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Potential of MSCA1 for isolating osteogenic cells in a chondrocyte population

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The aim of the present study was to elucidate the utility of Mesenchymal Stem Cell Antigen-1 (MSCA1) (tissue nonspecific alkaline phosphatase (TNAP)) as a potential marker for purification of human chondrocyte fraction with less heterogenous phenotype from those with osteogenic properties. Chondrocytes were isolated from human osteoarthritis cartilage and sorted according to MSCA1 expression by MACS (Magnetic-activated cell sorting) and FACS (Fluorescence-activated cell sorting) techniques (MSCA1^{high} and MSCA1^{low}), analyzed for gene expression, osteogenic and adipogenic differentiation capacities, and were compared between the sorted populations. Gene expression analyses revealed upregulation in osteogenic genes (*ALPL* and *RUNX2)* and significantly lower expression of chondrocyte-specific genes (*COL2A1*, *SOX9* and *MIA)* in sorted MSCA1^{high}, as compared to MSCA1^{low}. Expression of Aldehyde dehydrogenase isoform gene *ALDH1A2* was enriched in MSCA1^{low} chondrocytes. Stronger osteogenic differentiation was observed in MSCA1^{high}, as compared to the unsorted cells, and particularly, MSCA1^{low} chondrocytes. Expression of MSCA1 in human chondrocytes is biased in their commitment to the osteogenic lineage. We demonstrate that MSCA1 can be used as a marker for separation of cells, prone to differentiate towards osteogenic lineage from those, retaining chondrogenic phenotype. This may allow enrichment of a chondrogenic population and separation from undesirable osteoprogenitors.

Keywords MSCA1/TNAP, Osteoarthritis, Chondrocytes, MACS, Osteogenic differentiation

Abbreviations

MSCA1	mesenchymal stem cell antigen–1
TNAP	tissue nonspecific alkaline phosphatase
OA	osteoarthritis
BM	bone marrow
MSC	mesenchymal stem/stromal cells
Pi	phosphate
PPi	Inorganic pyrophosphate
HA	hydroxyapatite
ALDH	aldehyde dehydrogenase
ATRA	all–trans retinoic acid
FACS	fluorescence activated cell sorting
MACS	magnetic–activated cell sorting
CPC	hexadecylpyridinium chloride monohydrate
MFI	median fluorescence intensity
FGF	2–fibroblast growth factor 2
APC	Allophycocyanin
FBS	Fetal bovine serum
BSA	Bovine serum albumin
PS	penicillin and streptomycin
7	AAD–7–Aminoactinomycin D

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IBMX Isobutylmethylxanthine

Cartilage tissue is known to be highly susceptible to trauma and other types of damage, leading to tissue deterioration and development of osteoarthritis (OA). Cartilage regenerative capacities are very limited, since the cells residing in the tissue are not capable to restore the functional structure and integrity of the tissue. Possible technologies of repairing damaged cartilage include using different microfracturing, stem (stromal) cell/chondrocyte administration or scaffold-based tissue engineering, etc. The microfracturing procedure is based on breaking through the subchondral plate and allowing the migration of bone marrow (BM) cells into the defective area in the joint. The risk of the ossification and osteophyte formation urges to seek for possible joint trauma treatment alternatives, such as mesenchymal stem/stromal cell (MSC) based therapies. However, during chondrogenesis, MSCs tend to develop hypertrophic chondrocyte phenotype, leading to further osteogenicdifferentiation[1], similar to the process observed in the bone growth plate. Therefore, chondrocyteimplantation and cartilage tissue engineering remains among the most popular and attractive approaches so far[2]. However, recent histological and immunohistochemical evaluation of biopsies from clinically failed patients after matrix-assisted chondrocyteimplantation also revealed the abnormal calcification, and, more specifically, intralesional osteophyte formation within the cartilage defect area in all studied patients[3]. Chondrocyte hypertrophy along with manifestation of osteogenic properties seem to be major drawbacks, suggesting that a careful selection and purification of cartilage cells with superior chondrogenic properties will advance effectiveness of future cell-based therapeutic applications.

The enrichment of the desired population of progenitor and/or stem cells isolated from primary tissues is hampered by the lack of specific surface markers for elimination of unrelated and/or unwanted cells. Furthermore, the currently available markers identifying chondrogenic properties of isolated chondrocytes or differentiation capacity of stem/stromal cells are also insufficient as the main indicators remain the expression of SOX9, collagen type II and aggrecan[4]. Chondrogenic potential is being associated with CD49c, CD54, CD73, CD105 and some other surface markers[5–7], while approved autologous chondrocyte implantation (ACI) method Spherox, based on chondrocyte spheroid implantation has revealed acidic protein 1 (CRTAC1), glycosaminoglycans (GAGs) and aggrecan (ACAN)[8], though there is significant heterogeneity of those cell markers among donors.

Cell surface protein antigen-1 (MSCA1) has been characterized as an MSC (Mesenchymal Stem Cell) marker, indicating stemness and suitability for the isolation of highly purified BM-MSC (Bone marrow-MSC)[9–11]The expression of MSCA1 has been reported in several human adult tissues, including Wharton jelly[12], adipose tissue[13], cartilage[14]and bone marrow[15,16].

The same group of researchers, who characterized MSCA1 as a stem cell marker, later demonstrated that MSCA1 is identical to the tissue nonspecific alkaline phosphatase (TNAP), a cell membrane ectoenzymeknown to be expressed in liver, bone and kidney, as well as in embryonic stem cells[17]. As repeatedly demonstrated, TNAP is crucial for osteogenicdifferentiation and calcification[18–20]. Briefly, hydroxyapatite (HA) crystal formation begins within matrix vesicles[21], specific organelles that bud from the surface membrane of hypertrophic chondrocytes, osteoblasts and odontoblasts. Then break down of matrix vesicles membranes leads to spread of HA crystals into the extracellular matrix, among collagen fibrils. This process requires $Ca^{2+}$and inorganic phosphate (Pi). Inorganic pyrophosphate (PPi), which is formed mainly by the hydrolysis of ATP into AMP, inhibits HA formation. TNAP hydrolyzes PPiinto two Pi molecules and thus provides substrate for tissue mineralization[22]. Mutations in the TNAP gene are associated with accumulation of PPiand arrest of HA crystal formation leading to development of osteomalacia or hypophosphatasia[19,22].

In our previous study[23], we showed that chondrocytes with stronger chondrogenic phenotype were associated with Aldehyde dehydrogenase (ALDH) activity, measured using Aldefluor™ technique, and were enriched in the sorted ALDH + fraction. The ALDH gene isoforms *ALDH1A2* and *ALDH1A3*, encoding retinaldehyde dehydrogenases, responsible for all-trans retinoic acid (ATRA) synthesis, were enriched in the sorted (ALDH+) fraction, further suggesting that they are associated with the stronger chondrogenic phenotype in those cells[23]. We, therefore, in the present study also analyzed the expression of genes involved in ATRA metabolism in the $MSCA1^{high}$ and $MSCA1^{low}$ cell populations.

