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Multiparameter flow cytometry for the characterisation of extracellular markers on human mesenchymal stem cells

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Abstract

Extracellular surface proteins can be used to identify fully functional human mesenchymal stem cells (hMSCs) in a mixed population. Here a multiparameter flow cytometry assay was developed in order to examine the expression of several bone marrow-derived hMSC markers simultaneously at the single cell level. The multiparameter approach demonstrates a depth of analysis that goes far beyond the conventional single or dual staining methods. CD73, CD90 and CD105 were chosen as positive markers as they are expressed on multipotent hMSCs, whilst CD34 and HLA-DR were chosen as negative indicators. Single colour analysis suggested a population purity of 100%, in contrast, when analysed *via* the multiparameter method it was found that the $CD73^{+ve}/CD105^{+ve}/CD90^{+ve}/HLA\text{-}DR^{-ve}/CD34^{-ve}$ phenotype represented 94.49% ± 1.31% of the total cell population. Also, CD271 has recently been posited as a definite early stage hMSC marker but here we show it is not present on pre-passage cells, highlighting the need for careful marker selection.

Keywords: CD271, characterisation, extracellular markers, human mesenchymal stem cells (hMSCs), multiparameter flow cytometry,

Introduction

Human mesenchymal stem cells (hMSCs), also known as multipotent stromal cells, were first isolated from bone marrow and found to differentiate into adipocytes, chondrocytes and osteoblasts *in vitro* (Pittenger et al. 1999; Friedenstein et al. 1970). In addition, it is now known that hMSCs secrete multiple anti-inflammatory, angiogenic and immunomodulatory factors that serve to repair locally damaged tissue (Caplan 2007). For these reasons they are becoming a cell type of increasing interest for tissue engineering and regenerative medicine research. Currently there are over 150 active bone marrow-derived mesenchymal stem cell clinical trials with only a handful in phase 3; hence there is now a real need for robust product characterisation (http://www.clinicaltrials.gov/).

Mesenchymal stem cells are not unique to bone marrow; they have also been isolated from dental pulp, umbilical cord vein and adipose tissue (Williams and Hare 2011). The varied tissue sources and isolation techniques used raise the issue of whether the resulting cell populations are true hMSCs at all and whether the many published studies conducted are at all comparable (for a comprehensive review see (Rojewski et al. 2008)). Currently there is no single marker that defines an hMSC and therefore investigators will use a combination of markers to characterise a population. To address this concern the International Society for Cellular Therapies (ISCT) has proposed minimal criteria that define a true hMSC (Dominici et al. 2006). These include the ability to adhere to standard tissue culture plastic; differentiation into the adipocytes, chondrocytes and osteoblasts lineages; and expression of

specific surface antigen markers. These surface antigens include expression of CD90, CD73 and CD105; hMSCs must also lack the expression of CD45, CD34, CD14, CD79α and HLA-DR as they are indicators of non-stromal cell types. Recently this has been updated to include CD10, CD146 and GD2 as further positive markers (Rasini et al. 2013).

Recently, it has been suggested that another marker, CD271 (Low-Affinity Nerve Growth Factor Receptor), may in fact be specific for the colony-forming-unit-fibroblast (CFU-Fs) found in the stromal region (Jones et al. 2006; Kuci et al. 2010). Previous studies have demonstrated *via* fluorescent-activated cell sorting (FACS) that by collecting $CD271^{+ve}$ cells, the resulting population has a greater CFU-F capability when compared to a population of non-sorted cells. However, it is known to be down-regulated over prolonged culture *in vitro* suggesting it may be an efficient marker to initially select for bone marrow hMSCs but not as a positive phenotypic marker over multiple passages (Harichandan and Buhring 2011).

Flow cytometry is a high-throughput analytical technique that can be used to study protein expression properties of a cell *via* fluorescently labelled monoclonal antibodies. Most flow cytometers can measure the expression of several antigens provided that a sample is split for analysis using one or two fluorophore-labelled antibodies at a time. While technically straightforward, single or dual staining approaches do not provide the resolution of multiple antigens within the same population. Multiparameter (also known as multicolour) flow cytometry has been applied to clinical diagnostics and immunology, providing the high-resolution information needed to identify subtle phenotypic differences with statistical robustness (Peters and Ansari 2011). Unfortunately, multiparameter flow cytometry is not routinely used to define stem cell phenotypes, although a previous study has demonstrated the use of a seven-colour flow cytometry assay to examine the differential expression of markers

between freshly isolated bone marrow and umbilical cord samples (Martins et al. 2009). However, it is envisaged that greater uptake of multiparameter flow cytometry is expected to provide similar benefits to those achieved in the aforementioned fields of research.

Previous work by our laboratory has demonstrated that multiparameter flow cytometry can be used to characterise human embryonic stem cells (Brosnan et al. 2013). Here we show that this technique can be used to monitor five surface antigens that correspond to the hMSC phenotype. CD73, CD90 and CD105 were selected, as they are the most widely accepted surface markers of multipotency, additionally we also examine CD271 as a potential positive marker. CD34 and HLA-DR were selected as negative markers, as they indicate differentiation down the primitive hematopoietic and leukocyte lineages respectively.

