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Cellular therapy for cardiac tissue repair by haematopoietic and mesenchymal stem cells

Thérapie cellulaire de réparation tissulaire cardiaque par des cellules souches hématopoïétiques et mésenchymateuses

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Une des stratégies envisageables en thérapie cellulaire et génique est l'utilisation de cellules souches adultes isolées à partir de moelle osseuse. Les conditions optimales de culture des cellules souches mésenchymateuses humaines (CSMh) et murines (CSMm) ont été définies.

Nous avons observé que les CSM des deux espèces présentent une morphologie particulière qui permet de les isoler en cytométrie de flux sur les seuls paramètres de taille et de granularité. Le phénotype des CSM a été analysé par cytométrie de flux et nous pouvons observer que les CSMh sont $CD31^-$, $CD34^-$, $CD45^-$, $CD80^-$ et HLA-DR$^-$, tandis qu'elles expriment les antigènes CD73, CD90, CD105 et HLA-1.

En ce qui concerne les CSMm, le phénotype observé est: $CD34^-$, $CD45^-$, $CD11b^-$, $CD106^+$ et Sca-1$^+$.

Nous avons également voulu déterminer la fréquence en progéniteurs parmi les CSMm amplifiées *in vitro*. Dans ce but, nous avons tout d'abord évalué l'enrichissement en progéniteurs CFU-F (Colony-Forming Units – Fibroblast). Cette méthode consiste en une culture secondaire en milieu liquide optimisé pour le développement de colonies cellulaires d'origine mésenchymateuse. Nous avons ainsi pu observer une augmentation des CFU-F au cours des passages des CSM. Ensuite, nous avons mis au point une méthode permettant d'évaluer la fréquence en progéniteurs par mise en culture des CSM en dilution limite (CFUF-IC, Colony-Forming Units Fibroblast-Initiating cells). De nouveau, il est apparu que la fréquence en progéniteurs augmente au cours des passages successifs. Nous avons alors calculé que chaque CFUF-IC est à l'origine de ± 10 CFU-F, une valeur constante à chaque passage.

Des essais de différenciation ont également été réalisés et ont abouti à la différenciation des CSMm en adipocytes, chondrocytes et ostéocytes.

Enfin, un modèle de ligature de l'artère coronaire chez la souris a été développé ainsi que des marquages immunohistologiques sur le tissu cardiaque. Un anticorps monoclonal dirigé contre l'antigène CD31 nous permet de mettre en évidence l'angiogenèse, et deux autres anticorps dirigés contre l'α actinine et la connexine 43 marquent les cardiomyocytes de façon spécifique. La connexine 43 nous aide plus précisément à mettre en évidence les connexions entre cellules cardiaques. Dans le but d'injecter différents types de greffons dans ce modèle animal, nous avons également étudié le cycle cellulaire des cellules souches par coloration au Hoechst. Finalement, la fluorescence des CSMm isolées à partir de souris transgéniques exprimant l'EGFP a été analysée : nous avons pu constater qu'elle évolue de ± 30% dans les cellules médullaires primaires à plus de 90% pour les CSMm isolées et amplifiées par culture *in vitro*. La participation des cellules souches à la reconstruction tissulaire cardiaque de l'animal greffé pourra ultérieurement être analysée par la détection combinée de l'EGFP et de marqueurs myocardiques.

➤ Abstract

A conceivable strategy in cell and gene therapy is the use of adult stem cells isolated from bone marrow. We defined the optimal conditions for the culture of human and murine mesenchymal stem cells (hMSC and mMSC). We observed that the MSC of both species had a special morphology thanks to which it was possible to isolate them in flow cytometry on the basis of the size and granulosity parameter only. We analyzed the MSC phenotype by flow cytometry and observed that the hMSC were CD31$^-$, CD34$^-$, CD45, CD80$^-$ and HLA-DR$^-$ while they expressed CD73, CD90, CD105 and HLA-1 antigens. mMSC phenotype was CD34$^-$, CD45$^-$, CD11b$^-$, CD106$^+$ and Sca-1$^+$. We also wanted to determine the frequency in progenitors among the mMSC amplified *in vitro*. To do so, we first assessed the enrichment in CFU-F (Colony-Forming Units – Fibroblast) progenitors. This method consisted in a secondary culture in liquid medium optimized for the development of colonies of mesenchymal origin. We were able to observe an increase of the CFU-F during the MSC passages. Then, we developed a method in order to assess the progenitors frequency by culturing MSC at limiting dilutions (CFUF-IC, Colony-Forming Units Fibroblast-Initiating cells). Once again, we were able to notice that the frequency in progenitors increased during the successive passages. The ratio between the number of CFU-F and the frequency in progenitors amounted to ± 10. We also performed differentiation assays. We were able to differentiate the mMSC in fat cells, chondrocytes and osteoblasts. Finally, we developed a model of left coronary artery ligature in mice as well as immunohistochemical markers showing the antigens CD31, α-actinin and connexin 43. With the intent to inject various types of grafts in this animal model, we also studied the cell cycle of the stem cells by Hoechst staining. Fluorescence analysis of mMSC isolated from EGFP transgenic mice revealed that their fluorescence increased from ± 30% in marrow to more than 90% for the mMSC isolated and amplified via an *in vitro* culture.

➤ Introduction

The somatic stem cells (SC) existing in each organ are endowed with self-renewal abilities but can also differentiate into functional mature cells: they are particularly involved in the tissue regeneration [1,2]. The hematopoietic stem cells (HSC), taken from bone marrow, peripheral blood or umbilical cord blood, are at the genesis of all blood cells and are already widely used in the treatment of serious haematological and malignant affections. The mesenchymal stem cells (MSC) are isolated from marrow but recently also from umbilical cord blood[3]. They are able to differentiate into a variety of non-haematopoietic tissues of mesodermal origin such as bone, cartilage, tendon, fat cells, skeletal and cardiac muscle and medullary stroma[4]. The use of mesenchymal stem cells seems to be extremely promising, not only in repairing cellular therapy but also in transplantation and immune therapy.

The haematopoietic and mesenchymal stem cells potential opens up the application field in medicine, especially for cardiovascular diseases. These cardiovascular pathologies are the first killer worldwide. Unfortunately, the heart is not able to rebuild itself after a injury because cardiomyocytes are not able to significantly regenerate themselves *in vivo*. After an acute impairment of the heart tissue, one can actually observe the appearance of a non-contractile fibrous scar[5-7].

Hence, the scope of this study is to determine the haematopoietic (HSC) and mesenchymal stem cells (MSC) ability to contribute to the repair of the myocardial tissue injured by ischemia.
Though nowadays isolation procedures of HSC seem to be established, it is not the same for MSC. So we first had to optimize isolation, selection and amplification methods of these cells with repair potential.
Then, we studied the position of HSC and MSC in the cellular cycle. In fact, it has been demonstrated that changes related to the cellular cycle influence their ability to migrate and engraft in the marrow as well as in the injured myocardial tissue via the blood circulation[8,9].
Finally, we developed an infarcted myocardium model by left coronary artery occlusion in mice, as well as immunohistochemical methods enabling us to examine cardiac-specific markers. We will thus be able to study later on the impact of stem cells grafts injected in this mice model on cardiac tissue regeneration[10,11].

