Faculdade de Medicina da Universidade de

ESTABLISHMENT OF AN IN VIVO MODEL OF HUMAN HEMATOPOIESIS ON A THREE-DIMENSIONAL (3D) HUMANIZED STROMA

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To my brother Pedro

“Perfection is achieved, not when there is nothing more to add,
but when there is nothing left to take away”
Antoine de Saint - Exupéry

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Abstract

Umbilical cord blood hematopoietic stem cell transplantation (UCB-HSCT) is an increasing area both clinical and translational investigation. The main hurdle of this approach is a delayed engraftment (or even a graft failure) that accounts the most of the transplant-related mortality. To gain insight on the factors that may influence UCB engraftment is important to implement the clinical procedure. The interaction of hematopoietic progenitors with the stroma is the key process in this setting. Nevertheless, the establishment of a good *in vitro* model to evaluate three-dimensionally (3D) system part of the complex BM microenvironment are not well established.

In this study we have tried to develop an artificial BM niche by using a scaffold of Conduit-TCP (3D) together with human marrow mesenchymal stem cells (MSC). MSC were differentiated to stromal cells using the technology of classic long term bone marrow cultures (LTBMC). For this purpose, $CD34^+$ cells from UCB were cultured for 5 weeks in this 3D-BM niche construct (3D system), demonstrating the capacity of this construct to support the maintenance of immature progenitors in culture, besides the differences in colony formation between this 3D system and the conventional two dimensional cultures (2D system) were not statistically significant.

To complement the *in vitro* studies, we developed an *in vivo* experimental model to evaluate the engraftment capacity of this human-BM niche construct by implanting them subcutaneously in NOD/SCID mice after 4 weeks or 5 weeks of *in vitro* culture, showing better engraftment of the former group both at 3 and 6 weeks after transplantation of human UCB $CD34^+$ cells. When compared this approach to a control group where the 3D-BM niche was developed *in vivo* by implanting the scaffold with undifferentiated human MSC, the former group had higher engraftment of human hematopoietic cells ($CD45^+$) at 3 weeks postransplant on peripheral blood and 6 weeks on BM, with myeloid engraftment ($CD13^+$). The control group presented higher human lymphoid cells ($CD19^+$) when $CD34^+$ cells were transplant intravenously and lower human CD19+ cells with a predominant human myeloid graft when the HSC were subcutaneously transplanted.

The 6 weeks after transplantation an ex vivo study was performed. Immunohistochemistry and immunofluorescence assays demonstrated the homing and engraftment of the human cells into the 3D-BM niche in vitro constructs that were subcutaneously implanted. In addition, human MSC from the scaffolds contributed to form new cells in the microenvironment, contributing also to angiogenesis. By employing enhanced cyan

fluorescent protein-marked human MSC we were able to track them close to the endosteal zone.

In summary, the 3D-BM niche constructs allowed the growth, differentiation and migration of HSC in a 3D humanized environment effectively mimicking the natural *in vivo* bone marrow environment that allows the maintenance and proliferation of hematopoietic cells both *in vivo* and *in vitro*. This model permits the study of normal and pathological hematopoiesis and its relationship with the stroma.

Keywords: Hematopoietic stem cells – Three-dimensional system – Long term culture – Mesenchymal stem cells – Expansion - UCB $CD34^+$ cells - NOD/SCID mouse

Resumo

O transplante de células progenitoras/estaminais hematopoiéticas do sangue do cordão umbilical (SCU) é uma das terapias disponíveis para a clínica e uma área em expansão na investigação. O obstáculo principal da utilização destes progenitores está associado a uma lenta recuperação dos doentes, levando a um aumento no risco de falência do enxerto, incrementando a incidência de mortalidade nestes doentes. Esta limitação pode ser ultrapassada quando os factores que influenciam o sucesso do enxerto de SCU forem conhecidos, sendo crucial o estudo das interacções que ocorrem entre as células progenitoras e o seu ambiente (estroma).

Ainda não estão estabelecidos modelos experimentais empregando estromas a 3 dimensões (3D) que criam uma analogia ao complexo sistema que ocorre na medula óssea (MO) para estudos *in vitro*, sendo crucial o seu desenvolvimento.

Neste trabalho procurou-se estabelecer um "*nicho*" artificial de MO usando um biomaterial - Conduit-TCP (3D), juntamente com células precursoras mesenquimatosas humanas de medula óssea. Estas células foram diferenciadas a estroma utilizando técnicas clássicas de cultivo a longo prazo de medula óssea. Para atingir o objectivo do estudo as células $CD34^+$ de SCU foram cultivadas durante cinco semanas neste "nicho" a 3D, que demonstrou capacidade durante o tempo de cultura de manter em estado imaturo as células progenitoras hematopoiéticas. A produção de colónias (CFU-GM) provenientes destes sistemas é superior à produção de colónias quando comparados com o sistema de cultivo convencional a duas dimensões, contudo não são diferenças significativas. Para complementar a estudos *in vitro*, desenvolvemos um modelo experimental *in vivo* de forma a avaliar a capacidade de enxerto das células expandidas neste "nicho" artificial, para tal implantamos subcutaneamente este biomaterial proveniente de cultivos com 4 e 5 semanas em murganhos NOD/SCID (animais imunossuprimidos). Na terceira e sexta semana após o xenotransplante o grupo onde foram implantados os biomateriais com menos tempo de cultivo (4 semanas), apresentavam maior recuperação hematopoiética de células humanas ($CD45^+$). Quando se compara com o grupo standard em que o "nicho" foi construído *in vivo*, verificamos um maior nível de células humanas $CD45^+$ no sangue periférico nas três semanas pós-transplante e em medula óssea na sexta semana pós-transplante, com regeneração medular mielóide ($CD13^+$) para o grupo experimental. No grupo standard as células $CD34^+$ foram transplantadas 7 semanas após indução *in*

vivo das células mesenquimatosas a estroma em que se verificou maior enxerto de células $CD19^+$ quando as células $CD34^+$ foram transplantadas por via intravenosa, contudo quando subcutaneamente transplantadas esta linhagem linfóide diminuía.

Quando finalizadas as 6 semanas foram realizados estudos ex vivo por histologia, imunohistoquímica e imunofluorescência ao biomaterial subcutaneamente implantado e aos fémur dos animais dos diferentes grupos.

No caso dos grupos em que foram transplantados com o "nicho" (biomaterial+estroma+células$CD34^+$) construído *in vitro* as células progenitoras hematopoieticas conseguiram migrar (*homing*) deste sistema para a medula óssea dos animais.

Verificou-se regeneração de novo tecido no Conduit^{TM}TCP em que as células mesenquimatosas humanas não só contribuíram para a formação do estroma nestes implantes como também na angiogénese. Um dado muito interessante foi a confirmação de que estas células similarmente as células humanas hematopoiéticas também foram capazes de migrar para a medula óssea murina encontrando-se principalmente na zona endosteal

Em resumo, o nicho medular construído *in vitro* num sistema a 3D permitiu o crescimento, diferenciação e migração das células progenitoras hematopoieticas criando uma analogia ao ambiente da medula óssea humana, o que permitiu a manutenção e proliferação de células hematopoiéticas *in vivo* e *in vitro*. Este modelo permite o estudo da hematopoiese normal e patológica e sua relação com o estroma.

Palavras Chave: Células Precursoras Hematopoiéticas – Sistema a três dimensões – Cultivo a longo prazo – Expansão de células $CD34^+$ de Cordão Umbilical – Modelo murino NOD/SCID.

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AAD	Amino acid deaminase
APC	Allophycocyanine
BM	Bone marrow
B	B-lymphoid
BFU-E	Burst-forming unit – erythroid
CAFC	Cobblestone area forming cell
CFU-E	Colony-forming unit erythroid
CFU –F	Fibroblast colony-forming units
CFU - G	Colony forming unit granulocyte
CFU-GM	Colony forming unit – granulocyte, macrophage
CFU-GEMM	Colony forming that generates myeloid cells
CFU-Eo	Colony – forming unit-eosinophils
CFCs	Colony-forming cells
CMV	Cytomegalovírus
CFP	Cyan Fluorescente Protein
CXCR4	C-X-C chemokine receptor type 4
DAPI	4', 6-Diamidine-2' -phenyl indole, dihydrochloride
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium–low glucose - Gibco, Invitrogen, Paisely
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cells
EPCs	Endothelial progenitor cells
FITC	Fluorescein isothiocyanate

FBS	Fetal bovine serum
G0	Quiescence state
GVHD	Graft-versus-host disease
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplantation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HSC	Hematopoietic stem cell
IL-1	Interleukin-1
IL-10	Interleukin-10
IgG	Immunoglobulin
IFN-γ	Interferon gamma
ISCT	International Society of Cellular Therapy
LTBMC	Long-term culture initializing cell
LT-HSC	Long term repopulation hematopoietic stem cell
LIF	Leukemia inhibitory factor
LV	Lentiviral vectors
MSC	Mesenchymal Stem Cells
MNC	Mononuclear cell
NOD/SCID	non-obese diabetic/severe combined immunodeficient – NOD.CB17-Prkdc^{scid}/ NcrCrl
NK	Natural Killer
NBT/BCIP	Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate
PB	Peripheral blood
PL	Platelet lysates
PE	Phycoerythrin
PBS	Phosphate buffer saline
PerCPCy	Phycoerythrin-cyanine 5

SDF-1	Stromal cell-derived factor-1
ST-HSC	Short term repopulation hematopoietic stem cell
TCP	Tricalcium phosphate beta
T	T-lymphoid
UCB	Umbilical Cord Blood
VSV-G	Vesicular Stomatitis Virus
2D	Two dimensional
3D	Three dimensional

I - Introduction

1 – Stem cells

There is a difficulty to answer seemingly simple questions, as for example *How can a stem cell be defined?* This confusion can be partly attributed to the idiosyncratic terms and definitions founded in literature to describe stem cell, like “immortal”, “unlimited”, “continuous”, and “capable of extensive proliferation” joining the difficult to have standards protocols for identifying, isolating, and characterizing these cells[1].

Stem cells are capable of both self – renewal and ability to differentiate into different types of mature cells. Because of these characteristics they play essential roles in organogenesis during embryonic development and tissue regeneration[2].

Based on their species and tissue of origin, and their ability to differentiate it is possible to sub-classify stem cells in[3]:

- Totipotent cells, found in early embryos (1-3 days from oocyte fertilization), can originate all the embryonic tissues and placenta;
- Pluripotent embryonic stem cells (ESC) derived from blastocyts (days 4-14), that are compromised into embryonic tissues derived from the inner mass (ectoderm, mesoderm, and endoderm);
- Pluripotent adult stem cells, derived from fetal tissue or cord blood as example, can rise only to tissues belonging to one embryonic germ layer (ectoderm or mesoderm or endoderm), more limited potential for differentiation.
- Unipotent cells, giving rise to single lineage cells of certain tissues

The therapeutic use of ESC is still controversial not only because of their ethical problems[4] derived of their use but may also carry risk of neoplastic potential development (teratomas)[5]. Unlike the previous, adult stem cells have not shown tumorigenic potential, and can be obtained in adequate amounts for autologous or even allogeneic regenerative therapy, although they are more difficult to expand[6]. ESC cells may prove to be ideal for creating new organs after passing current ethical and technical minefields, and adult stem cells may be more useful for repairing damage to tissues by trauma, disease, or perhaps uncomplicated aging.

Adult stem cells are an essential component of tissue homeostasis, because they support ongoing tissue regeneration and maintain the balance between self-renewal and differentiation[6]. The best characterized of them is the hematopoietic stem cell (HSC), which is responsible for originating all circulating blood cell types[1]. Nevertheless, another bone marrow stem cell type that has recently attracted notable interest in the scientific

community is the Mesenchymal Stem Cell[7] (MSC) due to their unique and interesting properties, that we will address next

1.1 – Hematopoiesis and Hematopoietic stem cells

Hematopoiesis is a highly orchestrated process defined by the formation, development and maturation of all types of blood cells from a common precursor cell – hematopoietic stem cells[8] (HSC), figure 1.

Functionally, this extraordinary population was first described in the early 1950s using transplantation experiments with mouse models, which describe the ability of these cells to engraft and maintain hematopoiesis in lethally irradiated recipients[9]. Like a stem cell, HSC have the unique capacity to self – renewal and the potential to differentiate into different kinds of mature cells. However when a bone marrow (BM) or blood stem cell transplanted is performed, we can verify that this pool of cells are in different states: on one hand a finite-lived population or short term repopulation – HSC (ST-HSC) capable of yielding most of the major blood cell types in short time, and the long-term repopulation HSC (LT-HSC) that stable through the lifetime of the recipient. The later population is typically demonstrated by serial transplantation from cells of the first recipient into secondary and tertiary recipients.

Normally the HSC is located in a specific niche of BM, in a quiescence state (G0) that allows the maintenance of long term repopulating cells[10].

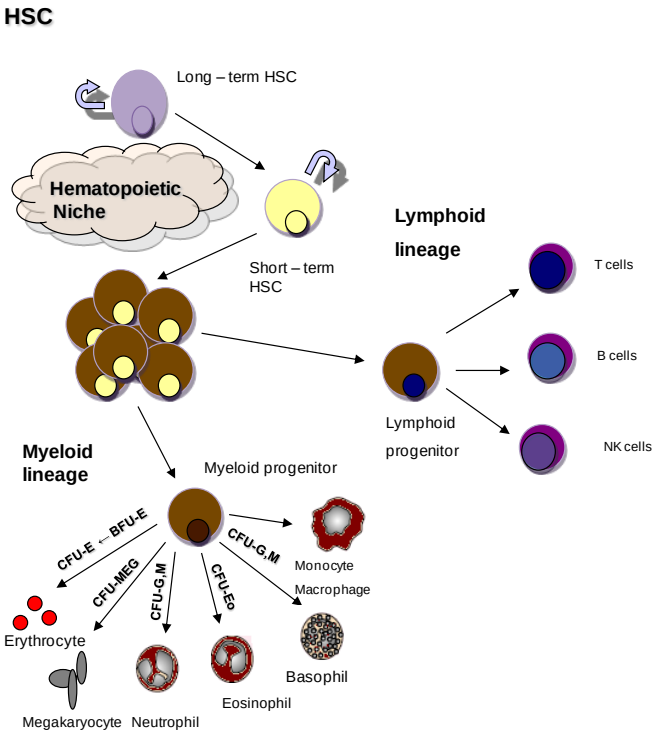

Figure1. Hematopoiesis: CFU – GM, colony forming unit – granulocyte, macrophage; CFU-MEG, colony-forming unit – megakaryocyte; BFU-E, burst-forming unit – erythroid; CFU-E, colony-forming unit erythroid; CFU-Eo, colony – forming unit-eosinophils.

2 – Stem cell Niche: Structure and Function

Normally adult stem cells reside in a specific microenvironment called the "niche" that plays a vital role in regulation of stem cell behavior to govern tissue homeostasis under diverse physiological and pathological conditions.

The hypothesis of an evidence for stem cell niche was brought up in 1978 by Schofield *et al*[11], describing the physiologically limited microenvironment that supports general genetic programs that maintain stem cells natures[12].

2.1 – Bone marrow niche

It is well known that adult bone has a unique microenvironment which allows the maintenance of hematopoietic stem cells[13]. Consequently is not unexpected that identification and characterization of the stem cells that regulate this process is an active field of investigation.

The research on bone marrow stem cells initiated in the 1960s, the pioneering studies *in vivo* by Till and McCulloch[14] where they describe classical spleen colony forming (CFU-S) assay, allowed to measure the ability of HSC to form large colonies in the spleens of lethally irradiated mice, as spleen colony forming units (CFU-S).

Subsequently, a number of cells fulfilling stem cell properties have been identified in BM, besides the hematopoietic stem cells:

- Endothelial progenitor cells (EPCs);
- Mesenchymal stem cells (MSCs);
- Multipotent adult progenitor cells;
- Marrow – isolated adult multilineage inducible cells.

Emergence of different definitions of BM niche and discrepancies in published results by the scientific community could be explained by the differences in the models employed and/or problems in using different experimental strategies when it came to identify these cells, leading assigned distinct names.

The establishment of the stem cell niche hypothesis first presented by Schofield in 1978, described the close association of HSC to an environmental niche essential for the maintenance of their self – renewal capacity and also regulating the differentiation and maturation processes[11]. When this association is disturbed, the HSC becomes committed to specific lines of blood cell development and loses its stem cell property. Consequently, BM stromal cells could play an important role in the homeostasis of the hematopoietic process.

Despite the success in identifying some components and signals of the HSC niche, validating the general concept that environmental factors, including soluble signals, specific cell-cell and cell-matrix interactions, as well as structural cues are vital for stem cell maintenance and function, the complete understanding of the extrinsic and intrinsic signals that influence the choice between self-renewal and differentiation of HSC remains elusive. That is due to the limited availability of appropriate ex vivo models which could mimic the complex niche organization[15].

During human embryogenesis the HSC are first presented in the yolk sac and in the aorta-gonad-mesonephros region, followed by fetal liver and spleen. With the beginning of gastrulation and organogenesis stem cells migrate and colonize tissue-specific niches, residing as a population of self – renewing cells, this is the most active period for the development migration/trafficking of stem cells[16].

