Differential Expression of Surface Markers in Mouse Bone Marrow Mesenchymal Stromal Cell Subpopulations with Distinct Lineage Commitment

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Abstract

Bone marrow mesenchymal stromal cells (BM MSCs) represent a heterogeneous population of progenitors with potential for generation of skeletal tissues. However the identity of BM MSC subpopulations is poorly defined mainly due to the absence of specific markers allowing in situ localization of those cells and isolation of pure cell types. Here, we aimed at characterization of surface markers in mouse BM MSCs and in their subsets with distinct differentiation potential. Using conditionally immortalized BM MSCs we performed a screening with 176 antibodies and high-throughput flow cytometry, and found 33 markers expressed in MSCs, and among them 3 were novel for MSCs and 13 have not been reported for MSCs from mice. Furthermore, we obtained clonally derived MSC subpopulations and identified bipotential progenitors capable for osteo- and adipogenic differentiation, as well as monopotential osteogenic and adipogenic clones, and thus confirmed heterogeneity of MSCs. We found that expression of CD200 was characteristic for the clones with osteogenic potential, whereas SSEA4 marked adipogenic progenitors lacking osteogenic capacity, and CD140a was expressed in adipogenic cells independently of their efficiency for osteogenesis. We confirmed our observations in cell sorting experiments and further investigated the expression of those markers during the course of differentiation. Thus, our findings provide to our knowledge the most comprehensive characterization of surface antigens expression in mouse BM MSCs to date, and suggest CD200, SSEA4 and CD140a as markers differentially expressed in distinct types of MSC progenitors.

Introduction

Bone marrow stroma is a complex tissue consisting of many cell types, which provide a microenvironment for haematopoiesis and also contribute to the maintenance and regeneration of skeletal tissues [1,2,3]. Perturbed function of stromal tissue in humans can cause severe defects of skeletal system [4,5,6,7] or dysregulation of haematopoiesis resulting in myelodysplasia and acute myeloid leukemia [8,9]. On the other hand, capacity of stromal cells to differentiate into osteoblasts and chondrocytes makes them an important source for tissue engineering [10,11] and provided differentiation of mesenchymal stromal cells (MSCs). The isolation of pure cell types from bone marrow stroma and their in vivo localization is hindered by the lack of known specific surface markers for those cells. To date only a combination of markers can define stem/stromal cells from bone marrow, and none of them is unique. In humans, CD146 [MCAM] has been proposed as a marker for osteogenic cells in stromal tissue capable of establishment of haematopoietic microenvironment, which together meet the definition of mesenchymal stem cell [14]. However, current methods of stromal cell isolation do not allow derivation of a pure population of stem cells and therefore cultured bone marrow stromal cells represent a mixture of their descendants, including progenitors of different types, hereafter referred as mesenchymal stromal cells (MSCs).

The isolation of pure cell types from bone marrow stroma and their in vivo localization is hindered by the lack of known specific surface markers for those cells. To date only a combination of markers can define stem/stromal cells from bone marrow, and none of them is unique. In humans, CD146 [MCAM] has been proposed as a marker for osteogenic cells in stromal tissue capable of establishment of haematopoietic microenvironment, which together meet the definition of mesenchymal stem cell [14].

Several approaches have been developed for in situ localization and lineage tracing of mesenchymal stem cells using mouse transgenic models. It has been shown that in the bone marrow perivascular cells expressing nestin-GFP reporter constitute the

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haematopoietic niche and contribute to skeletal tissues in lineage-tracing experiments [16]. Recently, Mx1-Cre has been proposed as a marker capable to label the cells that produce osteoblastic lineage life-long in the mice [17]. It was also noted, that Mx1-labelled cells only partially overlap with nestin positive population showing that cellular composition within those pools, each of which display mesenchymal stem cell properties, can be still complex.

Expression of surface antigens in cultured human bone marrow MSCs has been intensively studied by staining with existing antibodies [18,19,20], using proteomic approaches [21,22,23,24] and by analysis of their transcriptome [25,26]. In these works it has been noted that the levels of expression of some surface markers correlates with differentiation capacity of MSC. For example, CD106 and MSCA-1 mainly mark the adipogenic progenitors in cultured human MSCs, whereas ITGA11 is expressed on the cells with osteogenic capacity [20,26,27].