The aim of the present study was to elucidate the utility of MSCA1/TNAP as a potential marker for purification of chondrocyte fraction from chondrocytes with osteogenic properties. We have also analyzed levels and co-expression of MSCA1/TNAP with other stemness or differentiation related markers during osteogenesis and adipogenesis.

Results

Surface marker expression during osteogenic differentiation of chondrocytes

In order to analyze the changes of the expression of MSCA1 and other surface markers during osteogenic differentiation, the flow cytometry analysis of chondrocytes, differentiated for 21 days and control proliferating cells was performed (Fig. 1A). The ratios of these results (differentiated vs. control cells) are presented in Fig. 1B. Statistically significant increase in MSCA1 and CD49c levels in osteogenically induced chondrocytes was observed in both, % and MFI evaluation. The expression of CD166 and CD54 was about 100% in both differentiated and control chondrocytes (Fig. 1A, %), the percent ratio for these markers was equal to 1 (Fig. 1B, upper panel), while the MFI analysis revealed increased levels of those markers in the osteogenic differentiation (Fig. 1B, lower panel), which for the CD54 were statistically significant ($p < 0.01$). In contrast, osteogenic differentiation of whole, unsorted chondrocyte population led to significant depletion of CD46, CD55 and CD117 expression (particularly for the percentage ratios).

The expression of MSCA1 protein for two patient samples was further measured by flow cytometry every week during osteogenic differentiation. The MSCA1 expression ratio for both % and MFI values of differentiated