➤ **Materials and Methods**

Human mesenchymal stem cells (hMSC). After obtaining patient informed consent, fifty milliliters human bone marrow aspirates, taken from the iliac crest of normal donors, were diluted 1:1 with phosphate-buffered saline (PBS; Biowhittaker Europe) and centrifuged over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) at 500 *g* for 20 minutes at room temperature. Mononuclear cells were recovered from the gradient interface and washed with PBS. They were resuspended in Dulbecco's modified Eagle's medium containing 1 g/l of glucose (DMEM-LG; GIBCO) supplemented with 10% fetal bovine serum (FBS; Hyclone Perbio), 100 U/ml penicillin, 100 µg/ml streptomycin, and 25 µg/ml amphotericin B or in Mesenchymal Stem Cells Growth Medium (MSCGM, Biowhittaker Europe). Cells were plated at 50.000, 100.000 and 200.000 cells/cm^2. The cultures were maintained in a humidified atmosphere at 37°C and 5% CO_2. After 24 hours, nonadherent cells were removed by washing with phosphate-buffered saline (PBS) and then medium was changed twice a week. When cells were at 85% of confluence, they were washed with PBS and lifted by incubation in 0.1% trypsin (Biowhitakker) for 10 minutes at 37°C.

Human hematopoietic stem cells. After obtaining patient informed consent, cord blood (CB) samples were obtained following full-term vaginal delivery according to the guidelines established by the Ethical Committee of the University of Liège. Mononuclear low density cells were isolated by centrifugation over Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). CB CD34$^+$ cells were purified by immunomagnetic selection using MACS CD34 isolation kits (Miltenyi Biotech, Bergisch Gladbach, Germany) according to manufacturer's instructions. CD34$^+$ cell purity in the final product always

exceeded 97%.

Murine mesenchymal stem cells (mMSC). mMSCs were obtained from the inbred transgenic strain C57Bl/6-TgN(ACTbEGFP)1Osb (GFPtg) that ubiquitously expressed enhanced green fluorescent protein (Jackson Labs, Bar Harbor, ME). Animal experiments were performed in accordance with institutional guidelines and approved by the Animal Care Ethical Committee of the University of Liège.

Female and male mice 8 to 10 weeks old were individually killed by cervical dislocation. The bone marrow was flushed out of tibias and femurs. After washing by centrifugation at 900g for 8 min, cells were resuspended in Dulbecco's modified Eagle's medium containing 4,5 g/l of glucose (DMEM; GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin. To initiate an mMSC culture, cells from each bone were plated in a 12,5 cm^2 flask. The cultures were maintained in a humidified atmosphere at 37℃ and 5% CO$_2$. After 24 hours, nonadherent cells were removed by washing with PBS and then medium was changed every 3 or 4 days. When cells were at 85% of confluence, they were washed with PBS and lifted by incubation in 0.1% trypsin (Biowhitakker) for 10 minutes at 37℃.

Freezing cells. The cells were resuspended in FBS with 5% dimethylsulfoxide (DMSO) and frozen at - 80℃ for 24 hours, before being stored in liquid nitrogen. To recover frozen cells, the vials were quickly thawed to 37℃, and then plated in DMEM with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin.

Fibroblast colony forming units (CFU-F). At each passage, mMSCs were plated at 1000, 500, 50 and 5 cells/cm2 in DMEM with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin. The medium was changed every 3 days for a total of 21 days. The plates were fixed in methanol and stained with Hematoxylin-eosin at room temperature (RT) for 10 minutes. All visible colonies were counted.

Colony forming units fibroblast-initiating cells (CFUF-IC). At each passage, mMSCs were plated in 24 wells of 96 wells plate at 500.000 to 5 cells/cm2 in DMEM with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin. After two weeks, the number of positive wells was counted. The percentage of CFUF-IC was calculated using L-calc software (StemCell Technologies).

Differentiation. Cells were plated at 500 cells/cm^2 in 58-cm^2 dishes and incubated in DMEM with 10% fetal bovine serum (FBS; GIBCO), 100 U/ml penicillin, 100 µg/ml streptomycin for 10 days.

For osteogenesis, the cultures were then incubated in DMEM supplemented with 10% FCS, 10% HS, 100 U/mL penicillin, 100 µg/mL streptomycin, 12 mM L glutamine, 20 mM β-glycerol phosphate (Sigma, St Louis, MO), 50 ng/mL thyroxine (Sigma), 1 nM dexamethasone (Sigma), and 0.5 µM ascorbate-phosphate (Sigma). The medium was changed twice a week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained with Alizarin Red, pH 4.1 (Sigma) for 20 minutes at RT.

For adipogenesis, the cultures were incubated in DMEM supplemented with 10% FCS, 10% HS, 100 U/mL

penicillin, 100 g/mL streptomycin, 12 mM L-glutamine, 5 g/mL insulin (Sigma), 50 µM indomethacin (Sigma), 1×10^{-6} M dexamethasone, and 0.5 µM 3-isobutyl-1-methylxanthine (IBMX; Sigma). The medium was changed twice a week for 3 weeks. Cells were fixed with 10% formalin for 20 minutes at RT and stained with 0.5% Oil Red O (Sigma) in methanol (Sigma) for 20 minutes at RT.

For chondrocyte differentiation, a pellet culture system was used. Approximately 200.000 mMSCs (passage 7) were placed in a 15-mL polypropylene tube (Falcon, Bedford, MA), and centrifuged to pellet. The pellet was cultured at 37℃ with 5% CO2 in 500 µL chondrogenic media that contained 500 ng/mL bone morphogenetic protein-6 (BMP-6; R&D Systems, Minneapolis, MN) in addition to high-glucose DMEM supplemented with 10 ng/mL transforming growth factor β 3 (TGF-β 3), 10^{-7} M dexamethasone, 50 µg/mL ascorbate-2-phosphate, 40 µg/mL proline, 100 g/mL pyruvate, and 50 mg/mL ITS - Premix (Becton Dickinson, Bedford, MA; 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenious acid, 1.25 mg/mL bovine serum albumin, and 5.35 mg/mL linoleic acid). The medium was replaced every 3 to 4 days for 21 days. For microscopy, the pellets were embedded in paraffin. Five-µm sections were cut and stained with Toluidine blue Sodium Borate.

Cell cycle analysis with Hoechst 33342. Cells were plated in short term expansion culture in Iscoves' medium (IMDM) supplemented with 20% BIT (Stem Cell Technologies, Meylan, France), 2 mmol/L alanyl-glutamine, 1% (v/v) cholesterol-rich lipids, 1 mmol/L sodium pyruvate (all from Sigma), 100 U/mL penicillin, 100 µg/mL streptomycin and 5×10^{-2} mmol/L 2-mercaptoethanol (all from

Biowhittaker, Verviers, Belgium). Cells were stimulated for 72 hours by a combination of 3 cytokines (100 ng/mL each of stem cell factor (SCF), thrombopoietin (TPO; both from Amgen) and flt-3 ligand (FL; Peprotech, Boechout, Belgium)) or five cytokines (identical combination of cytokines plus 20 ng/mL of interleukin-6 (IL-6) and granulocyte-colony stimulating factor (G-CSF; both from Amgen)).

CB CD34+ cells, cultured CD34+ cells (with three or five cytokines) and mMSC (at each passage) were resuspend in a 5 µg/ml solution of Hoechst 33342 (Hst, Molecular Probes, Eugene,OR) in Hst buffer. Hst buffer consisted of Hanks Balanced Salt Solution (HBSS; Biowhittaker, Verviers, Belgium), 1g/L glucose, and 10% fetal calf serum (FCS, Biowhittaker, Verviers, Belgium). DNA histograms were derived from the Hoechst fluorescence.

Cell cycle analysis with propidium iodide. DNA staining was performed with PI by incubating cells in PBS 0,6% IGEPAL CA-630 (Sigma) containing 50 µg/mL PI and 1 mg/mL RNAse (Boehringer, Mannheim, Germany). After 30 min incubation at 4℃ in the dark, cells were analysed on a FACSort flow cytometer using FL-2 channel. The proportion of cells in the different phases of the cell cycle was determined using ModFit software. (Verity Software, Topsham, ME, USA).

