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Abdallah, Basem M; Al-Shammary, Asma; Skagen, Peter; Abu Dawud, Raed; Adyaye, James; Aldahmash, Abdullah; Kassem, Moustapha

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CD34 defines an osteoprogenitor cell population in mouse bone marrow stromal cells

Basem M. Abdallah a,c,⁎, Asma Al-Shammary a,¹, Peter Skagen a, Raed Abu Dawud d, James Adjaye b, Abdullah Aldahmash a, d, Moustapha Kassem a, d

a Molecular Endocrinology Laboratory (KMEB), Department of Endocrinology, Odense University Hospital and University of Southern Denmark, Odense, Denmark
b Institute for Stem cell Research and Regenerative Medicine, Faculty of Medicine, Heinrich Heine University, Duesseldorf, Germany
¹ Faculty of Science, Helwan University, Cairo, Egypt
b Stem Cell Unit, Department of Anatomy, College of Medicine, King Saud University, Saudi Arabia

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Bone marrow stromal cells (BMSCs, also known as bone marrow-derived mesenchymal stem cells) and their progenitors have been identified based on retrospective functional criteria. CD markers are employed to define cell populations with distinct functional characteristics. However, defining and prospective isolation of BMSCs and committed progenitors are lacking. Here, we compared the transcriptome profile of CD markers expressed at baseline and during the course of osteoblast and adipocyte differentiation of two well-characterized osteogenic-committed murine BMSCs (mBMSCBone) and adipogenic-committed mBMSCs (mBMSCAdipo), respectively. Bioinformatic analysis revealed the presence of a core set of canonical BMSC CD markers with comparable expression levels in mBMSCBone and mBMSCAdipo at baseline and during their differentiation. We identified 11 CD markers that are differentially expressed between mBMSCAdipo and mBMSCBone. Among these, we identified osteoprogenitor-associated CD markers expressed only in mBMSCBone, CD34, CD54, CD73, CD132, CD200, CD227 and adipoprogenitor-associated CD markers expressed only in mBMSCAdipo: CD53, CD80, CD134, CD141 and CD212. FACS analysis confirmed these results. We selected CD34 for further analysis. CD34 was expressed at baseline of mouse stromal cell line ST2, primary mBMSCs, mBMSCBone and its expression decreased during osteoblast differentiation. FACS-sorted CD34+ primary mBMSCs exhibited higher expression of 70% osteoblast-associated genes, and formed significantly higher heterotopic bone in vivo when implanted subcutaneously in immune-deficient mice compared with CD34− primary mBMSCs. Our results demonstrate that a set of CD markers can distinguish osteoprogenitor versus adipoprogenitor populations of mBMSCs. CD34 is suitable for prospective isolation of mouse bone marrow osteoprogenitors.

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1. Introduction

The stem cell subset contained within the bone marrow stromal cell population (BMSCs, also known as bone marrow-derived mesenchymal stem cells) is characterized by self-renewal capacity, clonogenicity in vitro, multipotent differentiation into mesoderm-type cells including osteoblast, adipocyte and chondrocyte and their ability to form bone and hematopoiesis-supporting stroma upon in vivo transplantation (Abdallah et al., 2005; Mabuchi et al., 2013). BMSCs are isolated based on adherence to the plastic surface of culture plates and the cells exhibited cellular and functional heterogeneity in culture. Thus, understanding cellular mechanisms of the beneficial therapeutic effects of BMSCs requires better definition of BMSCs in terms of phenotype and functional capacity. In this context, the minimal criteria (suggested by Tissue Stem Cell Committee of the International Society for Cellular Therapy) (Dominici et al., 2006) for defining BMSCs are based on a limited number of CD markers of variable sensitivity and specificity. Thus, there is a need for identifying CD markers that define BMSCs and their lineage committed populations. Such CD markers will be useful to use to isolate homogenous populations of BMSCs for clinical use.