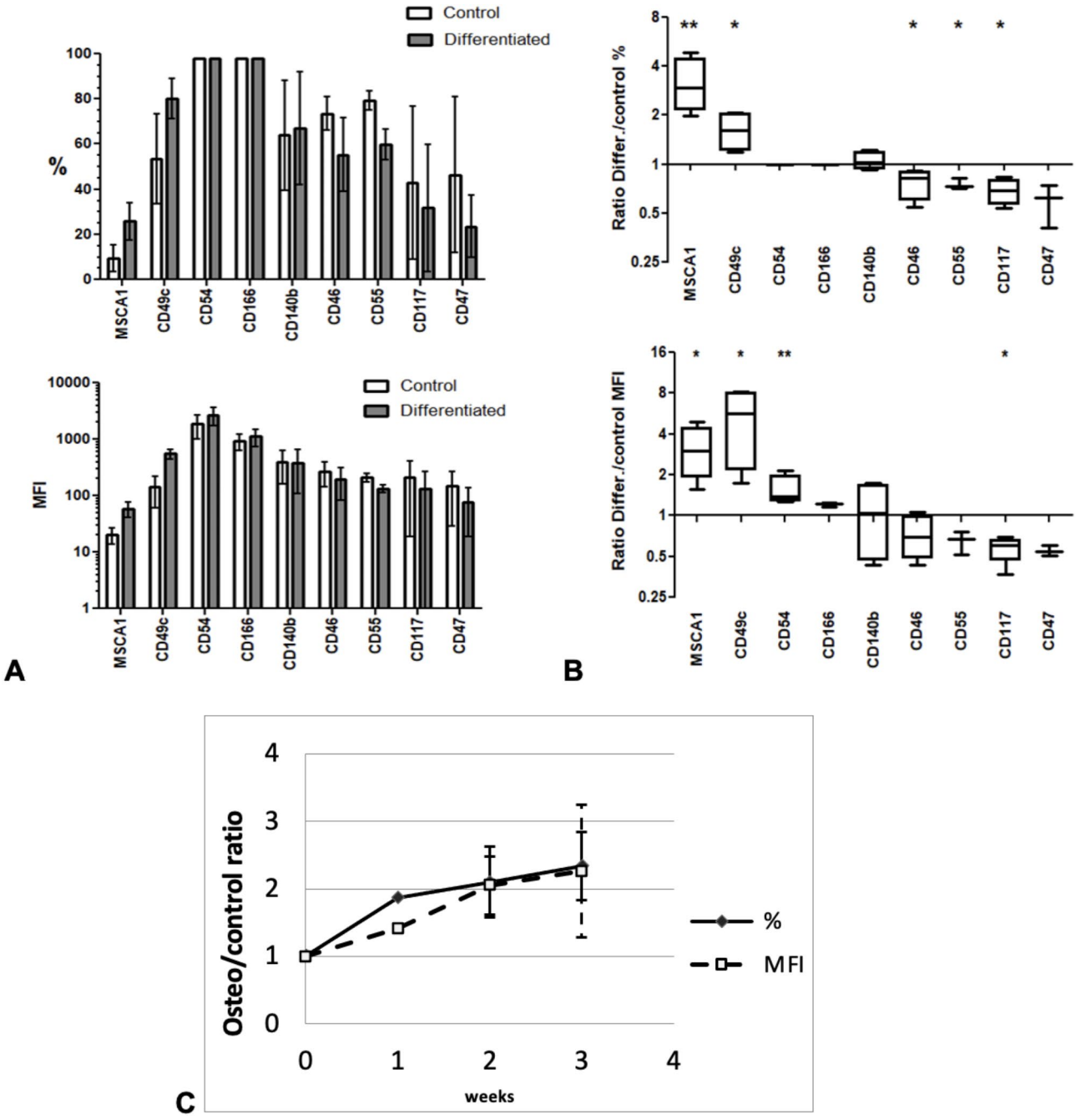


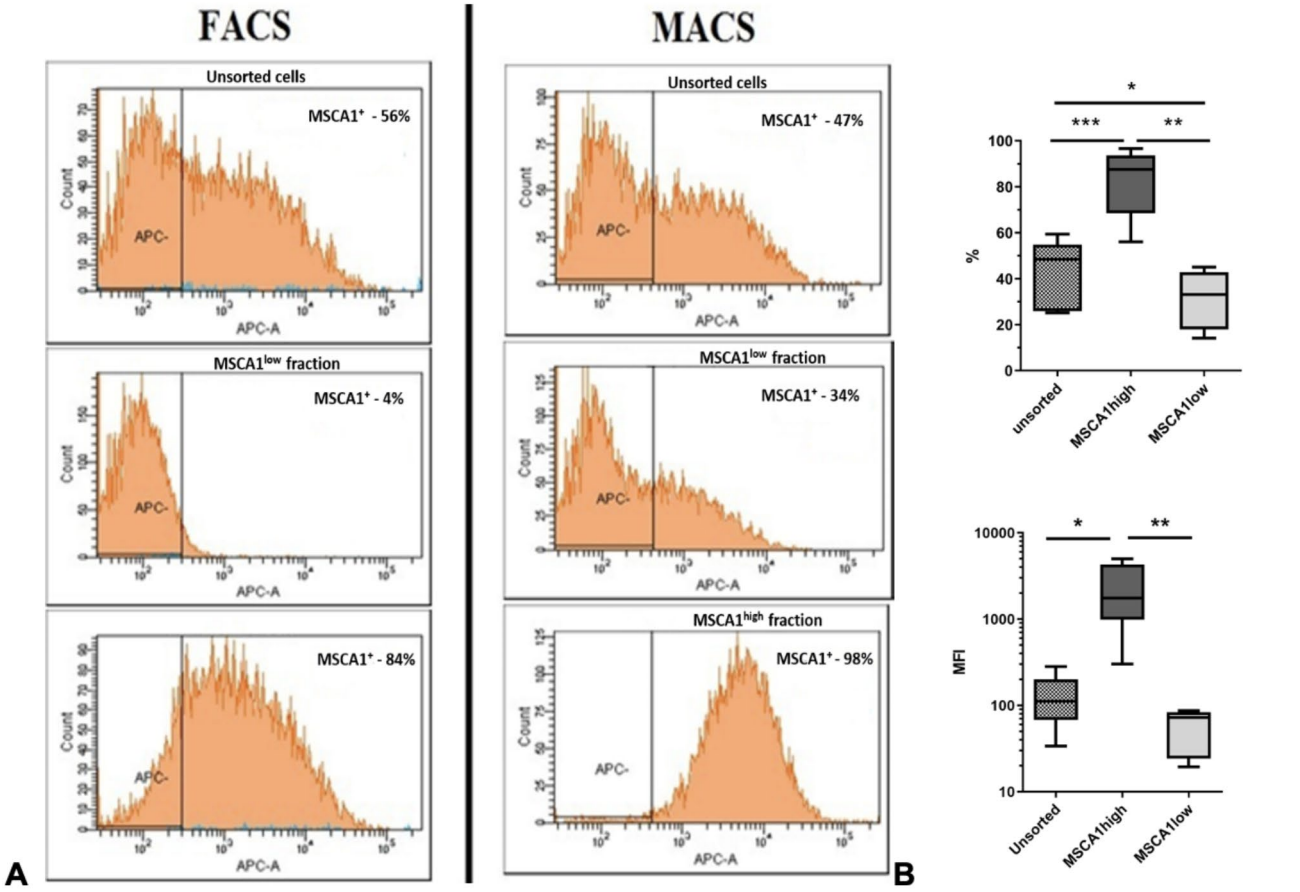
Fig. 1. Surface marker expression changes in whole, unsorted population of isolated chondrocytes that were either differentiated for 21 days or cultured under non-differentiating conditions for 21 days, analysed by flow cytometry. (**A**) Surface marker expression in 21 day-differentiated and control cells. Error bars represent SD. (**B**) Box plots show ratio for percentage and MFI value for every surface marker, measured on osteo-differentiated vs. control proliferating cells. Error bars show minimal to maximal values; $n = 3–5$. Statistical evaluation: paired t-test. *- $p < 0.05$, and **- $p < 0.01$. (**C**) Changes in MSCA1 expression ratio during 21 days of osteogenic differentiation. Error bars represent SD, $n = 2$.

cells vs. control cells are presented in Fig. 1C. Results show continuous increase in this ratio during 3-week of osteogenic differentiation process.

MSCA1 protein levels in chondrocytes and sorting (FACS and MACS)

MSCA1 levels in chondrocytes from different donors varied in a range between 5 and 30% (data not shown). During cultivation a tendency of slight increase in MSCA1 count was observed, but it was not significant (not shown).

Cell samples with the highest MSCA1% were sorted both by FACS and MACS (magnetic cell sorting), resulting in populations with different purity characteristics (Fig. 2A). FACS sorting gave very pure MSCA1^{low} fraction, while MSCA1^{high} fraction contained up to 16% of MSCA1^{low} cells. Whereas, MACS sorting of the same sample yielded a small, but very pure MSCA1^{high} fraction – up to 98% of cells were positive. MSCA1^{low} fraction, in contrast, was abundant, but still contained considerable portion of MSCA1^{high} cells (34% of MSCA1^{high} cells in MSCA1^{low} fraction vs. 47% of MSCA1^{high} cells in original chondrocyte sample before sorting). Repeated sorting procedures of MSCA1^{low} cells using new columns gave similar results with only slightly better depletion of MSCA1^{high} cells in MSCA1^{low} fraction (data not shown). MACS technique allowed easier sorting of larger



C

MFI-median	Unsorted	MSCA1^{high}	MSCA1^{low}	%	Unsorted	MSCA1^{high}	MSCA1^{low}
Donor 1	282.0	4988.0	87.0	Donor 1	59.4	96.6	45.0
Donor 2	120.0	3645.3	29.3	Donor 2	50.1	90.7	33.1
Donor 3	34.0	302.0	19.5	Donor 3	25.1	56.1	21.8
Donor 4	112.0	1674.0	72.5	Donor 4	48.4	87.5	40.5
Donor 5	102.0	1762.0	81.0	Donor 5	26.5	81.0	14.1

Fig. 2. Chondrocyte sorting according to MSCA1 expression ($n = 5$). (**A**) FACS (left) and MACS (right) sorting results of the chondrocytes from the same donor and same passage (cultivated for about 8 weeks before sorting). Flow cytometry results of unsorted population, MSCA1^{neg} (MSCA^{-}) and MSCA1^{pos} (MSCA^{+}) fractions. X axis – APC channel fluorescence intensity, Y axis – cell count. (**B**) Summarized flow cytometry results for MACS sorting of different cell preparations ($n = 5$) from different cell donors, presented as a percent of positive cells (%), above; and median fluorescence intensity (MFI), below. Error bars show standard deviation of the results. Statistical evaluation: two-way ANOVA. *- $p < 0.05$, ***- $p < 0.001$. (**C**) individual donor cell values for both, MFI and % detected after measuring. Abbreviations used: MSCA1 – Mesenchymal Stem Cell Antigen; FACS – Florescence-activated Cell Sorting; MACS – Magnetic-activated Cell Sorting; APC – Allophycocyanin.

cell amounts and resulted in higher yields and better cell viability: for instance, out of 1.4×10^6 of chondrocytes, 67% of viable cells were collected after sorting, including 40% (0.375×10^6) in MSCA1^{high} fraction and 60% (0.567×10^6) in MSCA1^{low} fraction. FACS sorting of 0.34×10^6 of chondrocytes from the same patient sample resulted in 52% of viable cells in total, including 48% (0.075×10^6) in MSCA1^{high} fraction and 62% (0.102×10^6) in MSCA1^{low} fraction. Therefore, MACS technique was used for further patient sample sorting in this study. Figure 2B shows these results both, for percentage of positive cells, and median fluorescence intensity (MFI value), which represents the intensity of the marker. In addition, individual values of every donor cell are provided in Fig. 2C.

