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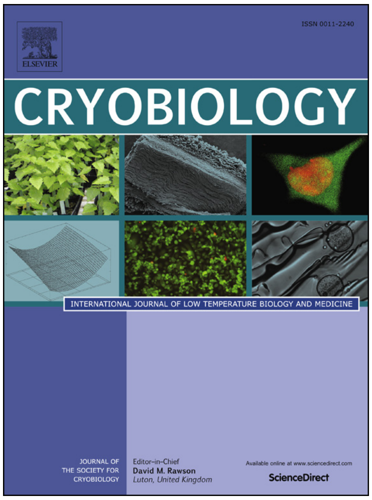

Brief communication

# The effects of cryopreservation on cells isolated from adipose, bone marrow and dental pulp tissues

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# THE EFFECTS OF CRYOPRESERVATION ON CELLS ISOLATED FROM ADIPOSE, BONE MARROW AND DENTAL PULP TISSUES

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**Keywords:**

MSC, Stem Cell, Adipose, Bone Marrow, Dental Pulp, Cryo-preservation, Liquid Nitrogen, Flow Cytometry

**Abbreviations:**

DMSO, dimethyl sulfoxide; ADSC, adipose-derived stem cell; BMSC, bone marrow stem cell; DPSC, dental pulp stem cell; MSC, mesenchymal stem cell; FACS, fluorescence-activated cell sorting; sqRT-PCT, semi-quantitative reverse transcriptase polymerase chain reaction; PDT, population doubling time

## ABSTRACT

The effects of cryopreservation on mesenchymal stem cell (MSC) phenotype are not well documented; however this process is of increasing importance for regenerative therapies. This study examined the effect of cryopreservation (10% dimethyl-sulfoxide) on the morphology, viability, gene-expression and relative proportion of MSC surface-markers on cells derived from rat adipose, bone marrow and dental pulp. Cryopreservation significantly reduced the number of viable cells in bone marrow and dental pulp cell populations but had no observable effect on adipose cells. Flow cytometry analysis demonstrated significant increases in the relative expression of MSC surface-markers, CD90 and CD29/CD90 following cryopreservation. sqRT-PCR analysis of MSC gene-expression demonstrated increases in pluripotent markers for adipose and dental pulp, together with significant tissue-specific increases in CD44, CD73 and CD105 following cryopreservation. Cells isolated from different tissue sources did not respond equally to cryopreservation with adipose tissue representing a more robust source of MSCs.

## INTRODUCTION

Non-hematopoietic stem cells have been described in many tissues and were originally termed fibroblastic colony-forming-units because they readily adhered to culture dishes and formed colonies of fibroblast-like cells [2, 20-23]. These cells are currently referred to as mesenchymal stem cells (MSCs) [1] because of their ability to differentiate into a variety of mesenchymal cell types such as adipose tissue, cartilage and bone [20-23]. MSCs were first identified in the bone marrow but have since been isolated from almost every tissue in the body [1, 8, 28]. Dental stem cells isolated from the gelatinous pulp region of the tooth were identified by Gronthos *et al* (2000), and have been employed for a wide range of regenerative therapies, most prominently neuronal and orthopaedic therapies [8, 13, 26]. Of the large number of tissues from which MSCs have been isolated adipose tissue is perhaps the most clinically useful, since this tissue contains relatively large numbers of stem cells (≤10%) when compared with the bone marrow (0.001-0.1%) that can be isolated in large volumes, relatively non-invasively with minimal patient discomfort [25, 28].

The capacity of mesenchymal stem cells (MSCs) to survive long-term storage and maintain their phenotype upon thawing is critical if they are to be banked and used for future therapeutic purposes. Reductions in cell viability and alterations in the expression of gene and phenotypic cell surface markers may have implications for the therapeutic application of MSC, including reduced functional and differentiation capacity. Cryostorage represents a physical insult to cells resulting in structural and molecular changes within cells. To protect cells from damage during the cryopreservation process and to maximise cell recovery cryoprotectants are incorporated within the freezing medium. The concentration of cryoprotectants added to the medium is one of the primary factors governing the survival of frozen cells. The majority of published cryopreservation protocols incorporate 10% DMSO in order to prevent the formation of intra- and extra-cellular crystals during the freezing process [4, 24]. However, recent studies have reported that the survival and number of colonies formed by MSCs is significantly decreased following cryostorage and that the magnitude of this decrease is inversely proportional to DMSO concentration [19]. Moreover, the use of DMSO as a cryoprotectant has been shown to be ineffective at protecting some cell types from cold shock (0 - +8°C) during the freezing process [11], highlighting the need for further investigation into the effects of cryostorage using 10% DMSO on MSC viability and self-renewal.