CD surface markers have been used to isolate different populations of mBMSCs with differences in their functions. For example: PDGFRα+/Sca-1+/CD45−/TER119− (Morikawa et al., 2009), CD34+/CD73+ (Akiyama et al., 2012), CD105+ (Goussietis et al., 2005), CD271+/CD90+/CD106+ (Mabuchi et al., 2013), CD271+/CD140a+ (Buhring et al., 2007) and CD49a (Gronthos et al., 2001) have been reported to define BMSCs. However, these markers do not distinguish...
BMSC stem cells from their committed progeny. To study the cellular heterogeneity of BMSC cultures, we have employed in previous studies a single cell cloning approach. We have demonstrated that human BMSCs contain committed progenitors with lineage-specific osteoblast differentiation capacity and in vivo bone forming ability (Larsen et al., 2010). Similarly, we have isolated and characterized from mouse BMSCs, two homogenous BMSC cell lines with unipotent differentiation capacities of either osteoblasts or adipocytes: mBMSCBone and mBMSCAdipo, respectively (Post et al., 2008). Interestingly, both cell lines share a similar pattern of the known surface markers of BMSCs. Thus, these cells are good models to determine surface marker expression associated with lineage commitment, using global methods of transcriptomic analysis or proteome analysis.

In the present study, we aimed at identification of BMSC lineage specific CD markers. We conducted a DNA microarray expression analysis and compared the global CD marker transcription profile of mBMSCBone and mBMSCAdipo during their differentiation course into osteoblasts and adipocytes, respectively. Data obtained from these two homogenous populations of mBMSCs enabled us to identify a set of CD markers that define osteoblast progenitor and adipocyte progenitor phenotype. Follow-up studies identified CD34 as a prospective marker suitable for isolating homogenous population of osteoblast progenitors directly from cultured mBMSCs with capacity for in vitro osteoblast differentiation and in vivo bone formation.

2. Materials and methods

2.1. Cell culture

Isolation and phenotypic characterization of mouse bone marrow derived spontaneously immortalized clonal cell strains mBMSCAdipo and mBMSCBone have been described previously (Post et al., 2008). Cells were cultured in DMEM (Gibco Invitrogen, USA) supplemented with 12% fetal bovine serum (FBS; Gibco Invitrogen, USA) and 1% penicillin/streptomycin (P/S) (Gibco Invitrogen, USA).

Mouse stromal cell line ST2 was obtained from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (ACC 333, Braunschweig, Germany). Cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (P/S) (all purchased from Gibco Invitrogen, USA) (Otsuka et al., 1999). Mouse bone marrow stromal cells (mBMSCs) were isolated from wild-type 8-week-old C57BL mice using a previously published centrifugation protocol (Peister et al., 2004). Cells were plated at a density of 1 × 10^6 cells/cm² in RPMI medium supplemented with 12% fetal bovine serum (FBS; Gibco Invitrogen, USA) and 1% penicillin/streptomycin (P/S) (Gibco Invitrogen, USA). After 48 h of culture, medium was removed, cells were washed with PBS and fresh medium was added.

2.2. Adipocyte differentiation

Cells were plated at 15,000 cells/cm² and cultured for 12 days in adipogenic-induction medium (AIM; DMEM supplemented with 9% horse serum, 450 μM 1-methyl-3-isobutylxanthine (IBMX), 250 nM dexamethasone, 5 μg/mL insulin (Sigma-Aldrich) and 1 μM rosiglitazone (BRL 49653, Cayman Chemical, Ann Arbor, Michigan)). The media was changed every three days.

2.3. Osteoblast differentiation

Osteoblastic differentiation was performed on cells plated at 15,000/cm² for 12 days in osteoblast-induction media (OB) containing αMEM supplemented with 10 mM beta-glycerophosphate (Calbiochem-Merck, Germany), 50 μg/mL l-ascorbic acid-2-phosphate (Wako Chemicals GmbH, Germany) and 10 nM dexamethasone (Sigma-Aldrich, Denmark). The medium was changed every three days during induction.

2.4. Ectopic bone formation in mice

To study the in vivo osteogenic capacity of sorted CD34+ or CD34− sub-populations, 5 × 10^6 cells were mixed with 40 mg hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder (Zimmer Scandinavia Albertslund, Denmark) and implanted subcutaneously in the dorsal surface of 2-month-old female in NOD/MrkBomTac-Prkdcscid mice (Taconic, Ry, Denmark). Implants (n = 4 implants/cell line) were recovered 8 weeks after transplantation, demineralized in EDTA solution (2.5% W/V), pH = 7.1, paraffin embedded, sectioned, and stained by eosin/hematoxylin as described previously (Justesen et al., 2004). Four μm sections were cut and stained with hematoxylin and eosin (H&E) (Biëlbørntens), The total bone volume per total volume was quantified as described previously (Abdallah et al., 2008).