MSCA1-sorted cells: gene expression analysis

The gene expression ratio of MSCA1^{high} vs. MSCA1^{low} sorted cells ($2^{-\Delta\Delta Ct}$) is presented in Fig. 3. Gene descriptions are presented in Table 1.

As anticipated, expression of gene encoding MSCA1, Tissue-Nonspecific Alkaline Phosphatase or TNAP (gene named *ALPL)* was significantly higher in the sorted MSCA1^{pos} fraction. Other genes of osteogenic markers *COL1A1* and *RUNX2* were also enriched in this fraction (up to 20-fold for *RUNX2*), but results were not so consistent among patients, therefore, not statistically significant (Fig. 3). Adipogenic marker *PPARG* also tended to accumulate in this fraction, but did not reach statistical significance, potentially because of impure MSCA1^{low} fraction. On the contrary, significant enrichment of main chondrogenic markers *COL2A1* (up to 50 times) and *SOX9* expression was determined in MSCA1^{low} fraction. Melanoma Inhibitory Activity protein (MIA) (or, alternatively, Cartilage-Derived Retinoic Acid-associated Protein -CD-RAP), which is highly characteristic for chondrocytes, encoded by gene *MIA* expression was also enriched in MSCA1^{low} fraction. Another gene, characteristic for chondrocytes, *SERPINA1* – tended to be associated with MSCA1^{low} fraction, but the results were more scattered, and therefore the differences were not statistically significant.

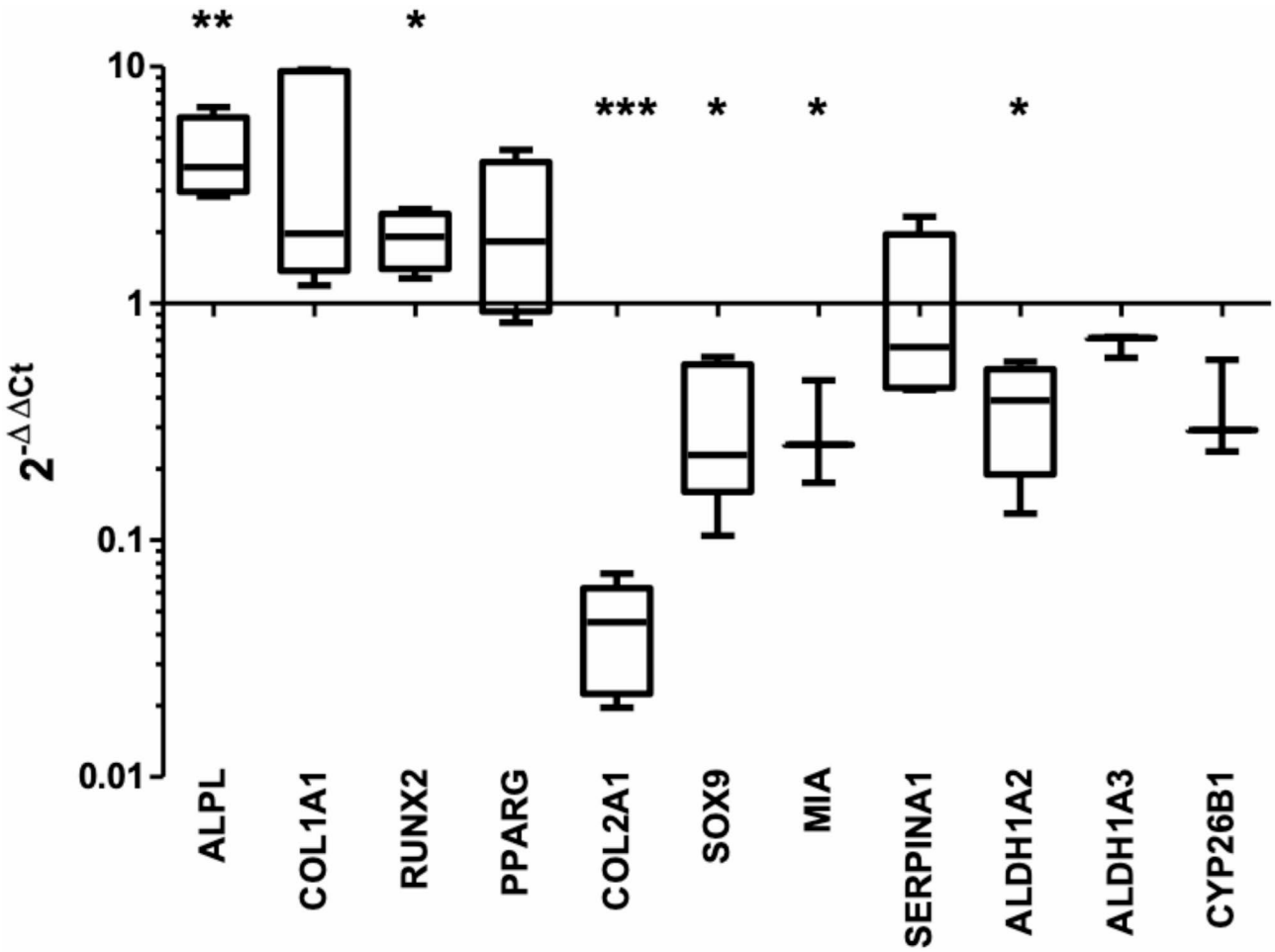


Fig. 3. Gene expression analysis of MSCA1-sorted chondrocytes normalized to reference gene RPS9 expression. Graphs show gene expression ratio for the sorted MSCA1^{high} vs. MSCA1^{low} chondrocyte populations ($2^{-\Delta\Delta Ct}$). The box length represents the interquartile range with a median. The whiskers represent the minimum and maximum data values, $n = 3$–5 ($n = 3$ for COL1A1, PPARG), statistical evaluation: paired t-test. *- $p < 0.05$, **- $p < 0.01$ and ***- $p < 0.001$. Description of tested genes is presented in Table 1.

Gene, assay ID	Encoded protein
RPS9 Hs02339424_m1	40 S ribosomal protein S9 (housekeeping gene)
COL2A1 Hs01060345_m1	Collagen type II, alpha 1
*COL1A1*Hs00164004_m1	Collagen type I, alpha 1
SOX9 Hs00165814_m1	Transcription factor SOX-9
SERPINA1 Hs00165475_m1	Alpha-1 antiproteinase, antitrypsin
MIA Hs00197954_m1	Melanoma inhibitory activity
RUNX2 Hs00231692_m1	Runt-related transcription factor 2
PPARG Hs01115513_m1	Peroxisome proliferator-activated receptor gamma
ALDH1A2 Hs00180254_m1	Aldehyde dehydrogenase 1 family, member A2
ALDH1A3 Hs00167476_m1	Aldehyde dehydrogenase 1 family, member A3
ALPL Hs01029144_m1	Alkaline Phosphatase
*CYP26B1*Hs01011223_m1	Cytochrom P450 26B1

Table 1. Gene primers used in the study.

