Immunophenotypic characterisation of ovine mesenchymal stem cells

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Running headline

Antibodies for sheep MSC immunophenotyping

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Key words

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Abstract

The clinical potential of multipotent mesenchymal stem cells (MSCs) has led to the essential development of analytical tools such as antibodies against membrane-bound proteins for the immunophenotypic characterization of human and rodent cells. Such tools are frequently lacking for emerging large animal models like the sheep that have greater relevance for the study of human musculoskeletal diseases. The present study identified a set of commercial nonspecies specific monoclonal antibodies for the immunophenotypic characterization of ovine MSCs. A protocol combining the less destructive proteolytic activity of accutase and EDTA was initially developed for the detachment of cells from plastic with minimum loss of cell surface antigens. A range of commercially available antibodies against human or rodent MSC antigens were then tested in single and multistain-based assays for their cross-reactivity to bone marrow derived ovine MSCs. Antibody clones cross-reactive to ovine CD73 (96.9% ± 5.9), CD90 (99.6% ± 0.3), CD105 (99.1 ± 1.5), CD271 (97.7 ± 2.0), and MHC1 (94.0% ± 7.2) antigens were identified using previously reported CD29, CD44, and CD166 as positive controls. Multistaining analysis indicated the colocalization of these antigens on MSCs. Furthermore, antibody clones identified to cross-react against white blood cell antigens exhibited either negative (CD117 (0.1% ± 0.1)) or low (MHCII (10.5% ± 16.0); CD31 (14.6% ± 4.2), and CD45 (39.4% ± 31.8)) cross-reactivity with ovine MSCs. The validation of these antibody clones to sheep MSC antigens is essential for studies utilizing this large animal model for stem cell-based therapies. © 2016 International Society for Advancement of Cytometry

Introduction

Multipotent mesenchymal stem cells (MSCs) have been defined as mononuclear spindle shaped clonogenic cells capable of tri-lineage differentiation and positive expression of cell surface markers including CD73, CD90 and CD105 (1).These cells have predominantly been isolated from bone marrow, based on methods that rely on early tissue culture plastic adherence, in contrast to the non-adherent hematopoietic stem cells and their related progenitors (2-3). There has been considerable interest in the potential clinical usefulness of these cells to treat diseases where natural healing is dysfunctional or inadequate. Indeed, both experimental studies in vitro and more limited clinical case series in large animal models suggest efficacy although their mechanism of action has not been completely determined (4–7). Fewer negative studies have been published although there is concern that regenerative medicine has not delivered as radical a step forward in therapeutic efficiency as originally promised. A major limitation to the field has been the diverse methodologies of therapeutic cell preparations which could explain variable results. Consequently, the scientific community has drawn up strict characterisation guidelines for MSC related studies in humans (1), which has been based on analytical tools such as antibodies against cell membrane receptors. Because no single cell surface marker defines an MSC, these guidelines suggest a panel of both positive and negative markers to evaluate cellular identity. Furthermore, there is also concern that small mammal models do not accurately reflect the characteristics of human disease (8), thus generating incentive for the use of large animal models, such as the horse and sheep, which bear a closer clinical parallel to man. Tools to identify cell surface markers in these species is limited, with early studies showing poor cross-reactivity of rodent-targetted antibodies (9–11). The advance of regenerative medicine would therefore benefit from immunophenotypic characterisation of MSCs from these species to enable standardisation and comparison of cell preparations used both experimentally and clinically.

The present study aimed to identify non-species specific monoclonal antibodies from commercial sources with the potential to cross-react with cognate antigens on ovine MSCs to standardise immunophenotyping for this animal model. The development of antibodies against novel cell surface markers is a technically elaborate process (12), whereas an alternative approach using commercially available antibodies for other species has been applied previously with success in horses (9–11) but only for a limited number of markers in the sheep (13). Our strategy was to test a large number of commercially available antibodies against well characterised ovine MSCs, and confirm experimentally their cross-reactivity to ovine MSCs.