2.5. Oil Red O staining

At day 12 of adipocyte induction, cells were fixed in 4% paraformaldehyde for 10 min at room temperature then stained with Oil Red O (Sigma, USA) to visualize the lipid content and droplet size. Briefly, cells were rinsed in 3% isopropanol solution and stained with filtered Oil Red O solution (0.5 g in 100% isopropanol) for 1 h at room temperature. Lipid accumulations were quantified by elution of Oil Red O in absolute isopropanol for 10 min at room temperature. The absorbance of the extracted dye was detected at 490 nm using a FLUO star Omega plate reader (BMG Laboratories, Denmark).

2.6. Alkaline phosphatase (ALP) staining

Cells were fixed with acetone/10 mM citrate buffer pH 4.2 (1:5:1 ratio) at room temperature for 5 min and incubated for 1 h at room temperature with ALP substrate staining solution containing 0.2 mg/mL Naphthol-AS-TR-phosphate dissolved in distilled water (1:5) and 0.417 mg/mL Fast Red dissolved in 0.1 M Tris buffer.

2.7. Alizarin red S staining (AR-S)

Calcium deposition, at day 12 of osteoblastic differentiation, was measured using Alizarin red staining. Osteoblasts were fixed with 70% ice-cold ethanol for 1 h at −20 °C before addition of AR-S (40 mM; Sigma-Aldrich) dissolved in distilled water, pH 4.17. The cells were stained for 10 min at room temperature. The level of calcium deposition was quantified by elution of AR-S following incubation in 10% cetylpyridinium chloride (Sigma-Aldrich) for 1 h at room temperature. The absorbance of the eluted dye was assessed at 570 nm in a FLUO Star Omega plate reader.

2.8. Microarray based gene expression analysis

mBMSCAdipo and mBMSCBone cells were cultured in triplicate under standard culture conditions as described above and differentiated into either adipocyte or osteoblast respectively. Total RNA were isolated at day 0, 1, 3, 6 and 9 during differentiation course. To perform global gene expression analysis, 500 ng quality-checked total RNA per sample in triplicate were amplified and labeled according to the manufacturer’s protocol (Illumina TotalPrep RNA Amplification Kit, Ambion, Austin, TX, USA,www.ambion.com). The resulting biotinylated cRNA was purified and hybridized to MouseRef-8 v2.0 Expression BeadChip (Illumina, San Diego, CA, USA, www.illumina.com) on the Illumina Beadstation 500 platform, followed by washing, blocking, staining with streptavidin-Cy3 and quantitative detection of the fluorescent image of the array as specified by the manufacturer. Raw data were processed using the Gene Expression Module version 1.8.0 provided with the GenomeStudio software (Illumina). This included background subtraction and normalization according to the ‘rank invariant’ algorithm. Genes were considered “expressed” if the
Corresponding 'Detection P-Value' given by the GenomeStudio software p<0.01. Differential gene expression analysis was computed using the 'Illumina Custom Model'. P-values of differentially expressed genes ('Diff P-Values') modified by the Benjamini and Hochberg false discovery rate (FDR) correction algorithm (Benjamini and Hochberg, 1995). Genes with 3 fold up- or down-regulated average signal intensities between the samples or groups of samples of interest were considered "differentially expressed" genes if the corresponding FDR-adjusted 'Diff P-Value' padj <0.05 and provided that the gene of interest was expressed in at least one of the samples under consideration.

2.9. Real-time polymerase chain reaction (RT-PCR)

RNA was extracted using TRIzol (Life Technologies; according to the manufacturer's instructions) and the first strand complementary DNA was synthesized from 1 μg of total RNA using a Revert Aid™ H minus first strand cDNA synthesis kit (Fermentas, Sweden). RT-qPCR was performed using an ABI StepOne™ Real-TIME PCR machine (Life Technologies/Applied Biosystems) with SYBR green (Applied Biosystems). The targeted primers and reference genes are shown in supplementary Table 1. The data were normalized to the geometric means of the reference genes β-actin and HPRT and analyzed using a comparative CT method where ΔΔ-CT is the difference between the CT values of the target and the geometric mean of the reference genes.

2.10. PCR array analysis

Total RNA was extracted from both sorted CD34+ and CD34- mBMSCs at baseline. Osteogenic RT² Profiler™ PCR array, containing 84 osteoblast-related genes (Qiagen Nordic), was performed for each sample in triplicates using SYBR® Green quantitative PCR method.

