the presence of hyaluronic acid and sialomucin.

Preparation of human Peripheral Blood Mononuclear Cells (PBMCs)

PBMCs were separated from buffy coats by centrifugation on a Ficoll Hystopaque density gradient. The buffy coats were prepared in the Blood Component Production and Validation Center, City of Science and Health of Turin, S. Anna Hospital from healthy donors, after informed consent, using an automated blood component separator (Compomat G5, Fresenius Kabi; Bad Homburg, Germany). Donors were negative for infectious markers (Hepatitis B and C, HIV 1-2 and Treponema pallidum) in accordance with Italian laws and European guidelines.

Co-culture MSC/T cells

On the basis of the experiments to be performed, BM, AF and PL-MSCs were plated in 6, 24 or 96 well plates or T-flasks ($25cm^2$) containing total PBMCs from an unrelated donor (the MSCs/T cell ratio was 1:10). To trigger T lymphocytes, PBMCs were stimulated with PHA (2.5 µg/mL).

The culture conditions were: 1) MSCs alone; 2) Unstimulated PBMCs; 3) PHA-stimulated PBMCs; 4) Co-culture of MSC-T cells with unstimulated PBMCs; 5) co-culture of MSCs with stimulated PBMCs.

After five days, co-cultures of the non-adherent cells were harvested and counted for total RNA extraction and molecular, cytofluorimetric and proliferative analyses.

The supernatants were collected to analyse Th1 and Th2 cytokine release analysis (IL-2, IL-12, INF-γ and TNF-α and IL-10, IL-17, IL-4, IL-6, respectively) performed by ELISA (Mabtech, ELISA Assay). MSCs were detached by scraper and collected for total RNA extraction and molecular analysis.

Proliferation assay

MSCs were plated in triplicates, after irradiation at 3000 rad, into 96-well plates at 2×10^4 cells/mL in 100 µL complete α-MEM medium and allowed to adhere to the plate for 24 hours. PBMCs, re-suspended at 2×10^5 cells/mL, were added to wells (in 100 µL volume) containing or lacking MSCs in the presence of the mitogen PHA (2.5 µg/mL). The MSC/PBMC ratio was 1:10. The experiments were performed using 3 preparations of MSCs from each source with PBMCs from an unrelated donor and 1 MSC preparation from each source with PBMCs from 3 additional unrelated donors. Co-cultures without PHA were used as controls. The culture was continued and ^{3}H-thymidine (1 µCi (0.037 MBq)) was added 4 hours before the end of the 72-hour culture. The cells were harvested and counted using a 1450 Microbeta TriLux apparatus (Perkin Elmer, Boston, MA). The T cell proliferation was represented as the incorporated radioactivity in counts per minute (cpm).

Cytofluorimentric analysis

PBMC characterization was performed using the following antibody (mAb) panels: anti-human CD45RA-FITC /CD45RO-PE/CD3-peridinin-chlorophyll protein cyanine 5.5 (PerCP-Cy5.5)/CD8-APC, CD45RA-FITC/CD45RO-PE/CD3-PercP-Cy5.5/CD4-APC, CD62L-FITC/CD27-PE/CD3-PerCP-Cy5.5/CD4-APC/CD8APCCy7,

CD45RA-FITC/CD27-PE/CD3-PerCP-Cy5.5/CD4-APC/CD8-APCCy7. All these antibodies were from Becton Dickinson.

Briefly, $2x10^5$-2.5 $x10^5$cells for each mAb panel, were stained with the appropriate amount of antibody according to the antibody titration as described by Rustichelli and colleagues [22] for 20 minutes.

The labeled cells were thoroughly washed with PBS 1X (200 g for 10 minutes) and analyzed on a FACSCanto II (Becton Dickinson) with the DIVA software program.

The percentage of positive cells was calculated using the unstained cells as a negative control and used to calculate the absolute number on the basis of the cell number counted after 5 days of co-culture (stimulated PHA-PBMCs + BM, AF and PL-MSCs) and mean fluorescence intensity (MFI) was analyzed on the positive cells.

Treg Analysis

For Treg analysis, the co-cultures were performed in 24 wells plates using 100,000 PBMCs in each condition. The Treg proportion was analyzed by flow cytometry analysis, labeling the cells with CD4-APC, CD25-PE and Fox-P3-FITC antibodies using a specific kit (Anti-Human Foxp3 Staining Set FITC, eBioscience). Preliminary experiments were performed on IL-2-stimulated PBMCs and only the percentage of total $CD4^+$/$CD25^+$/Foxp-3^+cells was analyzed without counting the cells in the culture. Then, considering T-proliferation results, the absolute number of Treg cells was evaluated in the co-cultures after counting the absolute number of cells present in the wells after 5 days.

STAT5B and Foxp3 expression were also analyzed at a molecular level by real time PCR as described below.

Evaluation of cytokine release by ELISA

The supernatants were collected for Th1 and Th2 cytokine release analysis (IL-2, IL-12, IFN-γ and TNF-α and IL-10, IL-17, IL-4, IL-6, respectively), performed by ELISA kit coated at home (Mabtech, ELISA Assay). Briefly, 96 well high protein binding ELISA plates (Nunc) were coated with mAb13A5 diluted, as indicated in the manufacturer's instructions, to 0.5 μg/mL in PBS, pH 7.4, and incubated over night at 4°C. After washing (PBS with 0.05% Tween 20), the plate was blocked for 1 hour at room temperature (rt) with a blocking solution (PBS with 0.05% Tween 20 containing 0.1% BSA). The standards of each cytokine were prepared by reconstituting the

content of each vial with incubation buffer (PBS with 0.05% Tween 20 containing 0.1% BSA) at the indicated dilution range.

Standards and samples were incubated at rt for 2 hours. Five washes were then performed. The plate was then incubated with mAb 39C3-biotin at 1 µg/ml in an incubation buffer for 1 hour at rt, washed 5 times and incubated for 1 hour at rt with Streptavidin-HRP. At the end of the procedure an appropriate substrate solution was used conferring at the reaction a coloring with an intensity (evaluated by a spectrophotometer at a wavelength of 450 nm) directly proportional to the cytokine concentration. Data were expressed as a cytokine concentration in pg/ml.

HPLC analysis of IDO activity

The chromatographic determination of tryptophan (Trp) to kynurenine (Kyn) was performed by HPLC assays in the Clinical Biochemistry Laboratory of the University Hospital - City of Science and Health of Turin.

The supernatant of MSCs alone and in co-culture were collected after 5 days and frozen at −20°C. Samples were thawed and 1mL was deproteinized by 100µl 30% trichloroacetic acid (Sigma-Aldrich, Italy). A 250µl amount of supernatant was added to 50µl of aqueous solution 49.4 µmol/L of Theophilline as an Internal Standard (IS, Sigma-Aldrich, Italy). Standard stock aqueous solutions (2.47 mmol/l for Kyn and 4.41 mmol/l for Trp, both from Sigma-Aldrich, Italy) were prepared and kept frozen at −80°C. Working standard solutions were made by appropriately diluting standard mixtures.

Separation was achieved on the HP1100 LC system (Agilent Technologies Italia S.p.A.) using a column Phenomenex Kynetex C18 100A (150mm X 4.6 mm, 5µ) by isocratic elution in 7 minutes. The mobile phase consisted of 50 mmol/l acetic-acetate (Sigma-Aldrich, Italy) buffer pH 4.6 and HPLC grade Methanol (VWR International PBI s.r.l, Italy) (85:15 v/v) at a flow rate of 0.9 ml/min at 40°C.

Eluates were monitored by diode array detector (DAD) set at λ 360 nm for Kyn and λ 275 nm for Trp and IS. Absorbances at λ 220 nm and λ 302 nm were also acquired: absorbance ratios were used for the identification and purity assessment of each peak. The sample injection was 50 µl.

The transcripts for IDO-1 and IDO-2 on MSCs were also analyzed by real-time PCR (assay IDs: Hs00984148_m1 and Hcg1646605_m1, respectively [Applied

Biosystems, Foster City, CA, USA]).

Molecular analysis on co-cultures

Total RNA was extracted from PBMCs and MSCs after 5 co-culture days, using the TRI-Reagent kit (SIGMA) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed with 5 µl of PCR buffer II 10X, 11 µl of $MgCl_2$ 25 mM, 2 µl reverse transcriptase MuLV 50U/l (murine Moloney leukemia virus), 1 µl of RNase inhibitor 20U/l, 5 l random hexamers 50 µM (Applied Biosystems), 1 µl l mix dNTPs 100 mM (Amersham) and dd-water in a final volume of 50 µl. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA) under the following conditions: 10 min at 20°C, 45 min at 42 °C and 5 min at 99°C for the enzyme inactivation; the cDNAs were stored at −80°.

The reverse transcription (RT) real-time PCR array was performed to quantify cytokines, chemokines, molecules for antigen uptake, signal transduction, regulators of T cell activation, regulators of Th1 and Th2 development and differentiation. Forward, reverse primer (- Sequence of primers used for real time PCR.) and Hydrolysis probe, were specific to each assay. 5µL of cDNA were added at 15 µL of reaction mix containing 900 nM of forward and reverse primer, 200 nM of Hydrolysis probe MGB or TAMRA and 1X of master mix in a final volume of 20 µL.

We used the Hs00984148_m1 and Hcg1646605_m1 assays, and TaqMan Universal PCR Master Mix, respectively (Applied Biosystems, Foster City, CA, USA) to detect the transcripts for IDO-1 and IDO-2 on MSCs.

The amplifications were performed on the ABI 7500 real-time PCR system (Life Technologies, Texas, USA) in a 96-well plate at 95°C for 10 min, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min.

The relative quantification of mRNA expression of selected genes was achieved by Taqman amplification. To normalize the PCR results, hypoxanthine phosphoribosyltransferase 1 (HPRT1) was used as a reference gene (assay HPRT1:Hs02800695_m1). Each sample was run in triplicate. Furthermore, in order to confirm that there was no DNA genomic contamination, a control PCR was performed with RNA before reverse transcription using the same primers and probe described above. Relative quantification of target genes expression was performed with the ΔΔCt

method.

Name	sequence
IFNγ-F	CTAATTATTCGGTAACTGACTTGA
IFNγ-R	ACAGTTCAGCCATCACTTGGA
IFNγProbe	6FAM-TCCAACGCAAAGCAATACATGAAC-TAMRA
Tbet-F	ACACGCATATCTTTACTTTCCAAGAA
Tbet-R	TCAGCTGAGTAATCTCGGCATTC
Tbet-Probe	6FAM-CCCAGTTCATTGCCGTGACTGCC-TAMRA
GATA3-F	TTCCCCAAGAACAGCTCGTT
GATA3-R	GGCTCAGGGAGGACATGTGT
GATA3Probe	6FAM-AACCCGGCCGCCCT-MGB
FOXP3-F	TCACCTACGCCACGCTCAT
FOXP3-R	ATTGAGTGTCCGCTGCTTCTC
FOXP3Probe	6FAMCTGGGCCATCCTGGA-MGB
STAT3-F	TCCTGGTGTCTCCACTGGTCTA
STAT3-R	TTCCGAATGCCTCCTCCTT
STAT3Probe	6FAM-CTCTATCCTGACATTCC-MGB
STAT6-F	TCCATCCCCCCGTATCAA
STAT6-R	GGCTCCTGGAAGGCTGACA
STAT6Probe	6FAM-CCTCTCCCCAGAAGAATCAGTCAACGTG-TAMRA
STAT5b-F	TGATTACAGTGGCGAGATCTTGA
STAT5b-R	GCCTGTGGCTTGGTGGTACT
STAT5bProbe	6FAM-CAACTGCTGCGTCATG-MGB
RORγ-F	CGGGCCTACAATGCTGACA
RORγ-R	GCCACCGTATTTGCCTTCAA
RORγ-PROBE	6FAM-CCGCACGGTCTTT-MGB

Table 3-1 - Sequence of primers used for real time PCR.

Statistical analysis

All the data obtained in this work were analyzed by Graph PAD Prism statistical software. The statistical tests used to compare the differences between the analyzed groups were chosen on the basis of the number of samples and the distribution of the data. The Kolmogorov-Smirnov test with Dalla-Wilkinson-Lille was used for the first analysis to verify the normality of the data distribution. The differences between paired samples were evaluated by the ANOVA test or Friedman’s test, respectively, whether data distribution was normal or not.

Dunn's multiple comparisons test in one-way ANOVA was used to compare the mean ranks of preselected pairs of columns with the mean ranks of a control column (PHA-PBMCs). Bonferroni's multiple comparisons test was used to compare each cell mean with the control cell mean on that row.
All statistical tests were considered significant for a $P<0.05$, highly significant for $p<0.001$ and very highly significant for $P<0.0001$.

RESULTS

MSC cultures

BM, PL and AF-MSC frozen samples, stored in nitrogen liquid were used for this study. BM-MSC aliquots were used from the second to fourth passages; AF-MSCs and PL-MSCs were used from the second to seventh passage in order to have the cells in the exponential phase of their cellular growth. Post-thawing cell viability was always >70% in all MSC samples. After 2-3 days post thawing, non-adherent cells and debris were discarded and single cells in BM-MSCs or colonies in AF-MSCs and PL-MSCs were left to expand until 70-80% of confluence.
We observed that the morphology after thawing was similar to that observed in the passage before freezing and maintained the immunophenotype characteristics. BM, AF and PL-MSCs used in this study were negative for hematopoietic antigens and for HLA-DR, and expressed more than 90% of CD90, CD73, CD105 and CD146 as shown in Figure 3-2 A, C, E. No statistical differences were observed among the three different media in terms of percentage or in terms of fluorescence means in the positive cells.
The MSCs obtained in the 3 different sources showed multipotent capacity as all samples used differentiated into osteoblasts, adypocytes and chondrocytes as shown in Figure 3-2 B, D, F.

MSC/T cell interaction

Proliferative assay

PBMCs alone showed fewer than 1000 cpm in these experiments.
Comparing the mean cpm of each group with the control condition, which was PBMCs stimulated with PHA (PHA-PBMCs), a decrease in proliferation was very highly significant in the presence of BM-MSCs ($p < 0.001$), highly significant in the presence

of AF-MSCs ($p < 0.01$) and significant in the presence of PL-MSCs ($p < 0.05$) in the co-culture conditions as shown in Figure 3-2.

No significant differences were observed between BM, AF and PL-MSCs.

These experiments showed that MSCs from different sources had a significant inhibitory effect on PHA-stimulated PMBC and a inhibitory, but not statistically significant, effect in induced Th1 effector cells, in all co-culture conditions. The proliferation data obtained on Th2 effector cells were not homogenous and were controversial. For this reason, we focused our experimental study on total PHA activated-PBMC.

A
FSC-A
FITC-A
PE-A
APC-A
FSC-A
CD90 FITC
CD73 PE
CD105 APC
FSC-A
CD45-34-14 FITC
HLA-DR PE
CD146 APC
C
FSC-A
FITC-A
PE-A
APC-A
FSC-A
CD90 FITC
CD73 PE
CD105 APC
FSC-A
CD45-34-14 FITC
HLA-DR PE
CD146 APC
E
FSC-A
FITC-A
PE-A
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CD45-34-14 FITC
HLA-DR PE
CD146 APC

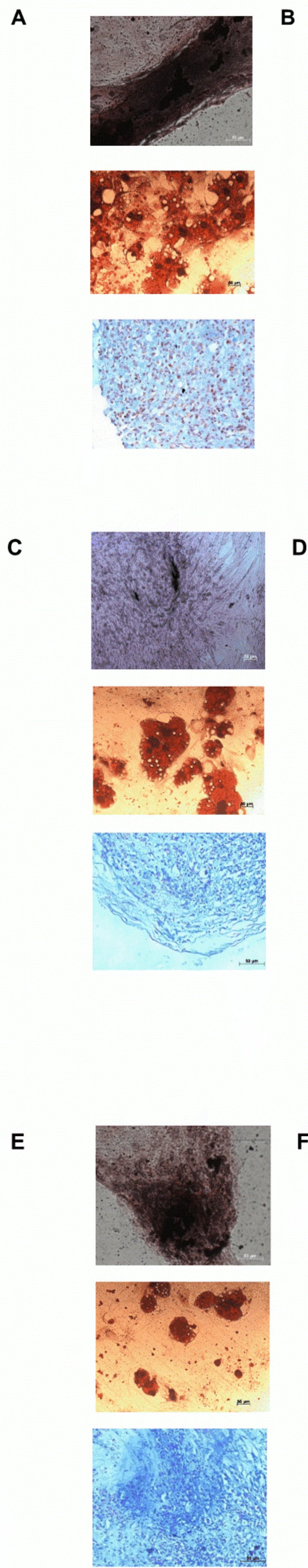

B
D
F

Figure 3-2 Immunophenotype and differentiative potential in BM, AF and PL-MSCs respectively in A and B; in C and D and in E and F. On the left, flow cytometry histograms show the histotype control in the top row and the characteristic immunophenotype of MSCs in the other histograms. No statistical differences between the 3 sources were observed. On the right, respectively in B, D and F: in the upper photographs, osteogenic differentiation shows differentiated cells containing mineralized matrices, which were strongly positive by Von Kossa; in the middle photographs adipogenic differentiation shows morphological changes in the formation of neutral lipid vacuoles containing numerous Oil Red O-positive lipid droplets; in the bottom photographs chondrogenic differentiation shows the presence of acid mucopolysaccharides and sulfated and carboxylated sialomucins positive for Alcian Blue.

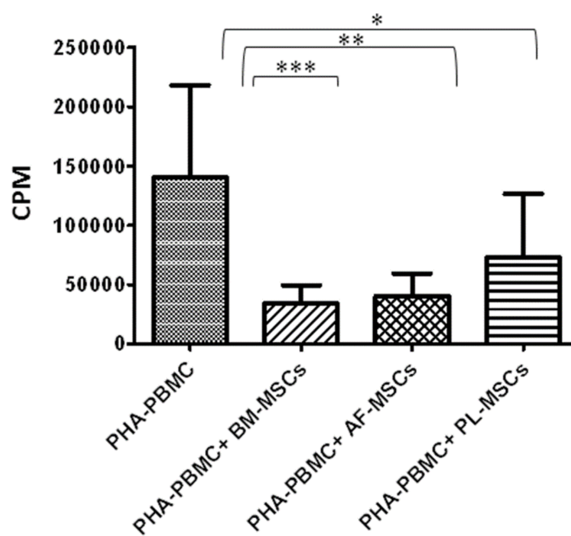


*Figure 3-3 Proliferative assay on stimulated PBMCs alone and in co-culture with BM, AF and PL-MSCs. The results reported are the average of 5 experiments of identical design. T cell proliferation cultures were performed in triplicate and results averaged. The bars show the SD. *, ** and ***indicates, respectively, significant, highly significant and very highly significant (p<0.05, p<0.001 and p<0.0001.) versus control cultures (PHA-PBMCs) without MSCs.*

T cell subsets determination

The multiparameter flow cytometric analysis allowed the identification of the following T subsets, based on the antibody combination used:

- naïve CD8+ T cells: CD45RA+ /CD3+/CD8+
- naïve Th cells: CD45RA+/ CD3+/CD4+
- memory CD8+ T cells CD45RO+/CD3+/CD8+
- memory Th cells: CD45RO+/CD3+/CD4+

The percentage obtained by cytofluorimetric analysis was used to calculate the absolute number based on the cell number counted after 5 days of co-culture (stimulated PBMCs + BM-, AF- and PL-MSC). The gating strategy using for the cytofluorimetric analysis is illustrated in Supplementary Figure 3-I.

We observed a high variability of naïve and memory T cells among the donors, so the standard deviations in the mean values were high but the modulation of the T cell subsets simultaneously analyzed in the different co-cultures was the same in all experiments. Indeed, in all the experiments, the memory T cell number was always higher than naïve T cell number in PHA-PBMCs, and after co-culture with MSCs, independently from the source, this ratio reversed in favor of naïve T cells (Figure 3-4 A and C), while the memory T subpopulation decreased (Figure 3-4 B and D).
Moreover, we observed that:

i. the increase of naïve T cells (CD3+/CD4+/CD45RA+ and CD3+/CD8+/CD45RA), observed in all co-cultures was statistically significant when the PHA-PBMCs were co-cultured with AF and PL- MSCs (Figure 3-4 A and C);
ii. the decrease of memory T cells (CD3+/CD4+/CD45RO+ and CD3+/CD8+/CD45RO+) was statistically significant in the co-cultures with PHA-PBMCs and BM-MSCs and PL-MSCs (Figure 3-4 B and D);

We also analyzed the subsets CD4+ T effector memory: CD4+/CD45RA-/CD27-/CD62L- and CD8+ T effector memory: CD8+/CD45RA-/CD27-/CD62L- and we observed that total effector memory T cells decreased in all co-culture conditions, but, interestingly, the Th subset (CD4+) remained unchanged while the CD8+ subset decreased especially in AF-MSC co-culture (data not shown).

Treg evaluation

Treg was evaluated considering their absolute number either in PBMCs alone in basal conditions and after stimulation with PHA, or in co-culture with MSCs isolated from the three sources.
An increase in absolute Treg numbers in all co-cultures experiments was observed. Also here, when each cell mean of each experiment with the control PHA-stimulated PBMCs was compared, we observed a significant increase in Treg absolute number in the co-cultures with AF-MSCs ($p < 0.05$) and PL-MSCs ($p < 0.05$) (Figure 3-4 E). No significant differences were observed in non-stimulated co-cultures.

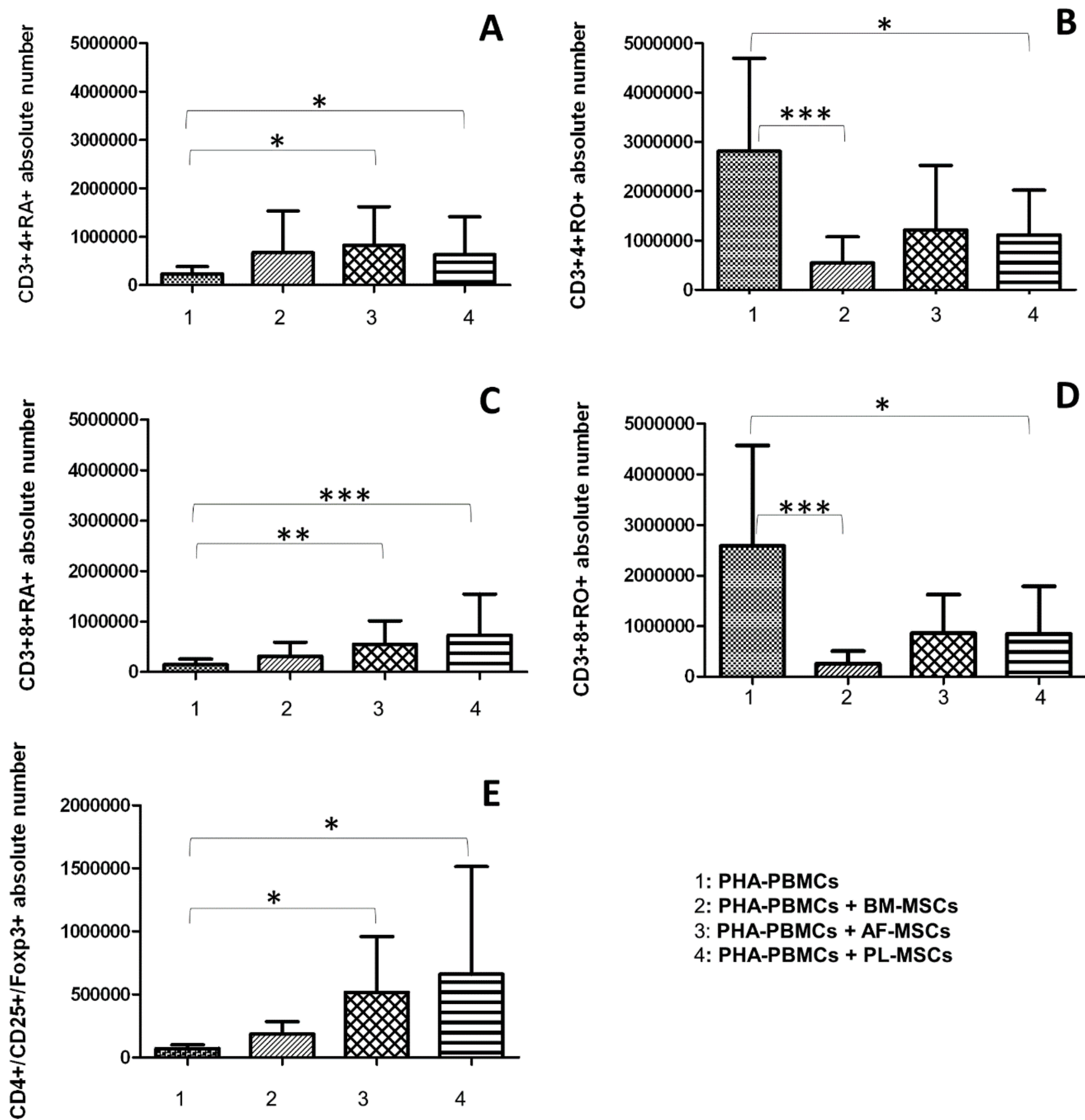
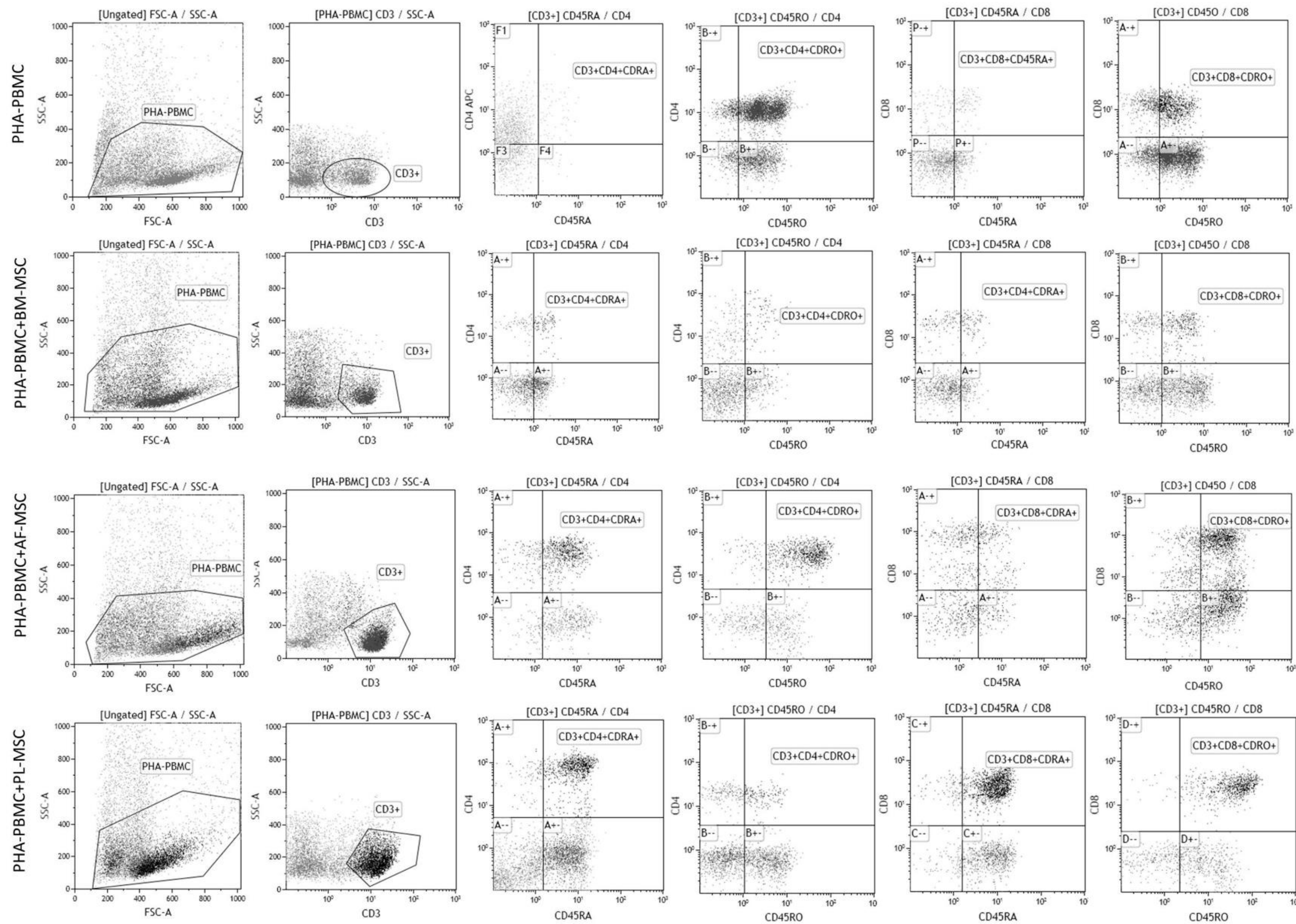


*Figure 3-4- Naïve and Memory T and Treg subsets in PMMC alone and in co-culture with BM, AF and PL-MSCs. In PHA-PBMCs, memory T cells were higher than naïve Th, and after co-culture, regardless of the source, this ratio was seen to reverse in favor of naïve T cells, especially naïve Th subsets (A and C). The CD8+ naive Th subset indreased in all co-culture conditions (C), while the CD8+ memory T subpopulation decreased (D). The bars show the SD and the symbols * , ** and ***indicate a statistically significant difference, respectively, with p<0.05, p< 0.01 and p< 0.001)).In PHA-PBMCs with AF and PL-MSCs, the Treg absolute number is significantly higher. (E). Also here the symbol * indicate a statically significant difference with p<0.05*



Supplemtau Figure 3-I Representative Dot Plots during cytofluorimetric analysis showing the gating strategy to evaluate T cells subsets in PHA-PBMCs alone (upper row) and in co-cultures with BM, AF and PL-MSCs. As it was shown on the upper side of each dot plot, we obtained the value of total percentage of CD3+/CD4+/CDRA+, CD3+/CD4+/CDRO+, CD3+/CD8+/CDRA+ and CD3+/CD4+/CDRO+. In order to obtain the absolute number of each T cells subsets, we cal-culate this percentage value on the total number of cells which were counted after five days of co-culture.

Cytokine release

PHA-stimulated PBMCs were used as the control and the range of analyzed cytokines was similar to that reported in literature [23–25].

MSCs constitutively produce: 1) Negligible levels of Th1 cytokines, except for IL-12 which was higher than IL-12 levels in PHA-PBMCs; 2) very low Th2 cytokines; 3) significant levels of IL-6 and IL-17.

As also reported in other papers (19-21), PHA-PBMCs showed high levels of IL-2, IL-12, TNF-α and IFN-γ which decreased in all co-culture conditions with MSCs.

In particular, IL-2 concentrations significantly decreased in all co-culture conditions as illustrated in Figure 3-5. Bonferroni's multiple comparison test showed a high statistical significance ($p<0.001$) in two experiments for the PHA-PBMCs vs PHA-PBMCs+ BM-MSCs and PHA-PBMCs vs PHA-PBMCs + PL-MSCs conditions, respectively. Interestingly, even though BM, AF and PL-MSCs constitutively produced higher levels of IL-12 than PHA-PBMCs, a decrease of IL-12 in all co-cultures (Figure 3-4) was observed. Dunn's multiple comparison test showed a significant difference in the presence of PL-MSCs ($p <0.05$). The high level of TNF-α produced by PHA-PBMCs significantly decreased in all MSC co-culture conditions, as shown in Figure 3-4. Dunnett's multiple comparison tests showed a significant decrease of TNF-α levels in BM, AF and PL-MSCs ($p <0.05$ in all samples). IFN-γ also significantly decreased in the co-cultures with MSCs. Dunn's multiple comparisons showed a significant difference in co-culture with AF-MSCs ($p <0.05$) and PL-MSCs ($p <0.05$) as shown in Figure 3-4.

PHA-PBMCs produced moderate amounts of IL-4 and IL-10, which increased in the presence of MSCS in all co-culture conditions.

Comparing the mean rank of each column with the mean rank of PBMCs, a significant increase of IL-4 in the co-cultures with AF- and PL-MSCs ($p <0.05$ and $p <0.05$, respectively) was observed, as shown in Figure 3-4. Similar results were obtained analyzing IL-10 release (Fig. 4 F): the increase of IL-10 was significantly higher in the co-culture with AF-MSC ($p < 0.05$) and PL-MSC ($p < 0.05$) (Dunn's multiple comparison test).

PHA stimulated PBMCs showed a moderate production of IL-17 and IL-6 which increased in all co-culture experiments with MSCs (Figure 3-4). Interestingly, these

two cytokines were produced constitutively by MSCs, BM, AF and PL-MSC and Dunn's multiple comparisons test showed a significant increase (P <0.05) of IL-17 in AF-MSC co-culture, as shown in the Figure 3-4.

