

MSCA-1/TNAP Selection of Human Jaw Periosteal Cells Improves their Mineralization Capacity

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Key Words

MSCA-1/TNAP • LNGFR • Osteogenic capacity • Jaw periosteal cells • Mineralization • Osteogenic marker

Abstract

Human jaw periosteum-derived cells (JPCs) represent an alternative cell source to bone marrow-derived mesenchymal stem cells for tissue engineering applications in the oral and maxillofacial surgery. In this study we investigated how far the presence or expression of human mesenchymal stem cell antigen-1/tissue non-specific alkaline phosphatase (MSCA-1/TNAP) and LNGFR (CD271) can be utilized to select and enrich the osteogenic progenitor cell fraction from the entire JPC population. Depending on their mineralization capacity, we classified the human isolated JPCs into mineralizing (mJPCs) and non-mineralizing JPCs (nmJPCs). Flow cytometric analyses revealed that undifferentiated mJPCs expressed MSCA-1/TNAP at significant higher levels than nmJPCs at day 5 and 10 of osteogenesis. Western blot analyses showed increased MSCA-1/TNAP expression levels in mJPCs during osteogenesis, whereas in nmJPCs MSCA-1/TNAP expression remained undetectable. Using the MSCA-

1 and LNGFR specific antibodies, we separated the positive and negative fractions from the entire mJPC population. In order to analyse the mineralization capacity of the MSCA-1⁺ and LNGFR⁺ cell subsets, we quantified the calcium deposition in both subpopulations in comparison to the respective negative subpopulations. The MSCA-1⁺/TNAP⁺ cell fraction showed a significant higher osteogenic capacity compared to the MSCA-1⁻/TNAP⁻ cell fraction whereas the LNGFR⁺ cell fractions did not differ in their osteogenic potential. Our findings suggest that MSCA-1 may represent a promising osteogenic marker for mJPC.

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Introduction

Tissue engineered bone has high clinical impact in the reconstruction of the alveolar and the jaw bone in the oral and maxillofacial surgery [1, 2]. Nearly 20 years ago, the research group of A.L. Caplan re-discovered the osteogenic potential of periosteal cells [3]. Bone

defects in the jaws will increase during the next years by the aging of the society and tissue engineering applications using jaw periosteum-derived cells (JPCs) represent a possible treatment modality for these patients [4]. In tibial periosteum-derived cells the osteogenic potential remains the same as age of donors increases [5, 6]. This is not the case for bone marrow mesenchymal stem cells (BMMSCs), which are known to lose their osteogenic potential with increases in age [7, 8]. Unfortunately, the human periosteum-derived heterogeneous population contains not only progenitor cells, but to a main part tissue fibroblasts, probably to a lesser part muscle cells and adipocytes. For this reason, analysis of the specific cell surface antigen profile of the periosteal progenitor cells is of great impact for the identification of suitable markers which could be used for cell separations and improved clinical applications. The benefit of an extensive enrichment or sorting by molecular markers is quite controversial, Arnsdorf and colleagues hypothesized that expanded periosteal cultures may be a source for tissue engineering applications without these tools [9]. Previously, we showed that not all isolated human JPCs are able to mineralize *in vitro* and we obtained mineralizing (mJPCs) and non-mineralizing JPCs (nmJPCs) deriving from different donors [10]. The observation that we obtain mineralizing and non-mineralizing JPCs could be a problem of tissue harvesting. The isolated cell population from the nmJPC group may contain either none or too few progenitors or any other cells which support the mineralization process. Up to date there are no specific markers which could help to reliably separate the different cell types *a priori*. In a previous study we could demonstrate that the stem cell marker LNGFR (low affinity nerve growth factor receptor - CD271) is induced during the first five days of osteogenesis and that it was expressed at higher levels in mJPCs in comparison to nmJPCs [10]. LNGFR could be used as an early surface marker of osteogenic capacity *in vitro*, however, it could not be allocated to a distinct type of (osteoprogenitor) cells.

In the present work we investigated the human mesenchymal stem cell antigen-1 (MSCA-1) expression in mJPCs and nmJPCs. Bühring and colleagues identified several monoclonal antibodies with superior selectivity for mesenchymal stem cells, including the monoclonal antibodies W8B2 against human mesenchymal stem cell antigen-1 (MSCA-1) [11, 12]. This antibody recognizes an antigen which was shown to be restricted to the CD271 bright population of BMMSCs [13, 14].