2.11. Flow cytometry for cells surface markers characterization

Cells were profiled for surface markers to identify their immunophenotype. Each cell type was single cell suspended and, incubated in FACS buffer containing pre-conjugated antibodies (see supplementary Table 2) for 20 min on ice. Following incubation, cells were washed twice with FACS buffer. All the flow cytometry performed using a Cell Lab Quanta SCMPL. The data were analyzed using Kaluza®1.2 software (Beckman Coulter, Denmark) and the expression of each CD markers on the cells was calculated based on the percentage and mean fluorescence intensity (MFI).

2.12. Fluorescence activated cell sorting (FACS)

mBMSCs in cell suspension washed with PBS−/− (Life Technologies). Cells were stained with pre-conjugated CD34-PE antibody for 20 min on ice. Non-specific binding was blocked by incubation in FACS buffer (Life Technologies) containing 0.5% BSA and 40 mM EDTA, (Sigma-Aldrich, Denmark). Following incubation, the unbound antibodies were removed using 3 ml wash of FACS buffer. The cells were sorted into 2 separate populations (CD34+ and CD34−) using a FACS Aria™ III (BD Biosciences, Denmark). Sorting gates were established based on the appropriate isotype control for CD34 antibody (supplementary Table 2). The sorted cell populations were then re-cultured for further experiments.

2.13. Alkaline phosphatase (ALP) activity assay

ALP activity was performed on at d6 of osteoblastic induction. Cell viability was determined using the Cell Titer-Blue cell viability assay according to the manufacturer’s instructions (Promega, USA) and the viability measured at 579/584 nm using a FLUO star Omega plate reader (BMG Laboratories). ALP activity was determined following incubation with 1 mg/mL of P-nitro phenyl phosphate in 50 mM NAHCO3 and 1 mM MgCl2 buffer (pH 9.6) at 37 °C for 20 min. The activity was stopped by addition of 3 M NaOH. The reaction absorbance was measured at 405 nm using a FLUO star Omega plate reader (BMG Laboratories) and ALP activity corrected for cell viability.

2.14. Cell morphology analysis by high content imaging

Cells were seeded in 96-cell carrier well-plates (PerkinElmer, Germany) and cultured in standard medium to assess differences in their morphology. Following one day of culture, the adherent cells were washed with PBS−/−, fixed in 4% Paraformaldehyde for 10 min at room temperature and washed three times with PBS. Cells were incubated with 1 μg/mL Alexa Fluor 568 Phalloidin (Molecular Probes, USA) for 40 min and washed twice with PBS. For nuclear visualization, cells were incubated in 1 μg/mL of DAPI (Sigma, USA) for 10 min at room temperature. The imaging of the cells and all the parameters analyzed were performed using an Operetta High Content Screening system (Perkin-Elmer, Germany). Images were analyzed using Harmony® High Content Imaging Software (PerkinElmer).

2.15. Statistical analysis

All experiments were performed in triplicate and at least 3 independent experiments were conducted. The data are presented as the mean ± SD. The statistical analysis was performed using Prism 5 (GraphPad). Student’s t-test was used for comparison between two groups and one way ANOVA was used for experiments with more than two groups. Differences were considered statistically significant at *p < 0.05, **p < 0.005 and ***p < 0.0001.

