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Molecules and Cells

Development of Novel Monoclonal Antibodies that Define Differentiation Stages of Human Stromal (Mesenchymal) Stem Cells

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Human mesenchymal stem cells (hMSC) are currently being introduced for cell therapy, yet, antibodies specific for native and differentiated MSCs are required for their identification prior to clinical use. Herein, high quality antibodies against MSC surface proteins were developed by immunizing mice with hMSC, and by using a panel of subsequent screening methods. Flow cytometry analysis revealed that 83.5, 1.1, and 8.5% of primary cultures of hMSC were double positive for STRO-1 and either of DJ 3, 9, and 18, respectively. However, none of the three DJ antibodies allowed enrichment of clonogenic hMSC from BMMNCs as single reagents. Using mass-spectrometric analysis, we identified the antigen recognised by DJ3 as CD44, whereas DJ9 and DJ18 recognized HLA-DRB1 and Collagen VI, respectively. The identified proteins were highly expressed throughout *in vitro* osteogenic- and adipogenic differentiation. Interestingly, undifferentiated cells revealed a sole cytoplasmic distribution pattern of Collagen VI, which however changed to an extracellular matrix appearance upon osteogenic- and adipogenic differentiation. In relation to this, we found that STRO-1$^{+/-}$/Collagen VI^{-} sorted hMSC contained fewer differentiated alkaline phosphatase^{+} cells compared to STRO-1$^{+/-}$/Collagen VI^{+} hMSC, suggesting that Collagen VI on the cell membrane exclusively defines differentiated MSCs. In conclusion, we have generated a panel of high quality antibodies to be used for characterization of MSCs, and in addition our results may suggest that the DJ18 generated antibody against Collagen VI can be used for negative selection of cultured undifferentiated MSCs.

INTRODUCTION

Bone marrow derived stromal (also known as skeletal- or mesenchymal) stem cells (MSC) are clonogenic, multipotent stem cells that can differentiate into mesodermal descendants including osteocytes, osteoblasts, chondrocytes, adipocytes and tenocytes (specialized fibroblasts) (Abdallah and Kassem 2008; Bianco et al., 2001). Human MSC are obtained from bone marrow aspirates taken from the iliac crest of normal donors, and represent a minor fraction of bone marrow derived mononuclear cells (BMMNC) with a frequency of 2-5 MSCs per 10^6 BMMNC (Minguell et al., 2001). Traditionally, MSC are isolated from BMMNC by their ability for selective adherence to plastic culture dishes (Kassem et al., 1991). However, MSCs obtained by this procedure are heterogeneous with only approximately 30% of the clonogenic cells being truly multipotent stem cells (Kuznetsov et al., 1997; Larsen et al., 2010).

MSC immunoselection utilizing specific MSC markers have therefore been used to enrich for hMSC (Gronthos et al., 2003), but a general lack of sensitive and specific hMSC markers remain a limiting factor for purifying clonogenic hMSC to homogeneity. During the last decades a few monoclonal antibodies (mAb) have been raised against surface proteins present on hMSC by injecting hMSC in immunocompetent mice. Haynesworth et al. (1992) obtained three antibodies SH2, SH3, and SH4 that were non-reactive with haematopoietic cells and mature osteoblasts suggesting specificity for early stage cells of the osteoblastic lineage. SH2 is known now to recognize endoglin (Barry et al., 1999) whereas both SH3 and SH4 react with CD73 (ecto-5′nucleotidase) (Barry et al., 2001). Similarily, the monoclonal antibody SB-10 was generated, and its antigen ALCAM is present on hMSC, though it seems to disappear upon differentiation as recognised by expression of alkaline phosphatase (ALP) (Bruder et al., 1997). Likewise, HOP-26 that

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identifies CD63 (Zannettino et al., 2003) is also expressed by hMSC before induction of ALP (Joyner et al., 1997). The STRO-3 antibody recognises an isoform of tissue-non-specific ALP (TNALP) that also identifies immature MSC and can enrich for multipotential MSC (Gronthos et al., 2007) whereas STRO-4 identifies the beta isoform of heat shock protein-90 (Hsp90beta) enabling MSC enrichment from human and ovine MSC (Gronthos et al., 2009). However, the STRO-1 mAb that was described by Simmons et al., in 1991 (Gronthos et al., 1994; Simmons and Torok-Storb, 1991) is still the antibody with the highest affinity and efficiency for isolating all clonogenic hMSC as a stand-alone reagent due to the high density of this antigen on the cell surface of hMSC (Gronthos et al., 2003). The epitope recognized by STRO-1 remains unidentified, and some contaminating Glycophorin-A^+ nucleated erythroid cells are present in the STRO-1^+ BMMNC fraction. Thus, further development of antibodies that specifically identify MSCs or its descendants are required to improve MSC isolation and characterization.

The aim of this study was to establish high affinity mAbs that could be used to improve isolation and characterization of human MSCs. In order to avoid culture and donor variations of hMSC, we employed an immortalized human telomerase reverse transcriptase transduced MSC line (hMSC-TERT) that has been developed by our group and expresses typical characertistics of hMSC both *in vitro* and *in vivo* (Abdallah et al., 2005; Simonsen et al., 2002). hMSC-TERT cells were thus employed to immunize mice for antibody development, where human skin fibroblasts were used for negative selection to enhance antibody specificity by eliminating common markers with hMSC. Following the generation of viable hybridomas, monoclonal antibodies were screened using flow cytometry, Western blotting, immuno(cyto/histo)chemistry, immunoprecipitation, MSC differentiation and enrichment. Suitable antibody candidates were then used for protein isolation followed by identification of the antigens recognized by these antibodies.