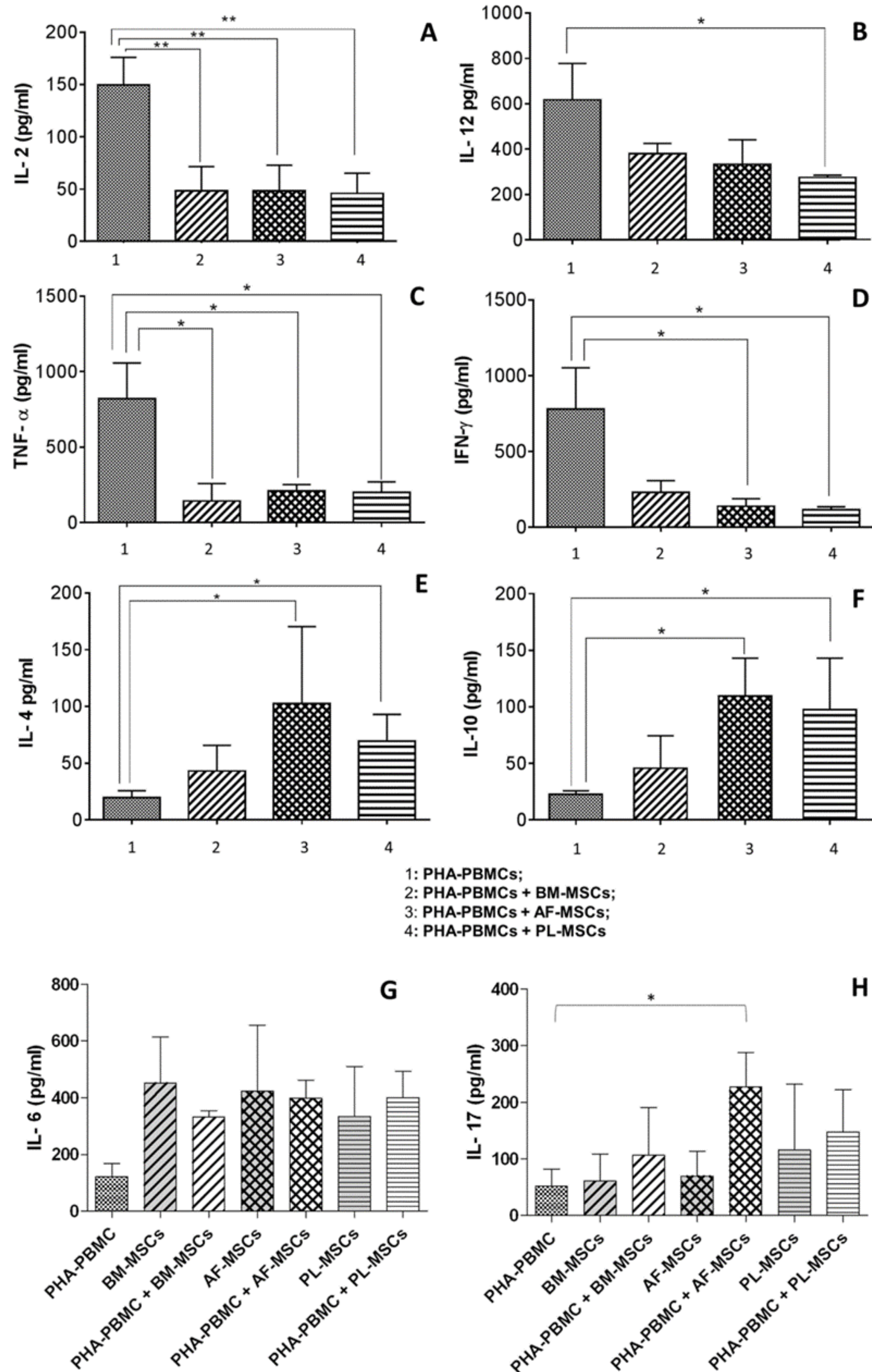


Figure 3-5 Cytokine release. IL-2 (A), IL-12 (B), TNF-α (C) , IFN-γ (D), IL-4 (E), IL-10 (F), IL-6 (G) and IL-17 (H) release detected on PHA-PBMCs and PHA-PBMCs co-cultivated with BM, AF and PL-

*MSCs. Each column represents the mean with the SD of 5 experiments. * indicates significant ($p<0.05$) versus control cultures (PHA-PBMCs) without MSCs.*

Master gene Expression: Th1, Th2, Th17 and Treg molecular pathway

It is well known that signature cytokines and master transcription factors play important roles in the differentiation of Th1/Th2/Th17/Treg cells. To elucidate the mechanisms underlying the regulatory effect of MSCs on the Th1/Th2/Th17/Treg paradigm, the mRNA of master transcription factors for Th1/Th2/Th17/Treg cell differentiation (IFNγ, T-bet, GATA-3, STAT-6, RORγ, STAT-3, STAT5b and Foxp3) was analyzed by RT-PCR. The molecular analysis performed after 5 co-culture days showed significant polarization vs Th2 and Th17 induction in all experiments in unstimulated PBMCs. The relative quantification calculated on unstimulated PBMCs showed a higher mRNA level of each analyzed transcript in the co-cultures with PL-MSCs vs PBMCs with BM- and AF-MSCs. The expression of GATA-3, STAT-6, RORγt, and STAT-3 mRNA increased in unstimulated PBMCs with BM-PL and AF-MSCs, while the expression of IFNγ, T-bet mRNA decreased (Figure 3-6). The Treg polarization is mixed with STAT5b mRNA expression that increased and FOXP3 mRNA expression that decreased in unstimulated PBMCs with BM-PL and AF-MSCs. When we performed the test in presence of PHA the results were different: the Th2 polarization with an increased expression level of mRNA of GATA-3, STAT-6 was present only in PHA-PBMCs with BM- and PL-MSCs. PHA-PBMCs with AF-MSCs only show a STAT-6 mRNA expression level increment. The Th17 polarization with increased expression of RORγt and STAT-3 mRNA was observed only in PHA-PBMCs with BM-MSCs, while PHA-PBMCs with PL-MSCs only show a RORγt mRNA expression level increment. Th1 and Th17 show mixed results: PHA-PBMCs with BM-MSCs show Th1 and Th17 polarization with an increased expression level of mRNA of IFNγ, T-bet, STAT5b and Foxp3; PHA-PBMCs with PL and AF-MSCs only show increased expression levels of mRNA of IFNγ; PHA-PBMCs with PL-MSCs only show an increased expression level of mRNA of STAT5b, and PHA-PBMCs with AF-MSCs only show increased expression levels of mRNA of FOXP3.

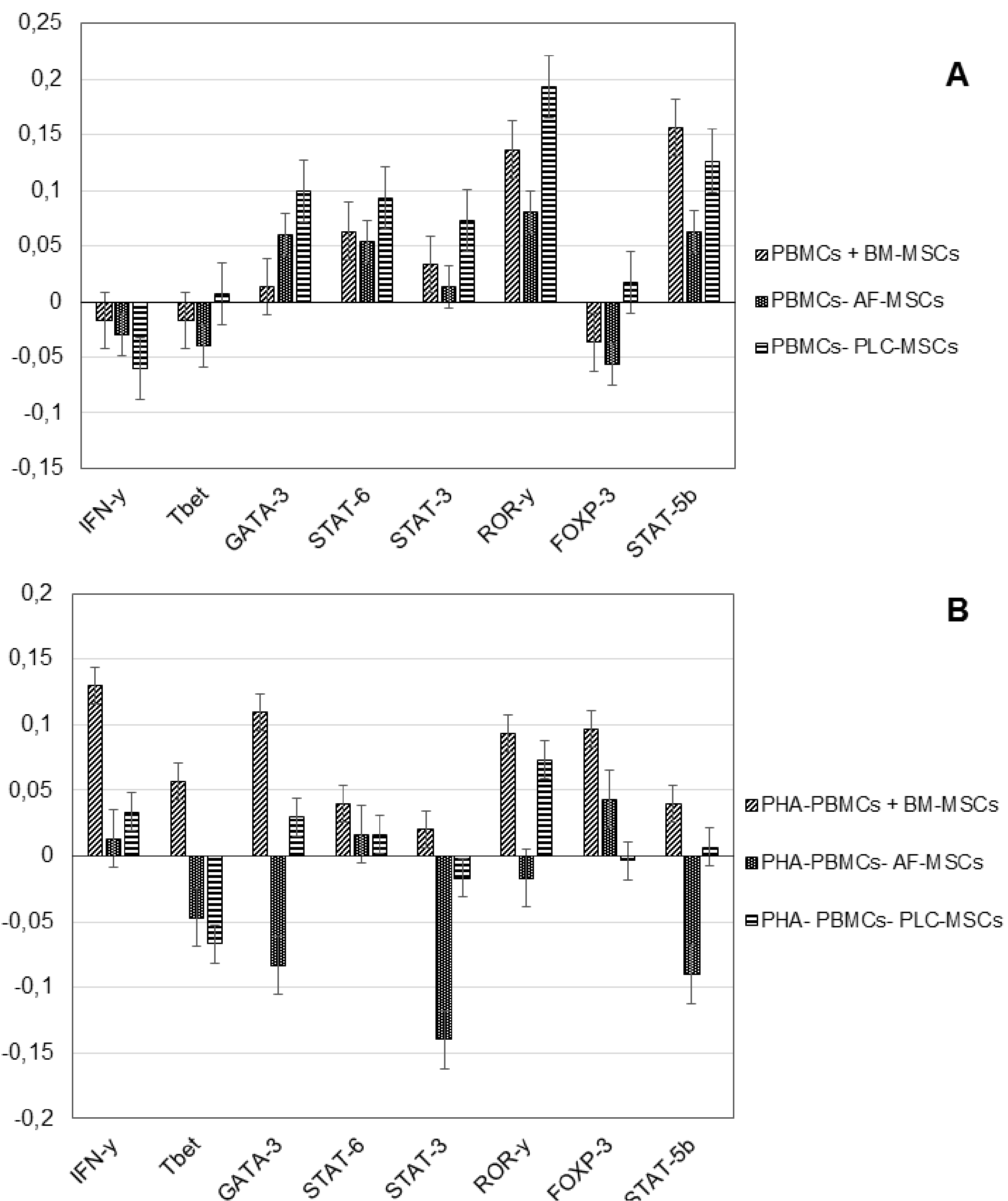


Figure 3-6 Master gene Expression: Th1. Th2. Th17, Treg molecular pathway.mRNA expression of IFN-γ, Tbet, GATA-3 In A) unstimulated PBMCs and in B) PHA-PBMCs with BM-, AF and PL-MSCs. The arbitrary unit was intended as: Ct Housekeeping gene MSCs/Ct target gene MSCs - Ct Housekeeping gene PBMCs/Ct target gene PBMCs.

IDO expression

We analyzed IDO-1 and IDO-2 mRNA expression on MSCs after 5 days' co-culture. We observed that in human MSC cultures, co-cultured PBMCs were responsible for a modulation of IDO at both gene and protein levels. Interestingly, unstimulated BM-MSCs do not express detectable levels of IDO-1 and IDO-2 transcripts (uncalculated Ct). However, after treatment with PBMCs, an expression of IDO-1 transcripts was

observed. By contrast, unstimulated AF and PL-MSCs already had detectable levels of IDO-1 transcripts in unstimulated conditions but also in this case we observed a stimulated effect by co-cultured conditions quantifiable in 2.3 and 1.3 log for AF and PL, respectively. This increment increases in co-culture with PHA-BPMC up to 2.57 and 1.8 log for AF and PL, respectively. The fold of increment of IDO-1 transcripts for MSCs derived from BM was not calculable because the initial level in single cultures was not detectable. Moreover, unstimulated human AF and PL-MSCs did not express detectable levels of IDO-2 transcripts. However, after 5 days' co-culture with PBMCs, the expression of IDO-2 transcripts was observed only in MSCs derived from PL. By contrast, we observed a stimulated effect by co-cultured PHA-PBMCs. This increment is quantifiable in 0.77 log for PL and undetectable for BM and AF.
However, to verify that IDO-1 and IDO-2 mRNA expression correlated with its activity, we also determined IDO activity by quantifying conversions of Trp to Kyn under the same experimental conditions. Both untreated BM, AF and PL-MSCs had negligible productions of Kyn, but co-culture with PBMCs and stimulated PHA-PBMCs induced an increase in Kyn and an total abolition of the tryptophan in the culture. In particular, we observed a Kyn mean value of 11.71±13.4; 5.12±6.83 and 10.63±4.64 mM in the co-culture with unstimulated PBMCs and 10.30±9.0, 12.08±9.6 and 7.0±3.9 mM in the co-culture with PHA-PBMCs and BM-MSCs, AF and PL-MSCs respectively. No statistical differences were observed among 3 MSC sources although AF produced lower levels of Kyn.

DISCUSSION

MSCs represent a promising tool for cell therapies in regenerative medicine for their multipotent, bystander and immunomodulant properties. An increasing number of Phase I, Phase II, or a mixture of PhaseI/II studies using MSCs, are in progress for a range of therapeutic applications. Most MSCs used in these clinical trials are isolated from BM and are considered safe and efficacious for their multipotent and immunomodulant properties (www.clinicaltrials.gov). However, the clinical applications of BM-derived cells is limited for the relatively invasive procedure for sample collection, the difficulties of obtaining a sufficient number of MSCs to appropriately perform studies, and a marked reduction in cell number, proliferation, and differentiation capacity with age [18]. The need therefore remains to identify a SC source that is safe, easily accessible, provides high cell yield and for which cell

procurement does not provoke ethical debate. We isolated multipotent SCs from AF [26] that showed greater proliferative and differentiative potential than BM-MSCs. We also isolated multipotent SCs, with similar characteristics to AF-MSCs, from term placenta (data not published).

For this study, BM, AF and PL-MSC aliquots were thawed and: i) immunophenotipic, proliferative and differentiative characteristics were not modified by cryopreservation, and ii) their immunomodulant effects on T cells of healthy donors were compared. We demonstrated that MSCs isolated from the three different sources are multipotent SCs with the immunophenotypic characteristics and differentiative potential established by guidelines by the Cellular Therapy Society [20], even though MSCs derived from fetal tissues have a greater proliferative capacity associated with the expression of embryonic markers [27].

The ability to modulate the alloreactive immune response has been documented for MSCs derived from human BM, and our knowledge, there are no comparative studies between BM-MSCs and AF and PL-MSCs, although single articles, with controversial results, describe immunomodulant properties of AF-MSCs [28, 29] and PL-MSCs [30–32].

Since T cells are the primary cells in adaptive immune response, we evaluated and compared the inhibitory effects of MSCs on total activated T cells with a potent mitogen (PHA). A T cell proliferation assay showed inhibitory effects on PHA-stimulated PMBC in all co-culture conditions. We also tested the effects of MSCs on naïve T cells induced to differentiate in Th1 and Th2 effector cells but in these experiments, different modulations in proliferative activity, were observed (data not shown). However, these results suggest that T cell inhibition might be closely related to an interaction of these cells with other cells from innate immunity (such as dendritic and NK cells) not present in co-cultures with Th1- and Th2- induced cells alone.

The inhibition of T cell proliferation was higher in co-cultures with BM-MSCs, but when the absolute number of Treg was analyzed, in the same experimental conditions, they were significantly higher in co-cultures with AF and PL-MSCs than in those with BM-MSCs.

Moreover, when we analyzed the various T subsets we observed a statistically significant increase of naïve T cells in the co-culture with PHA-PBMCs and AF and PL-MSCs and a decrease of memory T cells in the co-cultures with BM and PL-MSCs. Also, the presence of MSCs induced a reversal of the ratio (compared to stimulated

PHA-PBMCs) of these subsets in the co-cultures in favor of both $CD4^+$ and $CD8^+$ naïve T cell subpopulations.

The beneficial effects of MSCs in cell therapy could be explained by the paracrine action of secreted cytokines. For this reason, major cytokines associated with pro-inflammatory and anti-inflammatory functions were analyzed and compared in our experiments We observed a distinctly high concentration of IL-12 in MSCs, especially in BM and PL-MSCs. Independently from their origin, MSC also produced a moderate concentration of TNF-α and negligible amounts of IL-2 and IFN-γ. PHA-stimulated PBMCs showed high levels of Th1 cytokines as reported in Lee et al [23–25]. In all co-culture experiments, we observed a decrease of all Th1 cytokines. The MSC/T cell interaction might block Th1 polarization because this phenomenon was also found for IL-12 produced in high concentrations also by MSCs. It is interesting to note that IL-4 and IL-10, the major anti-inflammatory cytokines, significantly increased in co-cultures with MSCs in AF and PL-MSCs. The same effect was observed for Th17 cytokines. As the MSCs, independently from their sources, produced high levels of both IL-6 and IL-17, it is difficult to interpret the significant increase of these cytokines in the co-culture of PHA-activated PBMCs with AF-MSCs. IL-6 is a well-known immune modulator that also inhibits apoptosis in antigen-stimulated [33] and resting T cells by sustaining the expression of the anti-apoptotic molecule Bcl-2 [34]. The Inhibition of IL-6 produced by MSCs results in an additional decrease in the proliferation of activated T lymphocytes *in vitro* in co-cultures with MSCs [35] and increases apoptosis in neutrophils [36]. IL-17 is a cytokine that has attracted attention due to its involvement in chronic inflammation, having critical roles in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, psoriasis, inflammatory bowel diseases, diabetes, and multiple sclerosis [37]. Moreover, Guo *et al*, showed that fetal BM-derived MSCs promote the expansion of human Th17 cells, but still inhibit the production of Th1 cells in an *in vitro* assay using $CD4^+$ T cells stimulated with PHA and recombinant IL-2 [38]. Moreover, a recent work identified a new subset of IL-17^+ MSC capable of inhibiting *C. albicans* growth and attenuating cell-based immunosuppression [39]. The authors described IL-17^+ MSCs distinct from bulk MSC population, which were unable to upregulate Treg or downregulate Th17 cells, suggesting that IL-17 production in MSCs directly impairs MSC-based immunomodulatory functions.

The molecular data indicated that unstimulated PBMCs with BM, AF and PL-MSCs

promoted the expression of the signature cytokines and master transcription factors directed to Th2 and Th17 cells differentiation, but inhibited the cytokines and master transcription factors directed to the Th1 differentiation. The results of Treg polarization were mixed: unstimulated PBMCs with PL-MSCs seem directed to the differentiation of Treg with up-regulation of Foxp3 and STAT5b mRNA transcription levels; PBMCs unstimulated with BM- and AF-MSCs showed an up-regulation of STAT5b but down-regulation of FOXP3. The data indicated that PHA-PBMCs with BM-PL and AF-MSCs show mixed results about the role of the MSC effect of the signature cytokines and master transcription factors directed to the differentiation of Th1/Th2/Th17 and Treg cells probably due to a confounding effect of PHA stimulus. Moreover, we have to consider, as demonstrated by Fan *et al*, that the highest expression of mRNA encoding IL-2, IL-6, IL-10, TNF-α, and IFN-γ occurred almost at the same time (an average of 8 hours) after PHA-stimulation [40]. Based on this observation, the mRNA levels detected after 5 days of co-cultures with MSCs might be inversely related to the protein production. These results confirmed a high, intermediate and almost absent mRNA detection, in PHA-activated PBMCs co-cultured with BM, PL and AF-MSCs, respectively, which reflected data obtained in protein detection.

All together, in line with other authors [41], these data showed that MSCs inhibit or limit inflammatory responses and promote the mitigating and anti-inflammatory pathway with an increase of Treg. The inhibition is only on Th1 cells, leading to a paradoxical increase of pro-inflammatory Th17 cells. As already suggested by other authors, a mechanism that might explain the late stimulating effect of MSCs on pro-inflammatory Th17 cells is the up-regulation of IL-6 levels in the cultures, since IL-6 is a main mediator of Th17 cell differentiation [35, 38, 42]. Furthermore, in agreement with the results in this study, IL-6 might also inhibit the differentiation of Th1 subset [35]. The increase of Treg might also be induced by the high concentration of released IL10. However, from these studies conclusions cannot be drawn on the separate roles of the different cytokines in either mediating inhibition directly or via inducing Treg. (e.g. by IL-10).

In addition, IL-4 and IL-10 are indicative of a Th2-deviated immune response and might be produced by a cellular compartment other than from the T cells in PBMCs. It is therefore important to investigate the role of MSCs in contact with dendritic cells. Our preliminary results show that AF-MSCs block dendritic cell maturation to a

greater extent than BM and PL-MSCs.

Luo *et al*. have recently reported that PBMC proliferation was suppressed by the AF-MSCs in a dose-dependent manner and the inhibitory effect was caused by increased IL-10 and IDO induction after co-culture [43]. Moreover, IDO was also considered a key mediator of the PL-MSC immunosuppressive effect. For this study, IDO-1 and IDO-2 mRNAs were analyzed and were undetectable in basal MSCs (independently from the sources) but were significantly higher in co-cultures with PBMCs alone and in stimulated PHA-PBMCs. PL-MSCs produced higher IDO-1and IDO-2 mRNA.

In conclusion, analyzing MSC properties and their effects on T cells, AF-MSC showed a more potent immunomodulant effect on T cells than BM-MSCs and only a slightly higher effect than PL-MSCs.

This study shows that MSCs isolated from fetal tissues may be considered a good alternative to BM-MSCs for clinical applications, as recently illustrated by Fierabracci et al. and in Ullah et al. [44, 45]**.** The clinical trials website (www.clinicaltrials.gov) reports about 30 trials based on the use of 'cells derived from placenta' but most of these studies have an unknown status. We believe that the safety should be the main focus in cell therapy and stem cell research today, and that this also concerns MSCs isolated from fetal tissues. For this reason, further studies are needed to provide a complete understanding of the mechanisms underlying the immunomodulatory effects of AF and PL-MSCs, which will ultimately allow the development of new and more effective strategies for regenerative medicine and transplantation to treat a wide range of conditions.

A further fact that should not be overlooked is that AF-MSCs are isolated from AF harvested from women undergoing amniocentesis, a routine, but still seen as an invasive procedure used for pre-natal diagnosis at 14–16 weeks of pregnancy. Moreover, we only isolated AF MSCs clones from the most abundant samples that contained at least 6 mL of AF [19]. In all the other samples, we observed heterogeneous clones also presenting epithelial characteristics, which makes it difficult to set up a standard MSC isolation and expansion protocol from AF. On the other hand, although the method of isolation of MSCs from placenta could be critical [46], PL, is a more abundant discharged fetal tissue than AF and might well be considered an excellent source of MSCs without any ethical problems and may thus be considered a good alternative to BM-MSCs for clinical applications.

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Author Disclosure Statement

All the authors have reported no conflicts of interest and no competing financial interests exist.

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4 THE ROLE OF MSCS INFUSED IN BONE MARROW GRAFTS AND EVALUATION OF MSC ENGRAFTMENT AFTER ALLOGENEIC HSCT IN PEDIATRIC PATIENTS

AIM 2

4.1 PAPER II: IN VITRO MESENCHYMAL PROGENITOR CELL EXPANSION IS A PREDICTOR OF TRANSPLANT RELATED MORTALITY AND ACUTE GVHD III-IV AFTER BONE MARROW TRANSPLANTATION IN UNIVARIATE ANALYSIS: A LARGE SINGLE CENTER EXPERIENCE

ORIGINAL ARTICLE

In Vitro Mesenchymal Progenitor Cell Expansion is a Predictor of Transplant-related Mortality and acute GvHD III-IV After Bone Marrow Transplantation in Univariate Analysis: A Large Single-Center Experience

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ABSTRACT

Mesenchymal stromal cells (MSCs) are multipotent stem cells able to differentiate into mesenchymal origin tissue and support the growth of hematopoietic stem cells (HSCs). In order to understand the role of MSCs infused in bone marrow (BM) grafts, 53 consecutive patients were analyzed for engraftment, acute and chronic Graft versus Host Disease (GvHD), transplant related mortality (TRM), relapse incidence (RI), and overall survival (OS). The MSC content was measured as MSC expansion at the second passage.

When *in vitro* expanded MSC (cumulative population doubling at second passage, cPDp2) values were stratified according to the median value (2.2 fold increase), the univariate analysis showed a significant difference in transplant related mortality (TRM, 23% vs. 3.8%, P= 0.05.) and in acute GvHD III-IV incidence (12% vs. 4%, P= 0.04), while the multivariate analysis did not confirm its independent role. No clinical parameters in donors and recipients were identified as predictors of cPDp2 expansion. Our study suggests a role for short-term *ex-vivo* expanded MSCs in reduced aGVHD III-IV incidence and TRM in univariate analysis. A multicenter, larger study is warranted to conform these data.

Keywords: Mesenchymal stem cells, bone marrow transplantation, acute GvHD, Transplant Related Mortality

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation is the treatment of choice for many malignant and non-malignant disorders[1,2].

MSCs, also known as mesenchymal stromal/stem cells, are non-hematopoietic stem cells which were originally defined as self-renewing, multipotent progenitor cells with multilineage potential to differentiate into other types of cells of mesoderm origin [3]. These cells also provide support for the growth and differentiation of hematopoietic progenitor cells in bone marrow (BM) microenvironments and, in animal models, promote engraftment of hematopoietic cells[4,5]. In co-culture experiments with allogeneic lymphocytes, MSCs do not induce lymphocyte proliferation, interferon-γ production, or an up-regulation of activation markers[6,7]. Furthermore, MSCs suppress proliferation of activated lymphocytes *in vitro* in a dose-dependent, non-Human Leukocyte Antigen (HLA)-restricted, manner[6,8]. It has also been shown that stromal cells may be damaged by chemo-radiotherapy before Hematopoietic Stem Cell

Transplantation (HSCT)[9], and from a clinical point of view, data on previous studies showed how the add back of stromal cells entrapped in filters during HSCT provided an advantage in terms of reduced graft-versus-host disease (GvHD) and lower transplant-related mortality (TRM)[10].

To date, the human bone marrow fibroblast colony-forming units (CFU-F) and the adherent ratio are the easiest parameters of MSC content in the graft, but the role of MSCs to 1) expand in vivo, 2) maintain stemmess in vivo and later be able to differentiate into a committed lineage and 3) survive and engraft in the recipient is under investigation. Since the role of MSCs transplanted in the BM graft is still not fully understood, and the role of CFU-F is still under debate, we decided to study the in vitro expansion ability of MSCs and, then, to compare their role in reducing transplant toxicity, and improving survival.

PATIENTS AND METHODS

Patients

A retrospective study was carried out of fifty-three patients, who were treated by allogeneic bone marrow (BM) transplantation at our center-Regina Margherita Children Hospital, Pediatric Onco-Hematology and Stem Cell Transplant Division- between March 2009 and October 2013. Allogeneic donor BM samples were used for MSC isolation and expansion. The patients'parents/legal representative signed the informed consent. The study was conducted in compliance with the principles of the Declaration of Helsinki.

Bone Marrow collection and infusion

Hematopoietic stem cells from BM were infused in our patients from 22 unrelated and 31 related donors. The number of total nucleated cells (TNC), CD34+ cells, CD3+ cells, Colony Forming Unit- Granulocyte Monocyte (CFU-GM) and Burst Forming Unit-Erythroid (BFU-E) and Long Term Culture-Initiating Cells (LTC-IC) present in BM collection were analyzed, as previously described[11], and calculated pro patient Kg.

MSC preparation and in vitro expansion

The whole BM sample was directly plated in alpha-Minimum Essential Media (MEM [SIGMA- ALDRICH®, LTO Irvine, Ayrshire, UK]) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich®) at a seeding density of 10,000 cells/cm^2. After 7 days,

the non-adherent cells were removed and discarded. The adherent cells were re-fed every 5-7 days and, when confluence was reached, cells were detached and re-plated for a further 3-5 passages at 1,000 cells/cm^2 as previously described[12]. We considered MSCs at Passage 1 (P1) as the initial time-point when the cells were harvested and re-plated. We considered this value as an index to quantify stromal progenitors. The same cellular and culture conditions were maintained by the cellular plating of whole BM and during the expansion process. The following passages were coded with increasing numbers: P2, P3, etc. To evaluate cellular growth, the cell growth rate in terms of population doubling (PD) was computed using the formula (log N/log 2), where N indicated the rate (the cell number of the detached cells divided by the initial number of seeded cells) and the expansion in terms of cumulative PD (cPD). The cells were characterized as indicated by the International Society for Cellular Therapy[13] and minimal criteria were met for them to be defined as MSCs(Katia Mareschi et al. 2012c; Castiglia et al. 2014b)

In particular, at each passage the cells were analyzed for viability, immunophenotype, and proliferative potential and also they were differentiated in osteoblasts, chondroblasts and adipocytes as previously reported(Katia Mareschi et al. 2012c; Castiglia et al. 2014b).

Statistical analysis and Methods

The primary endpoint of this study was to evaluate the role of MSCs on reducing transplant related mortality (TRM) while the secondary endpoints were to evaluate: i) neutrophil and platelet engraftment, ii) GvHD II-IV cumulative incidence, ii) acute GVHD III-IV cumulative incidence, iv) chronic GvHD cumulative incidence, iv) 3-y relapse incidence (RI), and v) 3-year overall survival (OS). The RI was calculated for malignancies only.

Absolute neutrophil count (ANC) engraftment was defined as the first of three consecutive days of ANC $\geq 0.5 \times 10^9$/L, while platelets (PLT) engraftment was defined as the first of three consecutive days with PLT$\geq 50 \times 10^9$/L without transfusion. Acute and chronic GvHD were classified according to Seattle criteria[15,16]. TRM was defined as the probability of dying without recurrence. Relapse was considered the competitive event to compute TRM. RI was defined as the probability of disease recurrence. OS was defined as the probability of survival irrespective of the disease state. Graft failure was considered as a relapse for non-malignant disease, while for malignancies, graft

failure patients were censored. Patients were stratified according to the median value and each outcome was calculated according to these subgroups.

The acute GvHD II-IV, acute GvHD III-IV, chronic GvHD, TRM and RI cumulative curves were calculated by NCSS software, while the statistical differences were calculated by the Gray-test using the R-package[17]. The OS was calculated by Kaplan-Meier statistics[18]; the log-rank test[19,20] was used to calculate the p values. A p-value below 0.05 was considered as statistically significant.

RESULTS

A total of fifty-three consecutive patients underwent HSCT from both related (31, 58%) and unrelated donors (22, 41%). The median age at HSCT was 8.6 years (0.6-24.8) and the main indication for HSCT was acute leukemia (32 patients, 60%). Ten patients underwent HSCT for non-malignant disease (19%). Ten patients received HSCT in first complete remission (CR1, 19%), sixteen patients in CR2 (30%), three patients in CR3 (6%). Forty patients (75%) received a myeloablative conditioning regimen, the majority of donors were males (37, 75%), and the median donor age was 25 years (3-41). See Table 4-1for details.

A median of 5.1×10^8 total nucleated cells (TNC)/kg (range 1.7-15.8) and 5.8×10^6 CD34+/Kg (range 1.2-16.7) and 53.1 $\times 10^6$ CD3+/Kg (13-177.2) were infused.

	Patient characteristics	**N = 53 (100%)**
Gender M/F		30/23
Patient age		8.6 (0.6-24.8)
Disease	AML	10 (19%)
	ALL	22 (41%)
	Non Hodgkin Lymphoma	2 (4%)
	Non malignant disease	10 (19%)
	Hodgkin lymphoma	1 (2%)
	Myelodysplastic Symdrome	3 (6%)
	Neuroblastoma	5 (9%)
Disease status at HSCT		
	CR1	10 (19%)

	CR2	16 (30%)
	CR3	3 (6%)
	PR	5 (9%)
	Untreated/Present disease	19 (36%)
CMV	Negative	5 (9%)
	Positive	49 (92%)
HLA match	Identical Sibling	21 (40%)
	Unrelated 10/10	14 (26%)
	Unrelated ≤9/10	18 (34%)
Conditioning	Myeloablative	40 (75%)
	Nonmyeloablative	13 (24%)
GvHD prophylaxis	Cy-A	10 (19%)
	Cy-A+MTX	12 (23%)
	Cy-A+MTX+ATG	31 (58%)
Donor age		25 (4-57)
Sex mismatch		13 (24%)
		40 (75%)
TNC ($x10^8$)/kg		5.1 (1.7-15.8)
CD34+ ($x10^6$)/kg		5.8 (1.2-16.7)
CD3+ ($x10^6$)/kg		53.1 (13-177.2)
CFU-GM ($x10^4$)kg		19.9 (3.5-103.4)
BFU-E ($x10^4$)/kg		16.1 (3.9-59.9)
LTC-IC ($x10^2$)/kg		11.1 (0.6-144.5)

Table 4-1 Patient's characteristics: M: Male, F: Female, AML: Acute Myelogenous Leukemia, ALL: Acute Lymphoblastic Leukemia, CR1: first complete remissione, CR2: second complete remission, CR3: third complete remission, PR: partial remission, GvHD: Graft versus Host Disease, Cy-A: Cyclosporine-A, MTX: Methotrexate, ATG: polyclonal Anti-T Globulin, TNC: Total Nucleated Cells, CFU-GM: Colony Forming Unit-Granulocyte Monocyte, BFU-E: Burst Forming Unit Erythroid, LTC-IC: Long Term Culture-Initiating Cells

Isolation and analysis of MSCs

The time and the number of passages of MSC expansion was variable among patients, however we isolated and expanded MSCs until the second passage from all the patients with a median time for the P1 of 15 days (range: 11-20 days) , for the P2 of 28 days (range: 18-40) and for P3 of 38 days (range: 28-50). Isolated MSCs were negative for all hematopoietic antigens and for HLA-DR, and expressed more than 90% of CD90, CD73, CD105 and CD146 as showed in the Table 4-2. Isolated MSC were also able to differentiate into osteoblasts, adypocytes and chondrocytes[12,14].

Since all patient's MSC were cultured until the second passage, we considered cPDp2 a value to analyze the MSCs. As shown in Table 4-3 the ANC and PLT engraftment speed was not affected by the MSC growth. A trend for better OS was observed for patients having higher cPDp2 values (P>0.05). We observed a reduced TRM for patients having an HSCT content of cPDp2 above the 50th percentiles (P=0.05). A wisker plots graph shows the cPDp2 growth differences among TRM and non TRM patients (Figure 1). Acute GvHD II-IV was not significantly different among the groups (P=NS), while severe aGvHD III-IV was reduced in patients having a higher expansion of MSC (P=0.04.). The cGvHD incidence and the relapse incidence did not differ among the groups (P=NS).

	p1	p2
C90+	> 90% (91-100%)	> 90% (91-100%)
CD73+	> 90% (91-100%)	> 90% (91-100%)
CD105+	> 90% (91-100%)	> 90% (91-100%)
CD146+	> 80% (50-99%)	> 80 %(33-98%)
HLA-DR+	< 10% (0-8%)	< 2% (0-1%)
CD14+/34+/45+/19+	< 10% (0-8%)	< 2% (0-1%)

Table 4-2. MSC characteristics: immunophenotype analyzed by cytofluorimetric analysis

	CPDp2 above the median	CPDp2 below the median	P
ANC	20 (11-32)	20.5 (14-32)	NS
PLT	23 (12-191)	29 (21-125)	NS
TRM	3.8% (0-26)	23% (11-46)	0.05
RI	38% (23-62)	23% (11-46)	NS
GvHD II-IV	32% (18-56)	36% (21-60)	NS
GvHD III-IV	4% (0-27)	12% (4-34)	0.04
Chronic GvHD	21% (10-47)	23% (10-49)	NS
OS	60% (41-79)	54% (34-74)	NS

Table 4-3. Outcome of patients according to cPDp2 count. ANC: absolute neutrophil count, PLT: platelets, TRM: transplant related mortality, RI: relapse incidence, OD: overall survival

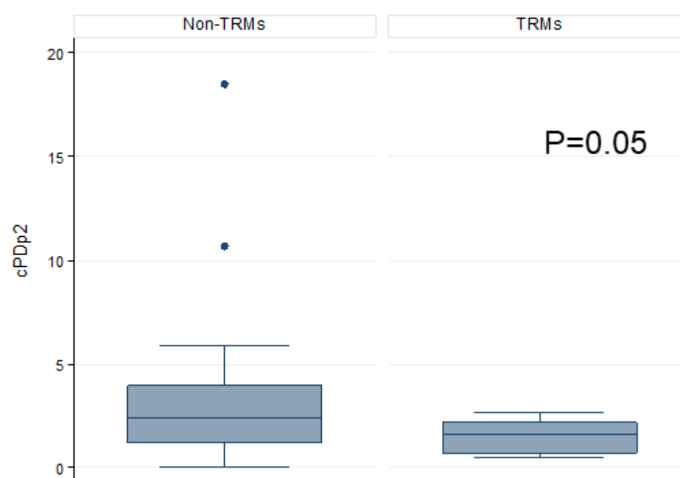

Figure 4-1. Wisker plots distribution of cPDp2 growth among TRM and non-TRM patients

Multivariate analysis

The multivariate model shows that cPDp2 growth values does not play a significant independent role on TRM risk (Hazard Ratio [HR] = 0.99, CI 95% 0.98-1.01), while the HLA match (unrelated donor with HLA match below 9/10 [HR = 3.23, CI 95% 0.33-32.2]) and the conditioning regimen (myeloablative [HR = 2.19, CI 95% 0.22-22.1]) seem to increase the risk of TRM even if the small numbers do not allow to reach the statistical significance. The GvHD III-IV risk shows a similar trend, even if the reduced number of the sample makes the interpretation even more uncertain.

Factors affecting cPDp2 growth

When we analyzed factors to assess a predictive role for higher cPDp2 content, no significant effects for the patient and donor age, gender, type of disease, conditioning regimen, TNC dose, CD34+ dose or CD3+ dose were observed, in particular no significant relation between donor age and expansion potential was observed.

Causes of death

Five patients having higher cPDp2 growth died. Two patients for disease progression (40%), one patient for post-transplant lymphoproliferative disease (20%), one for acute respiratory distress syndrome (ARDS, 20%) and one for acute GvHD (20%).
Ten patients having cPDp2 expansion below the median died. Four patients for disease progression (40%), one for pulmonary thrombosis (10%), one for ARDS (10%), one for fungal infection (10%), one for viral infection (10%) and finally one for acute GvHD (10%).