3. Results

3.1. Identification of predictive osteoblast and adipocyte CD markers

To identify CD markers associated with MSC lineage-commitment to osteoblasts and adipocytes, we employed the transcriptome profiles of two previously characterized committed osteogenic and adipogenic populations derived from mBMSCs: mBMSCBone and mBMSCAdipo, respectively (Post et al., 2008). As shown in Fig. 1A, the osteoblastic differentiation of mBMSCBone was evidenced by the ability of the cells to form mineralized matrix stained positive with Alizarin red as well as in vivo heterotopic bone. The adipogenic commitment of mBMSCAdipo was evidenced by the ability of the cell to form mature lipid filled adipocytes stained by Oil red O and the absence of heterotopic bone formation when implanted in vivo. A more detailed description of the two cell lines have been previously published (Post et al., 2008). Microarray based gene expression analysis was performed at different time points during the course of osteoblast and adipocyte differentiation of mBMSCBone and mBMSCAdipo, respectively. More than 80% (p < 0.005) of the up-regulated genes during osteoblast differentiation of mBMSCBone and 60% (p < 0.005) of the upregulated genes during adipocyte differentiation of mBMSCAdipo, were annotated according to their molecular function, as osteogenic or adipogenic-related genes, respectively (supplementary Tables 3 & 4). From the whole data set, we identified all the CD markers expressed by mBMSCBone (71 CD markers) and mBMSCAdipo (70 CD markers). Comparing the expression levels of CD markers at baseline in mBMSCBone versus mBMSCAdipo revealed the expression of a number of canonical CD markers characteristic of BMSCs to be expressed at similar level in both cell lines (Table 1). Some of these CD markers are known markers for BMSCs e.g. CD10, CD29, CD63, CD146, CD140a and some are new markers e.g. CD47, CD59, CD203, CD227. To identify a panel of CD markers associated with commitment to osteoblastic and adipogenic lineages, we selected CD markers that are expressed either in mBMSCBone or mBMSCAdipo during differentiation. Using this criterion, we identified CD markers that can possibly define osteoblastic progenitors (Fig. 1B) or adipocytic progenitors (Fig. 1C).
We verified the expression of these markers by performing FACS analysis for selected CD markers in mBMSCBone, mBMSCAdipo and primary cultured mBMSCs. As shown in Fig. 2 A&B, FACS analysis confirmed the presence of Sca-1, CD29, CD45 and CD140a in mBMSCBone, mBMSCAdipo and mBMSCs. Consistent with the transcriptome data (Fig. 1B), FACS analysis revealed higher expression of CD34 and CD200 by mBMSCBone versus mBMSCAdipo (Fig. 2A&B). Both CD34 and CD200 were expressed by primary mBMSCs. However, the levels of expression as estimated by MFI were 6 fold higher for CD34 compared with CD200. Based on these data, we chose CD34 for further studies.
Thereafter, we carried out FACS sorting of CD34 is expressed in osteoprogenitors and not mature osteoblastic cells. Both CD34 marker expression, cellular morphology and differentiation potential. CD34 related genes in CD34+ and CD34− real time PCR-based array analysis of the expression of 84 osteoblast-differentiation of mouse stromal cell line ST2, mBMSCBone and primary in CD34+ cells compared with CD34−Gene expression levels of early ( Runx2, Osterix) and late ( Pparγ and C/ebpα) and late ( p02, Apm1, Lpl) adipogenic markers as compared to CD34+ BMSCs.

### 4. Discussion

It is increasingly appreciated that cultured primary BMSCs are heterogeneous with respect to their differentiation potential and that these cultures contain multipotent BMSCs as well as lineage committed precursors (Post et al., 2008). Defining the cellular and molecular phenotype of lineage committed progenitors has been hampered by the absence of appropriate cell models for studying their biology. In the present study, we employed two committed progenitor cells that have been extensively characterized in our laboratory. Their progenitor phenotype is based on distinct cellular morphology and lineage restricted differentiation capacity in vitro and in vivo (Post et al., 2008) (Taipaleenniemi et al., 2011). Employing these two cell models, we identified a number of core CD markers that define mBMSC and CD markers associated with lineage commitment to osteoblastic or adipocytic cells. In addition, we demonstrated a strategy whereby one of these markers: CD34 can be employed to isolate prospectively a population of osteoprogenitors. Our studies demonstrate the feasibility of this approach to dissect the cellular heterogeneity of cultured BMSCs.

We identified a number of CD markers that are expressed in both osteoprogenitor and adipoprogenitor cells and thus can be employed as core markers of the BMSCs phenotype. Our data corroborate previous studies that employed alternative technologies to identify markers for multipotent BMSCs. Comparing gene expression and FACS analysis of osteoprogenitor and adipoprogenitor cells, we have identified CD markers that are exclusively associated with either the osteoblastic or adipocytic phenotype. The adipocytic phenotype-associated CD markers included CD53, CD80, CD141, CD134 and CD212. Only CD141 has previously been demonstrated to be expressed in human adipose tissue (Rymanen et al., 2014). On the other hand, osteoblastic phenotype-associated CD marker included CD54, CD34, CD200, CD132 and CD227. Among these, CD45, CD73, CD34 and CD200 markers have previously been reported to be expressed in osteoblasts (Reyes-Botella et al., 2000; Takedachi et al., 2012; Ode et al., 2013; Delorme et al., 2008). Reyes-Botella et al reported the expression of CD54 in cultured human osteoblasts isolated from normal mandibular bone. Human osteoblasts express CD73 and human periodontal osteoblasts stain positively for CD73 (Ode et al., 2013). Cell sorting of CD34+/CD34−cells from human BMSCs identify an osteoprogenitors that has the ability to differentiate into functional osteoblasts in culture (Chen et al., 1997). Additionally, CD200+ cells loaded on biphasic calcium phosphate (MBCP) ceramic disks and implanted subcutaneously in nude mice, showed higher ability for bone formation compared with unsorted cells (Delorme et al., 2008). The biological role of CD132 and CD227 markers are not known.