Here we demonstrate the power of a relatively simple and cost effective assay using a five-colour/two laser array for the analysis of extracellular marker expression on hMSCs. The multiparameter approach provides a simple way to determine putative hMSCs from a mixed population and for quality control that can be implemented to further facilitate process development.

Materials & Methods

Cell Culture

Human bone marrow derived mesenchymal stem cells (Lonza, Germany) were obtained from a single healthy donor after the patient provided informed consent. The local Ethical Committee approved the use of the sample for research. hMSCs passage 2-6 were cultured in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS; Hyclone, Belgium) and 2

mM L-glutamine (Lonza, UK); medium was replaced every 3 days. Cells were maintained in a humidified incubator at 37°C in air containing 5% v/v CO_2. The cells were passaged every 6 days using 3ml 0.25% w/w trypsin/EDTA (Lonza, UK) for 5 minutes at 37°C then quenched with 7ml growth medium. The cells were detached by gentle tapping and the cell suspension was centrifuged for 5 minutes at 220*g*. The supernatant was discarded and the remaining pellet was re-suspended in an appropriate volume of culture medium. The hMSCs were reseeded at $5x10^3$ cells/cm^2 in T-flasks.

Cell Separation

Bone marrow mononuclear cells (BM-MNC) samples, obtained from donors 080004A and 071475B with informed consent, were purchased from Lonza (US). Both samples were sorted using manual magnetic-activated cell sorting (MACS) (Miltenyi Biotech, UK) according to the manufacturer's instructions. Briefly, the BM-MNC were incubated with CD271-PE antibody and blocking agent for 10 minutes at 4°C. Then the samples were washed in buffer, centrifuged and the supernatant aspirated. The samples were then conjugated with anti-PE microbeads for 15 minutes at 4°C. A LS MACS column was used to isolate both the $CD271^{+ve}$ and $CD271^{-ve}$ cell fractions which were both plated into separate T-flasks.

Antibody Staining

Detached cells were suspended at $0.5x10^6$ cells/ml in growth medium and loaded onto a 96 well plate (200µl per well). The plate was centrifuged for 5 minutes at 220*g*. The aspirate was removed and the cells re-suspended and washed in flow cytometry staining buffer (R&D Systems, UK) and the centrifugation cycle repeated. The cells were stained for 30 minutes in the dark at room temperature with fluorescent monoclonal antibodies against CD34 (PE-CY5), CD73 (PE-Cy7), CD90 (APC), CD105 (PE) and HLA-DR (FITC, all from BD Biosciences, UK) in addition with the corresponding isotype controls. After incubation the

cells were washed twice with staining buffer as before. Finally, 200µl of staining buffer was used to re-suspend the samples before analysis. For CD45/CD271 staining BM-MNC were thawed and dual stained with both CD45 (APC-Cy7, BD Biosciences, UK) and CD271 (PE, Miltenyi Biotec, UK) antibodies using the same protocol as before. The gating strategy was performed in a similar manner to Jones et al, (2010) where the $CD271^{+ve}/CD45^{-/low}$ population was gated.

Flow Cytometry Acquisition and Analysis

All data was obtained using a Guava easyCyte 8HT flow cytometer (Merck Millipore, UK) equipped with 488nm and 640nm excitation running guavaSoft Incyte acquisition software (v2.5). A minimum of 10,000 gated (Forward scatter/Side scatter) events were recorded for each sample. Compensation values for spectral overlap were determined using anti-mouse Ig, κ antibody capture beads as follows (CompBeads, BD Bioscience, UK). A 2ul suspension of both the positive and negative capture beads was singly stained with each fluorescent antibody as before. For the rest of the protocol CompBeads were treated in the same way as the cells. Post-acquisition analysis and compensation was performed with FlowJo v7.6.5 (Treestar Inc, USA) software.

Differentiation

Differentiation was performed using established protocols (Chase et al. 2010). To induce osteogenic differentiation, cells were seeded into a twelve well plate at $5x10^3$ cells/cm^2 with Invitrogen StemPro Osteogenesis medium (Invitrogen, UK). The medium was changed every 3-4 days. After four weeks in culture, cells were washed and fixed in 4% (v/v) paraformaldehyde (PFA) for 5 minutes at room temperature. For calcium staining, a solution of 2.5% silver nitrate (Sigma, UK) was added and cells were incubated for 30 minutes at room temperature under an ultra violet light. Counterstaining for alkaline phosphatase (ALP)

was carried out with a solution containing Fast Violet B Salt with 4% (v/v) Napthol AS-MX Phosphate Alkaline Solution (Sigma, UK) for 45 minutes at room temperature in the dark. Cells were washed three times in distilled water and visualised under a light microscope (Nikon Eclipse TS-100).

To induce chondrogenic differentiation, cells were sub-cultured and re-suspended at $1.6x10^7$ cells/ml. Micromass cultures were generated by seeding a six well plate with 2 µl droplets of cell suspension and allowed to attach to the tissue flask surface in a humidified atmosphere for 30 minutes before adding pre-warmed Invitrogen StemPro Chondrogenesis medium (Invitrogen, UK). The medium was changed every 3-4 days. After 14 days in culture, cells were washed and fixed in 2% (v/v) PFA for 30 minutes at room temperature. Chondrocytes were stained with 1% (v/v) Alcian Blue in 0.1 M hydrochloric acid (HCl) solution for 30 minutes, washed three times with 0.1 M HCl, diluted with distilled water and visualised under a light microscope.