The hematopoietic niche on human adults is generally confined to the BM, and consist of diverse cells of the osteoblastic lineage[17] indifferent developmental stages that includes

preostoblasts, osteoblasts, osteocytes, together with collagen fibrils, and mineral deposits such calcium and phosphate[18]. In addition, the bone cavity also contains fibroblasts, macrophages, endothelial cells, and adipocytes.

It is difficult to discern the relative importance of each of these cells to maintain the hematopoietic niche. Only in recent years with the advent of mouse genetic models and live microscopy that permits to visualize the exact location, cellular composition, and associated molecular signals of the HSC niche decreased the gap of information, but the mechanism is still far from being fully understood.

At present, at least two distinct niches have been identified in the BM, responsible to play central roles in regulating stem cell proprieties[19]: the osteoblastic niche and the vascular niche[20]. Different experimental studies showed that according to HSC level of lineage commitment, the spatial distribution is distinct[15]. Precursors are mainly located in the central marrow region close to the sinusoids (*vascular niche*) that allows differentiation and ultimately mobilization into the peripheral circulation, and long-term HSC are predominantly localized in the endosteal region (trabeculae), in close contact with osteoblasts (*osteoblastic niche*), where the signaling events between these types of cells are crucial in maintenance and activation of stem cells[15, 18, 21], figure 2.

Osteoblastic cells also secrete cytokines that regulate stem cell function, including granulocyte–colony-stimulating factor (G-CSF), M-CSF, GM-CSF, interleukin-1 (IL-1), IL-6, among others[17, 18]

HSC trafficking, proliferation and survival is controlled by a complex signaling network produced by osteoblasts induced through the contact with HSC. Among them, Notch and Wnt signaling as well as the SDF-1/CXCR4 axis, are key components of the system.

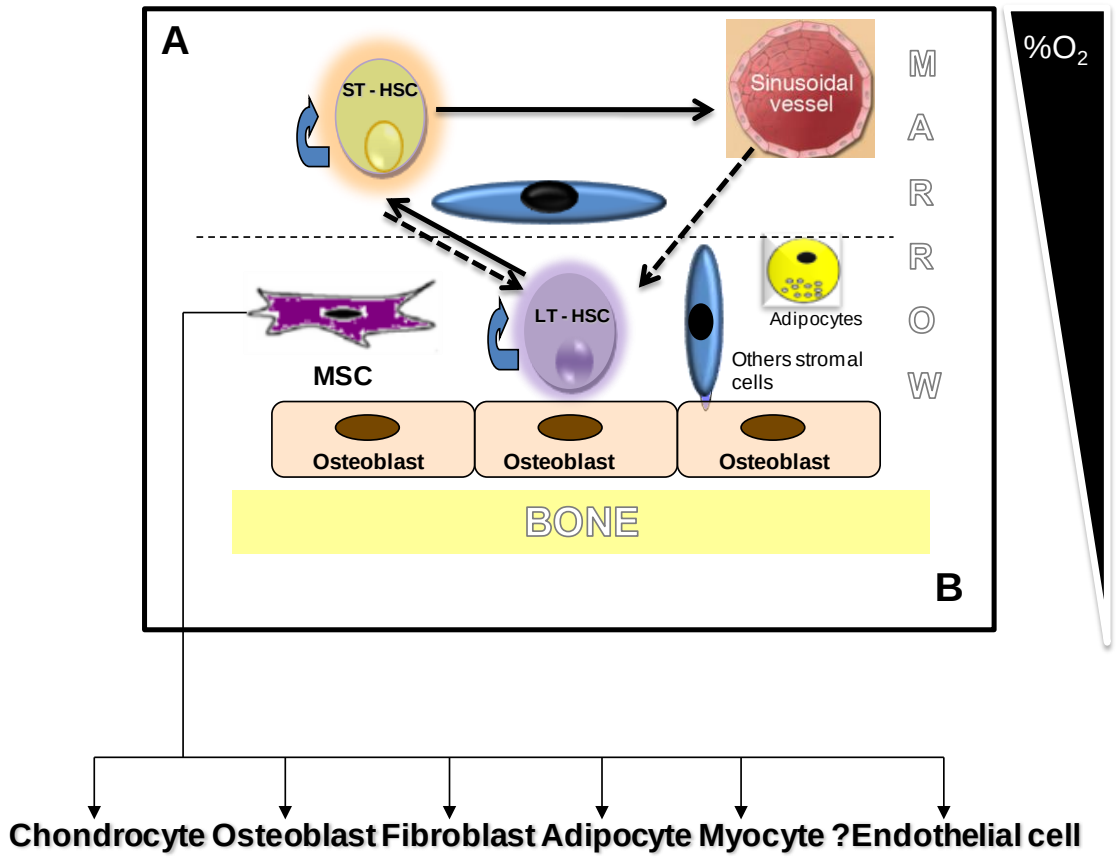

Figure 2. Model of hematopoietic stem cells niche: In bone marrow the primitive HSC reside either next to osteoblasts on the endosteal niche (compartment B), where most primitive and quiescent HSC reside, and in the vascular niche, where more committed HSC (ST-HSC) can be found adjacent to endothelial cells of sinusoidal vessels (comportment A), from where they can be released from the vascular niche into the systemic circulation.
It has been demonstrated that primitive hematopoietic cells in the BM are under a hypoxic microenvironment implying low oxygen levels are important in the maintenance of normal stem cell function[22].

2.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSC) are the precursor of most stromal cells of BM microenvironment, and thus a key component of the hematopoietic niche[23].
The first demonstration of MSC in BM was in the early 1970´s, by a pioneer work of Friedenstein and colleagues[24] that described a population of spindle-shaped cells *in vitro* were able to adhere to plastic surfaces and form colonies designed as fibroblast colony-forming units (CFU-F). This population represents only about 0.01-0.001%[25] of the total mononuclear cell (MNC) fraction in adult human BM, a proportion that varies with age and health[26].

MSC are not exclusively found in BM. Cells with MSC characteristics can be present in others tissues and organs such as fat, umbilical cord blood, placenta, muscle, liver or lung[27].

This population in the recent years has become objective of great scientific interest given their potential capacity to differentiate in mesoderm-type cells, including fibroblasts, osteoblasts, chodroblasts, and adipocytes[28], and for their *in vitro* expansion without compromising its genetic stability[29]. In addition, MSC have strong immunomodulatory activity both *in vitro* and *in vivo*, and are themselves immune-privileged[30,31] These cells can escape from host allogeneic response because they appear to be poorly immunogenic, since express low levels of major histocompatibility complex (MHC) class I and do not express MHC class II molecules[32].

Moreover, MSC do not express co-stimulatory molecules such CD40, CD80 or CD86 which also involved in the activation of T cells. In addition, they release bioactive molecules that were shown to inhibit not only T cells, but also B cells, NK cells, and monocyte-derived dendritic cells[25].

Some studies have demonstrated that the inhibitory effects of MSC *in vitro* were directed mainly by the level of cell proliferation, the T-cells are arrested at G1 phase, the MSC removed from the cultures allows the T-cells to restore the ability to produce IFN-γ in response to subsequent stimulus[33].

In the case of dendritic cells, MSC induce to tolerogenic phenotype since dendritic cells cultivated in the presence of these cells produce more IL-10 and inhibit their differentiation from monocytes[34]

2.2.1 Mesenchymal stem cell isolation, expansion and characterization

The first step to isolate MSC is generally the use of density centrifugation techniques to obtain the mononuclear fraction of BM[35]. Due to their characteristic plastic adherence, MNC are plated in culture flasks containing a standard culture medium and generally 10% of serum, although expansion media can be modified to improve the cell yield[36]. Thus, MSC are purified through successive passages where hematopoietic mononuclear cells are washed-out with the medium replaced. MSC has a characteristic spindle-shape *in vitro,* MSC was initially identified by a combination of this *in vitro* morphology, together with a characteristic immunophenotypic pattern and the demonstration of their *in vitro* multilineage differentiation potential[37, 38]. Despite the different efforts to extensively

characterize MSC surface immunophenotype, no single and specific MSC marker has been identified to date, table 1.

Table1. Surface antigen frequently identified during MSC isolation[38].

Human MSC	
Marker type	*Surface antigen*
Positive	Stro-1; CD13; CD29; CD44; CD73; CD 105 and CD106
Negative	CD11b; CD31; CD34; CD45; CD117
Variable	Sca-1; CD10; CD90; Flk-1

In order to minimize the increasing difficulty to compare and contrast study outcomes, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed, in 2006, the minimal criteria to define MSC based on phenotypic and functional characteristics[39].

- Must be plastic – adherent when cultured *in vitro* under standard conditions;
- Lack of expression of hematopoietic markers: CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA class II and express a specific pattern of surface antigen expression, namely CD105, CD73 and CD90;
- Must demonstrate under standard in vitro conditions the multipotent differentiation potential into osteocytes, adipocytes and chondrocytes (three different lineages)

2.3 – Three dimensional systems (3D)

In humans bone marrow is the principal and permanent hematopoietic organ where specialized microenvironments reside, called niches, regulate HSC. Anatomically, BM stroma includes virtually all nonhematopoietic cells types[40] reorganized in appropriated 3D architecture where the stromal cells and the extracellular matrix provide a physical and functional support that also regulates cell migration, proliferation, and differentiation through cell-to-cell and cell-to-substrate interactions[41], figure 3.

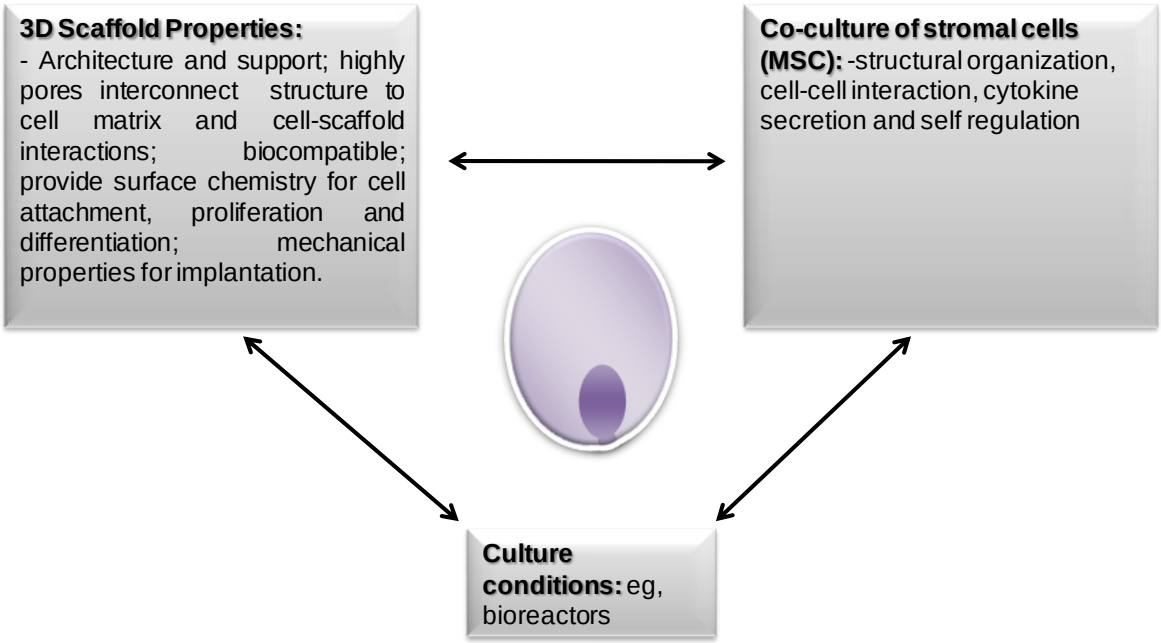

Figure 3. Schematic illustration of the parameters required to culture HSC in a 3D system that mimic an artificial niche. The bulk properties of the 3D Scaffold co-cultured with MSC in appropriate culture conditions try to recreate the physiological complexity of a stromal supportive tissue[41].

Recently, several studies have tried to mimic the BM microenvironment by 3D culture systems[42]. They have attempted to recreate the native BM environment by the addition of cytokines, growth factors or by the presence of co-cultured stromal of feeder cells. Employing 3D cell culture systems can induce more intensity cell-to-cell contacts between HSC and stromal cells which appear promising to produce the appropriate developmental niche for stem cells, and the growth and differentiation capacity compared to 2D cultures[43-46].

3 – Hematopoietic stem cell transplantation

Since the first successful hematopoietic cell transplant in the early 1950s, hematopoietic stem cell transplantation (HSCT) has been employed as an effective strategy to treat not only several hematologic malignancies but also congenital or acquired defects of hematopoiesis or immune function[47]. HSCT is generally classified according to the source of the cells into autologous, syngeneic or allogeneic HSCT.

Mobilized peripheral blood (PB) is the most common source of HSC for transplantation[48], since the procedure is easier and the donor can avoid entering in the operating room,

however the engraftment is shorter with PB when compared to BM[49]. Nevertheless, the use of PB is associated with a higher rate of graft-versus-host disease (GVHD), the main complication of allogeneic HSCT, due to the higher content of T-cells within the mobilized PB-graft[50].

In addition to the problems described from allogenic HSCT, the HLA-matched sibling donor is also a problem because just only 30% of patients have a potential sibling donor with adequate match with patient´s HLA loci. This problem is increased in non-caucasian patients[51].

3.1 – Umbilical cord blood stem cell transplantation

Since the first use of human umbilical cord blood (UCB) for HSCT in 1989[52], this modality has notably increased its importance due to the higher number of patients that are candidates for transplantation, and the lack of a suitable donor, as it has been already mentioned. Among the advantages[53] of UCB HSCT is the higher immunotolerance of the graft, so that a less stringent HLA-match is permitted, compared to BM or PB transplantation, without a higher incidence of GVHD. Nevertheless, UCB transplantation has the disadvantage of a delayed hematopoietic engraftment with a higher rate of graft failure, which is associated to an increased transplant-related mortality, mainly due to infection[54]. Strategies to enhance UCB engraftment include the in vitro expansion of CB cells, transplant of 2 UCB units, co-transplant of CD34+ cells from an haploidentic related donor, the co-transplant of MSC or the administration of the UCB progenitors via intrabone. Most of the above mentioned strategies are in an experimental phase[55-57].

3.2 – Mesenchymal stem cells and hematopoietic stem cell transplantation

MSC of BM origin are responsible to recycle the pool of most cellular components that supply hematopoietic microenvironment niche, including fibroblasts, osteoblasts, and adipocytes. In addition, they provide hematopoietic growth factors, facilitate cell-to-cell interaction, and elaborate matrix proteins that play a role in the regulation of hematopoiesis.

Despite an intensive preparation regimen, an adequate cell dose and complete donor chimerism poor haemopoietic function can be observed after HSCT[58, 59]. One of the critical points in haematopoietic regeneration following HSCT is the capacity of the recipient recovery the spatial organization of the bone marrow micro-environment[10].

MSC contribute in the context of HSCT[60] since can directly and indirectly regulate and controlled the maintenance of HSC. The MSC express a variety of α integrin and β – subunits and their monovalent associations that constitute receptors for extracellular matrix components including collagen, laminin, fibronectin and vitronectin who regulate the organization of the extracellular matrix[25]. In co – culture experiments, MSC form cell clusters with HSC, including megakaryocytes and osteoclast progenitors, providing a physical support for HSC[61].

MSC also modulate the immune response *in vitro* and *in vivo*, interacting with a broad range of immune cells including T-lymphocytes, B-lymphocytes, NK and dendritic cells. For all these properties, the therapeutic use of MSC is being explored in the HSCT setting for a number of complications, including graft failure or acute and chronic GVHD[62].

3.3 – *Ex vivo* models to study hematopoiesis

The first assays to study stem cell biology were introduced by Bradley and Metcalf with the semisolid culture system-colony-forming assay[63]. The primary aim of these assays was to obtain information on the developmental stages and differentiation potentials of HSC and also visualize their dynamics and kinetics.

However these methods do not recognize the presence of cells which have not matured enough to exhibit the characteristics of mature cells, for the temporary hematopoiesis (2-4 weeks) despite attempts to supplement with nutrients and cytokines, and most primitive stem cells fail to survive and proliferate[64].

When cultured in a suitable semi-solid matrix, such as methylcellulose supplemented with nutrients and cytokines, HSC or hematopoietic progenitors called colony-forming cells (CFCs) proliferate to form discrete cell clusters or colonies. Colony types include colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid (BFU-E), CFU-granulocyte, macrophage (CFU-GM) and CFU granulocyte, erythrocyte, macrophage, megakaryocyte (CFU-GEMM), the later are most commonly used, clinical transplantation, to provide a quantitative, high-throughput method to accurately measure HSC or hematopoietic progenitor clonogenic capability[65].

In attempts to more closely mimic hematopoiesis, Dexter *et al.* (in 1970s), developed the long-term bone marrow derived stromal culture who supported the production for several weeks of HSC[66].

Long-term culture initializing cell (LTC-IC) assay and Cobblestone area forming cell (CAFC) assay has been developed to recognize the presence of immature HSC in vitro.

LTV-IC cultures were developed with the aim to support the continued production of myeloid cells, and subsequently for the production of lymphoid cells[67]. The primitive progenitors can persist for more than 5 weeks and generate secondary CFU-C, while the committed progenitors present in the culture-flask are removed with medium changes, and susceptible of being assayed by semisolid cultures. This assay is a powerful procedure to quantify and distinguish cells at discrete stages of early hematopoietic cell differentiation[68].