MATERIALS AND METHODS

Cell lines and cell culture conditions

Normal human foreskin fibroblasts (kindly provided by Margit Aagaard; Aarhus University Hospital, Denmark (Ethical approval was obtained from the Regional Ethical Committee)) and osteosarcoma cell lines (MG63, SAOS, HOS, KHOS) were obtained from ATCC and cultured according to providers guidelines. Establishment and culture conditions for telemorase immortalized human mesenchymal stem cells (hMSC-TERT) have been described in previous reports (Abdallah et al., 2005; Simonsen et al., 2002). Bone marrow (BM) aspirates were obtained from the posterior iliac crest of normal adult volunteers (19-35 years old) following informed consent, according to procedures approved by the ethics committee of the Royal Adelaide Hospital, South Australia. Bone marrow mononuclear cells (BMMNC) were prepared as previously described (Gronthos et al., 2003). Primary BMSSC cultures were established from STRO-1 magnetic selected cells in α-MEM supplemented with 20% fetal calf serum, 2 mM L-glutamine and 100 mM L-ascorbate-2-phosphate as previously described (Gronthos et al., 2003). Colony efficiency assays cultures were terminated at day 14 and the number of CFU-F enumerated following staining with 0.1% (w/v) toluidine blue in 1% paraformaldehyde. Aggregates of >50 cells were scored as CFU-F derived colonies.

Osteogenesis was induced in hMSC-tert cells as described previously (Foster et al., 2005), and verified by expression of osteoblastic markers using immunofluorescence staining for CBFA1/Runx2 (1:100; R&D Systems) and osteocalcin (1:400; Biomedical Technologies). Adipogenic differentiation was performed as described previously and confirmed by oil-red o staining (Simonsen et al., 2002).

Mouse immunization procedure

At day 0 hMSC-TERT were washed ×3 in PBS (37°C), scraped off, washed extensively in PBS, and a single cell suspension was obtained by passage through a 27 gauge needle. Approximately 6×10^6 live hMSC-TERT (in 200 µl PBS) were injected i.p. in each of 5 NMRI mice. At day 38 and day 52 hMSC-TERT were prepared as described above, though cells were briefly (10 min.) fixed in Normal Buffered Formaldehyde [4% w/v in PBS (NBF)] and washed extensively in PBS prior to detachment. Mice were then boosted i.p. with 3×10^6 fixed hMSC-TERT at the respective days. Ten days after the third immunization a blood sample was obtained from each mouse and the serum fraction was serially diluted starting 1:10 in PBS/0.05%tween 20/1% BSA and tested for circulating antibodies against hMSC-TERT using the cyto-ELISAs described below. Normal mouse serum was included in the ELISA and reflected background levels of antibodies in mice. Based on the measured antibody titres, two mice were chosen and boosted four days prior to fusion with 6×10^6 fixed hMSC-TERT.

Monoclonal antibody (mAb) production

Fusions were performed according to Köhler and Milstein (1975) as modified by Reading (1982) with the myeloma cell line SP2/0-Ag14 using PEG 4000 as fusogen. Hybridoma supernatants were screened by the cyto-ELISAs described below for the presence of mouse immunoglobulins reactive with hMSC-TERT. Hybridomas positive in the screen were cloned several times using the standard limiting dilution procedure. The resulting hybridomas were further propagated and stored frozen at -80°C in FCS- containing 10% DMSO while the antibody containing supernatants were kept at -20°C or 4°C (0.05% azide added) until use.

Screening of hybridoma supernatants

Hybridoma supernatants were initially screened for reactivity against hMSC-TERT (positive screening) and human skin fibroblasts (negative screening) using cyto-ELISAs.

The cyto-ELISA setup was prepared by culturing hMSC-TERT and human fibroblasts in separate 96-well plates. Subconfluent cell layers were washed (×3) in PBS (37°C), fixed in 4% NBF (15 min.). Plates were then rinsed (×3) in PBS, blocked in PBS/1% BSA (15 min.) and washed (×4) in PBS/0.05% tween 20/0.37 M NaCl. Hybridoma supernatants were collected from culture plates and 50 µl was added to each well of both positive and negative screening plates prefilled with 50 µl washing buffer (PBS/0.05% tween 20/0.37 M NaCl) per well. Following o/n incubation at 4°C, plates were incubated with horseradish peroxidase conjugated rabbit-anti mouse immunoglobulins (P260, DAKO A/S, DK) diluted 1:4000 in PBS/1.0% BSA/0.05% tween 20, and developed using an enzyme-substrate mixture [4 mg ortho-phenylenediamine (Kem-En-Tec Diagnostics A/S), 4 µl H_2O_2 (35%) and 10 ml staining buffer (35 mM citric acid/ 67 mM Na_2HPO_4), pH 5.0] for 15 min. The colour reaction was ended by addition of 1 M H_2SO_4 and the optical densities were quantified using a Labsystems iEMS Reader MF at 492 nm.

Immunocytochemistry

hMSC-TERT and fibroblasts were grown on coverslips (Ther-

manox plastic, NUNC), gently rinsed in PBS (37°C), allowed to dry by air, and attached to slide glasses. For screening of DJ antibodies, coverslips were rinsed in TBS, permeabilized in TBS/0.5% tween 20, and blocked by pre-incubation with TBS/2% BSA for 10 min. Incubation with hybridoma supernatants (DJ 1-18) diluted 1:5 in TBS/1% BSA was performed o/n at 4°C, and sequential incubation with biotinylated goat anti-mouse immunoglobulin 1:200 (E433, DAKO A/S, DK) and HRP conjugated streptavidin 1:300 (P397, DAKO A/S, DK) followed by three-amino-9-ethylcarbazole (AEC) were used to detect DJ antibody specificity. Finally, nuclei were visualized by staining with Mayers hematoxylin, and slides were examined by light microscopy (Leica DMLB). For immunofluorescence, cells were stained directly in culture plates, processed as above, and primary antibody binding was detected by Alexa 488 or 555 conjugated donkey anti-mouse IgG antibodies (Molecular probes, Invitrogen). Alkaline phosphatase staining and quantification was performed as previously described (Gronthos et al., 2007).