We have identified CD34 as a potential marker for murine osteoprogenitor cells. CD34 is a cell surface glycoprotein that functions as an adhesion molecule and is commonly expressed on early hematopoietic stem cells (HSC) and their progeny as well as vascular endothelial cells (Krause et al., 1996; Muller et al., 2002). CD34 expression has been detected in a subset of human bone marrow stromal cells.

### Table 1

List of commonly expressed CD markers in mBMSCbone and mBMSCadipex cell lines.

<table>
<thead>
<tr>
<th>CD marker</th>
<th>Alternative name</th>
<th>Full name</th>
</tr>
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<tbody>
<tr>
<td>CD1d1</td>
<td>CD1d1 antigen</td>
<td>CD1d1</td>
</tr>
<tr>
<td>CD9</td>
<td>CD9 antigen</td>
<td>CD9</td>
</tr>
<tr>
<td>CD10</td>
<td>Membrane metallo-endopeptidase</td>
<td></td>
</tr>
<tr>
<td>CD29</td>
<td>Integrin beta 1 (fibronectin receptor beta)</td>
<td></td>
</tr>
<tr>
<td>CD47</td>
<td>Integrin associated protein</td>
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</tr>
<tr>
<td>CD49a</td>
<td>Integrin alpha 1</td>
<td></td>
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<tr>
<td>CD49e</td>
<td>Integrin alpha 5 (fibronectin receptor alpha)</td>
<td></td>
</tr>
<tr>
<td>CD59a</td>
<td>CD59 antigen</td>
<td></td>
</tr>
<tr>
<td>CD63</td>
<td>Lysosomal-associated membrane protein</td>
<td></td>
</tr>
<tr>
<td>CD71</td>
<td>Transferrin receptor protein 1</td>
<td></td>
</tr>
<tr>
<td>CD81</td>
<td>Target of the antiproliferative antibody 1</td>
<td></td>
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<tr>
<td>CD87</td>
<td>uPA-R</td>
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<td>CD97</td>
<td>CD97 antigen</td>
<td></td>
</tr>
<tr>
<td>CD104</td>
<td>Integrin, beta 4</td>
<td></td>
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<tr>
<td>CD108</td>
<td>VCAM-1</td>
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<tr>
<td>CD109b</td>
<td>LAMP-2</td>
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<tr>
<td>CD112</td>
<td>Poliovirus receptor-related 2 (herpesvirus entry mediator B)</td>
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<td>CD123</td>
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<tr>
<td>CD138</td>
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<td>CD140a</td>
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<tr>
<td>CD140b</td>
<td>Platelet-derived growth factor receptor, beta polypeptide</td>
<td></td>
</tr>
<tr>
<td>CD146</td>
<td>Melanoma cell adhesion molecule</td>
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</tr>
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<td>CD151</td>
<td>CD151 antigen</td>
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<td>CD164</td>
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</tr>
<tr>
<td>CD203c</td>
<td>Ectonucleotide pyrophosphatase/phosphodiesterase</td>
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<td>CD222</td>
<td>Insulin-like growth factor 2 receptor</td>
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<td>CD248</td>
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CD34+ mBMSCs exhibited enhanced osteoblastic differentiation capacity compared with CD34− cells or non-sorted parental primary mBMSCs as assessed by increased levels of ALP activity and formation of mineralized matrix visualized by Alizarin red staining (Fig. 4A&B). Gene expression levels of early (Runx2, Msx2, Dlx5, and Alp) and late osteoblastic markers (Oc, Omp and Bsp) were significantly up-regulated in CD34+ cells compared with CD34−cells (Fig. 4C). Furthermore, real time PCR-based array analysis of the expression of 84 osteoblast-related genes in CD34+ and CD34− cells at baseline revealed up-regulation of 70% of osteogenic genes in CD34+ cells compared with CD34− cells (3 folds, p < 0.05) (supplementary Table 5). This confirms enrichment in the osteoblastic molecular signature in CD34+ cells. We also implanted both CD34+ cells and CD34−cells; mixed with hydroxyapatite-tricalcium phosphate subcutaneously in immune-deficient mice for two months. No hematopoietic-supportive stroma was observed in the implants (Fig. 4D). Quantitative histological analysis of implants revealed 11.6 fold increase in the heterotopic bone formed by CD34+ cells compared to CD34− cells (p < 0.005) (Fig. 4D).