It is presently unknown whether post-thaw MSCs retain the same potential for regenerative therapeutic applications as their non-cryopreserved counterparts. The response of stem cells to cryopreservation can include a reduction in cell viability due to cold-shock and/or the toxic effects of DMSO, and changes in the expression of stem cell-related markers, cytoskeletal disassembly, delayed apoptosis, and osmotic and oxidative stresses [27]. These factors may have an influence on the functionality of MSCs and reduce their applicability for regenerative therapies. It has subsequently been suggested that the effects of cryopreservation may be responsible for the failure of a randomised phase III clinical trial using random donor MSCs in the treatment of steroid resistant graft-versus-host disease (NCT00366145) [7]. At present, much of the information concerning the effects of cryopreservation on MSCs is difficult to interpret as studies frequently isolate MSCs from different tissue sources and store them for variable periods of time [17, 22].

In the present study, we examined the effect of 14 days cryostorage on the viability, morphology, gene expression and immunophenotype of adipose-derived cells (ADCs), bone marrow cells (BMCs) and dental pulp cells (DPCs) derived from rats. The rat was used as a model since MSCs isolated from this species have a very similar cell-surface marker profile and differentiation potential when compared with human MSCs [9]. The use of a rat model also enabled the comparison of cells isolated from several tissues within the same animal, thereby limiting potential error caused by intra-species variation. A traditional graded freezing protocol (4°C, 1 hour; -20°C, 2 hours; -80°C, overnight; -136°C, 14 days) was adopted throughout this study since previous work has demonstrated that this method is no less effective in maintaining post-thaw viability of MSCs compared with controlled freezing, with consistent nucleation observed [18]. This work was undertaken to determine if cryopreservation with 10% DMSO affected the viability and the capacity of ADCs, BMCs and DPCs to survive fluorescence-activated cell sorting (FAC), which is arguably the most routinely used procedure for MSC isolation. The effect of cryopreservation on the cell-surface marker profile of MSCs was analysed using FACS to assess whether cryopreservation influenced the proportion of MSCs within heterogeneous cultures. Additionally, the expression profiles of pluripotent/multipotent genes between cryopreserved and non-cryopreserved cells were compared, as changes in these transcripts may alter their functionality and differentiation potential, and may therefore limit the clinical potential of these cells.

## METHODS

### Cell culture

Adipose, bone marrow and dental pulp tissues were isolated from six week old male Wister Hann rats (weight ~120g) (Aston University, Pharmaceutical Sciences Animal House, Birmingham, UK; ethical approval reference: BCHDent286.1471.TB). Cells were isolated from each tissue using a standard protocol [6].

### Cryopreservation of cell isolates

To prepare cells for cryostorage, ~80% confluent passage 1 cultures containing approximately 2.5 x $10^6$ cells per $75cm^2$ culture flask (Nunc, UK) were detached using 0.25% trypsin, 1mM $EDTA^{.}4Na$ (2.5g/L trypsin in 0.38g/L EDTA) (Gibco, UK), and centrifuged at 900g for a period of 5 minutes. The supernatant was aspirated and an equal volume of cells re-suspended in 0.4% Trypan blue solution to provide a cell viability count. 1 x $10^6$ cells were re-suspended in cryogenic medium [90% FBS containing 10 % dimethyl sulfoxide (DMSO)] [15] and the cell suspensions transferred to cryogenic vials that were prepared for liquid nitrogen storage by incubation at 4ºC for 1 hour, then at -20ºC for 2 hours and subsequently -80ºC overnight. Frozen cell suspensions were then transferred to liquid nitrogen storage. 10 vials of cryostored cells were recovered from storage by thawing in a 37ºC RS Galaxy S+ incubator (RS Biotech, UK) for ~5 minutes. To remove residual cryogenic medium prior to culture, the contents of each vial were transferred to 15mL Falcon® tubes containing 5mL α-MEM + 10% FBS and centrifuged at 900g for 5 minutes. Cell viability was measured immediately after thawing using the Trypan blue exclusion assay and approximately 2 x $10^5$ cells seeded in $25cm^2$ culture flasks (Nunc, UK). Cells were cultured to approximately 80% confluence, at which point Trypan blue cell counts were performed and population doubling times (PDT) calculated using the following equation:

$$PDT = T \ln2/\ln(Xe/Xb)$$

T = incubation time, Xb = cell number at the beginning of incubation, Xe = cell number at the end of incubation

### Cell viability during flow cytometry

To examine the capacity of cryostored cells to undergo flow cytometric cell sorting, cryostored and non-cryostored cells established at passage 2 were cultured until ~80% confluent (80% confluency was reached after 5 days for ADCs and BMCs, and after 7 days for DPCs). Cells were detached using 0.25% trypsin, 1mM $EDTA^{.}4Na$ (2.5g/L trypsin in 0.38g/L EDTA) (Gibco, UK), centrifuged at 900g for 5 minutes, neutralised with α-MEM + 10% FBS and the resulting suspensions transferred to 15mL Falcon® tubes. Cell suspensions were incubated in FACS buffer (sterile PBS + 1% FBS) and maintained under constant agitation using an orbital shaker, mimicking conditions experienced during flow cytometry. The number of viable cells was determined every 30 minutes during this incubation period at 4ºC for a total period of 5 hours by adding 0.4% Trypan blue solution to an equal volume of cell suspension and manually counting the cells using an improved Neubauer haemocytometer.