Materials and methods

Animal work

The study was carried out under approval from the Ethics and Welfare Committee of the Royal Veterinary College. Adult female English mule sheep were used to obtain bone marrow aspirates and blood under general anaesthesia. Briefly, sheep were given intravenous injections of 2 % xylazine (Rompun; Bayer Healthcare), ketamine (Ketaset; Fort Dodge Animal Health) and midazolam (Hypnovel; Roche) at doses of 0.1 mg, 2 mg and 2.5 mg respectively per kg of body mass. Anaesthesia was maintained with ~2% isoflurane gas (IsoFlo, Abbott Labs) to effect. Bone marrow (BM) was extracted from the iliac crest of the right tuber coxa into syringes with heparin at a concentration of 100 IU per 1 ml of aspirate using a 22G Jamshedi needle (CareFusion). BM aspirates of 10 ml were transferred to 5 ml of RPMI-1640 (Sigma) on ice. Additionally, a total of 50 ml of peripheral blood was obtained from the jugular vein into heparin sulphate containing tubes (BD Biosciences) for peripheral blood mononuclear cell isolation.

Cell culture

Bone marrow ovine mesenchymal stem cells (oMSCs) were isolated based on their adherence to tissue culture plastic (3). Bone marrow aspirates in RPMI-1640 were combined with an equal volume of alpha-MEM growth medium (Minimal Essential Medium alpha supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin; Gibco)) before being seeded to a 75 cm^2 tissue culture flask (Nunc) for 24 h and cultured in standard cell culture conditions at 37°C and 5% CO_2 in air. The following day, media was discared and attached cells gently washed up to 10 times with pre-warmed phosphate buffered saline (PBS; Gibco) to remove non-adherent cells. Flasks were incubated for a further 7 days in alpha-MEM with medium replaced every 48 hr to promote the emergence of colonies from adherent cells. Cells were detached from the flask by washing three times with pre-warmed PBS and incubating with %?? trypsin (Gibco) for 3-5 min with gentle agitation at what temperature??. Trypsin was inactivated with an equal volume of growth medium and cells recovered by centrifugation at 300 x *g* for 5 min. Isolated MSCs were re-suspended in growth medium and subsequently expanded at a seeding density of 1000 cells / cm^2 in 175 cm^2 tissue culture flasks (Nunc) for seven days and detached by trypsin treatment. Cells expanded at passage 2 were utilised for flow cytometry and trilineage analysis.

Trilineage differentiation

Trilineage differentiation was performed as previously reported (14), and briefly described here.

Osteogenesis

Osteogenic medium comprised Dulbecco's Modified Eagle's Medium (DMEM, low glucose) supplemented with 5 % FBS, 1 % antibiotics (all from Gibco), 10 nM dexamethasone (from), 5 mM β-glycerolphosphate and 50 µM ascorbate (all from Sigma Aldrich). For osteogenic induction, MSCs were seeded in 6-well plates in triplicate at a density of 5,000 cells per cm^2 in

control and osteogenic medium for 21 days with bi-weekly medium changes. Cultures were washed and fixed in 2 % paraformaldehyde (Sigma Aldrich) for 20 min before being stained with a solution of 2 % Alizarin Red S in water, pH 4.3 (Sigma Aldrich).

Adipogenesis

Adipogenic medium comprised DMEM (low glucose; 1g/ml) supplemented with 10 % FBS, 1 % antibiotics (all Gibco), isobutyl methyl xanthine (IBMX), indomethacin and dexamethasone (all Sigma-Aldrich). Cells were cultured in 24 well plates in triplicate for control and adipogenic media with bi-weekly medium changes for 21 days. Cultures were then washed with PBS and fixed in 2 % paraformaldehyde for 20 min before staining with Oil Red O (Sigma) as described in (14).

Chondrogenesis

Chondrogenic differentiation was conducted according to previously described protocol (15). Approximately 2-3x10^5 cells in growth medium were pelleted by centrifuging at 400 x *g* for 5 min in 15 ml tubes (Nunc). The cells pellets were then cultured in chondrogenic differentiation medium, which was composed of DMEM (high glucose) (Gibco) with 10% (v/v) ITS+ (BD Biosciences), 100 nM dexamethasone, 1 µM ascorbate-2-phosphate, 1 % sodium pyruvate and 10 ng/ml TGF-β3 (R&D Systems). Pellets were maintained for 21 days with bi-weekly medium changes, and then fixed in 2 % paraformaldehyde before being processed for paraffin embedding and sectioned to stain with haematoxylin and eosin, alcian blue and saffranin-O as described previously (15).