The CAFC can be scored sequentially assessing the percentage of confluence of the stromal layer and observing the presence of cobblestone areas, that represents areas of active *in vitro* hematopoiesis[69].

3.4 – *In vivo* models to study hematopoiesis

Considering that the *in vitro* assays can not sufficiently assess the engraftment potential of human HSC, the use of murine models of transplantation allows the study of basic principles of stem cell biology including immunophenotype, homing ability, engraftment kinetics, cytokine responsiveness and radiation sensitivity of repopulating cells.

The classical experiments of bone marrow transplantation were developed in the early 1950s showing that the infusion of BM from a donor could protect the recipients after lethal irradiation.

Human HSC activity can be tested by transplantation into immunodeficient mouse strains, which are unable to reject xenogenic cells. Principally, non-obese diabetic/severe combined immunodeficient – LtSz-scid (NOD/SCID) mice have proven to be a reliable recipient for detecting human hematopoietic – repopulating cells that differentiate into multilineage mature cells self-renew in mice. This is owed to their complete deficiency in alloreactive lymphocytes especially NK cells[70]. The level of human engraftment, in this model, after xenotransplantation of BM and UCB is higher (10x) than the classical transplants using SCID mouse.

Studies using the NOD/SCID/beta-2- microglobulin-null mouse strains have demonstrated a hierarchy of human repopulating cells supporting successive waves of human hematopoiesis in the mice. More recently assays show others models like, NOD/SCID/cγ$^{-/-}$ mice have been used as improved recipients for human HSCs, capable of complete reconstitution with human lymphocytes, even in the absence of additional human tissues. Even more promising has been the use of newborn mice with a profoundly impaired immune system (Rag-2$^{-/-}$Cγ$^{-/-}$), which results in a reproducible production of human B- and T-lymphoid and myeloerythroid cells[71].

II – Hypothesis and Aims

Hematopoietic stem cell transplantation (HSCT) is used to treat several disorders of the immunohematopoietic system. The conditioning regimen is toxic for hematopoietic stem cells (HSC) but also for the microenvironment.

A balance between the maintenance of the stem cell pool and the differentiation of HSC is mediated by soluble and membrane-bound cytokines, extracellular matrix components, and direct cell-to-cell contact.

Umbilical cord blood (UCB) units are being increasingly used for hematopoietic stem cell transplantation. The main limitation of UCB transplantation is a delayed engraftment. Thus, an adequate understanding of UCB stem cell homing and lodging in the hematopoietic niche is important to implement the procedure in the future.

Mesenchymal Stem Cells (MSC) are the key component of the hematopoietic niche since they are progenitors of most of the cell components of the BM microenvironment (osteoblasts, adipocytes, fibroblasts, etc). MSC regulate the niche both by secreting multiple soluble factors and by cell-to-cell contact.

In the present work we want to develop an in vitro 3D-model of human stroma using MSC and a biocompatible scaffold of Conduit^{TM}-TCP, able to maintain HSC proliferation and differentiation. Then, we will establish an in vivo model of human hematopoiesis by transplanting this humanized 3D niche subcutaneously into immunocompromised mice and further evaluate its ability to support hematopoiesis by administering CD34+ cells from UCB either IV or directly into the ectopic niche.

Specific objectives:

- To evaluate *in vitro* if human MSC and Conduit-TCP reproduce a functional stroma that is able to support the maintenance and proliferation of human HSC from UCB;

- To assess if the latter 3D-BM niche developed *in vitro* (MSC+Conduit-TCP+hHSC) when implanted subcutaneously in immunocompromised mice supports *in vivo* the homing and engraftment of human HSC;

- To evaluate if the 3D-BM niche (MSC+Conduit-TCP) induced *in vivo* supports the homing and engraftment of hHSC administered either intravenously and directly next to the subcutaneous implant.

IV - Materials and Methods

All the experiments of the present work were performed in the Cell Therapy Lab of the Hematology Department of the University Hospital and in the Animal Care Facility of the University of Salamanca.

1 – Human cell samples

Human cell samples were obtained from bone marrow (BM) aspirates of healthy donors collected at the University Hospital of Salamanca and fresh human umbilical cord blood (UCB) from the Barcelona Blood and Tissue Bank, strictly following their standard ethical criteria.

All samples from volunteer donors were obtained/proceeded after written informed consent had been obtained, in accordance with and approved by the Ethics Committee of the University Hospital of Salamanca.

Table 2. Characteristics of human samples used for the *in vitro* and *in vivo* studies

	BM Samples (n=3)	**UCB Samples (n=6)**
Age*	37,5 (29-46)	NI
Sex(M/W)	3/1	NI

M: Male; **W**: Woman *results are expressed by the median (range); NI – No identify

2 – Animals

To perform *in vivo* xenotransplantation assays a total of 18 six to eight weeks old female NOD.CB17*Prkdc*^{scid}/NcrCrl* (NOD/SCID) mice were purchased from Charles River Laboratories (Barcelona, Spain).

Animals were housed in microisolator cages with a maximum of 6 mice per cage in the animal facility of the University of Salamanca, maintained under constant conditions of temperature and humidity, 12 hours dark / light cycles, in sterility and asepsis ideals, and fed *ad libitum* with autoclaved food and water. They were maintained for at least one week before any experimental procedure. Those were performed following the Spanish and European Union guidelines (RD 1201/05 and 86/609/CEE, respectively) and after the approval of the local Bioethics Committee of the University of Salamanca

3 - MSC isolation, expansion and characterization

3.1 Bone marrow aspirates

Bone marrow (BM) aspirates were obtained from the posterior iliac crest aspiration under local anaesthesia from 6 healthy adult donors according to standard institutional procedures (University Hospital of Salamanca, Spain). BM were collected in sterile tubes (Venoject; Terumo®) with free heparin (without additives – 5000U/ml; Seromed Biochrom KG).

3.2 BM – MSC isolation and expansion

After confirming the absence of BM clots, samples were diluted 1:3 with Hanks Solution (Hank's Balanced Salt Solution, Gibco®, Invitrogen™). The low-density BM mononuclear cells (MNC) were separated by density gradient centrifugation at 500g for 30 minutes on solution of high density with low viscosity and low osmotic pressure[72] – Ficoll (Ficoll-Paque, density:1.077k, GE Healthcare BioSciences, Buckinhampshire, UK). Then, cells were washed twice with Hanks and counted. BM-MNC were seeded at a density of $1x10^6$ cells/cm^2 on plastic culture flasks (Corning Incorporated, Corning, NY, USA) with ***expansion medium*** consisting on DMEM (Dulbecco's modified Eagle's medium–low glucose - Gibco, Invitrogen, Paisely, UK) supplemented with 5% platelet lysate[36], heparin (200 IU/ml) and 1% penicillin/streptomycin (10^5U/ml/10^5µg/ml - Gibco, Invitrogen, Paisely, UK) and cultured at 37ºC and 5% CO2 in a humidified atmosphere. Platelet lysates (PL) were obtained from the blood Bank of the University Hospital of Salamanca (units that were discarded immediately after their expiration date) were pooled in a single culture supplement, frozen at -80ºC and subsequently thawed at 37ºC to obtain containing platelet-released growth factors from pooled platelet. Culture medium was replaced every 3-4 days and non-adherent cells were removed. By this procedure, MSCs were isolated due to their characteristic adherence to plastic surfaces. BM-MSCs cultures were maintained until they reached 70-80% of confluence. Afterwards, cells were washed with phosphate buffer saline (PBS)(GIBCO®, Invitrogen™ Corporation, Paisely, UK) and harvested with 0,05% Trypsin – EDTA (Gibco, Invitrogen Paisely, UK) for 5 minutes at 37ºC. The mixture was centrifuged at 300g for 10 minutes and supernatant was discarded. Cells were resuspended in 1 mL of expansion medium, counted and replanted into new flasks at $5x10^3$ cells/cm^2.

For MSC characterization, third passage cells were analyzed by flow cytometry following the minimal criteria set by International Society of Cellular Therapy[39], which also included

the demonstration of the multilineage (osteogenic, adipogenic and chondrogenic) differentiation potential.

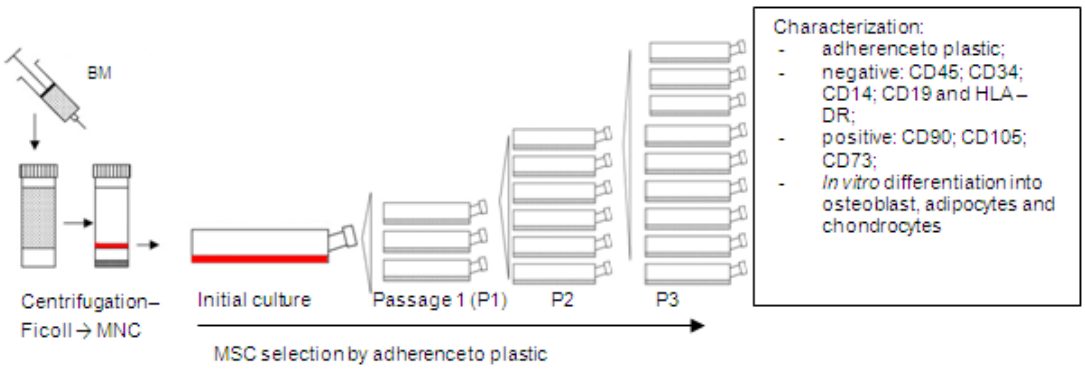

Figure 4. Scheme of BM – MSC isolation, expansion and characterization

3.3 Immunophenotyic characterization of MSC

At passage three, MSC were harvested as previously reported and resuspended in 100µL of PBS for their characterization by flow cytometry. Approximately $5x10^5$ cells per polystyrene tube (Falcon, Becton Dickinson Biosciences, San Jose, CA, USA) were incubated for 15 minutes with combinations of four-color conjugated-monoclonal antibodies (Table 3) and then washed and centrifuged 5 minutes at 700g with PBS for eliminate the excess of antibodies.

Monoclonal antibodies were conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), phycoerythrin-cyanine 5 (PerCP Cy5) and- allophycocyanine (APC) and combined as follows:

Table 3. Fluorochrome-conjugated antibodies for immunophenotype characterization of MSC of the samples

Tube	Antibodies	Quantity
Tube 1	Control	-
Tube 2	CD90/CD166/anti-HLA-DR/-	2µL/10µL/10uL
Tube 3	CD34/CD73/CD45/CD105	10µL/10µL/10uL/3µL
Tube 4	CD44/CD14/CD19/-	10µL/10µL/10uL

The control tube was done to correct the high auto fluorescence of the MSC (absence of antibody)

	Fluorochrome	Origin
CD90	FITC	all from Becton Dickinson Biosciences, San Jose, CA, USA
CD44	FITC	
CD34	FITC	
CD14	PE	
CD19	PerCP Cy5.5	
CD45	PerCP Cy5.5	
CD73	PE	
Anti-HLA-DR	PerCP Cy5.5	
CD166	PE	
CD105	APC	R&D Systems, Minneapolis, MN, USA

Table 4: Fluorochrome-conjugated antibodies for immunophenotype characterization of hMSC

The measurements were performed on a FACSCalibur equipped with four-color option (Becton Dickinson Biosciences, San Jose, CA, USA) and CellsQuest software (Becton Dickinson) was used for acquisition of a minimum of 50,000 events for each condition. Calibration procedures were perfomed before the acquisition of the records using previously established protocols provided by the Flow Cytometry Unit (University Hospital of Salamanca). Data were analyzed using the Infinicyt program (Cytognos, Salamanca, Spain).

3.4 Evaluation of Differentiation Capacity

3.4.1 Osteogenic differentiation – At fourth passage MSCs were plated at a concentration of 5,000 cells/cm^2 in a 9.6 cm^2 slideflask (Nunc, Denmark) with expansion medium to achieve 80% of confluence. Then cells were cultured in osteogenic medium (NH Osteodiff Medium; Miltenyi Biotec, Bergisch Gladbach, Germany) to induce there *in vitro* differentiation. Medium was replaced every 3-4 days during 10 days. At this time point osteogenesis was assessed by alkaline phosphatase activity detection. Detection of alkaline phosphatase expression was performed by staining with NBT/BCIP solution (Nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate - Roche, Basel, Switzerland) for 20-30 minutes and contrasted with hematoxylin (Merck KG1A, Darmstadt, Germany).

3.4.2 Adipogenic differentiation – Similarly to osteogenic differentiation, cells were grown in a slideflask until reached 80% of confluence, and subsequently incubated with the specific differentiation medium (NH Adipodiff Medium; Miltenyi Biotec) for 21 days replacing the culture medium twice a week. For adipocyte detection cells were stained with Oil-Red-O

solution (Certistain Merck KG1A, Darmstadt, Germany). Adipogenesis was confirmed by the observation of the accumulation of neutral lipids in fat vacuoles.

3.4.3 Chondrocyte differentiation – $5x10^5$ cells were placed in a 15 mL polypropylene tube (Corning Incorporated, Corning, NY, USA) and centrifuged. Pellet was cultured in 500µL chondrogenic medium (NH Chondrodiff Medium; Miltenyi Biotec) for 21 days changing the medium twice a week. Over this time, pellets were embedded in paraffin, cut into 5-µm sections and chondrocyte differentiation was demonstrated by methylene blue staining.
During the evaluation of the differentiation potential to osteoblast, chondrocyte and adypocytes also control cultures were used. In controls the MSCs were maintained in the same conditions as previously described but using only expansion medium. All cultures were incubated at 37ºC in a 5% CO_2 humidified atmosphere.
Slides were observed with an Olympus BX41TF microscope (Olympus Optical Co.,Tokyo, Japan).

4 – Transfection of MSC with lentiviral vectors (LV)

4.1 Plasmid DNA (pDNA):

- pLV-CMV-hGluc-Ires-CFP (6094bp) LV vector containing the Gaussia luciferase (Gluc) and the Cyan Fluorescente Protein (CFP), under the human cytomegalovírus (CMV) immediate-early promoter.
- psPax2 (10703bp) packaging vector containing viral polimerase and packaging genes.
- pLV-VSVG (5821bp) envelope vector encoding the G protein of the Vesicular Stomatitis Virus (VSV-G).

All vectors contain resistance to the antibiotic ampicillin that was used for bacterial selection, as indicated next.

4.2 Production and isolation of pDNA

The pDNA were produced using *Escherichia coli* DH5α following a transformation protocol, that consist in create a *heat shock*[73], incubating the bacteria and the DNA together on ice, placing them briefly at 42ºC and then back on ice.
Then bacteria were planted on Luria Bertini (LB) agar plates containing ampicillin (100mg/ml) and maintained overnight at 37ºC.

Only transformed bacteria that contain the plasmid could express beta-lactamase and can survive on the ampicillin plates, than was allowed their growth and consequently high –copy plasmid DNA. Plasmid purification was performed according to the QIAGEN® Plasmid Purification kit protocol (QUIAGEN). The concentration of purified pDNA solutions was assayed by spectrophotometry at 260 nm using a NanoDrop equipment and DNA integrity was confirmed by DNA agarose gels stained with ethidium bromide.

4.3 Production of lentiviral vectors (LVs)

Self-inactivating LVs containing the enhanced cyan fluorescent protein *(eCFP)* gene were generated by co-transfection the 293T cells using calcium phosphate transfection protocol.

$2x10^6$ 293T cells were seeded in 15 cm diameter cultures dishes (Falcon, Becton Diskinson Bioscience, San Jose, CA, USA), maintained with DMEM medium containing 10% fetal calf serum (FCS) (BioWhittaker, Lonza, Verviers, Belgium) and penicillin/streptomycin (pen/strep) in 5% CO_2 at 37° C incubator until they reached 40-50% of confluence (approximately 24 hours).

For 293T transfection, a total of 35µg of pDNA was added to each culture dish. The transfection medium included pLV-VSVG, psPax2 and pLV-CMV-hGluc-Ires-CFP vectors in calcium phosphate (for the DNA precipitation) and, buffer 2x HeBS* diluted in DMEM with penicillin/streptomycin.

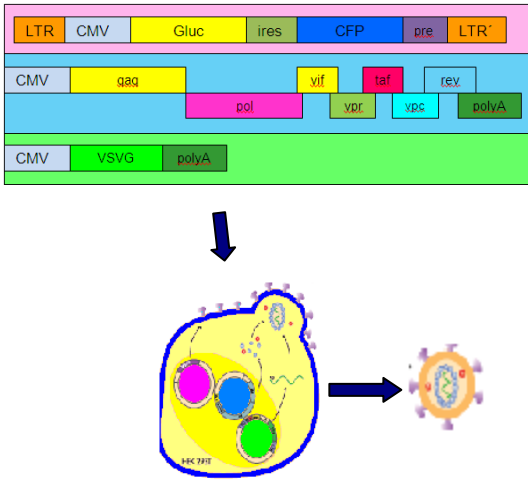

Figure 5. Second generation packaging construct of Lentivirus in 293T cells

*2xHeBS: 200mM NaCl, 10mM KCL, 1,5m $MNa_2HPO_4.2H_2O$, 12 mM dextrose and 50mM HEPES in milliQ water.