**Cell characterisation**

Cryostored and non-cryostored passage 2 ADCs, BMCs and DPCs were expanded *in vitro*, and after reaching ~80% confluence were analysed using fluorescence activated cell sorting (FACS). All analyses were performed on the same day. The presence of MSC surface antigens was determined using a FACSAriaII flow cytometer (BD Pharmingen, UK). MSC surface antigens CD29-APC (eBiosciences, 17-0291) and CD90-FITC (eBiosciences, 11-0900) were analysed using flow cytometry as previously described [5]. Semi-quantitative reverse transcription PCR (sqRT-PCR) was used to assess the expression of CD44, CD73 and CD105 in cryopreserved and non-cryopreserved cell cultures. For sqRT-PCR analysis ~80% confluent ADC, BMC and DPC cultures were isolated at passage 2 and compared with cryopreserved cells that had been cultured to ~80% confluency at the same passage. RNA was isolated using an RNeasy minikit (Qiagen, UK). RNA was reverse transcribed using an Omniscript RT kit (Qiagen, UK) according to the manufacturer’s instructions. The housekeeping gene GAPDH was used for normalisation. Primers details are listed in Appendix table 1.

**Statistical analysis**

All data was analysed using the statistical package SPSS 10.0 for Windows (SPSS Inc., USA). Statistical analyses were performed using one way analysis of variance (ANOVA), followed by the Bonferroni *post hoc*, $P<0.05$ was considered to indicate statistical significance. For all experiments $n$ indicates the number of experiments performed, with each experiment containing a total of three replicates.

## RESULTS AND DISCUSSION

Following cryopreservation recovered ADCs, BMCs and DPCs exhibited a fibroblast-like morphology (Fig. 1a). However, it cannot be ruled out that ultrastructural changes occurred, as was recently shown by James *et al* (2011) who described changes in ADC morphology following cryopreservation [10]. ADCs, BMCs and DPCs harvested at passage 1 all exhibited >95% viability before being place in cryostorage, which correlated with a previous study [3] (Fig. 1b). Following cryostorage the proportion of viable cells was not statistically (P<0.05) lower than when harvested, >90% for each cell type (Fig. 1b). The effects of *in vitro* expansion following cryopreservation on cell viability was found to be cell-type specific, with average numbers of viable cells in

ADC, BMC and DPC populations of 94%, 57% and 89% respectively immediately following post-thaw culture (Fig. 1c, 1d and 1e). The fact that significant ($P<0.05$) reductions in cell viability only became apparent following expansion and not immediately after thawing corroborates the findings of Naaldijk *et al* (2012), but demonstrates that the results were only significant ($P<0.05$) for BMCs [18]. Reductions in BMC viability following cryostorage may be related to the fact that bone marrow contains a relatively low proportion of stem cells (0.001-0.1%) when compared with adipose tissue (≤10%) and dental pulp (≤1%) [8, 24, 27]. Therefore, it is possible that the reduction in the viability of cells in the BMC population corresponded to nucleated non-progenitor cells, since these cells have been shown to have a comparatively high sensitivity to cryostorage when compared with MSCs [14]. Such non-progenitor cells may comprise MSCs that have undergone differentiation or other heterogeneous tissue components that not been successfully eliminated during cell culture. Suspension of the cells in FACS buffer, as would be required if the cells were to be used for cell sorting during flow cytometry resulted in a further and significant ($P<0.05$) reduction in cell viability for both BMCs and DPCs (Fig. 1d and e), but had no significant effect on ADCs (Fig. 1c). The reduction in BMC and DPC viability when maintained in a low nutrient FACS buffer may be a lasting result of cell damage occurring due to hyperosmotic stress, differences in the concentrations of intracellular salts, membrane alterations or the toxic effects of DMSO during the freezing process [16]. It is unlikely that these findings are a result of time spent in culture since both BMCs and ADCs took approximately the same time to reach ~80% confluence (5 days) with similar population doubling times observed (ADCs, 51.68hrs; BMCs, 53.85hrs), while DPCs took comparatively longer to reach confluence (7 days, with a PDT of 72.35hrs) but maintained a higher proportion of viable cells than BMCs following post-thaw expansion, and significantly ($P<0.05$) higher viability than BMCs when maintained in FACS buffer. The data collected in this study may support the hypothesis that adipose tissue contains a more robust source of MSCs that are able to survive out of culture for longer periods than MSCs isolated from bone marrow or dental pulp tissues [12]. These findings are interesting given that adipose tissue has recently been shown to contain a population of multi-lineage differentiating stress-enduring (Muse) cells that are able to endure extreme stresses such as hypoxia, serum deprivation, long term exposure to proteolytic enzymes such as collagenase, and low temperatures [12]. Similar cells with the ability to survive extreme stress within bone marrow and dental pulp tissues have not yet been described. The presence of a more robust source of MSCs in adipose tissue may have significant implications when selecting an appropriate stem cell source for regenerative therapies.