	Average ratio	StDev
ALPL	4,2689515	1,73500708
COL1A1	4,7637982	4,38056406
RUNX2	1,89965775	0,51530746
PPARG	2,23223838	1,63086305
COL2A1	0,04306164	0,02136394
SOX9	0,33092322	0,21015709
MIA	0,30078623	0,15519144
SERPINA1	1,01740975	0,89487926
ALDH1A2	0,3682768	0,18052555
ALDH1A3	0,67592803	0,07406053
CYP26B1	0,368863	0,18342313

Table 2. Gene expression average ratios.

Expression of three genes, associated with ATRA turnover, including *ALDH1A2*, *ALDH1A3* and ATRA-degrading enzyme *CYP26B1* was also analyzed in the MSCA1-sorted cells. Interestingly, they all tended to be enriched in MSCA1^{low} fraction, with *ALDH1A2* being significantly enriched along with chondrogenic markers *COL2A1* and *SOX9* (Fig. 3). Gene expression ratios are presented in Table 2.

MSCA1-sorted chondrocytes: adipo- and osteo-differentiation

As MSCA1^{high} fraction was enriched in osteogenic and adipogenic genes, we induced the unsorted and the sorted cells of both MSCA1^{high} and MSCA1^{low} fractions to differentiate towards 2 mesenchymal cell lineages – adipogenic and osteogenic. After 3 weeks, the cells were fixed and stained with Oil Red O (adipogenic differentiation) or Alizarin Red S (osteogenic differentiation). A representative staining of adipogenic differentiation of sorted chondrocytes is shown in Fig. 4A. No clear morphological differences in adipogenic differentiation of 21 days were observed between MSCA1^{high} and MSCA1^{low} populations. However, the quantitative evaluation of isopropanol-extracted Oil Red dye revealed somewhat stronger absorbance readings in the MSCA1^{high} fraction (Fig. 4B). In contrast, osteogenic differentiation showed considerable differences between all the three analyzed cell populations. MSCA1^{high} cell mineral deposits were stained considerably stronger with Alizarin Red S than those of unsorted population (Fig. 4C middle and upper panel, respectively), while MSCA1^{low} population stained very weakly, much less than the unsorted one (Fig. 4C lower and upper panel, respectively). In addition, quantitative evaluation of Alizarin Red S-stained and CPC (hexadecylpyridinium chloride monohydrate)-solubilized preparations confirmed the differences as statistically significant between MSCA1^{low} and MSCA1^{high} fractions, as well as between unsorted chondrocytes and MSCA1^{low} fraction (Fig. 4D).

Discussion

The intent of the present study was to investigate whether MSCA1 could be employed as a biomarker of a distinct cell population in cartilage. The MSCA1 protein levels differed among donors, and also changed during the cultivation in monolayer. Similarly, a varying expression of this protein among BM-MSCsfrom different donors have been previously described, while no correlation of its activity with age or gender of the donor has been observed[24].