Medium containing lentiviral supernatant was collected after 24 and 48 hours (in a 5% CO_2 incubator at 37ºC) pooled, filtered through a 0.45 mm pore filter, and frozen at -80ºC until used.

Virus titration was calculated by subsequently virus dilutions (Figure X) on a 6-well plate with 293T cells.

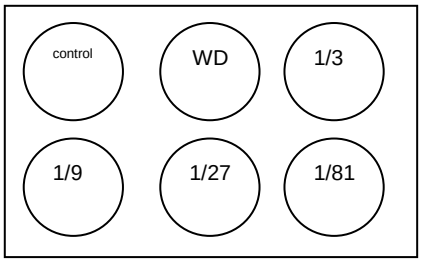

Figure 6. Different concentration of virus in the culture medium used for virus titration.

WD – without dilution

After 3 days of culture (5% CO_2 at 37ºC), the efficiency of transduction was determined by flow cytometry analysis of CFP expression on 293T cells as previously described. Data were analyzed using the Infinicyt® software calculating the percentage 293T cells in the first fluorescence channel. Titer was calculated using the following formula:

$$\frac{\text{Cell concentration at the day of transduction x virus supernatant dilution x \% CFP-positive cells}}{\text{Total volume.}}$$

4.4 Transduction of MSC

Passage 2 MSC were grown in a culture flask until subconfluence (around 50%) was observed. Then expansion medium was replaced by LV supernatants diluted 1:8 with DMEM with 10% FCS and peni/strep, resulting in a multiplicity of infection (MOI) of 1. To increase the efficiency of transduction 8µg/mL polybrene (hexadimethrine bromide, Sigma-Aldrich, Steinheim, Germany) were added.

After 24 hours, transduction medium was again replaced by fresh expansion medium (DMEM 10% FBS and peni/strep). Transduced hMSCs were maintained in standard conditions until they reached 90% of confluence. Cells were harvested and CFP^+ hMSCs were analyzed by flow cytometry to determine transduction efficiency.

5 - Isolation and characterization of hematopoietic Stem Cells (HSC - $CD34^+$) from UCB

A total of 10 fresh human UCB units were obtained from full-term normal delivers after parental donor consent, supplied by Barcelona Blood and Tissue Bank when were discarded for cryopreservation due to low number of cells. Heparinized cord blood was diluted (1:2) in isotonic 0,9% sodium chloride (sterile saline solution) and UCB mononuclear were separated by Ficoll density gradient and washed twice with saline solution. Then MNC were counted and $CD34^+$ progenitor cells were sorted by magnetic labeling using the human CD34 MicroBead Kit (Miltenyi Biotec) in accordance with the manufacturer's protocol. The purity of $CD34^+$ cells, after the isolation, was evaluated by flow cytometry using FITC-conjuG1ted CD34 (Becton Dickinson Biosciences, San Jose, CA, USA).

6 – Scaffold characteristics

Conduit® TCP Granules (DePuy Johnson & Johnson, Warsaw, USA) are commonly used as a substitute for bone defects repairment because is similar to the mineral phase that comprises 70% of adult human bone. It consists of a synthetic porous ceramic composed of tricalcium phosphate beta (β-TCP, $Ca_3(PO_4)_2$), forming 1.5-3mm diameter granules with interconnected pores that occupy 70% of volume and average pore diameter between 1 and 600µm. This sacaffold is resorbed through cell mediated events that are involved in the normal remodeling cycle of bone[74].

7 - *In vitro* cell culture experiments

Three groups in vitro were constituted to evaluate in vitro if the 3D-BM niche constructs (MSC+Conduit-TCP+HSC) were able to support HSC. On ***in vitro* group 1 (G1)**, approximately $2x10^5$/mL MSC-CFP^+ on 3rd passage were seeded onto a six well culture plate with 3 mL of expansion medium. On ***in vitro* group 2 (G2)** the same proceeded was done, but with the scaffold (16 Conduit-TCP granules per well). Were maintained in cultured on a incubator (37ºC with a humidified 5% CO_2 atmosphere) changing culture medium twice a week, until 80% of confluence was achieved. Then half of the granules (containing MSC attached inside) were transferred into new culture plates to have MSC growing in a real 3-D structure, while the other half remained on the original wells, constituted the ***in vitro* group 3 (G3)** (Figure 6)

7.1 Long-term bone marrow culture (LTBMC)

When MSC-CFP$^+$ achieved the confluence, expansion medium was changed by LTBMC* medium for 2 weeks to induce stromal differentiation, changing the medium twice a week. This medium can support both stromal cell-containing feeder layer (MSC), as well as a number of hematopoietic cells generated.

With a stroma formed, 2x10^5/mL CD34$^+$ cells from UCB were added for each well in the three groups and maintained in culture with LTBMC for 5 weeks. Formation of adipocytes, cobblestone areas and the stroma layer confluence was analyzed weekly[75].

Also half of the supernatant was weekly removed and replaced by fresh LTBMC medium. The harvested non–adherent cells were counted and 5x10^3 cells were planted in a 24-well culture plate (Costar, Corning NY, USA) with 0,5 mL of methylcellulose medium – Methocult H4534 (Stem Cell Technologies, Grenoble, France) to quantify the progenitor cell Colony Forming Unit–Granulocyte/Macrophage (CFU-GM). These cultures were incubated in a humidified atmosphere at 37ºC with 5% CO_2. After 14 days, CFU-GM colonies were scored with an inverted microscope following standard criteria for colony counting. Results were expressed as the number of CFU-GM per 10^5 cells seeded and the total number of CFU-GM present in the culture.

8 – *In vivo* experiments

To assess if the *in vitro* 3D-BM niche constructs were able to support *in vivo* homing and engraftment of HSC a xenotransplant murine model using female NOD/SCID mice was used. Animals were exposed to 300-cGy total body irradiation from a Cesium 137 source (Gammacell-200, Nordion International, Ottawa, ON, Canada) six to eight hours before the transplant. All the experimental procedures in the xenotransplantation setting were performed under anesthesia with a mixture of 90mg/kg dose of ketamine (Imalgène 500, Merial, Lyon, France) and 10 mg/kg of xylazine (Rompun 2%, KVP Pharma Healthcare, Kiel, Alemania) administered intraperitoneally.

*LTBMC: IMDM (Iscove´s Modified Dulbecco´s Medium, Gibco®, Invitrogen™ Corporation, Paisely, UK) to an osmolarity of 350mOsm/l supplemented with horse serum (PAA Laboratories, Pasching, Austria), 1% Hydrocortisone and 1% antibiotic. When mixed was filtered, aliquoted and frozen at -20ºC until use.

8.1 Study design

A total of 18 animals divided into 3 groups (n=6/group) were used to assess the level of human engraftment using MSC with Conduit® TCP Granules as bone marrow niche and $CD34^+$ cells from UCB as hematopoietic progenitor cells.
After anesthetized, each mouse was shaved and cleaned with 70% ethanol, three small (6 mm) incisions were made on the dorsum and a subcutaneous pocket was made between the dermis and the underlying tissue where were implanted the scaffolds, the incisions were sutured with nylon.

***In vivo* Group 1** → $2x10^6$MSC/ml were maintained in contact with the scaffold (2-3 ConduitTM-TCP granules placed in a 6-well plate) for 2 hours in the incubator at 37ºC, to facilitate the adhesion of the cells. Then, the mixture was subcutaneously implanted in the mice.
MSC on the scaffolds were *in vivo* induce to stroma during 7 weeks. Past this time the mice were irradiated with a sublethal dose (3Gy) as previously described, and injected either intravenously (IV) in the lateral tail vein or subcutaneously (SC) into the scaffold, $2x10^5$ $CD34^+$ cells/mouse resuspended in a total volume of 200µl of PBS.
Three and six weeks after transplantation samples from peripheral blood (PB) and bone marrow (BM) were obtained for human hematopoietic engraftment by flow cytometry.

***In vivo* Group 2 and 3** → NOD/SCID mice with 7 weeks were irradiated with a sublethal dose (3Gy) as previously described, and 6 hours after the irradiation were subcutaneous implanted the scaffolds - 3D-BM niche constructs providing from group 2 and 3 of the ***in vitro* cell culture experiments**. The only difference between both groups was that in **Group 2** the scaffolds was xenotransplanted after 4 weeks *in vitro* culture, and in Group 3 it was transplanted after 5 weeks.

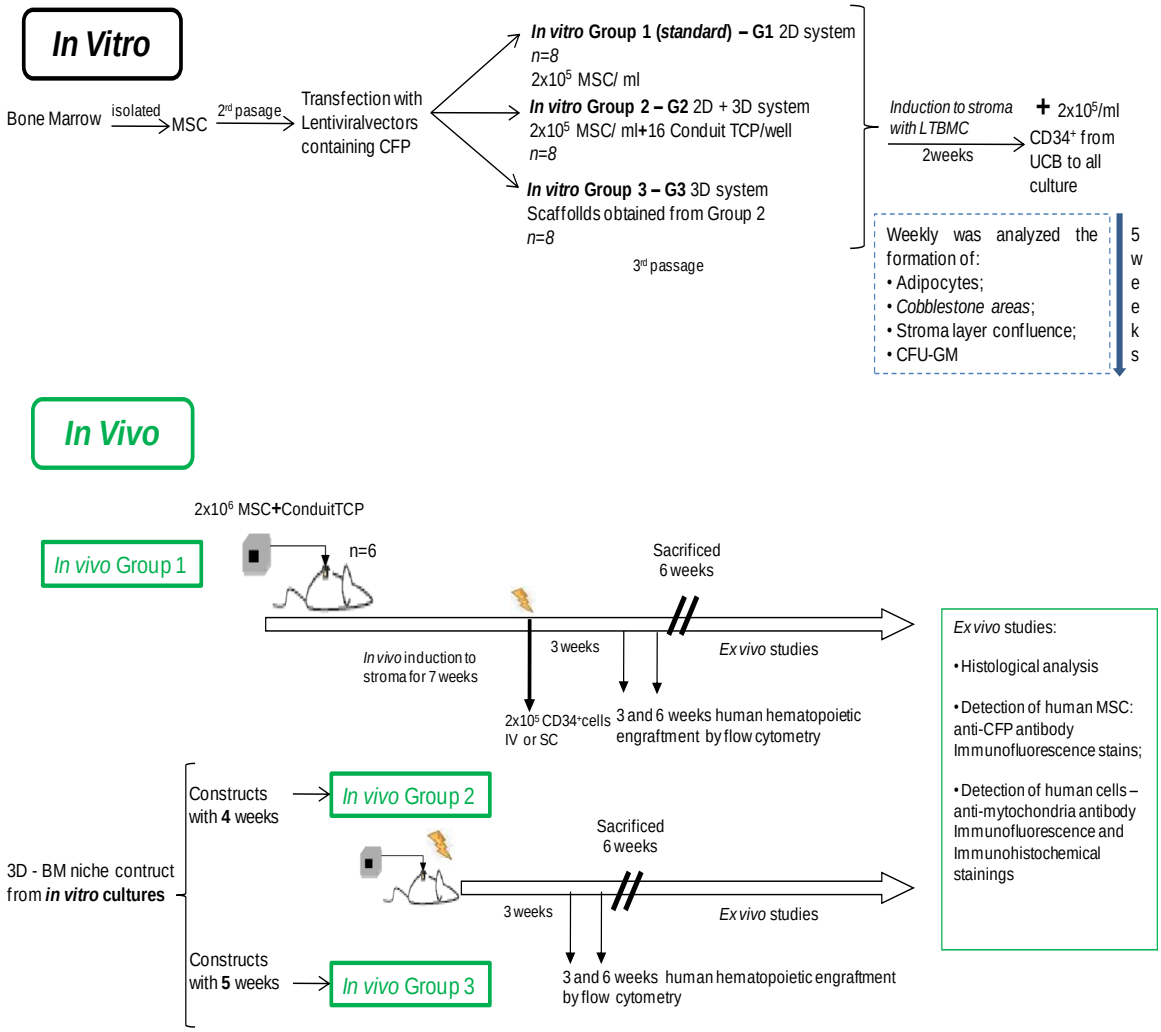

Figure 7. Scheme of *in vitro* and *in vivo* experimental design

8.2 Analysis of human hematopoietic engraftment by flow cytometry

Peripheral blood (PB) was aspirated from retroorbital sinus and bone marrow (BM) from the right femur were obtained 3 and 6 weeks after transplant the $CD34^+$ cells, to analyze the presence of human cells by flow cytometry. All samples obtained were prepared as single cell suspensions in PBS, subjected to lysis of erythrocytes with ammonium chloride, followed by a 5 minutes wash at 700g and then resuspended aG1in in PBS.

Human hematopoietic cells were distinguished from mouse cells by the expression of human CD45 antigen. For this purpose, samples were stained with FITC-conjugated anti–human CD45 (Miltenyi Biotec, Bergisch Gladbach, Germany). For detection of human myeloid and B-lymphoid cells samples were stained with PE-conjugated CD13 and APC-conjugated CD19 (all from Becton Dickinson Biosciences, San Jose, CA, USA), respectively. Both subpopulations were analyzed only within the human $CD45^+$ cell population. In all cases dead cells were excluded from the analysis, 10 min before acquisition, by ¡7-Amino-actinomycin D (7-AAD) (Becton Dickinson Biosciences, San Jose, CA, USA) staining.

8.3 Histological and Immunofluorescence analysis

Animals were sacrificed 6 weeks after the transplant by cervical dislocation after anesthetized with ketamina/xylazin. The femurs and 3D – BM niche implanted subcutaneously were dissected and fixed in 4% of paraformaldehyde at room temperature overnight. Samples were descalcified 24 hours for the femurs and 2 hours for the scaffolds, and then were embedded in paraffin. For the analysis, 5 µm thick sections were obtained in a Leica CM 2000 cryostat (Leica Microsystems, Nussloch, Germany) and placed onto glass slides.

For histological assays was performed hematoxylin and eosin staining to evaluate morphological changes.

8.3.1 Detection of human cells in NOD/SCID mice

Paraffin sections were de-paraffinized with xylene and then rehydrated in ethanol and water. For antigen retrieval, sections were subjected to 100ºC for 20 minutes in 10mM sodium citrate buffer (pH6), and then the nonspecific biding were blocked by 5% of normal donkey serum, bovine serum albumin and 0,2% of triton X-100 diluted in PBS for 60 minutes in constant agitation at room temperature.

The slides were incubated with a primary mouse monoclonal anti – GFP antibody (AbCam, Cambridge, UK) allowing the amplification of endogenous CFP signals of MSC, and also incubated with primary rabbit polyclonal anti- human mitochondria (AbCam, Cambridge, UK) to detect if expressing cells were from human origin. Primary antibodies were diluted in PBS following manufacturer's recommendations, and slides were incubated overnight at 4ºC.

Alexa Fluor448-conjugated anti- rabbit IgG and Alexa555 anti-mouse IgG were used as secondary antibodies (both from Invitrogen, Paisley, UK). After the final washes, 1 µg/ml 4', 6-Diamidine-2' -phenyl indole, dihydrochloride (DAPI) in PBS was added and the cells were incubated at room temperature for 5 min. Slides were viewed with a Leica DMI6000B fluorescence microscope (Leica Microsystems GmbH, Germany) equipped with camera system.

Protocol for immunohistochemical assay it is similar to the Immunofluorescence, but because the endogenous peroxidase activity is found in many tissues and can be detected by reaction with DAB substrate. The solution for eliminating endogenous peroxidase activity is by the pretreatment of the tissue section with hydrogen peroxidase prior to incubation of primary antibody.

It is used ABC - Avidin-Biotin Complex method (standard) for immunohistochemical staining. The peroxidase is then developed by the DAB because avidin is labeled with peroxidase, that produce different colorimetric end product.

9. Statistical analysis

For all statistical analysis, the SPSS 15.0 software (Chicago, IL, USA) was used. Median values and ranges were calculated for each variable. To explore the significance in the differences found between the different groups, the nonparametric Mann-Whitney U-test was employed. Differences were considered to be statistically significant when $p < 0.05$.

V – Results

1. BM-MSC isolation and expansion

Since MSC are recognized as adherent cells that are capable of extensive proliferation with a fibroblastic morphology and have the ability to differentiate into mesenchymal lineages, it was possible isolate this population according to their characteristics, figure 8. They were maintained until third passage and further characterized according to ISCT definition criteria.

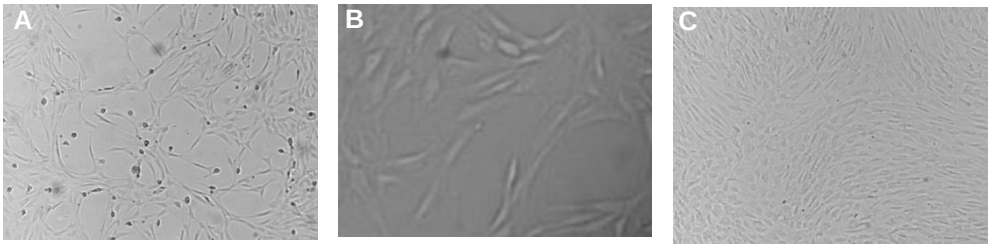

Figure 8. Sequential images of expanded hMSC culture. A – Initial culture morphology (40X); B – a single – shaped fibroblastic morphology (100X); C – evolution of the adherent cells until achieving confluence (40X).