DISCUSSION

The role of MSC progenitors in the setting of HSCT still needs clarifying and is still matter of debate. Lazarus *et al.* [21] were the first to show that it was safe to give HLA-identical MSCs to allogeneic HSCT recipients showing MSCs of donor origin in 2 out of 18 patients (2% to 14% of the MSCs obtained from BM culture). A further two papers dealt with the role of MSCs in two particular HSCT settings: Bernardo *et al.* (M. E. Bernardo et al. 2011) used MSCs at the time of HSCT transplantation in recipients of cord blood grafts. Although they did not notice any differences in hematological recovery or the rejection rate, there was a significant decrease of grade III and IV acute GvHD incidence in their study cohort when compared with controls (P=0.05). Ball *et al*[23] performed co-transplantation of MSCs after haploidentical HSCT showing how multiple MSC infusions were safe and effective in children with steroid-

refractory aGvHD, especially when employed early in the disease course. In addition, no increase of infections was documented.
Moreover, Kuzmina *et al.* [24], in a randomized prophylactic study, were able to show reduced acute GVHD II–IV from 33% to 5% for patients who received MSCs, but also in this series no improved survival was reported.
While all these studies agree on the safety and efficacy of MSC administration on GvHD, they do not offer any conclusive data on benefits for a better survival[10,24]. With the exception of very few studies, significant heterogeneity was observed in age, diagnoses, conditioning regimens, HLA matching, hematopoietic stem cell source, MSC source and indication (prophylaxis or treatment) and culture conditions (Rizk *et al* for reviews[25]).
Some considerations can be made if our findings are compared to other studies. Our data come from a pre-clinical study, while others are from clinical trials meaning that the MSC growth and expansion we observed were not affected by infections, drug toxicity, etc. that might well have a role on MSC activity after transplantation. Moreover data on MSC survival after HSCT are still lacking and difficult to obtain and very few studies have reported the engraftment of those cells, thus indicating a low engraftment of transplanted MSCs(Blazsek et al. 1999; E. M. Horwitz et al. 1999; Edwin M. Horwitz et al. 2002). However, in univariate analysis we demonstrated that the *ex-vivo* MSC progenitor expansion is statistically associated with both reduced GVHD III-IV occurrences and lower TRM while, as previous reported in clinical trials, no effect on overall survival was highlighted.
If numerous papers have reiterated the persistence of mesenchymal cells of recipient origin even after years from HSCT, a possible interpretation could be linked to a rapid effect of the transplanted MSCs within the graft that could be able to reduce the lymphocytes alloreactivity present in the bone marrow. This effect would then be subsequently lost due to the entrapment of MSCs in the pulmonary rather than splenic or hepatic reticuloendothelial system. In particular, two recent papers dealt on immunosuppressive ability of MSC: Klinker *et al.* were able to show a linear relation between MSC morphology and immunosuppressive activity while Bloom *et a*l. developed a reproducible assay to measure allogeneic MSC-mediated suppression of CD4+ lymphocytes, showing a suppression of T cell proliferation ranging from 27 to 88% according to standard MSC products [29-30].
When we focused on factors predicting a higher cPDp2 content, no significant effects

for the patient or donor ages, the donor relationship, gender, type or disease, conditioning regimen, TNC dose, CD34+ dose or CD3+ dose were observed, even when a subgroup analysis (related vs. unrelated donor) was carried out.

In conclusion, we found a direct relation between *ex-vivo* MSC expansion and clinical outcomes. In univariate analysis we have shown how the MSCs in the BM graft measured by *in vitro* cPDp2 is a clinical predictor of TRM and of severe acute GvHD. Despite lacking predictive patient-, donor- or transplant related factors to cPDp2, our study strengthens previous findings of the benefit of MSCs on HSCT outcomes and shows that short-term *ex-vivo* MSC expansion has a better prognostic value. A prospective study is ongoing to test MSC chimerism and survival following HSCT, while a larger multicenter study is warranted to confirm the role of MSC growth after HSCT.

Author Disclosure

All the authors have reported no conflicts of interest and no competing financial interests exist.

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4.2 Paper III: Analysis of Mesenchymal Stromal Cell Engraftment After Allogeneic HSCT in Pediatric Patients: A Large Multicenter Study

ORIGINAL ARTICLE

Analysis of Mesenchymal Stromal Cell Engraftment After Allogeneic HSCT in Pediatric Patients: A Large Multicenter Study

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ABSTRACT

Background: the mesenchymal stem cell (MSC) role after allogeneic haematopoietic stem cell transplantation (HSCT) is still matter of debate, in particular MSC engraftment in recipient bone marrow is unclear.

Methods: a total of 46 patients were analyzed for MSC and haemopoietic stem cell engraftment after HSCT. The majority of patients had bone marrow as stem cell source and acute leukemia was the main indication for HSCT. Mesenchymal and haemopoietic stem cell chimerism analysis was carried out through specific polymorphic tandemly repeated regions (STRs).

Results: in this series all patients reached complete donor engraftment, no evidence of donor derived mesenchymal stem cell engraftment was noted.

Conclusions: our data indicate that MSCs after HSCT remain of recipient origin despite: i) myeloablative conditioning, ii) the stem-cell source, iii) the interval from HSCT to BM analysis (3,4), iv) the underlying disease before HSCT, v) the patients' or the donors' age at HSCT.

INTRODUCTION

Bone marrow hematopoiesis takes place in defined niches where several cell types can be identified. Among these cells the mesenchymal stem cells (MSCs) are able to support hematopoiesis through the production of several growth factors. Bone marrow-derived MSCs retain the potential to differentiate readily into adipocytes and chondrocytes and, with variable efficiency, into a variety of further cell types (1). Furthermore, they can grow readily in culture, where they proliferate extensively with an adherent, fibroblastic-like morphology. Because MSCs support hematopoiesis and some studies have highlighted a beneficial for graft-versus-host disease (GvHD) treatment, it is of interest to determine whether these cells are susceptible to conditioning therapy, engraft with donor hematopoietic stem cells or depend on the graft source. This information will be necessary to understand in more detail the process of engraftment and, possibly, also the immunological events after allogeneic HCT. The aim of this study was to determine if donor-derived MSCs could be identified after HSCT in a heterogeneous group of patients and if there are any correlations with conditioning regimen (myeloablative vs. non-myeloablative), the source of the graft (bone marrow [BM], peripheral blood stem cells [PBSC] or cord

blood [CB]), the interval from HSCT, the patient's or the donor's age at HSCT.
Despite it is well known that literature data have shown how mesenchymal stromal cells remain of recipient origin in adult patients, we studied if this was also confirmed in a large heterogeneous pediatric population in more recent years.

Patient and methods

From March 2013 to March 2017 a total of 46 recipients of HSCT were analyzed for HSC and MSC chimerism (Table 1). The majority of our patients had HSCT for acute leukemia (ALL 45%, AML 37%), Twenty-seven received BM grafts (59%), 18 patients had PBSC as the stem cell rescue (39%), one patient had a CB rescue (2%). Thirty-seven patients received myeloablative conditioning (37, 80%), nine patients had non-myeloablative conditioning (9, 19%). Written consent was obtained from all the patients or their legal guardians.

Patients		46 (100%)
Gender	Male	29 (63%)
	Female	17 (37%)
Patient's age		13.5 (1-67)
Donor's age		25 (0-56)
Diagnosis	AML	17 (37%)
	ALL	21 (45%)
	Myelodysplastic syndrome	2 (4%)
	NHL	1 (2%)
	Neuroblastoma	1 (2%)
	Multiple myeloma	1 (2%)
	Nonmalignant disease	3 (6%)
Days after transplant		138 (29-1715)
Stem cell source	Bone marrow	27 (59%)
	Peripheral Blood	18 (39%)
	Cord Blood	1 (2%)
Conditioning	Myeloablative	37 (80%)
	Nonmyeloablative	9 (19%)
Hematopoetic cell chimerism		Full donor 100%

Mesenchymal cell chimerism		Full recipients 100%

Table 4-4 Details of patient' demographics. AML: acute myeloid leukemia, ALL: acute lymphoblastic leukemia, NHL: Non Hodgkin Lymphoma.

MSC Isolation

BM cells were plated and expanded in α-minimum essential medium (Cambrex Bioscience) with 10% of fetal bovine serum in 75 cm2 T-flasks, 2 mmol/L L-glutamine, and penicillin/streptomycin (1×).

MSC Differentiation

The culture was maintained at 37°C in a 5% CO2 atmosphere. After 5 to 7 days, nonadherent cells were removed, whereas the adherent cells were expanded until they reached confluence (14 to 20 d of culture). The MSCs were then harvested by treatment with trypsin/EDTA (Cambrex Bioscience) for 5 minutes at 37°C and used for further analysis.

MSC Flow Cytometry

The cells were then characterized according to the International Society for Cellular Therapy.2,3 Phenotypic characterization was performed using the following antibodies combination: anti-CD90 FITC, CD73 PE, CD34 FITC, CD14 FITC, CD45 FITC, HLA-DR PE, CD105 PC7, and CD19 APC (Miltenyi Biotec, Bologna, Italy). Data acquisition was performed using Navios (Beckman Coulter). Only cells negative for hematopoietic antigens (CD34, CD14, CD45) and for HLADR, and expressing >90% of CD90, CD73, and CD105, could be considered as MSCs and used for chimerism analysis (Figure 4-2 Representative immunophenotype of in vitro expanded mesenchymal cells. Light gray. histogram: positive reactivity for the specific antibodies against each CD marker. Dark gray histogram: isotype controls.).

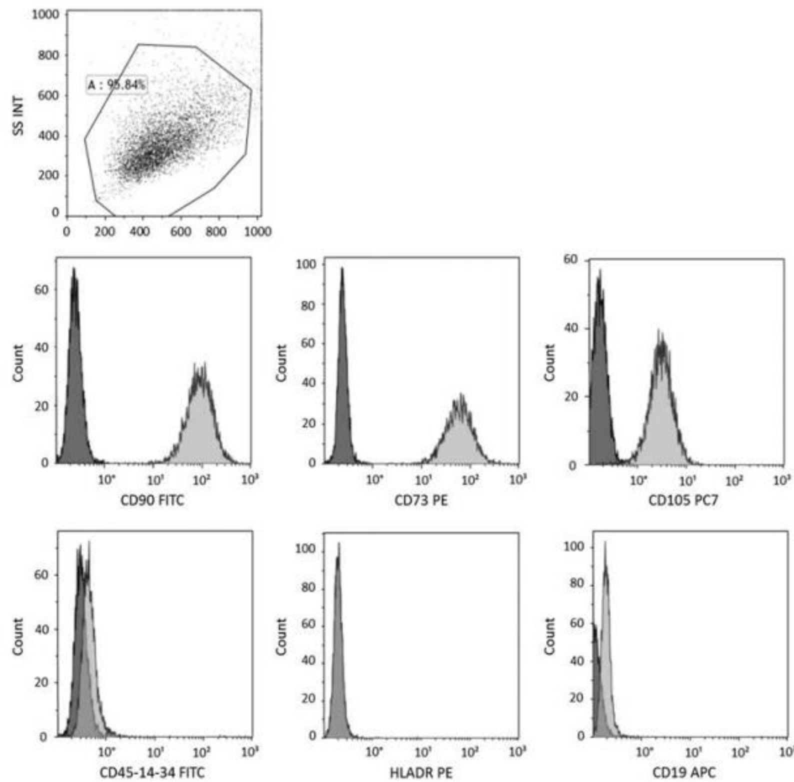

Figure 4-2 Representative immunophenotype of in vitro expanded mesenchymal cells. Light gray. histogram: positive reactivity for the specific antibodies against each CD marker. Dark gray histogram: isotype controls.

Chimerism Analysis

Specific polymorphic tandemly repeated regions (STRs) were amplified by means of the polymerase chain reaction (PCR) following the specific manufacturers' instructions. After cell detachment, nuclear DNA was extracted and amplified (AmpFISTR Identifiler kit; Applied Biosystems). This assay allows the amplification of 15 STR loci plus the sex-determining locus amelogenin (CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA, D2S1338 and D19S433) (X) that were resolved by capillary electrophoresis (3500 Ruo Genetic Analyzer, Applied Biosystems) and analyzed by comparing the genotype electropherograms of the BM recipient following HSCT (BM and mesenchymal cells) with those of the donor and the BMT recipient before HSCT.4 The same technique was used to test the engraftment of donor hematopoietic cells.

RESULTS

While all patients had complete donor hematopoietic engraftment (100%), full

recipient chimerism (100%) was observed in all patients when we analyzed the mesenchymal cell cultures despite: i) myeloablative conditioning; ii) the stem-cell source (BM vs. PBSC vs. CB); iii) the interval from HSCT to BM analysis (from day +29 to 1715); iv) the underlying disease before HSCT (malignant vs. nonmalignant), v) the patients' or the donors' age at HSCT. In particular, nested-PCR was not carried out because the clinical role of engraftment under 1% was considered clinically negligible.

DISCUSSION

The significance of the host nature of MSCs is still not clear. Although there is experimental evidence suggesting the presence of a common mesoderm cell, it is still controversial whether transient or durable engraftment of native donor-derived MSCs without *ex vivo* treatment can occur in allogeneic HSCT recipients. Other studies showed that, irrespective of the use of MSC co-administration, the post-transplant chimerism of BM-derived MSCs after allogeneic HSCT has been reported to remain of host origin, suggesting that the infused donor MSCs are either immunologically rejected or not capable of long-term engraftment in the host
BM microenvironment (5). These results, obtained mainly from BM of pediatric patients (30 patients, 65%), are consistent with the relative resistance of stromal cells to myeloablative conditioning therapy (including total body irradiation, 20 patients [43%]), so that regenerative demand can be satisfied by the surviving intrinsic MSCs. However, while the absence of donor-derived MSCs might also reflect an insufficient number of MSCs in the graft or a poor capability of transplanted MSCs to engraft, the transplantability of bone marrow stem cells has been demonstrated in patients with osteogenesis imperfecta (5). In contrast with results that we and others observed, a further aspect of interest is the clinical evidence that MSCs, also third parties, were shown able to i) treat acute GvHD (8) and ii) to prevent GvHD even after haploidentical or cord stem cell transplantation (9, 10). Therefore, the role of MSC after allogeneic stem cell transplantation remains to be defined.
If it is true that MSCs are able to control GvHD and if it is equally true that donor MSCs do not replace the patient's marrow stroma, perhaps the analysis of MSC should be targeted at the lymph node germinal centers where the auto-allo reactive T-cell clones might be responsible for triggering and maintaining the GvHD cascade.

Furthermore, the conventional method of HSC chimerism (PCR amplification of STRs followed by fragment analysis), has a 1–5% sensitivity limit which is probably insufficient for expected levels of MSC engraftment (11). However, BM cells were not analyzed by nested-PCR, since the sensitivity limit was considered clinically negligible.

To get a better understanding of the fate of donor-derived MSCs after allogeneic HSCT or some other MSC based therapy, the development of more sensitive methods to track infused MSCs is required.

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5 ANALYSIS OF NEW STEMNESS MARKERS, THEIR EXPRESSION AND THEIR EPIGENETIC REGULATION ON BM-MSCS

AIM 3

5.1 Paper IV H3K4me1 marks DNA regions hypomethylated during aging in human stem and differentiated cells

H3K4me1 marks DNA regions hypomethylated during aging in human stem and differentiated cells

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ABSTRACT

In differentiated cells, aging is associated with hypermethylation of DNA regions enriched in repressive histone posttranslational modifications. However, the chromatin marks associated with changes in DNA methylation in adult stem cells during lifetime are still largely unknown. Here, DNA methylation profiling of mesenchymal stem cells obtained from individuals aged 2 to 92 identified 18735 hypermethylated and 45407 hypomethylated CpG sites associated with aging. As in differentiated cells, hypermethylated sequences were enriched in chromatin repressive marks. Most importantly, hypomethylated CpG sites were strongly enriched in the active chromatin mark H3K4me1 in stem and differentiated cells, suggesting this is a cell type-independent chromatin signature of DNA hypomethylation during aging. Analysis of scedasticity showed that interindividual variability of DNA methylation increased during aging in MSCs and differentiated cells, providing a new avenue for the identification of DNA methylation changes over time. DNA methylation profiling of genetically identical individuals showed that both the tendency of DNA methylation changes and scedasticity depended on non-genetic as well as genetic factors. Our results indicate that the dynamics of DNA methylation during aging depend on a complex mixture of factors that include the DNA sequence, cell type and chromatin context involved, and that, depending on the locus, the changes can be modulated by genetic and/or external factors.

INTRODUCTION

Genomic DNA methylation is known to change during lifetime and aging (Jaenisch and Bird 2003). Some changes play important roles in development but others occur stochastically without any apparent biological purpose (Fraga 2009; Feil and Fraga 2012). These molecular alterations, which are known as the epigenetic drift, are currently being investigated as they have been proposed to account for many age-related diseases (Bjornsson *et al.* 2004; Heyn *et al.* 2013; Timp and Feinberg 2013). Various recent studies using 1.5K and 27K Illumina methylation arrays have identified a group of gene promoters in blood that become hypermethylated during aging (Christensen *et al.* 2009; Rakyan *et al.* 2010; Teschendorff *et al.* 2010; Bell *et al.* 2012; Fernandez *et al.* 2012). Interestingly, some of these studies have also shown that these DNA sequences are enriched in bivalent chromatin domains in embryonic stem cells (Rakyan *et al.* 2010; Fernandez *et al.* 2012; Heyn *et al.* 2012) and repressive histone marks such as H3K9me3 and H3K27me3 in differentiated cells (Rakyan *et al.* 2010), and that many of them are also frequently hypermethylated in cancer. However, drawing conclusions from some of these studies is limited by their low genome coverage (less than 0.1%) and the location of the sequences analyzed (mainly at gene promoters). Further studies using 450K methylation arrays and larger cohorts (Heyn *et al.* 2012; Hannum *et al.* 2013; Johansson *et al.* 2013) have, though, corroborated most of the previous observations with the 27K methylation arrays and have, in addition, identified new sets of genes that become hypermethylated and hypomethylated during aging in humans. Finally, a recent study that analyzed the genome wide DNA methylation status of newborns, middle-aged individuals and centenarians confirmed the results obtained with the methylation arrays and showed that aging is associated with overall hypomethylation, which primarily occurs at repetitive DNA sequences (Heyn *et al.* 2012). Most of the above studies were conducted with blood and, consequently, changes in cell heterogeneity during aging could have affected the results (Calvanese *et al.* 2012; Houseman *et al.* 2012). However, some genes presented consistent changes in different tissues which indicates that, in some cases, the changes truly are associated with aging (Rakyan *et al.* 2010; Horvath *et al.* 2012). Interestingly, Houseman and colleagues (Houseman *et al.* 2012) have recently reported an algorithm that, using the DNA methylation values of certain genes, estimates the relative proportion of the different blood cell types in a specific sample. This algorithm was successfully used by Liu and colleagues in a study to

identify DNA methylation alterations associated with rheumatoid arthritis (Liu *et al.* 2013).

In addition to the studies using blood, other works have identified specific DNA methylation signatures of aging in differentiated cell types including brain (Hernandez *et al.* 2011; Numata *et al.* 2012; Guintivano *et al.* 2013; Lister *et al.* 2013), muscle (Zykovich *et al.* 2014) and saliva (Bocklandt *et al.* 2011). Two studies have analyzed DNA methylation during aging in human adult stem cells: Bork and colleagues (Bork *et al.* 2010) used 27k methylation arrays to analyze the DNA methylation status of mesenchymal stem cells (MSCs) obtained from young (21–50 years) and old donors (53–85 years) and found similar DNA methylation changes over time during prolonged *in vitro* culture and *in vivo* aging. Using the same methylation arrays, Bocker and colleagues (Bocker *et al.* 2011) observed a bimodal pattern of methylation changes in older hematopoietic progenitor cells, with hypomethylation of differentiation-associated genes, as well as *de novo* methylation events resembling epigenetic mutations. Recent studies in mice have revealed a number of genome-wide alterations in DNA methylation (Taiwo *et al.* 2013) which might play an important role in the functional decline of hematopoietic stem cells during aging (Beerman *et al.* 2013). To study the role of DNA methylation in adult stem cell aging further, the present study used 450K methylation arrays to characterize the genome wide DNA methylation status of bone marrow MSCs obtained from individuals aged between 2 and 92. We then systematically compared our results with previously published data to identify the chromatin signatures associated with DNA methylation changes in adult stem cells and to determine whether these changes were also present in other tissues. Finally, we analyzed monozygotic twins of different ages to determine the effect of genetic factors on the DNA methylation changes during aging identified in our study.

RESULTS

Global DNA methylation profiling in adult stem cells

To identify DNA methylation changes during MSC aging we compared the DNA methylation status of 429789 CpG sites in 34 independently isolated MSC lines, obtained from individuals from 2 to 92-years old, using the Illumina Infinium® Human Methylation 450K BeadChip (**TABLE 5-1.** *Supplemental Table 1.Information available on donors and experiments carried out in the human mesenchymal stem cells included in the study*).

Using an empirical Bayes moderated t-test (see Materials and Methods) we first identified 64142 autosomal CpG sites which were differentially methylated (dmCpGs) (FDR<0.05) between MSCs obtained from young (ages ranging from 2 to 22) and elderly (aged between 61 and 91) individuals. Hierarchical clustering of all samples using the dmCpGs alone enabled each sample to be correctly allocated to its corresponding age group (**FIGURE 5-5-1**). Of the dmCpG sites, 18735 (29.20%) had become hypermethylated and 45407 (70.80%) hypomethylated with aging (**FIGURE 5-1B**).

To study, from a functional genomics point of view, the characteristics of these dmCpG sites we first determined their distribution within the different regions of the CpG islands (Wu *et al.* 2010). Interestingly, both hyper- and hypomethylated CpG sites were enriched in non-CGI (chi-square test; p<0.001, OR=2.58 and p<0.001, OR=1.76 respectively) (**FIGURE 5-1 C**) and in intragenic DNA regions (chi-square test; p<0.001, OR=1.23 and p<0.001, OR=1.34 respectively) (**FIGURE 5- 1 D**).

To validate the results obtained with the methylation arrays, we randomly selected 5 of the sequences previously identified and analyzed their methylation status by bisulfite pyrosequencing in an independent cohort of 46 MSCs obtained from individuals from 7 months to 80-years old (**TABLE 5 1).** In total in the validation phase we obtained information on the DNA methylation status of 950 CpGs. The sequences selected corresponded to the genes *HAND2* and *SIX2,* which become hypermethylated with aging, and to the genes *TBX15*, *PITX2* and *HOXA11*, which become hypomethylated. Bisulfite pyrosequencing results showed that all the sequences selected for validation displayed the same DNA methylation dynamics during aging as in the study samples (**Figure 5-1 E**).

Tissue-specific DNA methylation changes during aging

Global DNA methylation patterns are tissue/cell type-specific (Calvanese *et al.* 2012). To determine whether the CpG sites displaying DNA methylation changes during aging in adult stem cells are also affected in differentiated tissues, we used the same workflow described in the previous section to analyze the data obtained in previous aging studies which used the same methylation arrays with samples from blood (human whole blood from a mixed population of 426 Caucasian and 230 Hispanic individuals, with ages ranging from 19 to 101) and brain (neuronal and glial cells, from post mortem frontal cortex of 29 healthy individuals (14 male, 15 female, aged 32.6 ± 16.1) (Guintivano *et al.* 2013; Hannum *et al.* 2013). To reduce confounding factors in the Blood dataset, we first corrected for cellular heterogeneity (Houseman *et al.* 2012) to filter out only those associations which were the consequence of aging. Using this approach we identified 63512 hypermethylated and 60155 hypomethylated sequences in blood (FDR<0.05), 11603 hypermethylated and 14143 hypomethylated sequences in glial cells (FDR<0.05) and 5171 hypermethylated and 2380 hypomethylated sequences in neural cells (FDR<0.05). As in MSCs, hypomethylated cytosines in the differentiated cells preferentially occurred at both non-CGI regions (chi-square test; blood, p<0.001, OR=2.35; neural, p<0.001, OR=1.74; glial, p<0.001, OR=3.03) and at intragenic regions (chi-square test; blood, p<0.001, OR=1.11; neural, p<0.001, OR=2; glial, p<0.001, OR=1.89). However, in brain samples (neuronal and glial cells), hypermethylated cytosines occurred preferentially at both non-CGI regions (chi-square test; neural, p<0.001, OR=1.43; glial, p<0.001, OR=1.43) and at intragenic regions (chi-square test; neural, p<0.001, OR=1.1; glial, p<0.001, OR=1.1), while they occurred preferentially in both CGIs (chi-square test; p<0.001, OR=3.5) and at promoter regions (chi-square test; p<0.001, OR=1.49) in blood samples.

To identify possible cell type-independent DNA methylation signatures of aging, we created two additional datasets containing the hyper- and hypomethylated probes from selected subsets of the different tissues analyzed (**Figure 5-1F**). This approach showed only a small overlap between MSC and differentiated cells (42 hypomethylated and 38 hypermethylated), suggesting that systemic DNA methylation changes during aging are restricted to specific regions of the genome (**Figure 5-1F**).

Hypermethylated CpG sites during aging are associated with repressive chromatin marks

In blood, DNA hypermethylation during aging has been shown to occur at gene promoters enriched in repressive histone marks such as H3K9me3 and H3K27me3 (Rakyan *et al.* 2010). To identify possible chromatin signatures associated with DNA hypermethylation during aging in adult stem cells, we compared our methylation data with previously published data on a range of histone modifications and chromatin modifiers in 10 different cell types obtained from healthy individuals (see Materials and Methods). In the present study we found statistically significant associations with the repressive histone marks H3K9me3, H3K27me3 and EZH2 in most differentiated ENCODE cell lines (Fisher exact test; $p<0.001$) (**Figure 5-2**), which is in line with previously published data (Rakyan *et al.* 2010). To determine whether these observations can be extrapolated to other cell types, we used the same approach to analyze the CpG sites which are hypermethylated during aging in blood, neural and glial cells (Guintivano *et al.* 2013; Hannum *et al.* 2013) . The results showed that hypermethylated CpG sites in blood and brain were enriched in the same chromatin marks identified in the adult stem cells (**Figure 2**), suggesting that chromatin context is an important cell type-independent mark of DNA hypermethylation during aging. Analysis of the 38 commonly hypermethylated CpG sites in blood, MSCs and neural and glial cells also showed statistically significant associations ($FDR<0.05$) with the repressive histone marks H3K9me3, H3K27me3 and EZH2 found in some types of differentiated cells (**Figure 5-2**).

DNA hypomethylation during aging preferentially occurs at H3K4me1 rich sites

To identify chromatin marks associated with CpG sites hypomethylated in aged MSCs, we crossed the DNA sequences identified in our study with the same database of histone modifications and chromatin modifiers described in the previous section. Of note is the fact that hypomethylation largely occurred at regions occupied by the active histone mark H3K4me1 in most of the ENCODE cell lines ($FDR<0.05$) (**Figure 5-2**). To determine whether these associations occurred in differentiated cells, we used the same approach to analyze CpG hypomethylation during aging in blood, neural and glial cells (Guintivano *et al.* 2013; Hannum *et al.* 2013). Blood and brain samples showed similar enrichment patterns to those of the MSCs in that hypomethylated CpG sites were preferentially located at regions enriched in H3K4me1 (**Figure 5-2**).

Interestingly, the analysis of the 42 commonly hypomethylated CpG sites in blood, MSCs and neural and glial cells only showed statistically significant associations with H3K4me1 (FDR<0.05) (**Figure 5- 2A**).

Dynamics of interindividual DNA methylation variability during aging

As in most previous studies on DNA methylation and aging, our analytical design allowed the identification of DNA sequences showing a specific tendency to change (hyper- or hypomethylation) during aging, but not other putative DNA regions exhibiting no change tendency (i.e., sequences that do not become hyper- or hypomethylated with aging but rather show an increase or a decrease in interindividual variability). To address this issue, we carried out an alternative data analysis on our MSCs based on the aging-dependent behavior of interindividual variability (i.e. DNA methylation scedasticity). Interindividual variability was higher in MSCs obtained from older individuals than in those obtained from younger individuals (Figure 3A). Analysis of the scedasticity identified 16243 heteroscedastic CpG sites, of which 2437 were convergent and 13806 divergent. We also identified 124611 homoscedastic CpG sites, 68927 showing low interindividual variability in both young and old individuals (LV) and 55684 showing high interindividual variation in both populations (HV) (see Materials and Methods) (**Figure 5-3B, C**).

We studied these sequences from a functional genomics standpoint to identify factors associated with the behavior of DNA methylation changes during aging. We observed that divergent and HV CpG sites were preferentially enriched in non-CGIs (chi-square test; $p<0.001$, OR=1.59 and $p<0.001$, OR=1.58 respectively), and convergent and LV CpG sites in CGIs (chi-square test; $p<0.001$, OR=1.11 and $p<0.001$, OR=5.00 respectively) (**Figure 5-3D**). Both divergent and convergent sequences were more abundant in intragenic regions (chi-square test; $p<0.001$, OR=1.38 and $p<0.001$, OR=1.16 respectively), with HV being more frequently found in intergenic regions (chi-square test; $p<0.001$, OR=1.50), and LV in promoter regions (chi-square test; $p<0.001$, OR=3.62) (**Figure 5- 3D**).

To determine whether scedasticity behavior can also identify DNA methylation changes during aging in differentiated cells we repeated these same analyses on previously published blood DNA methylation data (Hannum *et al.* 2013). As Hannum *et al.*'s cohort contains DNA methylation data on more than 600 individuals, statistical

analyses were carried out using a Brown-Forsythe test (see Materials and Methods). To discount a possible effect of cell heterogeneity in the analysis of the scedasticity in blood, in addition to applying the algorithm described by Houseman et al. (Houseman *et al.* 2012), we carried out in silico functional analysis of the groups of genes established according to the behavior of the variance (see Materials and Methods). These analyses showed no significant associations between these groups of genes and any of the blood cell lineages examined. As in MSCs, interindividual variability was higher in blood obtained from older individuals than in blood obtained from younger individuals (Figure 4A). Furthermore, in line with the findings for adult stem cells, in differentiated cells, the analyses identified 19454 heteroscedastic CpG sites, of which 4037 were convergent and 15417 divergent. Of the homoscedastic CpG sites, 92074 showed LV in both young and old individuals and 92753 showed HV in both populations (**Figure 5-4B, C**).

The role of genetic factors on DNA methylation changes during aging

To study the role of genetic factors on DNA methylation changes during aging we used 450K methylation arrays to analyze the DNA methylation status of 24 monozygotic twins from two age groups (young, 21-22-yo and old, 58-66-yo). The effect of genotype was assessed comparing the Euclidean distance (ED) and the interindividual variability in methylation values between old and young monozygotic (MZ) pairs.

To reduce possible bias due to cell heterogeneity, DNA methylation data was corrected with the algorithm described by Houseman (Houseman *et al.* 2012). As in the larger cohort previously analyzed (**Figure 5-4**), interindividual DNA methylation variability substantially increased during aging in the MZ twins (**Figure 5-5A**). Interestingly, mean ED between MZ twins also increased (> 2-fold) with age in 46763 CpG sites (**Figure 5-5B)**, which indicates that, at these CpG sites, the increase in interindividual methylation variability depends, at least in part, on non-genetic factors. In 24782 of these sequences **(Figure 5-5B)** the increase in ED (> 2-fold) was higher than could be accounted for solely by interindividual variability, suggesting that, in these CpG sites, genetic factors play a less important role in the regulation of DNA methylation changes during aging. However, in 21908 of these sequences **(Figure 5-5B)**, the increase in ED (> 2-fold) was less than could be accounted for solely by interindividual variability, which suggests that, in contrast, at these CpG sites, genetic factors are more relevant

for the regulation of DNA methylation during aging.

Although the general trend was an increase in ED with age, for 22542 CpG sites ED between older MZ pairs decreased (> 2-fold) (**Figure 5-5B).** As the EDs between older MZ individuals are greater than those between younger MZs in more than half the sequences identified, our results support the notion that, in general, DNA methylation patterns diverge with age, even in genetically identical individuals. In 11624 sequences **(Figure 5-5B)** the decrease in ED (> 2-fold) was lower than could be accounted for solely by interindividual variability, which suggests that, in these CpG sites, genetic factors play a more important role in the regulation of DNA methylation changes during aging. In 10883 sequences **(Figure 5-5B)**, the decrease in ED was higher than could be accounted for solely by interindividual variability, indicating that in these CpG sites, genetic factors play a less important role in the regulation of DNA methylation during aging. As in the analysis of the previously published blood DNA methylation data, in silico functional analysis of the groups of genes identified in the monozygotic twins, suggested that, after correcting with the Houseman algorithm, cell heterogeneity had little impact on the Euclidean distances for changes in DNA methylation with age.

Comparative analysis of the interindividual variation and the EDs suggests that the effect of genotype on the regulation of DNA methylation changes during aging was locus-specific. Thus, to identify those DNA regions differentially affected by the genotype, we used Circos representations to study the genomic distribution of CpG sites which showed changes in ED with age (**Figure 5-5C**). The results demonstrated that whilst CpG sites showing a decrease, or no difference, in ED between young and old MZs presented a random distribution, those showing an age-dependent increase in ED were strongly enriched in subtelomeric DNA regions. The greatest changes occurred at chromosomes 11 and 19 and, in general, clustering occurred at the same genomic regions in both young and old twins.

To study the effect of the genotype on DNA methylation and its interindividual variability during aging we analyzed the Twins data using similar strategies to those described in previous sections, identifying 41987 hypermethylated, 56923 hypomethylated, 1018 convergent, 1635 divergent, 58680 HV and 59795 LV CpG sites (data not shown). The comparison of EDs between young and old MZ pairs for these groups of genes showed that the effect of genotype depended on the tendency and the scedasticity of the change (**Figure 5-5D**).

ED increased (>2-fold) with age in 9.5% of the hypomethylated and in 14% of the hypermethylated CpGs, suggesting that genetic factors have a greater effect on the former during aging **(Figure 5-5D)**. ED increased (>2-fold) in most (83.73%) of the divergent CpG sites and decreased (>2-fold) in most (66.7%) of the convergent CpG sites **(Figure 5-5D)**. However, changes in interindividual variability were higher than the increase or decrease in ED **(Figure 5-5D)**, which indicates that genetic factors play a role in the regulation of DNA methylation of these DNA regions during aging. Interestingly, ED also increased in most of the HV and LV sequences (>2-fold) during aging **(Figure 5-5D).** Furthermore, in most CpG sites, the increase in ED between the MZ twins was higher than the interindividual variability changes during aging **(Figure 5-5D)**, suggesting that genotype has little effect on epigenetic drift in homoscedastic DNA regions.