Comparison of reagents for cellular detachment

A systematic comparison of cell detachment reagents was performed to identify the optimal conditions that yielded MSCs with homogeneous morphometric characteristics (forward and side scatter properties on the flow cytometer) while preserving the integrity of cell surface proteins for cross-reactive antibodies. The cellular detachment reagents tested were 0.25% Trypsin (Gibco); Accutase (StemPro); EDTA (what molarity?) in Hank's buffer (Gibco); a five part combination comprising 3 parts of Accutase and 2 parts of EDTA in Hank's buffer. Cultures of $1x10^6$ MSCs (per 75 cm^2 flask) (Nunc) were washed three times with pre-warmed PBS before incubating with 3 ml of each cell detachment reagent for 3-5 min in an incubator at 37°C. The reagents were then diluted with an equal volume of growth medium and detached cells transferred to 50 ml tubes (Falcon) tube for recovery by centrifugation at 300 x *g* for 5 min. In the case of EDTA, a cell scrapper (Falcon) was used to fully detach the cells. Cell pellets were resuspended in 2 % paraformaldehyde for 30 min at room temperature. This fixation step was aimed to minimise alteration in the expression levels of cell surface antigen after cell detachment and subsequent procedures. The fixed cells were washed twice with PBS and centrifuged at 350 x *g* for 5 min before analysis by flow cytometery as detailed below.

Single colour antibody staining

The total mAbs tested are tabulated in Table 1. Fixed cell pellets were resuspended at a concentration of 3 × 106 cells in 1 ml of staining buffer that comprised PBS supplemented with 10% FBS, sheep serum, goat serum (giving a final concentration of 30% serum) with 100 mg/ml bovine serum albumin (all from Sigma-Aldrich, Dorset, UK). Ovine peripheral blood mononuclear cells were isolated from systemic blood samples were used as controls to confirm cross-reactivity of antibodies that did not cross-react to oMSCs (Fig. 1, Supporting Information).

All staining procedures were conducted in non-adherent round-bottom 96 well plates (Nunc). A 100 µl volume of suspension containing $3x10^5$ cells in staining buffer was transferred to each well in triplicate (??) for each antibody and for controls that consisted of an unstained and an isotype control antibody. Antibodies were used at the suppliers recommended dilutions. Sample incubation with antibody was performed with gentle agitation for 1 hr at room temperature protected from light. Samples were then transferred to polystyrene FACS tubes (Thermo Fischer) and washed twice by centrifugation at 350 x *g* for 5 min and re-suspending in 4 ml of PBS (Gibco). Cells were finally re-suspended in 250 µl of PBS and retained on ice protected from light untill analysis by flow cytometery.

Multi-colour cell staining

Multicolor staining was performed to confirm the co-localisation of cross-reactive antibodies on the same cell and to eliminate the possibility of staining by negative markers. A CD29-FITC mAB (green channel) was paired with CD271-Alexa 674 mAB (red channel) to evaluate co-localisation of phycoerythrin (PE, yellow channel)-conjugated CD73, CD90, CD105 and CD166 mAbs. The readings of single mAb stained MSCs were used for compensation calculations post hoc in flow cytometery analysis software FlowJo (Tree Star, OR) as described below.

Flow cytometery

Flow cytometry was performed on a BD FACS Calibur instrument (BD Bioscience, Cambridge, UK). The instrument was calibrated before each analysis with the CaliBRITE 3 FACS Comp beads (BD Biosciences) according to the manufacturer's instructions. For cellular analyses, unlabeled MSCs suspended in PBS in FACS tubes (12 × 75 mm2 BD Biosciences) were used to adjust the voltages and amps to position the population in the center of the scatter dot-plot

based on forward and side scatter properties. Simultaneously, fluorescence channels were adjusted to place the unstained population within the first decade (order of magnitude) of the log intensity axis. Acquisition event counts was set for a total of 5 × 104 events. Sample cell suspensions were gently agitated before being analyzed at a low suction rate (60 µl/min). Further details are provided in MIFlowCyt (Supporting Information).

