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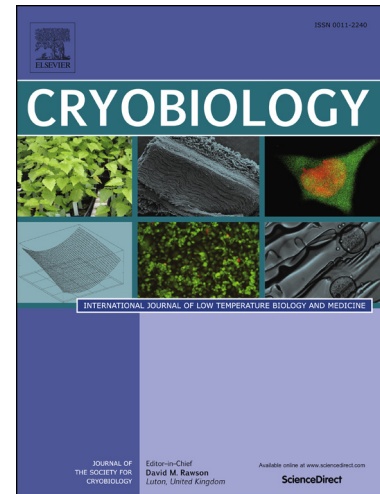
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THE EFFECTS OF CRYOPRESERVATION ON CELLS ISOLATED FROM ADIPOSE,
BONE MARROW AND DENTAL PULP TISSUES

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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

23 **ABSTRACT**

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

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47 **INTRODUCTION**

48 Non-hematopoietic stem cells have been described in many tissues and were originally termed fibroblastic
49 colony-forming-units because they readily adhered to culture dishes and formed colonies of fibroblast-like cells
50 [2, 20-23]. These cells are currently referred to as mesenchymal stem cells (MSCs) [1] because of their ability to
51 differentiate into a variety of mesenchymal cell types such as adipose tissue, cartilage and bone [20-23]. MSCs
52 were first identified in the bone marrow but have since been isolated from almost every tissue in the body [1, 8,
53 28]. Dental stem cells isolated from the gelatinous pulp region of the tooth were identified by Gronthos *et al*
54 (2000), and have been employed for a wide range of regenerative therapies, most prominently neuronal and
55 orthopaedic therapies [8, 13, 26]. Of the large number of tissues from which MSCs have been isolated adipose
56 tissue is perhaps the most clinically useful, since this tissue contains relatively large numbers of stem cells
57 ($\leq 10\%$) when compared with the bone marrow (0.001-0.1%) that can be isolated in large volumes, relatively
58 non-invasively with minimal patient discomfort [25, 28].

59
60 The capacity of mesenchymal stem cells (MSCs) to survive long-term storage and maintain their phenotype
61 upon thawing is critical if they are to be banked and used for future therapeutic purposes. Reductions in cell
62 viability and alterations in the expression of gene and phenotypic cell surface markers may have implications for
63 the therapeutic application of MSC, including reduced functional and differentiation capacity. Cryostorage
64 represents a physical insult to cells resulting in structural and molecular changes within cells. To protect cells
65 from damage during the cryopreservation process and to maximise cell recovery cryoprotectants are
66 incorporated within the freezing medium. The concentration of cryoprotectants added to the medium is one of
67 the primary factors governing the survival of frozen cells. The majority of published cryopreservation protocols
68 incorporate 10% DMSO in order to prevent the formation of intra- and extra-cellular crystals during the freezing
69 process [4, 24]. However, recent studies have reported that the survival and number of colonies formed by
70 MSCs is significantly decreased following cryostorage and that the magnitude of this decrease is inversely
71 proportional to DMSO concentration [19]. Moreover, the use of DMSO as a cryoprotectant has been shown to
72 be ineffective at protecting some cell types from cold shock (0 - +8°C) during the freezing process [11],
73 highlighting the need for further investigation into the effects of cryostorage using 10% DMSO on MSC
74 viability and self-renewal.

75 It is presently unknown whether post-thaw MSCs retain the same potential for regenerative therapeutic
76 applications as their non-cryopreserved counterparts. The response of stem cells to cryopreservation can include
77 a reduction in cell viability due to cold-shock and/or the toxic effects of DMSO, and changes in the expression
78 of stem cell-related markers, cytoskeletal disassembly, delayed apoptosis, and osmotic and oxidative stresses
79 [27]. These factors may have an influence on the functionality of MSCs and reduce their applicability for
80 regenerative therapies. It has subsequently been suggested that the effects of cryopreservation may be
81 responsible for the failure of a randomised phase III clinical trial using random donor MSCs in the treatment of
82 steroid resistant graft-versus-host disease (NCT00366145) [7]. At present, much of the information concerning
83 the effects of cryopreservation on MSCs is difficult to interpret as studies frequently isolate MSCs from
84 different tissue sources and store them for variable periods of time [17, 22].