Flow cytometry analysis. Cells were washed and the supernatant was eliminated. For murin MSC analyse, five µl MAbs anti CD45-(IgG2bκ, BD Biosciences), CD34 (IgG2bκ, BD Biosciences), CD11b (IgG2bκ, BD Biosciences), CD106 (VECAM 1) (IgG2bκ, BD Biosciences) or Sca1 (IgG2bκ, BD Biosciences) were added to the pellet and cells were incubated

30 minutes at 4℃. Cells were washed and 5 µl Allophycocyanin (APC)-conjugated goat anti-rat Ig were added to the pellet and cells were incubated 30 minutes at 4℃. Cells were washed and fixed in PBS 1% formaldehyde. For human MSC analyse, five µl Mabs anti-CD34 (IgG1, BD Biosciences), CD45 (IgG1, Iotest) HLA-Dr (IgG1, Immunotech), CD80 (IgG1, BD Biosciences), CD31 (PECAM) (IgG1, BD Biosciences), HLA-1 (IgG1, BD Biosciences), CD73 (SH3, SH4) (IgG1, BD Biosciences), CD90 (Thy-1) (IgG1, BD Biosciences) and CD105 (Endoglin) (IgG1, Serotec), were added to the pellet and cells were incubated 30 minutes at 4℃. Cells were washed and fixed in PBS 1% formaldehyde prior to flow cytometry analysis. Samples were analyzed on a FACSort.

Myocardial infraction. Myocardial infraction was induced in female C57Bl/6 at 2 months of age. Under general anesthesia, mouse were intubated, and positive pressure ventilation (30 mL / min) was maintained. The mouse heart was exposed through a 1-cm left lateral thoracotomy. The left anterior descending coronary artery of each mouse was ligated by passing an 8-0 silk suture. The chest wall, muscle layers, and skin were then closed with interrupted 6-0 silk suture. Intubation was discontinued and the mouse was allowed to recover on a heated platform.

Immunohistochemical analysis. Hearts of female C57Bl/6 at 2 months of age were collected and embedded in Tissue-Tek OCT (Sakura). The samples were cut to yield 10-µm thick cryostat sections, and fixed in 10% methanol for 10 min at -20℃. Myocytes were identified with a mouse monoclonal antibody directed against sarcomeric α-actinin (EA-53, 1/1600, Sigma) followed by revelation with ARK™ (Animal Research Kit) Peroxidase (Dako). Slides were also stained with a rabbit polyclonal anti-connexin 43 antibody (1/2000, Sigma), followed by revelation with a peroxidase polyclonal goat anti-rabbit antibody (Dako). Endothelial cells were studied with a rat anti mouse CD31 (PECAM-1) monoclonal antibody (557355, BD Biosciences) for 60 min at RT followed by revelation with a rabbit anti rat / Biotin (E468, 1/400, DAKO) for 30 min at RT and after with streptavidin / HRP antibody (P397, DAKO, 1/500) for 30 min at RT. Imaging was performed with a confocal microscope. Cytospin preparations of murine and human MSC were realized and fixed for 10 min in acetone. We examined the expression of the three above-mentioned antigens on the MSC according to the same protocol.

➤ Results

Amplification and characterization of human MSC. Adhesion of primary mononuclear marrow cells was dependent of the FBS lot used. After initial screening, we used the same FBS lot for all further experiments. A better cellular amplification was obtained in MSCGM than in DMEM-LG supplemented with 10% FBS. We analyzed the phenotype of the marrow mononuclear cells by flow cytometry. Cells were negative for the CD34, CD80, CD105, CD73 and CD90 antigens. The HLA-I and –II antigens showed a positivity of 71.9% and 22.33% respectively. The marrow mononuclear cells were also positive for the CD45 (77.02%) and CD31 (39.83%) haematopoietic markers. At the second passage, we already obtained the described phenotype for human MSC. One could actually see that the expected negative antigens were really negative (CD31, CD34, CD45 (Fig.1 B), CD80, HLA-Dr). Moreover, HLA-1 (Fig.1 C), CD73, CD90 and CD105 antigens were strongly positive (Table 1). The appearance of a large size, high-density cellular population and undetectable in primary marrow cells was detected in expanded MSC (Fig.1 A). It has indeed been reported that MSC had a particular morphology and that it was possible to isolate them on the only criterion of the size. That's why we recorded the results for the cells as a whole but also for a large size (FSC^{high}) and high-density (SSC^{high}) superior population during our flow cytometry analyses. The FSC^{high} SSC^{high} population showed more quickly the expected phenotype for MSC.

Table 1: Study of human cultured MSC surface antigen expression.

Passage	CD31	CD31 FSC^{high} SSC^{high}	CD34	CD34 FSC^{high} SSC^{high}	CD45	CD45 FSC^{high} SSC^{high}	CD80	CD80 FSC^{high} SSC^{high}	HLA-DR	HLA-DR FSC^{high} SSC^{high}
0	39,83%		4,73%		77,02%		3,95%		22,33%	
2	1,92%	1,76%	0,93%	0,21%	0,93%	0,21%	0%	0%	0,5%	0%

Passage	HLA-1	HLA-1 FSC^{high} SSC^{high}	CD73	CD73 FSC^{high} SSC^{high}	CD90	CD90 FSC^{high} SSC^{high}	CD 105	CD105 FSC^{high} SSC^{high}
0	71,9%		0%		0%		0%	
2	79,25%	92,59%	84,53%	92,84%	92,15%	92,84%	71,08%	83,58%

A.