Data analysis

Sample data output generated from flow cytometry was analysed with FlowJo software version 10.0.7 (Tree Star Inc). Gating was applied to exclude debris or multi cellular aggregates by selecting the central most population of single cells with highly similar morphologic and cytometeric characteristics. Isotype control readings were used against fluorescence readings to determine fluorescence shift. Outputs of positive and negative percentages from different experiments were combined to obtain a mean with standard deviation for positive and negative readings. Compensation was conducted post hoc with FlowJo using single stain analysis readings of mAbs. The resulting data was presented in 2 dimensional plots with quadrant percentages.

Results

Characterisation of bone marrow derived ovine MSCs

A 10 ml aspirate of sheep bone marrow yielded ~$2x10^5$ plastic adherent cells after 7 days of primary culture. At this stage, these cells exhibited spindle shaped morphology (Fig. 1A) and were able to form colony forming units by 7–10 days when seeded at a limiting density (Fig. 1B). After further expansion, the oMSCs were capable of differentiating to adipogenic, osteogenic, and chondrogenic lineages (Figs. 1C–1F).

Effects of cell detachment methods on ovine MSC scatter properties

Data comparing forward and side scatter readings from different detachment reagents are presented as contour plots in figure 2. The cells detached with trypsin presented a single, morphometrically (size) and cytometerically (granularity) homogeneous population (Figure 2A). Cells detached with Accutase displayed a more dispersed scatter profile compared to trypsin with cellular aggregates appearing in the upper right corner of the scatter plot (figure 2 B). EDTA in Hank's buffer caused further heterogeneity as observed by the appearance of two morphometerically different but cytometerically similar populations (Figure 2C). This reagent also required a two-fold longer incubation time compared to the enzymatic methods and the need for total detachment encouraged by the use of a cell scraper in the case of fully confluent cultures. Furthermore, not all EDTA detached cells were spherical, but had retained their native spindle-shaped morphology (data not shown). The combination of Accutase and EDTA in Hank's buffer resulted in a scatter profile of oMSCs that was similar to that obtained with trypsin (Figure 2 D) but with less cell debris present.

Single color flow cytometric analysis

oMSCs were positive for CD73 (clone AD2; 96.9% ± 5.9), CD90 (99.6% ± 0.3), CD105 (99.1 ± 1.5), CD271 (97.7 ± 2.0), and MHC1 (94.0% ± 7.2) (Fig. 3). In contrast, the oMSC populations were negative for mouse-antihuman CD117 (clone 104D2; 0.1% ± 0.1). Inconsistent low levels of cross-reactivity with other putative negative markers were observed: these were mouse-anti-sheep CD31 (clone CO.3E1D4; 14.6% ± 4.2); mouse- antisheep MHC class II (clone 37.68; 10.5% ± 15.9); and mouse-antihuman CD45 (clone 1.11.32; 39.4% ± 31.8).

Multi-color flow cytometeric analysis

A single oMSC cell line prepared from a primary culture (not subjected to cell passage) was used to assess co-localisation of positive phenotypic markers. The results of this analysis are shown in figure 5. All positive markers evaluated in this experiment were observed to be co-expressed on all gated cells. The negative markers CD34 and MHC Class II were not detected in these cells.

Discussion

This study has identified a panel of cross reactive monoclonal antibodies (mAbs) against cell surface markers (73, 90, 105, 271, MHC-I) on ovine mesenchymal stem cells (MSCs). In addition, antibodies cross-reactive to sheep CD31, CD34, CD45, CD117 and MHC-II were also identified for negative markers (<1% positive) of MSCs. The low expression of CD31 and MHC-Class II was encouraging in suggesting minimal contamination with white blood cells. These were positive against PBMCs but another white blood cell marker, using the mouse-antihuman CD34 (QBEND/10), was not observed to cause a significant shift against PBMCs, possibly due to confinement of the antigen to hematopoietic progenitor cells in bone marrow that were not tested here. Interestingly, a high expression of CD45 was detected in one of four primary cell cultures tested, which we speculate was due to unusually high contamination of cultures with adherent non-MSCs.