Materials and Methods

Cell cultures

Biopsies of human jaw periosteum were obtained during routine maxillofacial interventions. A reliable separation of the outer (fibrous tissue) from the inner layer (cambium layer containing the progenitor cells) of periosteum was not possible. Samples from overall 12 donors were included in this study in accordance with the local ethical committee (411/2005V), following informed consent. JPCs were isolated in a main digestion step for 90 min by type XI collagenase (1500 U/ml, Sigma, Steinheim, Germany) for 60-90 min and then plated in 75 cm² cell culture flasks. The first medium change after primary adhesion of the cells was after 10-14 days. After reaching confluence JPCs were cultured in D-MEM: F-12 (Invitrogen-BioSource Europe, Nivelles, Belgium) containing 10% FCS (Sigma-Aldrich, Steinheim, Germany), penicillin/streptomycin and fungizids (Biochrom, Germany). Periosteal cells from the fifth to seventh passages were used for these experiments (for details [15]).

We classified the isolated and expanded cells into two groups: 1) non-mineralizing JPCs (nmJPCs or osteogenic non responders), which were not able to calcify *in vitro* (n = 6); 2) mineralizing JPCs (mJPCs, osteogenic responders), which were able to calcify *in vitro* (n = 6). These two cell strains were derived from different donors and classification in m/nmJPCs followed after detection of calcium precipitates by alizarin stainings at day 20 of *in vitro* osteogenesis (cells were in the third passage). nmJPCs showed an alizarin-negative staining after cell culture for 20 days with osteogenic supplements (percentage of mineralization 0%). mJPCs clearly showed an alizarin-positive (reddish dye) staining after the same period of osteogenic differentiation (percentage of mineralization 50-90%). Due to the limited cell numbers it was not possible to make all substantial analyses described in this paper with the same donor cells. Some donor cells were used for one part of the experiments and others for the other part. However, only results from patient cells that behaved identically concerning the mineralization capacity were averaged (mJPC/nmJPC). For MACS separation only mJPC were used (see flow diagram).

Differentiation experiments

For the differentiation experiments, mJPCs and nmJPCs were cultured in D-MEM/10% FCS, 4 µM dexamethasone, 10 mM β-glycerolphosphate and 100 µM L-ascorbic acid 2-phosphate (Sigma-Aldrich, Steinheim, Germany), referred to as OB medium. Cells were treated with these components for a period of 20 days. The culture medium was replaced every two days. As controls, we used untreated cells that were cultivated in the absence of any osteogenic components for the same time period.

Flow cytometry analysis of MSCA-1/TNAP expression

After cell detachment with 0.5% trypsin and washing with PBS, cell pellets were incubated with 60 µl GAMUNEX 10% (human immune globulin solution) for 15 min at 4°C to block unspecific labelling. After an additional wash step, up to 10⁴ cells were incubated with 20 µl of MSCA-1-reactive antibody

W8B2 (culture supernatant) [16] for 15 min at 4°C. Cells were washed twice with FACS buffer (PBS/1% BSA/0.01% Na₃N) and incubated with the secondary polyclonal goat anti-mouse immunoglobulins (Dako, Hamburg, Germany) which were PE labelled for 15 min at 4°C. After two wash steps with FACS buffer, cells were analysed on a FACSCanto flow cytometer (Becton Dickinson, Heidelberg, Germany). Data were processed using the FCS Express software (DeNovo, Ontario, Canada). As negative controls, we incubated the cells with the secondary goat anti-mouse antibodies.