To induce adipogenic differentiation, cells were seeded in a twelve well plate at $1x10^4$ cells/cm^2 with Invitrogen StemPro Adipogenic medium (Invitrogen, UK). The medium was changed every 3-4 days. After three weeks in culture, cells were washed and fixed in 2% (v/v) PFA for 30 minutes at room temperature. For adipogenic staining, cells were washed three times with PBS and incubated with 0.3% (v/v) Oil Red O solution in 99% isopropanol for 30 minutes at room temperature. Cells were washed three times in distilled water and visualised under a light microscope.

Results and Discussion

CD271 separation

Previous work has speculated that CD271 (LNGFR) may be a specific bone marrow hMSC marker (Alvarez-Viejo et al. 2013; Jones et al. 2010). On this basis we performed MACS on two frozen BM-MNC populations using antibodies against CD271 and both resulting cell fractions were plated. After 3 weeks of culture there were no cells found within the $CD271^{+ve}$ sorted plates **(Fig. 1a)**, in contrast a large adherent cell population was observed in the $CD271^{-ve}$ plates (**Fig. 1b)**. The $CD271^{-ve}$ population expressed the CD73, CD90, CD105 (≥99.9% in all cases) and did not express CD34 (5.37%) and HLA-DR (5.14%) **(Fig. 1c)**.

Previous reports have demonstrated a rare population of $CD271^{+ve}/CD45^{-/low}$ cells found in bone marrow aspirates (Jones et al. 2010). Therefore we performed dual-staining flow cytometry for CD45/CD271 on the BM-MNCs. The gating strategy used to analyse the population was performed in a similar manner to previously published reports (Cox et al. 2012; Jones et al. 2006). Similarly to the MACS technique we could not detect a significant number of $CD271^{+ve}$ cells (0.16% ± 0.02%) or the presence of a discrete population in the $CD271^{+ve}/CD45^{-/low}$ gate (**Fig. 1d)**.

These findings are supported by the comprehensive proteomic profiling conducted by the US Food and Drug Administration (FDA) which only found CD271 expression in one out of four donor samples (Mindaye et al. 2013). This variability of CD271 expression has also been reported by other groups (Alvarez-Viejo et al. 2013; Siegel et al. 2013).

Single Colour Staining

To validate the panel of markers for the multiparameter study the hMSC samples were first labelled with single antibodies against the markers of interest **(Fig. 2)**. The ISCT suggests ≥95% purity for expression of positive markers. Through this method, single staining results showed a highly enriched sample of the three positive markers CD73, CD90 and CD105 (100%). Additionally they demonstrated little expression of the negative markers CD34 and HLA-DR (<3.5% in both cases). Due to this being outside of the ISCT range for negative markers (≤2%) we then sought to develop a multiparameter approach to find the true expression of $CD73^{+ve}/CD105^{+ve}/CD90^{+ve}/HLA\text{-}DR^{-ve}/CD34^{-ve}$ in the population.

Multiparameter Flow Cytometry

Many publications regarding hMSC characterisation *via* flow cytometry employ either single or double stained populations. Although these techniques validate the expression of surface markers they do not give a representation of the population when taken together. Therefore the population was next examined using the multiparameter approach. All dual analyses of the different combinations of CD73, CD90 and CD105 showed a >99% positive population similar to the previous single colour analyses. In all cases >90% of the population fell within the $HLA\text{-}DR^{-ve}/CD34^{-ve}$ gate with the overall average at 95% **(Fig. 3)**. Taken together, these plots show that the population is a highly pure sample of hMSCs with most cells expressing the correct positive markers and lacking the selected negative markers.

Serial gating was then performed to further analyse the hMSC population **(Fig. 4)**. From the initial starting populations, defined from the forward and side scatter, each marker combination was examined in turn until a full cell surface marker phenotype was established.

225 The $CD73^{+ve}/CD105^{+ve}$ population was first gated, and as with the dual analysis over 99% of
226 the population expressed this combination. This quadrant was then analysed for CD90 and
227 HLA-DR. As expected, the cell population fell within the $CD90^{+ve}/HLA\text{-}DR^{-ve}$ quadrant.
228 Finally the resulting cells were gated for CD34 and HLA-DR. As seen previously in Fig 2,
229 the population was found in the $HLA\text{-}DR^{-ve}/CD34^{-ve}$ quadrant. From this the number of cells
230 with the surface marker expression to be $CD73^{+ve}/CD105^{+ve}/CD90^{+ve}/HLA\text{-}DR^{-ve}/CD34^{-ve}$
231 was calculated to be 94.49% ± 1.31% of the original gated population **(Table 1.)**. This is
232 lower when compared to results from the single staining where the population was considered
233 to be 100% positive for a mesenchymal phenotype indicating multiparameter analysis with
234 serial gating allows for a more stringent analysis and indicates the purity of the hMSC
235 population.
236
237 Throughout this study the cell surface markers were chosen based upon the ISCT
238 recommendations (Dominici et al. 2006). CD73, CD90 and CD105 are all expressed on
239 hMSCs even through extended culture, making them an ideal combination for cell
240 identification (Bernardo et al. 2007). CD105, also known as endoglin, is a receptor for TGF-
241 β_1. It is known that these receptors are mediators of cell proliferation and differentiation
242 down the osteoblast and chondrocyte lineages whilst inhibiting the adipogenic lineage
243 (Roelen and Dijke 2003). Upon differentiation down these lineages CD105 is down regulated
244 making it a valuable marker of cell multipotency (Jin et al. 2009).
245
246 Human MSCs are costly to isolate and culture, so by taking a multiparameter approach the
247 phenotype analysis requires fewer cells and reagents, as well as reduced operator time
248 representing a real cost saving for laboratories. For the multicolour assay fewer samples of
249 cells than the usual single staining were prepared, one 5-isotype control, one unstained tube