Immunohistochemical staining

Cells were grown in large scale, washed extensively in 37°C PBS, and detached by scraping. Following centrifugation (10 min, 4°C and 1,000 rpm^{-1}), cell-pellets were fixed in 4% NBF for 24 h at room temperature, embedded in paraffin, and sliced into 4 μm sections. Additionally, we used tissue array blocks comprising a panel of normal and abnormal fetal and adult human tissues (kindly provided by Henrik Daa Schrøder, Department of Pathology, Odense University Hospital, Denmark) for further testing of DJ 3, 9, and 18. Immunohistochemical staining was performed as described previously (Andersen et al., 2009), including block of endogenous peroxidase activity and antigen demasking by boiling in TEG buffer [10 mM Tris (SIGMA T-1378)/0.5 mM EGTA (Merck 8435), pH 9.0]. In case of tissue arrays IHC, we blocked endogenous biotin using a biotin-avidin kit system (DAKO A/S, DK), and TEG demasking was performed at 60°C for 24 h to prevent damage of tissues enriched for collagens. The remaining part of the immunohistochemistry procedure was performed as described above for immunocytochemistry. For each tissue examined, at least three sections were stained and each section was independently examined by two individuals. Based on tissue origin, morphology and localization in the tissue, the pathologist characterized staining patterns and the cell types stained.

IgG purification and isotype determination

Protein G affinity chromatography was performed according to the manufacturers recommendations (Amersham Biosciences, UK) using 5 ml HiTrap Protein G columns attached to an Äkta Fast Performance Liquid Chromatography system (Amersham Biosciences, UK). In order to avoid cross-contamination in purification of IgG from different hybridomas, new matrices were used for each culture supernatant. The isotype of generated antibodies were determined by an Amersham Kit as recommended by manufacturer.

Flow cytometry

Trypsinized hMSC-TERT and human fibroblasts were re-suspended at 0.5×10^6 cells/ml, incubated in blocking buffer (15 min.; Hanks balanced salt solution, 0.5% BSA, 5% normal human serum, 5% FCS) on ice while shaking. Cells were spun down (5 min, 1,200 rpm^{-1}, 4°C) and incubated 1 h on ice with protein-G purified primary DJ antibodies diluted in washing-buffer (Hank's balanced solution/5% FCS). Antibody rosetted cells were incubated with FITC conjugated rabbit anti-mouse Ig (F0261, DAKO A/S) diluted 1:20 or Donkey anti-mouse IgG-Alexa 488 (1:200; Molecular Probes), and analyzed on a FACScan instrument (Becton Dickinson). Secondary cultures of normal hMSC were prepared as single cell suspensions by trypsin/EDTA digest and incubated with antibodies identifying STRO-1 and either DJ3, DJ9 or DJ18 for one hour on ice. After washing, the samples were incubated with goat anti-mouse IgG_1-FITC and IgM-PE antibodies (Southern Biotechnology Associates Inc., USA) as secondary detection agents for 45 min on ice. Following washing, the cells were subsequently analyzed using an Epics®-XL-MCL flow cytometer (Beckman Coulter, USA).

Immunoprecipitation and microsequencing

Approximately, 2-4 $\times$ 10^7 MG63 or hMSC-TERT cells were lysed in a 0.1 ml volume of 1% NP40-TSE in TSE Buffer pH 8.0 or PBS/5 mM triton X-100 including complete protease inhibitor cocktail (Complete™ Mini, Roche). In some experiments, mixtures were briefly sonicated before they were chilled on ice, for 30-120 min, and microfuged at 14,000 $\times$ *g* for 15 min at 4°C. Dynabeads M-450 (sheep anti-mouse IgG, DYNAL, Norway) were washed $\times$ 3 in PBS/5 mM triton X-100 and 10^7 beads were rosetted by protein G-purified DJ antibodies (3-8 μg) or washing buffer (o/n, 4°C, end-over-end mixing). Lysates were first pre-cleared with sheep anti-mouse IgG coated magnetic Dynal beads, and then resuspended in Dynal beads pre-incubated with antibody for 2-24 h at 4°C. The Dynal beads were pelleted using a magnetic particle concentrator, then washed extensively with 1% NP40-TSE or PBS/5 mM triton X-100, and resuspended in NuPage LDS sample buffer (1×) (Invitrogen™) ± reducing conditions. Samples were loaded on a 10% acrylamide gel (some experiments for DJ18) or a NuPAGE 4-12% Bis-Tris pre-cast gel (DJ3, 9 and 18), and the gels were either stained with Coomassie blue reagent or silver stained or immediately used for immunoblotting according to manufacturers recommendation. Protein bands were excised and subjected to trypsin digestion at 37°C before being analyzed by mass spectrometry using an LTQ-FT (Kratchmarova et al., 2005) instrument (Thermo Electron) or an Applied Biosystems 4700 Proteonomics Analyser with TOF/TOF optics. The protein sequences were submitted to the data search based program MASCOT (Matrix Science Ltd., UK). Moreover, we performed DNA sequence analysis as previously described (Gronthos et al., 2007) to identify the antigen recognized by DJ3.

Western blotting

Proteins in gels were transferred to a PVDF membrane (Hybond-P, Amersham pharmacia biotech) in line with the protocol (Xcell II™) provided by Invitrogen. Following transfer, PDVF membranes were blocked for 15 min in PBS/0.05% tween 20/0.37 M NaCl, incubated o/n at room temperature with primary DJ antibody (hybridoma supernatants) diluted 1:2 in washing buffer, and then washed three times. Secondary horse radish peroxidase labelled rabbit anti-mouse immunoglobulin (P0260, DAKO A/S) (diluted 1:1000 in PBS/0.05% tween 20/0.37 M NaCl) was added (1 h at 4°C), and excess antibody was removed by washing four times in PBS/0.05% tween 20/0.37 M NaCl and one time in 0.05 M acetate buffer, pH 5.0 for 15 min. Immunocomplex formation was visualized by incubation with AEC developing solution as described for immunocytochemistry.

Statistical analysis

All analyses comprised 2-6 independent experiments (n), and two-tailed *t*-tests were performed [GraphPad Prism (5.0a Mac version) software] to test significance ($\alpha = 0.05$) of data.