Western blot analysis of MSCA-1/TNAP expression

For western blot analysis, cells were harvested on ice. After centrifugation, pelleted cells were dissolved in 60 µl lysis buffer (1% NP40; 150 mM NaCl; 50 mM Tris/HCL, pH 8.0 plus protease inhibitors (Complete, EDTA-free, Roche, Mannheim, Germany)) and were incubated overnight at -70°C. Cell debris were removed by centrifugation and protein concentrations of the supernatants were measured using a colorimetric assay (RD DC Protein Assay, Bio Rad, Germany) according to the manufacturer's instructions. After addition of reducing Laemmli sample buffer, a total of 100 µg of nondenaturated cell lysate from the cellular extracts was separated by SDS-PAGE (8%) and transferred onto nitrocellulose membranes. The membranes were analyzed by immunoblotting using monoclonal mouse anti-MSCA-1 (culture supernatant 1:2) and anti-GAPDH (Abcam, Cambridge, UK) specific primary antibodies overnight at 4°C. Binding of the primary antibodies was detected with peroxidase-conjugated goat anti-mouse secondary antibodies (Santa Cruz Biotechnology, USA) and visualized by the enhanced chemiluminescence method (GE Healthcare, Freiburg, Germany).

Magnetic labelling and separation of MSCA-1/TNAP^{+/+} and CD271^{+/+} cell fractions

MSCA-1/TNAP^{+/+} and CD271^{+/+} cell fractions were separated from the whole cell population of mJPC using the human anti-MSCA-1 and anti-CD271 MicroBead Kit (Miltenyi Biotec, Germany). Briefly, up to 2x10⁷ total cells were incubated with 40 µl blocking reagent and 40 µl anti-MSCA-1 or anti-CD271 MicroBeads for 20 min in the refrigerator (2-8°C). After washing and centrifuging cell suspension was applied onto the magnetic MS column using pre-separation filters to remove cell clumps. The unlabeled cell fractions passed through the column, MSCA-1⁺/CD271⁺ cells were retained within the column. After washing with 500 µl PBS/0.5% BSA/2mM EDTA three times, the column was removed from the separator and the positive fraction was collected. 4x10⁴ cells (MSCA-1⁺ or CD271⁺ cell fraction) were plated per well of a six-well plate for quantitative analysis of calcium deposition. After an overnight incubation to allow the cells to adhere, culture medium was removed and cells were treated with osteogenic stimuli (dexamethasone, ascorbic acid, β-glycerophosphate) for 20 days.

Quantitative analysis of the mineralization capacity of MSCA-1/TNAP^{+/+} and CD271^{+/+} cell fractions

Calcium deposition of JPCs was detected by alizarin staining. Therefore, cells were fixed in ice-cold methanol for 2 min, air-dried and stained with alizarin (40mM) for 20 min. After intensive washing (four times with deionized H₂O and gentle rocking for 5 min), cells were rinsed in PBS, dehydrated with ice-cold ethanol and air-dried. Differentiated cells containing mineral deposits were stained bright red.

Quantitative analysis of Alizarin Red staining was performed using a commercial osteogenesis quantification kit (Chemicon International) and following manufacturer instructions. In brief, the Alizarin dye could be extracted from the stained monolayer by adding 10% acetic acid to each well and shaking for 30 min. The monolayers were detached by a cell scraper and transferred to microcentrifuge tubes. After heating at 85°C for 10 min, cooling on ice for 5 min and centrifuging at 20000xg for 15 min, supernatants were transferred to fresh tubes and neutralized with 10% ammonium hydroxide. Mineral deposit quantification was performed by determining optical density values at 405 nm of a set of known standards and unknown samples.

Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was performed using two sample t-tests for data with a normal distribution. A value of p<0.05 was considered significant.

Results

Flow diagram illustrating the selected experimental design

For better understanding of the selected experimental design a flow diagram was generated (Fig. 1). The heterogeneous population of periosteal cells isolated from different donors underwent osteogenic differentiation for 20 days. The osteogenic responder cell group was able to mineralize *in vitro* (mineralizing JPCs = mJPC, total sample number n = 6), as shown by the alizarin staining, whereas the non-responder cell group remained alizarin negative (non-mineralizing = nmJPC, total sample number n = 6). Both cell groups were analysed by FACS and western blot concerning MSCA-1 expression. Additionally, only mJPCs were separated by MACS using the MSCA-1 and the CD271 specific antibody. MSCA-1^{+/+} and CD271^{+/+} cell fractions underwent osteogenesis and mineralization potential of the respective cell fractions were photometrically quantitated.

Fig. 1. Flow diagram illustrating the experimental design. MACS = magnetic activated cell sorting, FACS = fluorescence activated cell sorting, WB = western blot, mJPC = mineralizing jaw periosteal cells, nmJPC = non-mineralizing jaw periosteal cells, MSCA-1 = mesenchymal stem cell antigen-1.