85 In the present study, we examined the effect of 14 days cryostorage on the viability, morphology, gene
86 expression and immunophenotype of adipose-derived cells (ADCs), bone marrow cells (BMCs) and dental pulp
87 cells (DPCs) derived from rats. The rat was used as a model since MSCs isolated from this species have a very
88 similar cell-surface marker profile and differentiation potential when compared with human MSCs [9]. The use
89 of a rat model also enabled the comparison of cells isolated from several tissues within the same animal, thereby
90 limiting potential error caused by intra-species variation. A traditional graded freezing protocol (4°C, 1 hour; -
91 20°C, 2 hours; -80°C, overnight; -136°C, 14 days) was adopted throughout this study since previous work has
92 demonstrated that this method is no less effective in maintaining post-thaw viability of MSCs compared with
93 controlled freezing, with consistent nucleation observed [18]. This work was undertaken to determine if
94 cryopreservation with 10% DMSO affected the viability and the capacity of ADCs, BMCs and DPCs to survive
95 fluorescence-activated cell sorting (FACS), which is arguably the most routinely used procedure for MSC
96 isolation. The effect of cryopreservation on the cell-surface marker profile of MSCs was analysed using FACS
97 to assess whether cryopreservation influenced the proportion of MSCs within heterogeneous cultures.
98 Additionally, the expression profiles of pluripotent/multipotent genes between cryopreserved and non-
99 cryopreserved cells were compared, as changes in these transcripts may alter their functionality and
100 differentiation potential, and may therefore limit the clinical potential of these cells.

101 **METHODS**

102 **Cell culture**

103 Adipose, bone marrow and dental pulp tissues were isolated from six week old male Wister Hann rats (weight
104 ~120g) (Aston University, Pharmaceutical Sciences Animal House, Birmingham, UK; ethical approval
105 reference: BCHDent286.1471.TB). Cells were isolated from each tissue using a standard protocol [6].

106 **Cryopreservation of cell isolates**

107 To prepare cells for cryostorage, ~80% confluent passage 1 cultures containing approximately 2.5×10^6 cells per
108 75cm^2 culture flask (Nunc, UK) were detached using 0.25% trypsin, 1mM EDTA:4Na (2.5g/L trypsin in
109 0.38g/L EDTA) (Gibco, UK), and centrifuged at 900g for a period of 5 minutes. The supernatant was aspirated
110 and an equal volume of cells re-suspended in 0.4% Trypan blue solution to provide a cell viability count. $1 \times$
111 10^6 cells were re-suspended in cryogenic medium [90% FBS containing 10% dimethyl sulfoxide (DMSO)] [15]
112 and the cell suspensions transferred to cryogenic vials that were prepared for liquid nitrogen storage by
113 incubation at 4°C for 1 hour, then at -20°C for 2 hours and subsequently -80°C overnight. Frozen cell
114 suspensions were then transferred to liquid nitrogen storage. 10 vials of cryostored cells were recovered from
115 storage by thawing in a 37°C RS Galaxy S+ incubator (RS Biotech, UK) for ~5 minutes. To remove residual
116 cryogenic medium prior to culture, the contents of each vial were transferred to 15mL Falcon[®] tubes containing
117 5mL α -MEM + 10% FBS and centrifuged at 900g for 5 minutes. Cell viability was measured immediately after
118 thawing using the Trypan blue exclusion assay and approximately 2×10^5 cells seeded in 25cm^2 culture flasks
119 (Nunc, UK). Cells were cultured to approximately 80% confluence, at which point Trypan blue cell counts
120 were performed and population doubling times (PDT) calculated using the following equation:

