Human mulipotential mesenchymal/stromal stem cells are derived from a discrete subpopulation of STRO-1^{bright}/CD34$^-$/CD45$^-$/glycophorin-A- bone marrow cells

Magnetic and flow cytometry-based methods were used to characterize clonogenic stromal cells in human bone marrow. STRO-1^{bright} stromal cells were found to lack expression of CD34, CD45 and glycophorin-A markers associated with hematopoietic progenitor cells. These studies support the view that these are two distinct stem cell compartments in adult bone marrow.

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Recent studies have identified a mesodermal derived multipotent stem cell population with the capacity to develop into a diverse array of cell lineages representative of mesodermal (osetoblasts, chondrocytes, adipocytes, myoblasts, cardiomyocytes, endothelial cells), endodermal (hepatocytes) and ectodermal (neurons) derived tissues.[1,2] In addition, studies in rodent models indicate the existence of a common stem cell population from which both hematopoietic and stromal elements can develop.[3] It is possible, therefore, that the stromal, vascular and hematopoietic components of bone marrow (BM) are derived from a common multipotent stem cell. However, despite these findings, the existence of a multipotent stem cell or common stromal/hematopoietic stem cell still hasn't been satisfactorily demonstrated in adult human BM.[4]

Until now, the isolation of clonogenic BM stromal stem cells (BMSSC) has largely relied on traditional methodologies based on density gradient separation of mononuclear cell populations and their capacity to adhere to plastic when grown in media supplemented with fetal calf serum. More sophisticated studies have attempted to isolate and characterize BMSSC according to their expression of various markers. However, antibody reagents with restricted specificity for BMSSC have yet to be developed. We previously showed that clonogenic BMSSC are contained within the minor cell fraction of human BM that expresses high levels of the STRO-1 antigen (STRO-1^{bright}).[5,6] In the present study, we examined whether the STRO-1^{bright} BMSSC population could be further distinguished from their hematopoietic counterparts. We used normal adult human bone marrow aspirates (subject age 20-35) obtained with the subjects' informed consent under the approved guidelines of the Human Ethics Committee of the Royal Adelaide Hospital. Bone marrow mononuclear cells (BMMNC) were prepared by density gradient separation essentially as previously described.[5] The total STRO-1$^+$ BMMNC population was isolated using magnetic activated cell sorting (MACS) which essentially contained all the clonogenic BMSSC population (Figure 1A) as previously described.[5, 6] The STRO-1$^-$ fraction was then labeled with murine monclonal antibodies (IgG) specific to human CD45 and Glycophorin-A as previously described for the isolation of human multipotent adult progenitor cells (MAPC).[1] All cell fractions were then cultured under serum-deprived conditions which are known to stimulate the *in vitro* growth of both BMSSC[5, 7] and MAPC[1] in the presence of PDGF-BB and EGF (Figure 1A).

Data confirmed that the adherent stromal cells were only contained within the STRO-1$^+$ fraction and that no adherent cell growth was evident in the STRO-1$^-$, STRO-1$^-$/CD45$^-$/glycophorin-A$^-$ or STRO-1$^-$/CD45$^+$/glycophorin-A$^+$ fractions.

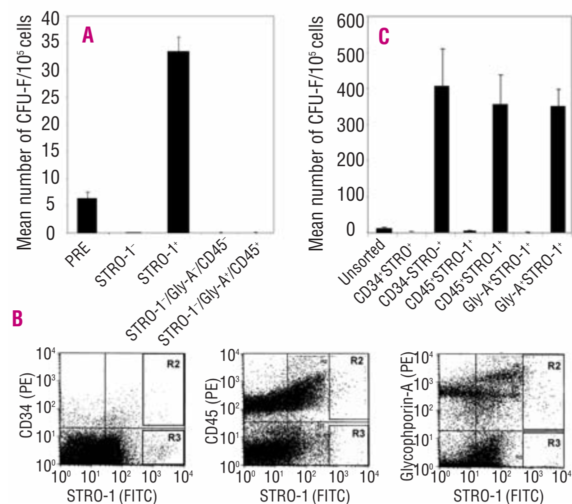

Figure 1. Clonogenic BMSSC are contained within the STRO-1$^+$ fraction. (A) Bone marrow mononuclear cells were isolated by MACS based on their expression of the STRO-1 antigen. The STRO-1$^-$ cell fraction was subsequently incubated with antibodies specific to CD45 and Glycophorin-A then re-selected by MACS. All cell populations PRE (unfractionated), STRO-1$^-$, STRO-1$^+$, STRO-1$^-$/CD45$^+$/Glycophorin-A$^+$ and STRO-1$^-$/CD45$^-$/Glycophorin-A$^-$ were plated under serum-deprived conditions as previously described.[4-6] Data are presented as the average number of day 14 CFU-F per 10^5 cells plated ± standard error of the mean from 4 different marrow donors. (B) Bone marrow mononuclear cells were isolated by MACS based on their expression of the STRO-1 antigen labeled with FITC. The STRO-1$^+$ cell fraction was subsequently incubated with antibodies specific to either CD34, CD45 or glycophorin-A labeled with PE. The histograms show the different STRO-1^{bright} subpopulations in region 2 (R2) STRO-1^{bright}/CD34$^+$, STRO-1^{bright}/CD45$^+$, STRO-1^{bright}/Glycophorin-A$^+$ and region 3 (R3) STRO-1^{bright}/CD34$^-$, STRO-1^{bright}/CD45$^-$, STRO-1^{bright}/glycophorin-A$^-$ that were isolated by FACS. (C) The different STRO-1^{bright} subpopulations isolated from regions were plated under serum-deprived conditions as previously described.[4-6] Data are presented as the average number of day 14 CFU-F per 10^5 cells plated ± standard error from 4 different marrow donors.