TABLE 5-1. *Supplemental Table 1.Information available on donors and experiments carried out in the human mesenchymal stem cells included in the study*

Sample ID	Methylation assay	Age (years)	Gender	Nº Passages	Lesion	Extraction	Isolation	Culture
MSC-1	1	2	female	5	OS	BM aspirates	ficoll	hMSC medium
MSC-2	1	2	female	5	OS	BM aspirates	ficoll	hMSC medium
MSC-3	1	3	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-4	1	3	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-5	1	6	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-6	1	6	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-7	1	9	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-8	1	10	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-9	1	10	male	5	OS	BM aspirates	ficoll	hMSC medium
MSC-10	1	10	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-11	1	10	male	4	OS	BM aspirates	ficoll	hMSC medium
MSC-12	1	12	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-13	1	12	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-14	1	14	female	4	OS	BM aspirates	ficoll	hMSC medium
MSC-15	1	18	male	4	HD	BM aspirates	ficoll	hMSC medium
MSC-16	1	19	male	5	HD	BM aspirates	ficoll	hMSC medium
MSC-17	1	22	male	4	HD	BM aspirates	ficoll	hMSC medium
MSC-18	1	22	male	4	HD	BM aspirates	ficoll	hMSC medium
MSC-19	1	22	male	6	HD	BM aspirates	ficoll	hMSC medium
MSC-20	1	22	male	5	HD	BM aspirates	ficoll	hMSC medium
MSC-21	1	28	male	4	HD	BM aspirates	ficoll	hMSC medium
MSC-22	1	29	male	5	HD	BM aspirates	ficoll	hMSC medium
MSC-23	1	61	female	6	OP	Bone Scraping	ficoll	hMSC medium

MSC-24	1	63	male	6	OA	Bone Scraping	ficoll	hMSC medium
MSC-25	1	70	female	6	OP	Bone Scraping	ficoll	hMSC medium
MSC-26	1	77	female	6	OP	Bone Scraping	ficoll	hMSC medium
MSC-27	1	79	female	7	OP	Bone Scraping	ficoll	hMSC medium
MSC-28	1	80	male	6	OP	Bone Scraping	ficoll	hMSC medium
MSC-29	1	86	female	6	OP	Bone Scraping	ficoll	hMSC medium
MSC-30	1	87	female	6	OP	Bone Scraping	ficoll	hMSC medium
MSC-31	1	87	male	6	OP	Bone Scraping	ficoll	hMSC medium
MSC-32	1	89	female	7	OP	Bone Scraping	ficoll	hMSC medium
MSC-33	1	91	female	8	OP	Bone Scraping	ficoll	hMSC medium
MSC-34	1	92	female	7	OP	Bone Scraping	ficoll	hMSC medium
MSC-35	2	0.7	male	4	HD	thoracotomy	whole BM	hMSC medium
MSC-36	2	1	male	3	HD	thoracotomy	whole BM	hMSC medium
MSC-37	2	2	female	3	HD	BM Aspiration	percoll	hMSC medium
MSC-38	2	3	male	1	HD	thoracotomy	whole BM	hMSC medium
MSC-39	2	3	male	1	HD	BM Aspiration	whole BM	hMSC medium
MSC-40	2	4	male	4	HD	thoracotomy	whole BM	hMSC medium
MSC-41	2	5	male	4	HD	BM Aspiration	whole BM	hMSC medium
MSC-42	2	6	male	2	HD	thoracotomy	whole BM	hMSC medium
MSC-43	2	7	male	4	HD	BM Aspiration	ficoll	hMSC medium
MSC-44	2	7	male	2	HD	BM Aspiration	whole BM	hMSC medium
MSC-45	2	7	male	5	HD	BM Aspiration	whole BM	hMSC medium
MSC-46	2	8	male	4	HD	BM Aspiration	percoll	hMSC medium
MSC-47	2	12	male	4	HD	BM Aspiration	whole BM	hMSC medium
MSC-48	2	12	female	6	HD	thoracotomy	whole BM	hMSC medium
MSC-49	2	13	male	4	HD	BM Aspiration	whole BM	hMSC medium

MSC-50	2	13	female	4	HD	BM Aspiration	whole BM	hMSC medium
MSC-51	2	17	male	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-52	2	19	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-53	2	21	male	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-54	2	21	male	5	HD	BM Aspiration	ficoll	hMSC medium
MSC-55	2	21	female	3	HD	BM Aspiration	percoll	hMSC medium
MSC-56	2	29	female	1	HD	BM Aspiration	ficoll	hMSC medium
MSC-57	2	34	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-58	2	36	male	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-59	2	37	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-60	2	39	male	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-61	2	42	-	5	-	-	ficoll	hMSC medium
MSC-62	2	44	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-63	2	46	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-64	2	46	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-65	2	47	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-66	2	53	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-67	2	54	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-68	2	55	male	5	-	-	ficoll	hMSC medium
MSC-69	2	56	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-70	2	59	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-71	2	59	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-72	2	61	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-73	2	61	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-74	2	65	female	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-75	2	68	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium

MSC-76	2	69	male	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-77	2	75	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-78	2	75	female	2	OS	BM Aspiration	Attachment of cells	hMSC medium
MSC-79	2	75	male	2	HD	BM Aspiration	ficoll	hMSC medium
MSC-80	2	80	male	2	HD	BM Aspiration	ficoll	hMSC medium

Methylation assay 1: 450K Methylation array
Methylation assay 2: Bisulfite pyrosequencing validation

HD: healthy donor
OS: orthopedic surgery
OA: hip osteoarthritis
OP: osteoporotic hip fracture

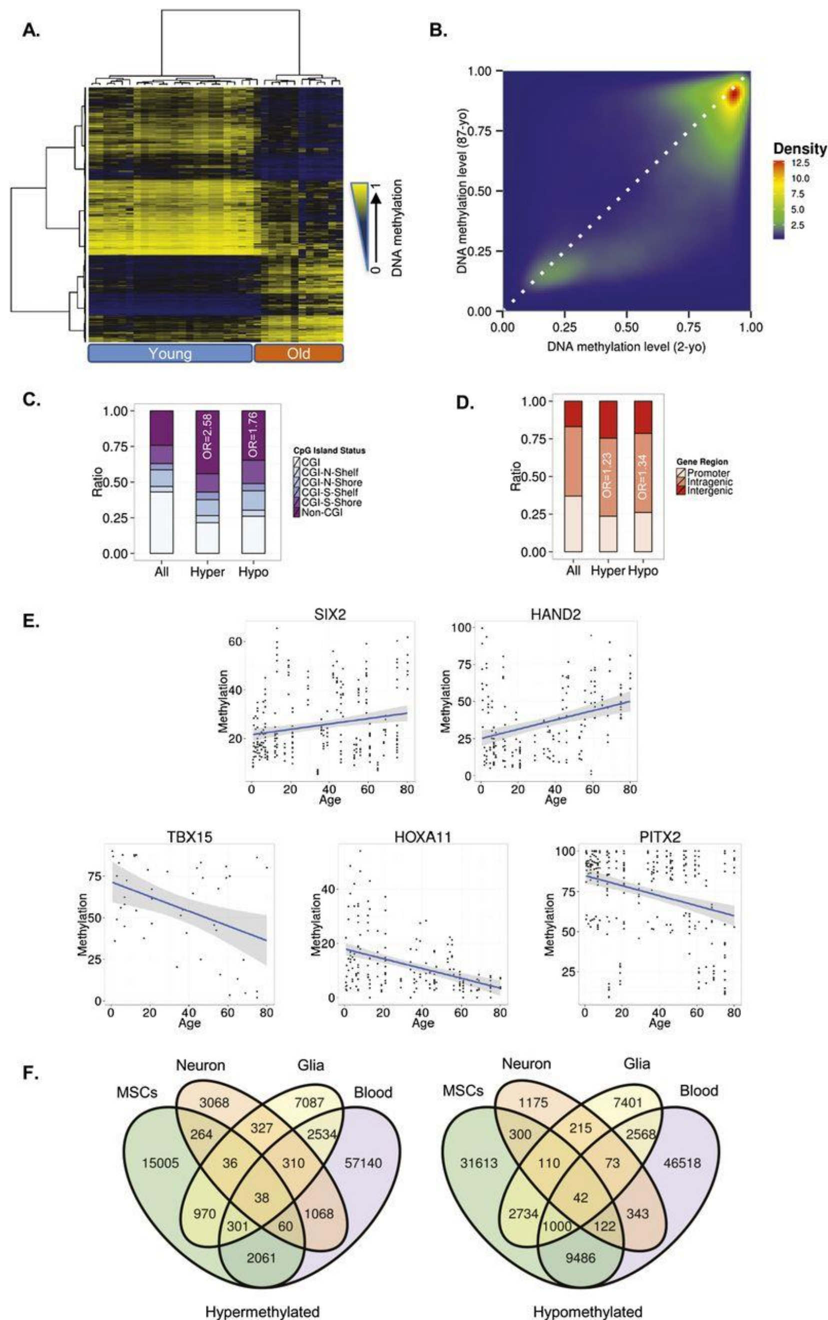

FIGURE 5-5-1 *Dna methylation changes during msc aging. (a) unsupervised hierarchical clustering and heatmap including the 15000 most variable cpg sites with differential dna methylation between young and old mscs. average methylation values are displayed from 0 (blue) to 1 (yellow). (b) density plot for differentially methylated cpg sites between representative young (two years old; 2-yo) and old (87 years old; 87-yo) mscs. (c) distribution of differentially methylated cpgs relative to cpg island. (d) relative distribution of differentially methylated cpgs across different genomic regions. (e) examples of aging-specific cpg methylation in particular genes further validated by pyrosequencing in an independent set of samples. for each of the genes of interest, a scatter plot of the percentage of methylation obtained for each sample and cpg of interest is shown. the two genes at the top show an age-dependent hypermethylation tendency, while the three genes at the bottom show hypomethylation with respect to age. each point represents a single observation for a given sample and cpg of interest. the blue line represents a linear model fit. a 0.95 confidence interval of the fitted model is shown in gray. (f) venn diagrams showing the number of cpg sites (hyper- and hypomethylated) shared by the different tissues*

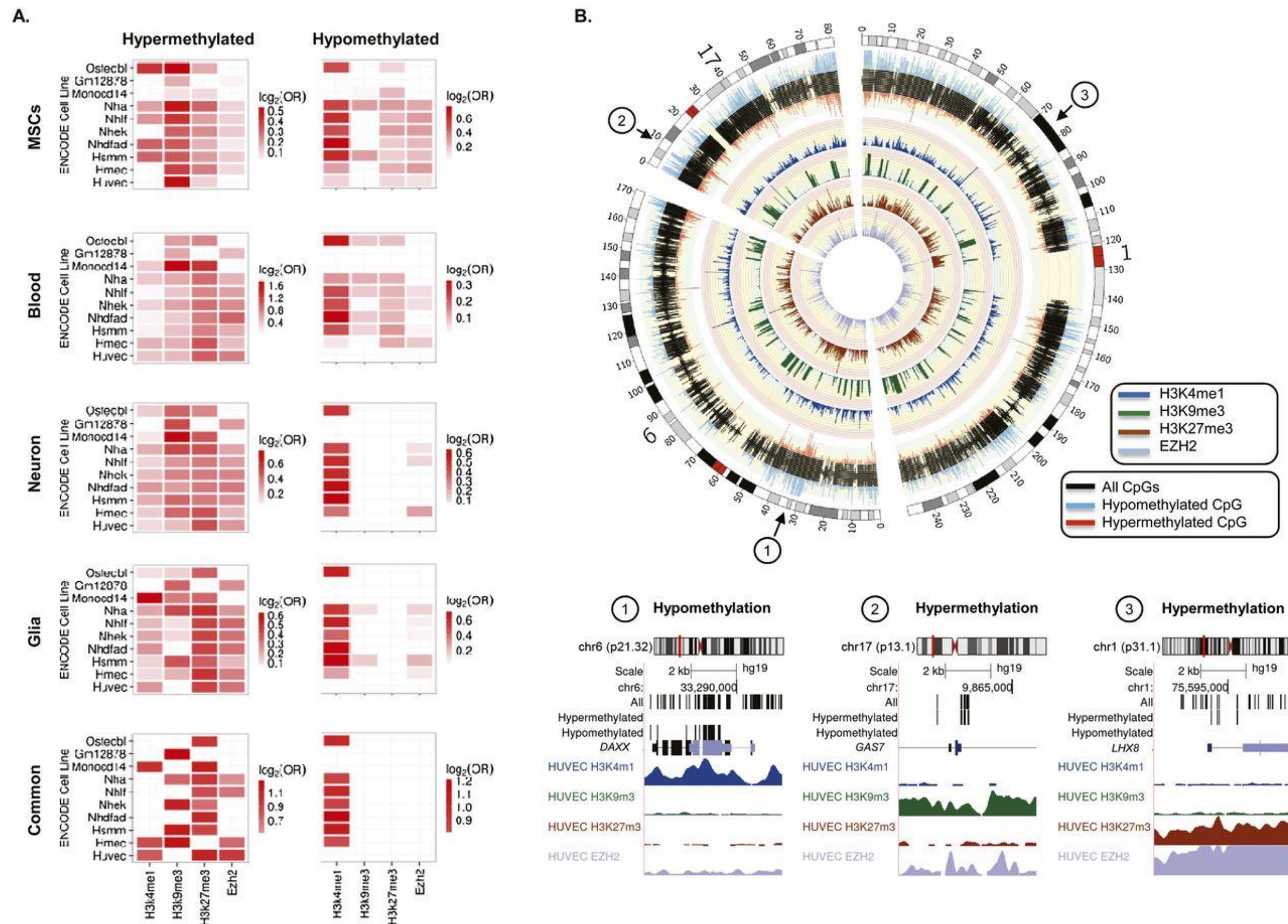

FIGURE 5-5-2 *Chromatin signatures associated with DNA methylation changes during aging. (A) Heatmaps showing significant enrichment of hyper- and hypomethylated CpG sites—identified in MSCs, blood, neurons, and glia—with different histone marks and chromatin modifiers contained in the UCSC Genome Browser Broad histone track from the ENCODE Project. Color code indicates the significant enrichment based on log2 odds ratio (OR). (B) Circular representation of three representative chromosomes (1, 6, and 17), indicating whether the CpGs were hypermethylated (red) or hypomethylated (blue) during MSC aging. Inner tracks display chromatin marks (H3K4me1, H3K9me3, H3K27me3, and EZH2) generated for HUVEC cells and associated with differentially methylated regions during aging. Broad histone peak information was averaged in 200-kbp genomic windows and represented as histogram tracks. Three examples of hypo- and hypermethylated DNA regions associated with specific chromatin signatures are displayed below.*

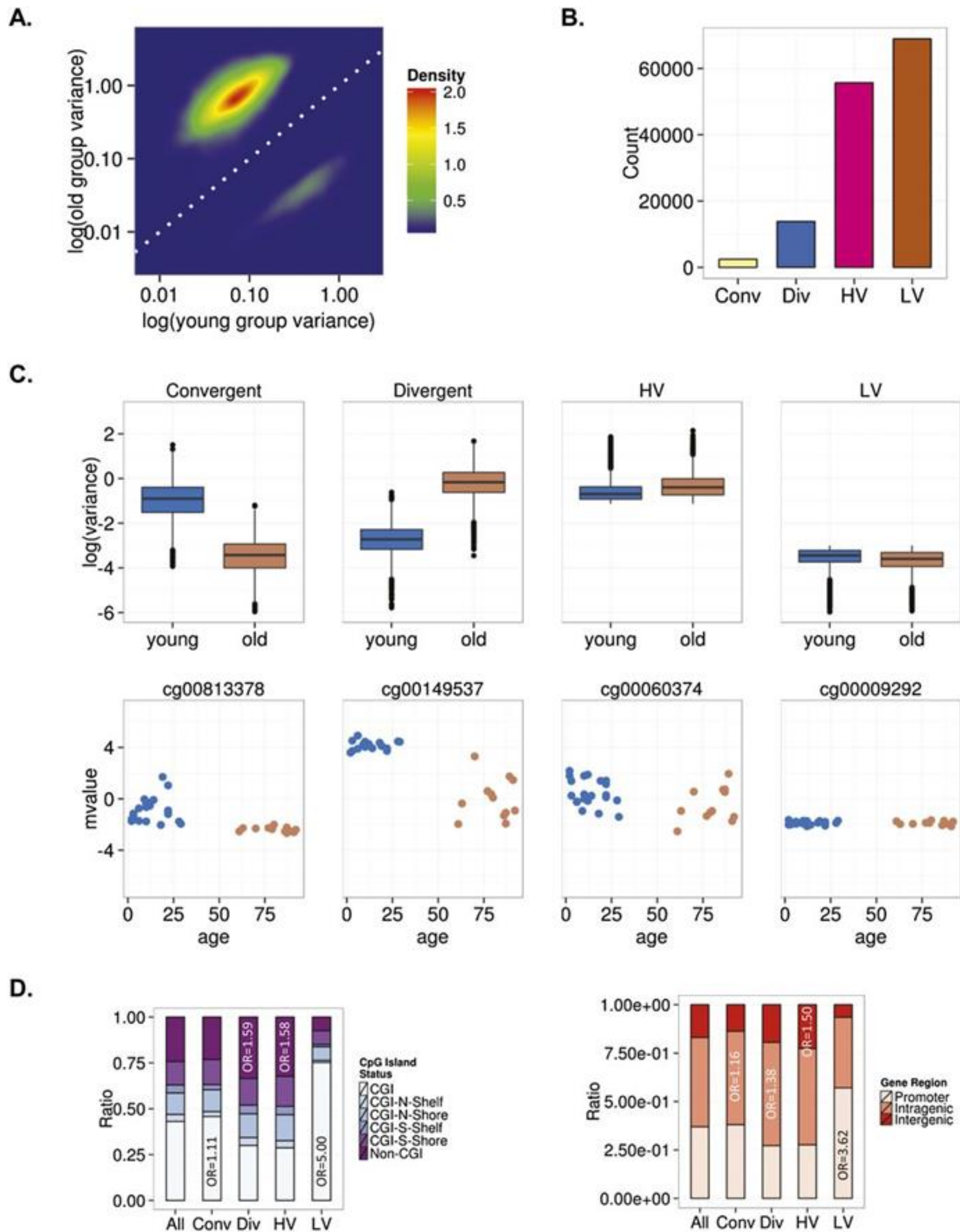

FIGURE 5-5-3 *Interindividual DNA methylation variability during MSC aging. (A) Density plot for CpG sites showing significant changes in variance in young and old MSCs. (B) Bar plot showing the number of age-dependent heteroscedastic (convergent and divergent) and homoscedastic (high [HV] and low [LV] variability) CpG sites in MSCs. (C) Box plots showing the classification of CpG sites into different groups based on the aging-dependent behavior of the interindividual variability. Representative examples of CpG sites for each group are shown below (mvalue: relative methylation values). (D) Distribution of homoscedastic and heteroscedastic CpGs relative to CpG island status and relative distribution across different genomic regions.*

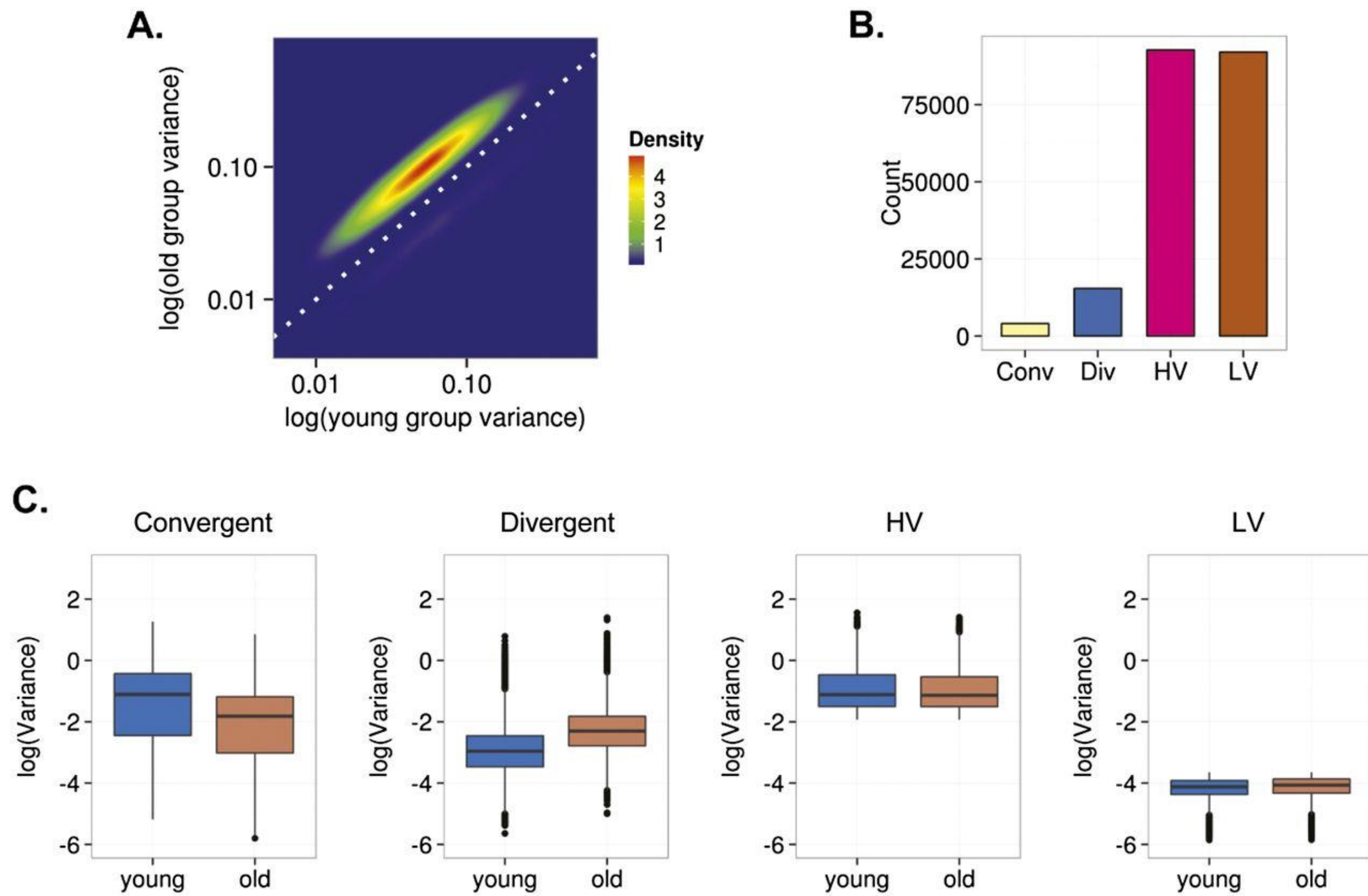

FIGURE 5-4 *Interindividual DNA methylation variability during aging of blood cells. (A) Density plot for CpG sites showing significant changes of variance in young and old individuals. (B) Bar plot showing the number of age-dependent heteroscedastic (convergent and divergent) and homoscedastic (high [HV] and low [LV] variability) CpG sites. (C) Box plots showing the classification of the CpG sites in different groups based on the aging-dependent behavior of the interindividual variability.*

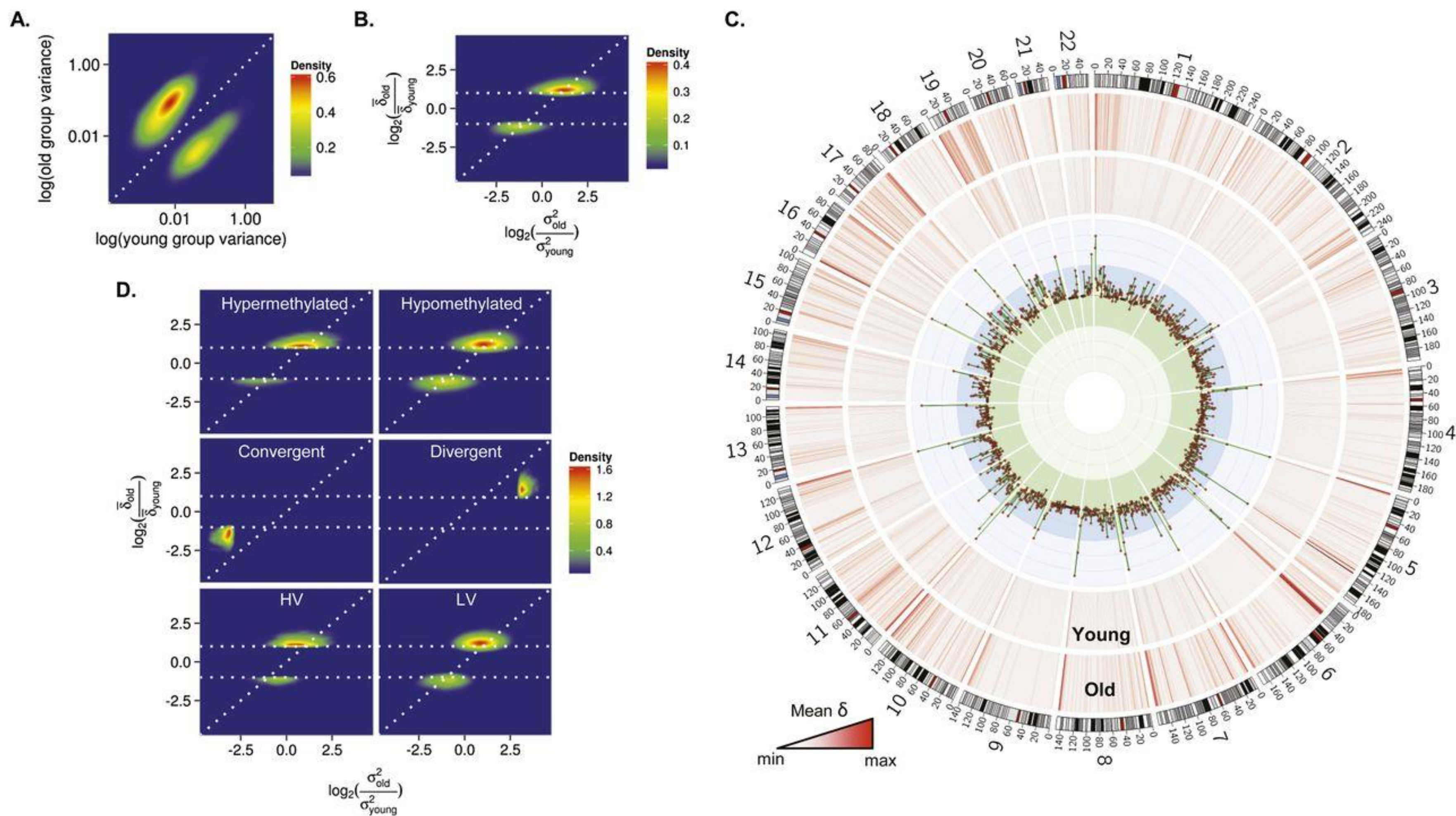

FIGURE 5-5-5 *Role of genetic factors in interindividual dna methylation variability during aging. (a) density plot for cpg sites showing significant changes of methylation variance in the blood cells of mz twins during aging. (b) density plot for comparison between the mean euclidean distance (δ) and the interindividual variability (σ2) in methylation values between old and young mz twins. the horizontal dotted lines represent a twofold change in the δ between mz twins. (c) circular representation of genome-wide cpg sites showing differences in the between methylation values of young and old mz twins. δ was averaged using a 2-mbp window size. inner tracks show genomic regions where the was higher (blue region) or lower (green region) in old compared with young mz twins. (d) density plots for comparison between the and the σ2 in methylation values between old and young mz twins, in hypermethylated, hypomethylated, heteroscedastic, and homoscedastic cpgs.*

DISCUSSION

Recent studies have shown that DNA methylation is altered during aging in a number of differentiated cell types (Rakyan *et al.* 2010; Teschendorff *et al.* 2010; Bell *et al.* 2012; Fernandez *et al.* 2012; Heyn *et al.* 2012; Numata *et al.* 2012; Guintivano *et al.* 2013; Hannum *et al.* 2013; Johansson *et al.* 2013). Here, we studied the dynamics and the context of DNA methylation changes during aging in human adult stem cells as they have been proposed to play an important role in aging (Sharpless and DePinho 2004). Indeed, a recent study in mice showed that epigenomic alterations of the DNA methylation landscape contribute to the functional decline of hematopoietic stem cells (HSCs) during aging (Beerman *et al.* 2013). To analyze our DNA methylation data, we first used an analytical strategy similar to that used in most of the previous studies on DNA methylation and aging (i.e. linear models). Using this approach, we identified 18735 CpG sites which were hypermethyated and 45407 which were hypomethylated during aging in MSCs, which provides support for the idea that, as in blood (Heyn *et al.* 2012), aging is associated with global DNA hypomethylation in MSCs. In addition, we validated 5 of the genes indentified through the methylation arrays (*HAND2*, *SIX2*, *TBX15*, *PITX2,* and *HOXA11*) by bisulfite pyrosequencing, using an independent sample set of 46 MSCs obtained from individuals from 7 months to 80-years old. The results corroborated the data obtained from the methylation arrays and suggest that our genome-wide data can be extrapolated to independent sample sets of MSCs. *HAND2* and *SIX2* genes code for transcription factors and have been also found hypermethylated in several cancer types (Rauch *et al.* 2006; Tong *et al.* 2010; Jones *et al.* 2013). In contrast, the genes which are hypomethylated during MSC aging, *TBX15*, *PITX2,* and *HOXA11,* code for transcription factors involved in several differentiation and developmental processes (Singh *et al.* 2005; Gross *et al.* 2012; Gage *et al.* 2014). Interestingly, 80 of the differentially methylated sequences identified in the MSCs were present in both blood and brain, which is in line with previous observations that suggest the existence of systemic DNA methylation changes during aging (Rakyan *et al.* 2010; Heyn *et al.* 2012). However, as many of the sequences were not common to different tissues, our data indicate that, as has recently been proposed (Christensen *et al.* 2009; Day *et al.* 2013) systemic changes are restricted to specific loci, and cell type plays an important role in the regulation of DNA methylation changes over time.

The factors determining the behavior of DNA methylation during aging have received

much attention during the last few years. Recent works have shown that genes which are hypermethylated in blood during aging are associated with the presence of bivalent chromatin domains in embryonic stem cells (Rakyan *et al.* 2010; Teschendorff *et al.* 2010; Fernandez *et al.* 2012; Heyn *et al.* 2012) and with repressive histone marks (H3K27me3/H3K9me3) in differentiated cells (Rakyan *et al.* 2010). Our data indicate that the same repressive histone marks in differentiated cells are also present in sequences in those MSCs which are hypermethylated during aging, implying that, independent of morphogenic potential and/or cell type, these repressive histone marks are associated with DNA methylation gain during aging. Of note, our data provide new evidence that sequences which are hypomethylated in MSCs and differentiated cells during aging are strongly enriched in the active chromatin mark H3K4me1, which suggests that this histone modification is a cell type-independent chromatin signature of DNA hypomethylation during aging. Interestingly, H3K4me1 has recently been associated with enhancers (Rada-Iglesias *et al.* 2010), genomic regions that play a fundamental role in cis-regulation of gene function. In addition, a recent study has shown that DNA hypomethylation within specific transposable elements is associated with tissue-specific enhancer marks, including H3K4me1, suggesting that these sequences might play an important role in tissue-specific epigenetic gene regulation (Xie *et al.* 2013), which implies that H3K4me1-associated DNA hypomethylation could play a role in the deregulation of gene expression during aging (Bahar *et al.* 2006). Further parallel studies analysing DNA hypomethylation in enhancers and gene expression during aging should shed light on this matter. Collectively, our data indicate that, although there are few altered DNA sequences which are common to different cell types, the chromatin signatures associated with DNA hyper- and hypomethylation during aging were similar for different tissues, supporting the notion that chromatin context is associated with the dynamics of systemic DNA methylation changes during aging. The reasons why the repressive histone marks H3K27me3/H3K9me3 favor hypermethylation and the active histone mark H3K4me1 promotes hypomethylation during aging are not known. A simple explanation could be the preferential location of DNA methyltransferases (DNMTs) at repressive chromatin regions (Jeong *et al.* 2009). Repressive chromatin regions could be predisposed to becoming hypermethylated due to the abundance of DNMTs. In contrast, active chromatin regions would be more susceptible to losing methylation

because the low levels of DNMTs at these regions make it more difficult to maintain DNA methylation patterns after mitosis. This possibility is supported by the fact that postmitotic tissues such as brain (Numata *et al.* 2012; Guintivano *et al.* 2013) and muscle (Zykovich *et al.* 2014) present far fewer hypomethylated sequences during aging than highly mitotic cells such as blood and MSCs. Further studies analyzing the genome wide distribution of DNMTs during aging are needed to support this possibility.

One possible limitation of our study is the purification and the *in vitro* culture of MSCs (Calvanese *et al.* 2008; Choi *et al.* 2012), although this should have no great impact when comparing young and old MSCs as both sets of samples were cultured under exactly the same conditions. Moreover, cell heterogeneity, which is a major issue in DNA methylation studies (Houseman *et al.* 2012; Guintivano *et al.* 2013), has less impact in relation to MSCs as they are more homogeneous than blood cell populations. However, to minimise the impact of cell heterogeneity in our analysis of blood we corrected DNA methylation data with a recently published algorithm (Houseman *et al.* 2012), which yielded slightly different sequences to those previously proposed, suggesting that some of the DNA changes previously identified might be cell-type dependent. Another limitation of our study is that the differences in the number of individuals analyzed and different data analyses undertaken make difficult the interpretation of the comparison of age-dependent DNA methylation changes in different cell types. However, the conserved pattern of chromatin signatures in stem and differentiated cells suggests that H3K9me3/H3K27me3 and H3K4me1 are truly tissue-independent histone marks of DNA hyper- and hypomethylation respectively during aging.

As in most previous studies on DNA methylation and aging, CpG sites showing DNA methylation changes during lifetime associated with a specific tendency (i.e. hyper- or hypomethylation) were identified. However, using this analytical approach means that other possible changes occurring at CpG sites displaying high interindividual variability in both young and old individuals and/or age-dependent interindividual variability are overlooked. To address this issue, we re-analyzed the DNA methylation data to characterize the age-dependent interindividual variability (i.e. scedasticity).

Using this approach we identified 16243 heteroscedastic (2437 convergent and 13806 divergent), and 55684 homoscedastic CpG sites with high (HV) and 68927 with low

(LV) interindividual variability. Most of these CpG sites were not identified through linear model analysis, leading us to suggest that DNA methylation changes during aging might be more frequent than has previously been thought. Interestingly, although there were some CpG sites that converged during aging, most of the heteroscedastic changes were divergent, providing support for the notion that interindividual DNA methylation variability increases during lifetime (Gemma *et al.* 2013; Ong and Holbrook 2013). Although the behavior adults stem cell populations during aging is still poorly understood (Pollina and Brunet 2011), the clonal expansion or decline of specific cell populations could affect the interpretation of changes of interindividual DNA methylation variability with aging. As it has been proposed that the number of MSCs declines with age (Stolzing *et al.* 2008), it is possible that the increase in interindividual variability might in fact be even larger than was observed in our study. Functional genomics analyses of the groups of CpG sites established according to the behavior of the variance revealed that low variable CpG sites were enriched in CpG islands and gene promoters. As DNA methylation occurring at CpG island promoters has been proposed to play an important role in gene regulation (Bird 1986; Bird and Wolffe 1999; Calvanese *et al.* 2012), our results could indicate that the DNA methylation involved in gene regulation is protected against the stochastic epigenetic changes that occur during lifetime (Feil and Fraga 2012). Interestingly, analysis of the interindividual variability of DNA methylation during aging in blood, showed that, as in adult stem cells, the DNA methylation patterns of differentiated cells also diverge with age, thereby supporting the notion that a systemic epigenetic drift occurs during the lifetime of higher organisms (Feil and Fraga 2012; Issa 2014). To confirm that the sequences identified in blood after correcting with the Houseman algorithm were not affected by cell heterogeneity, we carried out in silico functional analysis to discard a possible blood cell lineage-dependent regulation. The analyses showed no meaningful associations, which further supports our contention that, after correcting with the Houseman algorithm, cell heterogeneity had a minor impact on our blood DNA methylation data.