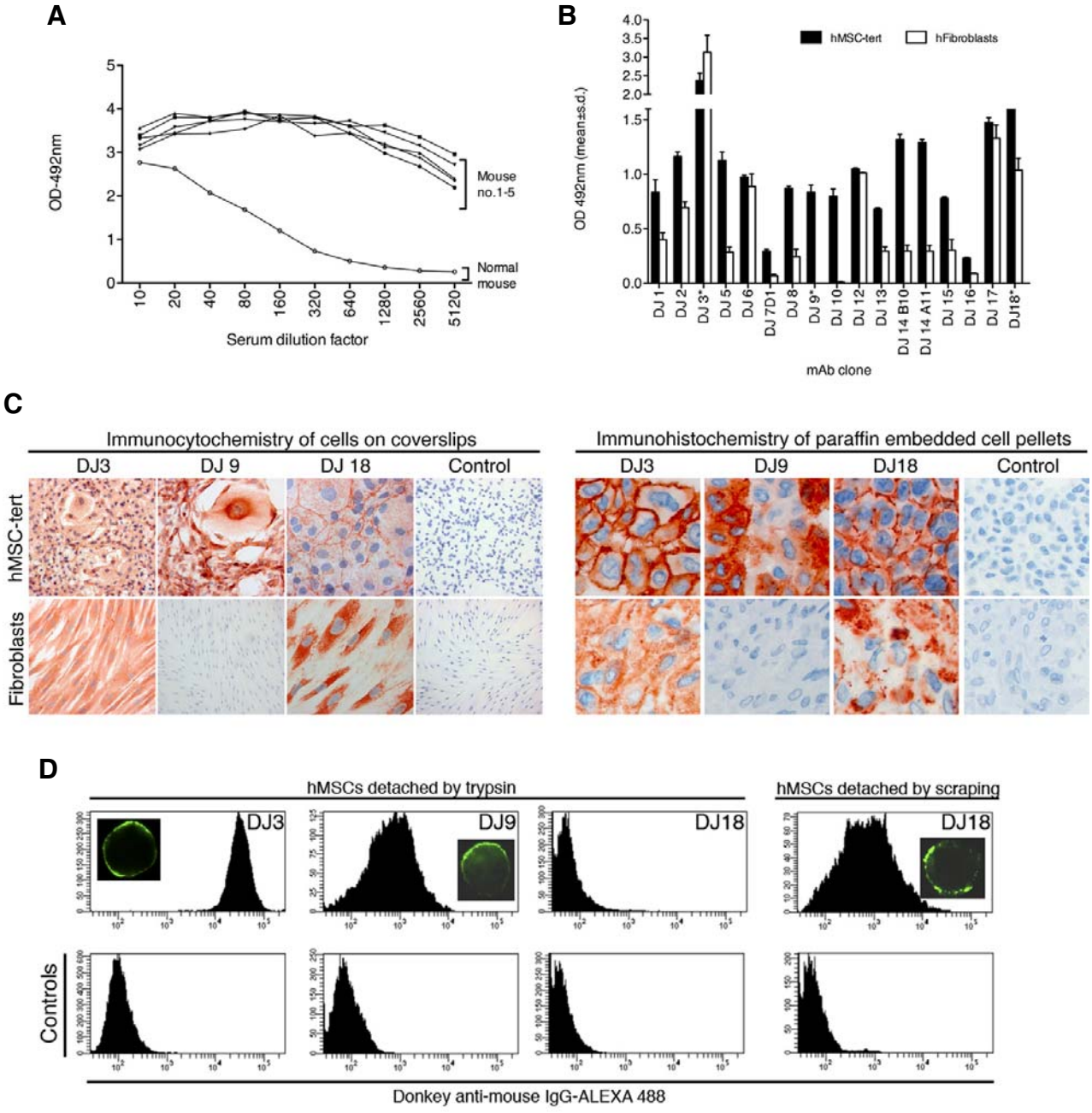

Fig. 1. Generation and screening of DJ antibodies. (A) The antibody titer in serum from mice immunized three times with MSCs was determined by a MSC cyto-ELISA. Normal mouse serum was used as background. (B-D) Screening of 17 DJ hybridoma supernatants for reactivity with MSCs or skin fibroblasts using (B) cyto-ELISAs, (C) immunocytochemistry of cultured cells, and immunohistochemistry of paraffin embedded pellets of cultured cells. (D) Flowcytometric analysis and immunofluorescence microscopy (inserts) of live MSCs stained with DJ antibodies.

RESULTS

Generation of mAbs against hMSC

Mice immunized three times raised circulating antibodies against hMSC-TERT as measured by an established cyto-ELISA (Fig. 1A). Two mice were boosted a fourth time and their spleens were used for hybridoma production following a standard procedure (Kohler and Milstein, 1975; Reading, 1982; Skjoedt et al., 2010). Stable clones reactive with hMSC-TERT were re-cloned three times, designated with a specific DJ number, and then tested for reactivity against both hMSC-TERT and skin fibroblasts using cyto-ELISAs (Fig. 1B). All seventeen antibodies were further tested for use in immuno(cyto/histo) chemistry (only selected results shown, Fig. 1C). Based on their distinct reactivity patterns in the cyto-ELISAs, ICC, and IHC (Figs. 1B and 1C), we selected three mAbs, DJ 3, 9, and 18, for further analysis described below.