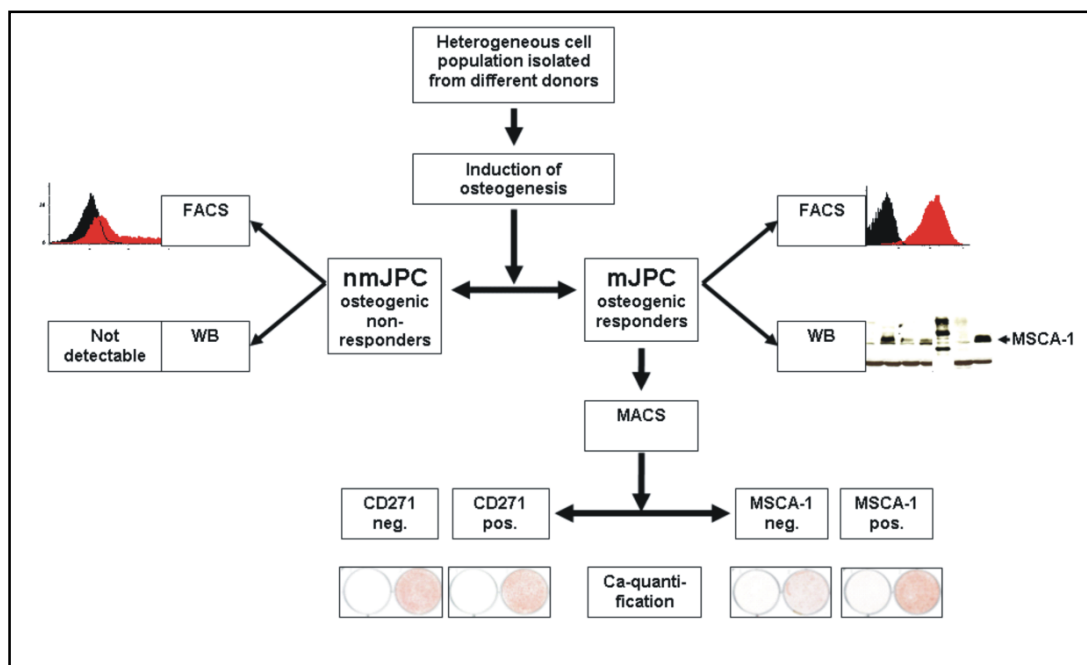
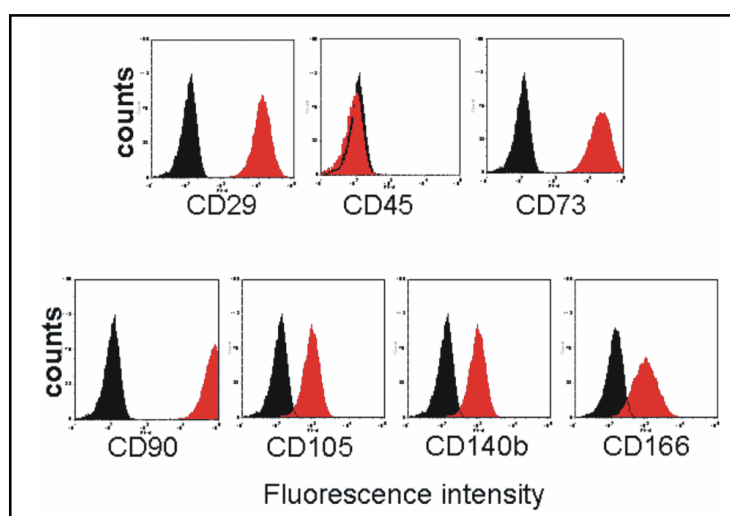


Fig. 2. Stem cell marker profile of human jaw periosteal cells. The phenotype of expanded human JPCs was analysed by flow cytometry ($n=4$) to compare a stem cell marker profile associated with bone marrow mesenchymal stem cells (BMMSCs). Representative histograms of CD29, CD45, CD73, CD90, CD105, CD140b, CD166 are shown. JPCs were positive for CD29, CD73, CD90 and negative for CD45. JPC expression of CD105, CD140b and CD166 varied between 50-100%.