Previous reports have demonstrated that genetic factors play an important role in the regulation of DNA methylation during aging (Heijmans *et al.* 2007; Coolen *et al.* 2011; Gertz *et al.* 2011; Bell *et al.* 2012). To determine whether the effect of genotype is different depending on the intrinsic behavior of the DNA changes during aging at each

specific CpG site, we analyzed the DNA methylation status of monozygotic twins of different ages. The results showed that interindividual variability increased with aging, in agreement with the notion that epigenetic drift during lifetime occurs even in genetically identical individuals (Fraga *et al.* 2005; Wong *et al.* 2010; Pirazzini *et al.* 2012; Talens *et al.* 2012; van Dongen *et al.* 2012). However, our results also showed that the DNA methylation status of some CpG sites may converge during lifetime. Specifically, the analysis of genetically identical individuals revealed that the effect of genotype depended on the intrinsic behavior of the DNA methylation changes during aging. For example, although the mechanisms underlying methylation convergence are still largely unknown, our MZ data indicate that genetic factors must be involved, at least in part, as the intrapair changes were similar to, or even less than, the interindividual variations. In addition, in contrast to the convergent and divergent CpG sites, genotype seems to play a less important role in whether the CpG sites display high or low interindividual variability, as evidenced by the fact that the increase in ED in the homoscedastic sequences for MZ twin pairs during aging was higher than the differences explained by interindividual variability. Of particular note is the finding that genotype had the lowest effect on the CpG sites, displaying high interindividual variability in young and old individuals, evidenced by the increase Euclidean distance in MZ twins during aging being similar to or even higher than the increase in interindividual variability. Our results indicate that these CpG sites, which have received little attention until now, might be important targets of environmental and/or stochastic epigenetic variation during development and aging. Although we have reduced the effect of cell heterogeneity and immune status over time (Allegretta *et al.* 1990) using the Houseman algorithm (Houseman *et al.* 2012) and by performing several functional in silico analyses of the groups of the genes showing age-related changes in Euclidean distance, we cannot completely discount a partial effect of these in our results.

Our data indicate that the differences in the effect of genotype on DNA changes during lifetime depend largely on the genomic region involved, which is in agreement with previously published data (Wong *et al.* 2010). In line with this, the greatest DNA methylation changes for MZs were clustered at subtelomeric DNA regions, which suggests that the regulation of DNA methylation at these sequences is largely independent of genetic factors. Interestingly, subtelomeric DNA methylation has been

shown to be affected by environmental factors (unpublished observations). It is worth noting that, although for most CpG sites the ED in young twins was lower than for older twins, they still clustered in the same subtelomeric regions, providing support for the previous proposal that epigenetic drift starts early in life (Martino *et al.*; Kaminsky *et al.* 2009; Ollikainen *et al.* 2010; Wong *et al.* 2010) and accumulates during lifetime at particular CpG sites that, for still unknown reasons, evade the control of genetic factors (Fraga 2009).

Collectively, our results indicate that the dynamics of DNA methylation during lifetime in humans is associated with a complex mixture of factors. These include the DNA sequence itself, tissue type and, in particular the chromatin context, where repressive histone modifications such us H3K9me3 and H3K27me3 are related to DNA hypermethylation and, most notably, the active histone mark H3K4me1 is related to DNA hypomethylation. Finally, depending on the locus, the changes appear to be modulated by genetic and/or external factors.

MATERIALS AND METHODS

Isolation and culture of MSCs

MSCs were purchased from Lonza (Verviers, Belgium), Millipore (Billerica, MA, USA), and Inbiobank. (San Sebastian, Spain) or directly obtained from young and elderly donors. After informed consent, bone marrow aspirates were obtained from one group of patients and, from a second group, bone scrapings were obtained following hip replacement surgery. Mononuclear cells were isolated by Ficoll density centrifugation (400 g, 25 min, 20C), washed twice by sedimentation with phosphate buffer (300 g, 5 min) and the cells resuspended in MSC medium (DMEM plus 10% FBS) and seeded into culture flasks (Nunc, Roskilde, Denmark) at 1.5x10e5 cells/cm2 and allowed to adhere for 24 hours. MSCs were then cultured (37C, 5% CO2) in MSC medium. DNA methylation analyses were carried out at cell passages 4-6 (**Supplemental Table 1**).

MZ twins samples

Genomic DNA from 24 samples from the Italian Twin Registry, corresponding to 12 pairs of MZ twins, were extracted from buffy coats following standard procedures. Two different age groups were included for array-based DNA methylation profiling; one included individuals between 21 and 22 years old (*young* MZ twins), and the other individuals between 58 and 66 (*old* MZ twins). The sample distribution by gender was the same in both groups.

Genome-wide DNA methylation analysis with high-density arrays

Microarray-based DNA methylation profiling was performed with the Illumina Infinium® Human Methylation450 BeadChip (Bibikova *et al.* 2011). Bisulfite conversion of DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) following the manufacturer's procedures, with the modifications described in the Infinium Assay Methylation Protocol Guide. Processed DNA samples were then hybridized to the BeadChip (Illumina), following the Illumina Infinium HD Methylation Protocol. Genotyping services were provided by the Spanish "Centro Nacional de Genotipado" (CEGEN-ISCIII)" (www.cegen.org).

Datasets of blood and brain samples

DNA methylation data of blood (Hannum *et al.* 2013) and brain (neuron and glia) (Guintivano *et al.* 2013) samples produced with the Illumina Infinium Human

Methylation450 were used to compare with the results obtained in MSCs. DNA methylation β value data was downloaded from GEO accession numbers GSE40279 and GSE41826. The data analysis workflow is outlined in **Supplemental Fig. S1.**

Illumina Infinium450k data preprocessing

IDAT files from the Illumina Infinium 450k DNA methylation microarray were processed further using the R/Bioconductor package *minfi* (Hansen and Aryee). In order to adjust for the different probe design types present in the 450k architecture, red and green signals from the IDAT files were corrected using the SWAN algorithm (Makismovic *et al.* 2012). No background correction or control probe normalization was applied. Probes where at least two samples had detection p-values over 0.01 were filtered out. In accordance with Du *et al.* (Du *et al.* 2010), both beta values and m-values were computed and employed across the analysis pipeline. M-values were used for all the statistical analyses, assuming homoscedasticity (with the exception of the blood heterogeneity adjustment), while beta values were mostly used for the intuitive interpretation and visualization of results.

Filtering confounding probes

Probes located in the X/Y chromosomes were removed from the dataset when differential methylation profiles were analyzed. Probes that had been found to cohybridate with probes in the sex chromosomes (Lemire *et al.* 2013) were also removed. We used the information from the SNP137Common track from the UCSC browser (Sherry *et al.* 2001) in order to remove those probes with an SNP located inside their 2bp central region.

Batch effect correction

Multidimensional Scaling (MDS) was employed to detect whether there was any significant batch effect depending on the different 450k plates which comprised the experiments. When there was, the ComBat method implemented in the R/Bioconductor package *sva* (Leek *et al.*) was used to adjust the datasets accordingly, employing the variable *age* as the outcome of interest and the sample plate as a batch covariate.

White blood cell heterogeneity adjustment

Methylation data for the Blood and Twins datasets was adjusted for blood cell heterogeneity using the method described in Houseman et. al. (Houseman *et al.* 2012).

In order to feed this method, we used the original 27k database of purified white blood cell subtypes included in the original implementation of the algorithm. The correction was performed on the beta values due to the fact that the 27k database was expressed using those units. M-values were obtained from the corrected beta values for subsequent downstream analyses.

Detection of differentially methylated probes

For the MSC dataset, the 34 samples were divided into two groups: *young* (ages ranging from 2 to 22) and *old (*ages between 61 and 92). Similarly, samples in the Twins dataset were divided into *young* (ages ranging from 21 to 22) and *old* (age between 58 and 66). For the neuron and glia datasets, the two groups were defined by taking those individuals whose age was below the 33rd percentile (*young*) and above the 66th percentile (*old*). Blood samples were not divided into groups, and the age predictor was used as a quantitative covariate. For the MSCs, Twins, neuron and glia datasets, significant methylation of a probe was determined by the moderated t-test implemented in the R/Bioconductor package *limma* (Smyth 2005). Probes in the blood dataset were tested with a linear regression. A linear model, with methylation level as response and *age* as the only predictor, was used on all the datasets. P-values were corrected for multiple testing using the Benjamini-Hochberg method for controlling the false discovery rate (FDR). A significance level of 0.05 was employed to determine differentially methylated probes. An additional threshold of effect size was applied, meaning that only those probes with the strongest differences between groups (the top 70%) were selected. The application of this threshold is essential to remove those differences prone to coming from technical artifacts, and consequently ensure a more biologically sound statistical data analysis (Pan *et al.* 2005). Our threshold was adjusted according to the differences in M-values between groups in the brain and MSC datasets and the slope coefficients extracted from the blood dataset linear regression model.

Analysis of variability trends

To analyze aging-dependent behavior of DNA interindividual variability (i.e. DNA methylation scedasticity), two groups, corresponding to *young* (samples where *age* was below the 33rd percentile) and *old* (those where *age* is above the 66th percentile) individuals, were selected for all the datasets. This separation allows the method to

focus on the global tendency of the variability, and be less dependent on a fixed, underlying model. A Brown-Forsythe test for the equality of variances was used to determine which probes in the blood dataset had significantly different variability in methylation between the two groups. For the remaining datasets, and due to the small number of available samples and low statistical power for conducting a variance test, a simple descriptive approach was used, labeling a probe as having a significant difference in methylation variability when the absolute value of the base-2 logarithm of the change of the variances for the two groups was greater than 3 fold. We did not apply any threshold of effect size for any of the datasets. For the blood dataset, p-values were corrected for multiple testing using FDR (Benjamini-Hochberg method) and a significance level of 0.05 used to determine which probes had a significant trend in variability. Two special subsets of probes with no significant trends in variability were generated: one, named HV (High Variance), for those probes with variance values above the 75th percentile of the whole set of variances for both the *young* and *old* sample groups, and one named LV (Low Variance), generated with those probes where both young and old variances were below the 25th percentile.
The *in silico* functional analysis and interpretation of the groups of genes established according to the behavior of the variance in blood was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) and the "Gene ontology" and “UP_TISSUE" categories (Dennis *et al.* 2003; Huang da *et al.* 2009).

Measuring intra- and interindividual distance

Euclidean distances between twins were computed for every probe in the original Twins dataset, using beta-values. In a simple scenario, the Euclidean distance accounts for the absolute difference between the beta values of the two siblings. Differences in distances were computed as the base-2 logarithm of the fold change between the average Euclidean distance for the young and old sample groups.

Histone enrichment analysis

In order to analyze the enrichment of a histone mark on a given subset of probes, we used the information contained in the UCSC Browser Broad Histone track from the ENCODE project (Bernstein *et al.* 2005; Bernstein *et al.* 2006; Mikkelsen *et al.* 2007; Guttman *et al.* 2010; Ernst *et al.* 2011) **(Supplemental Table 26)**. Histone peak data for every histone modification and chromatin modifier in hESCs and 10 different cell

types obtained from healthy individuals were downloaded from the UCSC Browser. Small peaks were discarded when they were completely contained within wider peaks. Following the ENCODE Broad Histone Methods description, discrete intervals of ChIP-seq fragment enrichment were identified using Scripture, a scan statistics approach, under the assumption of uniform background signal (http://genome.ucsc.edu//cgi-bin/hgTrackUi?db=hg19&g=wgEncodeBroadHistone). For each combination of cell line and mark, a 2x2 contingency table was built to determine its association with the input subset of probes. Probes in the array were classified according to whether they belonged to the subset or not, and whether they intersected with a significant broad peak for the given combination of cell line and mark. A Fisher exact test was used to determine if the given subset of probes was significantly enriched for each combination of cell line and mark. P-values were corrected for multiple testing using FDR (Benjamini-Hochberg method) and a significance level of 0.05 was used to determine which probes had significant enrichment. The base-2 logarithm of the Odds Ratio was used as a measure of effect size.

Genomic region analysis

The probes in the microarray were assigned a genomic region according to their position relative to the transcript information extracted from the R/Bioconductor package *TxDb.Hsapiens.UCSC.hg19.knownGene* (Carlson). A probe was said to be in a *Promoter* region if it was located inside the first exon, the 5'-UTR or a region up to 2kbp upstream of the transcription start site (TSS) of any given transcript. Similarly, a probe was labeled as *Intragenic* if it was inside any intron or any exon other than the first. *Intergenic* probes were determined as those not falling into either of the two previous categories. According to this definition, a probe could be in both a *Promoter* and an *Intragenic* region at the same time for different transcripts. A contingency table was built for each selected subset of probes and a given genomic region, with one variable indicating whether a given probe belonged or not to the subset, and the other indicating whether a given probe was labeled with the selected region. Significance of the association was determined by a Pearson's chi-squared test with Yates' continuity correction. A significance level of 0.05 was used to determine whether a subset was dependent with respect to a given genomic region. Odds Ratio was used as a measure of effect size.

CpG Island status analysis

The CpG island locations used in the analyses were obtained from the R/Bioconductor package *FDb.InfiniumMethylation.hg19* (Triche 2013) The generation procedure of these CpG Islands is described by Wu and colleagues (Wu *et al.* 2010). *CpG shores* were defined as the 2kbp regions flanking a CpG Island. *CpG shelves* were defined as the 2kbp region either upstream or downstream of each CpG shore. Probes not belonging to any of the regions thus far mentioned were assigned to the special category *Non-CpG Island*. Each probe was assigned to only one of the categories. A 4x2 contingency table was constructed for every subset of probes in order to study the association between the given subset and the different CpG Island categories. A chi-squared test was used to determine if any of the categories had a significant association with the given subset. For each of the CpG Island status levels, a 2x2 contingency table was defined and another chi-squared test was used to independently evaluate the association of the given subset with each status level, a significance level of 0.05 being employed for all tests. Effect size was reported as the Odds Ratio for each of the individual tests.

Microarray background correction

Although it is sometimes referred to as a genome wide solution, the Infinium450k microarray only covers a fraction of the entire genome. In its 27k predecessor, the probes were mainly located at gene promoter regions, while in addition to the promoter probes, the Infinium450k includes probes located inside genes and in intergenic regions (Dedeurwaerder *et al.* 2011).

The irregular distribution of probes can lead to unwanted biases when studying whether a selected subset of probes is enriched with respect to any functional or clinical mark. A reference to the background distribution of features was included in every type of statistical test performed in order to prevent our conclusions from being driven by the irregular distribution of probes. In qualitative tests (CpG Island status, genomic region or histone mark enrichment), the contingency matrix was built to represent the background distribution of the microarray. Thus, any significant result would indicate a departure from the fixed background distribution, and ignore any manufacturer bias.

Circos data track smoothing

In order to plot the CpG information on the Circos genome-wide graphs, smoothing was applied to our data. Broad histone peak information from UCSC was averaged by partitioning the genome into intervals of 200kbp and assigning to each a score corresponding to the average of the broad peak scores found within it. CpG locations were not smoothed. Distances in the Twins dataset were averaged using a 2Mbp window size.

Bisulfite pyrosequencing

DNA methylation patterns of representative dmCpGs during aging were analyzed by bisulfite pyrosequencing in an independent sample set of 46 MSCs obtained from individuals of different ages (**Supplemental Table 1**). Bisulfite modification of DNA was performed with the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's instructions. The set of primers for PCR amplification and sequencing were designed using the specific software PyroMark assay design (version 2.0.01.15). Primer sequences were designed to hybridize with CpG free sites to ensure methylation-independent amplification **(Supplemental Table 27)**. After PCR amplification of the region of interest with the specific primers, pyrosequencing was performed using PyroMark Q24 reagents, and vacuum prep workstation, equipment and software (Qiagen). A linear regression model was fitted to the pyrosequencing methylation data using *age* as a predictor.

Data analysis workflow

All the necessary steps for upstream and downstream analyses were defined and implemented using the Snakemake tool (Köster and Rahmann 2012). This tool helps data scientists to generate a reproducible and inherently parallel processing pipeline. The source code of the workflow is included as Supplemental Material.

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5.2 Paper V: Human endogenous retroviruses -H and -K expression in human mesenchymal stem cells as potential markers of stemness

TITLE PAGE

HUMAN ENDOGENOUS RETROVIRUSES -H AND -K EXPRESSION IN HUMAN MESENCHYMAL STEM CELLS AS POTENTIAL MARKERS OF STEMNESS.

Short running title: HERV-H and K as potential stemness markers

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ABSTRACT

The “HERV” (human endogenous retroviruses) are endogenous retroviruses that are inserted into the germ cell DNA of primate over 30 million years ago. The core transcription factors are involved in pluripotency, including POU class 5 homeobox 1 (OCT-4), sex determining region Y-box 2 (SOX-2), and NANOG homeobox (NANOG) representing approximately 80% of the LTRs and regulating the expression of the 50 most highly expressed HERV-H proviruses.

Our aim was to evaluate *pol* gene expression of HERV-K and HERV-H in Mesenchymal stem cells (MSCs) that are adult multipotent stem cells during their expansion.

MSCs were isolated from BM of healthy donors and expanded until the 5th passage in □-MEM with 10% fetal bovine serum. HERV-K, -H pol gene, NANOG, OCT-4, SOX-2 and GAPDH expression was quantified by real-time PCR in MSCs during the expansion.

HERV-K and HERV-H expression was always higher at p1 compared to other passages and this difference reached a high statistical significance when passage p1 was compared with passage 3. In addition, NANOG, OCT-4 and SOX-2 expression at p1 was significantly higher than the expression at p3. Spearman’s test demonstrated a strong correlation between the expression of HERV-K and HERV-H and expression of NANOG, OCT-4 and SOX-2

Our findings showed that HERV-K and –H were concurrently expressed with pluripotency biomarkers NANOG, OCT-4 and SOX-2. These findings might suggest that the HERVs pol genes play an important role in the differentiation of the MSCs and should be considered as new markers of stemness or differentiation for MSCs.

KEYWORDS:

Mesenchymal stem cells, endogenous retroviruses, NANOG, OCT-4 , SOX-2

INTRODUCTION

The human endogenous retroviruses (HERVs) are endogenous retroviruses that are inserted into the germ cell DNA of humans over 30 million years ago (Sverdlov, 2000). They are able to modify the cell gene expression and their inserted Long terminal repeats (LTRs) can act as alternative promoters to stimulate expressions of nearby genes and cause the activation of oncogenes or inactivation of tumor suppressor genes (Götzinger et al., 1996; Katoh and Kurata, 2013). HERV-H and K transcripts are expressed in ESCs/iPSCs at higher levels than in differentiated cells (Fuchs et al. 2013; Santoni, Guerra, e Luban 2012b). The core transcription factors including pluripotency factors such as POU class 5 homeobox 1 (OCT-4), sex determining region Y-box 2 (SOX-2), and NANOG homeobox (NANOG) occupy approximately 80% of the LTRs and regulate the expression of the 50 most highly expressed HERV-H proviruses. Furthermore, Naive embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are associated with elevated transcription of HERVH, so their expression may be an indicator of pluripotency (Santoni, Guerra, e Luban 2012b; J. Wang et al. 2014b). Moreover, LTR7/HERV-H is one of the most over-expressed transposable elements (TE) seeding NANOG and POU5F1 binding sites throughout the human genome (Kunarso et al., 2010).

Mesenchymal stromal cells (MSCs) are adult stem cells with extensive self-renewal capacity and multipotent cells (Mareschi et al., 2016). SOX-2, NANOG and OCT-4 are three transcription factors able to maintain the stem cell pluripotency and self-renewal (Xiao et al., 2017). Based on these considerations, our aim was to assess *pol* gene expression of HERV-K and HERV-H in MSCs and to investigate their possible role as pluripotency markers. The purpose of this study was to evaluate *pol* gene expression of HERV-K and HERV-H in MSCs at different passages by real-time PCR.

MATERIAL AND METHODS

BM-MSC isolation and expansion

Human bone marrow (BM) samples were collected after submission of written consent, in accordance with the ethics committee of the hospitals Ospedale Infantile Regina Margherita-Sant'Anna-Mauritian order, which approved the collection of the samples and according to the Declaration of Helsinki. Written informed consent was taken from all the patients and Ethics committee of the hospitals Ospedale Infantile

Regina Margherita-Sant'Anna-Mauritian order, approved the collection of the samples used in this study

BM cells were harvested from the iliac crest of adult or pediatric Caucasian donors who underwent BM collection for a familiar allo-BM- transplantation in the Pediatric Oncohematology, Stem Cell Transplantation and Cell Therapy Division, City of Science and Health of Turin, Regina Margherita Children Hospital, Turin, Italy. When available, an unfiltered BM collection bag was also used (Baxter Healthcare, Deerfield, IL) and was normally discarded before the BM infusion. The bag was washed three times with 1x phosphate-buffered saline (PBS) (Lonza, Versviers, Belgium), and the cells were collected and washed at 200g for 10 min. An aliquot of whole BM was counted and plated directly in T25 or T75 flasks (Becton Dickinson, Franklin Lakes, NJ, USA) at $1x10^4$ cells/cm^2. The culture medium was □-MEM (Biochrome, Berlin, Germany) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich), 2 mmol/L L-glutamine (Sigma-Aldrich), penicillin/streptomycin 1X (Euroclone, Pero, MI, Italy). The culture was maintained at 37°C in a 5% CO2 atmosphere. After 5–7 days, non-adherent cells were removed, and the adherent cells were re-fed every 3–4 days. To expand the isolated cells, the adherent semi-confluent monolayer was detached with trypsin/EDTA (Sigma-Aldrich) for 5 min at 37°C and expanded for several passages until they no longer reached confluence (Mareschi et al., 2006).

MSC analysis and characterization

BM MSCs used for this study were analyzed for viability, immunophenotype, and differential and proliferative potential to verify the characteristics of the MSCs. To analyze the immunophenotype, flow cytometry analysis was performed on MSCs using the following antibodies: anti-CD90 fluorescein isothiocyanate (FITC), CD73 phycoerythrin (PE), CD34 FITC, CD14 FITC, CD45 FITC (Beckman Coulter) and CD105 PC-7 and CD146 APC (Miltenyi Biotec, Bologna, Italy). Details of the cytofluorimetric analysis are described below. To analyze multipotent capacity, MSCs isolated from BM were cultured in osteogenic (StemCell Technologies), adipogenic (StemCell Technologies), and chondrogenic (Lonza, Cologne, Germany) media for 21 days, according to the manufacturer's instructions. Briefly, 5,000 and 10,000 cells, for control samples and differentiation experiments, were seeded in a six-well plate for osteogenic and adipogenic culture conditions, respectively. Osteogenic differentiation was demonstrated by the accumulation of crystalline hydroxapatite on Von Kossa

staining, and adipogenic differentiation, by the presence of intracellular lipid vesicles assessed with oil red O. MSC chondrogenic differentiation was achieved as previously described and was evaluated by alcian blue staining, which identifies the presence of hyaluronic acid and sialomucin (Mareschi et al., 2016).

Reverse transcription and relative quantification by real-time PCR

Total RNA was extracted from MSCs using the automated extractor Maxwell (Promega, Madison, WI) using simply RNA Blood Kit protocol without modification. One microgram of total RNA was reverse-transcribed with 8 µl of buffer 10X, 4.8 µl of $MgCl_2$ 25 mM, 2 µl ImpromII (Promega), 1 µl of RNase inhibitor 20U/l, 0.4 µl random hexamers 250 µM (Promega), 2 µl mix dNTPs 100 mM (Promega) and dd-water in a final volume of 20 µl. The reaction mix was carried out in a GeneAmp PCR system 9700 Thermal Cycle (Applied Biosystems, Foster City, CA, USA) under the following conditions: 5 min at 25 °C, 60 min at 42 °C and 15 min at 70 °C for the inactivation of enzyme; the cDNAs were stored at −80° until use.

Relative quantification of mRNA expression of selected genes was achieved by means of Taqman amplification and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was chosen as a reference gene, using the ABI PRISM 7500 real time system (Life technologies, Texas, USA). The expression of HERV-K, -H pol gene, NANOG, OCT-4, SOX-2 and GAPDH was quantified by real-time PCR. Approximately 100 ng cDNA were amplified in a 20 µl of total volume reaction containing 2.5 U ampli-Taq Gold DNA polymerase (Applied Biosystem, USA), 1.25 mmol/l MgCl2 and 500 nmol of specific primers and 200 nmol of specific probe were used: (KPOLF-5'-CCACTGTAGAGCCTCCTAAACCC-3')(KPOLR-5'-TTGGTAGCGGCCACTGATTT-3') and probe (KPOLP-6FAM-CCCACACCGGTTTTTCTGTTTTCCAAGTTAA-TAMRA); HERV-H primers (HPOLF-5'- TGGACTGTGCTGCCGCAA-3') (HPOLR-5'-GAAGSTCATCAATATATTGAATAAGGTGAGA-3') and probe (HPOLP-6FAM-TTCAGGGACAGCCCTCGTTACTTCAGCCAAGCTC-TAMRA); GAPDH specific primers (GAPDHF-5'-CCAAGGTCATCCATGACAAC-3') (GAPDHR-5'-GTGGCAGTGATGGCATGGAC-3') and probe (GAPDH-6FAM-TGGTATCGTGGAAGGA-3' MGB); NANOG primers (NANOGF-5'-GCCAGGATGGTCTCGATCTC-3')(NANOGR-5'-GGTGGCTCACGCCTGTAAAT-3') and probe (NANOGP-6FAM-TGACCTTGTGATCCACCCGCCTC –TAMRA); OCT-4 primers (OCT4F-5'-

ACCCACACTGCAGCAGATCA-3’) (OCT4R-5’-CACACTCGGACCACATCCTTCT-3’) and probe (OCT4P-6FAM-CCACATCGCCCAGCAGCTTGG –TAMRA) and SOX-2 primers (SOX2F-5’-TGCGAGCGCTGCACAT-3’) (SOX2R-5’-GCAGCGTGTACTTATCCTTCTTCA-3’) and probe (SOX2P-6FAM- CCGGCGGAAAACCAAGACGCT –TAMRA). The established assays use the probe and primers designed by Primer ExpressTM software version 3.0 (Applied Biosystems, Foster City, USA).

The amplifications were in a 96-well plate at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60°C for 1 min. Each sample was run in triplicate. Relative quantification of target genes expression in patients compared with normal samples was performed with the ΔΔCt method and the relative results are expressed in corresponding arbitrary units (AU).

Statistical analysis

Statistical analyses were performed using the Prism software (GraphPad Software). The Shapiro-Wilk test was used to verify the normality of the data distribution. Because the MSCs are heterogeneous cells which can express different levels of stemness markers on the basis of the donor age for instance, we compared the different gene expression between paired samples at the different passages by paired t test. Multivariate analysis was excluded because there was no homogeneity of variance between the groups. Pearson’s test was used to analyze the correlation between HERV-H and HERV-K expression and the embryonic markers. All statistical tests were considered significant at $p< 0.05$, highly significant at $p< 0.001$, and very highly significant at $p < 0.0001$.

RESULTS

For quantitative evaluation of *pol* gene expression of HERV-K, HERV-H, NANOG, OCT-4 and SOX-2 real-time PCR by TaqMan system was used. The threshold value Ct, computed for each of the genes as the average of 3 determinations, was used to measure the amount of resulting PCR products.

The study group enrolled 15 BM-MSCs from passage 1 to 5 (p1-p5). Because retro-transcription efficiency was equivalent in all samples, as suggested by GAPDH expression, the hypothesis that the strong variations of signal intensity between BM-MSCs could be related to differences in HERV-K, –H pol, NANOG, OCT-4 and SOX-2 transcriptional levels was formulated.

Relative expression of HERV-K and HERV-H pol gene in MSCs at different passages (*p1-p5)*

HERV-K expression was always higher at p1 than at other passages, as shown in Figure 5-6 Analysis by real-time quantitative RT-PCR of HERV-K gene expression at the different passages of analyzed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with $p<0.05$ and $p<0.01$. This difference reached a high statistical significance with p =0.0071 when passage p1 was compared with passage 3. Conversely, no statistical significances were observed when passage p1 was compared with passages 2, 4 and 5 (p values were respectively: 0.1523, 0.3328 and 0.4712). Also, HERV-H expression was higher at p1 than at any other passages, as shown in Figure 5-6

Moreover, this difference reached a statistical significance with p = 0.049 when passage p1 was compared with passage 3. Conversely, no statistical significance was reached when passage p1 was compared with passages 2, 4 and 5. (p values were respectively: 0.0744, 0.4436 and 0.4278)

Relative expression of NANOG, OCT-4 and SOX-2 in MSCs at different passages (p1-p5)

NANOG expression always resulted higher at p1 than at any other passages reaching a statistical significance when NANOG expression was compared between p1 and p3 (p=0.0398) as shown in Figure 5-8 . Analysis by real-time quantitative RT-PCR of NANOG gene expression at the different passages of analysed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with $p<0.05$ and $p<0.01$. P values calculated by paired t test between NANOG expression at p1 and respectively at p2, p4 and p5 were 0.9572, 0.4828 and 0.2724)

Furthermore, OCT-4 expression at p1 was significantly higher than the expression at p3 (p = 0.0151), as shown in Figure 5-9. Conversely, no statistical significance was observed when passage p1 was compared with passages 2, 4 and 5 (p values were respectively: 0.1639, 0.0821 and 0.142 .

As shown in Figure 5-10, SOX-2 expression was more significantly higher at p1 than at p3 (p=0.0011). No statistically different differences were observed for SOX-2 expression between p1 and p2, p4 and p5 (p = 0.1810, 0.0054, and 0.0667 respectively

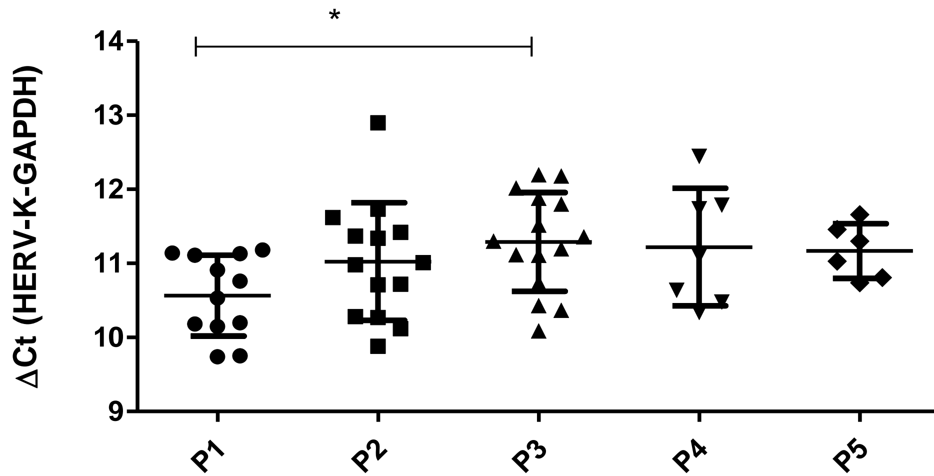

*Figure 5-6 Analysis by real-time quantitative RT-PCR of HERV-K gene expression at the different passages of analyzed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with p<0.05 and p<0.01.*

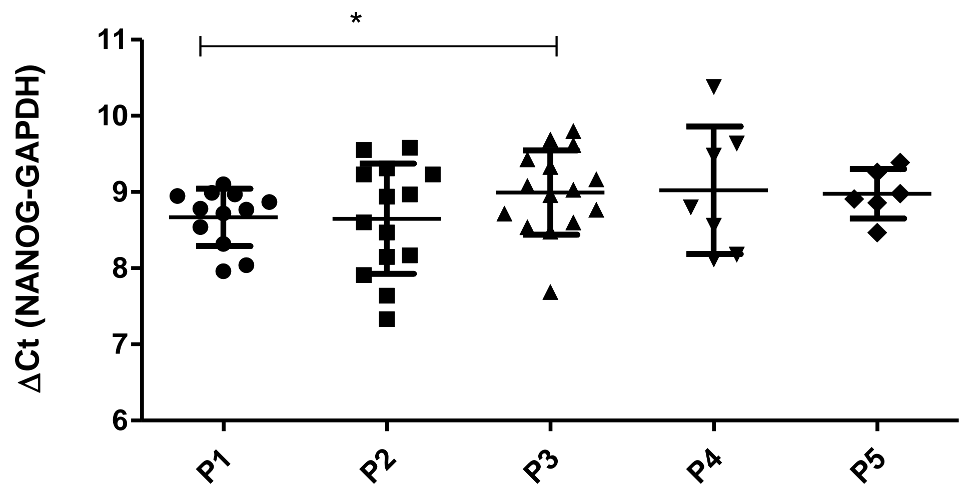

*Figure 5-7. Analysis by real-time quantitative RT-PCR of HERV-H pol gene expression at the different passages of analyzed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with p<0.05 and p<0.01.*

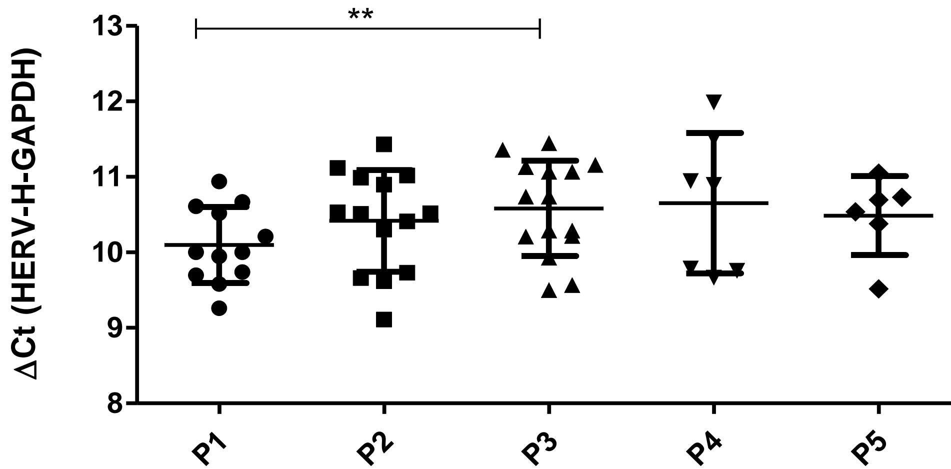

*Figure 5-8 . Analysis by real-time quantitative RT-PCR of NANOG gene expression at the different passages of analysed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with p<0.05 and p<0.01.*

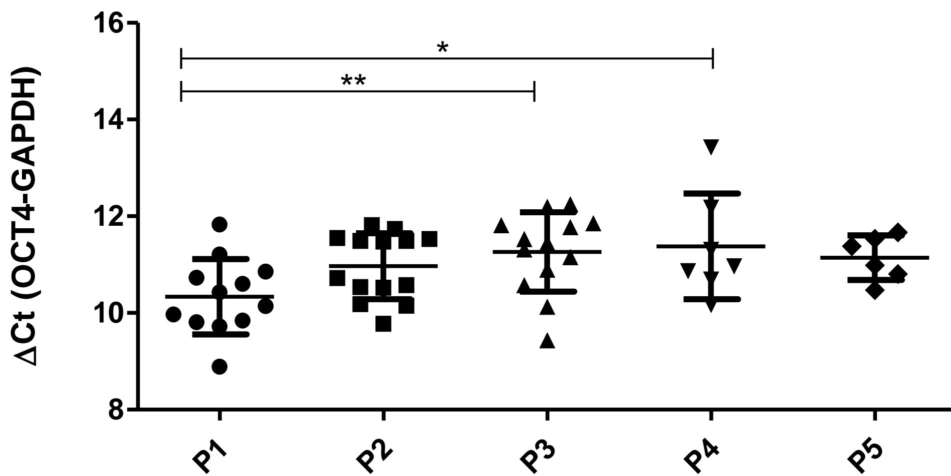

*Figure 5-9. Analysis by real-time quantitative RT-PCR of OCT-4 gene expression at the different passages of analysed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with p<0.05 and p<0.01.*

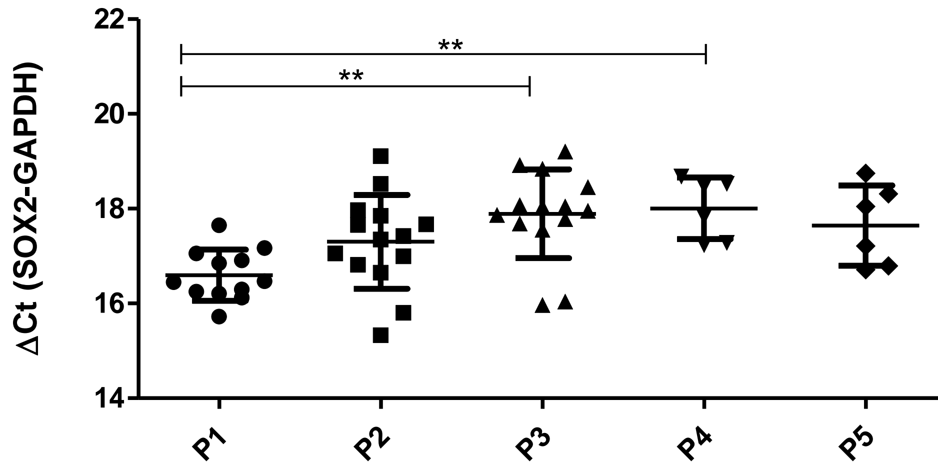

*Figure 5-10 Analysis by real-time quantitative RT-PCR of SOX-2 gene expression at the different passages of analysed MSCs. The symbol * and ** indicate, respectively, a statically significant difference with p<0.05 and p<0.01.*

Correlation analysis of HERV-K and -H expression with Pluripotency biomarkers NANOG, OCT-4 and SOX-2 expression

Moreover, a correlation between the HERV-H and HERV-K pol gene expression levels, and the NANOG, OCT-4 and SOX-2 expression was analyzed. Pearson's test demonstrated a strong correlation between the expression of HERV-K and HERV-H and expression of NANOG, OCT-4 and SOX-2 (table 1). In particular, HERV-H was significantly correlated with: - HERV-K expression at the passage 2, 3, 4 and 5; NANOG expression at the passage 2 and 3 with p values $<0.0001$and at the passage 4 with p <0.05; OCT-4 expression at all passages; SOX-2 at the passage 3 and 5 with p value<0.05. HERV-K was also significantly correlated with NANOG and OCT-4 expression at the passage 2, 3, 4 and 5.