It is important to examine the effect of cryopreservation on MSC gene expression to provide an insight into molecular changes that occur following cell freezing, and may shed light on the self-renewal and differentiation capacity of cells following banking. This study presents novel data demonstrating the influence of cryostorage on the expression of MSC-associated markers in ADC, BMC and DPC populations, evaluated using sqRT-PCR gene expression analysis and FACS profiling. These analyses showed relatively high levels of expression of MSC-associated markers CD73, CD90 and CD105 for ADC cultures, CD44 and CD105 for BMC cultures, and CD73 and CD44 for DPC cultures following cryopreservation (Fig. 2 and 3). Genes associated with stem cell maintenance and pluripotency such as Klf4, Lin28 and Nanog were also increased following cryopreservation of ADC and DPC cultures (Fig. 3). Interestingly, in this study a reduction in cell viability following cryopreservation of DPCs and ADCs coincided with a proportional increase in the number of MSC markers, potentially implying positive MSC selection during cryostorage. The finding that the expression of pluripotent markers in BMC cultures was not altered following cryopreservation and subsequent culture, coupled with the appreciable reduction in the overall viability of BMC cultures may indicate that the population of stem cells present within bone marrow isolates decreased following cryopreservation, and that this reduced viability was not solely due to the loss of non-progenitor cells [14]. These results suggest that MSCs present within the bone marrow may be more susceptible to hyperosmotic damage resulting from cryopreservation. Additionally, ADCs and DPCs showed increases in the expression of transcription factors first identified with the maintenance of multi-potency and self- renewal in embryonic stem cells, such as Nanog, Lin28 and Klf4. These results indicate that cryopreservation may alter the expression profile of genes associated with maintaining multipotency, thereby having an effect on the relative "stemness" of heterogeneous adipose and dental pulp cultures.

This study has shown that cryopreservation in a routinely used cryopreservation medium (10% DMSO) led to reduced cell viability and an altered FACS profile, which has implications when comparing freshly isolated and cryopreserved cells using flow cytometry, and may influence the clinical translatability of cryopreserved cells. Post-thaw viabilities of cells isolated from different tissue sources differed significantly, with adipose tissue containing a more robust stem cell source than dental pulp and bone marrow. Additionally, cryopreservation increased the proportion of MSC surface markers in ADC, BMC and DPC cultures, and genes associated with self-renewal and multipotency in ADC and DPC cultures, which is potentially indicative of a decline in heterogeneity and subsequent increase in the relative proportion of MSCs after thawing. These data indicate that cells isolated from different anatomical locations do not respond equally to cryopreservation and that adipose tissue may be a more viable source MSCs for cell banking. Further studies will need to be conducted using human cells to corroborate these findings.

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**Conflict of interest** The authors have no conflicts of interest.

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## FIGURE LEGENDS

**Figure 1.** (a) Representative phase contrast photomicrographs comparing passage 2 ADSCs, BMSCs and DPSCs that had been cryopreserved (cryo) in FBS + 10% DMSO for 14 days with cells of the same passage that had not experienced cryopreservation. (b) Viability was measured before cells were placed into cryostorage and immediately after thawing. Following cryostorage cells were thawed and cultured until ~80% confluent. Following the *in vitro* expansion of cryostored and non-cryostored cells at passage 2, percentage viability was measured for (c) ADSCs, (d) BMSCs and (e) DPSCs while maintained in FACS buffer over a period of 270 minutes to mimic conditions experience during cell selection (mean ±SD, n=10). $^*P<0.05$. Trypan blue cell counts performed every 30 minutes to determine proportional cell viability.