Mouse bone marrow MSCs are particularly difficult to characterize due to the low proliferative activity of the cells in vitro and substantial contamination of cultures by the cells of haematopoietic origin [28,29]. At the same time, experience from other stem/progenitor cell types, such as haematopoietic or embryonic, shows that expression of surface epitopes in mouse cells does not always mirror their human counterpart [30,31]. Since mouse model provides an important platform to study fundamental properties of bone marrow MSCs and is often used for preclinical studies, we were encouraged to perform an extensive study of surface markers in murine MSCs. Previously our group generated a transgenic mouse with inducible expression of SV40 Large T-antigen for conditional immortalization of somatic cells including bone marrow MSCs [32]. Importantly those cells did not change the immunophenotype after long-term culturing, which makes them useful for studies of surface markers.

In this report, we characterized the expression of surface markers in conditionally immortalized bone marrow MSC lines using a large panel of antibodies. As a step further we characterized clonally derived MSC progenitors with distinct differentiation potential aiming to find specific markers for MSC subsets, which we could confirm in cell sorting experiments.

Materials and Methods

Cell Isolation and Culture

Bone marrow MSCs were derived from transgenic mice with a modified tetracycline-inducible SV40 Large T-antigen generated previously by our group [32]. All protocols related to animal experiments were performed accordingly to the German Animal Welfare Legislation, and the Animal Welfare Officer(s) appointed for the facility oversaw them. For cell isolation bone marrow was flushed from tibia and femurs and plated to cell culture dishes in growth medium DMEM containing 1 g/L D-glucose (Gibco, Life Technologies) supplemented with 10% fetal calf serum (FCS, PAA). After reaching confluency the cells were passaged by diluting 1:2 without or with adding 10−7 M Dexamethasone (Dex) and 1 μg/ml Doxycycline (Dox, both from Sigma) to induce the expression of T-antigen. Conditionally immortalized BM MSCs were routinely cultured in the growth medium in the presence of Dex/Dox and passed every 3–5 days with dilution 1:5–1:10. Cellular cloning was performed by manual dilutions by plating the cells at 1 or 3 cells per well of 96-well plate. After 2 weeks the plates were checked for clones rising from single cells, which were further collected and passed in 96-well plates using multi-channel pipette. The clones were screened for their potential to differentiate into osteogenic and adipogenic lineages in 96-well plates, at least in triplicates. The clones with distinct differentiation potentials were selected and further expanded.

Differentiation of BM MSCs

For differentiation assays, BM MSCs were deinduced by Dex/Dox withdrawal for 3 days to stop cell proliferation, and then differentiation conditions were applied to the cells. Osteogenic differentiation was performed in the growth medium by adding 10−8 M Dex, 300 μM ascorbic acid and 10 mM β-glycerophosphate. After 2 weeks the cells were fixed with ice-cold methanol for 10 min, stained with 2% Alizarin Red at pH 4.3 for 10 min and dried. For quantification of the efficiency of differentiation the dye was extracted from the stained samples using 4 M guanidine chloride solution at 37°C overnight, and the optical density of extract was measured at 490 nm. For adipogenic differentiation 10−7 M Dex, 5 μg/ml insulin and 5 μg/ml Trogilitazone were added to the growth medium for 7 days. The cells were fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 30 min at room temperature and stained with Oil Red (0.3% solution in isopropanol mixed 3:2 with water and filtered) for 5 min. To estimate the efficiency of differentiation the rate of adipocytes was counted using phase contrast microscope at least in three fields of view. Chondrogenic differentiation was done in pellet culture prepared from 105 cells in the medium containing DMEM with 4.5 g/L D-glucose, 1% FCS, 10 ng/ml TGF-β1 (Peprotech), 10−7 M Dex, 6.25 μg/ml apo-transferrin, 6.25 μg/ml insulin and 50 μg/ml ascorbate-2-phosphate. After 3 weeks the pellets were fixed in 4% formaldehyde in PBS for 10 min at room temperature, then paraffin embedded and sectioned. The sections were rehydrated and stained with anti-aggrecan antibody (1:100, Chemicon) for 1 hour at 37°C. The staining was visualized using Vectastain ABC kit (Vector Labs) according to manufacturer recommendations. All chemicals were purchased from Sigma, unless otherwise stated.