The expression levels of NANOG, OCT-4 and SOX-2 were plotted among them. Pearson's test demonstrated a strong correlation between the expression of NANOG, OCT-4 and SOX-2 (data not shown), supporting the idea that an asynchrony between the genes was reached.

DISCUSSION

The aim of our study was to evaluate gene expression profile of HERV-H and K in MSCs at different passages. Our findings showed that these endogenous viruses were associated to the expression of the pluripotency biomarkers NANOG, OCT-4 and SOX-2. These results might suggest an important role of HERV pol genes in the MSC differentiation.

	HERV -H vs NANOG		
Passage	Pearson r	P value (two-tailed)	P value summary
P1	0.1952	0.5889	ns
P2	0.9582	< 0.0001	***
P3	0.8487	< 0.0001	***
P4	0.8867	0.0078	**
P5	0.6408	0.121	ns

HERV -K vs NANOG		
Pearson r	P value (two-tailed)	P value summary
0.1206	0.74	ns
0.6999	0.0053	**
0.7135	0.0028	**
0.883	0.0084	**
0.7601	0.0473	*

	HERV -H vs OCT-4		
Passage	Pearson r	P value (two-tailed)	P value summary
P1	0.289	0.3887	ns
P2	0.8728	< 0.0001	***
P3	0.8278	0.0005	***
P4	0.9036	0.0053	**
P5	0.903	0.0053	**

HERV -K vs OCT-4		
Pearson r	P value (two-tailed)	P value summary
0.2517	0.4554	ns
0.7599	0.0016	**
0.6993	0.0078	**
0.8917	0.007	**
0.9016	0.0055	**

	HERV -H vs SOX2		
Passage	Pearson r	P value (two-tailed)	P value summary
P1	0.8785	0.05572	ns
P2	0.2668	0.3187	ns
P3	0.563	0.0452	*
P4	-0.04541	0.9319	ns
P5	0.7578	0.0484	*

HERV -K vs SOX2		
Pearson r	P value (two-tailed)	P value summary
0.8877	0.05147	ns
0.2454	0.3977	ns
0.2877	0.3192	ns
0.5596	0.2691	ns
0.1625	0.5908	ns

	HERV -H vs HERV-K		
Passage	Pearson r	P value (two-tailed)	P value summary
P1	0.5853	0.0586	ns
P2	0.7175	0.0039	**
P3	0.5949	0.0193	*
P4	0.7536	0.049	*
P5	0.8608	0.0129	*

*Table 5-2 Comparison of the expression levels of pol gene HERVs with pluripotency biomarkers NANOG, OCT4 and SOX calculated by Pearson Correlation Test. Pearson r and p value are showed and *, ** and *** indicate, respectively, if the correlation is significant (. p< 0.05), highly significant (p< 0.001), and very highly significant (p < 0.0001), ns= not significant.*

Recently Santoni et al (Santoni, Guerra, e Luban 2012b) described a strong association between the HERV-H genomic location and H3K4me3-modified histones, suggesting that HERV-H contributes to pluripotency in human ES and in some iPS

cells. HERV-H expression was high in these pluripotent stem cells. In addition to the binding of NANOG, OCT-4, and SOX-2 to the HERV-H promoter, HERV-H RNA decreased in ES cells differentiated in a manner that was proportional related to the expression of NANOG and OCT-4 (Santoni, Guerra, e Luban 2012b). Moreover, NANOG, OCT-4, and SOX-2 are or bound to the LTRs of transcriptionally active HERV-H proviruses, or within the 2 kB (Ohnuki et al., 2014). In accordance with Santoni, we demonstrated that HERV-H and –K pol gene expression is correlated with NANOG, OCT-4, and SOX-2 expression. HERV-H and K can be exploited as a reliable marker of MSCs cell pluripotency, as well as an indicator of the degree of "stemness". SOX-2 was more stably expressed during the differentiation than NANOG or OCT-4 expression and its expression did not correlate with that of HERV-H (Santoni, Guerra, e Luban 2012b).

In accordance with our data Fuchs and colleagues demonstrated the same kinetics of HERV-K and OCT-4 and NANOG expression during reprogramming of the iPSCs (Fuchs et al., 2013) in response to activation of the LTR5; and Ohnuki and colleagues demonstrated the same kinetics of HERV-H and OCT-4 and SOX-2 expression during reprogramming of the iPSC (Ohnuki et al., 2014). HERV-H activation is inversely correlated with the DNA methylation status, and the activated copies are marked with transcriptionally active histone marks (H3K4me1/2/3, H3K9ac, H3K36me3, and H3K79me2), while the repressive marks, (H3K9me3 and H3K27me3) are rare (Izsvák et al., 2016). In order to drive transcription in pluripotent stem cells, TEs should contain transcription factor (TF) binding sites for factors expressed in these cells. The genomic expansion of HERV-H over time was likely facilitated by the presence of core key pluripotent TF binding sites in the LTR. The pluripotency factors NANOG, OCT-4, KLF4, and LBP9/Tfcp2l1 all bind to active LTR7s of HERV-H and drive transcription of HERV-H derived transcripts. Transactivation of LTR5_Hs/HERV-K by pluripotency master transcription factor POU5F1 (OCT-4) at hypomethylated long terminal repeat elements (LTRs) represents the most recent genomic integration sites of HERV-K retroviruses. Moreover, this mechanism induces HERV-K expression during normal human embryogenesis, beginning with embryonic genome activation at the eight-cell stage, continuing through the stage of epiblast cells in preimplantation blastocysts, and ceasing during hESC derivation from blastocyst outgrowths (Glinsky 2015; Göke et al. 2015b). Grow et al. (Grow et al., 2015) reported also an unequivocal experimental evidence

demonstrating the presence of HERV-K viral-like particles and Gag proteins in human blastocysts, consistent with the idea that HERVs are functionally active during early human embryonic development

In conclusion, our study demonstrates that human HERV-K and -H elements show the same kinetics of pluripotency biomarkers NANOG, OCT-4 and SOX-2. We could conclude that the pol gene of HERV-K and -H should be considered as new markers of stemness or differentiation.

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WORKS IN PROGRESS

6 PAPERS IN DRAFT

6.1 INACTIVATED PLATELET LYSATE SUPPORTS PROLIFERATION AND IMMUNODULANT CHARACTERISTICS OF MESENCHYMAL STROMAL CELLS IN GMP CULTURE CONDITION

6.1.1 INTRODUCTION

For all these reasons there has been a growing use of BM-MSCs as Advanced Therapy Medicinal Products (ATMP) which has led to production processes that need to meet Good Manufacturing Practice (GMP). The use of xenogeneic protein free GMP-compliant growth media is a prerequisite for clinical MSC isolation and expansion. Human platelet lysate (HPL) has been efficiently implemented into MSC clinical manufacturing as a substitute for foetal bovine serum (FBS). As the use of human-derived blood materials alleviates immunologic risks, but not the transmission of blood-borne viruses, the aim of our study was to test an even safer alternative than HPL to FBS: HPL subjected to pathogen inactivation by psoralen (iHPL) and study its effectcs on MSCs immunomodulant properties. In a recent study performed in the "Stem Cell Transplantation and Cellular Therapy Laboratory" at the Regina Margherita Hospital (directed by Dr Franca Fagioli), we demonstrated that iHPL is safer than HPL and represents a good, GMP-compliant alternative to FBS for MSC clinical production which is even more advantageous in terms of cellular growth and stemness (Castiglia et al. 2014c). On the base of our findings about iHPL and as in literature emerged a possible decrease of immunomodulant properties of MSCs cultured in HPL (Abdelrazik et al. 2011) we want to test iHPL's effects on immunomodulatory properties of MSCs.

In this study MSCs cultivated in two different culture conditions, which are alpha-MEM with 10 % of FBS and iHPL, were compared for their immunomodulant properties. In particular, we focused on the interaction of two groups of MSCs, FBS-MSC and iHPL-MSC, with T lymphocytes (Ly).as described in the paper 3.1. The effects of MSC cultured with the three different culture supplement on Ly, were analysed by using in vitro co-culture system.

In particular, the following were tested:

- the effect on proliferation of total Ly;

- the effect on proliferation of naïve T lymphocyte subsets induced to differentiate versus Th1 and Th2 Ly;
- presence of Treg;
- immunophenotype of different T cell subsets (naïve, memory, effector, Th1 and Th2 lymphocytes)
- cytokine release and master gene expression to verify Th1, Th2 and Th17 polarization
- production of IDO a potent immunomodulant soluble factor

6.1.2 MATERIAL AND METHODS

We used the same experimental design described in the paragraph 3.1 (Figure 3-1)

6.1.3 RESULTS

6.1.3.1 *MSC characteristics*

Thawed FBS-MSCs and iHPL-MSC, grew and reached confluence within a few days. Prior to use, the cells were analysed for immunophenotype and multipotent characteristics. MSCs, independently from the culture condition were negative for CD45, CD34 and CD14 and HLA-DR and were positive (over 95%) for CD90 (a membrane glycoprotein, also called Thy-1), CD105 (endoglin) and CD73. CD146 (cell surface glycoprotein MUC18) was also tested and was positive in all samples. No statistical differences were observed among the three groups in terms of both positive cell percentages and fluorescence means of the positive markers.

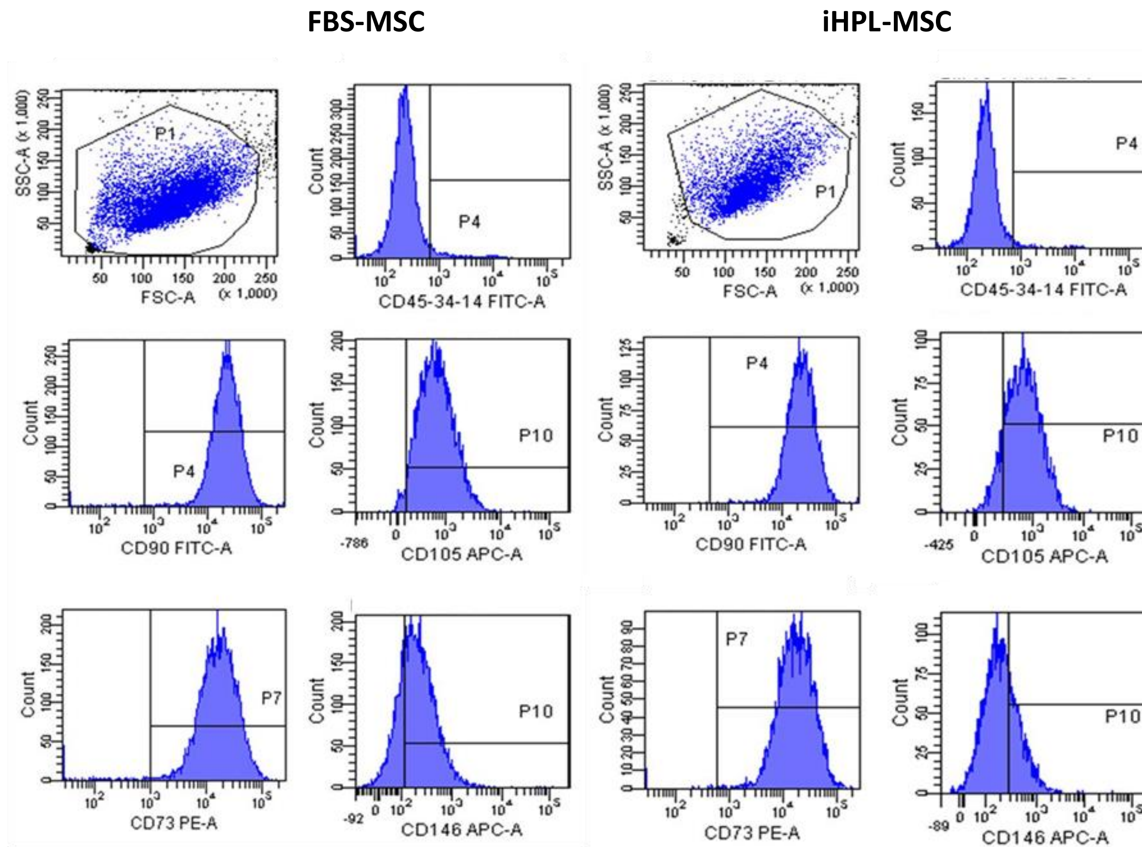

Figure 6-1 - Immunophenotype of a representative FBS-MSC and iHPL-MSC culture post thawing

6.1.3.2 *MSC/T cell interaction and Proliferative assay*

The 3H-thymidine incorporation data of each experiment (n=5), expressed as a mean cpm of the triplicate (Table 3).

Comparing the mean cpm of each group with the control condition which was PBMC stimulated with PHA (PHA-PBMC), a decrease in proliferation was significant in the presence of FBS-MSCs (p=0.038) and almost significant (0.056) in the presence of iHPL-MSCs. By contrast, as shown in Figure 12, no significant differences were observed between FBS-MSC and iHPL-MSC. However, in Th1 and Th2 induced PBMCs, different modulations in proliferative activity, were observed, without significant differences between FBS-MSC and HPL-MSC.

	PHA-PBMC	PHA-PBMC + FBS-MSC	PHA-PBMC + iHPL-MSC
exp 1	197073	43584	77978
exp 2	92286	38120	48739
exp 3	234100	10353	26051
exp 4	137472	27698	32542
exp 5	40394	49800	33306
	Th1	**Th1 + FBS-MSCs**	**Th1+ iHPL-MSCs**
exp 1	52144	32774	24543
exp 2	40118	19003	49142
exp 3	42839	14820	9858
exp 4	54344	29772	32755
exp 5	29800	40275	28159
	Th2	**Th2 + FBS-MSCs**	**Th2 + iHPL-MSCs**
exp 1	39879	81740	80821
exp 2	146513	16384	33592
exp 3	123789	17778	31428
exp 4	26815	89537	95636
exp 5	32248	70408	31718

Table 3 - Mean cpm of the triplicate proliferative test in PBMC

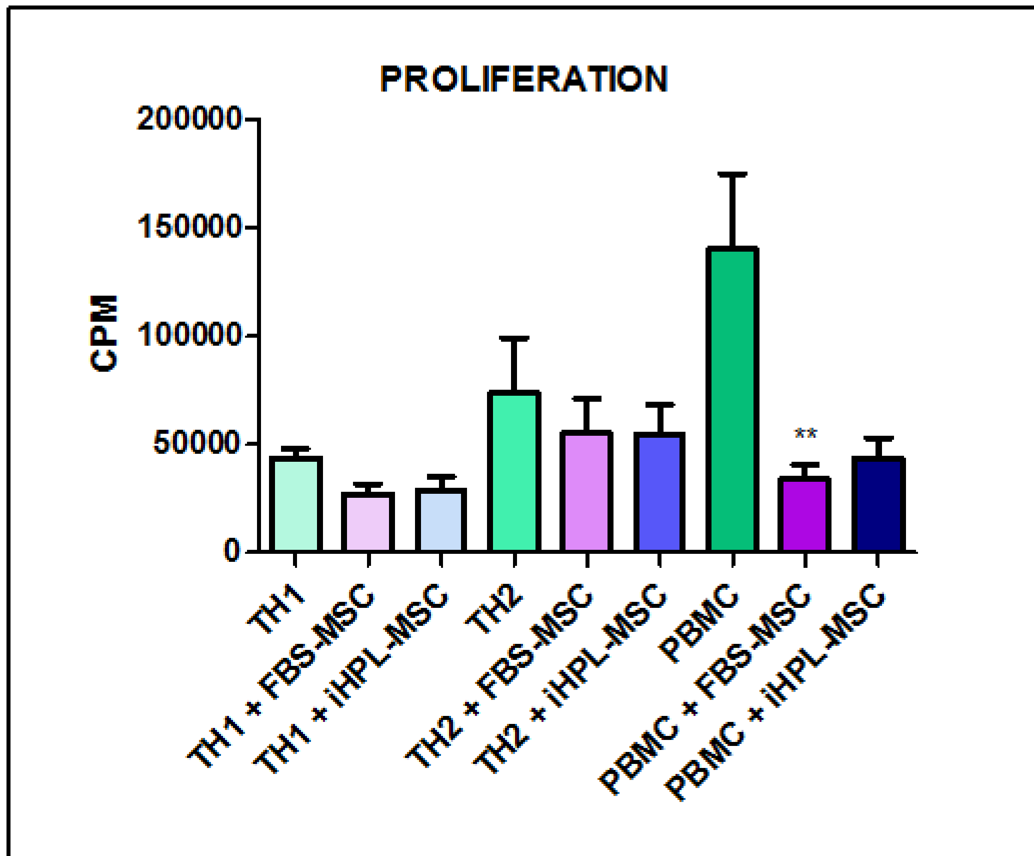

Figure 6-2 - Proliferative assay on stimulated PBMCs and on induced Th1 and Th2 cells alone and in co-culture with FBS-MSC o iHPL-MSC

6.1.3.3 *T cell subsets determination*

The multiparameter flow cytometric analysis allowed the identification of the following T subsets, based on the antibody combination used:

- naïve cytotoxic T cells : $CD45RA^+$ /$CD3^+$/$CD8^+$
- naïve Th cells: $CD45RA^+$/ $CD3^+$/$CD4^+$
- memory cytotoxic T cells $CD45RO^+$/$CD3^+$/$CD8^+$
- memory Th cells: $CD45RO^+$/$CD3^+$/$CD4^+$

The percentage obtained by cytofluorimetric analysis (was used to calculate the absolute number based on the cell number counted after 5 days of co-culture (stimulated PBMC+FBS-MSC and PBMC+iHPL-MSC). The gating strategy was shown in Figure 13. The data, obtained from 11 experiments, are summarized in Table 4. The percentage of these subsets was strictly related to the variability of the donors. Despite we obtained a variable number in the stimulated PBMC, we reaching a strong

statistical significance in both the co-culture PBMC+FBS-MSC and PBMC+iHPL-MSC in comparison to the single culture of stimulated PBMC. Interestingly, in all experiments the different subsets showed the same modulation trend. In particular, in PHA-PBMC, memory T cells were higher than naïve Th, and after co-culture with both FBS-MSC and iHPL-MSC, it was observed that:

1. This ratio reversed in favour of naïve T cells, especially naïve cytotoxic subsets (Figure 14 B and D).
2. Both CD4 and CD8 memory T cell subset significantly decreased (Figure 14).
3. No statistical significant differences were observed between co-culture FBS-MSC and iHPL-MSC

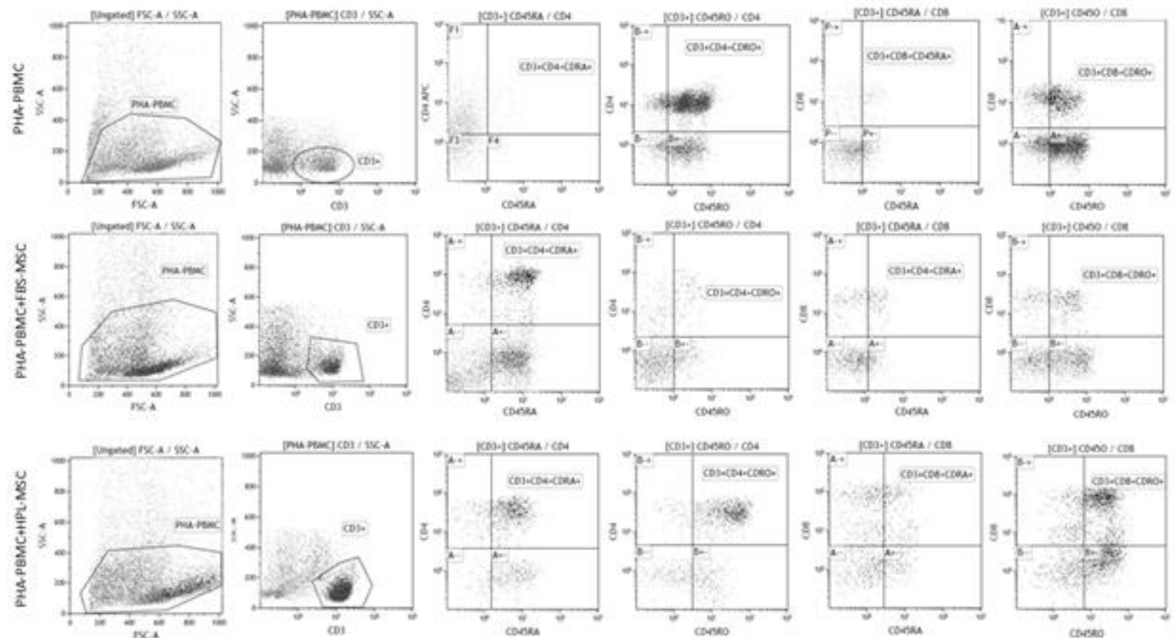

Figure 6-3- Gating strategy to evaluate the percentage of T lynfocyte subsets.

	PBMC	PBMC FBS-MSC	PBMC iHPL-MSC		PBMC	PBMC FBS-MSC	PBMC iHPL-MSC
CD3+/4+/RA+	130570	2827355	3331692	**CD3+/4+/RO+**	4453356	1507565	2395575
	146850	95889	---		781242	213431	---
	156200	136080	283170		634769	503496	353970
	300000	600000	2015000		5340000	1560000	2788500
	240000	273000	480000		3315000	168000	1205000
	465000	605000	975000		6090000	340000	355000
	280000	480000	350000		592200	320000	142000
	32250	44000	50000		462250	110000	102000
	19500	29250	23750		1131000	65250	32500
	271050	1676880	1434000		3315000	778260	705600
	485550	695565	471000		2772900	418860	125400
	PBMC	**PBMC FBS-MSC**	**PBMC iHPL-MSC**		**PBMC**	**PBMC FBS-MSC**	**PBMC iHPL-MSC**
CD3+/8+/RA+	54801	904650	1398042	**CD3+/8+/RO+**	2388395	244200	394497
	327184	96307	---		799084	117374	---
	95604	105144	523875		131013	82902	179221
	280000	348000	604500		7120000	822000	1384500
	45000	189000	320000		3975000	238000	810000
	300000	285000	525000		3705000	130000	145000
	168000	236000	225000		3948000	120000	80000
	21500	36000	44000		268750	52000	54000
	29250	31500	31250		936000	69750	36250
	97500	605880	1068000		992550	640560	651600
	173550	576225	517800		1187550	280800	206400

Table 6-1 Table 4 - Naïve, memory, effector T subsets

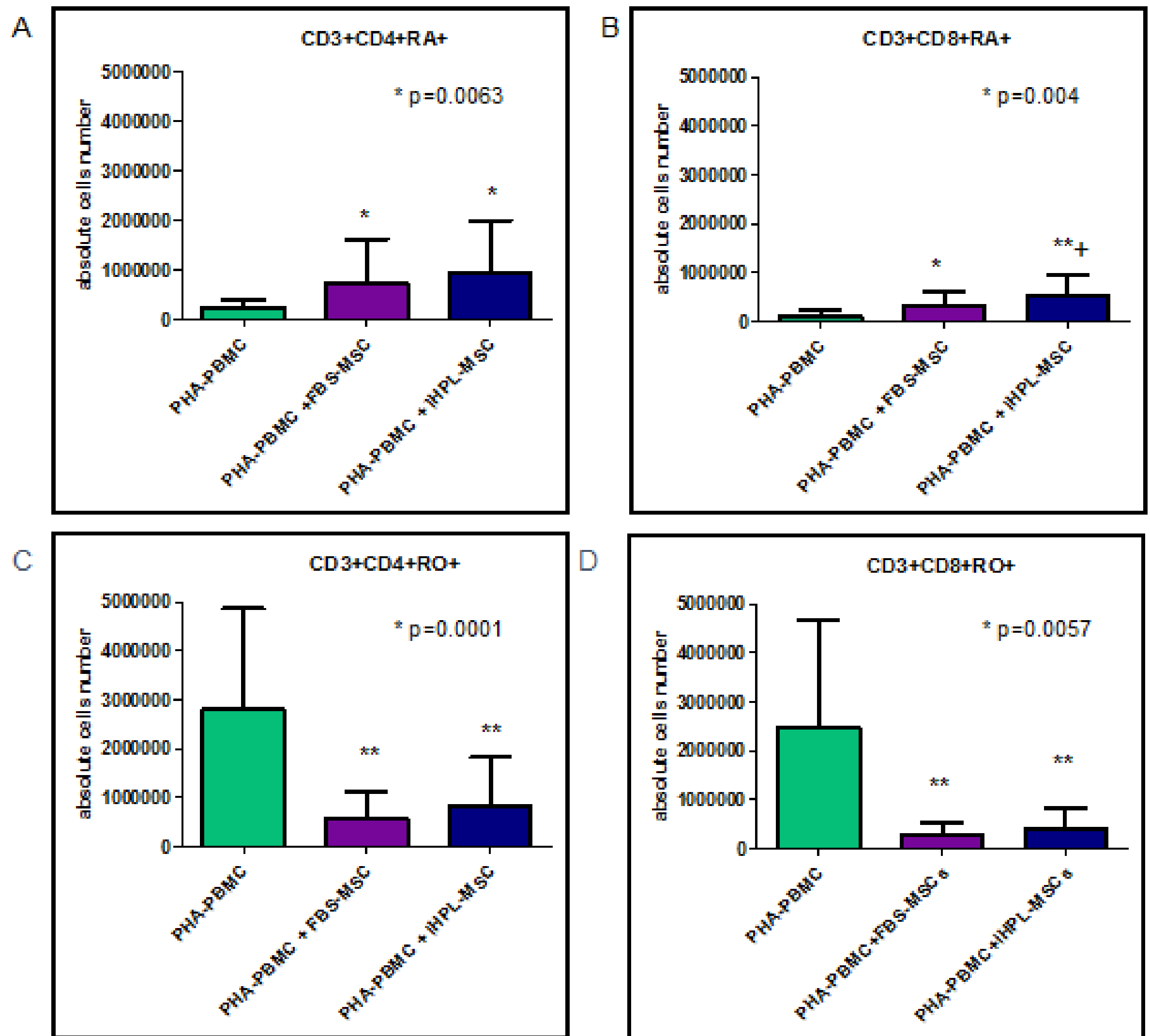

Figure 6-4 - Naïve and Memory T subsets

6.1.3.4 *Treg evaluation*

Preliminary experiments were performed on PHA-PBMC. Only the percentage of cells concurrently positive for CD4/CD25/Foxp-3, without counting the cells in the culture were analysed. In this set of experiments (N=3) a variable modulation depending of the different MSC types was observed, but based on some considerations obtained on T proliferation data, for a proper evaluation of T reg cells, the absolute number of T reg analysis was considered the best value to the analysis.

As PHA is usually used to induce *in vitro* T reg, and as in this study other co-culture experiments to trigger T lymphocytes with PHA were developed, T reg evaluation was evaluated considering their absolute number in either PBMC alone in basal conditions

and after stimulation with PHA, or in co-culture with MSCs cultured in alpha-MEM + 10% of FBS or 10% of iHPL (Table 5).

An increase of absolute T reg number in all co-cultures experiments was observed. Although the number of samples is limited for a statistical analysis, as shown in Figure 15, we observed an increase in T reg absolute number in both the PBMC+FBS-MSC and PBMC+iHPL-MSC co-culture without differences between the two experimental conditions. No significant differences were observed in non-stimulated co-cultures.

	No PHA			PHA		
	EXP1	**EXP2**	**EXP3**	**EXP1**	**EXP2**	**EXP3**
	absolute cell number after 5 days					
PBMC	600000	3000000	3300000	10072000	11000000	8700000
PBMC+FBS-MSC	7400000	---	4000000	7400000	3800000	6000000
PBMC+iHPL-MSC	7000000	3100000	4800000	9000000	6000000	6900000
	CD4+/CD25+/FoxP3+ (%)					
PBMC	---	39.5	10.2	1.5	16.4	5.9
PBMC+FBS-MSC	12.9	---	28	18.3	19.4	20
PBMC+iHPL-MSC	4.1	25.8	20.7	27.4	14.4	12.4
	CD4+/CD25+/FoxP3+ absolute cell number					
PBMC	---	48000	3300	37520	96000	78300
PBMC+FBS-MSC	66600	---	32000	125800	129200	300000
PBMC+iHPL-MSC	14000	65100	72000	36000	198000	227700

Table 6-2-Treg evaluation on 3 experiments

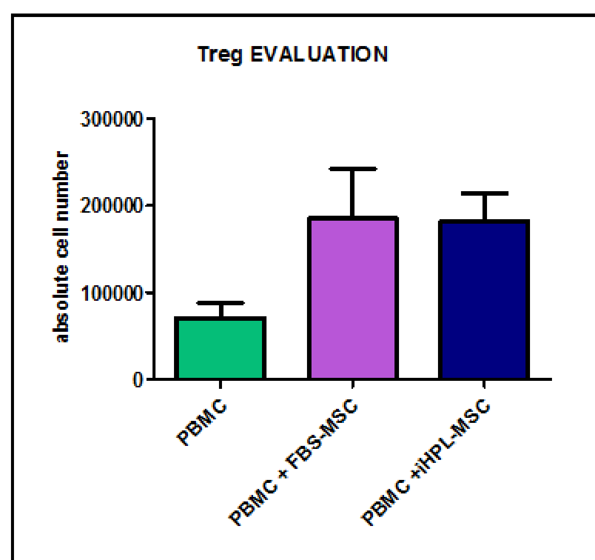

Figure 6-5– Treg absolute number in PMMC with and without PHA, alone and in co-colture with FBS-MSC or iHPL-MSC

6.1.3.5 *Th1/Th2/Th17 immunomodulation*

PHA-stimulated PBMC was used as the control and the range of analysed cytokines analysed were similar to that reported in literature (C.-L. Lee et al. 2011).

6.1.3.6 *MSC constitutive production of cytokines*

As shown in Table 6, MSCs constitutively produce:

- Negligible levels of Th1 cytokines, except for IL-12 which was higher than IL-12 levels in PHA-PBMC;
- Very low Th2 cytokines;
- Significant levels of IL-6 and IL-17.

ELISA release (pg/ml)		**FBS-MSC**	**iHPL-MSC**	***PHA-PBMCs***
Th1 cytokines	**IL-2**	12	10	150
	IL-12	1170	1245	617
	TNF-α	131	6	821
	IFN-γ	37	36	781
Th2 cytokines	**IL- 4**	14	13	20
	IL- 10	9	8	22
Th17 cytokines	**IL- 6**	323	303	123
	IL- 17	101	167	52

Table 6-3- Mean values obtained MSCs alone cultured in alpha-MEM + 10% of FBS or 10% of iHPL (the values of PHA-PBMCs are also reported in the last column).

6.1.3.7 *Th1 cytokine release*

As also reported in literature (C.-L. Lee et al. 2011) PHA stimulated PBMCs showed high levels of IL-2, IL-12, TNF-α and IFN-γ and in all co-culture conditions we observed a strong decrease in cytokine release (Table 7).

Statistical analysis performed (Friedman's test) didn't reveale any significant differences between FBS-MSCs and iHPL-MSCs.

	PHA-PBMC	PHA-PBMC + FBS-MSCs	PHA-PBMC + iHPL-MSCs
IL-2	119	25	26
	163	71	33
	167	50	46
IL-12	500	425	235
	550	380	320
	800	335	285
TNF-α	569	274	231
	1036	54	104
	859	99	99
IFN-γ	981	144	189
	890	259	347
	470	290	244

Table 6-4- Th1 Cytokines release (pg/ml) analysed by ELISA assay

In particular, IL-2 concentrations significantly decreased in co-culture with FBS-MSC ($p = 0.02$) and not significantly in those with iHPL-MSC. No statistical differences were underlined between the co-culture conditions (Figure 16A). The stimulated PBMC produced high levels of IL-12. Interestingly, even if FBS and iHPL-MSCs constitutively produced higher levels of this cytokine than PHA-PBMCs (Table 7) we observed a reduction of IL-12 in the two co-culture conditions (Figure 16 B).

The high level of TNF-α produced by PHA-stimulated PBMCs decreased in all MSC co-culture conditions as shown in Figure 16 C. Also IFN-γ decreased in the co-cultures with MSCs Figure 16 D.