2. Analysis of immunophenotype and differentiation capacity

Cell surface antigen expression of MSC in third passage was analyzed by flow cytometry. This cells did not express hematopoietic lineages markers such as CD14, CD19, CD34, CD45 and neither HLA-DR. They were positive for CD73, CD90, CD105, CD166 and CD44 thereby demonstrating an characteristic MSC immunophenotypic profile, Figure 9.

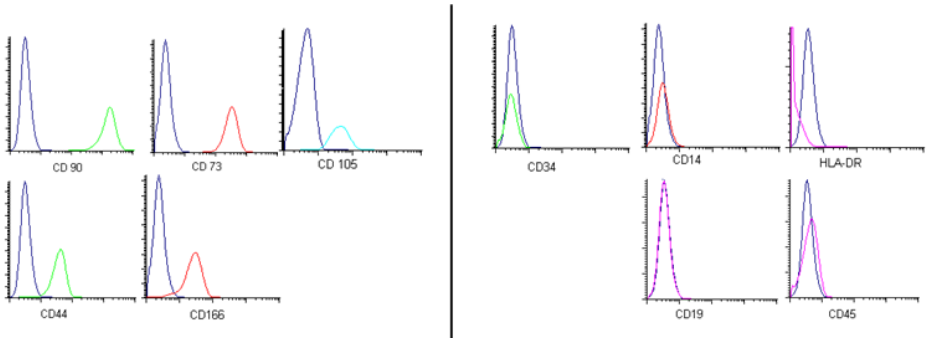

Figure 9. Flow cytometry characterization of hMSC – representative diagrams were obtained from confluence cultures of hMSC at passage 3, in the left represents the positive markers and in the right represents the negative markers. Blue stain represents unlabeled control cells (absence of antibodies).

The tri-lineage differentiation ability of MSC cultures was tested at passage 3 upon culture in osteogenic, adipogenic, and chondrogenic medium. In vitro differentiation was demonstrated in all cases, after specific assessment of alkaline phosphatase activity, oil-red-O, and type II collagen staining, respectively, figure 10.

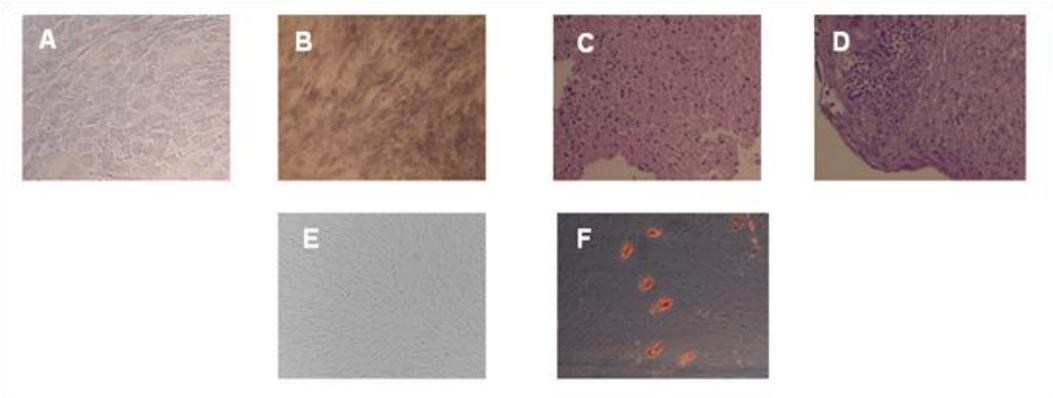

Figure 10. Differentiation potential after exposure MSC to differentiation medium. Osteogenic differentiation was confirmed by (A,B) cytochemical staining for alkaline phosphatase. (C,D) immunocytochemical staining for collagen type I demonstrating chondrocyte differentiation. (E,F) Lipid-filled adipocytes detection by oil red O staining. Control MSC maintained in expansion medium (A,C,E) and in differentiation medium (B,D,F). Data are representative of three independent experiments.

3. Efficiency transfection of MSC with lentiviral vectors containing CFP protein

The MSC used in this study had high efficiency presented in all cases expression of CFP higher than 90% when analyzed by flow cytometry, figure 11.

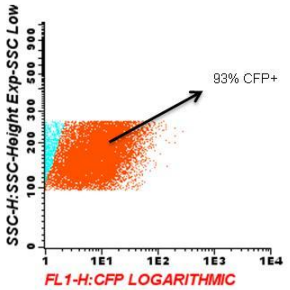

Figure 11: Representative case of MSC - CFP^{+} cells observed by flow cytometry

4. HSC characteristic

The average volume of the UCB used was 337ml (range 289-675 ml, including anticoagulant). The median recovery of MNCs was 137,5x10^6cells/unit (range 122-168x10^6cells). In the figure 12 illustrate the percentage of purified $CD34^+$ cells that were used for *in vitro* and *in vivo* assays, isolated through immunomagnetic beads was higher than 90% when analyzed by flow cytometry.

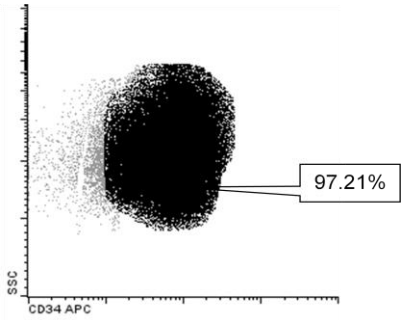

Figure 12. Percentage of $CD34^+$ cells in a representative case. Cells were isolated by immunomagnetic beads and purity analyzed by flow cytometry.

5. *In vitro* cell cultures experiments

To mimic bone marrow stroma 2x10^5 MSC/ml were seeded on Conduit TCP. This concentration was selected based on optimal cell attachment frequency (data not provided), growth rate and growth cavity[43]. After 7 days of stromal induction with LTBMC, 2x10^5 $CD34^+$ cells were seeded onto stromal cell-Conduit TCP constructs. Identical cultures were also performed on 2D plates (without the scaffolds of Conduit-TCP) to establish if 3D biomimetic niche can support the maintenance and proliferation of HSC, Figure 13.

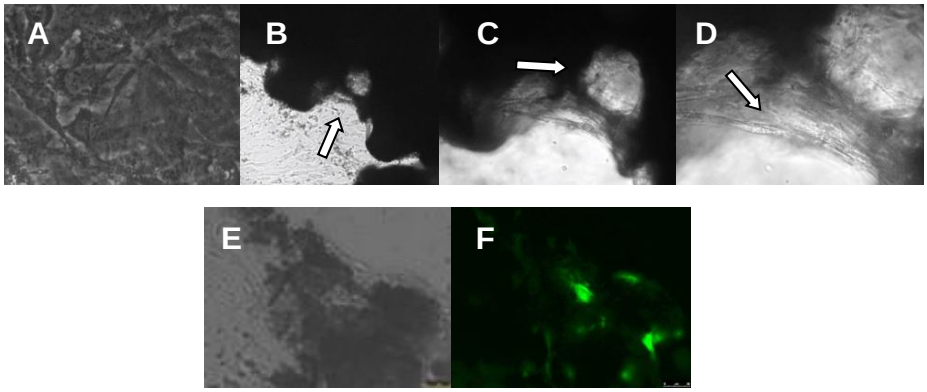

Figure 13. MSC culture in the biomimetic 3D-BM niche. After 7 days of induction, morphological analyses on the stroma of different experiments were performed using an inverted microscopy. A - Stromal layer confluence on 2D plates – control (100X), sequential images (B,C and D – 10X, 20X and 40X) were the cells grew along and inside the scaffold cavity.
Scaffold in brightfield (E) and in fluorescence (F) MSC-CFP^{+} inside the scaffolds, 40 X

5.1 Long-term bone marrow culture (LTBMC)

To confirm the maintenance of hematopoiesis *in vitro* under different experimental conditions, layer confluence, presence of cobblestone areas, adipocytes and clonogenic cell assay from LTBMC were evaluated weekly during the 5 weeks.

5.1.1 Stromal layer confluence

During the culture time the stromal layer confluence in the control group (G1) always reached a high percentage of confluence (95-100%). On the other groups the stromal layer behavior was different. In group 2 (G2) – *2D+3D system,* the confluence was lower when compared with the control group with a decrease of 20% from the fourth week. MSC attached into the scaffolds were observed during the five weeks of culture, figure 14.
The third in vitro group (group G3 – *3D system*) was not scored for stromal layer confluence or the presence of *cobblestone areas* and adipocytes because the objective of this group was to just had the stroma inside the scaffold (3D niche), and therefore those standard LTC parameters cannot be quantified in this setting with an inverted microscope.

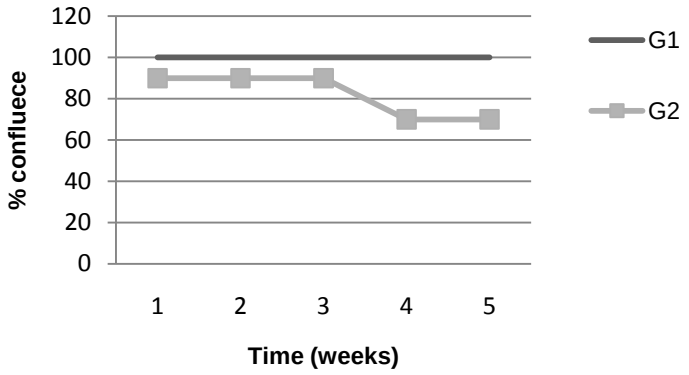

Figure 14. Adherent stromal layer confluence evaluation during the five weeks. Data represents the median. G1=control (MSCs without the scaffold); G2=2D/3D system (adherent stromal layer/Conduit TCD).

5.1.2 Presence of *cobblestone areas* and adipocytes

Adipocytes appeared from the first week in both groups (G1 and G2) and remained until the end of the *in vitro* experiments, figure 15. In G1 (control) the percentage of adipocytes was always superior when compared with G2 (with scaffold), with an increase in week 2 and maintained until week 5. Nevertheless, in G2 the number of adipocytes decreased from the third week, which was related to the decrease of the stromal layer. To score adipocytes an inverted phase – contrast microscopy (20X) was used and assigned with 0 – absence of adipocytes, 1 – one adipocyte in 10 fields, 2 – one adipocyte in 2 fields and 3 – two adipocytes in 1 field (number of adipocytes per field).

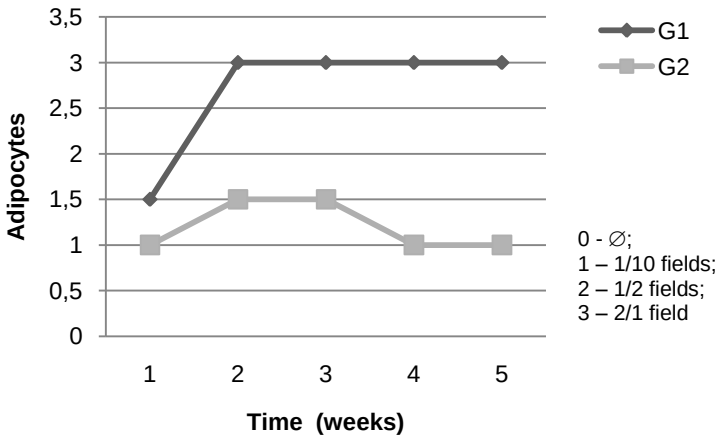

Figure 15. Score of adipocytes cells observed in LTBMC, during the five weeks, with respect to different culture conditions. Data represents the median. G1=control (MSCs without the scaffold); G2=2D/3D system (adherent stromal layer/Conduit TCP).

Cobblestone areas – forming (CAFC) assay was performed to assess the proliferative capacity of $CD34^+$ cells when cultured on different experimental conditions, figure 16.
At different time – points appeared small colonies of round cells that grew underneath the stromal layer.
The cobblestone areas were scored using the same criteria used to score adipocytes. In control group (G1) CAFC appeared in the second week. In some cases it was impossible to count because the hematopoietic cells covered the entire microscopic field. In G2, CAFS appeared one week latter and in a lower percentage when compared to G1. A decrease of CAFS was observed in the fifth week was which was consistent with the results previously described.

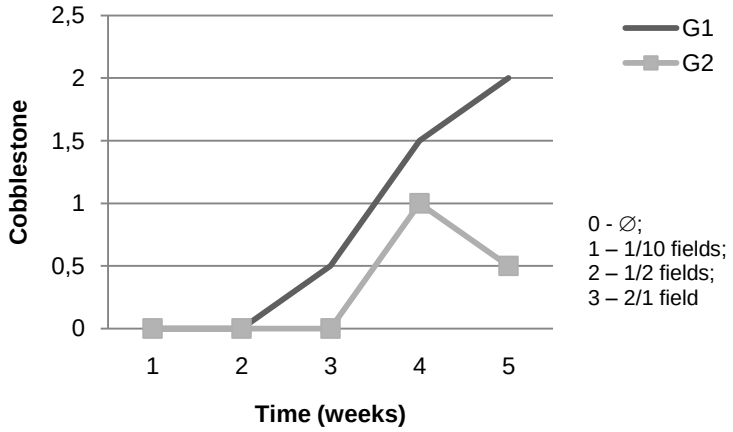

Figure 16. CAFC observed in a LTBMC during the five weeks, in the different culture conditions. Data represents the median. G1=control (MSCs without the scaffold); G2=2D/3D system (adherent stromal layer/Conduit TCP).

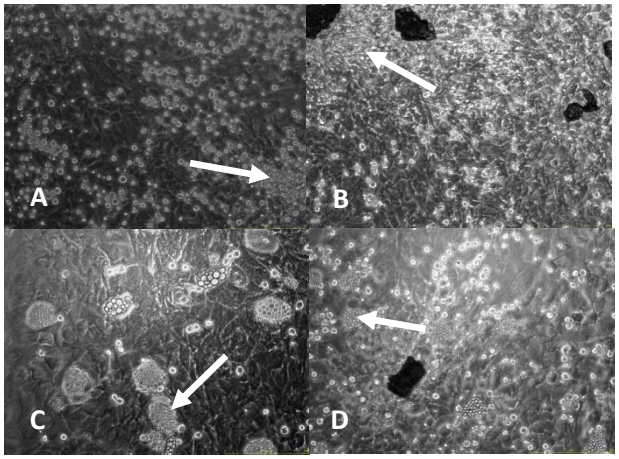

Figure 17. Long term bone marrow culture 5 weeks after seeding $CD34^+$ cells. Cobblestone area formed in 2D system and in 2D/3D system - adherent layer co-cultured with Conduit TCD (A, B) dark cells associated with a fat cells (C, D) together with phase-birth supernatant cells, 10X

5.1.3 Cell-culture in vitro assays

5.1.3.1 Number of cells produced from long-term bone marrow culture (LTBMC)

In order to ascertain how the different co-culture systems affect cell numbers produced from LTBMC, supernatants from the different experimental groups were weekly counted (Figure 18). As it is shown, total number of non - adherent cells from 2D/3D culture-systems increased during the five weeks of LTBMC, and in last week was significantly higher ($p<0,01$) compared with other groups.

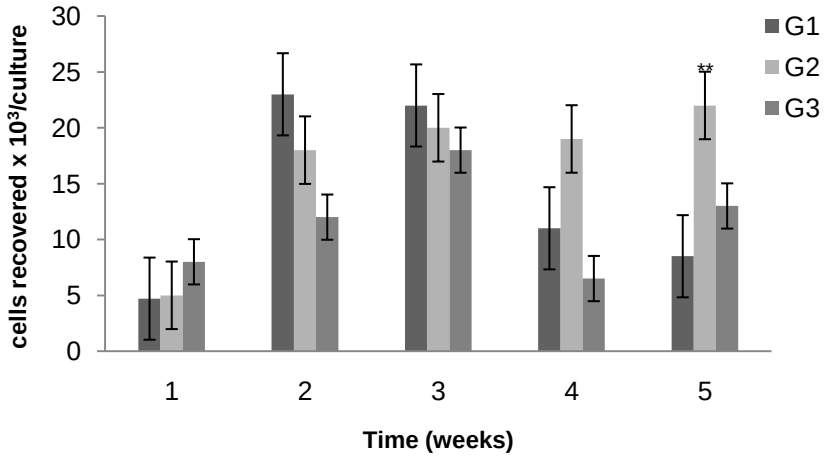

Figure 18. Long – term bone marrow culture data. Cells recovered during the 5 weeks of culture. Data represents the median (range). G1=control (MSC without the scaffold); G2=2D/3D system (adherent stromal layer/Conduit TCD); G3=3D system (just Conduit TCP).

5.1.3.2 Measurement of CFU-GM from LTBMC experiments

Colonies of CFU-GM in standard methylcellulose assays were performed from cells released to the supernatant during the five weeks of LTBMC in each experimental group. Results are shown in table 5. Data suggest that all groups have similar CFU-GM content during the five weeks. Number of colonies decreased in the second and increased in fourth week, and dropped in the fifth week.

Table 5. CFU-GM/10^3 weekly produced from supernatant cells of different assays.