and four multicolour test samples with an additional six wells for the compensation beads. In contrast the conventional single staining method would require at least twice this number to achieve a less stringent characterisation. This is important for autologous cell therapies, especially for older patients, where cell quantity may be limited and fewer cells are available for analysis.

Differentiation

To demonstrate multipotency of the hMSCs differentiation into chondrocytes, adipocytes and osteoblasts was performed using well-established techniques. To induce chondrogenic differentiation the cells were plated as micromass cultures onto tissue culture plastic with chondrogenic differentiation media. Positive Alcian Blue staining demonstrates the deposition of sulphated proteoglycans, typically secreted by chondrocytes into the extracellular matrix **(Fig. 5a)**. After 14 days of adipogenic differentiation, treated hMSCs accumulated lipid vacuoles that are characteristic of adipocytes. The presence of lipid vacuoles was confirmed *via* Oil Red O staining **(Fig. 5b)**. No lipid vacuoles were seen in the control samples. Finally, the osteogenic differentiation potential of the hMSCs was demonstrated following 21 days of culture in differentiation media. The treated cells showed alkaline phosphatase activity as seen by Fast Violet B Salt with 4% (v/v) Napthol AS-MX Phosphate Alkaline staining (red) and calcium deposition (black) **(Fig. 5c)**.

By demonstrating the ability for trilineage differentiation into adipocytes, chondrocytes and osteoblasts as well as adherence to tissue culture plastic this fulfils the criteria outlined by the ISCT for the maintenance of hMSC phenotype.

Conclusion

The aim of this study was to develop a multiparameter flow cytometry assay to analyse hMSCs at the single-cell level. Fresh bone marrow aspirates are a heterogeneous population containing several subpopulations of distinct cell types from which the hMSCs are isolated. A major obstacle for characterisation is that to date there is no single marker that defines an hMSC. As such, researchers use a panel of indicators to measure population purity creating a need for these multiparameter approaches.

Multiparameter flow cytometry provides a way to determine cell phenotype based on surface proteins in a way that goes beyond the current single colour methodologies. With this method we were able to identify a population of hMSCs that express the cell surface marker panel as suggested by the ISCT. Our multiparameter assay gives a stringent analysis of the population, as each cell must pass through three separate gating strategies to be considered a fully functional hMSC. Our results show that the triple gated population was 94.49% ± 1.31% for the $CD73^{+ve}/CD105^{+ve}/CD90^{+ve}/HLA\text{-}DR^{-ve}/CD34^{-ve}$ expression phenotype, compared to what was considered to be 100% pure *via* the single staining approach. Even though this is just under the threshold value set by the ISCT we believe single stain analysis will give an overestimation of the purity of the population whereas the multiparameter method allows for full single-cell phenotype analysis thus a more stringent cell classification.

Studies have suggested CD271 to be a specific marker for early passage bone-marrow hMSCs. Our findings from the flow cytometry study and MACS conducted here have indicated there is little to no expression of this marker in the cells studied here. Given the low abundance of this specific antigen and the need for a large number of cells for therapeutic use

it is concluded that CD271 may not be an appropriate marker choice on which to base the isolation of hMSCs.

Whilst multiparameter flow cytometry within the stem cell community is still not widely performed recent reports suggest that this technique will become increasingly important(Zimmerlin et al. 2010; Zimmerlin et al. 2012; Khalili et al. 2012). This is attributed to a growing demand to measure multiple parameters in a single sample, thus maximising the information gathered and enhancing the depth of collected data.

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Table 1. The samples were initially gated on the single cell population followed by expression of CD105/CD73. This was then further gated on HLA-DR/CD90 and HLA-DR/CD34 respectively to assess, from the starting population, the percent of cells with the $CD105^{+ve}/CD73^{+ve}/CD90^{+ve}/HLA\text{-}DR^{-ve}/CD34^{-ve}$ phenotype. ± values represent the SE, $n \geq 3$ in all cases.