Previous attempts at immunophenotyping ovine MSCs have been reported (13,16–18). These are based on negative selection criteria by using CD29 and CD44 as positive markers and CD31 and CD45 as negative markers, (18,19). Although this combination is sufficient in discriminating fibroblasts against hematopoietic cells, both CD29 and CD44 are not specific to MSCs, which significantly limits cellular identification by this method. In another report. In another report (17) the identities of the clones used to assess the wider range of positive

markers were not provided, thus limiting wider use in sheep studies. This is particularly relevant to CD73 and CD105, whose clones displayed variable degrees of binding affinity in our study. For instance, the mouse-anti-human CD73-PE (clone AD2) was observed to cause lesser fluorescence intensity on the flow cytometer compared to the mouse-antirat CD73-PE (clone TY/23) in cells derived from the same primary culture, most likely due to lesser affinity to the antigen. Similarly, only one mAb against CD105 (rat-antimouse clone MJ7/18) out of four tested positively cross-reacted with oMSCs. In contrast, all four mAbs showed strong affinity to human MSCs.

An important consideration to the expression of cell surface markers is the procedure used to detach cell from plastic. Although trypsin provided morphometrically a highly homogeneous cell population, its replacement with a combination of Accutase and EDTA was also found to consistently yield populations of morphometrically homogeneous MSCs with good preservation of cell surface markers observed by flow cytometry. Importantly, however, it negated the possible harsher affects of trypsin on cell membrane proteins.

The flow cytometery data also provides validation to the method of whole bone marrow plating for MSC isolation (3). Current techniques for the preparation of cell therapy products from bone marrow has employed density centrifugation or red blood cell lysis and recently, a convenient alternative in the form of direct plating of whole bone marrow for 24 hours followed by removal of debris and red blood cells to obtain adherent colony forming cells (3). While the latter method risks contamination by adherent non-MSCs, the findings presented here suggest this to minimally impact the population's characteristics as a whole.

The panel of clones proposed in this study represent an important “tool-kit” to immunophenotypically characterise oMSC populations when investigating the effects of oMSCs in large animal models such as sheep. Our investigations would suggest that the best cell

surface markers for defining oMSC populations should be CD90 and CD105 (>99% positive), CD73 and CD271 (>95%), while being <1% positive for CD117.

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341 **Table 1.** Monoclonal antibodies tested against oMSCs

Marker	Antigen	Antibody clone	Host	Reactivity	Cross-reactivity reported	Catalogue identity and supplier	Conjugated fluorochrome
Cross Reactive mAbs							
CD29	Integrin, beta 1	MEM-101A	Mouse	Human	No	CD2901 Life Technologies	FITC
CD44a	glycoprotein receptor for hyaluronic acid	25.32	Mouse	Sheep	Human, Goat, Cow	MCA2219F Abdserotec	FITC
CD73	5'-nucleotidase	TY/23	Mouse	Rat	No	550741 BD Biosciences	PE
CD90	Thy-1 cell surface antigen	5E10	Mouse	Human	No	555593 BD Biosciences	PE
CD105	Endoglin	MJ7/18	Rat	Mouse	No	562759 BD Biosciences	PE
CD166[a]	Activated leukocyte cell adhesion molecule	3A6	Mouse	Human	No	559263 BD Biosciences	PE
CD271	Neural Growth Factor receptor	C40-1457	Mouse	Human	No	560877 BD Biosciences	Alexa-Fluor® 647
MHC-I	Major Histocompatibility Complex, Class I	Bu8	Mouse	Human	No	MCA2509F Abdserotec	FITC
MHC-II	Major Histocompatibility Complex, Class II	37.68	Mouse	Sheep	Bovine	MCA2226F Abdserotec	FITC
CD31	Platelet endothelial cell adhesion molecule type 1	CO.3E1D4	Mouse	Sheep	Bovine, Goat	MCA1097F Abdserotec	FITC
CD45	Lymphocyte Phosphatase-Associated protein	1.11.32	Mouse	Sheep	Bovine, Goat, Dog	MCA2220F Abdserotec	FITC
CD117	Hematopoietic cell surface glycoprotein	104D2	Mouse	Human	No	MCA1841PET Abdserotec	RPE