Cell Surface Marker Screening

BM MSCs were characterized using BD Lyoplate Mouse Cell Surface Marker Screening Panel (BD Biosciences, cat. 562208), which contains 176 monoclonal antibodies and correspondent isotype controls. The staining was done according to manufacturer protocol with minor modifications. Conditionally immortalized MSCs were expanded, and then Dex and Dox were omitted from the growth medium for 3 days to deinduce T-antigen expression. The cells were dissociated using PBS with 10 mM EDTA, washed, filtered through 40 μm nylon mesh and distributed into U-bottom 96-well plates in the concentration 2.5·103 cells per well in PBS+2%FCS. The incubation with primary antibodies was done in 50 μl (0.5 μg antibodies per 106 cells) for 30 min at +4°C, followed by two times washing. After that the cells were incubated with the biotinylated secondary antibodies (goat anti-rat 1:400, goat anti-arnemenian hamster 1:800, goat anti-syrian hamster 1:400 or goat anti-mouse 1:400) for 30 min at +4°C. After the wash, incubation with Alexa647-Streptavidin conjugate (1:4000) was done for 30 min at +4°C. The cells were fixed with 2% formaldehyde solution in PBS. The measurement was done using flow cytometer BD LSRII with High-Troughput Screening 96-well plate loader (HTS FACS, BD).

FACS Sorting

A similar staining protocol was applied for other flow cytometry measurements and cell sorting; the antibodies used for this work were rat anti-mouse CD200 (BD Pharmingen, 552512), rat anti-mouse CD140a (BD Pharmingen, 558774) and mouse anti-mouse SSEA4 (BD Pharmingen, 560073). FACS sorting was done using...
Western Blot
Whole cell protein extracts were prepared by freezing and thawing of cells in the extraction buffer (20 mM Hepes pH 8.0, 350 mM NaCl, 10% glycerol, 0.1% Tween-20, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% Protease Inhibitor Cocktail, all Sigma). 20 μg of proteins were resolved in PAGE gel and transferred to nitrocellulose membrane using semi-dry blotting. Blocking was done overnight in 5% milk in PBS with 0.1% Tween-20 at 4°C. The membranes were incubated with primary antibodies (mouse monoclonal against T-antigen, 1:1000, Santa Cruz) for 1 hour at room temperature, washed and incubated with secondary horseradish peroxidase-conjugated goat anti-mouse antibodies (1:2000, Pierce Thermo Scientific) in the same conditions. Detection was performed using SuperSignal West Femto Substrate kit (Pierce Thermo Scientific).

Results
Characterization of Conditionally Immortalized Mouse Bone Marrow MSCs
To establish expandable MSCs we isolated cells from the bone marrow of transgenic mice carrying a modified system for tetracycline-regulated expression of SV40 Large T-antigen, which has been reported previously by our group [32,33]. The expression of Large T was induced in primary MSCs in the presence of two ligands, Dexamethasone and Doxycycline (Dex and Dox), resulting in rapid cell proliferation (Fig. 1A, B). The conditionally immortalized cells could be passaged practically indefinitely, whereas uninuced cells had limited growth potential and exhibited signs of senescence after 1–3 passages [32]. After removing Dex/Dox from the medium, T-antigen was not expressed anymore (Fig. 1C), and consequently the cells stopped dividing, showing that immortalization was reversible. Deinduced cells could be differentiated into osteogenic, adipogenic and chondrogenic lineages (Fig. 1D) by applying culture conditions described for MSCs, indicating that immortalization did not affect the differentiation potential of MSCs.