	Week 1	Week 2	Week 3	Week 4	Week 5
G1 (control)	32,3 (15,5-78)	6,0 (0,2-27)	5,6 (0,4-40)	190 (100-310)	1,8 (0-27)
G2 (2D + 3D system)	29,8 (0,08-58)	13,2 (0,2-34)	16,3 (0,07-30)	165 (100-300)	3,7 (0-26)
G3 (3D system)	42,2 (18-72)	5,1 (0,2-14)	13 (0-39)	100 (100-310)	3,2 (0-20)

G1=control (MSC without the scaffold); G2=2D/3D system (adherent stromal layer at 2D conventional system with Conduit TCD to create a 3D niche); G3= 3D system (just Conduit TCP with the stroma inside the scaffold). Data represents the median (range).

6 *In vivo* experiments

To verify if human bone marrow (3D niche) from the *in vitro* cultures have homing capacity in vivo, xenotransplantation experiments were performed in sublethally irradiated NOD/SCID mice

6.1 Human hematopoietic cell engraftment in xenotransplanted NOD/SCID mice

Percentage of human CD45+ cells in PB was higher in group 2 (11.6%), that included animals that received a subcutaneous implant of 3D BM niche after 4 weeks in culture, compared to group 1- *in vivo* construction of 3D BM niche (8.9%) and Group C – animals with a subcutaneous implant of *in vitro*-developed3D BM niche after 5 weeks in culture (6.19%), table 6.

Table 6. Percentage of human hematopoietic cells (hCD45) in peripheral blood (PB) at 3 weeks after xenotransplanted.

	%hCD45 PB
G1 n=5	8,9 (7,07-10,58)
G2 n=5	11,76* (8,63-16,74)
G3 n=6	6,19† (5,01-10,54)

Data represents the median (range)

*Higher% of hCD45 cells ($p<0,05$) when compared with group 1 (G1) - *in vivo* 3D niche construct; †Lower % of hCD45 ($p<0,05$) when compared with group 2 (G2) – xenotransplant the *in vitro* 3D niche construct (MSC/$CD34^+$/scaffold) past 4 weeks in culture. Group 3 (G3) the same that G2 but with the construct in culture for 5 weeks.

At 6 weeks after the xenotransplant PB and BM were analyzed. Engraftment levels were slightly higher in PB from group 1. By contrast in BM engraftment was significantly higher in G2, table 7.

Additionally, in BM and PB human cells committed to both lymphoid (positive for CD19) and myeloid lineages (positive for CD13) were detected. Myeloid cells were the predominant population within the human population in both *in vitro* 3D niches constructs groups (2 and 3), with a higher percentage for group 2. In Group 1 (*in vivo*) B-cells were the predominant human cell population in both compartments (BM and PB) with significant differences when compared to group 2, table 8

Table 7. Percentage of human hematopoietic cells in BM and PB in NOD/SCID mice determined 6 weeks after transplantation.

	%hCD45 PB	BM
G1 n=5	7,35* (6-13)	5,45 (2-11)
G2 n=4	3,74 (2-6)	11,76* (9-17)
G3 n=6	6,33 (6-8)	5,63†† (1-7)

Data represents the median (range)

Peripheral Blood (PB):

*Higher percentage of human CD45 cells (p<0,05) when compared with G2

Blood Marrow (BM):

*Significant differences of human CD45 cells percentage (p<0,05) when compared with G1;

†Low percentage of human CD45 cells (p<0,01) when compared with G3

G1 (group 1) - *in vivo* 3D niche construct; G2 (group 2) – xenotransplant the *in vitro* 3D niche construct (MSC/CD34$^+$/scaffold) past 4 weeks in culture. G3 (Group 3) the same that GB but with the construct in culture for 5 weeks

Table 8. Percentage of human lineage-positive cells expressing hCD19 and hCD13 in BM and PB of NOD/SCID mice 6 weeks after transplantation.

	PB		BM	
	%hCD19	%hCD13	%hCD19	%hCD13
G1 n=5	4,39 (0-32)	1,28 (1-3)	16,98* (0-88)	2,23 (0-18)
G2 n=4	1,11 (0-11)	4,47* (2-6)	0 ---	9,52 (3-26)
G3 n=6	0,82 (0-3)	1 03 (0-4)	0 ---	7,35 (2-42)

Data represents the median (range)

Peripheral Blood (PB):

*Higher percentage of human CD13 cells (p<0,05) when compared with G3;

Blood Marrow (BM):

*Significant differences of percentage of human CD19 cells (p<0,05) when compared with G3;

G1 (group 1) - *in vivo* 3D niche construct; G2 (group 2) – xenotransplant the *in vitro* 3D niche construct (MSC/CD34$^+$/scaffold) after 4 weeks in culture. G3 (Group 3) the same that G2 but with the construct in culture for 5 weeks.

In order to know whether IV $CD34^+$ cells could migrate to the 3D-BM niche constructs and produce hematopoietic chimerism, on group 1, *in vivo 3D niche construct*, human hematopoietic cells ($CD34^+$) were transplanted intravenously (IV) in 4 mice and subcutaneously (SC) within in 2 mice. Despite no significant differences detected lower percentage of human $CD19^+$ cells were observed in cases with $CD34^+$ subcutaneously injected (table 9)

Table 9. Percentage of human hematopoietic cells and there lineage on group 1, BM and PB, in NOD/SCID mice determined 6 weeks after transplanted.

	Group 1					
	BM			PB		
	%hCD45	%hCD13	%hCD19	%hCD45	%hCD13	%hCD19
IV n=4	9,40	17,62	25,71	9,26	0,63	5,54
SC n=2	3,49	0,89	1,12	6,48	2,38	ND

Data represents the median (range)

IV – intravenously, SC – subcutaneously, BM – bone marrow and PB – Peripheral Blood.

7. *Ex vivo* studies

From the total of the 18 mice used, two were eliminated from the experimental study because premature death of animals with no possibility to perform histologic assessment in these two cases, corresponding to a yield of 89% induction.

Yield = number of animals at the end / number of animals at the beginning = 16/18

= 0.89

Median Animal weights were: Group A = 18,90 (17-22), Group B= 19,90 (19.23) and Group C= 20,35 (18-22) without significant differences between experimental groups.

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7.1 Macroscopic examination

Macroscopic and microscopic assessment of the subcutaneous implants and femurs was performed. Macroscopic observations o the skin showed in all groups high vascularization especially in Conduit TCP areas.

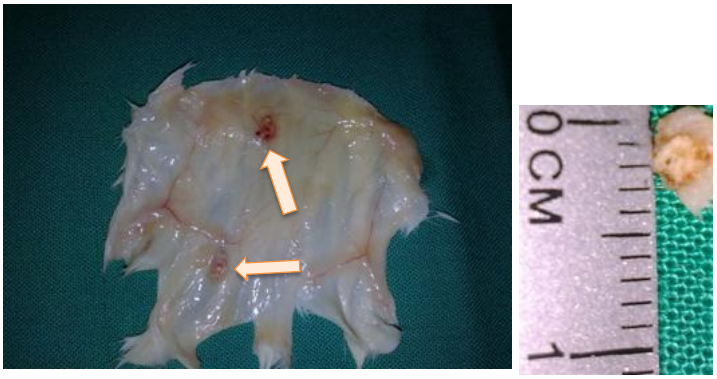

Figure 19. Representative macroscopic image of murine skin. Arrows mark the areas where were implants were located. Right image illustrate a scaffold of approximately 3 mm of diameter.

7.2 Histological analysis

In all groups the 3D-BM niche construct appeared in the histological analysis integrated within the surrounding tissue and without an evident foreign body reaction (no obvious inflammation).

Blood vessels were found inside implanted scaffolds especially in groups with scaffolds derived obtained after “in vitro culture” (Groups 2 and 3). Non-physiological spaces originated from the scaffolds were also found, which were surrounded by a layer of cells adhered to the biomaterial and hematopoietic cells.

In the implanted 3D-BM niche construct from in vivo group 1, the tissue formation showed morphology similar to murine tissue with a higher quantity of adipocytes (fat), figure 20. In all three groups had within the tissue scaffolds some cells with fusiform morphology (mesenchymal), predominantly in the in vivo groups 2 and 3, figure 21 and 23 respectively.

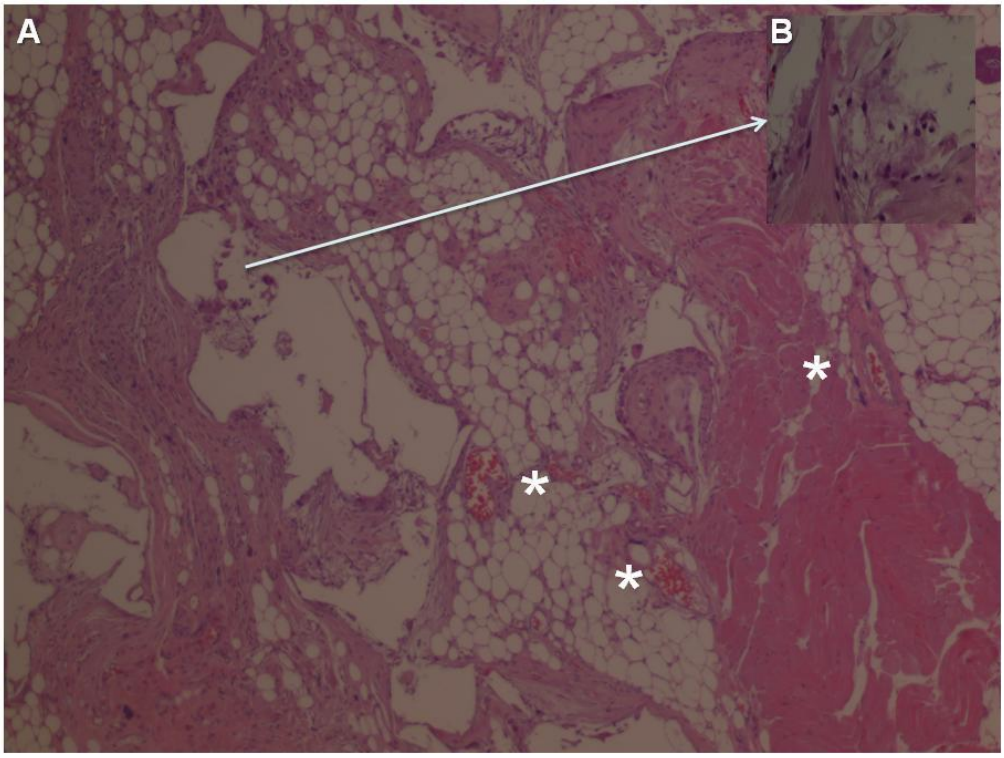

Figure 20. Analysis of *in vivo* 3D-BM niche constructs into the subcutaneous tissue of immunocompromised mice (group 1). The scaffolds with MSC were subcutaneous implanted on NOD/SCID mice and induced to form stroma for 7 weeks, *in vivo.* Then, intravenously and subcutaneously with $CD34^+$ cells from UCB were transplanted. Six weeks later mice were sacrificed and *ex vivo* studies were performed on the implants.. Scaffolds integrated well with surrounding tissues constituted mainly by fibroblastic cells figure B (100X). The morphology of tissue constructs were similar to murine tissue. A high number of adipocytes and small and large blood vessels were observed(*****), figure A (10X).

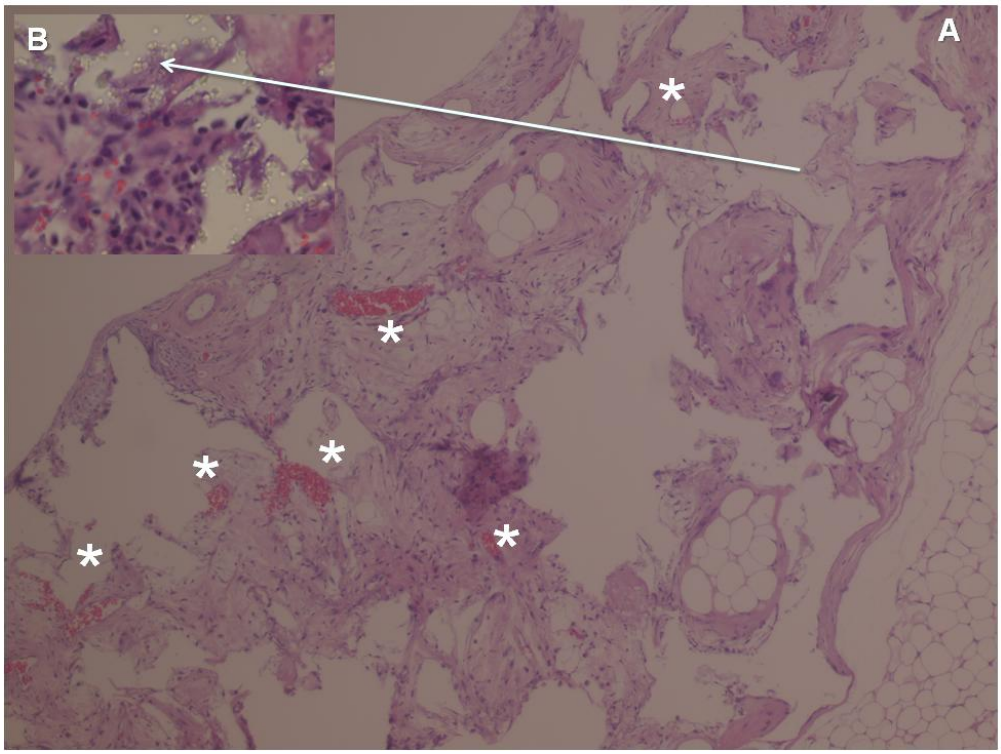

Figure 21. Histology representative of group 2 - *in vitro* 3D-BM niche constructs 6 weeks after being subcutaneously implanted in NOD/SCID mice. The 3D-BM niche obtained from cultures 4 weeks cultures were maintained for 6 weeks on the mice. The scaffolds were decalcified for 2 hours, then paraffin-embedded, and finally stained with hematoxylin/eosin. In figure A (10X) the scaffolds are sourrounded by polyclonal cells (maybe MSC) and also rounded cells suggestive of hematopoietic cells. figure B (100X). The morphology of tissue constructs is different from the murine tissue. Many small and large blood vessels can be detected (*****)

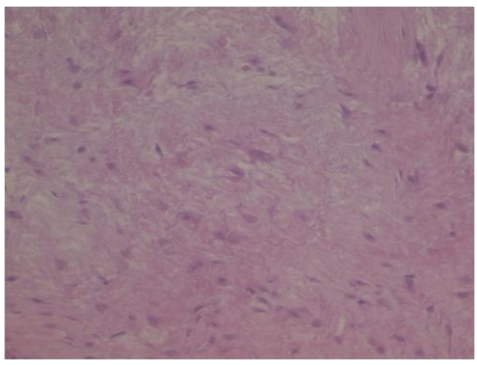

Figure 22. Histology representative of group 2 - *in vitro* 3D-BM niche constructs 6 weeks after subcutaneous implant in NOD/SCID mice. With a magnification x 100 a total integration of the scaffold within the surrounding tissue, where sometimes formed a capsule of fibroblastic cells.

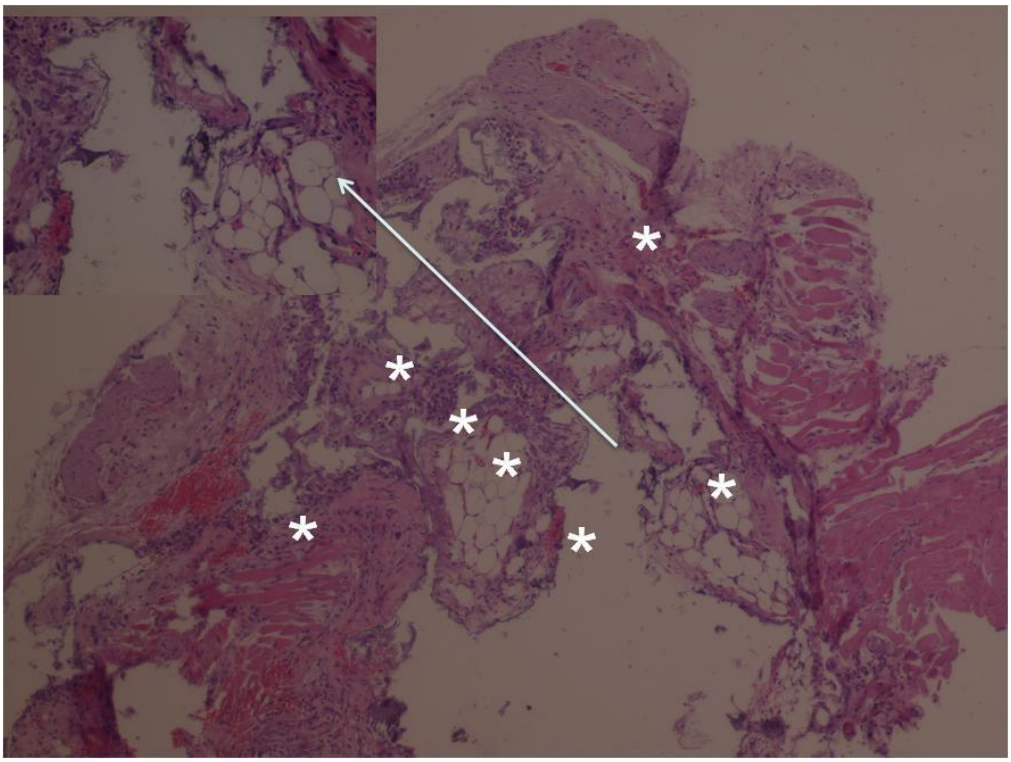

Figure 23. Histological stain representative of group 3 - *in vitro* 3D-BM niche constructs 6 weeks after subcutaneous implantation in NOD/SCID mice. The 3D-BM niche constituted by MSC and HSC ($CD34^+$) from *in vitro* long term cultures of 5 weeks were implanted in immunocompromised mice and maintained for 6 weeks. Can be observed that the implants integrated in the tissue with the presence mostly of polygonal morphology cells, many blood vessels and new tissue formation

7.3 Localization and spatial distribution of human cells (hematopoietic and MSC) on 3D-BM niche constructs and in the murine BM compartment

In order to verify tissue scaffold formation from human cells, slides were stained with an anti-human mitochondria antibody. Because the constructs were formed with MSC - CFP a monoclonal anti – CFP antibody was used to confirm in situ the presence of human MSC.