Non-Cross Reactive mAbs							
CD73	5'-nucleotidase	AD2	Mouse	Human	No	550257 BD Biosciences	PE
CD105	Endoglin	43A3	Mouse	Human	No	60039AD.1 Stem Cell Tech.	Alexa Fluor® 488
CD105	Endoglin	SN6	Mouse	Human	Horse.	MCA1557A488T Abdserotec	FITC
CD105	Endoglin	266	Mouse	Human	Horse.	MCA1557FT Abdserotec	FITC
MHC-I	Major Histocompatibility Complex, Class I	2G5	Mouse	Human	Dog.	MCA2189F Abdserotec	FITC
MHC-I	Major Histocompatibility Complex, Class I	CVS22	Mouse	Horse	No	MCA1086PE Abdserotec	RPE
MHC-II	Major Histocompatibility Complex, Class II	28.1	Mouse	Sheep	Bovine	MCA2225F Abdserotec	FITC
CD34	Hematopoietic Progenitor Cell Antigen	QBEND/10	Mouse	Human	Monkey	SFL547PE Abdserotec	RPE
Isotype controls							
Isotype control	Anti-mouse IgG1: FITC with anti-rat IgG2a: RPE with anti-rat IgG1: Alexa Fluor® 647		Mouse		No	TC022; Abdserotec	FITC/RPE/Alexa-Fluor® 647
Isotype control	Anti-mouse IgG1		Mouse		Rat	MCA928F; Abdserotec	FITC

342 1. All mAbs used in this study are tabulated above. FITC, fluorescein isothiocyanate; RPE, R-phycoerythrin.

343 a Reported previously by [13] to be cross-reactive against multipotent sheep MSCs.

Figure legends / captions

Figure 1 Characteristics of ovine mesenchymal stromal cells. (A) Early passage oMSCs with a fibroblastic spindle morphology. (B) The oMSCs formed single cell derived colonies when seeded at clonal densities (100 cells) in a 6 cm petri dish for 10-14 days. (C-D) Adipogenic differentiation assay showing positive Oil red O staining in induced samples. (E-F) Osteogenic differentiation assay indicating positive calcium specific Alizarin Red S staining in induced cultured. (G-I) Chondrogenic differentiation of oMSC shown in paraffin embedded sections with (top) hematoxylin and eosin, (centre) alcian blue stain for GAGs (centre) and (below) safranin-O GAG specific stain.