To characterize the expression of surface markers in mouse bone marrow MSCs we used conditionally immortalized lines from two individual mice (#6472 and #6652) for screening using an antibody panel BD Lyoplate and high-throughput flow cytometry. The immortalization was deinduced by Dex/Dox withdrawal for 3 days before the experiment to avoid influence of T-antigen expression to the cell properties. The Mouse Cell Surface Marker Screening Panel contains 176 specific monoclonal antibodies and corresponding isotype controls. Amongst the markers present in the panel we identified 13 antigens that were homogeneously and highly expressed on the surface of bone marrow MSCs, with more than 85% of cells in the positive gate (Table 1, Fig. 1E). These included CD9 (Tetraspanin 29), CD24 (Heat Stable Antigen), CD29 (Integrin beta-1), CD44 (Hyaluronic acid receptor), CD47 (Integrin-associated protein), CD49e (Integrin alpha-5), CD81 (Tetraspanin 28), CD98 (Slc3a2), CD106 (Vcam1), CD138 (Syndecan-1), CD147 (Basigin), Crl1 (complement component 3b/4b receptor 1-like), Sca1 (Stem Cell Antigen 1).

We detected 20 markers, which were moderately expressed or showed heterogeneous staining within MSC population with more than 5% of cells in the positive gate (Table 1, Fig. S1).

All the other antibodies used for the screening did not show a positive staining (143 markers). Notably, we did not detect expression of haematopoietic markers, such as CD11b, CD19, CD34, CD45, corresponding to characteristics of MSCs and confirming the absence of contamination with the cells of blood lineages. The immunophenotype of bone marrow MSCs was essentially reproduced in two lines that we used for the screening.

The complete results of the screening are shown in (Fig. S1, S2).

Characterization of Bone Marrow MSC Subpopulations
We aimed to dissect a population of MSCs at the single-cell level and characterize progenitors committed to different lineages focusing on the osteogenic and adipogenic properties. For this purpose we performed cellular cloning of conditionally immortalized MSCs and established clonally derived subpopulations from MSC lines derived from two individual mice (#6472 and #6652). The clones were screened for their potential to differentiate into osteocytes and adipocytes (Fig. 2A). The differentiation assays were done in at least 3 independent experiments and only completely reproducible results were considered for the analysis. We identified bipotential clones capable for both types of differentiation (OA), as well as monopotential progenitors for osteo- and adipogenic lineages, O and A, respectively (Fig. 2B, C).

To characterize the expression of surface markers in different types of MSC progenitors we performed a screening by staining with Lyoplate Antibody Panel and flow cytometry, considering only those antigens that we found to be positive or moderately positive/heterogeneous in the initial MSC lines (33 antibodies). For each MSC subsets (OA, O, A) we chose 4 representative clones, two originating from each of two mice (in total 12 clones).

All clones were positive for the markers, which were highly and uniformly expressed in the original MSC lines, listed in Table 1. Of a particular interest was to analyze the expression of markers that showed heterogeneous staining in the parental lines. Most of those antigens exhibited similar intensity of staining to the MSC lines and amongst the clones. But we also detected differences in the expression of three antigens between MSC subsets, and among them CD200 (OX-2), SSEA4 (stage-specific embryonic antigen 4) and CD140a (platelet-derived growth factor receptor alpha), (Fig. 2D, E). CD200 was expressed higher in all clones with osteogenic potential (OA and O) than in the A monopotent clones, 3.3- and 2.7-fold (P<0.01), respectively, by comparing the Mean Fluorescence Intensity (MFI). Conversely, SSEA4 was represented more in the A clones with MFI of 1.9- and 2.2-fold higher relatively to OA and O clones (P<0.01). A similar pattern
Figure 1. Characterization of conditionally immortalized mouse bone marrow MSCs. (A) An improved tet-inducible system for conditional expression of Large T-antigen. In the presence of Dex/Dox irtTA-GBD* fusion protein can be translocated to the nucleus and activate transcription of Large T. (B) MSCs were isolated from bone marrow of transgenic mice and expanded in the immortalization conditions (Phase Contrast, 20 x). (C) Western blot showing induction of T-antigen in primary MSCs (“−−” cells before induction, “+DD” induced cells, “−DD” cells deinduced for 3 days). (D) Conditionally immortalized MSCs maintained differentiation potential in vitro into osteo-, adipo- and chondrogenic lineages. (E) Flow cytometry results for the markers highly expressed in MSCs, as shown by the staining with BD Lyoplate Antibody Screening Panel. Abbreviations: Dex – Dexamethasone, Dox – Doxycycline, irtTA – improved reverse tetracycline transactivator, GBD* - mutated glucocorticoid-binding domain, TAg – Large T-antigen, CAGGs and PGK – constitutively active promoters, tet-tk – tetracycline operator and minimal promoter, IRES – internal ribosome binding site, Puro and Hygro – puromycin and hygromycin resistance genes.