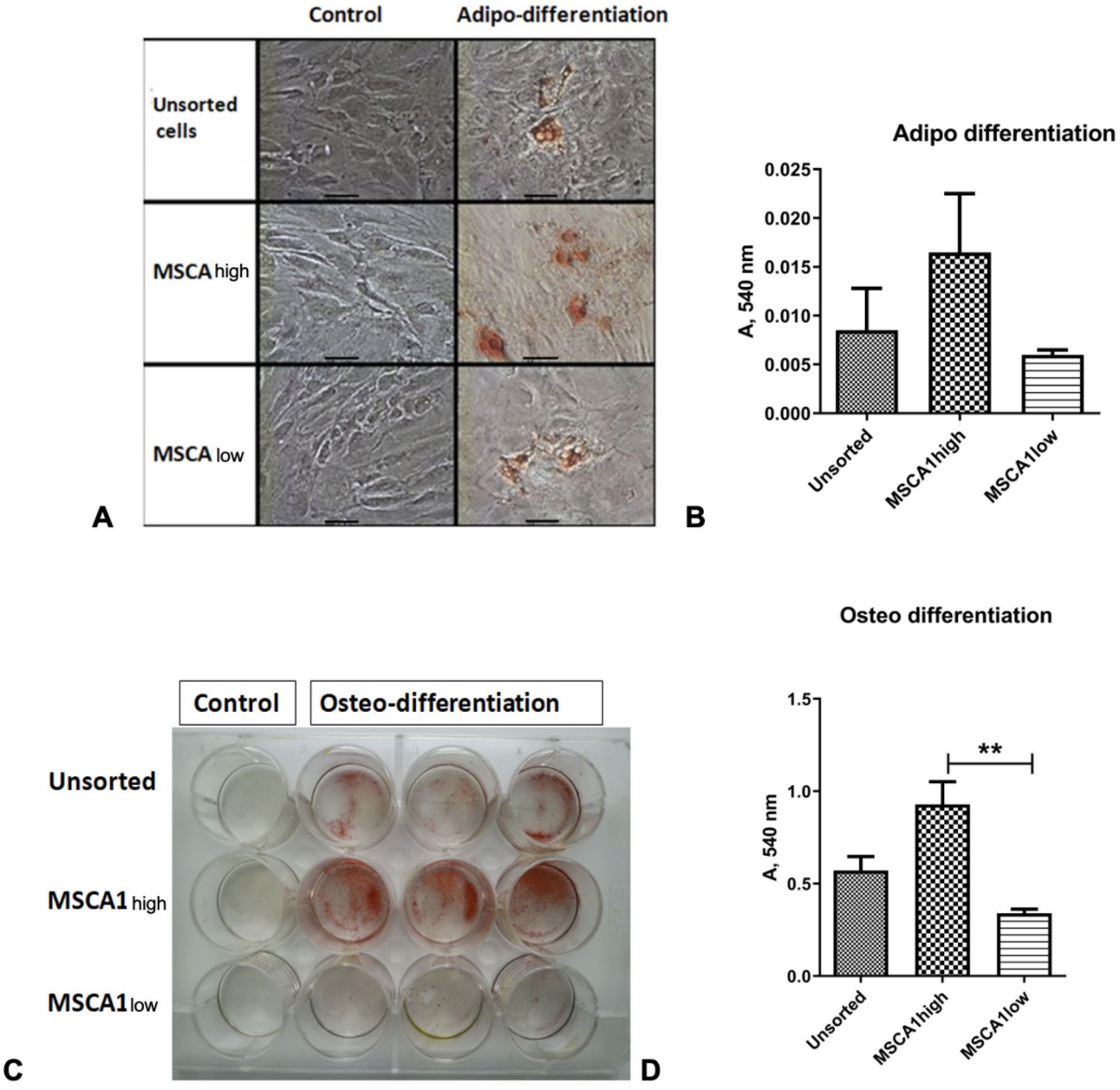


Fig. 4. Adipogenic and osteogenic differentiation of unsorted and MSCA1-sorted chondrocytes. (**A**, **B**) Cells, stained with Oil Red O after 21-day of adipogenic differentiation. (**A**) Microscopic view, magnification x200, scale bar – 100 µm. (**B**) Quantitative evaluation of adipogenic differentiation – absorbance at 540 nm of isopropanol-extracted Oil Red O dye, normalized to control reads, for unsorted, MSCA1^{high} and MSCA1^{low} cells, spectrophotometry ($n = 5$). (**C**, **D**) Osteogenic differentiation for 21 days. (**C**) Macroscopic view of the unsorted and the sorted MSCA1^{high} and MSCA1^{low} cell cultures, stained with Alizarin Red S in 12-well plate. (**D**) Quantitative evaluation of Alizarin Red S-stained and CPC-solubilized preparations, absorbance at 540 nm, normalized to control reads, spectrophotometry ($n = 5$). Error bars represent SD. Statistical evaluation: ANOVA, Tukey's pod test. *- $p < 0.05$, **- $p < 0.01$. CPC - hexadecylpyridinium chloride monohydrate.

Firstly, we analyzed the changes in MSCA1 protein expression during osteogenic differentiation by flow cytometry and found significantly increased expression of this enzyme after 3 weeks, as compared to control, non-differentiated cells (Fig. 1).

The expression of CD49c and CD54 proteins, previously associated with chondrogenic potential of chondrocytes[5,7], similarly to MSCA1, tended to be enriched in the osteogenically stimulated chondrocytes (Fig. 4B), while CD46, CD47, CD55, and CD117, on the contrary, were more characteristic for the non-differentiated control cells. CD46 and CD55 are cytoprotectivecomplement regulatory proteins that function in the prevention of complement-mediated damage[25], while their upregulation has been observed in the joints with arthritis, and in IL-1 treated cartilage[26]. CD47 is a cell surface signaling receptor for thrombospondin-1 and the

counterreceptor for signal regulatory protein-α (SIRPα), functioning as an immune checkpoint molecule, the antiphagocyticsignal to macrophages[27].

CD117 (tyrosine-protein kinase KIT) is mainly characterized as hematopoietic stem cell marker, but its expression is documented in various other cells, including MSCs[28–30].

CD166 was commonly described as a MSC marker, but several studies demonstrated, that expression of this surface protein was enhanced during osteogenic and adipogenic differentiation of BM-MSCs[31–33]and downregulated during chondrogenic differentiation of these cells[24]. Therefore, the suitability of CD166 as a marker of non-differentiated hBM-MSCshas been questioned. In our study, almost all cells were CD166-positive in both cell populations, while MFI of CD166 was only slightly increased after osteogenic differentiation of cartilage-derived cells vs. non-differentiated cells (Fig. 1).

Noteworthy, our previous results showed that stronger ALDHactivity and stronger chondrogenic phenotype correlated with more abundant CD54 expression counts in chondrocytes[23]. Moreover, CD49c and CD54 have been previously reported to be associated with chondrogenic potential by other authors[5,7]. Therefore, these proteins cannot be attributed to the specific osteogenic differentiation markers, but they seem to rather characterize a specific cell population. This suggests that MSCA1 identifies a unique cartilage derived cell population and other markers do not correspond to the same cell group.

For the cell separation, MACS or FACS were used in our study. Previous studies demonstrated that cell yield and viability differs between these methods[34], and even between different magnetic separation techniques (MACS vs. EasySep)[35]. Similarly to the previous reports[14], magnetic cell sorting in our experiments resulted in rather high numbers of MSCA1^{high} cells, up to 34%, in the MSCA1^{low} fraction which could affect the differences of gene and protein expression in sorted fractions in the present study (Fig. 2). Potentially, the detected differences in certain tested marker expression would be more pronounced, if the higher purity of the separated MSCA1^{high} and MSCA1^{low} fractions could have been achieved.

Gene expression analysis in the sorted chondrocytes showed that expression of genes, associated with osteogenic and adipogenic potential, namely *ALPL*, *RUNX2*, *COL1A1* and *PPARG*, were enriched in MSCA1^{high} fraction (Fig. 3) even without any induction of differentiation, which indicates bias in their commitment to the osteogenic lineage.

Osteogenic induction led to remarkably stronger differentiation towards osteogenic lineage in MSCA1^{high} cells, as demonstrated by the considerably more intensive staining of calcium deposits, observed in this cell population, as compared to the general non-sorted cell population, and particularly to the sorted MSCA1^{low} cell fraction (Fig. 4C and D). In the latter, accumulation of calcified nodules was very weak. Taken together, these data demonstrate considerably elevated functional osteogenic potential of MSCA^{high} chondrocytes. Differences in adipogenic differentiation capacity were less pronounced.

Previous studies[14], in contrast, report no association between the *ALPL* expression levels and mineralization, suggesting that this inconsistency might have occurred due to the presence of FGF-2 during the chondrocyte expansion.

Several studies also observed the association of MSCA1 with osteogenicdifferentiation potential, including mineralization in periosteal cells of human jaw[34–36]and bone marrow[24]. Furthermore, inhibition of TNAPin human and murine osteoblasts lead to a decreased level of osteocalcin expression[20,37].

Induction of adipogenesis led to an increase of MSCA1 activity in human adipose tissue derived MSCs and defined the MSCA1^{high}cell subset being the most committed cells toward white and briteadipogenesis[13]. Our results also showed somewhat stronger *PPARG* expression in non-differentiated MSCA1^{high} chondrocytes (Fig. 3) and a tendency of a stronger chondrocyte adipogenic differentiation in the MSCA1^{high} fraction (Fig. 4A and B).

In this study, statistically significant enrichment of chondrogenic marker expression was observed in MSCA1^{low} subpopulation (Fig. 3). Especially big difference between fractions was found for *COL2A1*, the gene encoding the most characteristic and abundant hyaline and articular cartilage protein. Chondrocytes in MSCA1^{high} fraction demonstrated the extremely low expression of the *COL2A1* gene, sometimes at the edge of detection level, which along with the reduced expression of *SOX9* indicates that while acquiring osteogenic characteristics, this subpopulation is strongly biased against chondrogenic commitment. Chondrogenic capacity of sorted chondrocytes would have been of interest in this study, however, due to low amounts of cells collected after sorting the fractions, it was impossible to implement. Chondrogenic differentiation protocol requires large amounts of cells (at least 0,5-1 × 106/sample) and thus long cultivation and passaging of sorted chondrocytes, which affects their phenotype and results in dedifferentiation. Therefore, the redifferentiation of sorted and expanded chondrocytes following multiple passages can hardly reflect their chondrogenic capacity and thus cannot be comparable to osteogenic and adipogenic capacity immediately following sorting. Nevertheless, this would be of interest to study in the future studies.