DJ3 and DJ18 recognized their corresponding antigen in

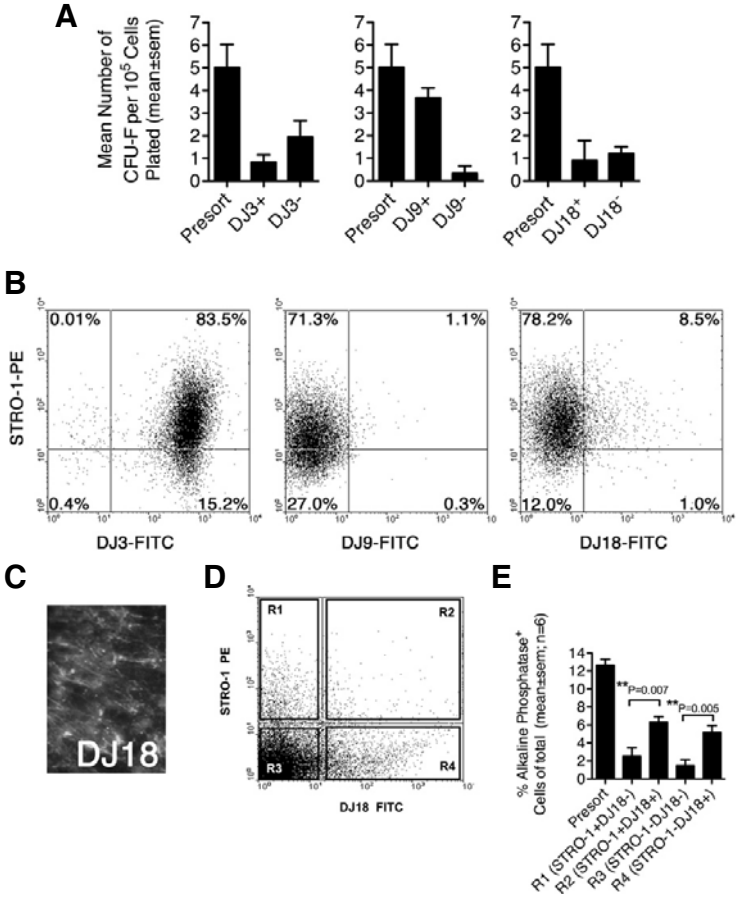

Fig. 2. Testing DJ antibodies on BMMNCs and STRO-1+ selected BMMSCs. (A) The number of colony forming fibroblasts (CFUs) was determined in cultures of unfractionated and DJ+ or DJ- selected human BMMNCs. Aggregates of > 50 cells were scored as CFU-F derived colonies 14 days after plating. (B) Flow cytometric analysis for co-expression of STRO-1 and DJs in primary cultures of STRO-1+ MACS selected BMMNCs (BMMSCs). (C) Immunocytochemistry of cultured BMMSCs using DJ18. (D) STRO-1+ selected cells were cultured for 3 passages and stained with the DJ18 antibody using flow cytometry. (E) Primary cultures of STRO-1+ selected cells were FACS sorted into different STRO-1/DJ18 subpopulations (R1, STRO-1^+/DJ18^-; R2, STRO-1^+/DJ18^+; R3, STRO-1^-/DJ18^-; R4, STRO-1^-/DJ18^+) that were further cultured before staining with Alkaline phosphatase which identifies more differentiated cells in the cultures.

hMSC-TERT and human skin fibroblasts. DJ9 exclusively reacted with hMSC-TERT (Figs. 1B and 1C). This was also seen by flow cytometry on live hMSC cells, which additionally confirmed a cell surface localization for all three DJ antigens (Fig. 1D). However, DJ18 antigen was mainly localized to the extracellular matrix of confluent MSC (Fig. 1C), and preservation of the membrane tethered DJ18 antigen was clearly increased by scraping as compared with trypsin treatment in the flow cytometry protocol (Fig. 1C). Isotype analysis showed that DJ 3, 9, and 18 belong to the immunoglobulin G1κ, G2aκ, G2bκ isotypes, respectively (data not shown), and protein G affinity chromatography was used for their purifications (data not shown).

DJ antibody reactivity against MSC in BMMNC and primary BMMSC cultures

Our previous studies have shown that MACS cell sorting of bone marrow mononuclear cells (BMMNCs) using the STRO-1 antibody is able to enrich (90.0 ± 7.86) for clonogenic CFU-F (Gronthos et al., 1994). However, none of the three DJ antibodies demonstrated any capacity to enrich for cells forming CFU-Fs (Fig. 2A). In combination with the STRO-1 mAb, we next evaluated by flow cytometry whether any of the three DJ antibodies could subfractionate cultured STRO-1+ BMMSC. We found that 83.5, 1.1, and 8.5% of BMMSC were double positive for STRO-1 and either of DJ 3, 9, and 18, respectively (Fig. 2B). However, immunocytochemistry revealed that all cultured BMMSCs expressed the protein recognized by DJ18 (Fig. 2C). Thus, as DJ18 identified all BMMSC in ICC, only a subfraction of BMMSCs by flowcytometry, and at the same time did not enrich for CFU-Fs from BMMNCs, we speculated if expression of this protein on the membrane could identify differentiated contaminants in the STRO-1+ BMMSC cultures. We tested this by examination of alkaline phosphatase activity in different STRO-1/DJ18 primary BMMSCs subsets isolated by FACS (Fig. 2D-E). Both STRO-1^+/DJ18^+ and STRO-1^-/DJ18^+ cell fractions contained higher numbers of alkaline phosphatase staining cells as detected by immunocytochemistry as compared with STRO-1^+/DJ18^- and STRO-1^-/DJ18^- cell fractions, respectively (Fig. 2E). This indicates that localization of the DJ18 protein on the membrane identifies more differentiated cells in the heterogenic BMMSC cultures, and may suggest that the DJ18 antibody can be used for negative selection of undifferentiated BMMSCs.

Expression of DJ antigens during *in vitro* osteogenic and adipogenic differentiation of hMSCs

We next assessed the temporal expression of the DJ3, DJ9, and DJ18 antigens during osteogenic differentiation of hMSC-TERTcells. Osteogenic differentiation was confirmed by expression of Runx2/CBFA1 and osteocalcin (Fig. 3A). All three antigens were expressed in undifferentiated MSCs. DJ3 seemed to be upregulated early in osteogenic differentiation, but then decreased in intensity hereafter, although it was still highly expressed when differentiation was completed at day 9. In contrast, we found the DJ9 antigen to be focally expressed in the differentiating MSCs at day 3 and 6, whereas a more diffuse localization was observed at day 9. The DJ18 antigen was highly expressed at all time points (Fig. 3A), but a dramatic shift from the cytoplasm/membrane to the cell interstitial region was