Stem cell marker profile of human jaw periosteal cells

Pittenger and Dominici defined CD29 ($\beta 1$ -integrin), CD73 (Ecto-5'-nucleotidase), CD90 (Thy-1) and CD105 (endoglin) to be indicative (but not definitively so) of MSC phenotype. MSC do not express the haematopoietic marker CD45 (leucocyte cell antigen) and express the adhesion molecules CD166 (ALCAM) and CD140b (PDGF-RB) [11, 17-20]. By flow cytometry, we determined the expression of these stem cell markers to characterize the phenotype of our human jaw periosteal cells. Independent from their mineralization capacity, JPCs were positive for CD29, CD73, CD90 and negative for

CD45. CD105, CD140b and CD166 expression varied between 50-100%. Representative histograms from $n = 4$ experiments of each marker are shown in Fig. 2.

Flow cytometry analysis of MSCA-1/TNAP expression in mJPCs versus nmJPCs

MSCA-1/TNAP expression was analysed by flow cytometry at day 5 and 10 of osteogenesis. At later time points (day 20) mJPCs calcified and cells could not be detached from the plastic flasks to get a single cell suspension as required for FACS analysis. mJPCs and nmJPCs showed a clearly different MSCA-1/TNAP expression pattern. In untreated control cells from

Fig. 3. Fluorescence intensity of MSCA-1/TNAP expression in mJPCs vs. nmJPCs. MSCA-1/TNAP expression in untreated and OB-treated mJPCs and nmJPCs (A) cultured for 10 days was analysed on a FACSCanto flow cytometer. Median fluorescence intensity (MFI) of MSCA-1/TNAP protein expression in mJPCs vs. nmJPCs was evaluated after day 5 and 10 (B) of osteogenesis. * $p < 0.05$; ** $p < 0.001$. We found significantly higher MSCA-1/TNAP expression levels in untreated controls and OB treated mJPCs ($n = 4$) than in nmJPCs ($n = 4$).

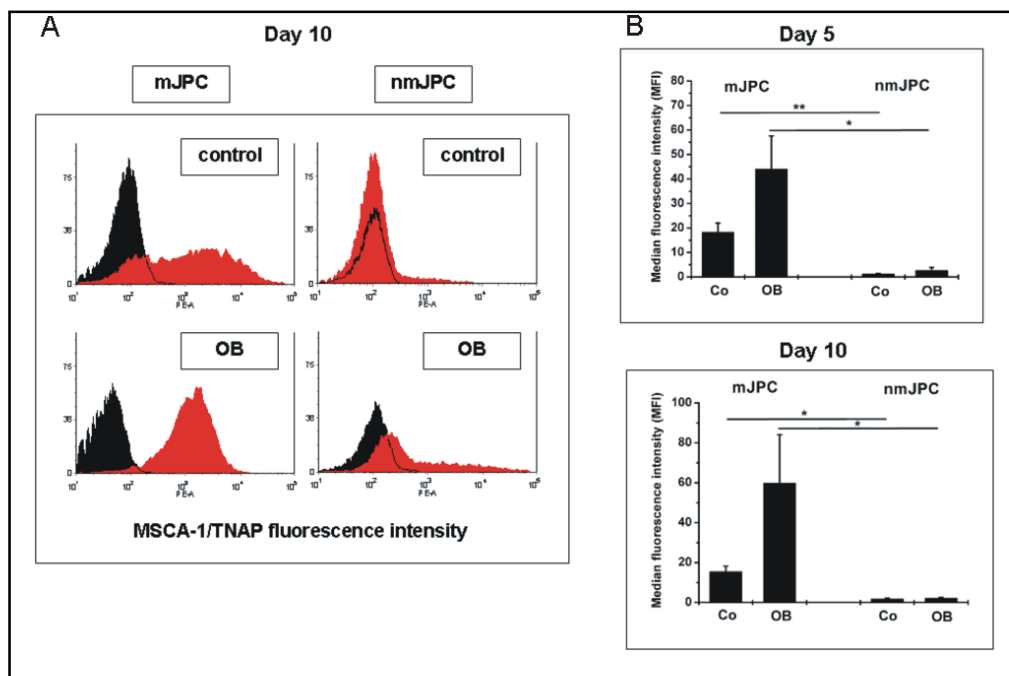
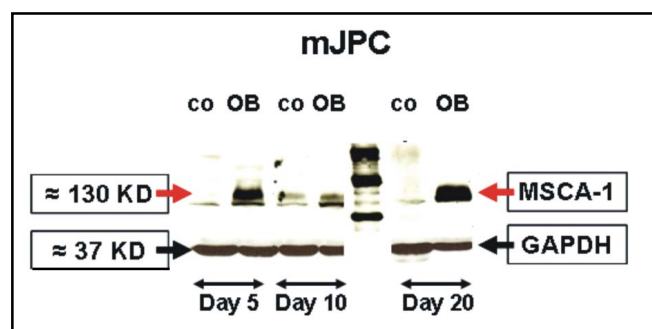