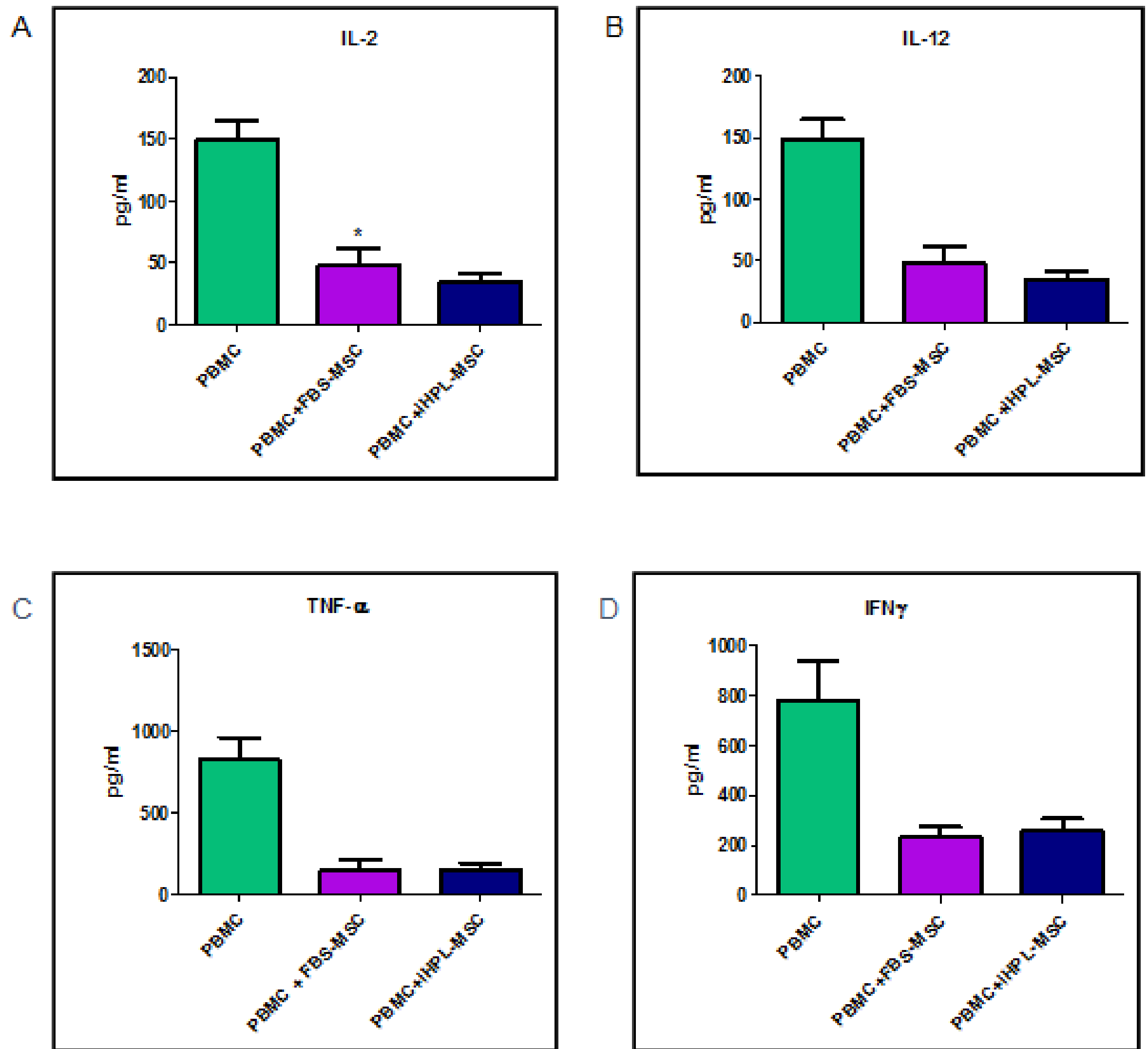

Figure 6-6 IL-2 (A), IL-12 (B), TNF-α (C) and IFN-γ (D) release detected on PHA-PBMC and PHA-PBMC co-cultivated with FBS-MSc and iHPL-MSC.

6.1.3.8 *Th2 cytokines release*

Th2 cytokine levels (IL-4 and IL-10) measured by ELISA are reported, for each experiment in **Errore. L'origine riferimento non è stata trovata.**. PHA-stimulated PBMC produced moderate amounts of these cytokines, which increased in the presence of MSC in all co-culture conditions.

Comparing the mean rank of each column with the mean rank of PBMC, a significant increase of IL-4 in FBS-MSC (p=0.0278) but not in iHPL-MSC. No significant differences were obsreve between the two culture conditions (Figure 17 A).

	PHA-PBMCs	PHA-PBMC + FBS-MSC	PHA-PBMC + iHPL-MSC
IL-4	18	69	33
	26	34	30
	14	27	23
IL-10	19	24	35
	26	78	96
	22	35	95

Table 6-5. Th2 cytokines release (pg/ml) analysed by ELISA

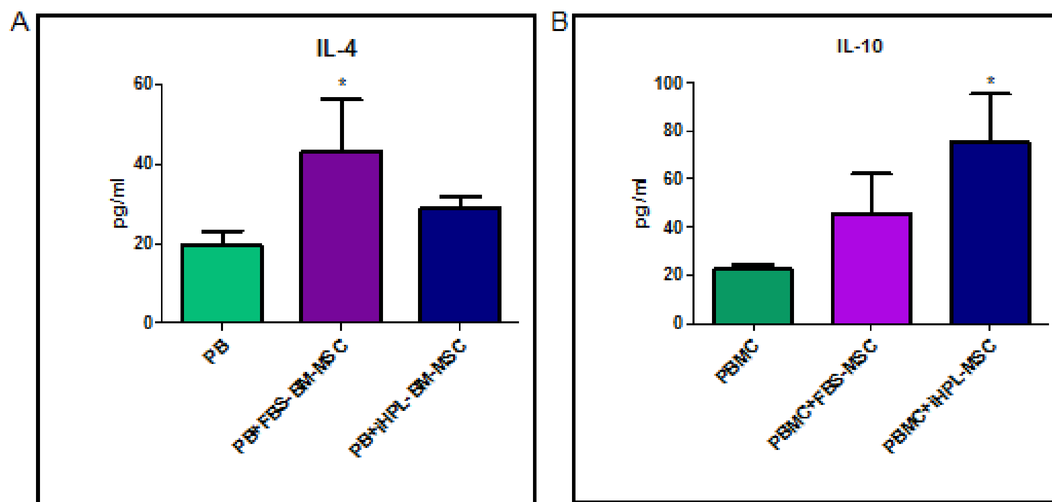

Figure 6-7 IL-4 (A) and IL-10 (B) release detected on PHA-PBMCs and PHA-PBMC co-cultivated with BM, AF and PL-MSCs

At the contrary, as illustrated in Figure 17B, Dunn's multiple comparison test showed that the increase of IL-10 was significantly higher in the PBMC + iHPL-MSC (p=0.0278) but not in the co-culture with FBS-MSC. Also in this case no significat difference were observed between the two experimental condition.

6.1.3.9 *Th17 cytokine release*

PHA stimulated PBMCs showed a moderate production of IL-17 and IL-6 which increased in all co-colture experiments with MSCs. The data are reported in Table 9.

	PHA-PBMC	PHA-PBMC + FBS-MSC	PHA-PBMC + iHPL-MSC
IL-6	72	345	394
	159	345	368
	137	308	334
IL-17	26	61	49
	45	55	98
	85	204	85

Table 6-6 Th17 cytokine release (pg/ml) analysed by ELISA

Dunn's multiple comparisons test showed a significant increase (p=0.0278) of IL-6 but not in IL-17 level in PBMC+iHPL-MSC co-culture and not significant in FBS-MSC and iHPL-MSC co-culture for both IL-6 and IL-17. No statistical difference between the two co-culture conditions were observed (Figure 18 A and B).

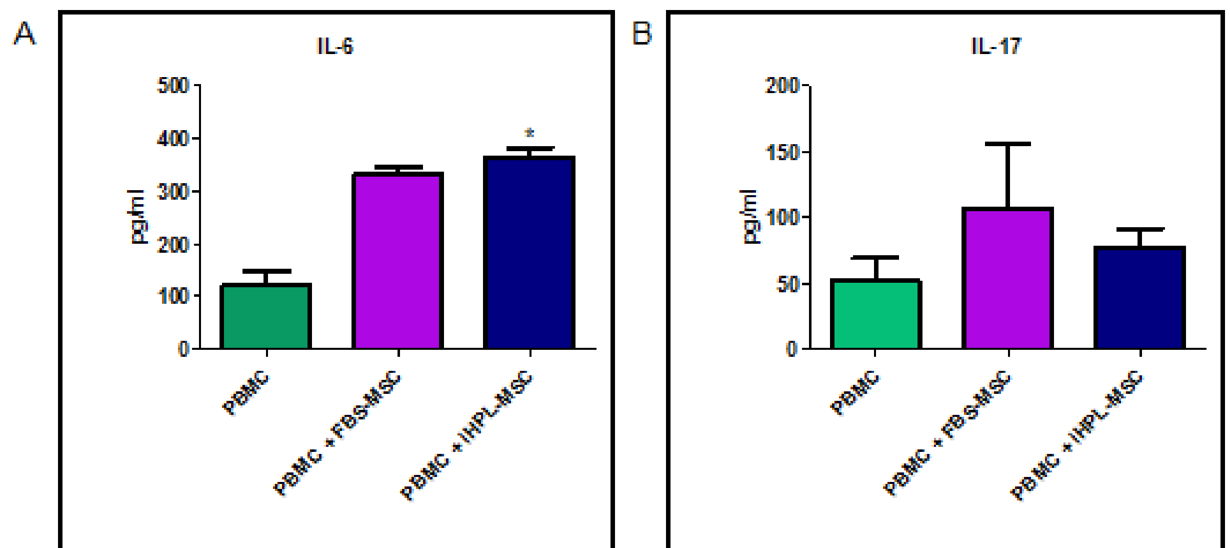

Figure 6-8 IL-6(A) and IL-17 (B) release detected on BM, AF and PL-MSCs and on their respective co-cultures with PHA-PBMC

As these two cytokines were also produced constitutively by MSC, their determination were also considered for a further statistical analysis.

6.1.3.10 *IDO expression*

We analyzed IDO-1 and IDO-2 mRNA expression on MSCs alone and in the co-cultures conditions after 5 days.. We observed that the presence of unstimulated and PHA-PBMC, induced a modulation of IDO at both gene and protein levels. Interestingly, IDO-1 and IDO-2 transcripts were not detectable in FBS-MSC and iHPL-MSC in basal condition (uncalculated Ct) but compared in the co-culture conditions. The fold increase of IDO-1 transcripts for MSC was not calculable because the initial level in single cultures was not detectable. Making a CT difference and expressing the result in logarithm we can say that in FBS-MSC co-cultures, IDO1 expression is more stimulated than than in iHPL-MSC co-culture (Table 10)

	Ct iHPL-MSC + PBMC	Ct FBS-MSC + PBMC	(Ct iHPL-MSC) – (Ct FBS-MSCs)	Fold increase (LOG)
EXP1	35,32	33,84	1,48	0,45
EXP2	24,36	23,37	0,99	0,30
EXP3	31,17	29,58	1,59	0,48

Table 6-7 Table 10- Summary of results

However, to verify that IDO-1 and IDO-2 mRNA expression correlated with its activity, we also determined IDO activity by quantifying conversions of Trp to Kyn under the same experimental conditions. Both untreated FBS-MSC and iHPL-MSC had negligible productions of Kyn, but the co-cultures with unstimulated and PHA-PBMC induced an increase in Kyn and so in IDO activity. In particular, we observed a Kyn mean value of 6,29±11,21 and 6,32±5.79 mM, respectively in the co-cultures of FBS-MSCs and iHPL-MSCs with unstimulated PBMC and 11,71±8,08 and 6,6±4,80 mM in the co-cultures with PHA-PBMC with FBS-MSC and iHPL-MSC, respectively. No statistical differences were observed in the co-culture condition with FBS and iHPL- MSC.

6.1.3.11 *Summary of results*

Table 11 summarized the results obtained in all experiments of this study. The symbols "+++", "++" and "+" or "-/+" indicate the expression or effect grade as very

strong, high and moderate, statistically significant, respectively.

	FBS - MSC	iHPL- MSC
MSC characteristics		
MSC immunophenotype*	+++	+++
Proliferative potential*	+	+++
Pluripotency marker expression (protein)	+	+++
MSC Th1 cytokine production	++	++
MSC Th2 cytokine production	-/+	-/+
MSC Th17 cytokine production	++	++
Effect on T-cells		
Inhibition T proliferation*	+++	+++
Naïve T helper Increase	++	++
Naïve T cytotoxic Increase	++	++
Memory T helper Decrease	++	++
Memory T cytoxic Decrease	+++	+++
Treg increase*	++	++
Th1 cytokine decrease*	+++	++
Th2 cytokine release increase*	++	+++
Th17 cytokine release increase*	++	++
IDO expression	+++	++

Table 6-8 - Summary of results (indicate that data are statistically significant)*

6.1.4 DISCUSSION

Regenerative medicine is of growing interest in biomedical research and in this context, MSCs are a promising tool for cell therapies for their multipotent, bystander and immunomodulant proprieties. For these reasons, MSCs are used for a very wide range of therapeutic applications, the majority of which are in Phase I, Phase II, or a mixture of PhaseI/II studies. MSCs used in these clinical trials are isolated from BM and are considered safe and efficacious for their multipotent and immunomodulant proprieties. Moreover, this study shows that iHPL itself is a safe and efficient MSC culture supplement for robust MSC culture, thus offering certain advantages compared to FBS, especially in terms of cell growth and stemness maintenance and

without causing variations in terms of immunomodulatory properties. For these reasons, it represents a good GMP-compliant alternative to animal serum for MSC clinical production confirming recent data reported in the literature (Fekete, Gadelorge, et al. 2012; Capelli et al. 2007; Schallmoser et al. 2009). As the risk of transmission of infective agents not routinely tested, or for which no tests are available remains, HPL quality and safety had to be greatly improved. This was done with photochemical treatment by Amotosalem and UVA, a technology that is efficient against the vast majority of known pathogens and which might also prevent the transmission of unknown pathogens (Tice et al. 2007; Irsch e Lin 2011; Allain et al. 2005; Prowse 2013; Tsetsarkin et al. 2013).

For this study, BM -MSC cultured in alpha-MEM + 10%of FBS or 10% of iHPL (FBS-MSC and iHPL-MSC) aliquots were thawed and: i) immunophenotipic (Figure 11), proliferative and differentiative characteristics were not modified (data not shown) by cryopreservation, and ii) their immunomodulant effects on T cells of healthy donors were compared. The results as summarized in Table 10 and in accordance with literature showed that MSCs cultured in FBS and iHPL are multipotent SCs with the immunophenotypic characteristics and differentiative potential established by guidelines by the Cellular Therapy Society (Dominici et al. 2006), even if, as demonstraded in a recent study conducted in our laboratories, MSCs cultured in alpha-MEM + 10% iHPL have a greater proliferative potential associated with the presence of embryonic markers. As emerged from the analyses of the pluripotency markers, such as Oct-3/4 and NANOG, these proliferative and differentiative properties of the iHPL-MSC might be linked to more immature stemness in comparison with FBS-MSC. Oct-3/4 is a key transcription factor for the maintenance of pluripotent and self-renewing phenotype in undifferentiated ESCs. NANOG, a homeodomain protein present in pluripotent human cells, plays a critical role in the regulation of the cell fate of the pluripotent inner cell mass during embryonic development, maintaining the pluripotent epiblast and preventing differentiation to the primitive endoderm. These observations indicate that iHPL-MSC contain a subpopulation of multipotent SC, which might be the precursors of MSC, with a more primitive phenotype than those of FBS-MSC (Castiglia et al. 2014).

The ability to modulate the alloreactive immune response has been documented for

MSCs derived from human BM; concurrently comparative studies between FBS-MSC and HPL-MSC were already performed and indicate thad HPL, used as supplement for MSCs media, supports immune modulation at least to the same extent than FBS in addition to its role during MSC isolation and expansion (Flemming et al. 2011; Bernardo et al. 2007). No comparative studies of immunomodulation were performed between FBS-MSC and MSC cultured in Inactivated Human Platelet Lysate (iHPL-MSC) which is even safer and more GMP compliant than HPL for MSC expansion.

Since T-cells are the primary cells in adoptive immune response, an evaluation and comparison of the inhibitory effects of MSCs on total activated T-cells with a potent mitogen (PHA) and on naive T-cells induced to differentiate in Th1 and Th2 effector cells. A T-cell proliferation assay showed inhibitory effects on PHA-stimulated PMBC in all co-culture conditions, while the proliferation data obtained in induced Th1 and Th2 effector cells were not homogenous and are controversial. For this reason, we focused our experimental study on total PHA activated-PBMC. However, these results suggest that T cell inhibition might be strictly related to an interaction of these cells with other cells from innate immunity (such as dendritic cells and NK) not present in co-cultures with Th1- and Th2- induced cells alone.

The inhibition of T cell proliferation was slightly more evident in the co-cultures with FBS-MSCs than in those with iHPL-MSCs, but no significant differences were found between the two experimental conditions. Moreover, when the absolute number of Treg was analysed, in the same experimental conditions, no significant differences emerged between FBS-MSC and iHPL-MSC co-cultures. Again, when we analyzed the different T subsets, we observed a statistically significant increase of naïve T cells as well as a strong decrease of memory T cells in both the co-cultures with FBS-MSCs and iHPL-MSCs. Indeed, the presence of MSCs induced a reversal of the ratio (compared to stimulated PHA-PBMCs) of these subsets in the co-cultures in favor of both CD4+ and CD8+ naïve T cell subpopulations.

An important mechanism by which MSC have beneficial effects in cell therapy is the paracrine action of secreted cytokines. Major Cytokines associated with pro-inflammatory and anti-inflammatory functions were analysed and compared. A noteworthy finding was a distinctly high concentration of IL-12 in MSCs.

Independently from the culturing conditions, MSCs also produced a moderate concentration of TNF-α and negligible amounts of IL-2 and IFN-γ. PHA-stimulated PBMC showed high levels of Th1 cytokine as reported in Lee et al (Lee et al. 2011). In all co-culture experiments, we observed a decrease of all Th1 cytokines. The interaction MSCs/T cells might block the Th1 polarization because this phenomenon was also found for IL-12 produced in high concentrations also by MSCs. It is interesting to note that IL-4 and IL-10, the major anti-inflammatory cytokines, increased significantly in co-cultures with FBS-MSCs and iHPL-MSCs. In particular, the increment of IL-4 was statistically significative in the co-cultures with FBS-MSC, and not in that with iHPL-MSC, and by contrast for IL-10 the increment of this cytokine was significant in the co-culture with iHPL-MSC and not in those with FBS-MSC. This difference between the two co-culture conditions could be explained by the fact that IL-4 inhibits IL-10 production (Yao et al. 2005). Consequentially, where there was a more significant increase in IL-4, IL-10 levels were lower and vice versa.

The same effect was observed for the Th17 cytokines. Moreover, the MSCs, independently from the culturing condition, produced high levels of both IL-6 and IL-17, so it is difficult to interpret the significant increase of these cytokines in the co-culture with PHA-activated PBMCs. IL-6 is a well-known immune modulator that also inhibits apoptosis in antigen-stimulated (Rochman, Paul, e Ben-Sasson 2005) and resting T cells by sustaining the expression of the anti-apoptotic molecule Bcl-2 (Teague et al. 1997). The Inhibition of IL-6 produced by MSCs results in an additional decrease in the proliferation of activated T lymphocytes in vitro in co-cultures with MSC (Najar et al. 2009) and increases apoptosis in neutrophils (Raffaghello et al. 2008). IL-17 is a cytokine that has attracted attention due to its involvement in chronic inflammation, having critical roles in the pathogenesis of autoimmune diseases, such as rheumatoid arthritis, psoriasis, inflammatory bowel diseases, diabetes, and multiple sclerosis. IL-17 also contributes to host defence against extracellular and intracellular pathogens, including Aspergillus fumigatus, Cryptococcus neoformans and Candida albicans (C. albicans) (Yang et al. 2013). The data of this study contrast with the usual immunosuppressive effect of MSCs, as exemplified by the inhibitory effects found on Th1 cell and Th17 cell differentiation (Ghannam et al. 2010). Moreover, a recent work identified a new subset of IL-17+

MSC capable of inhibiting C. albicans growth and attenuating cell-based immunosuppression (Yang et al. 2013). The authors described, IL-17+ MSCs, distinct from bulk MSC population, which were unable to upregulate Treg or downregulate Th17 cells, suggesting that IL-17 production in MSCs directly impairs MSC-based immunomodulatory function. Interestingly, similar to the IL-17 produced by Th17 cells, Yang and others found that MSC-produced IL-17 possesses an antifungal effect. All together these data showed that MSCs inhibit or limit inflammatory responses and promote the mitigating and anti-inflammatory pathway with an increase of Treg, in accordance with other authors (Maccario et al. 2005). The inhibition is only on Th1 cells, leading to a paradoxical increase of pro-inflammatory Th17 cells. As already suggested by other authors, a mechanism that could explain the late stimulating effect of MSC on pro-inflammatory Th17 cells is the up-regulation of IL-6 levels in the cultures, since IL-6 is a main mediator of Th17 cell differentiation (Najar et al. 2009; Liu et al. 2009). Furthermore, in agreement with the results in this study, IL-6 might also inhibit the differentiation of Th1 subset (Najar et al. 2009). The increase of Treg might be induced also by the high concentration of IL-10 released in the co-cultures, especially in iHPL-MSC. However, from these studies conclusions cannot be drawn on the separate roles of the different cytokines in either mediating inhibition directly or via inducing Treg. (e.g. by IL-10).

In addition, IL-4 and IL-10 are indicative of a Th2-deviated immune response and might be produced by a cellular compartment different from the T cells in PBMC.

Another key mediator of the PL-MSC immunosuppressive effect was IDO. For this study IDO-1 mRNA was analysed and, although the results still have to be confirmed, it was undetectable in basal MSC (independently from the sources) but significantly higher in co-cultures. In particular, performing a relative quantification of IDO1 mRNA we can say that in FBS-MSC co-cultures its expression is slightly higher than in iHPL-MSC co-cultures. To deepen this data we whant to verify that IDO-1 mRNA expression correlated with its activity: we determined IDO activity by quantifying conversions of Trp to Kyn under the same experimental conditions. Both untreated FBS-MSCs and iHPL-MSCs had negligible productions of Kyn, but co-cultures with PBMC and with stimulated PHA-PBMCs induced an increase in Kyn and so in IDO activity. Also in this case we observed a more relevant IDO activity in the co-cultures with FBS-MSCs than

with iHPL-MSC.

On the base of our last result it could seem that iHPL has an action slightly less immunomodulat than FBS on MSC. Della Chiesa et all demonstrated that Tryptophan catabolism mediated IDO plays a central role in the regulation of T-cell-mediated immune responses. In this study, was also demonstrated that natural killer (NK)-cell function can be influenced by IDO. Indeed, l-kynurenine, a Trp-derived catabolite resulting from IDO activity, was found to prevent the cytokine-mediated up-regulation of the expression and function of specific triggering receptors responsible for the induction of NK-cell-mediated killing (Della Chiesa et al. 2006). This was in accordance with Abdelrazik et all, who showed that MSCs expanded in HPL had a reduced capacity to inhibit NK and T-lynphocyte proliferation. For this reason they may be less effective than FBS-MSC in the control of immune-mediated pathologies, particularly GVHD. On the other hand HPL-MSC appeared particularly usefull in regenerative medicine (Abdelrazik et al. 2011).

The advantage of HPL in obtaining MSCs in vitro expansion is related not only to the increment of proliferation but also to the fact that, different from FBS, HPL does not induce antibody responses in patients (Lange et al. 2007; Muller et al. 2006) and its inhactivation with with psoralen makes it even safer than HPL blocking and inactivating the replication of viruses, bacteria and leukocytes in PLT concentrates (Tice et al. 2007; Lin et al. 1997; van Rhenen et al. 2003). The fact that we and other (Abdelrazik et al. 2011) found differences in immunomodulatort properties of FBS-MSC and HPL/iHPL-MSC that, in our case, are not are not significant, offer an interesting clue regarding possible functional differences in MSC output and clinical applications and protocols.

In Conclusion, for each experiment a contingency table is given (Table 10- Summary of results) where the MSC proprieties and their effects on T-cells are considered as strong, higher or moderate and it can thus be stated that :

- iHPL show a greater proliferative, differentiative and stemness potential than FBS-MSC (Castiglia et al. 2014)
- Both FBS-MSC and iHPL-MSC showed a potent immunomodulant effect on T-cells without strong significant differences between them.
- This study show that iHPL, used as medium supplement, may be considered a

good alternative to FBS for a GMP-compliant MSC expansion.

The development of new strategies for the large scale production of these cells, according to current regulations, including good manufacturing practice (GMP), represents a fundamental step to allow their use in effective therapeutic approaches. The use of iHPL as an alternative to FBS to isolate and expand MSC confirmed that it is possible to obtain a number of MSCs for clinical doses which manteined intact all their characteristics, including their immunomodulant properties. The application of iHPL as medium supplement, further reduce manufacturing time, limiting the passages and reducing the starting volume of BM. Moreover the PI treatment did not modify the characteristics of HPL, made it safer and more suitable for MSC isolation and expansion for clinical use and might be a requirement for GMP MSC expansion.

This study might well open the way for new ambitious projects which will allow the identification of new and more advantageus way to culture MSCs, which was safer than those commonly used, in order to create a bio-bank of ready to use MSCs for clinical use. The possibility of banking MSCs isolated from BM or other sources in GMP conditions in an AIFA (Agenzia Italiana del Farmaco – the Italian Medicine Agency) accredited Cell Factory might represent a new scenario for their clinical use in cell therapy protocols providing a continuous supply of cells to treat patients with acute GVHD after allogeneic hematopoietic stem-cell transplantation, solid organ transplantation or in inflammatory and autoimmune diseases.

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6.2 ANALYSIS OF EPIGENETIC PROFILE ASSOCIATED WITH STEMNESS MARKERS IN MESENCHYMAL STEM CELL CULTURES

6.2.1 INTRODUCTION

Histone modifications (acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation) control structural chromatin compaction and, in turn, regulate gene transcription. Acetylation of histones H3 and H4 and methylation of histone H3 lysine (K4, K36, K79) are generally associated with active gene transcription within euchromatin, while gene silencing is correlated with methylation at H3K9, H3K27, and H4K20 promoting the formation of compact heterochromatin structure. Stem cell chromatin is in dynamic state with global DNA hypomethylation and global increase in acetylation of histones H3 and H4 with involvement of histone deacetylases (HDACs) representing an open chromatin structure. Several studies provide evidence for different chromatin organization in embryonic SCs genome that contains domains with "bivalent" chromatin modifications of both histone H3K4me3 and H3K27me3 that mark a number of transcription factors and developmental genes, which are repressed by the H3K27me3 and transcriptionally "poised" by the H3K4me3 for rapid activation in response to differentiation signals]. Global reorganization of chromatin occurs during the differentiation programs leading to global lack of DNA methylation and activation of cell-type specific genes. Transcription factors such as Oct3/4, Sox2, and Nanog play essential role in maintenance of SCs pluripotency and self-renewal by their involvement in activation of genes for survival/proliferation and repression of such target genes that will be only activated during cell differentiation.(Savickienė et al. 2017; Nakayama et al. 2001) In this context, we analysed histone marks in MSCs derived from adult and paediatric BM donors during the expansion. We analysed both MSCs isolated expanded with traditional method in presence of FBS both using the method in GMP conditions in presence of human platelet Lysate (HPL). We focused on the analysis of H3K4me 3 and H3K27me3 at the promoter regions associated with Oct3/4, Sox2 and Nanong. Histone marks define the specific states of chromatin: histone 3 lysine 4 trimethylation (H3K4me3) is a marker of active state corresponding to gene transcription activation in the promoter, histone 3 lysine 27 trimethylation

(H3K27me3) is a marker of silenced state corresponding to gene transcription silencing

6.2.2 MATERIAL and METHODS

6.2.2.1 *Isolation of BM-MSCs*

BM cells were harvested from the iliac crest of adult or pediatric donors who underwent BM collection for a related patient after informed consent. We used a part of the BM initially dedicated to transplantation collected after submission of written consent, in accordance with the ethics committee of the hospitals Ospedale Infantile Regina Margherita-Sant'Anna-Mauritian order, which approved the collection of the samples and according to the Declaration of Helsinki.

The whole BM sample was then equally split into 2 cellular culture conditions: a-MEM (SIGMA-ALDRICH®, LTO Irvine, Ayrshire, UK) containing 10% of FBS (FBS-MSC), or iHPL (iHPL-MSC). The seeding density of the initial whole BM was at 10,000 cells /cm2 as previously reported (Mareschi et al. 2012d).

After 7 days, the non-adherent cells were removed and discarded. The adherent cells were re-fed every 5-7 days and, when they reached confluence, were detached, counted and re-plated for a further 3-5 passages at 1,000 cells/cm2. We considered MSCs at Passage 1 (P1) the first time the cells were harvested and re-plated. The cellular condition was maintained from the cellular plating of whole BM and during the expansion process. We indicated the following passages with increasing numbers: P2, P3 etc. On the basis of our experience and our previous reported data (Mareschi et al. 2012) we found that three passages suffice for clinical applications , althought we analysed the cells to P4 or p5.

6.2.2.2 *Colony-forming unit fibroblasts*

To quantify MSC precursors, we performed a colony forming unit fibroblast (CFU-F) test. The BM cells were plated directly in a-MEM containing 10% FBS, HPL or iHPL at densities of 10,000 cells/cm2 or 100,000 cells/cm2 in a six-well plate. MSC clonogenic precursors were scored macroscopically after 15 days from seeding, and clusters of >50 cells were considered colonies. All experiments were performed in duplicate and

by two different operators. The CFU-F means were expressed as fibroblastic clones obtained from 1 million BM cells (CFU-F/10^6 cells).

6.2.2.3 *MSC cellular growth evaluation*

To evaluate cellular growth, the cell growth rate was expressed in terms of population doubling (PD) by means of the formula (log N/log 2), where N is the cell number of the detached cells divided by the initial number of seeded cells and the expansion in terms of cumulative PD (cPD).

6.2.2.4 *MSC cytofluorimetric analysis*

To analyze the immunophenotype, flow cytometer analysis was performed on adherent cells at each passage. Briefly, 200,000 cells were incubated with the appropriate amount of antibody according the specific antibody titration as described in Rustichelli et al. (25) for 20 min with anti-CD90 fluorescein isothiocyanate (FITC), CD73 phycoerythrin, CD34 FITC, CD14 FITC, CD45 FITC (Becton Dickinson, San Jose, CA, USA), CD 105 antigen-presenting cells (APC) and CD146 APC (Miltenyi Biotec srl, Bologna, Italy). The labeled cells were acquired by means of FACScanto II (Becton Dickinson) with use of the DIVA software program. The percentage of positive cells was calculated through the use of cells stained with immunoglobulin FITC/phycoerythrin/ APC as a negative control, and mean fluorescence intensity was analyzed on the positive cells

6.2.2.5 *Quantitative real-time PCR (RT-QPCR) assay*

RNA extraction and real-time polymerase chain reaction Total RNA was extracted with the use of RNeasy Plus Mini Kits (Qiagen, Austin, TX, USA). Reverse-transcription polymerase chain reaction (RT-PCR) was carried out with the use of the high-capacity complementary DNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. RNA and complementary DNA concentrations were measured with the use of a GeneQuant pro spectrometer (Amersham Biosciences, GE Healthcare, Pittsburgh, PA, USA). We performed RT-PCR to detect the transcripts for human Oct-4 and NANOG through the use of specific assays (assay ID: OCT-4 (POU2F2)

Hs00231269_m1; NANOG: Hs04260366_g1; HPRT1:Hs02800695_m1) and Taq-ManUniversal PCRMasterMix (Applied Biosystems). Comparative cycle threshold (DDCT) experiments were performed according to the manufacturer's specifications in a total reaction volume of 25 mL. All experiments were performed in three replicates. To normalize the PCR results, we used hypoxanthine phosphoribosyltransferase 1 (HPRT1) as the housekeeping gene. We performed the Relative Quantification (RQ) method, which is based on the expression levels of a target gene versus a housekeeping gene to compare different RT-PCR experiments For each set of experiments, we analyzed Nanog and Oct-4 messenger RNA expression levels in MSCs obtained in three different culture conditions at P3, and the
FBS-MSC condition was used as reference sample.The amount of mRNA was normalized to GAPDH, the level of mRNA expression was calculated based on the PCR cycle number (Ct), the relative gene expression level was determined using the delta delta C_t method.

6.2.2.6 *Chromatin Immunoprecipitation Assay (CHIPs)*

The chromatin immunoprecipitation assay and quantitative real-time polymerase chain reaction (qPCR) quantification were performed as previously described (Guglielmotto et al. 2016). Cell cultures were cross-linked with 1% formaldehyde in PBS for 10 minutes at 37°C. The reaction was stopped by adding glycine to a final concentration of 125 mM at Room Temperature (RT). Crosslinked spinal cords were washed three times in cold PBS containing proteinase inhibitors and then collected in 1 ml of cell lysis buffer (5 mM Pipes pH 8, 85 mM KCl and 0.5% NP-40). After 10 minutes of incubation on ice, nuclei were collected by centrifugation and lysed with 400 µl of nuclei lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA and 1% SDS). The lysates were incubated on ice for 10 minutes and then sonicated 20 times for 20 s at 30% amplitude with SonoPlus HD2070 sonicator (Bandelin). Small portion of sonicated chromatin (25 µl) was used to verify that the average size of DNA fragments was in the range of 250–500 bp. 1 µg of sheared chromatin for each immunoprecipitation was diluted in IP buffer (16.7 mM Tris-HCl pH 8, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X100) and incubated with 0.5 µg of antibodies against Nurr1 and NFkB p65 (Santa Cruz Biotechnology), histones H3K4me3 and H3K27me3 (Active Motif) in a BSA pre-treated 96-well dish at 4°C overnight on an

orbital shaker. Samples with IgG antibody (Santa Cruz Biotechnology) were run in parallel as negative controls. The following day, 30 µl of 50% Protein A SepharoseTM 4 Fast Flow (GE Healthcare) slurry was added and incubated for 2 hrs at 4°C to capture the immune complexes. Proteins and DNA not specifically associated with the beads were removed by sequentially washing with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8 and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8 and 500 mM NaCl), LiCl washing buffer (0.25 M LiCl; 1% deoxycholate sodium salt, 1 mM EDTA, 10 mM Tris-HCl pH 8 and 1% NP-40) and twice with Tris-EDTA buffer (10 mM TrisHCl pH 8, 1 mM EDTA) at 4°C for 5 minutes each wash. The immunoprecipitated DNA-protein complexes were purified using 10% Chelex® 100 Resin (BioRad) for 10 min at 95°C. Proteins were digested by incubating each sample with 20 µg of Proteinase K (Thermo Fisher Scientific) for 30 min at 55°C and then 10 min at 95°C to obtain Proteinase K inactivation, thus achieving DNA purification.
Quantification of ChIP enriched DNA was performed by real-time PCR using iTaq Universal SYBR Green Supermix (Bio-Rad). The enrichment of target sequence in the immunoprecipitated samples was normalized on input samples (1% of total chromatin used per IP). ChIP primers for OCT4, Nanog and SOX2 derived from Barrand et al., 2010.