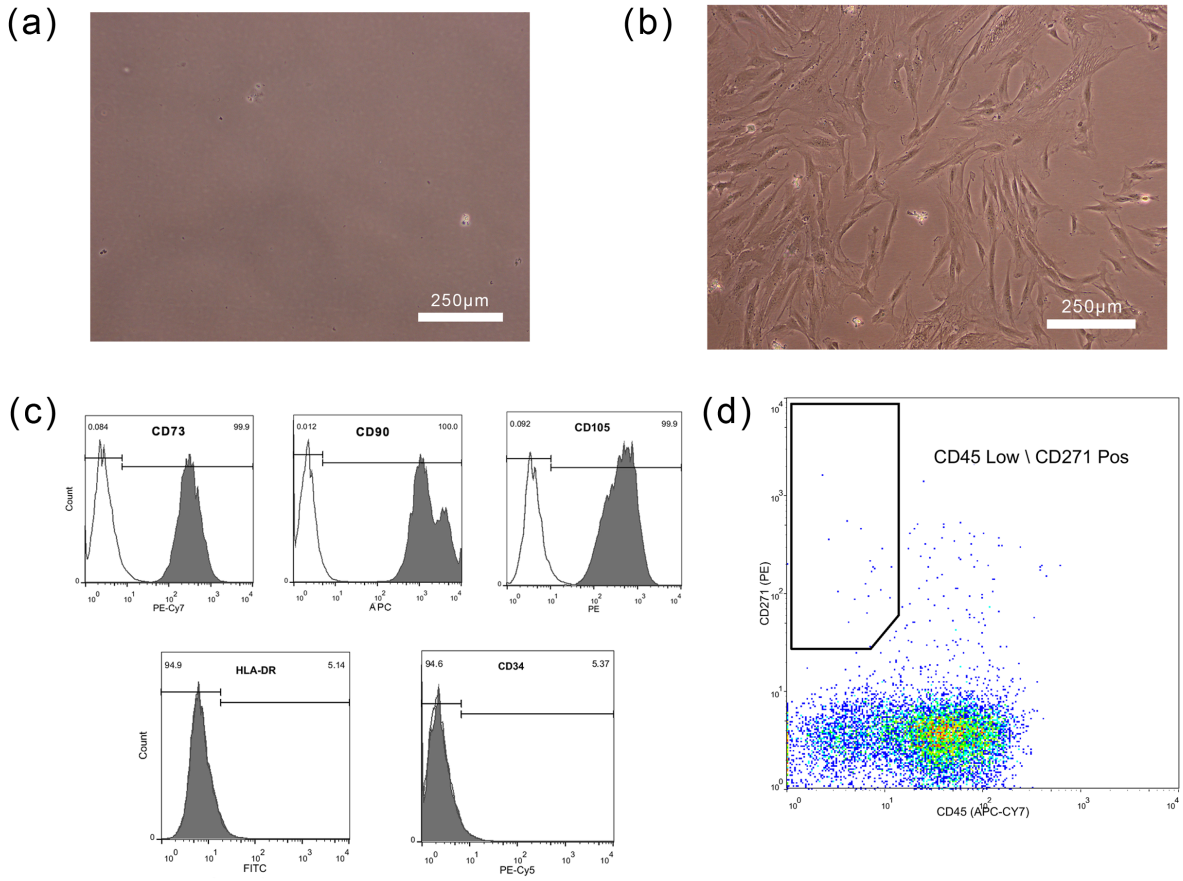
Figure 1. CD271 separation and analysis of bone marrow mononuclear cells. **(a)** Image of tissue culture flask 21 days following $CD271^{+ve}$ MACs separation population showing no cells. **(b)** $CD271^{-ve}$ fraction shows appearance of adherent stromal cells. Scale bar = 250μm. **(c)** Flow cytometry analysis of hMSCs obtained from the $CD271^{-ve}$ fraction shows expression of CD73, CD90 and CD105 whilst lacking CD34 and HLA-DR. **(d)** Dual staining for CD271/CD45 shows an insignificant number of $CD271^{+ve}$ cells, 0.16% ± 0.02% (n = 3).

Figure 2. Single colour flow cytometry results for expression of selected markers of hMSCs (filled). Isotype gating (outline) was set to 95%. Positive markers CD73 (PE-Cy5), CD90 (APC), CD105 (PE) compared to negative markers CD34 (PE-Cy7) and HLA-DR (FITC). Graphs are representative of 4 separate experiments.

Figure 3. Five colour analysis of a single population of hMSCs. The cell surface markers CD73, CD90 and CD105 show >99% positive expression. Negative markers, CD34 and HLA-DR, were found in the negative quadrant. Isotype gating was set to 95%. Each dot plot is accompanied by bar charts that represent each quadrant showing the mean of the samples (error bars indicate the SE). $n \geq 3$ in all cases.

Figure 4. Serial gating of hMSCs showing co-expression of the surface markers. The initial analysis region used forward and side scatter to determine the population of interest. This was then gated for the $CD73^{+ve}$ and $CD105^{+ve}$ cells. Sequential gating was used to determine the $CD90^{+ve}$/HLA-DR^{-ve} and the HLA-DR^{-ve}/$CD34^{-ve}$ population. From the initial gate 95% of cells had the $CD73^{+ve}$/$CD105^{+ve}$/$CD90^{+ve}$/HLA-DR^{-ve}/ $CD34^{-ve}$ cell surface expression phenotype.