Fig. 4. Western blot analysis of MSCA-1/TNAP expression. MSCA-1/TNAP protein expression in untreated and OB-treated mJPCs ($n=3$) was detected after day 5, 10 and 20 of osteogenesis. The detected molecular size of the MSCA-1/TNAP binding protein was shown to be between 130-140 KDa. As loading controls, GAPDH (37KDa) protein expression was detected in the same lysates. MSCA-1/TNAP specific signals in nmJPCs remained under the detection limit. In contrast, differentiating mJPCs revealed strong MSCA-1/TNAP signals following osteogenic treatment.



the mJPC group, we detected a strong MSCA-1/TNAP^{bright} expression (on average 79% at day 10, representative histograms in Fig. 3A upper panel), whereas undifferentiated nmJPCs revealed a much weaker MSCA-1/TNAP^{bright} expression (on average 35% at day 10, representative histograms in Fig. 3A upper panel). OB-treated mJPCs showed a clear-cut shift so that almost all cells were MSCA-1/TNAP positive (on average 93% at day 10, representative histograms in Fig. 3A lower panel). In contrast, OB-treated nmJPCs were only 23% on day 5 and 45% on day 10 positive (representative histograms in Fig. 3A lower panel).

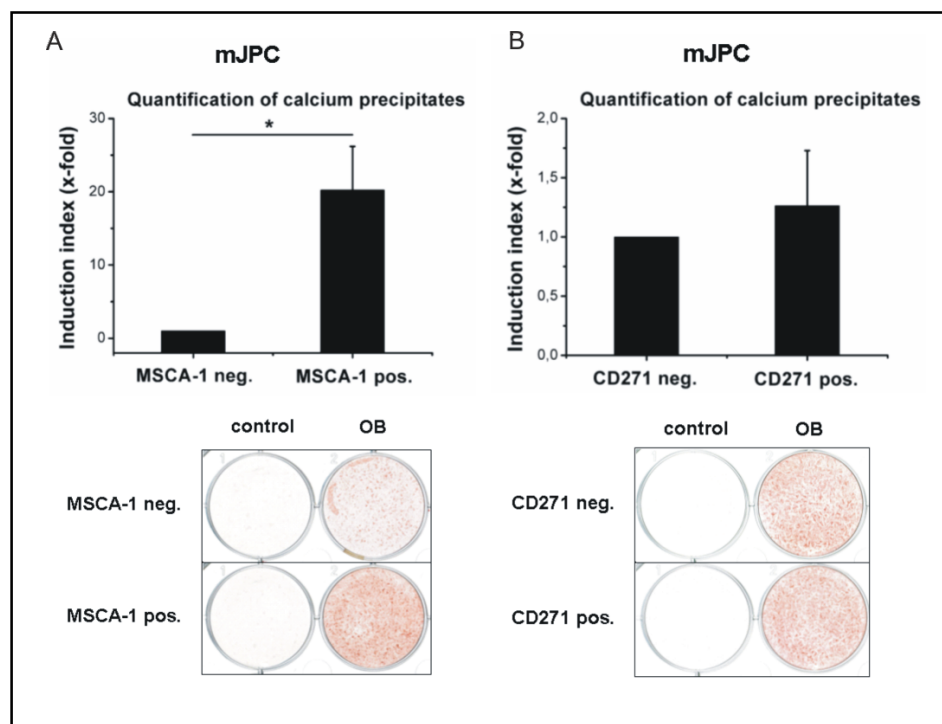
Quantitative analysis of the median fluorescence intensity (MFI) revealed significantly higher MSCA-1/TNAP expression levels in untreated controls and OB treated mJPCs ($n = 4$) than in nmJPCs ($n = 4$). Untreated mJPC controls showed at day 5 and 10 significant higher