6.2.3 RESULTS

6.2.3.1 *MSCs characteristics*

We isolated MScs from BM of 3 donors (BM-1, BM-2 and BM-3) with an average age of 18 years (min and max values: 12 and 24) and we analyzed their cellular growth , immunophenotype, mRNA expression of stemness markers and endogenous retroviruses and their epigenetic modification.
After 15 days from the starting seeding, we counted the CFU-Fs in the all the MSCs batches and the results are summarized in the Table 6-9.
As already previously demonstrated (Katia Mareschi et al. 2012d) also here we observed that MSCs from BM seeded at 10.000 cell/cm^2 had a higher clonogenic capacity than MSCs seeded at 100.000 cells/cm^2 . also HPL

	seeding	10.000 cells/cm^2		100.000 cells/cm^2	
	supplement	HPL	FBS	HPL	FBS
samples	**BM 1**	3	2	16.5	18
	BM 2	17	4.5	31	36
	BM 3	3	2.5	11	15
		CFU-Fs/1*10^6			
	mean	76.7	30.0	19.5	23.0

Table 6-9 CFU-F counted after 15 days from the seeding at the 10.000 cells/cm^2

No differences were observed in term of CFU-Fs in HPL and in FBS (p value >0.05 calculated by t test)

6.2.3.2 *MSC growth and immunophenotype*

MSCs cultivated in HPL showed a higher proliferative capacity than MSCs in FBS. Although the statistical analysis on 3 samples were not significant, these date were in accordance with data obtained in one previously work

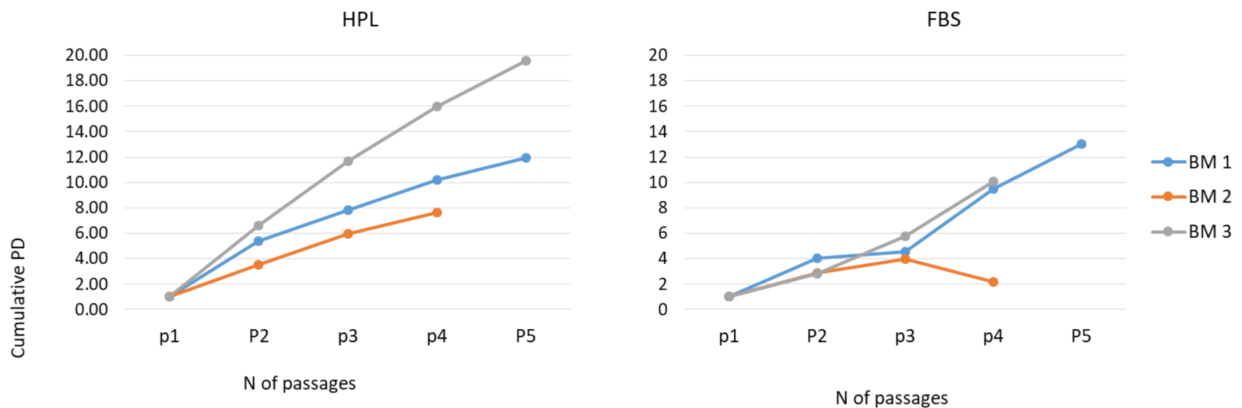

Figure 6-9. Cellular growth expressed as cumulative Population Doubling (cPD) of BM-MSCs cultivated in HPL (left) and in FBS (right)

They showed all the mesenchymal stem cells characteristics as described in (K. Mareschi et al. 2006). They were negative for the hemopoietic markers CD45, CD14, CD34, CD19 and HLA-DR while they were positive for expression major than 90% of CD90, CD73, CD105, as showed in the Figure 6-9

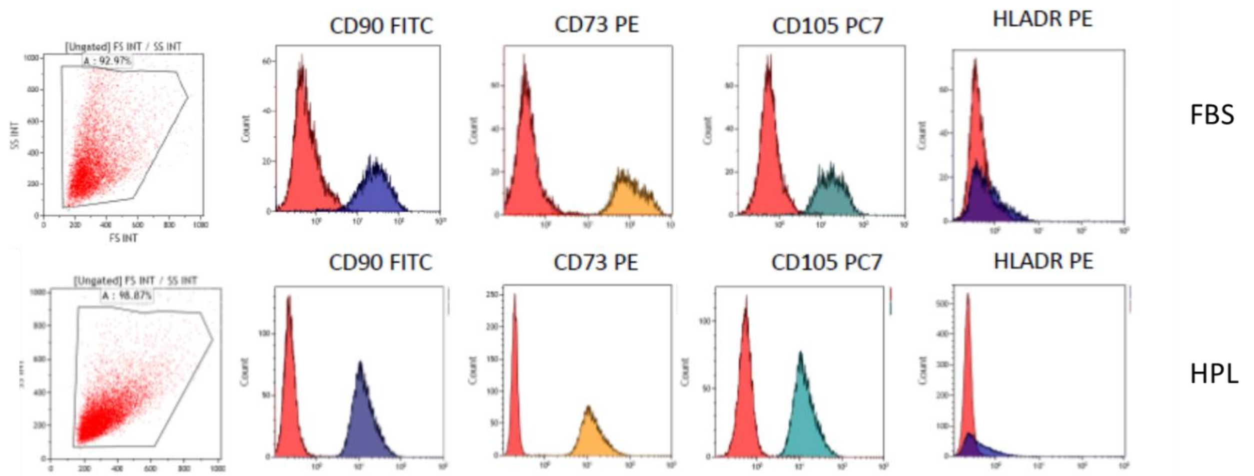

Table 6-10. Immunophenotype analysis . Flow cytometry histograms of the histotype control in the red and the characteristic immunophenotype of MSCs in the colors. No statistical differences between FBS and HPL were observed.

6.2.3.3 *RT and real time PCR*

The MSCs were analyzed during the expansion until 4th or 5th passager for the expression of mRNA of stemness marker such us NANOG, OCT-4 and SOX-2 and on the basis of data described in the paragraph 6.1 we also analyzed the molecular expression of HERV-H and HERV-K.

We analyzed the data obtained by the RT-PCR on the single samples and we observed in all analyzed MSC batches a correlation between Nanog and OCT-4 and HERV-K and HERV-H molecular both all BM-MSCs and both in conditions with FBS and HPL. These correlation confirmed the data presented in the paragraph 6.1

We used the cells at the first passage as the reference sample and the expression of stemness markers was expressed as fold change of gene expression levels calculated as the relative mRNA expression amount of a target gene in comparison at a reference sample, normalized .

We observed a variable trend of their expression during the expansion as reported in the Figure 6-10

6.2.3.4 *Epigenetic analysis*

In order to explore whether cell cultures could have an impact on cellular reprogramming changing epigenetic modifications, the samples previously described

were analysed for chromatin immuniprecipitation assay following from Real Time PCR (ChIP-qPCR) using antibodies against the activatory histone mark, H3K4me3 and the repressive histone mark, H3K27me3.

We observed a variable expression of H3K4me3 and H3K27me3 levels at Nanog, *OCT-4 and SOX-2* promoters as showed in the figures Figure 6-11 , 7-6 and 7-7 .

For example, BM 1 sample maintained in FBS H3K4me3 and H3K27me3 levels are very similar at *OCT4* promoter in the first passage. We observed already an increased signal of H3K27me3 in the 2rd passage until to 5th passage, while H3K4me3 decreased for two passages and returned to basal level at 4th passage. In cells maintained in HPL, the first passage had an higher signal of H3K4me3 respect to H3K27me3, while the ratio between the two histone marks revealed an increase in the repressive histone mark, H3K27me3 (Fig.). These results suggest that the first passage preserved active chromatin state at the OCT4 promoter because we found both histone marks, while the other passages showed an increase for repressive histone mark, H3K27me3 indicating a progressive shift versus silence chromatin state.

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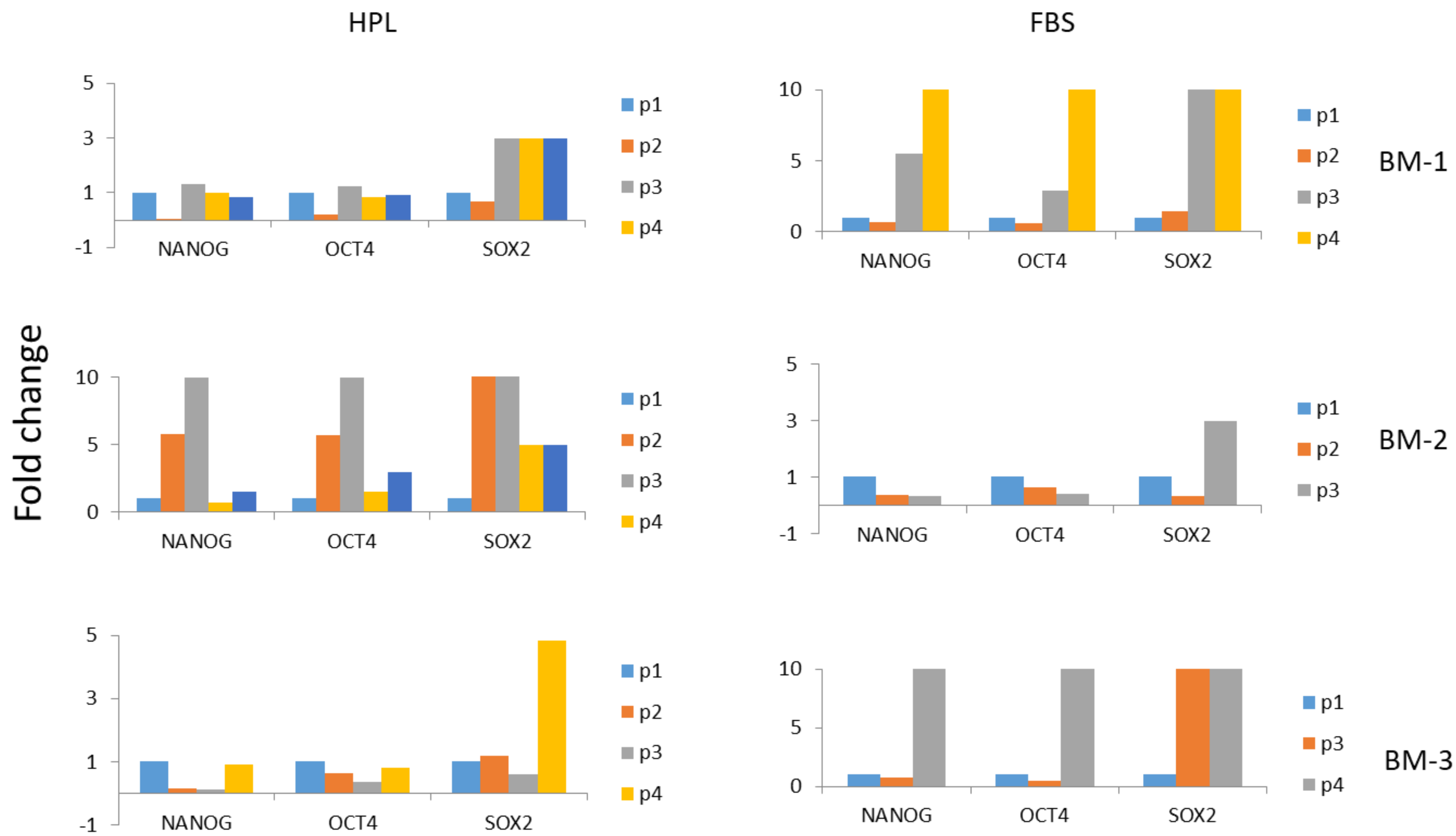

Figure 6-10 mRNA expression as fold change on BM samples cultivated in FBS and HPL

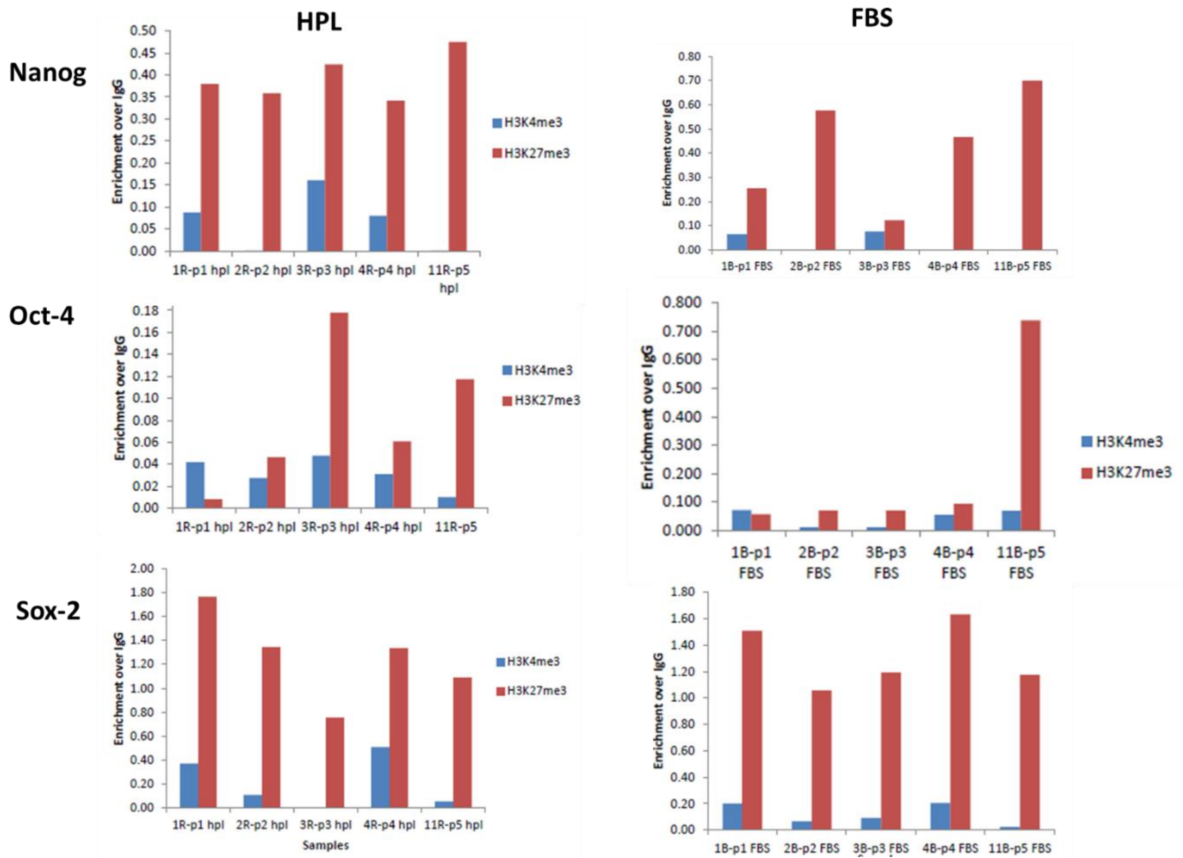

Figure 6-11 Histone modification profiles in the regulatory regions of OCT4, NANOG, SOX2 and GAPDH on batch BM- 1.

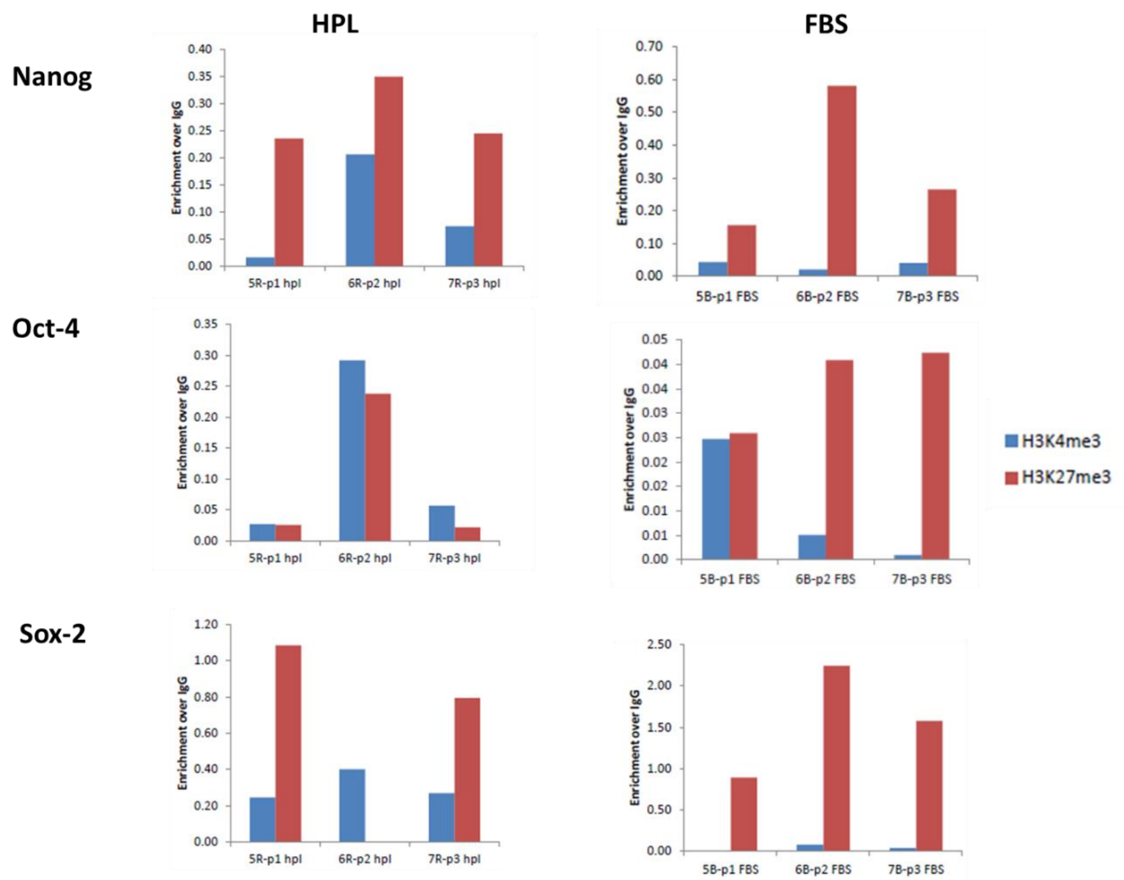

Figure 6-12 Histone modification profiles in the regulatory regions of OCT4, NANOG, SOX2 and GAPDH on batch BM- 2.

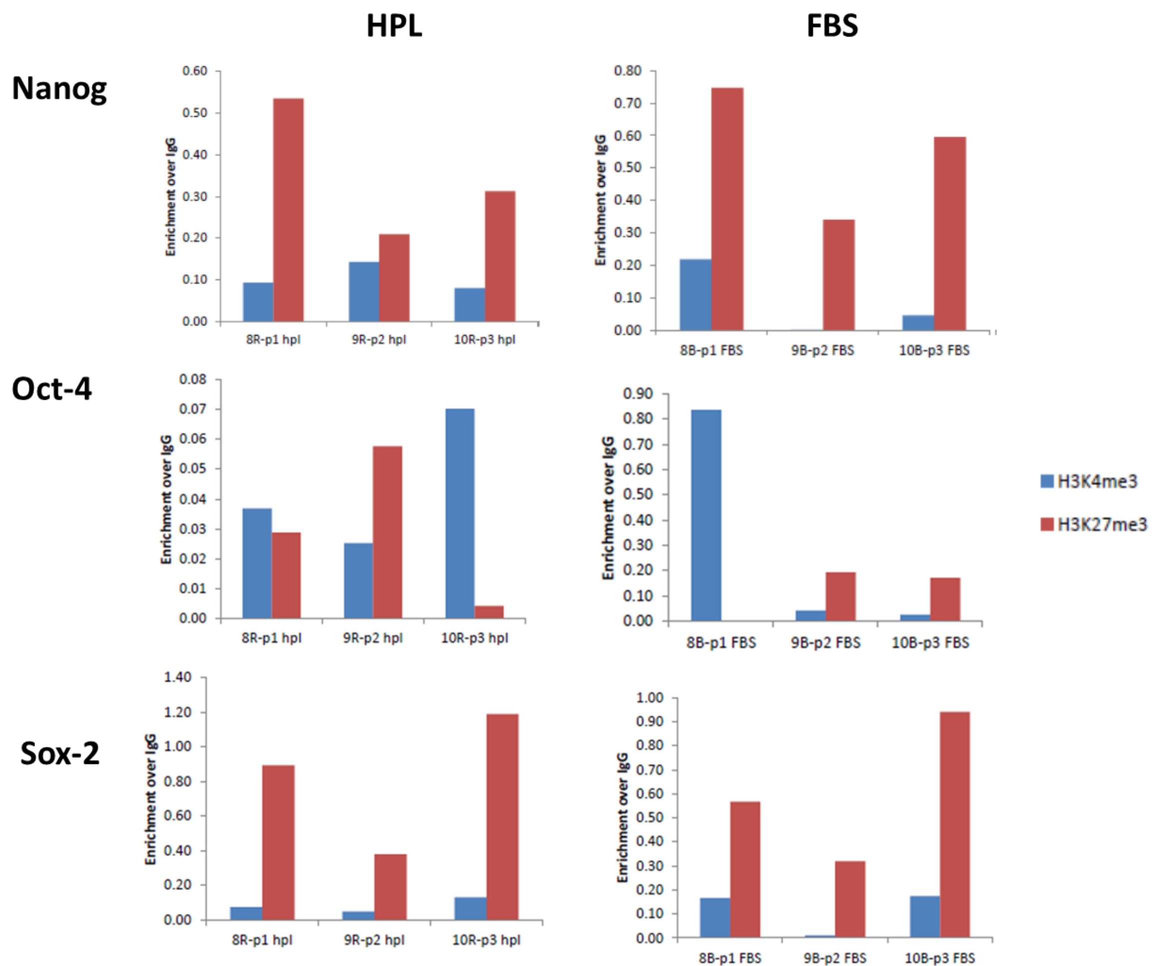

Figure 6-13 Histone modification profiles in the regulatory regions of OCT4, NANOG, SOX2 and GAPDH on batch BM- 21.

6.2.4 CONCLUSIONS

–MSCs are a population of cells extremely heterogonous and have an interindividual variability of DNA methylation. As previoscly reported (Paragraph 6.1) ,the dynamics of DNA methylation during aging depend on a complex mixture of factors that include the DNA sequence, cell type and chromatin context involved, and that, depending on the locus, the changes can be modulated by genetic and/or external factors. Endogenous retroviruses such us HERV-K and –H should be considered as new markers of stemness or differentiation for MSCs while at the light of our results the activatory histone mark, H3K4me3 and the repressive histone mark, H3K27me3 cannot be considered good marker to analyse the stemness maintaince during the expansion of MSCs in vitro. These preliminary data suggest us to identify specific regulatory regions different from NANOG, OCT-4 and SOX-2 to identify stemness

stage.

All these data support the current hypothesis that MSCs could act by secreting paracrine factors in a “hit-and-run” scenario including the secretion not only of cytokines and other soluble factors but also extracellular vesicles that can contain cargos that include peptides, proteins, metabolites, microRNAs, and even mitochondria. Our future perspectives focus on the study of secretome obtained by MSCs expanded in GMP conditions to obtain a pharmaceutic product safer and extremely effective and efficient for multiple clinical use

6.2.5 REFERENCES

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CONCLUSIONS

7 THESIS SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Regenerative medicine is of growing interest in biomedical research and in this context, MSCs are a promising tool for cell therapies for their multipotent, bystander and immunomodulant properties. For these reasons, MSCs are used for a very wide range of therapeutic applications, the majority of which are in Phase I, Phase II, or a mixture of PhaseI/II studies.(see www.clinicaltrial.gov) Most MSCs used in these clinical trials are isolated from BM and are considered safe and efficacious for their multipotent and immunomodulant properties. However, the clinical application of BM-derived cells is limited for the relatively invasive procedure for sample collection, the difficulties of obtaining a sufficient number of MSCs to appropriately perform studies, and a marked reduction in cell number, proliferation, and differentiation capacity with age (K. Mareschi et al. 2006).

The majority of pre-clinical and clinical studies, which are going on, are incipient. They analyzed the results of treatment in few months or years, not allowing long-term analysis. In these early results, MSC therapy has not shown adverse effects, being designated safe by FDA. Several studies have showed that MSCs after infusion do not engraft in any tissue, emphasizing the paracrine mechanism of action (Parekkadan e Milwid 2010). However, if the treatment is continuous or the MSC administration is repeated, MSCs can be found in the injured tissue. When MSC is engrafted or even when there is an in situ administration, MSC may differentiate. In a model of glomerulonephritis, MSCs differentiate to adipose cells in the kidney. In situ administration of human autologous stem cell therapy in a patient with lupus nephritis has induced angiomyeloproliferative lesions at the sites of injection and haematuria (Thirabanjasak, Tantiwongse, and Thorner 2010).

All bioactive molecules secreted by MSCs are prone to stimulate cancer cells to proliferate and migrate. Some works correlate MSCs to suppression of tumour growth, others to supporting it. Several questions may be related to its discrepancy: animal host, timing of injection of MSCs, differences in tumour models, etc. In addition, MSCs may differentiate to tumour associated fibroblast (TAF), cells that

support tumour growth (Spaeth et al. 2009). Thus, MSC therapy should exclude patients with cancer family history.

Also, the immunosuppression level of MSC therapy cannot be controlled. Once MSCs are injected intravenously, immunosuppression is achieved. MSC therapy may compromise the host's defence against infections agents (Sundin et al. 2006).

The cellular therapy is the principal focus of the laboratory where I have been working from several years and in my PhD project I investigated the immunophetopype, immunomodulant properties and epigenetic aspects of MSCs for their clinical use. We routinely isolated MSCs from BM of healthy donors who underwent BM collection for a familiar allo-BM- transplantation but also isolated MSC from alternative sources of foetal origin such us amniotic fluid and placenta.

As we need to identify a SC source that is safe, easily accessible, provides high cell yield and for which cell procurement does not provoke ethical debate we compared BM, AF and PL-MSC. We showed that MSCs isolated from the three different sources are multipotent SCs with the immunophenotypic characteristics and differentiative potential established by guidelines by the Cellular Therapy Society (Dominici et al. 2006), even if MSCs derived from foetal tissues have a greater proliferative potential associated with the presence of embryonic markers. To verify whether these proliferative and differentiative properties of the AF and PL-MSCs might be linked to more immature stemness in comparison with those of BM-MSC, the expression of pluripotency markers, such as Oct-3/4 and NANOG were analysed. Oct-3/4 is a key transcription factor for the maintenance of pluripotent and self-renewing phenotype in undifferentiated ESCs. NANOG, a homeodomain protein present in pluripotent human cells, plays a critical role in the regulation of the cell fate of the pluripotent inner cell mass during embryonic development, maintaining the pluripotent epiblast and preventing differentiation to the primitive endoderm. Moleular analysis showed that both AF- and PL-MSCs (but in particular AF-MSCs) possess high expression of these pluripotency markers compared to BM-MSC. These observations indicate that AF and PL contain a subpopulation of multipotent SC with a primitive phenotype and, which might be the precursors of MSC. This interesting aspect is most likely due to the early embryological origin of the AF and PL.

In Conclusion, for each experiment a contingency table is given where the MSC

properties and their effects on T-cells are considered as strong, higher or moderate and it can thus be stated that Figure 7-1:

- AF and PL-MSCs show a greater proliferative, differentiative and stemness potential than BM-MSCs
- AF-MSC showed a more potent immunomodulant effect on T-cells than BM-MSCs and only a slightly higher effect than PL-MSCs

This study show that MSCs isolated from foetal tissues may be considered a good alternative to BM-MSCs for clinical applications.

Further studies are needed to provide a complete understanding of the mechanisms underlying immunomodulatory effects of AF and PL-MSCs, which will ultimately allow the development of new and more effective strategies for regenerative medicine and transplantation to treat a wide range of conditions. A further fact should not be overlooked is that AF-MSCs were isolated from AF harvested from women undergoing amniocentesis, a routine procedure used for pre-natal diagnosis at 14–16 weeks of pregnancy and this is an invasive procedure. Moreover, we only isolated AF MSCs clones from the most abundant samples that contained at least 6 mL of AF (Mareschi et al. 2009). In all the other samples, as we observed heterogeneous clones also presenting epithelial characteristics, it is difficult to set up a standard MSC isolation and expansion protocol from AF. On the other hand, PL, which is an abundant discharged foetal tissue, may be considered an excellent source of MSCs without any ethical problems.

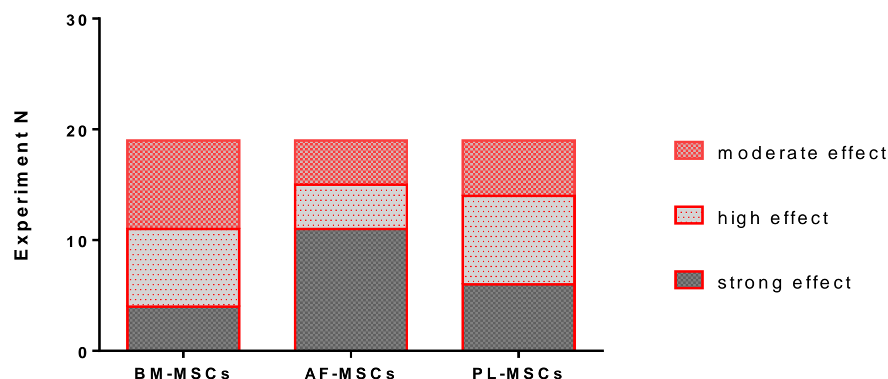

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Figure 7-1 Summary of the results. MSC characteristics and the immunomodulant effects on T-cells were classificated as moderate, high or strong (as indicated in legend) and each column (BM, AF and PL-MSCs) represents the experiment number divided for their effects.

The development of new strategies for the large scale production of these cells, according to current regulations, including good manufacturing practice (GMP), represents a fundamental step to allow their use in effective therapeutic approaches. Therefore, by using HPL obtained from a pool of platelets of healthy donors, which is safer and more advantageous than FBS for GMP production, an attempt was made to ameliorate the isolation and expansion of PL-MSCs following the criteria used for BM-MSC clinical protocol (Castiglia et al. 2014). Studies are still underway in the Regina Margherita Cell Laboratory to compare immunomodulant effects. This study might well open the way for new ambitious projects which will allow the identification of new, ethically acceptable, readily available sources of SC for cellular therapy and the creation of a new bio-bank of ready to use MSC. The possibility of banking MSCs isolated from placenta in GMP conditions in an AIFA (Agenzia Italiana del Farmaco – the Italian Medicine Agency) accredited Cell Factory might represent a new scenario for their clinical use in cell therapy protocols providing a continuous supply of cells to treat patients with acute GVHD after allogeneic hematopoietic stem-cell transplantation (HCST), solid organ transplantation or in inflammatory and autoimmune diseases.

During the activity of my PhD, we obtain useful data to clarify some mechanisms of action at the cellular, molecular and epigenetic level of MSCs isolated from BM, AF and PL to use for clinical use.

We showed that:

- MSCs isolated from foetal tissues may be considered a good alternative to BM-MSCs for clinical application, because AF and PL-MSC were considered multipotent stem cells with the immunophenotypic characteristics and differentiative potential established by guidelines by the International Society of Cellular Therapy , a greater proliferative potential associated with the presence of embryonic markers and great immunomodulant properties.
- The immunomodulant properties of MSCs is maintained when FBS is substituted from inactivated HPL to have a method safer and more advantageous for large scale expansion in GMP conditions
- BM-MSCs have an important role in reduced aGVHD III-IV incidence and TRM and remain of recipient origin after HSCT despite: i) myeloablative conditioning, ii)

the stem-cell source, iii) the interval from HSCT to BM analysis (3,4), iv) the underlying disease before HSCT, v) the patients' or the donors' age at HSCT)

- MSCs are a population of cells extremely heterogonous and have an interindividual variability of DNA methylation. The dynamics of DNA methylation during aging depend on a complex mixture of factors that include the DNA sequence, cell type and chromatin context involved, and that, depending on the locus, their changes can be modulated by genetic and/or external factors.
- Endogenous retroviruses such us HERV-K and -H should be considered as new markers of stemness or differentiation for MSCs
- The preliminary data obtained from ChIP-seq experiments showed that the activator histone mark, H3K4me3 and the repressive histone mark, H3K27me3 cannot be considered good markers to analyse the stemness maintenance during the expansion of MSCs in vitro and suggest us to identify specific regulatory regions different from NANOG, OCT-4 and SOX-2 to identify stemness stage.

All these data support the iHPL represents a good, GMP-compliant alternative to FBS for MSC clinical production which is more advantageous in terms of cellular growth and stemness and preserve the immunomodulant proprieties and stemness marker. Moreover, our data also support the current hypothesis that MSCs could act by secreting paracrine factors in a "hit-and-run" scenario including the secretion of not only cytokines and other soluble factors but also extracellular vesicles that can contain cargos that include peptides, proteins, metabolites, microRNAs, and even mitochondria. Our future perspectives focus on the study of secretome obtained by MSCs expanded in GMP conditions to obtain a pharmaceutic product safer and extremely effective and efficient for multiple clinical use

8 APPENDIX : PUBBLICATIONS

1) Bandino A, Lupino E, Compagnone A., Meli F, Mighetto L, Castiglia S, Mareschi K. and Fagioli F. 4 N,N'-tetramethylene bisacetamide display anti-fibrotic activity and downregulate SNAI1, α-SMA and Fibronectin in human Mesenchymal Stem Cells, submitted

2) Mareschi K , Montanari P., Rassu M. , Galliano I., Daprà V., Adamini A., Castiglia S., Fagioli F., Bergallo M. Human endogenous retroviruses -H and -K expression in human mesenchymal stem cells as potential markers of stemness, submitted

3) Bergallo M, Daprà V, Galliano I, Calvi C, Berger M, Montanari P, Mareschi K, Fagioli F.Mir-155 Expression is downregulated in Hematopoietic Stem Cell Transplantation Patients with Epstein-Barr virus Infection

4) Castello L, Leone M , Adamini A, Castiglia S, Mareschi K, Ferrero I, De Gobbi Marco, Carnevale-Schianca F, Fagioli F, Berger M. Analysis of Mesenchymal Stromal Cell Engraftment after Allogeneic HSCT in Pediatric Patients: a Large Multicenter Study J Pediatr Hematol Oncol in press

5) Berger M., Mareschi K., Castiglia S., Rustichelli D., Mandese A., Migliore E., Fagioli F. In Vitro Mesenchymal Progenitor Cell Expansion is a Predictor of Transplant-related Mortality and acute GvHD III-IV After Bone Marrow Transplantation in Univariate Analysis: A Large Single-Center Experience (2018) J Pediatr Hematol Oncol in press

6) Castiglia S, Adamini A, Rustichelli D, Castello L., Mareschi K., Pinnetta G., Mandese A., Ferrero I, Mesiano G., Fagioli F.Cytokines induced killer cells produced in good manufacturing practices conditions: identification of the most advantageous and safest expansion method in terms of viability, cellular growth and identity. DOI : 10.1186/s12967-018-1613-5. JTRM-D-18-00205.2

7) Bergallo, M., Montanari, P., Mareschi K., Merlino, C., Berger, M., Bini, I., Daprà, V., Galliano, I., Fagioli, F. Expression of the pol gene of human endogenous retroviruses HERV-K and -W in leukemia patients (2017) Archives of Virology, 162 (12), pp. 3639-3644. DOI: 10.1007/s00705-017-3526-7

8) Bergallo, M., Montanari P., Mareschi K., Rassu, M., Galliano, I., Ravanini, P.. A novel TaqMAMA assay for allelic discrimination of TLR9 rs352140 polymorphism (2017) Journal of Virological Methods, 243, pp. 25-30.DOI:10.1016/j.jviromet.2017.01.015

9) Bergallo, M., Merlino, C., Montin, D., Galliano, I., Gambarino, S., Mareschi K., Fagioli, F., Montanari, P., Martino, S., Tovo, P.-A. Development of a Low-Cost Stem-Loop Real-Time Quantification PCR Technique for EBV miRNA Expression Analysis (2016) Molecular Biotechnology, 58 (8-9), pp. 540-550. DOI: 10.1007/s12033-016-9951-0

10) Mareschi K., Castiglia, S., Sanavio, F., Rustichelli, D., Muraro, M., Defedele, D., Bergallo, M., Fagioli, F. Immunoregulatory effects on T lymphocytes by human mesenchymal stromal cells isolated from bone marrow, amniotic fluid, and placenta (2016) Experimental Hematology, 44 (2), pp. 138-150.e1. DOI:10.1016/j.exphem.2015.10.009

11) Bergallo, M., Galliano, I., Montanari, P., Gambarino, S., Mareschi K., Ferro, F., Fagioli, F., Tovo, P.-A., Ravanini, P. CMV induces HERV-K and HERV-W expression in kidney transplant recipients (2015) Journal of Clinical Virology, 68, pp. 28-31. DOI: 10.1016/j.jcv.2015.04.018

12) Fernández, A.F., Bayón, G.F., Urdinguio, R.G., Toraño, E.G., García, M.G., Carella, A., Petrus-Reurer, S., Ferrero, C., Martinez-Camblor, P., Cubillo, I., García-Castro, J., Delgado-Calle, J.U., Pérez-Campo, F.M., Riancho, J.A., Bueno, C., Menéndez, P., Mentink, A., Mareschi K., Claire, F., Fagnani, C., Medda, E., Toccaceli, V., Brescianini, S., Moran, S., Esteller, M., Stolzing, A., De Boer, J., Nistico, L., Stazi, M.A., Fraga, M.F. H3K4me1 marks DNA regions hypomethylated during aging in human stem and differentiated cells (2015) Genome Research, 25 (1), pp. 27-40. DOI: 10.1101/gr.169011.113