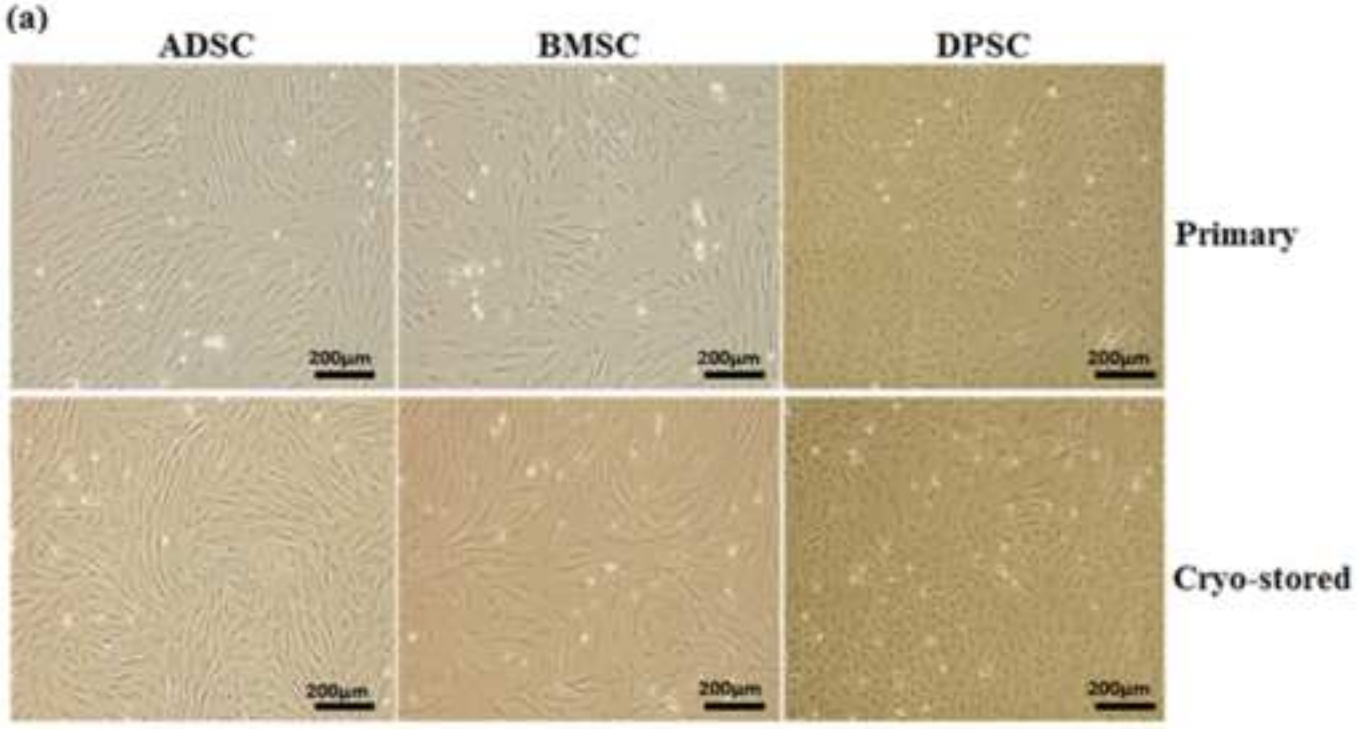
**Figure 2.** (a) Representative flow cytometry profiles for cryopreserved and non-cryopreserved ADSC, BMSC and DPSC populations established at passage 2. Y-axis represents the percentage of positive cells within a manually defined area that was selected to exclude dead/clumped cells. (b) Analysis of the proportion of CD29, CD90 and CD29/CD90 cell surface antigens presented by cryopreserved (cryo) and non-cryopreserved cells derived from (i) adipose, (ii) bone marrow and (iii) dental pulp established at passage 2 (mean ±SD, n=10). $^*P<0.05$

**Figure 3.** Comparative sqRT-PCR analysis of (a) pluripotency and (b) multipotency markers on cryopreserved and non-cryopreserved cells derived from adipose (AD), bone marrow (BM) and dental pulp (DP) established at passage 2. All cells were cultured until ~80% confluent prior to RNA isolation (mean ±SD, n=10). $^*P<0.05$

## APPENDIX

**Table. 1** DNA sequences, annealing temperatures, and cycle numbers for primers used in the sqRT-PCR reaction. All primers were designed using Primer Blast software (http://ncbi.nlm.nih.gov/tools/primer-blast/) and manufactured by Invitrogen, UK.