7.3.1 immunohistochemistry analysis of 3D- BM niche constructs

Human cell were detected by their positivity to anti-human mitochondria antibody showing an intense brown reactivity spots on the cytoplasm. Spindle-shaped cells surrounding the scaffolds were manly human cells. Human positive cells were also found in the basement membrane surrounding blood vessels formatted in the scaffolds, figure 24.

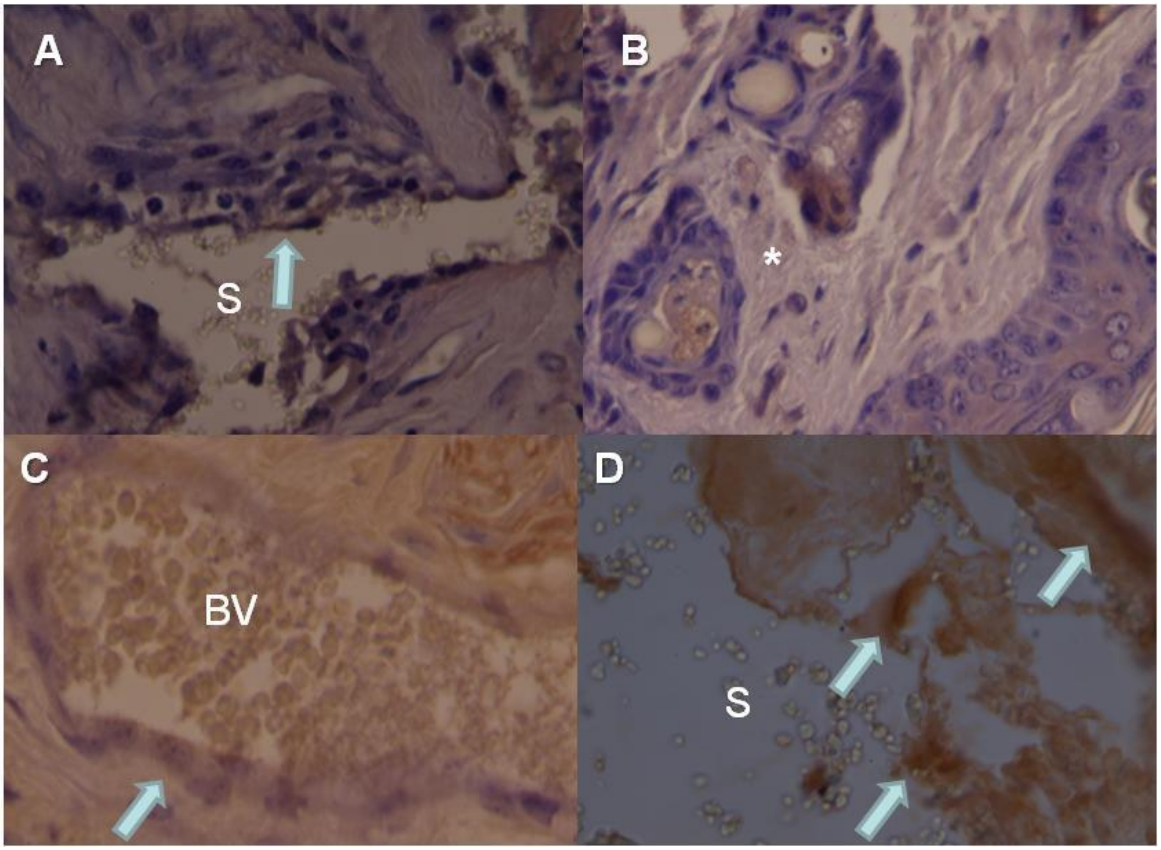

Figure 24. Subcutaneous 3D-BM niche constructs staining with anti-human mitochondria antibody. Immunohistochemical analysis of 3 µm paraffin cross-section through Conduit TCP containing mitochondria positive cells (human) stained with DAB showed positive brown spots on the cytoplasm. (A) (D) positive cells with polygonal morphology in contact with the scaffold (40X) and with (B) other morphology (40X). (C) human cells associated within vascular structures (100X).
S – scaffold; BV – blood vessels; (A), (B) – *in vitro* Group 1; (C) – *in vitro* Group 2; (D) – *in vitro* Group 3; (A, B and C) were contrasted with hematoxylin.

7.3.2 3D-BM niche constructs immunofluorescence analysis

In other to confirm immunohistochemical results and to detect simultaneously human mesenchymal stem cells and hematopoietic cells immunofluorescence studies were carried out. Cells with polygonal morphology expressed CFP proteins (green) and were mitochondria positive (red) confirming the presence of human MSC on the scaffolds after 13 weeks of subcutaneous implants on group 1 and 6 weeks on group 2 and 3. The MSC contributed to tissue scaffold formation (figure 25, B and E) as well as blood vessels, figure 25 - H and I.

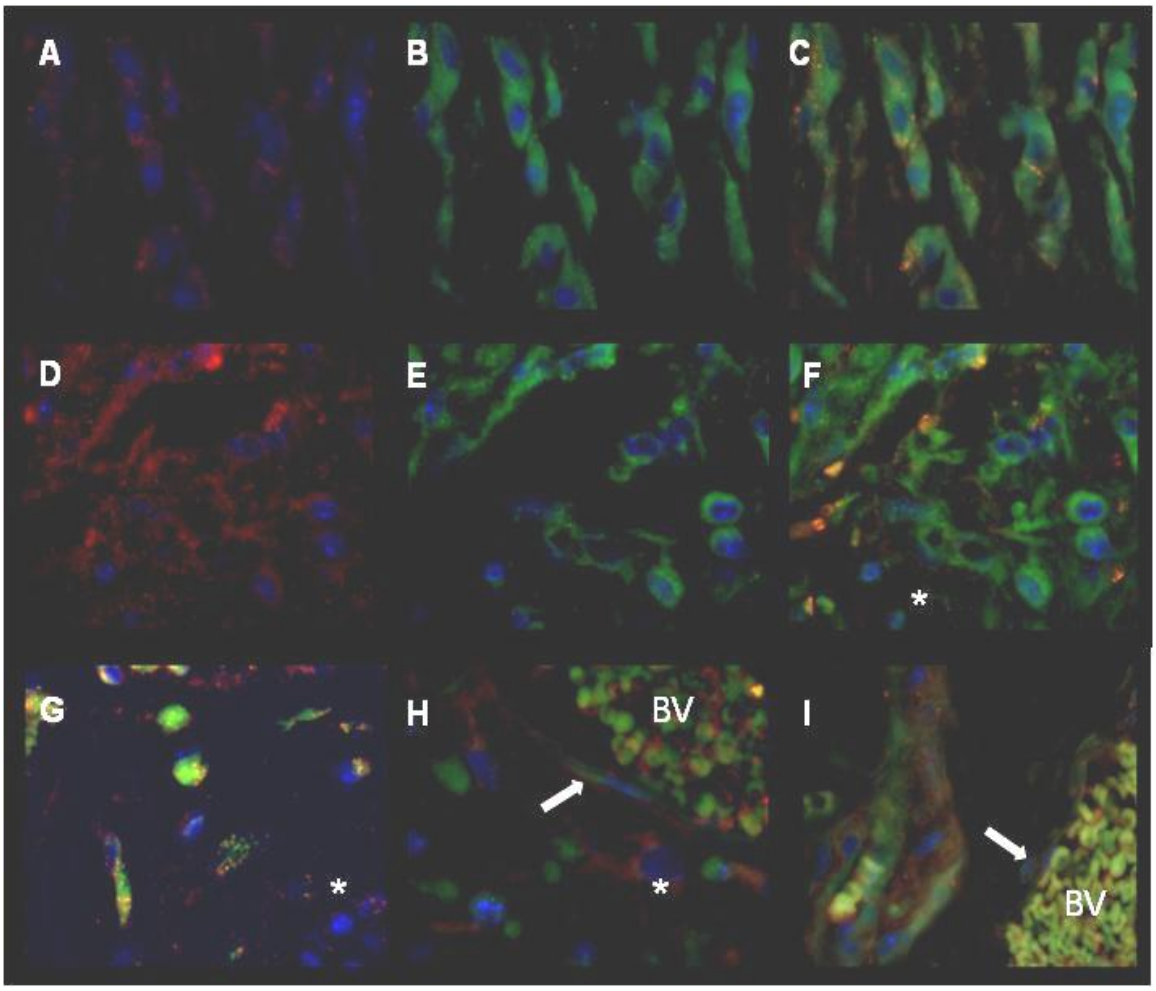

Figure 25. Subcutaneous 3D-BM niche constructs analysis with anti-human mitochondria and anti – CFP antibody. Immunofluorescence representative analysis of 3 µm paraffin cross-section through Conduit TCP containing MSC- CFP^+ cells (green) (B)(E) showing engraftment in Conduit TCP of human MSC (green and red)(C) (F) . Positive mitochondria (red) (*)CFP negative cells were identified suggesting the presence of possible human hematopoietic cells. DAPI (blue) was used as nuclear counterstaining in all panels. Magnification x 100. Arrows indicate human MSC. BV – Blood vessels; (A) (D) DAPI and Mitochondria; (B)(E) DAPI and CFP^+ cells; (C)(F)(G)(H)(I) overlay DAPI (blue), Mitochondria (red) and CFP^+ (green) cells.
(A)(B)(C)(H)(I) – *in vitro* group 2; (D)(E)(F) – *in vitro* group 3; (G) – *in vitro* group

7.3.3 Immunohistochemical and immunofluorescence analysis of murine femurs.

Immunohistochemical assays analysis showed that human hematopoietic cells were present within mouse bone marrow, with the highest proportion observed in the G2 mice.
Also anti-mitochondria antibody positive cells with fibroblastic morphology (human MSC) were observed in the endosteal zone (Figure 26 C) and in close relationship with human hematopoietic cells (Figure 26 D).
When immunofluorescence analysis was formed data confirmed immunohistochemical findings, showing hematopoietic cells distributed within mouse BM (Figure 25 E and F).
Also with the immunofluorescence approach showed human MSC in endosteal zone (Figure 27 and 28).
For both assays (immunohistochemical. and immunofluorescence.) were realized negative controls for protocol validation (Data not provided).

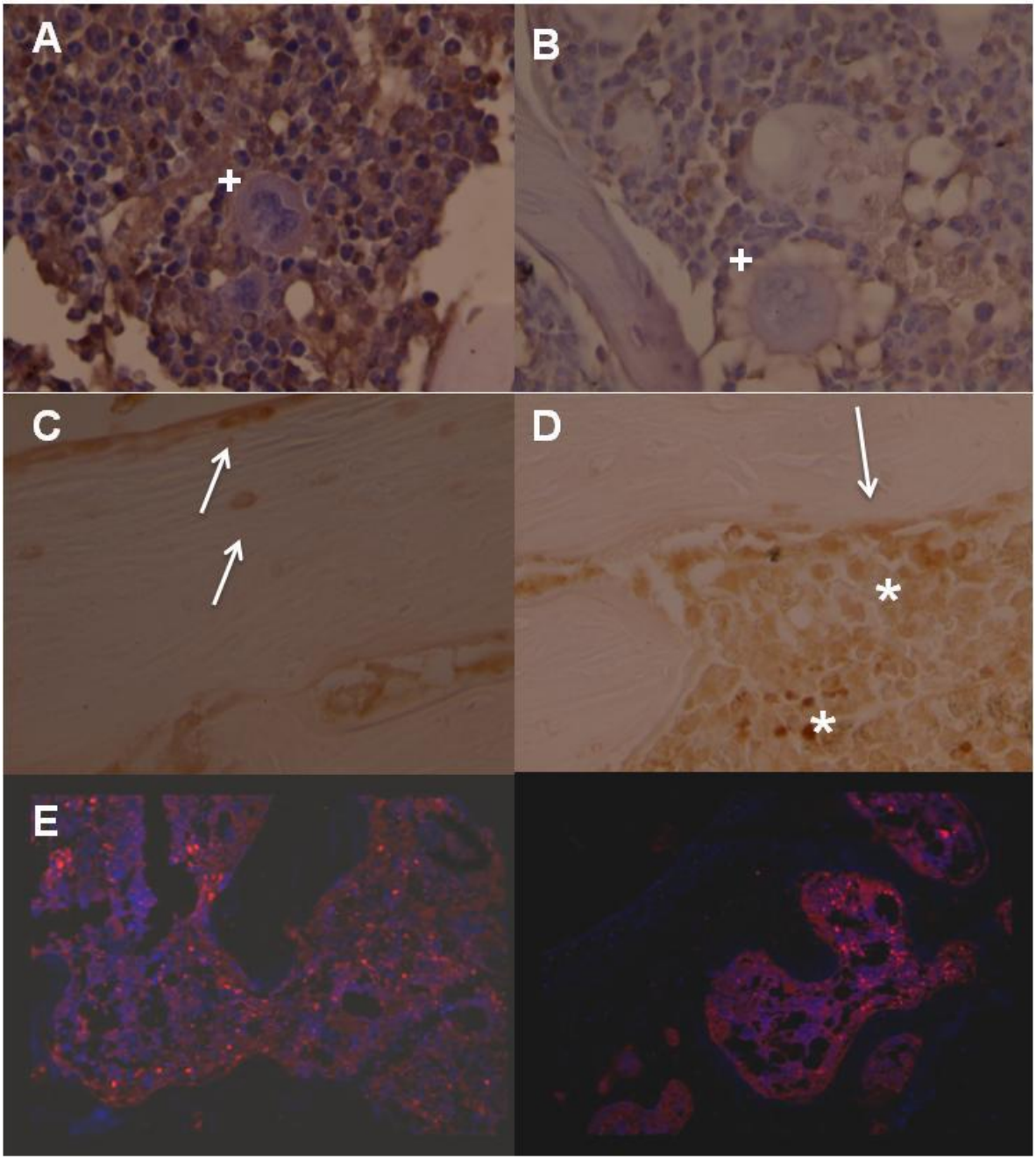

Figure 26. In situ visualization and spatial organization of human hematopoietic and MSC in the BM compartment of NOD/SCID mice at 6 weeks after xenotransplatation. Representative bone/bone marrow of 3 µm paraffin cross section were immunohistochemically stained using anti-human mitochondria (dark brown spots) and immunofluorescence stained using anti-mitochondria (red) and anti-CFP antibodies (green). (A) large quantities of mitochondria positive cells were possible to identify on trabecular bone of group 2 when compared with group 1 (B). Osteoblast positive cells were found in bone tissue (C) mitochondria positive cells (*) in closely proximity to bone (D) the results confirmed human cells (red) on murine trabecular bone (E)(F). DAPI (blue) was used as nuclear counterstain in all panels. (A) (D) counterstained with hematoxylin; (E)(F) overlay DAPI (blue), anti- human mitochondria (red) positive cells. (A)(B) Magnification x 40 and (C)(D)(E)(F) x100.