As mentioned above, our previous results showed that *ALDH1A2* and *ALDH1A3*genes, responsible for ATRA synthesis, were associated with stronger chondrogenic phenotype in ALDHactivity-sorted chondrocytes[23]. In agreement to that, we hereby also found that sorted MSCA1^{low} cells had stronger expression of *ALDH1A2* and *ALDH1A3* genes, along with upregulation of ATRA-degrading enzyme *CYP26B1* gene (Fig. 3). As the chondrogenic markers are also enriched in the same fraction, ATRA-associated enzymes again showed association with chondrogenic phenotype in the present study.

Previous study showed capability of MSCA1^{high}/CD56$^{+/-}$ sorted BM-MSCs to differentiate into cells of osteogenic, adipogenic, chondrogenic, myogenic, neuronal, and pancreatic lineages, emphasizing that effective chondrogenesis is restricted to the MSCA1^{high}/CD56^{high}MSC subset[9]. In contrast, our results showed almost no expression of *COL2A1* in MSCA1^{high} population, furthermore, other chondrogenic markers were also significantly enriched in the MSCA1^{low} fraction. Those discrepancies between the studies might have occurred

due to the differences in MSCA1 role in BM-MSCs vs. chondrocytes, suggesting that it potentially indicates different cell phenotypes in those two populations.

There is an increasing body of evidence that combinations of several distinct cell populations are beneficial and even essential in tissue engineering. For instance, for the treatment of osteochondral defects, application of a construct comprising both, articular cartilage- and subchondral bone- derived differentiated cells was suggested[38,39]. In addition, osteochondral constructs become increasingly used as cartilage and joint models in vitro. Appropriate hybrid tissue constructs/organoids could be formed using distinct cell types with the established profiles, e.g., joint cartilage constructs could be formed from osteogenic and chondrogenic differentiation committed cells of different origins. Our results show an advantage of using MSCA1, where cartilage-derived cells could be separated according to their differentiation potential, and both populations from the same individual patient sample used to form such constructs.

Taken together, we conclude that MSCA1/TNAP is a robust biomarker to identify and sort cartilage-derived cells prone to differentiation towards osteogenic lineage in the MSCA1^{high} fraction from MSCA1^{low} chondrocytes, that possess high *COL2A1* expression and other cartilage biomarker enrichment. Results of this study suggest that MSCA1/TNAP in chondrocytes can be used as a biomarker to predict their osteogenic capacity without tedious and long-lasting differentiation procedure. Furthermore, it could be considered for therapeutic applications of chondrocytes for treatment of cartilage lesions or OA, and might help to increase the chondrogenic capacity and avoid undesired mineralization by depleting the culture of MSCA1^{high} cells, prone to osteogenic differentiation and mineralization (osteophyte formation) from the cells retaining chondrogenic phenotype. This may allow for enrichment of chondrocytes and depletion of osteoprogenitors. Finally, these two populations may be used in desired combination for creation of osteochondral tissue constructs and organoids.

Methods

Chondrocyte isolation and culture

Samples of cartilage ($n=5$) were obtained from the knee joints of patients with OA removed during the joint replacement surgery at "Republican Vilnius University Hospital", according to the Study Protocol approved by the Lithuanian Bioethics Committee (No. 158200–12–270-63), following informed consents of the patients (Table 3). Lateral tibia and femur compartments were used for chondrocyte isolation, with the depth of the cut approximately 2–5 mmthickness. Chondrocytes were isolated as described previously[23]. Briefly, cartilage specimens were cut from areas without visible lesions, incubated overnight in DMEM (Gibco, Germany, 21885-025) with 1% antibiotics penicillin and streptomycin (PS) (Sigma Aldrich, USA, P4333-100mL) in 37 °C humidified incubator with 5% CO_2, digested for 60 min with pronase (Sigma Aldrich, USA, 9036-06-0), washed and digested with collagenase type II (Biochrom, Germany, C2-22-BC), gently shaking at 37 °C for 3 h. The harvested cell suspension was seeded at density 20,000–30,000 cells/cm^2 in tissue culture flasks in culture medium – DMEM with 1 g/L glucose, 10% FBS (Fetal bovine serum) (Merck, Germany, S 0615) and 1% PS. At sub-confluence, cells were harvested by trypsin/EDTA digestion (Gibco, Germany, 25200056) and re-plated for further expansion or analysis. The medium was changed twice a week.

Cell surface marker expression

Isolated chondrocytes (passage 2–3) were cultivated in monolayer from day 1 up to 12 weeks, then trypsinized, washed with PBS (Biochrom, Germant, L 182-01) + 1% BSA (Bovine serum albumin) (Sigma Aldrich, USA, A2153-100G) and stained with specific fluorochrome-conjugated antibodies on ice in the dark for 30 min. Concentration for each antibody was optimized and used according to the manufacturer's instructions, then washed again and stained with 7AAD (7-Aminoactinomycin D) (Invitrogen, USA, A1310) at RT for 20 min to exclude the dead cells from the analysis. Non-specific staining was assessed using appropriate isotype fluorochrome-conjugated controls; samples were also stained with 7AAD. Cells were analyzed with cytometer/sorter FACSAria (Becton Dickinson, USA).

All monoclonal antibodies and isotype controls, used in this study (Table 4), were directly conjugated to fluorochromes. Gates for the specific surface markers were placed according to the appropriate isotype controls. Data was analyzed with BD FACSDiva software. Fluorescence Activated Cell Sorting (FACS) of cells labeled with anti-MSCA1-APC-conjugated antibodies was carried out on flow cytometer/sorter FACSAria (Becton Dickinson, USA) according to the manufacturers' instructions.

Cell sorting

Chondrocytes (passage 3–4) were sorted according to MSCA1 expression by two different techniques: (1) FACS, as described in previous section; and (2) Magnetic-activated cell sorting (MACS) with magnetic particles, columns and equipment from Miltenyi Biotech, Germany, using 1 million of each donor cells for

Sample	Sex	Year of birth	Height/weight	OA grade (K&L)
Donor 1	F	1957	160/88	3
Donor 2	F	1946	159/100	4
Donor 3	M	1943	152/92	4
Donor 4	F	1945	162/87	4
Donor 5	M	1949	170/94	3

Table 3. OA cartilage donor information.