Figure 1



(b)

| | ADC | BMC | DPC |
|---|---|---|---|
| Viability Before Cryostorage | 98±3 | 95±4 | 98±5 |
| Viability Immediately After Thawing | 96±5 | 90±8 | 94±7 |

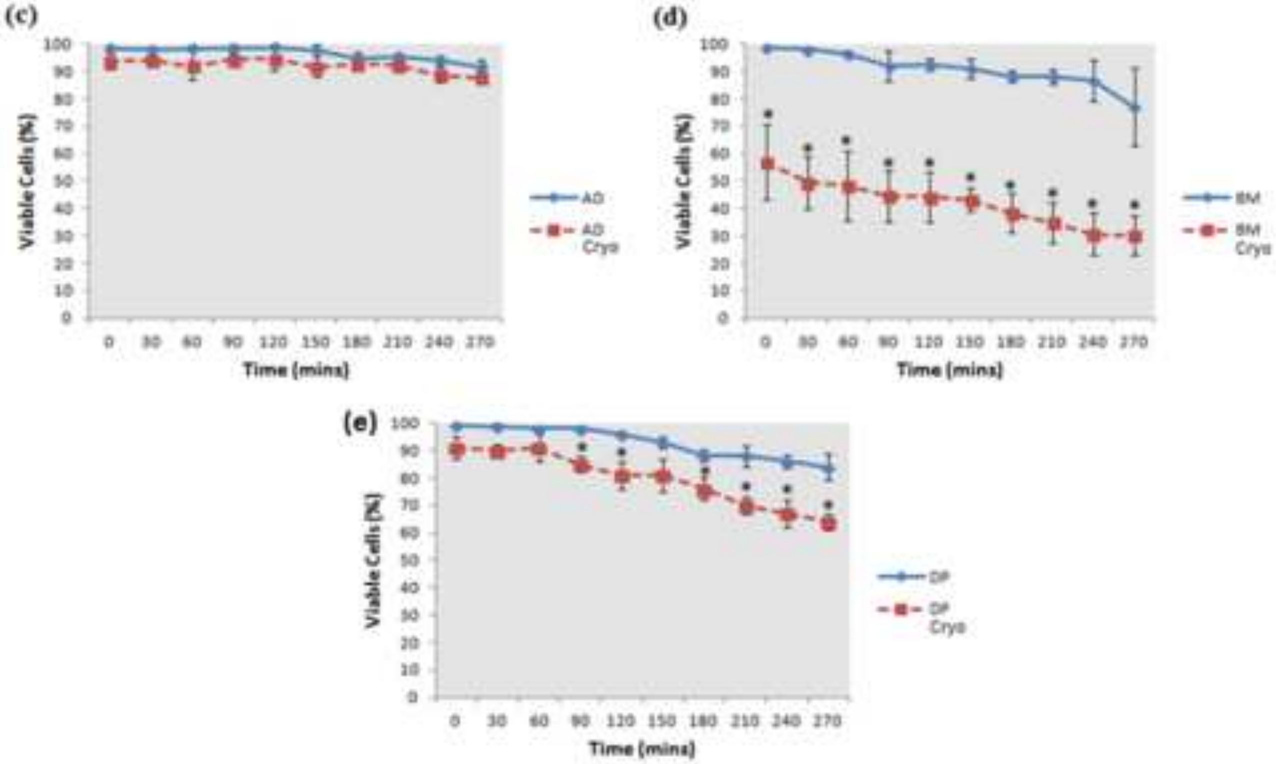


Figure 2

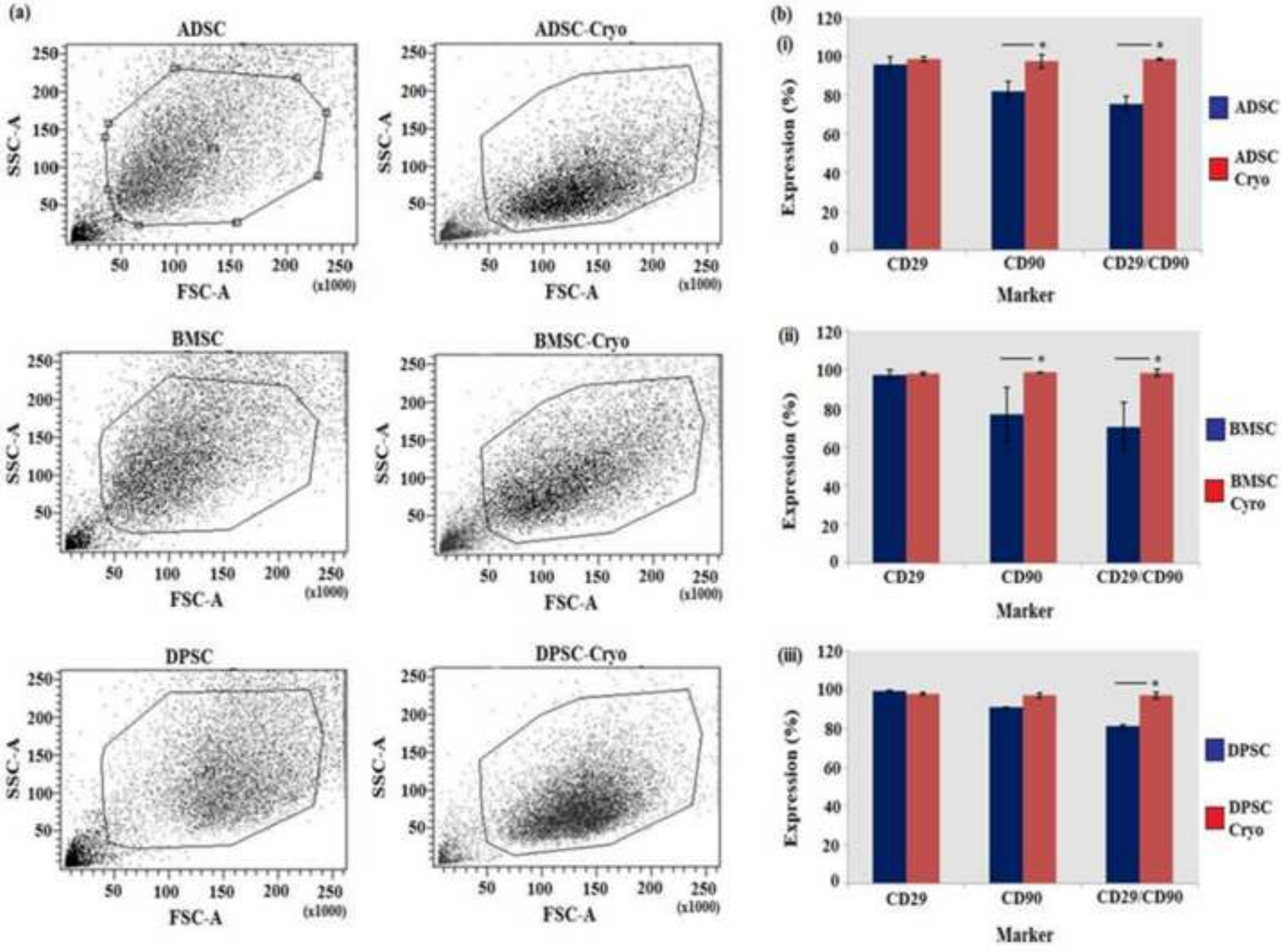
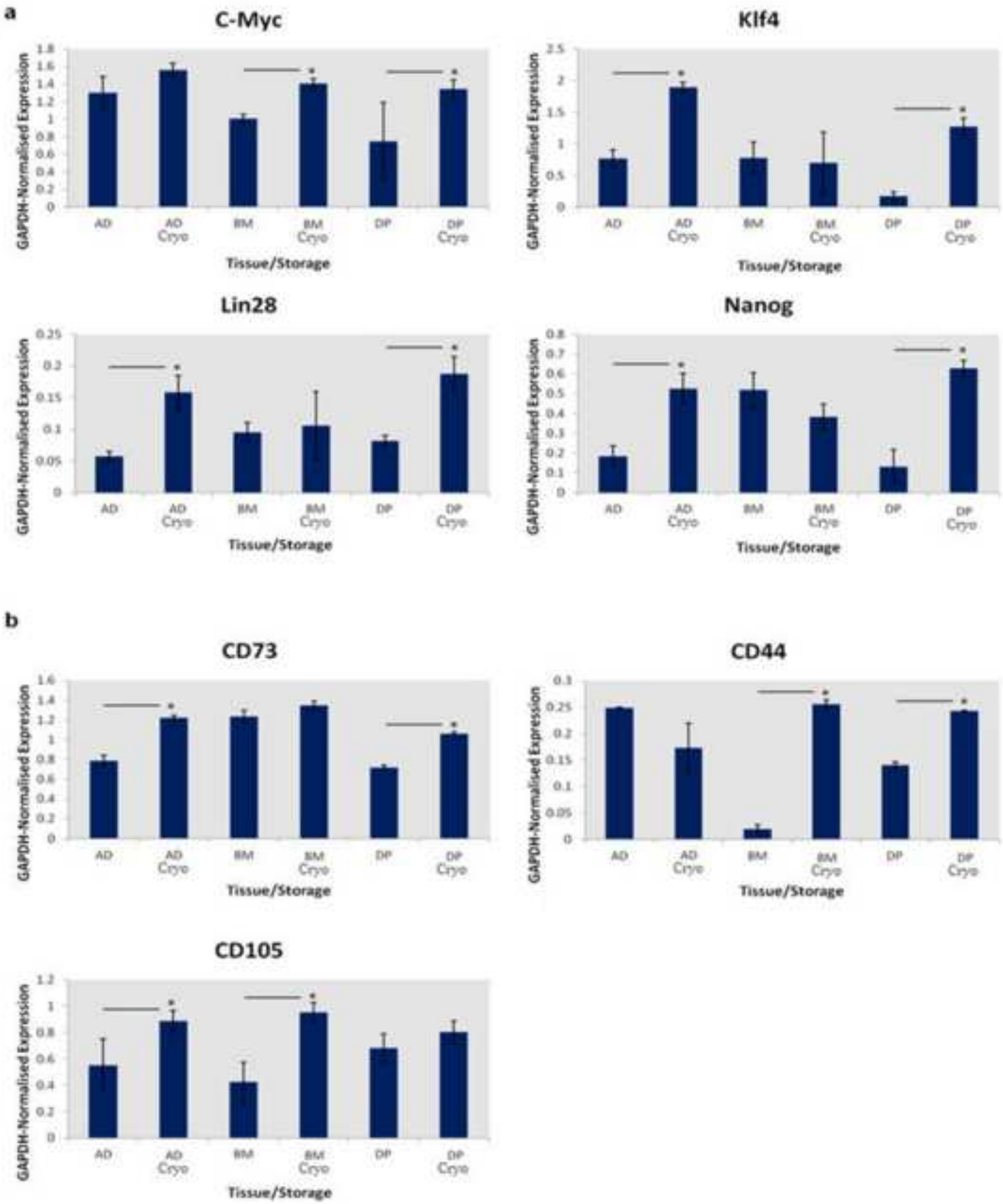