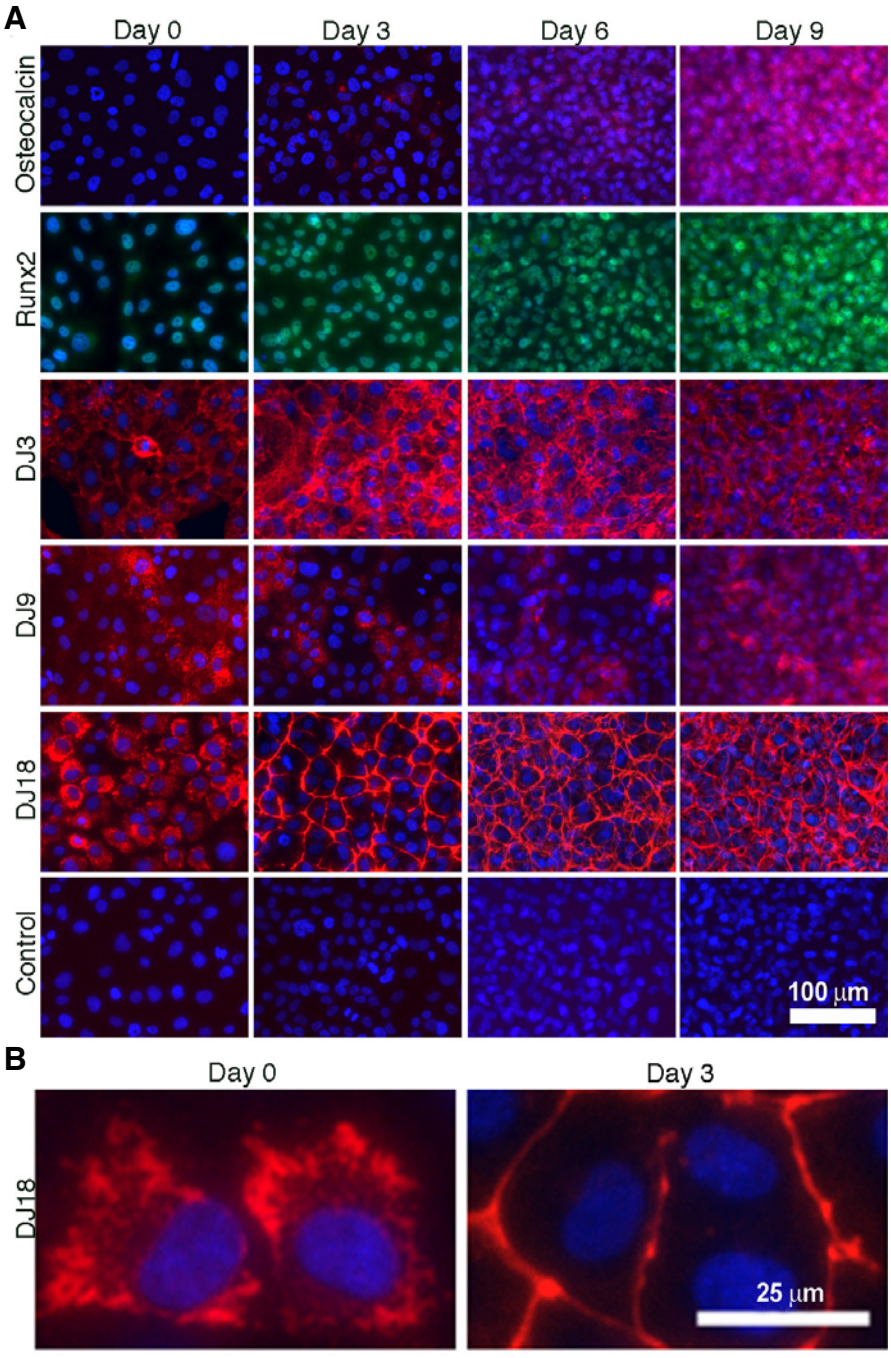

Fig. 3. DJ reactivity during osteogenic differentiation of MSCs. (A-B) MSCs were cultured until confluence (Day 0), and then cultured for 9 days during osteogenic differentiation conditions. Runx2 and Osteocalcin verified osteogenic differentiation.

observed immediately upon differentiation (Fig. 3B) indicating possible extracellular secretion. We, next examined whether this pattern of localization for the DJ18 protein was similar during adipogenic differentiation of MSC (Fig. 4). Indeed, we found that the DJ18 protein resided in the cytoplasm of the undifferentiated MSCs, whereas differentiated MSCs exhibited a clear extracellular distribution of the DJ18 antigen. This further suggest, that the DJ18 antibody recognizes its corresponding antigen on the surface of only differentiated MSCs and supports it's use for negative selection of undifferentiated MSCs as proposed above.

Distribution of DJ antigens in different skeletal tissues

To confirm the DJ reactivity patterns *in vivo*, we tested the DJ antibodies on formaldehyde fixed sections of normal trabecular bone and different osteogenic-, and chondrogenic-derived tumors. As seen in Supplementary Fig. S1 DJ3, 9, and 18 each resulted in unique staining patterns revealing differential cell reactivity among different tissues. None of the three mAbs recognize adipose cells residing in the marrow cavities, but osteocytes located in lacunae of surrounding trabeculae bone were positive for DJ3 and DJ18. In regions of new bone formation, DJ18 stained ECM surrounding osteoblasts, whereas neoplastic osteoblasts were identified by DJ3. Neither chondroblasts

