In vitro and *in vivo* Characterization of Adult Bone Marrow Neural Crest Stem Cells and their Implication in Hematopoietic Support

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Introduction

Human bone marrow is composed of hematopoietic stem cells (HSC) and stromal cells that are supporting HSC's activity. Stroma compartment contains mesenchymal stem cells (MSC) and many other cells types constituting helpful microenvironment for hematopoiesis process.

Hematopoietic Stem Cell (HSC) niches are defined as cellular and molecular microenvironments that regulate stem cell function together with stem cell autonomous mechanisms. Many different cell types have been characterized as contributors to the formation of HSC niches, however, several constituents of HSC niches are unclear, and contribution by as yet undefined cells have been speculated.

Mendez-Ferrer and collaborators strongly suggested that nestin-positive mesenchymal stem cells (MSC) are spatially associated with HSC and highly expressed several factors for HSC maintenance. They also shown that nestin-positive MSC are able to grow as non adherent mesenspheres.

We recently demonstrated that mouse nestin-positive MSC was a mixed population mainly composed of neural crest stem cells (NCSC). Concerning spheres formation, we recently showed that non adherent spheres were an enriched population of neural crest stem cells (the same observations were carried out with rat bone marrow stromal cells by Shi and collaborators).

Based on these information, it is tempting to ask if NCSC are supporting HSC maintenance and proliferation by being part of HSC niches.

Background

The adult bone marrow is composed of two types of stem cells: **Hematopoietic stem cells** (HSC) are responsible for blood cells regeneration and **stromal stem cells** (or bone marrow stromal cells, BMSC) constitute a support for hematopoiesis and bone marrow (BM) homeostasis. This second population regroup two main types of cells which are mesenchymal stem cells (MSC) for the most part, and recently-discovered neural crest-derived stem cells (NCSC).

The *figures* in this part describe the procedure we followed so as to bring out NCSC in mouse bone marrow cell population. Clonal approach allowed us to characterize different NCSC specific markers in order to distinguish them from MSC : **Nestin, p75 ^{NTR} and Sox10**.

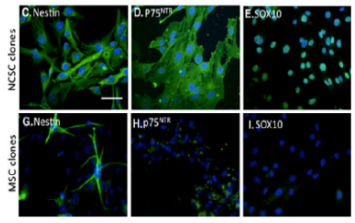


Fig. 1. Immunostaining of mouse mesenchymal and neural crest-derived clones using NCSC specific markers Nestin, p75^{NTR} and Sox10.

More interestingly, we have shown that only NCSC were able to form spheres in presence of EGF and bFGF.

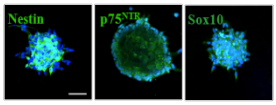
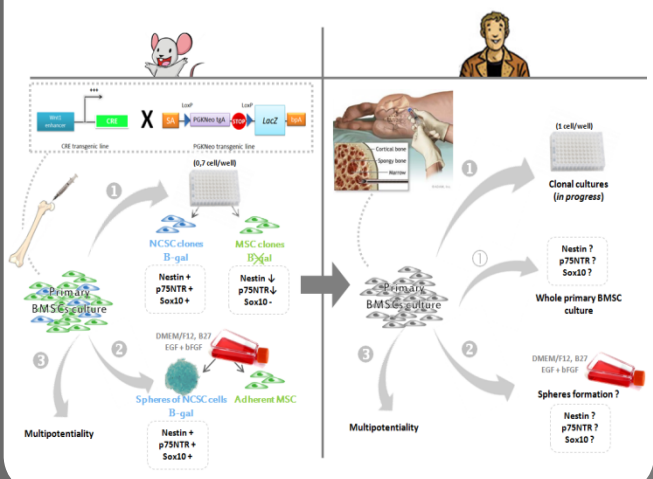


Fig. 2. Immunostaining performed on NCSC-derived spheres using NCSC specific markers.

MSC are able to differentiate into mesenchymal tissues like chondrocytes, adipocytes, osteocytes, smooth muscles, neuron-like cells and glial cells. NCSC can differentiate into almost the same panel of cell types; **however, they can generate melanocytes but are not able to generate adipocytes**.