(a)
ADSC
ADSC-Cryo
BMSC
BMSC-Cryo
DPSC
DPSC-Cryo
SSC-A
FSC-A
50
100
150
200
250
(x1000)
P4
(b)
(i)
(ii)
(iii)
Expression (%)
0
20
40
60
80
100
120
CD29
CD90
CD29/CD90
Marker
*
ADSC
ADSC Cryo
BMSC
BMSC Cyro
DPSC
DPSC Cryo

Figure 3


a
C-Myc
GAPDH-Normalised Expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*
Klf4
GAPDH-Normalised Expression
0
0.5
1
1.5
2
2.5
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*
Lin28
GAPDH-Normalised Expression
0
0.05
0.1
0.15
0.2
0.25
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*
Nanog
GAPDH-Normalised Expression
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*
b
CD73
GAPDH-Normalised Expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*
CD44
GAPDH-Normalised Expression
0
0.05
0.1
0.15
0.2
0.25
0.3
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*
CD105
GAPDH-Normalised Expression
0
0.2
0.4
0.6
0.8
1
1.2
AD
AD Cryo
BM
BM Cryo
DP
DP Cryo
Tissue/Storage
*
*

| Gene Name | Sequence (5’ → 3’) | Annealing Temperature (°C) | Cycle Number | Accession Number |
|---|---|---|---|---|
| **Normalisation** | | | | |
| GAPDH | F-CCCATCACCATCTTCCAGGAGC;<br>R-CCAGTGAGCTTCCCGTTCAGC | 60.5 | 21-27 | NM_017008 |
| **Pluripotent Markers** | | | | |
| Klf4 | F-ATCATGGTCAAGTTCCCAGC;<br>R-ACCAAGGACCATCGTTTAGG | 60.5 | 35 | NM_052713 |
| C-myc | F-CTTACTGAGGAAACGGCGAG;<br>R-GCCCTATGTACACCGGAAGA | 60.5 | 35 | BC091699 |
| Nanog | F-TATCGTTTTGAGGGGTGAGG;<br>R-CAGCTGGCACTGGTTTATCA | 60.5 | 35 | NM_001100781 |
| Lin28 | F-TTTCTTGTTTCCCCCAAATG;<br>R-AGAGGGGCTGGTTGTAAGGT | 60.5 | 35 | NM_001109269 |
| SOX-2 | F-ATACAAGGGAATTGGGAGGG;<br>R-AAACCCAGCAAGAACCCTTT | 60.5 | 25 | NM_001109181 |
| **Multipotent Markers** | | | | |
| CD44 | F-TGGGTTTACCCAGCTGAATC;<br>R-CTTGCGAAAGCATCAACAAA | 60.5 | 33-37 | NM_012924.02 |
| CD105 | F-TTCAGCTTTCTCCTCCGTGT;<br>R-TGTGGTTGGTACTGCTGCTC | 60.5 | 41-45 | NM_001010968 |
| CD73 | F-GGACTGATTGATCCCCTCCT;<br>R-TTGTCCCTGGATTTGAGAGG | 60.5 | 25 | NM_002526 |
| CD29 | F-AATGGAGTGAATGGGACAGG;<br>R-TCTGTGAAGCCCAGAGGTTT | 60.5 | 25 | NM_017022.2 |
| CD90 | F-AGCTCTTTGATCTGCCGTGT;<br>R-CTGCAGGCAATCCAATTTTT | 60.5 | 26 | NM_012673 |