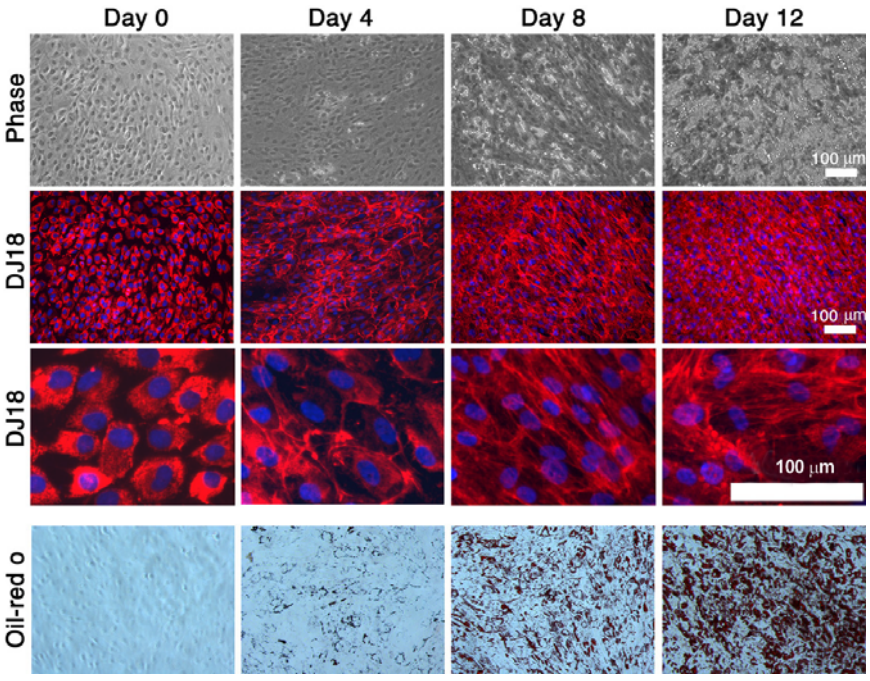

Fig. 4. DJ18 reactivity during adipogenic differentiation of MSCs. MSCs were cultured until confluence (Day 0), and then cultured for 12 days during adipogenic differentiation conditions. Lipid-filled vacuoles (see phase pictures) reflecting adipogenesis increased in number during differentiation and was verified by oil-red O staining (Bottom).

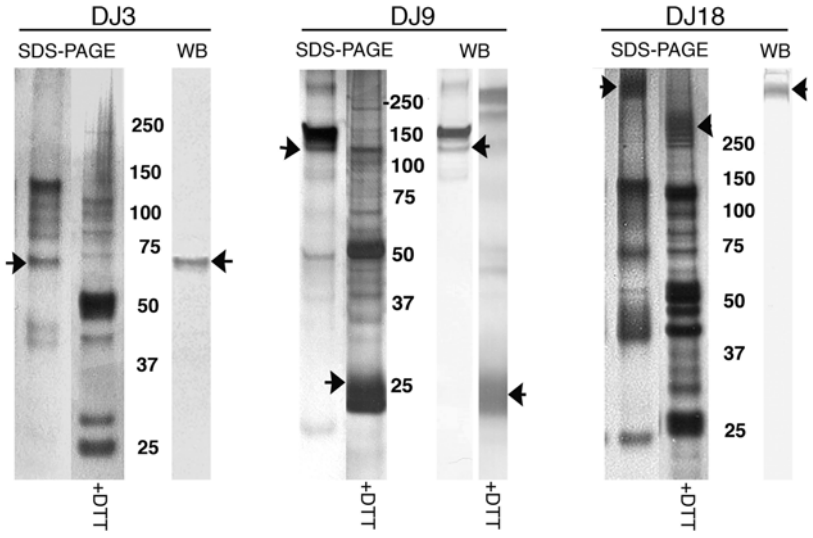

Fig. 5. Purification of proteins recognized by DJ antibodies. Proteins recognized by DJ antibodies were purified by immunospecific affinity chromatography (DJ3 and DJ18) or magnetic Dynalbead purifications of MSC lysates. Isolated proteins were run in SDS-PAGE (4-12%) followed by Western blotting (WB). Specific DJ protein bands (arrows) were determined from control samples where the primary antibodies have been omitted from purification methods (data not shown).

nor chondrocytes were identified in any sections of normal tissue, though some neoplastic chondroblasts were stained positive with DJ18. The three DJ mAbs were also tested by flow cytometry for staining of four different osteosarcoma cell lines (MG63, SAOS, HOS, KHOS). DJ3 clearly identified its antigen on all four cell lines, DJ18 only reacted (weakly) with MG63 cells, whereas DJ9 lacked reactivity to any of the cell lines (Supplementary Fig. S2). On *fetal tissue sections* comprising the spine region and upper extremities (Supplementary Fig. S1), the three DJ antibodies recognized completely different structures. DJ9 showed an intense staining of a few non-skeletal structures, and some cells located in the perichondrium and in surrounding connective tissue (Supplementary Fig. S1). In contrast, the antigen corresponding to DJ18 was distributed throughout the perichondrium and different types of connective tissue, but also in regions with high densities of chondroblasts (Supplementary Fig. S1). Though, except for a few cells, DJ18 appeared only in relation to the extracellular matrix. DJ3 did not show any reactivity to the cells or structures residing in the perichondrium, whereas the adjacent mesenchyme and regions corresponding to the growth-zones of the ribs stained positive with DJ3 (Supplementary Fig. S1).

Additionally, we tested DJ3, DJ9, and DJ18 on a large panel of different non-skeletal tissues distributed throughout the human body (Supplementary Figs. S3 and S4). Overall, DJ3 seems to recognize several cell types including epithelial cells, stromal cells, endothelial cells and some hematopoietic cells, whereas DJ9 mainly localized to lymphocytes, endothelia, and macrophages. The DJ18 stained almost any structure except for epithelial cells, and as for the cultured MSCs the DJ18 anti-

gen seemed to be part of the extracellular matrix (Supplementary Figs. S3 and S4).

Identification of proteins recognized by DJ 3, 9, and 18

We employed several approaches to identify antigens recognized by the three DJ antibodies. Initially, Protein G purified DJ Mabs were coupled to CNBr activated Sepharose, and protein lysates from hMSC-TERT were then subjected to immunospecific affinity chromatography (data not shown). All eluted fractions containing isolated proteins were then run and visualized on a 4-12% SDS-PAGE gel followed by Western blotting (WB) (Fig. 5). DJ3 reacted with a protein of approximately 70 kDa whereas DJ18 identified a high molecular mass protein situated far above the 250 kDa protein marker (Fig. 5). However, no specific bands were observed for DJ9 using this approach (data not shown). We therefore tested if the protein recognized by DJ9 could be purified using magnetic beads (Fig. 5). Although several bands existed in the DJ9 WB mainly due to contaminating IgG and BSA, we recognized DJ9 specific bands at approximately 120- and 25 kDa in unreduced and reduced samples, respectively (Fig. 5).