B. CD45   **C.** HLA-1
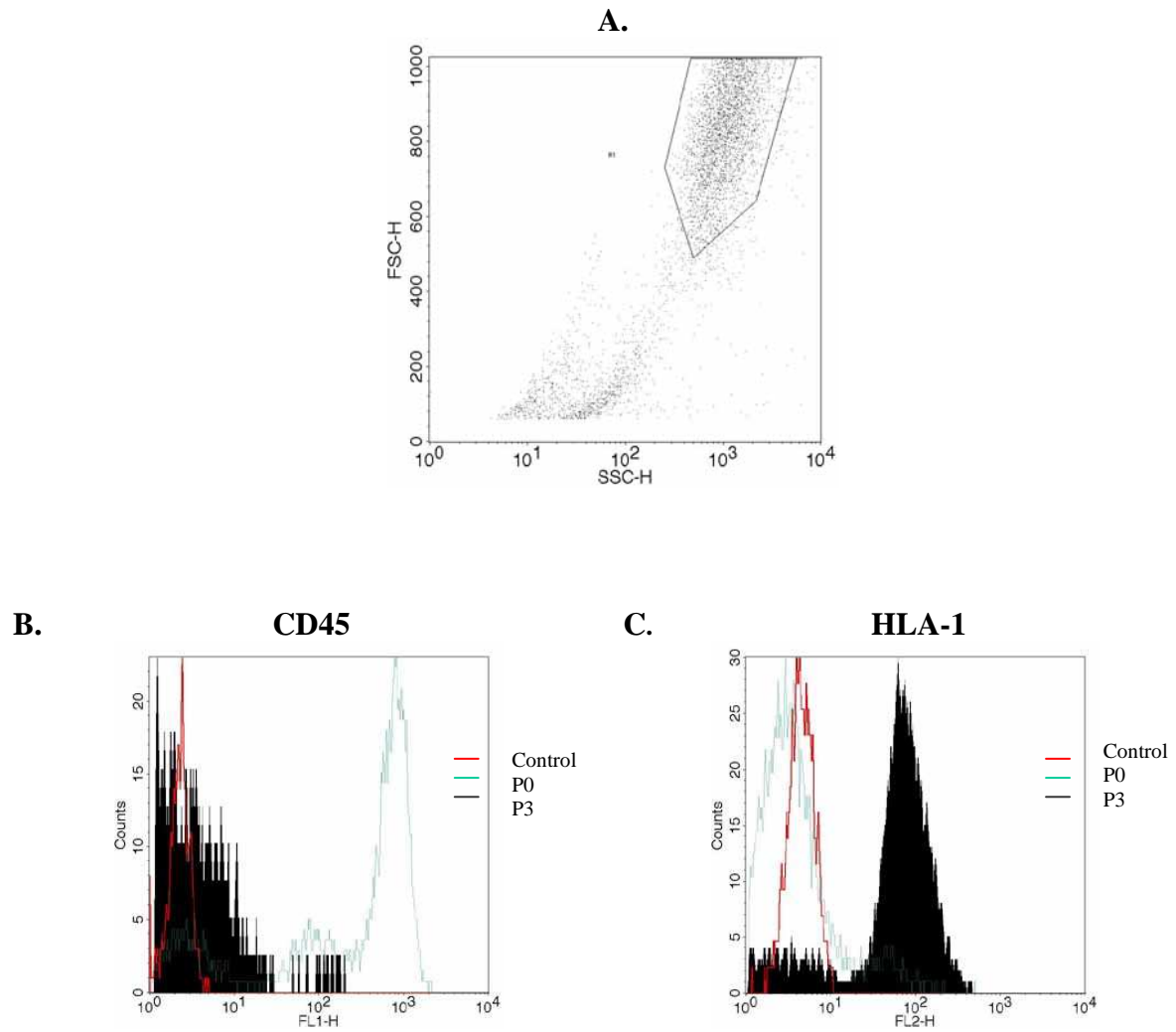

Figure 1: Flow cytometry analysis of hMSC **A.** Scatter plot depicting the FSC^{high} SSC^{high} MSC population **B.** and **C**. CD45 and HLA-1 expression in FSC^{high} SSC^{high} population of hMSC.

Amplification of murine MSC. After testing various culture mediums, serums and cellular density, we observed that the best murine MSC amplification efficiency was obtained with DMEM + 10% FBS + 1%PS. As for the optimum cellular density, it amounted to 800000 cells/cm^2 from marrow cells, and decreased to 40000 cells/cm^2 from passage 1 to 5 and to 16000 from the 6th passage onwards. It was possible to plot a growth curve and the calculated generation time was 8 days (Fig.2 A). No declining phase could be observed until the 15th passage, which was close to the growth lines observed in the continuous growth. We also drew up culture curves by comparing a high and a low cellular density culture (Fig.2 B-C). We noticed that the best amplification efficiency was obtained for the highest cellular concentrations.
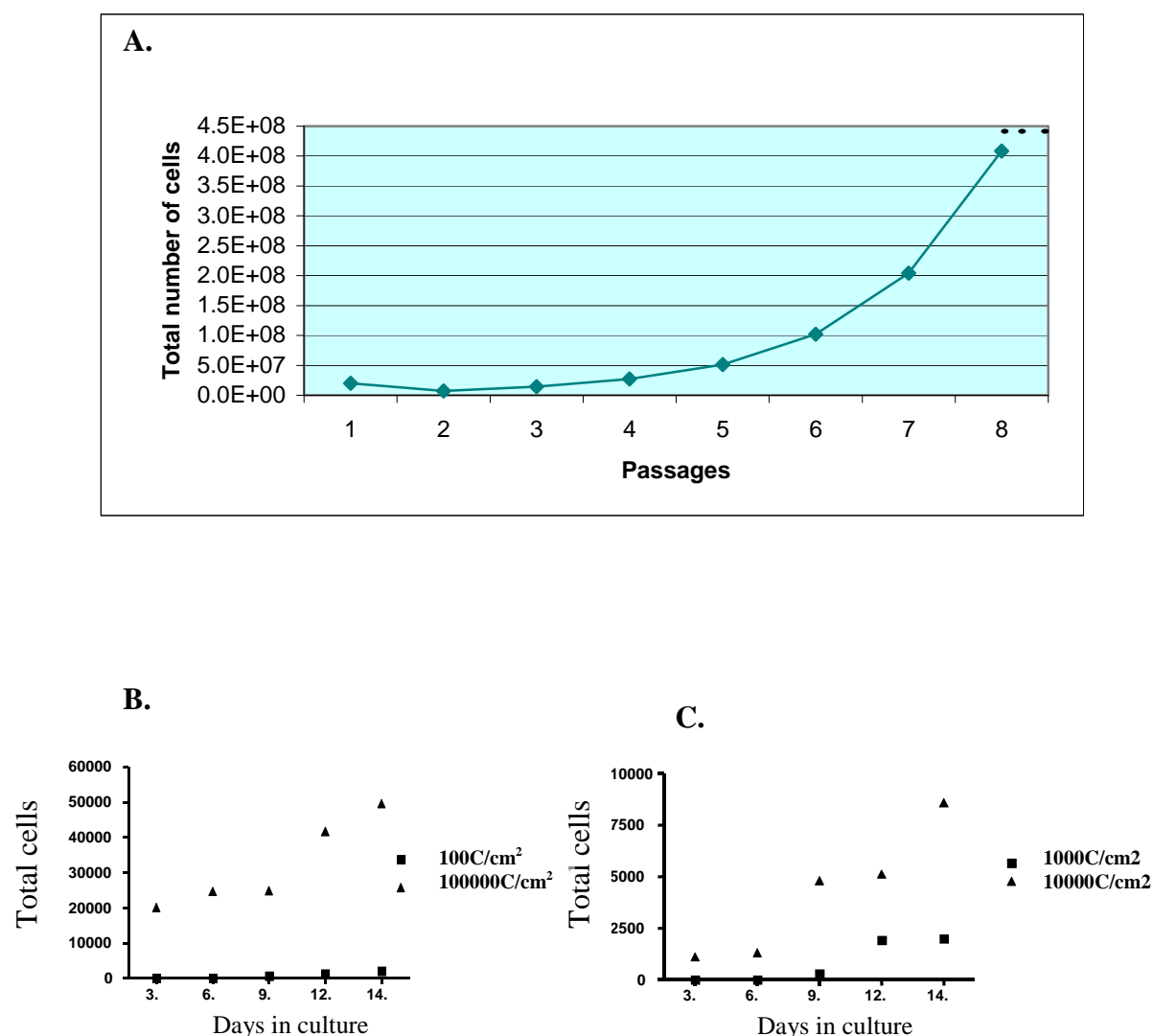
Figure 2: **A**. Growth curve obtained during the successive passages of the murine MSC. **B.** Growth curve obtained at P1 for cellular densities of 100 and 100000 cells/cm^2. **C.** Growth curve obtained at P3 for cellular densities of 1000 and 10000 cells/cm^2.