Based on those different data, we will apply similar approaches on human adult bone marrow samples in order to assess the presence of NCSC inside the human bone marrow stroma.



NCSC in human bone marrow

❖ NCSC specific markers

While waiting for the obtention of clonal populations, immunostainings and RT-PCR experiments were performed on whole BMSC cultures in order to evaluate the expression of NCSC specific markers like Nestin, p75^{NTR}, and Sox10. Tuj1 staining (which recognize immature neuron-characteristic β3-tubulin) was also tested. We can conclude that all human BMSC express Nestin, Tuj1 and p75^{NTR} starting from low passages. However, they do not express Sox10 (even if low level of Sox10 expression was observed at the mRNA level) (*Figure 3* and *4*).

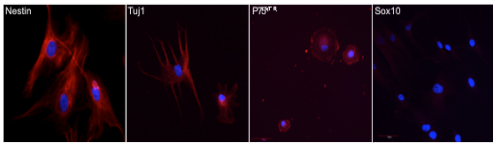


Fig. 3. Immunostaining of human BMSC using NCSC markers like Nestin, Tuj1, p75^{NTR} and Sox10.

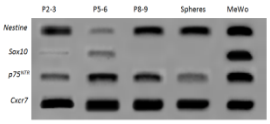
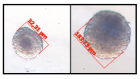
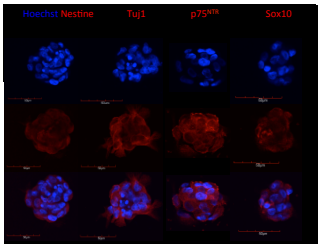


Fig. 4. RT-PCR performed with human BMSC at different passages and for NCSC specific markers.

❖ Sphere-forming ability

NCSC are described as cells able to grow as spheres in specific culture medium; which is a good tool in order to isolate NCSC from MSC which don't present this property.
Interestingly, human BMSC also present this property.
Indeed, **0,1 ± 0,03%** of human cells are able to grow as spheres (with a diameter of 30 to 180 µm) (*figure 5*).
Moreover, re-adherent primary/secondary spheres present an increasing growth potential in order to give rise secondary/tertiary spheres (graph below).
We thus carried out spheres' characterization using immunofluorescence (*figure 6*) and RT-PCR (*figure 4*).





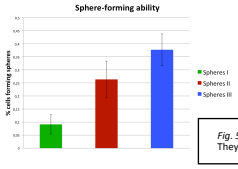


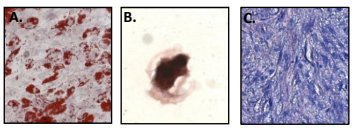
Fig. 5 and 6. NCSC spheres present a diameter range between 30 and 180µm. They are positive for NCSC markers Nestin, Tuj1 and p75^{NTR} but negative for Sox10.

The same markers were tested in RT-PCR. In *figure 4*, we observed that *Cxcr7* is strongly expressed by human BMSC and also by spheres.

❖ Differentiation potential of human BMSC

We applied diverse protocols to induce human BMSC differentiation into adipocytes, osteocytes and chondrocytes. *Figure 7* illustrates their multipotentiality.
Moreover, we are carrying out melanocyte and adipocyte differentiation experiments in spheres, to see if sphere-derived population present different abilities compared with whole BMSC.

Fig. 7. Differentiation experiments in BMSC population.
A . Adipocytes
B. Osteocytes
C. Chondrocytes



Perspectives

The main objective of this project will be to analyze the role of adult bone marrow NCSC in HSC niches and specifically in HSC maintenance and proliferation. Three axes will be developed in this project:

1) **Isolation and characterization of NCSC from human adult bone marrow.**
   - Isolation and characterization using clonal approach
   - Isolation and characterization using sphere-forming approach
   - Confirmation of neural crest identity by injection in avian embryo
2) **In Vitro characterization of NCSC support to HSC maintenance and proliferation.**
   - Co-culture of NCSC and HSC
   - NCSC secretome analyses and molecular characterization of HSC support
3) **In Vivo characterization of NCSC support to HSC maintenance and proliferation.**
   - Localization of NCSC in adult long bones
   - Cell transplantation in NOD/SCID mice