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Table 1. Immunophenotype of conditionally immortalized mouse BM MSCs.

| Marker | Alternate name | % of positive | Ref |
|--------|----------------|---------------|-----|
| **Highly expressed** | | | |
| CD9    | Tspan29, Tetraspanin 29 | 99.1 | [25] |
| CD24   | HSA, Heat Stable Antigen | 98.6 | [18]* |
| CD29   | Itgb1, Integrin beta-1 | 90.7 | [25, 18,50]* |
| CD44   | Hyaluronate receptor | 99.0 | [25,51, 18,50]* |
| CD47   | Itgp, Integrin-associated protein | 97.6 | [21,25] |
| CD49e  | Itga5, Integrin alpha-5, Fibronectin receptor | 97.5 | [25,51, 18]* |
| CD81   | Tspan28, Tetraspanin 28 | 97.8 | [25,51, 18]* |
| CD98   | Slc3a2, Solute Carrier family 3 member 2 | 95.3 | [21] |
| CD106  | Vcam1, Vascular Cell Adhesion Molecule 1 | 92.1 | [25, 28]|
| CD138  | Sdc1, Syndecan-1 | 94.8 | N |
| CD147  | Bsg, Basigin | 94.0 | [51] |
| Crl1   | Complement component (3b/4b) receptor 1-like, Crry, p65 | 93.8 | N |
| Sca1   | Ly6a, Stem Cell Antigen-1 | 85.3 | [18,50,52]*, +/- [28]* |
| **Moderately or heterogeneously expressed** | | | |
| CD13   | Anpep, Aminopeptidase N | 6.4 | [25] |
| CD51   | Itgav, Integrin alpha-v, Vitronectin receptor | 52.8 | [21,25, 18]* |
| CD61   | Itgb3, Integrin beta-3 | 30.7 | [25, 18]* |
| CD71   | Tfrc, Transferrin receptor | 55.1 | [21, 18]* |
| CD73   | Ntse, Ecto-5'-nucleotidase | 28.1 | [25] |
| CD80   | T-lymphocyte activation antigen CD80 | 20.1 | [18]* |
| CD95   | Fas, TNF receptor superfamily member 6 | 11.8 | [18]* |
| CD107b | Lamp2, Lysosomal-Associated Membrane Protein 2 | 9.7 | [21] |
| CD119  | Ifngr1, Interferon Gamma Receptor Alpha | 60.1 | [53] |
| CD120a | Tnfrsf1a, TNF Receptor superfamily member 1a | 11.2 | [54] |
| CD120b | Tnfrsf1b, TNF Receptor superfamily member 1b | 38.9 | [25, 18]* |
| CD140a | Pdgfra, PDGF Receptor alpha | 14.5 | [25,51, 18,52]* |
| CD172a | Sirpa, Signal-Regulatory Protein alpha | 7.8 | [55] |
| CD200  | Ox-2 | 16.0 | [25] |
| IFNγR1b | Interferon gamma receptor 1, beta chain | 8.5 | [53] |
| Sdc4   | Syndecan-4 | 58.5 | N |
| Ly-51  | Enpep, Glutamyl Aminopeptidase, CD249 | 34.6 | [56] |
| H-2Kb  | H-2Kb MHC class I alloantigen | 60.5 | [50]* |
| H-2Db  | H-2Db MHC class I alloantigen | 67.0 | N |
| SSEA-4 | Stage Specific Embryonic Antigen 4 | 11.2 | [25, 46]* |