MSCA-1/TNAP expression levels than the nmJPC controls (day 5: 18.32 ± 3.66 versus 1.27 ± 0.21 , $p < 0.05$ (Fig. 3B, upper panel); day 10: 15.47 ± 2.7 versus 1.77 ± 0.36 , $p < 0.05$ (Fig. 3B, lower panel). Induction of osteogenesis lead to a MSCA-1/TNAP upregulation in both cell groups, however, expression levels in mJPCs were significant higher than in nmJPCs (day 5: OB-treated: 44 ± 13.56 versus 2.66 ± 1.18 , $p < 0.05$; day 10: OB-treated: 59.84 ± 24.23 versus 2.22 ± 0.36 , $p < 0.05$; Fig. 3B upper and lower panel).

Western blot analysis of MSCA-1/TNAP expression

Using the same antibody as for FACS analysis we established the MSCA-1/TNAP specific western blot ($n = 3$). As shown in Fig. 4 we detected a protein with a molecular weight of approximately 130 Kd. Differentiating mJPCs revealed strong MSCA-1/TNAP signals following

Fig. 5. Quantitative analysis of the mineralization capacity of MSCA-1/TNAP^{+/−} and CD271^{+/−} cell fractions. mJPCs were magnetically labelled using the MSCA-1/TNAP (A, n=4) and CD271 (B, n=4) specific MicroBeads and the obtained positive and negative cell fractions were separated from the entire untreated mJPC population. Both cell fractions were plated onto 6-well plates and underwent osteogenesis for 20 days. Calcium deposition was quantified after Alizarin Red staining of the MSCA-1/TNAP^{+/−} and CD271^{+/−} cell fractions. Representative pictures of the stainings are shown beneath the diagrams. *p<0.05. The MSCA-1⁺/TNAP⁺ but not the CD271⁺ cell fractions revealed significant higher amounts of calcium precipitates in comparison to the MSCA-1/TNAP[−] cell fractions.



osteogenic treatment with OB medium. MSCA-1/TNAP protein expression in untreated mJPC controls remained under the detection limit or showed very weak signals. Specific MSCA-1/TNAP signals in cell lysates from undifferentiated and differentiated nmJPCs could not be detected by western blot.

Quantitative analysis of the mineralization capacity of MSCA-1/TNAP^{+/−} and CD271^{+/−} cell fractions

Using the MACS technology MSCA-1/TNAP^{+/−} and CD271^{+/−} cell fractions (n=4 for each group) were isolated from the heterogeneous periosteal population, seeded onto 6-well plates and underwent osteogenesis. At day 20 of differentiation mineralization capacity of both cell fractions from mJPCs was analysed. Both cell fractions showed a positive alizarin staining, however, the MSCA-1/TNAP⁺ cell fraction revealed significant higher amounts of calcium precipitates in comparison to the MSCA-1/TNAP[−] cell fraction (induction index: 20.22 ± 5.96 in comparison to the MSCA-1/TNAP[−] cell fraction which was set as 1, n=4, Fig. 5A). In contrast to these results the CD271⁺ and CD271[−] cell fractions showed no significant differences regarding their osteogenic potential (induction index of CD271⁺ cell fraction 1.26 ± 0.47 in comparison to the CD271[−] cell fraction which was set as 1, n=4, Fig. 5B).

Discussion

Previously we demonstrated that not all isolated human jaw periosteal cells are able to mineralize *in vitro* [10], however, the identification of a well-defined, lineage homogeneous cell population and the enrichment of bone progenitor cells from the initial heterogeneous population would be an important step to improve cell-therapy based bone transplantations. Searching of patient files points that the mineralization capacity of human JPCs (tested by Alizarin and von Kossa stainings) was not age-dependent or controlled by the patient history. Analogue to published data considering age independency of the analysed osteogenic potential of periosteal cells, we made the same observation. The examination of all patient cells tested in the past 3 years in our laboratory showed an average age of the mJPC population of 46,5 whereas that of the nmJPC population was 50,7 (for each group n=25, the difference was not significant).