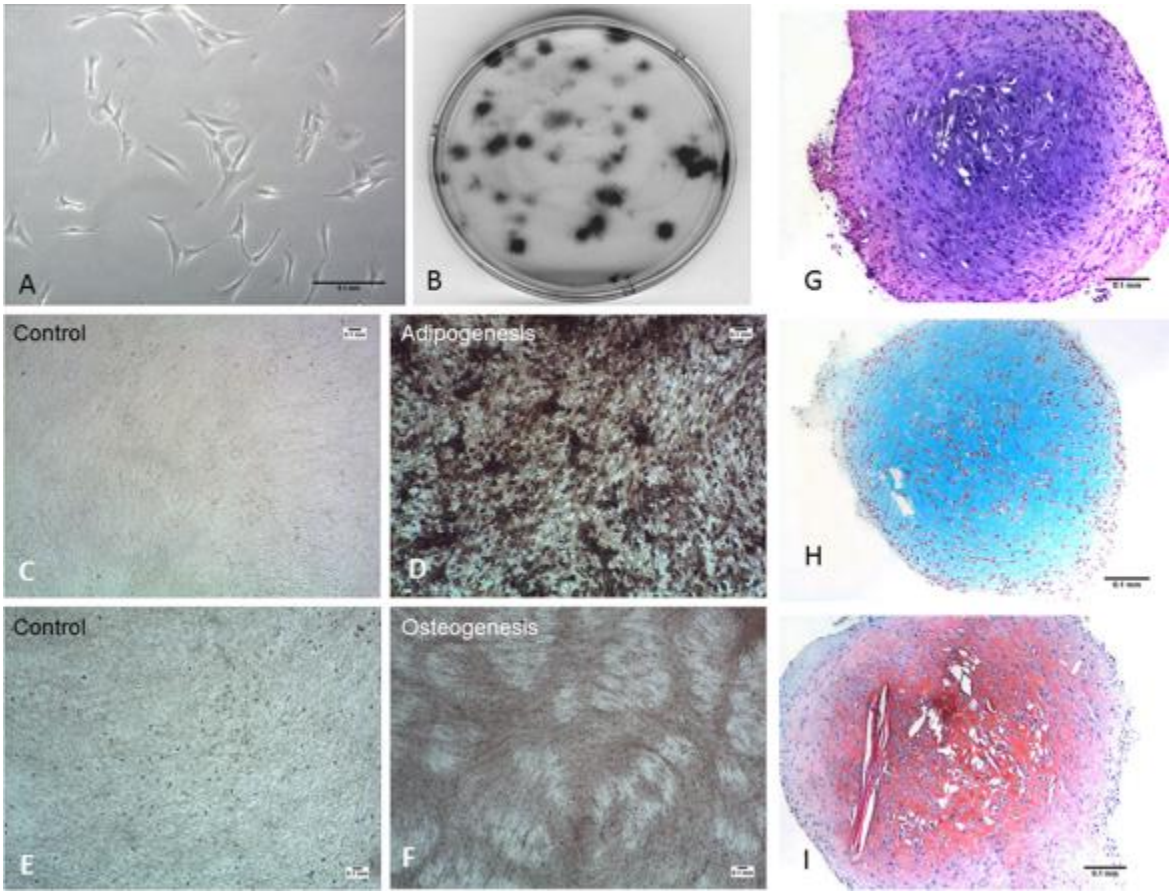


Figure 2 . Comparison of cell detachment reagents. Contour plots at 5 % increments presenting the scatter profiles of cells detached from culture flasks with trypsin, Accutase, EDTA in Hank's buffer and a 3:2 mix of Accutase and EDTA. (A) Cells detached with trypsin exhibited a compact and homogeneous scatter profile. (B) Accutase derived cells displayed a relatively wider spread of cells than trypsin. (C) Cells detached with EDTA displayed further heterogeneity by the appearance of two population of cells with equal cytoplasmic densities but differing sizes. (D) A 3:2 (v/v) combination of Accutase and EDTA resulted in a scatter profile of cells highly similar to trypsin with a characteristically compact and homogeneous profile. Furthermore, the quantity of larger aggregates or duplets confined to the top axis was less in the 3:2 mixture of Accutase and EDTA than these reagents alone.