Figure 5. Histology of differentiated mesenchymal stem cells to demonstrate multilineage potential. **(a)** Phase-contrast microscopy of micromass cultures differentiated down the chondrocyte lineage. The cells were fixed and stained with Alcian Blue. **(b)** Microscopy of cells following 14 days of adipogenic differentiation. The samples were fixed and stained with Oil Red O. **(c)** Phase-contrast microscopy of cells following 21 days of osteogenic differentiation. Following fixation the samples were stained using Fast Violet B Salt with 4% (v/v) Napthol AS-MX Phosphate Alkaline Solution. Magnification 10x. Scale bar = 250μm.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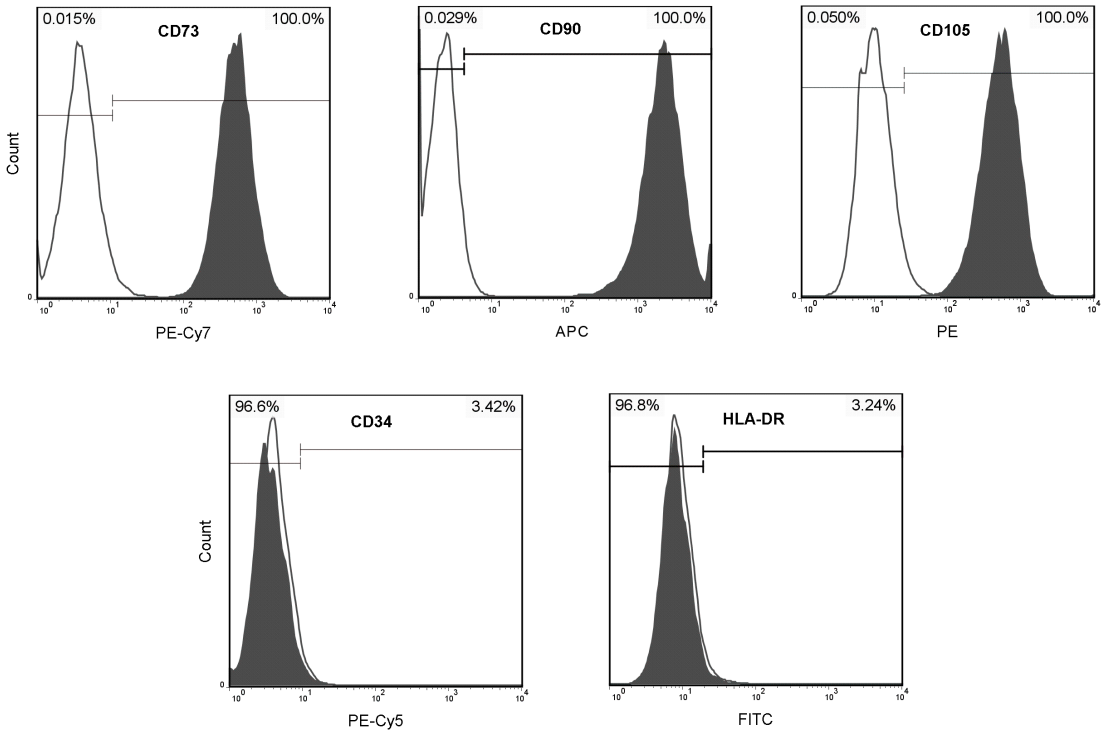
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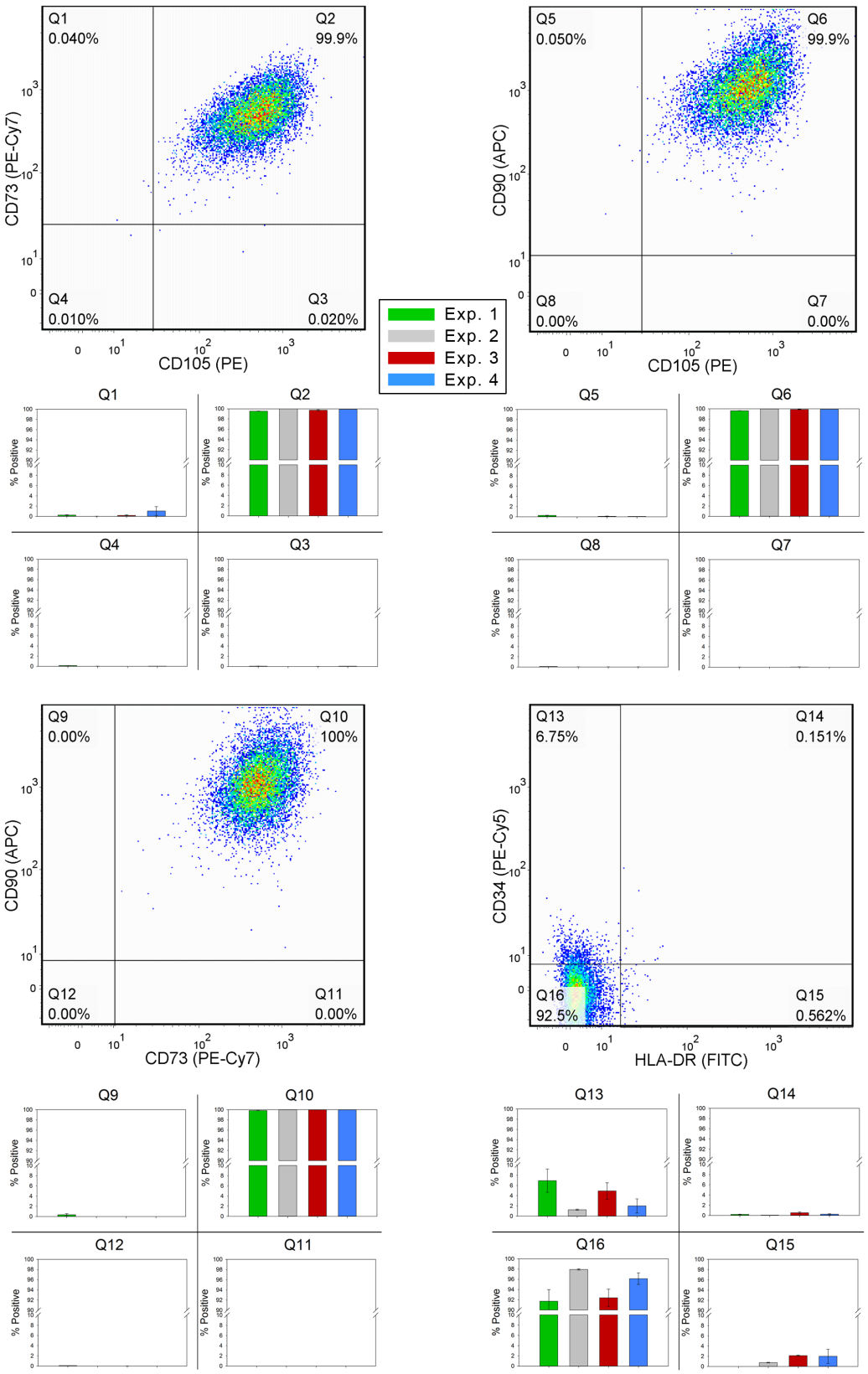
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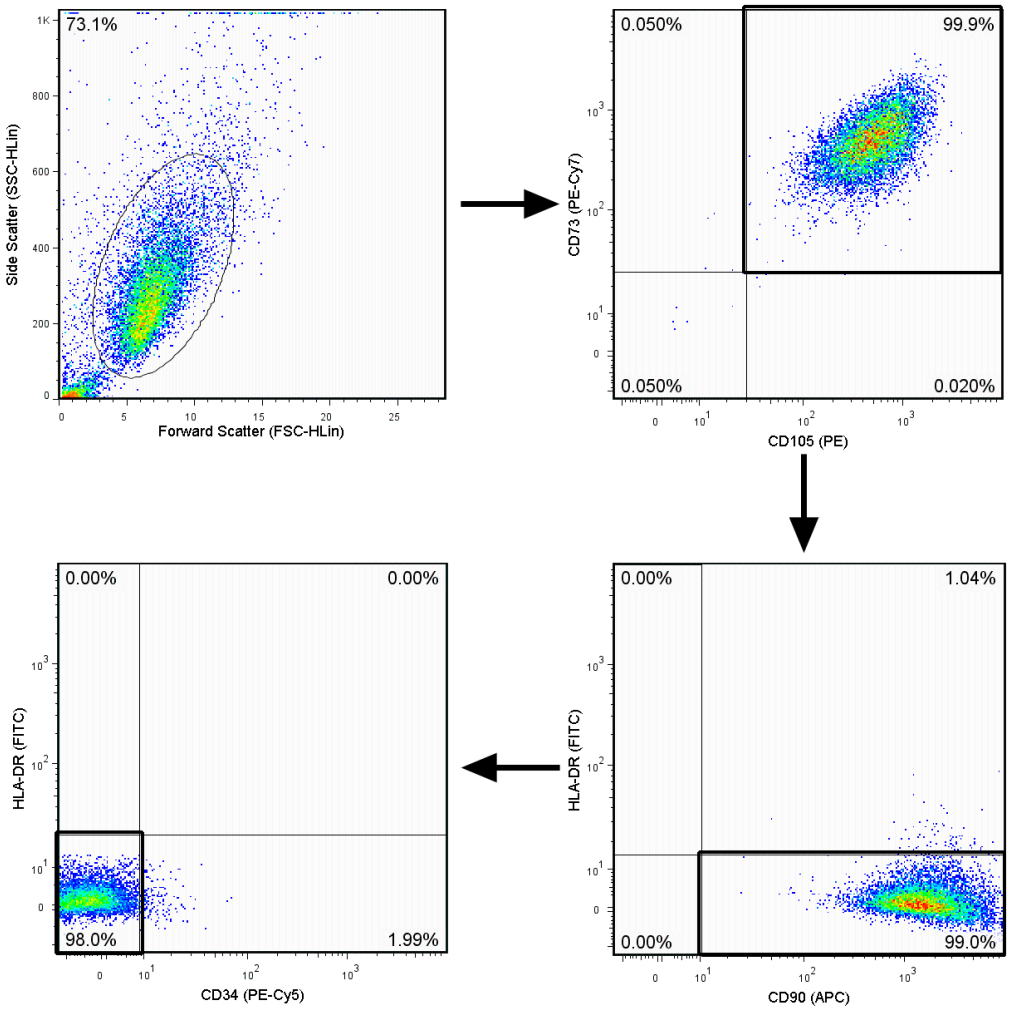
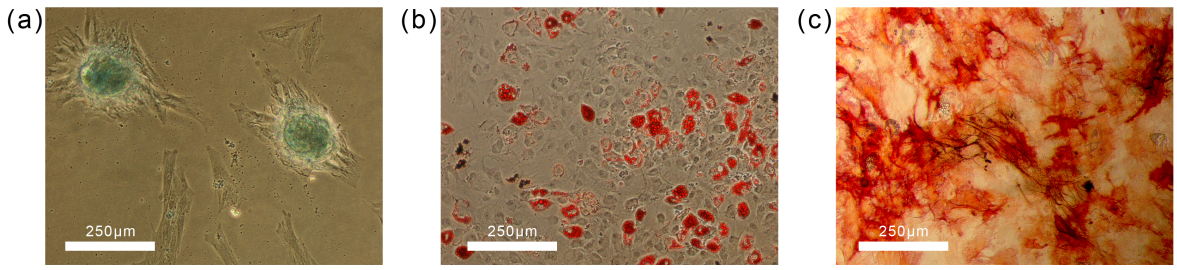
0.00%
CD90 (APC)
CD73 (PE-Cy7)
Q13
6.75%
Q14
0.151%
Q16
92.5%
Q15
0.562%
CD34 (PE-Cy5)
HLA-DR (FITC)