*reported for mouse BM MSCs. +/- reported as inconsistent level of expression between MSC preparations. N - not reported for expression in MSCs before.
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A

Conditionally immortalized MSCs

Cellular cloning

Osteogenic differentiation
Adipogenic differentiation
Master plate

B

|   | Bipotential clone | Osteogenic clone | Adipogenic clone |
|---|------------------|------------------|------------------|
| OA| ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| Oil Red 4x | ![Image](image4.png) | ![Image](image5.png) |
| Total screened | 6652 | 40 | 17 | 8 | 3 | 68 |

C

| Line | Number of clones | Total screened |
|------|------------------|----------------|
| OA   | 6652 40 17 8 3 68 |
| OA   | 6472 52 36 10 7 105 |

D

Clone OA

CD200: 73.6
SSEA4: 45.6
CD140a: 16.8

Clone O

CD200: 87.7
SSEA4: 10.3
CD140a: 28.3

Clone A

CD200: 3.7
SSEA4: 78.4
CD140a: 91.9

E

CD200

Mean Fluorescence Intensity

SSEA4

Mean Fluorescence Intensity

CD140a

Mean Fluorescence Intensity

Clone type

*P < 0.01
**P < 0.05
***P < 0.07
(n=4)
A high level of CD200 expression is associated with increased adipogenic capacity, as determined by qPCR and FACS analysis of sorted subpopulations. CD200high cells showed a significant increase in adipogenic markers compared to CD200low cells, with a 2.2-fold higher expression of CD200. SSEA4 and CD140a expression also correlated with the adipogenic potential, with CD140ahigh cells exhibiting a 4.5-fold increase in CD140a compared to CD140alow cells. Additionally, SSEA4high cells showed a 1.9-fold increase in SSEA4 expression compared to SSEA4low cells.

Expression of Markers during Differentiation of Bone Marrow MSCs

In order to evaluate the expression of lineage-specific markers in subpopulations sorted for CD200, SSEA4, and CD140a, we performed differentiation experiments with osteogenic and adipogenic conditions. CD140ahigh cells revealed a higher osteogenic potential with a 4.5-fold increase in osteogenic markers compared to CD140alow cells. Conversely, SSEA4high cells showed a significant decrease in adipogenic markers, with a 2.2-fold decrease in Plin4 and a 1.4-fold decrease in PPARγ.

Discussion

Functional studies of specific cell types are inevitably based on the ability to visualize the cells in the tissue context or to isolate pure cell populations and estimate their homogeneity. Isolation of HSCs expressing the set of well-established surface antigens [34,35] and localizing intestinal stem cells using Lgr5 reporter [36] provided extraordinary examples of breaking through in these contexts.
fields of research. Unfortunately, those stem cell types are exceptions among many others existing in mammalian body. Despite accumulating knowledge on the location and functions of bone marrow mesenchymal stem cells [14,16,17], a concise view on their identity is still missing and furthermore the hierarchy of their descendants is not defined.

In vitro culture systems represent a model to study cell properties and can provide sufficient cell numbers for biochemical characterization, although caution should be taken because of possibly introduced artifacts. It has been previously noted that cultured human BM MSC are changing their differentiation properties and expression of markers during passaging [27,37,38], which hampers the studies of their properties and makes characterization of BM MSC subtypes complicated. Moreover, mouse BM MSCs are difficult to expand [28,39] and therefore are not well described. We applied conditional immortalization for BM MSC expansion by isolating the cells from transgenic mice carrying inducible SV40 Large T-antigen [32,33]. This system enables expression of Large T in the presence of two compounds, Dexam and Dox, which is reversible after withdrawal of ligands. In our previous work we have shown that immunophenotype of conditionally immortalized MSCs is not changed after prolonged culturing and upon induction/deinduction [32]. These observations motivated us to perform an extensive characterization of the expression of surface antigens in conditionally immortalized BM MSCs. Here we report the results on BM MSC immunophenotyping using 176 antibodies, and to our knowledge this is the most comprehensive study of surface markers expressed in mouse bone marrow MSCs to date (Fig. 1, Table 1, Fig. S1, S2). Among 33 antigens that we detected in conditionally immortalized MSCs, 13 markers have not been previously characterized in mouse BM MSCs. Additionally, 3 markers (CD138, Gr1, Sdc4) have not been reported for BM MSCs neither from mice nor from other species.