### 3.4. Reduced adipogenic potential of sorted CD34+ mBMSCs

Adipocyte differentiation was evaluated by determining the number of oil red O stained mature lipid filled adipocytes as well as gene expression levels of adipogenic markers. As shown in Fig. 5A&B, CD34+ BMSCs exhibited reduction in the number of mature adipocytes formed (p < 0.0001) as well as significant reduction in early (Pparγ and C/ebpα) and late (p02, Apm1, Lpl) adipogenic markers as compared to CD34− BMSCs.

#### 3.2. Identification of CD34 as a marker for osteoprogenitors

We first investigated mRNA expression of CD34 during osteoblast differentiation of mouse stromal cell line ST2, mBMSCbone and primary mBMSCs. In all three BMSCs cell lines, CD34 was found to be down regulated during osteoblast differentiation (Fig. 2C), suggesting that CD34 is expressed in osteoprogenitors and not mature osteoblastic cells. Thereafter, we carried out FACS sorting of CD34+ and CD34− populations from cultured primary BMSCs (Fig. 3A) and compared their CD marker expression, cellular morphology and differentiation potential. Both CD34+ and CD34− populations expressed comparable levels of Sca-1 and CD140a, while CD146 was exclusively expressed by CD34− cells (Fig. 3B). Quantitative cell morphology using high content imaging analysis revealed a larger cytoplasmic area and reduced roundness of CD34+ cells compared to CD34− (Fig. 3C).

#### 3.3. Increased osteogenic potential of CD34+ mBMSCs in vitro and in vivo

CD34+ mBMSCs exhibited enhanced osteoblastic differentiation capacity compared with CD34− cells or non-sorted parental primary mBMSCs as assessed by increased levels of ALP activity and formation of mineralized matrix visualized by Alizarin red staining (Fig. 4A&B). Gene expression levels of early (Runx2, Msx2, Dlx5, and Alp) and late osteoblastic markers (Oc, Omp and Bsp) were significantly up-regulated in CD34+ cells compared with CD34− cells (Fig. 4C). Furthermore, real time PCR-based array analysis of the expression of 84 osteoblast-related genes in CD34+ and CD34− cells at baseline revealed up-regulation of 70% of osteogenic genes in CD34+ cells compared with CD34− cells (3 folds, p < 0.05) (supplementary Table 5). This confirms enrichment in the osteoblastic molecular signature in CD34+ cells. We also implanted both CD34+ cells and CD34− cells; mixed with hydroxyapatite-tricalcium phosphate subcutaneously in immune-deficient mice for two months. No hematopoietic-supportive stroma was observed in the implants (Fig. 4D). Quantitative histological analysis of implants revealed 11.6 fold increase in the heterotopic bone formed by CD34+ cells compared to CD34− cells (p < 0.005) (Fig. 4D).
human placental stromal cells (Garcia-Pacheco et al., 2001) and human adipose tissue derived stromal cells (ADSCs) (Mitchell et al., 2006). Interestingly, cultured human BMSCs were considered negative for CD34 expression by the International Society for Cellular Therapy (Dominici et al., 2006). However, several lines of evidence suggest that CD34 defines a sub-population of BMSCs. First, studies that reported negative expression of CD34 in BMSCs examined cultured BMSCs on plastic surfaces (Lin et al., 2012) which may explain the loss of CD34 expression as it has been reported in cultured expanded BMSCs from human bone marrow (Kaiser et al., 2007), adipose tissue (Planat-Benard et al., 2004) and thymus (Mouiseddine et al., 2008). Second, sorted CD34+ from freshly isolated human bone marrow showed high frequency for colony-forming unit-fibroblast (CFU-F) formation which is a functional marker of multipotent BMSCs (Kaiser et al., 2007). Third, the development of Stro-1 antibody that identifies multipotent human BMSCs, was developed based on immunization with human CD34+ BMSC fraction (Simmons and Torok-Storb, 1991; Ning et al., 2011). Finally, our own data presented in the current manuscript demonstrate that CD34+ is enriched in committed osteoprogenitors. Previous studies and our own