423


73.1%
Side Scatter (SSC-HLin)
Forward Scatter (FSC-HLin)
0.050%
99.9%
0.050%
0.020%
CD73 (PE-Cy7)
CD105 (PE)
0.00%
1.04%
0.00%
99.0%
HLA-DR (FITC)
CD90 (APC)
0.00%
0.00%
98.0%
1.99%
HLA-DR (FITC)
CD34 (PE-Cy5)

424


(a)
250μm
(b)
250μm
(c)
250μm

425

426

% Expression from Multiparameter Data

		Subset 1: CD105 / CD73		→	Subset 2: HLA-DR / CD90		→	Subset 3: HLA-DR / CD34		% with full phenotype
Exp (n=3)	1	CD105- / CD73+	0.24±0.06		HLA-DR- / CD90+	98.80±0.05	→	HLA-DR- / CD34+	7.06±2.31	91.32±2.04%
		CD105+ / CD73+	99.53±0.03	→	HLA-DR+ / CD90+	1.22±0.05		HLA-DR+ / CD34+	0.00±0.00	
		CD105+ / CD73-	0.06±0.02		HLA-DR+ / CD90-	0.00±0.00		HLA-DR+ / CD34-	0.05±0.02	
		CD105- / CD73-	0.16±0.02		HLA-DR- / CD90-	0.00±0.00		HLA-DR- / CD34-	92.87±2.28	
Exp (n=4)	2	CD105- / CD73+	0.00±0.00		HLA-DR- / CD90+	99.37±0.06	→	HLA-DR- / CD34+	1.14±0.03	98±0.09%
		CD105+ / CD73+	100.00±0.00	→	HLA-DR+ / CD90+	0.61±0.05		HLA-DR+ / CD34+	0.01±0.00	
		CD105+ / CD73-	0.00±0.00		HLA-DR+ / CD90-	0.00±0.00		HLA-DR+ / CD34-	0.19±0.03	
		CD105- / CD73-	0.02±0.01		HLA-DR- / CD90-	0.00±0.00		HLA-DR- / CD34-	98.63±0.02	
Exp (n=4)	3	CD105- / CD73+	0.01±0.00		HLA-DR- / CD90+	97.37±0.12	→	HLA-DR- / CD34+	5.46±2.20	91.90±2.40%
		CD105+ / CD73+	99.80±0.16	→	HLA-DR+ / CD90+	2.60±0.13		HLA-DR+ / CD34+	0.00±0.00	
		CD105+ / CD73-	0.21±0.14		HLA-DR+ / CD90-	0.00±0.00		HLA-DR+ / CD34-	0.00±0.00	
		CD105- / CD73-	0.01±0.01		HLA-DR- / CD90-	0.02±0.01		HLA-DR- / CD34-	94.57±2.20	

		CD105- / CD73+	0.05±0.00		HLA-DR- / CD90+	99.10±0.05	→	HLA-DR- / CD34+	0.00±0.00	
Exp (n=4)	4	CD105+ / CD73+	99.90±0.00	→	HLA-DR+ / CD90+	0.91±0.05		HLA-DR+ / CD34+	0.00±0.00	96.72±0.68%
		CD105+ / CD73-	0.02±0.00		HLA-DR+ / CD90-	0.00±0.00		HLA-DR+ / CD34-	2.31±0.64	
		CD105- / CD73-	0.04±0.01		HLA-DR- / CD90-	0.00±0.00		HLA-DR- / CD34-	97.70±0.65	