EGFP expression in murine MSC in vitro. We wanted to obtain a homogenous population of MSC expressing EGFP. We thus estimated the expression of this fluorochrome in the C57Bl/6 EGFP+ mice marrow cells, as well as during the various passages of the MSC cultured *in vitro*. To begin with, we examined the cells with a fluorescent confocal microscope (Fig.3 A-B), and we noticed that not all the cells were fluorescent. In order to quantify these results, we analyzed the cells by flow cytometry. Thirty percent of the cells were fluorescent in transgenic mice marrow as well as in the MSC cultures from the 1st to the 5th passage. Then, the percentage of fluorescent cells increased up to more than 85% (Fig. 3C).
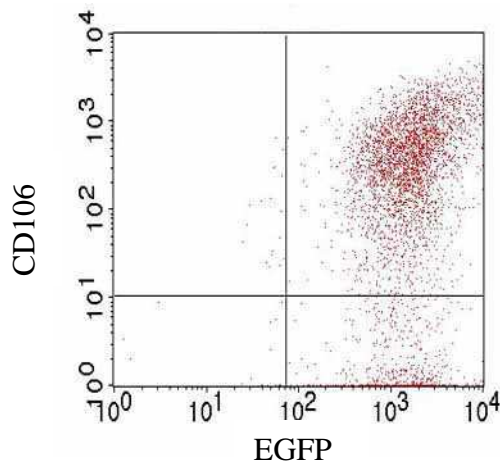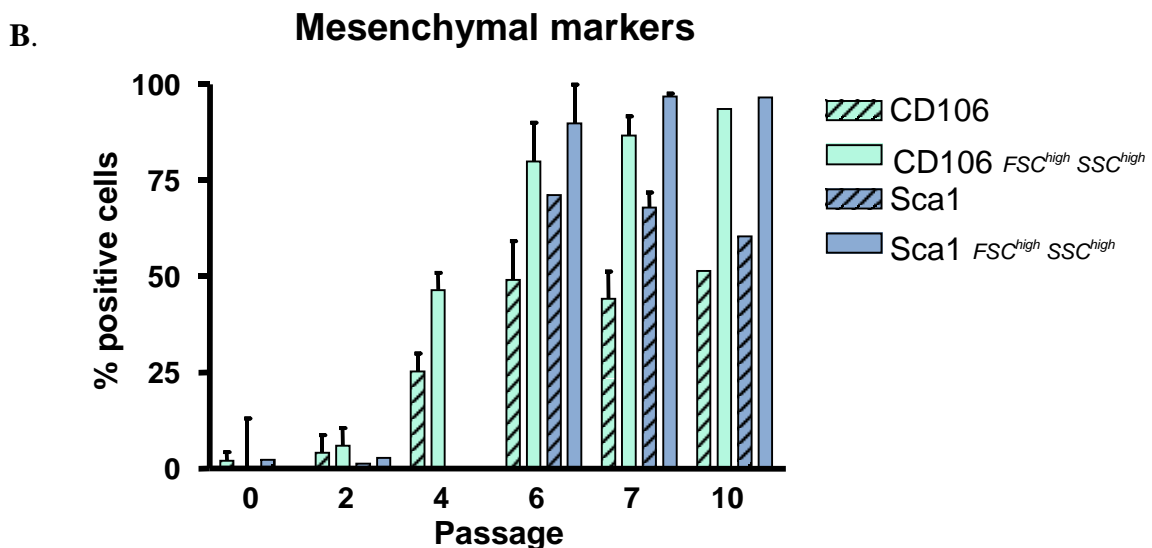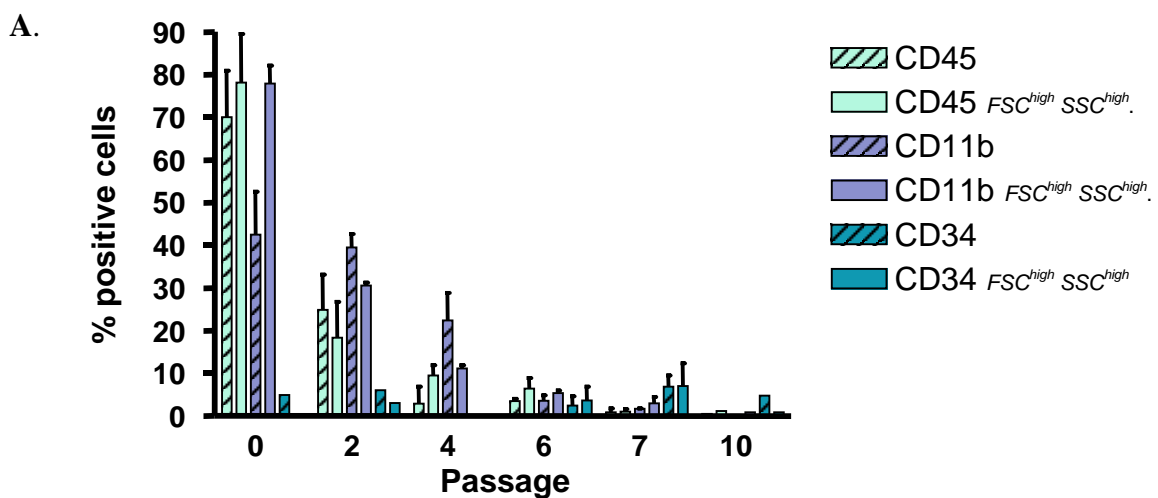
A.
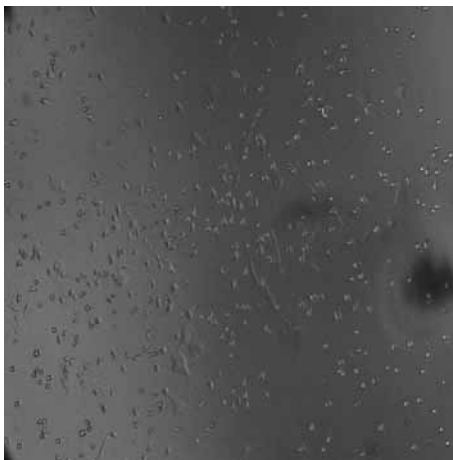
B.
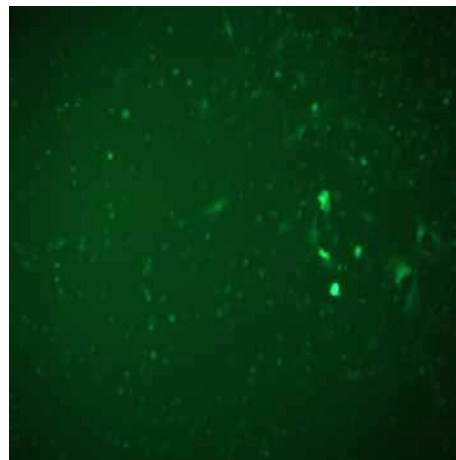
C.

Figure 3: **A.** Visualization with sight field microscopy, **B.** with fluorescence microscopy, of C57Bl/6 EGFP+ mice MSC at P3. **C.** Flow cytometry analysis of the expression of C57Bl/6 EGFP+ mice MSC at P8, of EGFP and of the CD106 marker in the murine MSC.

Epitope analysis. We studied the expression of various antigens at the surface of murine marrow cells and also during the various culture passages of mice MSC. The appearance of a large size, high-density cellular population FSC^{high} SSC^{high} was also detected. This population more quickly showed the expected phenotype for the MSC. We could see that the CD45 and 11b markers expression was high in marrow and strongly decreased as passages progressed, until being less than 5%. As for the CD34 antigen, it remained slightly expressed during all the passages (Fig.4 A). The CD106 and Sca-1 antigens were very slightly detected in marrow and strongly increased, up to more than 95%, at the 8th passage in the FSC^{high} SSC^{high} population (Fig.4 B). This phenotype corresponded to the expression of the expected antigens for the murine MSC.

Hematopoietic markers

A.

CD45
CD45 FSC^{high} SSC^{high}.
CD11b
CD11b FSC^{high} SSC^{high}.
CD34
CD34 FSC^{high} SSC^{high}

B. **Mesenchymal markers**

CD106
CD106 FSC^{high} SSC^{high}
Sca1
Sca1 FSC^{high} SSC^{high}

<u>Figure 4</u>: **A.** Evolution of the expression of the expected negative antigens in murine MSC and **B**. of the expected positive antigens in murine MSC, for all of the cells and for the FSC^{high} SSC^{high} population.

12.