Conditionally immortalized BM MSC have practically indefinite proliferative potential, which allowed us establishing clonally derived subpopulations. We found progenitors with distinct differentiation potential among BM MSC subpopulations (Fig. 2), which confirms heterogeneity of MSCs reported by others [38,40,41,42]. By comparing immunophenotypes of clonally derived progenitors we showed that osteogenic cells (with OA and O properties) expressed higher level of CD200 (OX2) and lower levels of SSEA4 and CD140a (PDGFRα) than non-osteogenic (A clones). Our sorting experiments confirmed that expression of those markers is distinctive for subpopulations with different efficiencies of osteogenesis and adipogenesis (Fig. 3).

We showed that high expression of CD200 marks the progenitors with higher potential for osteogenesis in expense of adipogenic capacity. Our observations are in a good agreement with the molecular function of CD200, which is to mediate osteoblast-osteoclast interaction and to take part in regulation of balance between bone formation and resorption [43,44]. Lee et al. [43] reported that CD200 enhanced differentiation of primary calvarial osteoblasts upon overexpression or after adding a soluble form of CD200 to the cells. Our experiments showed that CD200 was upregulated during osteogenesis in BM MSCs, which may indicate positive autoregulation during differentiation. Another study suggested CD200 as a marker for multipotent human BM MSCs characteristic for undifferentiated cells [25]. The authors described a reduction in CD200 level after osteogenic and adipogenic differentiation of BM MSCs, which contradicts to our data and observations of the others [43]. We cannot explain this discrepancy, however the difference in the role of CD200 in human and mouse MSCs cannot be completely excluded.

We found that SSEA4 was characteristic for the cells with higher adipogenic potential and lower osteogenic potential, which was the opposite distribution to CD200 within BM MSC population. Detection of SSEA4 in our study is remarkable since this molecule represents a globo-series glycosphingolipid and would not be detected by transcriptomic or proteomic methods [45]. Expression of SSEA4 in human and mouse BM MSCs was previously described [46], and it was shown as heterogeneous in expanded primary BM MSCs. SSEA4 positive cells were found to be tripotent (osteo-, adipo-, chondrogenic), although with a low mineralization efficiency, but unfortunately, a side by side comparison with SSEA4 low expressing cells hasn’t been done.

We identified CD140a to be higher expressed in adipogenic monopotent progenitors than in osteogenic, and further confirmed higher adipogenic efficiency of CD140a-high subpopulation, but osteogenic property of CD140a-high and CD140a-low cells was variable. Therefore, CD140a expression level did not correlate with the capacity for the alternative differentiation pathway, in contrast to the other markers analyzed in our work. Indeed, we observed that CD140a was expressed among MSCs independently of SSEA4 level (Fig. S4). CD140a was used for prospective isolation of MSCs from mouse bone marrow [15]. In this work, CD45<sup>-</sup>TER119<sup>-</sup> cells from bone marrow were separated into subpopulations with different expression of PDGFRα and Sca1 and checked for differentiation potential. Interestingly, only PDGFRα<sup>+</sup> cells exhibited adipogenic potential (both Sca1<sup>+</sup> and Sca1<sup>-</sup>). This observation supports our data obtained using in vitro cultured cells. Additionally, we found that CD140a reached high level on the early stage of adipogenesis, also in the OA clones that had initially a low level of CD140a, and then dropped. Similar to this result, during adipogenic differentiation of mouse embryonic stem (ES) cells PDGFRα expressing cells were giving rise to adipocytes, but not PDGFRα<sup>+</sup> [47]. It was also shown that adding PDGF inhibited differentiation of pre-adipocytes 3T3-L1 [48] and blocking of PDGF receptor by antibodies promoted adipogenesis in MSCs [49]. Taking together these data and our results, we suggest that high level of PDGFRα marks adipogenic precursors at the early stage of commitment, but has to be downregulated to proceed to terminally differentiated state.