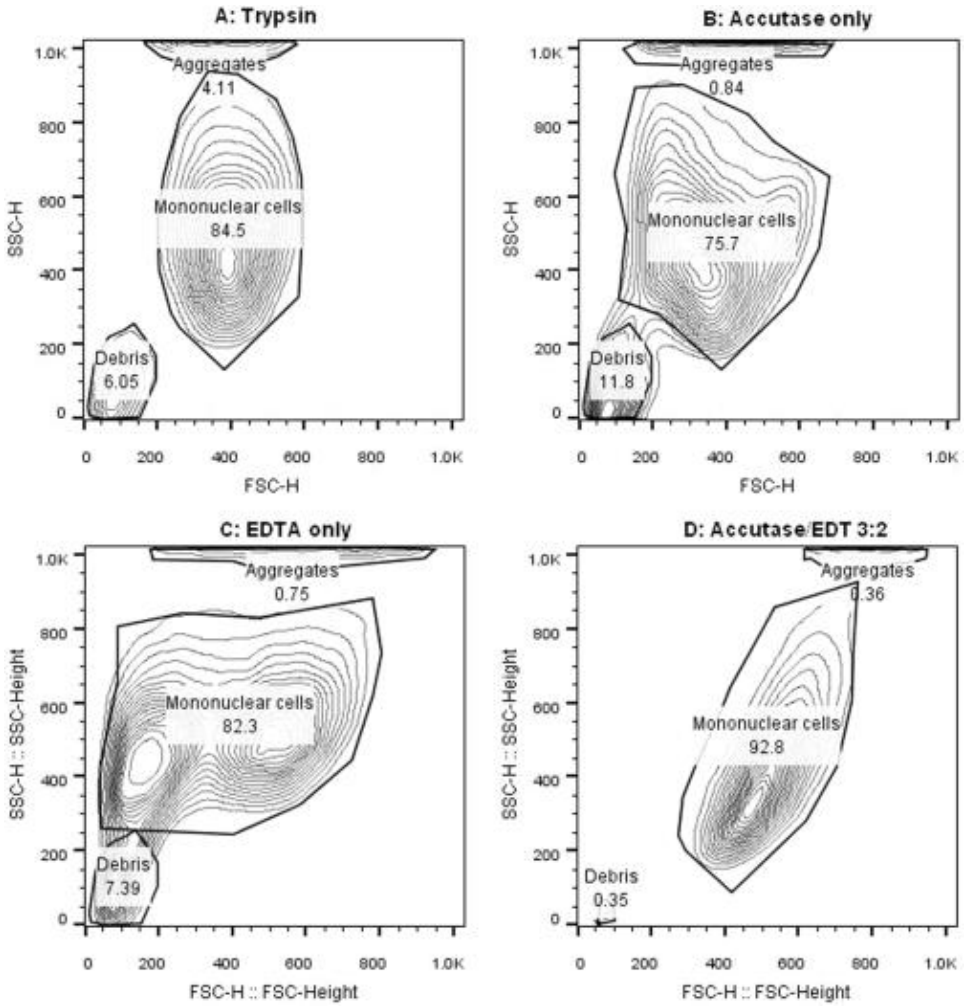
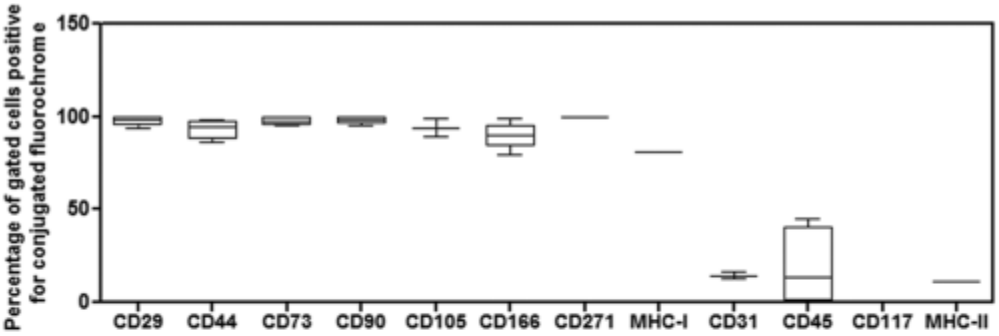


Figure 3. Single stain analysis of mAb cross-reactivity to human MSCs. (A) Forward and side scatter plot of human MSCs detached by the Accutase/EDTA combination. (B) Overlay of cells only (red) and isotype control labelled cells (blue). Reading obtained for different mAbs (orange) are for (C) CD29, (D) CD44, (E) CD105 (clone 43A3), (F) mouse-human CD73 (clone AD2), (G) rat-mouse CD73 (clone TY/23), (H) mouse-human CD90 (clone 5E10) and mouse-human CD166.



Antibody	Number of experiments	Mean percent of positive events	SD	Mean percent of negative events	SD
CD29 FITC	6	99.04	1.98	0.99	1.97
CD44 FITC	6	99.42	0.35	0.56	0.35
CD73 RPE	6	96.87	5.88	3.16	5.87
CD90 RPE	4	99.63	0.26	0.40	0.28
CD105 RPE	3	99.07	1.53	0.93	1.55
CD166 PE	6	93.40	6.76	6.61	6.75
CD271 Alexa® 647	2	97.70	1.98	2.33	2.00
MHC1 FITC	2	94.00	7.21	6.01	7.21
CD31 FITC	2	14.60	4.24	85.40	4.24
CD45 FITC	4	39.43	31.81	60.58	31.82
CD117 RPE	2	0.06	0.06	99.95	0.07
MHC-II FITC	3	10.45	15.90	89.57	15.91

Figure 4. Multi-stain analysis of mAb cross-reactivity to oMSCs. (**A**) A contour plot based scatter profile of primary oMSCs. (**B**) Dual-stain analysis with CD29-FITC and CD271-Alexa 647 displaying co-localization of the two markers on the oMSCs. This pair was used in triple stain analyses of samples to confirm colocalization with CD73 (**C**, **D**); CD90, (**E**, **F**); CD105 (**G**, **H**); CD166 (**I**, **J**). Additionally, a lack of costaining between CD271-Alexa 647 and MHC-II negative marker was seen in (**K**).