Assay for fibroblast colony-forming units and colony-forming units fibroblast initiating cells. Thanks to the CFU-F and CFUF-IC colony-forming units assays performed at each passage, we were able to assess the proliferative ability and the presence of the *in vitro* cultured MSC progenitors. During the CFU-F assays, classically used to assess MSC cultures, we did not systematically obtained a linear relationship between the number of seeded cells and the number of counted colonies. That's why we developed a second assay in order to assess the progenitor frequency: it consisted in a two-weeks culture in 96 well plates at various cellular densities (limiting dilution). However, with these two methods, we observed an increase of the MSC frequency during the successive passages (Fig.5 A-B). Starting with $1 \ 10^6$ initial marrow mononuclear cells, the results obtained were $2,29 \ 10^2$ CFU-F formed from eGFP+ mice marrow, $2,98 \ 10^3$ from cells at the first passage, $3,72 \ 10^3$ at the second passage, $1,35 \ 10^4$ at the third passage, $7,35 \ 10^3$ at the fourth passage, $5,38 \ 10^4$ at the sixth passage, $1,36 \ 10^5$ at the seventh passage, $1,35 \ 10^5$ at the eighth passage and $1,76 \ 10^5$ at the ninth passage. As for the CFUF-IC tests, the calculated frequency of MSC amounted to 1/261700 in the EGFP+ mice marrow, 1/18806 at the first passage and 1/7276 at the second one, 1/1957 at the third passage, 1/1451 at the fourth passage, 1/246 at the fifth passage, 1/83 at the sixth passage and 1/42 at the seventh passage, 1/89 at the eighth passage and 1/100 at the ninth one. We also wanted to check if the EGFP gene was expressed in the CFU-F formed at the various passages. All colonies detectable in visible light were fluorescent when observed with a fluorescent confocal microscope. We could thus assume that the progenitors were fluorescent and that they transmitted the EGFP coding gene to their lineage. We calculated that each CFUF-IC gave rise to $9,66 \pm 7,02$ CFU-F, a value which remained constant at each passage.
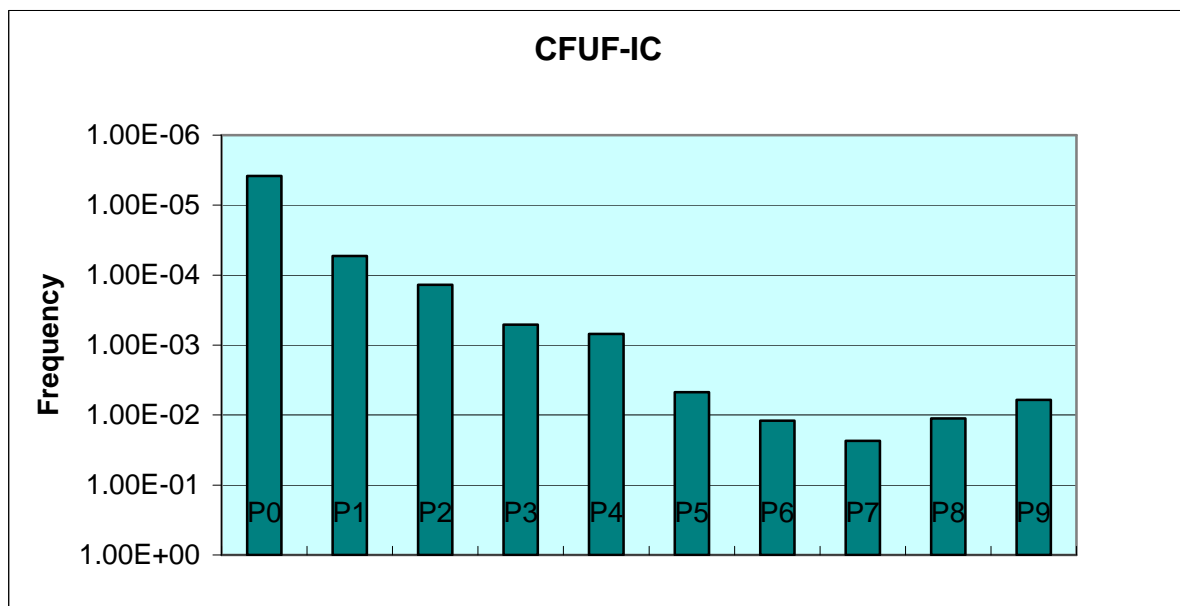
13.

A.

CFU-F

B.
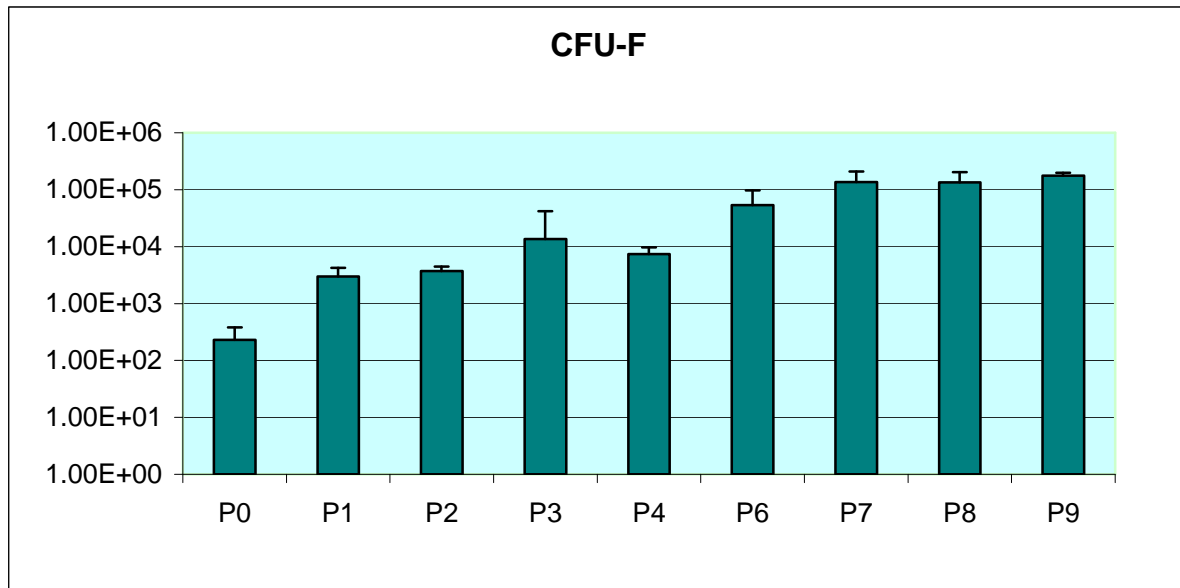
CFUF-IC

Figure 5: **A**. Number of CFU-F formed at each passage starting with 10^6 marrow mononuclear cells**. B.** Frequency in MSC progenitors at each passage, estimated by CFUF-IC tests.

Differentiation assays. We wanted to assess the MSC ability to differentiate into three tissues of mesodermal origin. MSC isolated from EGFP⁺ mice differentiate into mineral cells or fat cells when they are placed in the adequate induction medium. The fat cells are showed by Oil Red O staining of the lipid vesicles (Fig. 6A-B), and the osteoblasts differentiation by Alizarin Red staining (Fig. 6C-D). We were also able to induce MSC differentiation into chondrocyte cells. When MSC are placed in a particular differentiation medium, they form a micromass and produce proteoglycans stained by Toluidine blue (Fig. 6E-F).

A.

B.

C.

D.

E.