In conclusion, we performed a comprehensive characterization of surface marker expression in mouse bone marrow MSCs and their subsets using conditionally immortalized cells. Moreover, we were able to identify the markers differentially expressed in the distinct types of BM MSC progenitors, CD200 in osteogenic and
Figure 4. Expression of CD200, SSEA4 and CD140a during MSC differentiation. (A) Clonally derived conditionally immortalized MSCs were differentiated into osteocytes (upper panel, Alizarin Red staining) or adipocytes (lower panel, Oil Red staining). Undifferentiated state, early time point and late differentiation were analyzed (0, 3, 10 days of osteogenic induction, and 0, 3, 5 days of adipogenic induction). (B) Flow cytometry results of the expression of CD200, SSEA4 and CD140a in the representative OA clone during differentiation time course (0, 3, 10 days of osteogenic treatment).
SSEA4 and CD140a in adipogenic cells. Our results conform to the data obtained using primary cells and mice [15,43,47], and provide an important insight to the identity of BM MSCs and cellular composition of this cell population. We believe that our findings would be effective for the studies of bone marrow stroma functions and understanding biology of mesenchymal progenitor cells; further investigations are anticipated.

Supporting Information

Figure S1 Expression of surface markers in conditionally immortalized mouse BM MSCs. Expression of 176 markers was checked by staining with antibodies and flow cytometry of two MSC lines after de-induction of immortalization. The results for 13 highly and 20 moderately/heterogeneously expressed antigens are shown as histograms and percents of cells in the positive gate are indicated (for one of the lines). Line – IgG control, colored histogram – antibody staining. (TIF)

Figure S2 The complete results of surface markers screening in conditionally immortalized mouse BM MSCs. Three 96-well plates containing antibodies, which were used for the staining, are depicted (the empty wells contained isotype controls or nothing). The color indicates the result of the measurement as shown; the average percentage of cells in the positive gate was calculated from the screening of two individual lines. (TIF)

Figure S3 Expression of surface markers in the BM MSC subsets sorted for CD200, SSEA4 and CD140a. The subpopulations of BM MSCs were sorted from two individual conditionally immortalized lines for high and low expression of the markers above, passaged for at least 5 times and each of them was checked for the levels of all those three markers by flow cytometry. The Whisker box plots show the ratio of the Mean Fluorescence Intensity (MFI) in the population sorted for high level to MFI in the one sorted for low expression of (A) CD200, (B) SSEA4 and (C) CD140a. The dashed line represents ratio = 1, i. e. equal expression. The results are summarized from two independent measurements of subsets sorted from two lines. Statistical significance was calculated using Student’s t-test. The cells sorted for high expression of CD200, SSEA4, CD140a maintained increased level of those markers as compared to the cells sorted for low expression. Additionally, CD200high subpopulation exhibited lower level of SSEA4 compared to CD200low, whilst SSEA4high had decreased CD200 expression relatively to SSEA4low. (TIF)

Figure S4 Co-expression of markers CD200 and SSEA4 or CD140a and SSEA4 in conditionally immortalized BM MSCs. Conditionally immortalized BM MSCs were stained with combinations of antibodies for CD200 and SSEA4 (upper panel) or CD140a and SSEA4 (lower panel). The gating has been done according to unstained control and stainings with individual antibodies combined with all secondary reagents to exclude unspecific staining. BM MSCs were mostly composed of the subpopulations with CD200high SSEA4low and CD200low SSEA4high immunophenotypes and high expression of both markers was exclusive. Expression of CD140a was detected within SSEA4high and SSEA4low subsets. A percentage and Mean Fluorescence Intensity for PE and APC staining are shown for the described subpopulations. (TIF)

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Author Contributions

Conceived and designed the experiments: MR KA. Performed the experiments: MR. Analyzed the data: MR. Contributed reagents/materials/analysis tools: MR KA. Wrote the paper: MR KA.

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