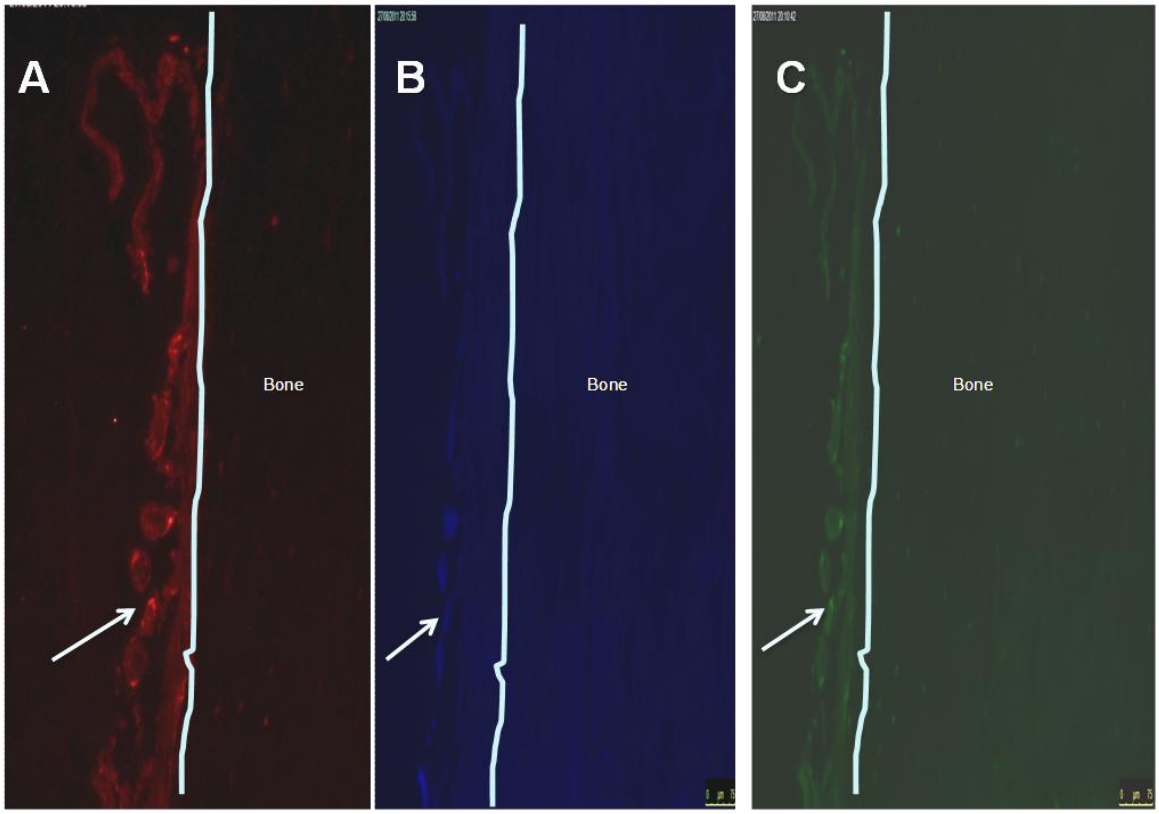

Figure 27. In situ visualization of human MSC in the murine bone from the in vivo group 2 –3D-BM niche constructs with. (A) positive cells for human mitochondria were detected (red) that were also CFP^+ cells, representing human MSC. (B) DAPI (blue) was used as nuclear counterstain. Magnification x 40.

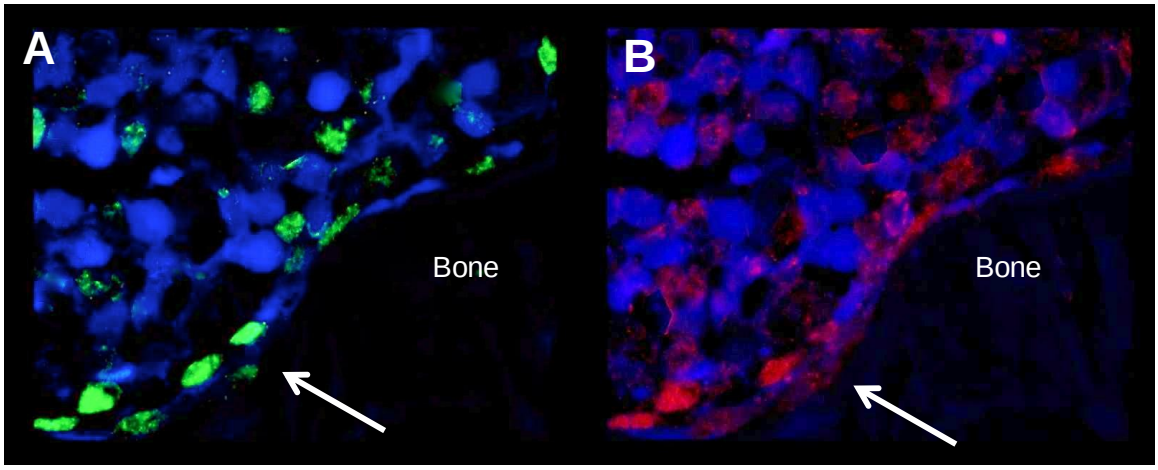

Figure 28. Site-specific differentiation of human eCFP-MSC in the murine BM compartment. The eCFP-MSC was determined by immunofluorescente staining for lineage-specific antigens. The MSC-CFP^+ (green) were located in endosteal zone (A) and positive human cells (red) anti-human mitochondria antibody (B). DAPI (blue) was used as nuclear counterstain. Magnification x 100.

VI - Discussion

After the description of the hematopoietic stem cell "niche" that regulates the "stemness" of HSC, its study has deserved an increasing scientific interest.

HSC are not independent units, they need to reside in a dynamic microenvironment where cell-to-cell interactions, soluble factors, and three-dimensional spaces provide the ultrastructure for their survival. The cellular constituents forming the HSC niche in the BM are still unclear, wherever different studies show that osteolineage cells controls the niche size[17].

On the other hand, after the first successful transplantation in 1989[76], umbilical cord blood has been used as an established source for HSCT, although its use is clinically limited by the low number of progenitor cells. *Ex vivo* expansion has become an alternative to increase the cell dose available for transplants[77].

The most promising model of HSC expansion involves the use of a three-dimensional (3D) culture system[43] that provides a better alternative to the classic static cultures where high doses of cytokines have to be added to allow the proliferation and differentiation of many of the progenitor and precursor pool on UCB[78].

Hypothetically the 3D culture systems try to reproduce the in vivo hematopoietic microenvironment, but at least three components[41] are necessary:

- soluble factors (cytokynes);
- supportive cells and adhesion molecules;
- three – dimensional geometry.

It has been demonstrated that MSC in culture promote the expansion and differentiation of HSC by secreting cytokines[25] including a set of well-established interleukins, leukemia inhibitory factor (LIF), Flt-3 ligand, SCF, G-CSF and GM-CSF, and express surface molecules, including intercellular adhesion molecule and vascular cell adhesion molecule to behave as *"feeder layers"* implicated in the regulation of hematopoiesis.

Systemic studies of co-transplantation of HSC with MSC in the context of HSCT[25] have shown to increase chimerism or/and accelerate hematopoietic recovery in animal models and in humans[59].

One of our first objectives was to develop an *in vitro* 3D-model with human stroma cells induced from human MSC and a biocompatible scaffold – Conduit^{TM} – TCP, able to support the maintenance and expansion of $CD34^{+}$ cells providing from UCB. Some reports indicate that co-cultures with MSC can efficiently support[79] the expansion and differentiation of HSC without the need of adding exogenous cytokines[44] since they can provide an effective microenvironment (with both soluble and non soluble signals).

Previous results from our group[80] suggests that the Conduit™ – TCP can provide a 3D system similar to human bone able to support the expansion and differentiation of human MSC when compared to other biomaterials, and at the same time differentiate into osteogenic linage creating an incipient bone matrix.

Other studies demonstrated the capacity of maintaining and expanding HSC *in vitro* using a scaffold with human mesenchymal stem cells, however few studies demonstrate the *in vivo* functionality of these constructs[42]. For this reason we have developed an experimental model based on a biomimetic niche formed by a biocompatible and osteoconductive biomaterial - Conduit™ – TCP- and human MSC differentiated into mature stromal cells (3D system) that could maintain and expand HSC in long term cultures. This 3D system was compared with the conventional two dimensional culture system.

The data provided from long – term marrow cultures (LTMBC) confirmed the maintenance of hematopoietic cells *in vitro* under different experimental conditions (2D, 2D+3D and only 3D system), where the stromal layer confluence, the presence of *cobblestone areas* and adipocytes were detected during the five weeks of culture. The formation of an osteogenic niche inside the scaffolds was demonstrated by detecting MSC-CFP$^+$.

On the group presented with the two systems (stromal layer cultured with Conduit-TCP), *cobblestone areas* appeared one week later than in group control (only 2D system), this can be explained by the capacity of HSC to migrate to specific niches and to be retained into the scaffold by their adhesion to the stroma[45]. For this reason *cobblestone area* results for this group could be underscored because the active areas of hematopoiesis inside of scaffolds could not be detected.

It has been demonstrated that co-transplantation of MSC with CD34$^+$ cells from human UCB results in the acceleration of the engraftment[81]. These results are related to the role of MSC in control of the hematopoietic niche. However, animal models could be more reliable in order to analyzed LT-HSC behavior.

Primitive human hematopoietic cells can be assayed on the basis of their ability to repopulate immune-deficient NOD/SCID mice, using a xenotransplantation model that provides a powerful tool to characterize long-term engraftment and multi-lineage differentiation of human HSC[82].

To assess the LT-HSC and verify the functionality of the *in vitro* 3D-BM niche construct *in vivo*, different experimental niches were implanted subcutaneously in irradiated NOD/SCID mice. The constructs were prior developed from *in vitro* cultures during 4 and 5 weeks (groups 2 and 3), respectively. Both groups were compared to the control group

(group 1) in which the niche was directly developed in vivo by the subcutaneous delivery of the scaffold and MSC.

At third week after xenotransplantation human hematopoietic cells ($CD45^+$) were detected both in PB in all experimental groups, with higher human chimerism in the group where the scaffolds from 4-weeks of *in vitro* cultures were implanted. These data are in agreement with the in *vitro* experimental results where a decrease of CFU-GM formation and *cobblestone* area in cultures with five weeks was observed, suggesting that an higher number of LT-HSC were presented inside 3D constructs that stay in culture for 4 weeks and then during the 5^{th} week they began to differentiate.

The percentage of human hematopoietic cells at the two time points of analysis (3^{rd} and 6^{th} week) was similar on *in vivo* group 1 (control) and group 3 where the scaffolds providing from *in vitro* cultures with five weeks, but with differences in the percentages of myeloid and lymphoid cells. In this regard, in group 1 both cell types were detected whereas in group 3 the CD13+ (myeloid) subset accounted of most of the human cells observed.

When results of human chimerism were analyzed at 6 weeks after transplant we observed that, in BM the highest proportion of human hematopoietic cells was detected in G2 but by contrast this group showed the lower expression in PB. This feature is difficult to interpret but perhaps a higher proportion of immature progenitors can migrate from the construct to BM and longer follow up could result in higher proportion of human cells in PB.

Ours data suggest that ex vivo expansion of $CD34^+$ (UCB) using *in vitro* 3D-BM niche construct leads to myeloid differentiation, but with a closer look to the group where the niche was constructed *in vivo*, the percentage of human lymphoid cells detectable on the mice transplanted with $CD34^+$ cells subcutaneously was lower compared with those who were transplanted intravenously. It can be hypothesized that this effect is not the result of the ex vivo expansion of HSC but can be related with the scaffold used on the assays which leads to the predominant myeloid differentiation. There is not any prior study in the literature exploring the ability of Conduit™ TCP as 3D scaffold to support *in vitro* or *in vivo* the hematopoiesis. Most of the studies have focused on assessing its osteogenic potential[74] combined with human MSC *in vivo*, showing new ectopic bone formation after 4 weeks of implantation, with the differentiation of MSC into osteoblasts in vivo[83]. This biomaterial provides a skeletal support for the growth of osteogenic cells creating enough space for new bone formation.

Analyses of human hematopoietic cells by flow cytometry in our system provides information on the engraftment kinetics for $CD45^+$ cells and cell subsets, but does not

allow the assessment of the spatial distribution of these cells. For this reason, 6 weeks after transplantation mice were sacrificed and the implants as well as the BM from femurs ex vivo evaluated.

Firstly, as it would be expected, Conduit^{TM}-TCP was not rejected after subcutaneous implantation, since no inflammatory response was observed from a morphological and histological point of view. As mentioned, this is not surprising, specially in an immunocompromised host. Blood vessels, immature connective tissue and adherent cells with polygonal morphologies were found within the implants. However there were several differences among the groups. For instance, the presence of adipocytes and connective tissue was characteristic in the group in which niche was formed *in vivo*, whereas on *in vitro* constructs more blood vessels, capillaries and hematopoietic cells were detected, and the fusiform cells formed an encapsulating cell layer surrounding the scaffold.

The finding of more adipocytes in the control group, where the MSC were induced for 7 weeks in the ectopic implant may be related to differences in the methodology that included a different time of contact between MSC and CD34^{+} cells. It has been shown that microenvironment behavior is influenced by its contact with hematopoietic cells.

The identification of the phenotype was possible because the human MSC that are responsible for the formation of 3D-BM niche were genetically marked. The engraftment and differentiation of MSC-CFP^{+} cells were determined by immunofluorescence staining. These cells were located on the blood vessels basement that seemed to be involved in the formation of new tissue. In all groups positive MSC-CFP^{+} were detected in 3D-BM niche constructs but it was not possible to identify if these elongated cells were in contact with the scaffolds because the scaffold material could not be seen. This problem was minimized with immunohistochemical stains that allowed locating the positive human cells stained with anti-mitochondria antibody. Our findings showed the human origin of these cells in the tissue.

Human mitochondria positive cells and at the same time negative to CFP were identified as possible human hematopoietic cells.

The routes of human MSC administration have been investigated in previous studies on murine models, where they can be an important factor in the reconstitution of functional human hematopoietic microenvironment. The visualization of human MSC in murine BM compartment it was not observed when these cells were intravenously administered, since most of them are trapped in the microvasculature of the lungs preventing their homing to BM. When these cells are intra-bone transplanted neither can be detected in the

contralateral bone to the one that received the transplant, suggesting minimal migrations, as opposed to human HSC.

Our data show that *in vitro* group 2, bone marrow showed the presence of human MSC-CFP^+ cells coming from the 3D-BM niche construct that had been subcutaneously implanted and contradict previous findings about the capability of migration of MSC. Furthermore this population of cells was preferentially located on the surface of the bone (endosteal region) and in close relationship with human hematopoietic cells. It could be suggested that this hematopoietic cells could have attracted MSC to the murine BM and bone. Immunohistochemical analyses lead us to identify positive human cells with the morphology spindle-shaped of osteocytes extending into the canaliculi bone.

Previous reports have demonstrated the therapeutic potential of bone marrow transplantation in children with severe osteogenesis imperfect[84], suggesting that mesenchymal cells can engraft and function in skeletal sites and differentiate into functional osteoblasts[85].

The fact that in the only group where human MSC were detected in BM was the group where 3D-BM niche constructs were implanted after 4 weeks in culture (in vivo group 2), may be related to the results of *in vitro* experiments where the in vitro hematopoetic activity was higher in the LTBMC established for 4 weeks.

It will be interesting to assess in the future the differences in gene expression depending on the condition of culture (2D versus 3D system) [86].

Since MSC have been demonstrated to contribute for tissue repair, it will be also interesting to study if in this *in vitro* experimental model MSC can provide a "trophic activity"[87] capable of producing a variety of factors, as cytokines and peptides that influence on biological functions, including angiogenesis. Stromal derived factor 1(SDF1)[88] is expressed by osteoblasts, endothelial cells and BM stroma cells has been involved in the regulation of primitive HSC both in vivo an in vitro[89]. This cytokine plays an important role in cell trafficking and homing of $CD34^+$ stem cells, SDF1/CXCR4 (receptor for SDF1) are essential for MSC migration to the sites of injury. *In vitro* studies[90] indicate that the addition of MSC to damaged stromas could improve SDF1 secretion and correlates with higher level of CXCR4 expression on CD34+ cells.

Perhaps, the most shining results of the result of the present study is to show that 3D-BM niche constructs can expand HSC from UCB *in vitro* and these cells can engraft in the murine model. Even these cells "in vivo" can expand and migrate to murine BM. However it should be remind that the Conduit-TCP can be responsible of a decrease in the human lymphoid compartment.

HSC can differentiate following a hierarchical process that is often dictated by intrinsic actions of hematopoietic transcription factors[91].

The Notch signaling pathway[89] is one of the most important regulate cell fate in a variety of cellular systems. Some transmembrane glycoprotein receptors such, Jagged 1, Jagged 2 and Delta 1, 3 and 4 initiate the activation of Notch signaling[92]. Some reports show that Delta 1 and Jagged1, have differential effects in human lymphoid differentiation[93].

Future studies in Notch signals may be important to verify why the $CD34^+$ cells from UCB loss or reduce their capacity to differentiate in lymphoid lineage in this culture system. These feature future studies can illustrate the inter-relationship between the niche and stem cells and could lead us to evidence the molecular events that regulate the transition from multipotency to unipotency, during lymphopoiesis. Progenitors undergo a stepwise lineage restriction process where they can express multiple lymphoid-affiliated genes, such as those enconde TdT, IL-7Rα and EBF, in addition to RAG1[94] could be over or down expressed.

In summary, the 3D-BM niche constructs allowed the growth, differentiation and migration of HSC in a 3D humanized environment effectively mimicking the natural *in vivo* bone marrow environment that allows the maintenance and proliferation of hematopoietic cells both *in vivo* and *in vitro*. This model permits the study of normal and pathological hematopoiesis and its relationship with the stroma.

VII - Conclusions

1. Constructs of Conduit TCP loaded with human BM stroma derived from MSC are able to support the *in vitro* maintainance and proliferation of $CD34^+$ cells from UCB.

2. The 3D-BM niche constructs obtained from 4-weeks cultures when implanted subcutaneously in NOD/SCID mice allow the in vivo homing, lodgment and engraftment of human hematopoietic cells.

   2.1. The human MSC from these 3D implants are responsible of new tissue formation (contributing also to angiogenesis) on the ectopic bone. From the implants they can migrate to the murine BM of the femur, preferentially homing in endosteal region and in close relationship with human hematopoietic cells.

3. 3D-BM niche constructs can regulate HSC behavior depending on the administration route: when intravenously injected, UCB progenitors preferentially differentiate into human B-lymphoid cells whereas they form mainly myeloid cells when injected subcutaneously.

VIII - References

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