F.
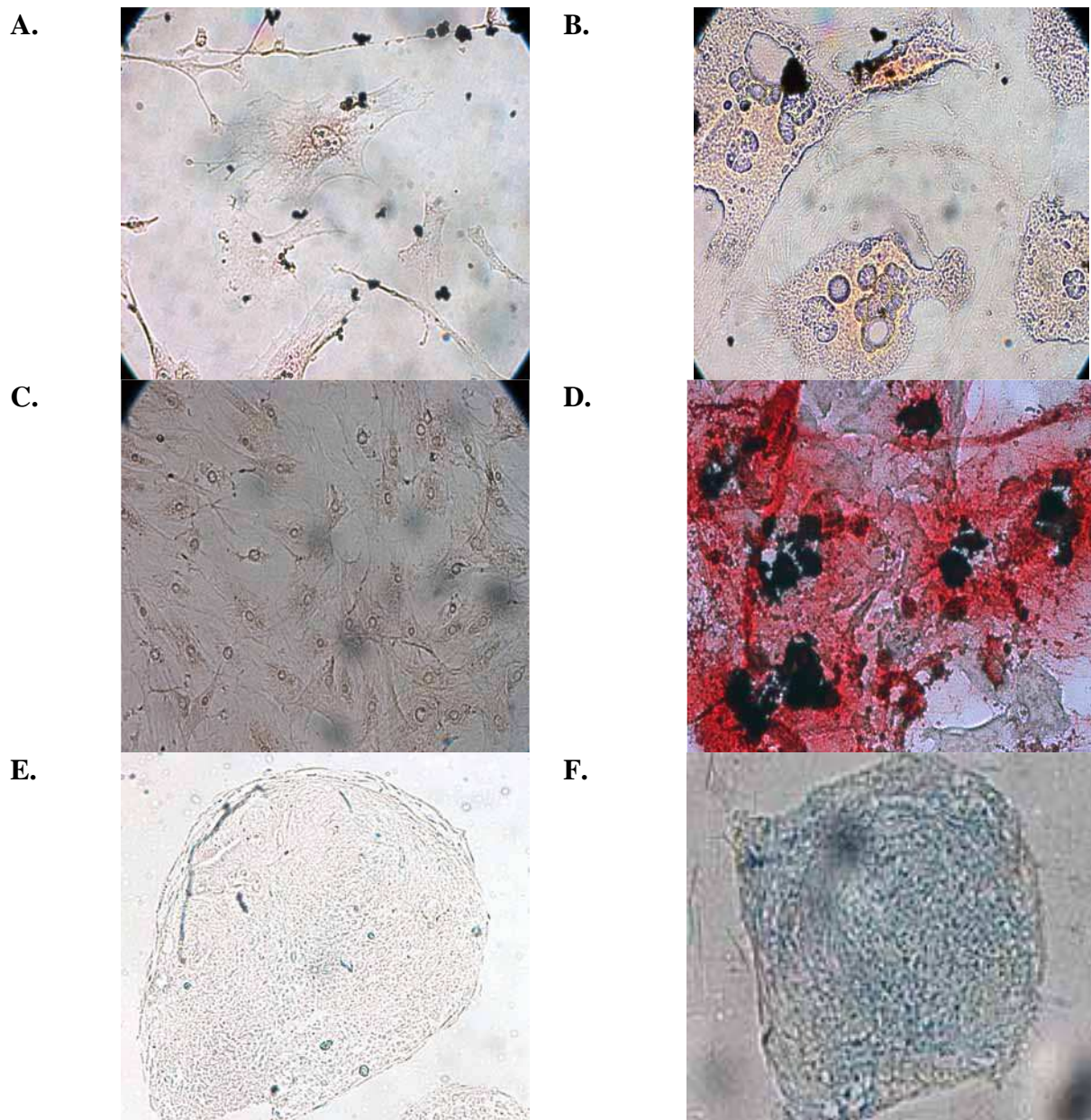
Figure 6: mMSC differentiation at the 7th passage. **A**. Control culture incubated in DMEM+10 % FBS and stained with Alizarin Red. **B**. Induction of osteoblasts differentiation and stained with Alizarin Red (Magnification 40X). **C**. Control culture incubated in DMEM+10 % FBS and stained with Oil Red O. **D**. Induction of fat cells differentiation and stained with Oil Red O (Magnification 20X). **E**. Control culture incubated in DMEM+10% FBS and stained with Toluidine blue. **F**. Induction of chondrocytes differentiation and stained with Toluidine blue (Magnification 20X).

Cell cycle analysis with Hoechst 33342. We analyzed the cell cycle of murine and CD CD34$^+$ MSC in flow cytometry after having stained them with Hoechst. For the mice MSC, we found, at each passage, 89% of cells in G0/G1 when they were recovered at 85% of confluence. As far as the analysis of the CB CD34$^+$ was concerned, 96% of the cells were in G0/G1 when they were newly isolated, and this result dropped to 65% after a short-term culture with SCF, TPO and Flt3, and to 61% when IL6 and G-CSF were added to the previous cytokines combination. These results were validated by the cell cycle analysis via staining with propidium iodide. We studied MSC and HSC position in the cell cycle because it has been demonstrated that cell cycle can have an impact on their ability to migrate and engraft in the marrow and in other tissues such as the heart.

Immunohistochemical analysis and myocardial infarction. We developed immunohistochemical markers on hearts of healthy C57Bl/6 mice. The CD31 (PECAM-1) antigen (Fig.7 A) allows us to observe the heart capillaries. Later on, this marking on ligatured mice hearts will enable us to identify capillary changes in reaction to the ischemia. Two antibodies directed against specific proteins of the myocardial cells were chosen. The anti-α actinin antibody (Fig.7 B) shows a secondary protein of the cardiomyocytes cytoskeleton that contributes to the contraction of these cells. The anti-connexin 43 antibody (Fig.7 C) is directed against a protein contributing to the connections between cardiac cells. It will not only enable us to see if the HSC and MSC engrafted in the animal model differentiate into cardiomyocytes but also if they possibly connect with the neighbouring cells.
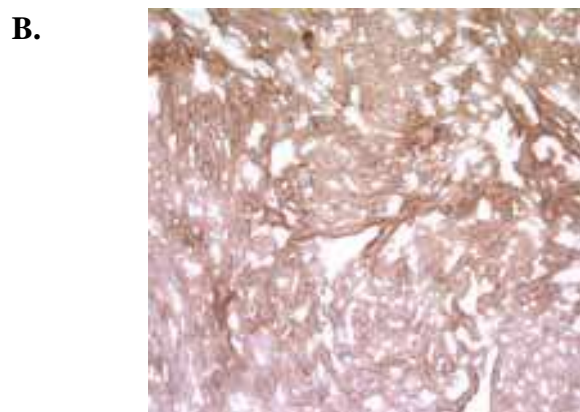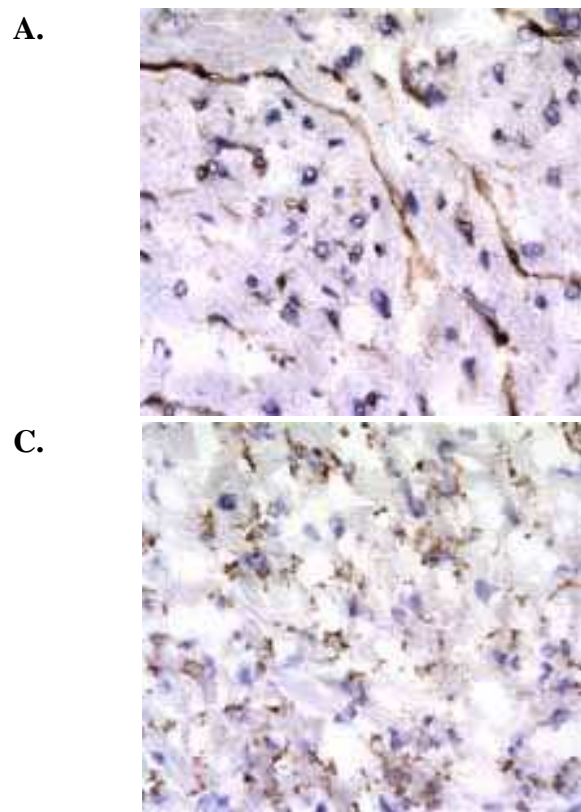
A.

B.

C.

Figure 7: Immunostaining on healthy C57Bl/6 mice heart (Magnification 50X) **A**. Anti-CD31 antibody diluted at 1/500. **B**. Anti-α-actinin antibody diluted at 1/1600. **C**. Anti-connexin 43 antibody diluted at 1/2000.