Antibody	Isotype	Clone	Producer
CD46 FITC	IgG1	3F1	BD Pharmigen
CD47-PE(R-Phycoerythrin)	IgG1	B6H12	BD Pharmigen
CD49a-PE	IgG1	SR84	BD Pharmigen
CD49c-PE	IgG1	C3II	BD Pharmigen
CD54-APC (allophycocyanin)	IgG1	51-10C9	BD Pharmigen
CD55-PE	IgG2a	NaM-16-4D3	Santa Cruz Biotech
CD140b-APC	IgG1	18A2	BioLegend
CD166-PE	IgG1	3A6	BioLegend
Notch1-APC	IgG1	MHN-519	eBioscience
MSCA-1- APC	IgG1	W8B2	Miltenyi Biotec
Isotype control-PE	IgG1	-	Santa Cruz Biotech
Isotype control-PE	IgG2a	MOPC-173	Biolegend
Isotype control-APC	IgG1	-	Santa Cruz Biotech
Isotype control-FITC	IgG1	-	Santa Cruz Biotech

Table 4. Antibodies used in the study.

every sorting method. For MACS sorting the following materials were used: anti-MSCA1 antibody, conjugated with APC; magnetic particles, conjugated to anti-APC antibodies; LS columns; VarioMACS instrument (all from Miltenyi Biotech, Germany) according to manufacturer's protocols. Briefly, cells were labeled with anti-MSCA1-APC antibody and evaluated with flow cytometer/sorter FACSAria as described in previous section. Then stained cells were incubated with anti-APC magnetic particles, washed with PBS and separated on LS columns attached to VarioMACS instrument. Two different fractions – MSCA1-positive and MSCA1-negative were collected from the columns and then evaluated with flow cytometer. To discriminate cells with positive or negative fluorescence signal from sorted positive and negative fractions, we introduced designation $MSCA1^{high}$ and $MSCA1^{low}$, respectively, for sorted populations and $MSCA1^{high}$ and $MSCA1^{low}$ for cells with positive and negative fluorescence signal, respectively.

Part of the sorted cells were washed with PBS, immediately frozen in liquid nitrogen and stored at −80 °C for gene expression analysis.

Cell differentiation

Adipogenic differentiation

Chondrocytes (passage 3–4) were seeded at a density of 3×10^4 cells/well in 12-well plates and cultured in culture medium until they reached confluence, then for 3 weeks in adipogenic differentiation medium, prepared from DMEM with 1 g/L glucose, supplemented with 20% FBS, 1 µM Dexamethasone (Sigma Aldrich, USA, D4902-25MG), 0.5 mM IBMX (Isobutylmethylxanthine) (Biosource, USA, I5879-100MG) and 60 µM Indometacin (Sigma Aldrich, USA, I7378-10G). Control cells were simultaneously cultured in culture medium. Adipogenic and control medium was changed twice a week. After differentiation, lipid droplets in the cells were stained with Oil Red O (Carl Roth, Germany, C.I. 26125) and visualized by inverted light microscope. For quantitative evaluation, Oil Red O staining was eluted with isopropanol and the absorbance was read at 540 nm, using spectrophotometer Spectramaxi3.

Osteogenic differentiation

Chondrocytes (passage 3–4) were seeded at a density of 3×10^4 cells/well in 12-well plates, cultured in culture medium until reaching subconfluence, after which treated with osteogenic differentiation medium, prepared from DMEM with 4.5 g/L glucose, supplemented with 10% FBS, 0.1 µM dexamethasone (Sigma Aldrich, USA, D4902-25MG) and 0.17 mM ascorbic acid phosphate (Sigma Aldrich, USA, 49752-10G). After 3 days, β-glycerophosphate (Santa Cruz Biotechnology, USA, sc-203323 A) was added to the osteogenic differentiation medium (final concentration 10 mM), and differentiation was performed for another 1–3 weeks. Control cells were simultaneously cultured in culture medium. Osteogenic and control medium was changed twice a week. Qualitative evaluation of osteogenesis was performed by light microscope after cell staining with Alizarin Red S (Carl Roth, Germany, 4476.1). For quantitative evaluation, calcified nodules were solubilized for 1 h at RT with 10% of hexadecylpyridinium chloride monohydrate (CPC) solution in 10 mM sodium phosphate (pH 7.0) at RT for 1 h. The absorbance was read at 540 nm, using spectrophotometer Spectramaxi3.

RNA extraction and RT-qPCR

RNA extraction from harvested chondrocytes (approx. 1 million cells of $MSCA1^{high}$ and 1 million cells of $MSCA1^{low}$) was performed using RNeasy Mini Spin columns (Qiagen, Germany, 74106) according to the manufacturer's instructions. RNA concentration and purity were measured with the NanoPhotometer Pearl (Implen, Germany). RNA samples were treated with DNase I (ThermoFisher Scientific, Lithuania, K1671) and cDNA synthesis was performed using the MaximaFirst Strand cDNA Synthesis Kit (ThermoFisher Scientific, Lithuania, K1671) according to the manufacturer's protocols. PCRs were performed using Maxima Probe qPCR Master Mix (2x) (ThermoFisher Scientific, Lithuania) and Stratagene MX-3005P detection instrument

(Agilent Technologies, USA). The TaqMan Gene Expression Assays (Applied Biosystems, USA) for 12 genes were used for gene expression analysis. Gene primers used in the study are presented in Table 1. RPS9 was used as housekeeping gene, as it was the only stably expressed gene in presented chondrocyte cultures. B2M was additionally tested, however the gene remained instable due to cytokines secreted by cells. The PCR reaction volume was 25 µL with 0.5 µL of 20x Taqman Gene Expression Assay mix. All reactions were run in triplicates. Cycle conditions were as follows: initial denaturation step for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C for denaturation, and 60 s at 60 °C for annealing and extension. Each RNA sample was controlled for genomic DNA contamination by reactions without reverse transcriptase (RT-) and reagent contamination was checked by the reactions without template (NTC). Relative mRNA levels were expressed as $2^{-\Delta Ct}$ and the fold change in gene expression in sorted fractions ($MSCA1^{high}$ vs. $MSCA1^{low}$) was expressed as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were analyzed using GraphPad Prism 5 software and paired samples t-test was used on ΔCt (dCt) values of $MSCA1^{high}$ and $MSCA1^{low}$ sorted cells. A paired t-test was used for surface marker expression ratio of osteo-differentiated vs. non-differentiated cells. Two-way ANOVA was used for the analysis of MACS sorting of different cell preparations from different cell donors. ANOVA with Tukey's post-hoc was used for quantitative evaluation of adipogenic and osteogenic differentiations. P values less than 0.05 were considered statistically significant.

Data availability

The data supporting these findings can be found at Innovative medicine centre, Department of Regenerative medicine.

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Author contributions

A.U. conceptualization, study design, acquisition of data, article draft; I.U. acquisition of data, article draft; E.Bag. study design, acquisition of data, analysis and interpretation; A. Zen. Acquisition of data, result analysis; N. Por. Patient enrolment and sample collection; E.Ber. conceptualization, study design, critical review.

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Declarations

Competing interests

The authors declare no competing interests.

Ethics approval of research on humans or animals and patient consent

All procedures with the donor tissues were performed in accordance with the Bioethical Permission (No. 158200-14-741-257) approved by the Vilnius Regional Biomedical Research Ethics Committee. All patients approved the donation of their samples and signed patient consent form.

Additional information

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