To characterize our jaw periosteal cells we examined a panel of common stem cell surface markers – however, the expression of CD29⁺/CD73⁺/CD90⁺ and partly CD105⁺/CD140b⁺/CD166⁺ surface markers (Fig. 2) was not dependent on their mineralization capacity. The question rose up, whether it would be possible to enrich *a priori* the (unknown) progenitor cell population by MACS sorting using common stem cell markers. For this reason

we choose the low-affinity nerve growth factor receptor (LNGFR - CD271) as a known surface stem cell marker. LNGFR was described as a selective marker for the purification and phenotypic characterization of BMMSC [21]. In former studies we could show by FACS analysis a significantly increased LNGFR surface expression in mJPCs versus nmJPC during the first five days of osteogenesis. By western blotting analysis much stronger LNGFR signals were detected in mJPC in comparison to those from nmJPC [10]. However, despite of stronger LNGFR induction in mJPC we could not demonstrate in the present study a significant difference regarding the osteogenic capacity of the CD271⁺ enriched cell fraction in comparison to the CD271⁻ one (Fig. 5B). For this reason LNGFR does not represent an appropriate surface marker for MACS enrichment of periosteal progenitor cells. Probably, LNGFR induction during osteogenesis is an epiphenomenon however, the role of LNGFR should be further elucidated. Definitively, LNGFR had in our study no influence on the osteogenic capacity of mJPC. Moreover, we demonstrated that the expression pattern of the newly defined human stem cell marker MSCA-1 (mesenchymal stem cell antigen-1) was quite different in mJPCs compared to that in nmJPCs (Fig. 3A and 3B). The close relation of MSCA-1 and LNGFR is characterized by the fact that the monoclonal antibody W8B2 against human MSCA-1 recognizes an antigen which was shown to be restricted to the LNGFR bright population of BMMSCs [13, 14]. In a recently published paper the mesenchymal stem cell antigen MSCA-1 was identified as tissue non-specific alkaline phosphatase (TNAP), which is highly expressed in embryonic stem cells and bone cells as well [22]. This target gene identification is in line with previous measurement data from our group, which showed that AP (alkaline phosphatase) gene induction was significantly higher in mJPCs during the whole osteogenic differentiation process compared to nmJPCs [10]. Although FACS analyses revealed much higher MSCA-1/TNAP

expression levels even in untreated mJPC controls (Fig. 3A), western blot signals in JPC lysates from the undifferentiated state were detected only in exceptional cases (Fig. 4) based on higher sensitivity of the flow cytometry approach. This is consistent with de Bari's examination which describes an almost undetectable expression of alkaline phosphatase in undifferentiated tibial periosteal cells [5]. These findings indicate that *a priori* magnetic separations of untreated JPCs using the MSCA-1/TNAP antibody turn out to be difficult due to the poor positive fraction obtained. In our experience the percentage of MSCA-1/TNAP positive undifferentiated mJPCs was higher than in nmJPCs, however, the yielded cell number was not sufficient for extensive experiments. Under osteo-inductive conditions, mJPCs showed strongly upregulated MSCA-1/TNAP expression levels both by FACS and western blot analyses. Based on this fact and on the observation that the MSCA-1/TNAP positive cell fraction showed stronger mineralization capacity, it would make more sense to further expand MSCA-1/TNAP positive cell fractions after MACS separation.

Taken together, identifying of specific markers to hallmark the periosteal progenitors is essential to improve tissue engineering applications using this stem cell type. Our data implicate the MSCA-1/TNAP relevance for its utility as a separation marker for periosteal progenitor cells due to the fact that MSCA-1 seems to influence the osteogenic potential of mJPC (Fig. 5A). Further analysis on molecular level should be conducted to fully characterize the MSCA-1/TNAP⁺ cell population and its osteogenic potential. Ongoing work is focused on optimizing this approach to get higher cell yields which enable the performance of more profound analyses.

Our findings suggest that the MSCA-1⁺/TNAP⁺ enriched human JPC population exhibits higher osteogenic capacity than the MSCA-1⁻/TNAP⁻ and the CD271⁺ cell fraction. This procedure could help to improve clinical bone tissue engineering applications using this stem cell type.

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