Fig. 2. Expression of CD34 by mBMSCs. CD marker profile of mBMSC cell lines including, primary mBMSCs, mBMSCBone and mBMSCAdipo. (A) FACS analysis of Sca-1, CD29, CD34, CD73, CD105, CD45, CD140a and CD200 was performed at baseline. (B) The mean fluorescence intensity (MFI) of the expressed CD markers. (C) Real time PCR analysis of CD34 mRNA expression during osteoblast differentiation of primary mBMSCs, mBMSCBone and stromal cells ST2. Data are mean ± SD of three independent experiments. ns = not significant, "p < 0.05, ""p < 0.005 and """"p < 0.0001 compared to primary mBMSCs. #p < 0.05, and ###p < 0.0001 as compared between mBMSCBone and mBMSCAdipo.
demonstrate that CD34 expression is rapidly down regulated in cultured BMSCs and thus it is more suitable for isolating osteoprogenitors from freshly isolated bone marrow mononuclear cells (Sato et al., 1999; Zanjani et al., 1998; Cao et al., 2005).

Comparing CD markers expression between CD34+ and CD34− mBMSCs revealed similar pattern of expression of CD markers known to be expressed by BMSCs. Interestingly, CD146 was enriched in CD34+ cells. CD146 has been identified as a stemness marker for

Fig. 3. CD34 as a selective marker for osteoprogenitors cells. FACS cell sorting of CD34− and CD34+ derived from primary mBMSCs. (A) Gating strategy used in sorting the negative and the positive populations of CD34. The gates were set according to the isotype control of CD34 Ab. (B) FACS analysis of the non-sorted primary mBMSCs, sorted CD34− and CD34+ for known BMSC markers. The isotype controls represented in red line histogram, dark blue for the non-sorted mBMSCs, green for CD34− and light blue for CD34+. (C) Cell morphology analysis by high content imaging Operetta® system of the sorted populations compared with the non-sorted cells. Scale bars indicate 100 μm. Data are mean ± SD of three independent experiments. ns = not significant, *p < 0.05, **p < 0.005 and ***p < 0.0001 compared to non-sorted cells. #p < 0.05 and ##p < 0.005 as compared between CD34+ and CD34− cell populations.
isolating human BMSCs with high osteogenic capacity in vitro and in vivo (Sorrentino et al., 2008; Sacchetti et al., 2007; Ulrich et al., 2015). We have previously shown that CD146 is enriched in hBMSCs clones with high bone forming capacity (Larsen et al., 2010). Interestingly, CD146 failed to isolate cells with the BMSCs phenotype (Chou et al., 2012). Thus, our data suggest CD146 expression is not sufficient to identify murine BMSCs, but it can be employed as an additional CD marker, in combination with CD34 to isolate bone marrow osteoprogenitors.

CD34+ BMSCs formed heterotopic bone in vivo which corroborate their enrichment in osteoprogenitors. While in the present study, we employed CD34 as a marker that defines a specific lineage within BMSCs, it is plausible that CD34 plays a role in osteoblast differentiation. CD34 plays an important role in hematopoietic stem cell (HSC) biology as demonstrated in CD34 deficient mice that exhibits enhanced proliferation and blocked differentiation of hematopoietic progenitor cells (Nielsen and McNagny, 2008). Few studies have investigated the role of CD34 in BMSCs biology (Lin et al., 2012). Comparing CD34−
versus CD34+ cells derived from cultured murine BMSCs revealed a correlation between CD34 expression and vasculogenic and angiogenic capacity in vivo (Copland et al., 2008). Also, in humans, the angiogenesis-related genes were significantly enriched in CD34+ cells (Suga et al., 2009).

5. Conclusions

We performed global microarray analysis to compare the transcription profile of CD markers expression during the course of osteoblast and adipocyte differentiation of our previously established osteogenic-committed mBMSCs (mBMSCBone+) versus adipogenic-committed mBMSC (mBMSCAdipo−) respectively. Our data provide a set of CD markers that distinguish osteoprogenitor versus adipoprogenitor populations derived from mBMSCs. Among these markers, we have identified CD34 as a potential single surface marker for isolating a homogenous population of osteoprogenitors from mouse BMSCs.

**Conflicts of interest**

The authors declare that they have no conflicts of interests.

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**Appendix A Supplementary data**

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.scr.2015.09.005](http://dx.doi.org/10.1016/j.scr.2015.09.005).
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