The immunophenotype of clonogenic BMSSC was then examined by subdividing the STRO-1^{bright} cell fraction, according to co-expression with either CD34, CD45 or Glycophorin-A using dual colour fluorescence activated cell sorting (FACS) (Figure 1B).[5,6] Each cell fraction STRO-1^{bright}/CD34$^+$, STRO-1^{bright}/CD34$^-$, STRO-1bright/CD45$^+$, STRO-1^{bright}/CD45$^-$, STRO-1^{bright}/glycophorin-A$^+$ or STRO-1^{bright}/glycophorin-A$^-$, was subsequently plated under serum deprived conditions as described above. As seen in Figure 1C, the STRO-1^{bright} clonogenic stromal precursor cells lacked expression of CD34, CD45 and Glycophorin-A. These findings are consistent with those of Reyes *et al.* who, using a negative selection strategy, reported that MAPC are also contained within the BMSSC/MSC fraction of bone marrow lacking expression of CD45 and Glycophorin-A.[1,8] Furthermore, our study also demonstrates that the lack of CD34, CD45 and Glycophorin-A expression by purified BMSSC is not attributed to an *in vitro* artefact. Importantly, the isolation methodology in the present study allows to distinguish between BMSSC and hematopoietic stem/progenitor populations. These findings support the view that at least two distinct stem cell compartments are present in aspirates of adult human BM.

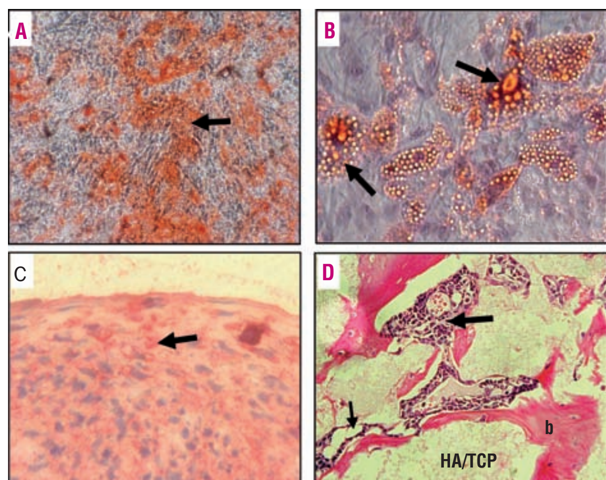

Figure 2. Developmental potential of purified BMSSC. Preparations of primary BMSSC cultures derived from either STRO-1^{bright}/CD34^{-}, STRO-1^{bright}/CD45-, or STRO-1^{bright}/glycophorin-A- FACS isolated cells were re-cultured either in osteogenic, adipogenic or chondrogenic inductive conditions as previously described.[4-6] After three weeks of multi-differentiation induction, the STRO-1^{bright}/CD34^{-}, STRO-1^{bright}/CD45^{-}, or STRO-1^{bright}/Glycophorin-A- cell populations all demonstrated the capacity to form (A) Alizarin positive mineral deposits (arrows), (B) Oil Red O positive lipid (arrows), and (C) a collagen type II matrix (arrows) at similar levels. Furthermore, cultured BMSSC established from either STRO-1^{bright}/CD34^{-}, STRO-1^{bright}/CD45^{-}, or STRO-1^{bright}/glycophorin-A- isolated cells also demonstrated the potential to form ectopic bone (b) with associated adipose tissue (small arrow) and hematopoietic marrow (large arrow) when transplanted with HA/TCP carrier into immunocompromised mice (D).

The multi-lineage differentiation potential of cultured BMSSC derived from each STRO-1^{bright}/CD34^{-}, STRO-1bright/CD45^{-} and STRO-1^{bright}/glycophorin-A^{-} cell fraction was subsequently examined both *in vitro* and *in vivo*. The sorted different STRO-1^{bright} cell populations were found to form mineral producing osteoblasts, lipid containing adipocytes and collagen type II synthesizing chondrocytes when cultured under osteogenic, adipogenic and chondrogenic inductive conditions *in vitro* respectively (Figure 2A-C) according to previously published methods.[5,6,9] Parallel studies demonstrated that the STRO-1^{bright}/CD34^{-}, STRO-1^{bright}/CD45^{-}, or STRO-1^{bright}/glycophorin-A^{-} cell populations were all capable of forming lamellar bone structures and vascularized fibrous tissue supporting adipose formation and a hematopoietic active BM, following subcutaneous transplantation with hydroxyapatite/tricalcium phosphate (HA/TCP) particles into immunocompromised mice (Figure 2D) as previously described.[5,6,10] Furthermore, there were no measurable differences in the growth potential or differentiation capacity of STRO-1^{bright}/CD34^{-}, STRO-1^{bright}/CD45^{-} and STRO-1^{bright}/glycophorin-A^{-} selected BMSSC populations. Therefore, it seems that a rare population of highly proliferative multipotent BMSSC, previously identified by clonal analysis[5, 9, 10] share a similar phenotype to the recently identified MAPC population. While the identification of different multipotent stem cells is encouraging for the future development of novel therapies for regenerative medicine, the role of these different stem cell populations in adult BM under steady state conditions, and the precise relationship between each cell type, must still be determined.

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