Relevant gel bands (10% acrylamide for DJ18) were then excised and subjected to mass spectrometry (Supplementary Figs. S5-S8). Accordingly, DJ9 was identified to recognize an epitope on the major histocompatibility complex, class II, DR (HLA-DRB1) (Supplementary Fig. S5) a result we confirmed by immunocytochemistry using another antibody against HLA-DR (Supplementary Fig. S5). Likewise, we found DJ18 to react with Collagen VI (Supplementary Fig. S6) and verified this using a commercially available antibody against Collagen VI on tissue sections of human bone marrow trephines (Supplementary Fig. S7). The antigen corresponding to DJ3 was however not identified by mass spectrometry, likely due to obscuring IgG molecules. Instead we used DNA sequence analysis of the DJ3 antigen cDNA using a BMSC cDNA retroviral expression library as previously described (Gronthos et al., 2007), and found it to be homologous with CD44 (data not shown). This result was further confirmed by flow cytometric analysis of DJ3 expressing FDCP1 clones with the CD44 specific antibody H9H11A along with the DJ3 antibody (Supplementary Fig. S8).

DISCUSSION

In this study, we have generated and characterized three high affinity antibodies DJ3, DJ9, and DJ18 that recognize proteins of cultured human MSCs and have demonstrated their usefulness in identifying subpopulations in heterogenous hMSC cultures. In specific, we found that the DJ18 antibody may be used for negative selection of cultured, but undifferentiated MSCs. Finally, we have identified the proteins recognised by these antibodies. The DJ antigens identified as CD44, HLA-DR, and Collagen VI, were achieved by mass spectrometry and DNA sequence analysis, in agreement with the protein- size and -tissue distribution known for CD44 (Rudy et al., 1993), HLA-DR (Triantafilou et al., 2000), and Collagen VI (Doliana et al., 1990), confirming the specificity of the DJ3, DJ9, and DJ18 antibodies.

The cell surface glycoprotein CD44 is known to be expressed on MSCs and differentiated osteogenic descendants (Liu et al., 2008), and is often used in conjunction with other molecules to confirm MSC identity (Bianco et al., 2001). However, CD44 is a widely distributed molecule and not specific for MSC (Delorme et al., 2008). In agreement, the present study revealed that our DJ3 antibody, which recognizes CD44, was unable to enrich for MSCs from BMMNC due to its reactivity to haemopoietic cells. Furthermore, DJ3 recognized cells throughout osteogenic differentiation, and it reacted extensively with human skin fibroblasts as well. The DJ9 corresponding antigen, HLA-DR is normally expressed in antigen presenting cells, and not MSCs (Delorme et al., 2008). However, expression of HLA-DR in cultured MSCs has been described previously as a result of induction by interferon-γ (Chan et al., 2008) or basic fibroblast growth factor (Sotiropoulou et al., 2006) present in the culture medium. Thus, the expression of HLA-DR in telemorase immortalized MSCs, but not primary derived MSCs, may reflect the long term *in vitro* culture of these cells.

Collagen VI expression by hMSC has not previously been demonstrated at the protein level in undifferentiated MSCs. Our study showed that Collagen VI is is expressed at high levels and localized in the cytoplasm of undifferentiated MSCs, whereas it resides in the membrane/extracellular matrix of MSCs differentiated into the osteogenic- and adipogenic lineage as well as in mature bone. Previous studies have demonstrated Collagen VI at the mRNA level in MSCs during early chondrogenic differentiation, but it was downregulated upon differentiation (Muratoglu et al., 1995; Xu et al., 2008). As Collagen VI was absent in the cytoplasm of differentiated MSC in the present study, it is likely that Collagen VI mRNA levels also herein was decreased in osteogenic and adipogenic differentiation. Accordingly, it may be that de novo synthesis of Collagen VI is blocked by MSC differentiation, and instead existing Collagen VI is transported to the exterior of the cells. In agreement, we also found that cultures of primary STRO-1+ selected cells expressed Collagen VI in the cytoplasm, whereas STRO-1+ selected cells with Collagen VI in the membrane represent a more differentiated cell population.

As such, our results may indicate that Collagen IV is absent in native MSCs, but it is expressed and localized in the cytoplasm of activated (or cultured) MSCs and immediately secreted into the ECM upon MSC differentiation. As such, Collagen VI may be involved in MSC cell fate determination as also described for this protein in cardiac myofibroblast differentiation (Naugle et al., 2006). Altogether, these results may suggest that the DJ18 antibody against Collagen VI could be used to negatively select for cultured, but undifferentiated human MSCs.

Although our three antibodies offer high quality reagents for characterizing MSCs in a diverse panel of assays, it was not possible to employ them for MSC enrichment from BMMNCs. It is believed that cultured MSCs are different from native MSCs found *in vivo*, and MSCs that have been cultured (especially for a long period of time) may have lost many proteins normally associated with the immature MSC phenotype (Pontikoglou et al., 2008). Generation of novel antibodies with an ability to enrich for native MSCs, may thus be achieved by immunizing animals with short-term expanded primary derived MSCs combined with negative screening using long-term cultured MSCs. Alternatively, the decoy immunization strategy with parallel injections of long-term and short-term cultured MSCs in the footpads may provide novel MSC enrichment antibodies. This latter protocol has been used by others, to produce antibodies against un-differentiated embryonic stem cells (Choi et al., 2008). Additionally, it is likely that the many antibodies produced herein against ECM may have been avoided by using trypsinized MSCs or purified membranes hereof instead of MSCs detached by cell scraping.

In conclusion, we have generated and carefully characterized some new high affinity antibodies against CD44, HLA-DR, and Collagen VI that may be used by various immunological techniques to study human mesenchymal stem cell biology. In addition, we suggest that collagen VI localization in the cytoplasm of

MSCs may be used as a marker of cultured undifferentiated MSCs.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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