We also checked expression of these three antigens on the third passage hMSC (Fig.8 A-B) and on the seventh passage mMSC (Fig.8 C-D), i.e. at the time when we consider to have a pure MSC population. To this end, we carried out immunohistochemical staining on cytospin preparations of MSC. For the two species, we only noted positivity of the connexin 43 marker. However, this marking is diffuse and does not show an organization similar to that observed on myocardial slices. On the other hand, these cells did not express the antigens CD31 and α actinin.

Then, we ligatured the left coronary artery in the C57Bl/6 mouse in order to be able to reproduce left ventricle infarctions. We will draw up survival curves for these animals as well as immunohistochemical markings and recordings of the parameters on the heart function after ligature.
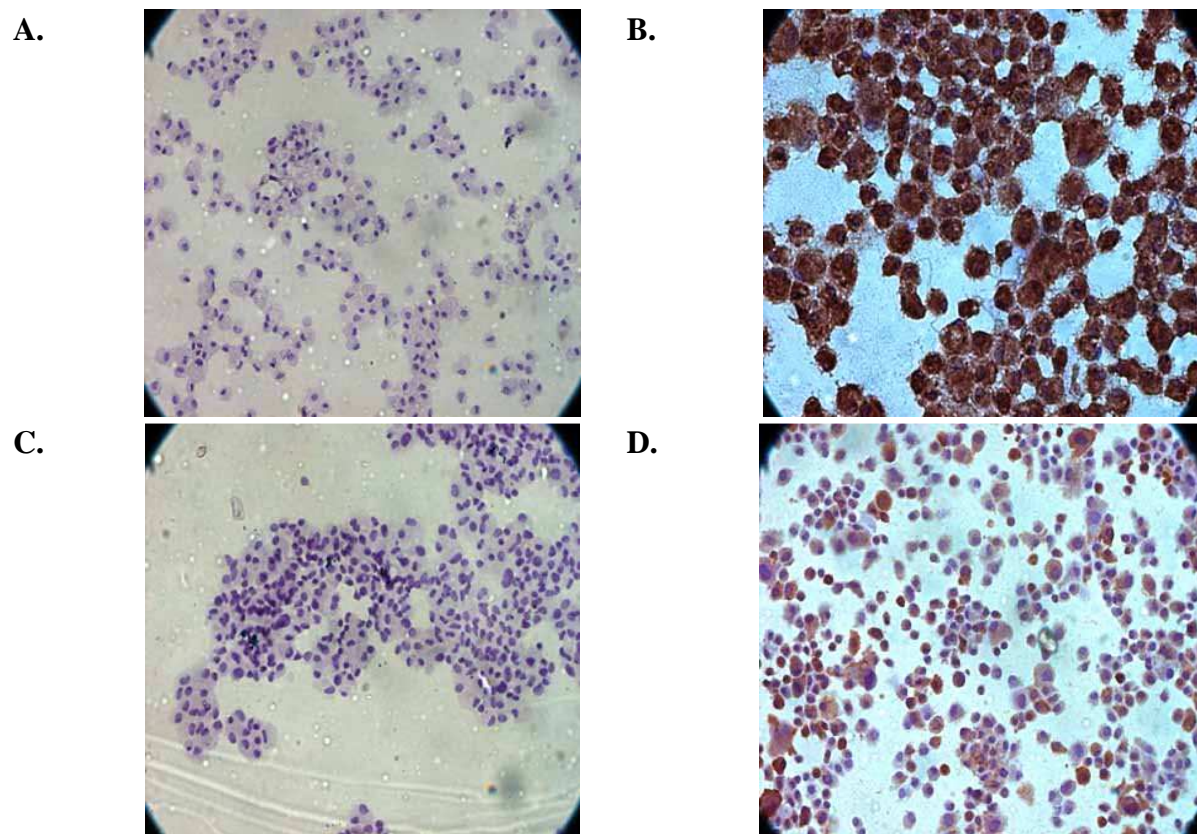
Figure 8: Immunostaining on cytospin preparations of MSC. **A.** Control slide on hMSC. **B.** Anti-connexin 43 antibody diluted at 1/2000 on hCSM. **C.** Control slide on mMSC. **D.** Anti-connexin 43 antibody diluted at 1/2000 on mCSM.

> **Discussion**

There is a substantial interest concerning marrow adult stem cells for their possible application in biology and their potential in cellular therapy. As HSC have already been the subject of an in-depth study, our work focused more on the MSC characterization[12].

In that study, we observed that both human MSC (hMSC) and murine MSC (mMSC) showed a clearly distinguishable morphology. In fact, large size and high-density cells appeared after an *in vitro* expansion. This population was not present in primary marrow cells. As described previously, it was thus possible to obtain a homogenous MSC population on the basis of MSC's size not only for human but also for murine MSC[13].

hMSC and mMSC are very sensitive to the cellular density at which they are plated. We noticed that the highest cellular density gave the best rate of cellular amplifications. We could determine a generation time of 8 days[14].

One of the methods used to characterize MSC is the analysis of cell surface antigens. For the hMSC, we confirmed the absence of the CD31, CD34, CD45, CD80, HLA-Dr antigens, while the HLA-1, CD73, CD90 and CD105 antigens were highly positive. The mMSC analysis showed that the CD34, CD45 and CD11b antigens were slightly expressed while the CD106 and Sca-1 were present on more than 90% of the cells[15].

In order to have a more objective opinion on the presence of stem cells, we carried out various functional tests on mMSC. We started by assessing the presence of progenitors through the mMSC ability to form fibroblastic colonies. Afterwards, we developed a test enabling us to assess the frequency of progenitors. It consisted in a secondary culture in liquid medium optimized for the development of mesenchymal colonies. These two methods allowed us to observe an increase in the frequency of progenitors up to the 7th passage. The ratio between the CFU-F number and the frequency in progenitors assessed by CFU-IC was 9,66 $\pm$ 7,02. We can thus consider that one mesenchymal progenitor originates 10 precursors who will each give birth to a fibroblastic colony.

The culture time needed to obtain a homogenous MSC population is one of the main differences during the *in vitro* amplification of these two species of MSC. The study of the phenotype by flow cytometry actually shows that a culture made up of more than 90% of MSC is obtained after two passages for the hMSC, while 7 passages are necessary with mMSC. Moreover, the results of MSC phenotype analysis as well as the CFU-F and CFU-IC functional tests do not show any significant difference after the 2nd passage for the hMSC and after the 7th passage for the mMSC. We can thus assume that the culture purity in MSC does not improve beyond these two limits.

The development of left coronary artery ligature in mice is an interesting model that will help us to study the impact of stem cells administration on cardiac regeneration. We will be able to assess the participation of various cell grafts such as HSC, MSC as well as the association of these two cellular types in the myocyte regeneration. We will also be able to determine if the differentiation of MSC in contractile cells, via culture with 5-azacytidine[16], and this prior to the graft, allows a

better repair of the infarcted areas than undifferentiated MSC. We will finally assess the influence of the cell cycle position on the cells homing ability. The cells contribution to the cardiac reconstruction will be studied by immunohistochemistry. Angiogenesis will be detected by the presence of the CD31 antigen. The myocardial markers, alpha-sarcomeric actin and connexin 43 will be studied. The presence of connexin 43 will indicate a possible communication, indeed synchronization, between the differentiated stem cells and the cardiac cells. Previous works demonstrated that, when they are injected in an ischemic heart, the muscle satellite stem cells do not synchronize thus involving a fibrillation[17]. Fetal cardiomyocytes seem to survive when they are injected in the heart tissue but this raise ethical problems[18].

The use of marrow stem cells seems to be an interesting field of investigation in the treatment of cardiovascular diseases by cell therapy[19].

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