$$121 \text{ PDT} = T \ln 2 / \ln(X_e / X_b)$$

122 T = incubation time, X_b = cell number at the beginning of incubation, X_e = cell number at the end of incubation

123 **Cell viability during flow cytometry**

124 To examine the capacity of cryostored cells to undergo flow cytometric cell sorting, cryostored and non-
125 cryostored cells established at passage 2 were cultured until ~80% confluent (80% confluency was reached after
126 5 days for ADCs and BMCs, and after 7 days for DPCs). Cells were detached using 0.25% trypsin, 1mM
127 EDTA:4Na (2.5g/L trypsin in 0.38g/L EDTA) (Gibco, UK), centrifuged at 900g for 5 minutes, neutralised with
128 α -MEM + 10% FBS and the resulting suspensions transferred to 15mL Falcon[®] tubes. Cell suspensions were
129 incubated in FACS buffer (sterile PBS + 1% FBS) and maintained under constant agitation using an orbital
130 shaker, mimicking conditions experienced during flow cytometry. The number of viable cells was determined

131 every 30 minutes during this incubation period at 4°C for a total period of 5 hours by adding 0.4% Trypan blue
132 solution to an equal volume of cell suspension and manually counting the cells using an improved Neubauer
133 haemocytometer.

134 **Cell characterisation**

135 Cryostored and non-cryostored passage 2 ADCs, BMCs and DPCs were expanded *in vitro*, and after reaching
136 ~80% confluence were analysed using fluorescence activated cell sorting (FACS). All analyses were performed
137 on the same day. The presence of MSC surface antigens was determined using a FACSARIAII flow cytometer
138 (BD Pharmingen, UK). MSC surface antigens CD29-APC (eBiosciences, 17-0291) and CD90-FITC
139 (eBiosciences, 11-0900) were analysed using flow cytometry as previously described [5]. Semi-quantitative
140 reverse transcription PCR (sqRT-PCR) was used to assess the expression of CD44, CD73 and CD105 in
141 cryopreserved and non-cryopreserved cell cultures. For sqRT-PCR analysis ~80% confluent ADC, BMC and
142 DPC cultures were isolated at passage 2 and compared with cryopreserved cells that had been cultured to ~80%
143 confluency at the same passage. RNA was isolated using an RNeasy minikit (Qiagen, UK). RNA was reverse
144 transcribed using an Omniscript RT kit (Qiagen, UK) according to the manufacturer's instructions. The
145 housekeeping gene GAPDH was used for normalisation. Primers details are listed in Appendix table 1.

146 **Statistical analysis**

147 All data was analysed using the statistical package SPSS 10.0 for Windows (SPSS Inc., USA). Statistical
148 analyses were performed using one way analysis of variance (ANOVA), followed by the Bonferroni *post hoc*,
149 $P < 0.05$ was considered to indicate statistical significance. For all experiments n indicates the number of
150 experiments performed, with each experiment containing a total of three replicates.

151 **RESULTS AND DISCUSSION**

152 Following cryopreservation recovered ADCs, BMCs and DPCs exhibited a fibroblast-like morphology (Fig. 1a).
153 However, it cannot be ruled out that ultrastructural changes occurred, as was recently shown by James *et al*
154 (2011) who described changes in ADC morphology following cryopreservation [10]. ADCs, BMCs and DPCs
155 harvested at passage 1 all exhibited >95% viability before being placed in cryostorage, which correlated with a
156 previous study [3] (Fig. 1b). Following cryostorage the proportion of viable cells was not statistically ($P < 0.05$)
157 lower than when harvested, >90% for each cell type (Fig. 1b). The effects of *in vitro* expansion following
158 cryopreservation on cell viability was found to be cell-type specific, with average numbers of viable cells in

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

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

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

217 adipose tissue may be a more viable source MSCs for cell banking. Further studies will need to be conducted
218 using human cells to corroborate these findings.

219

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

223

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298 **FIGURE LEGENDS**

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

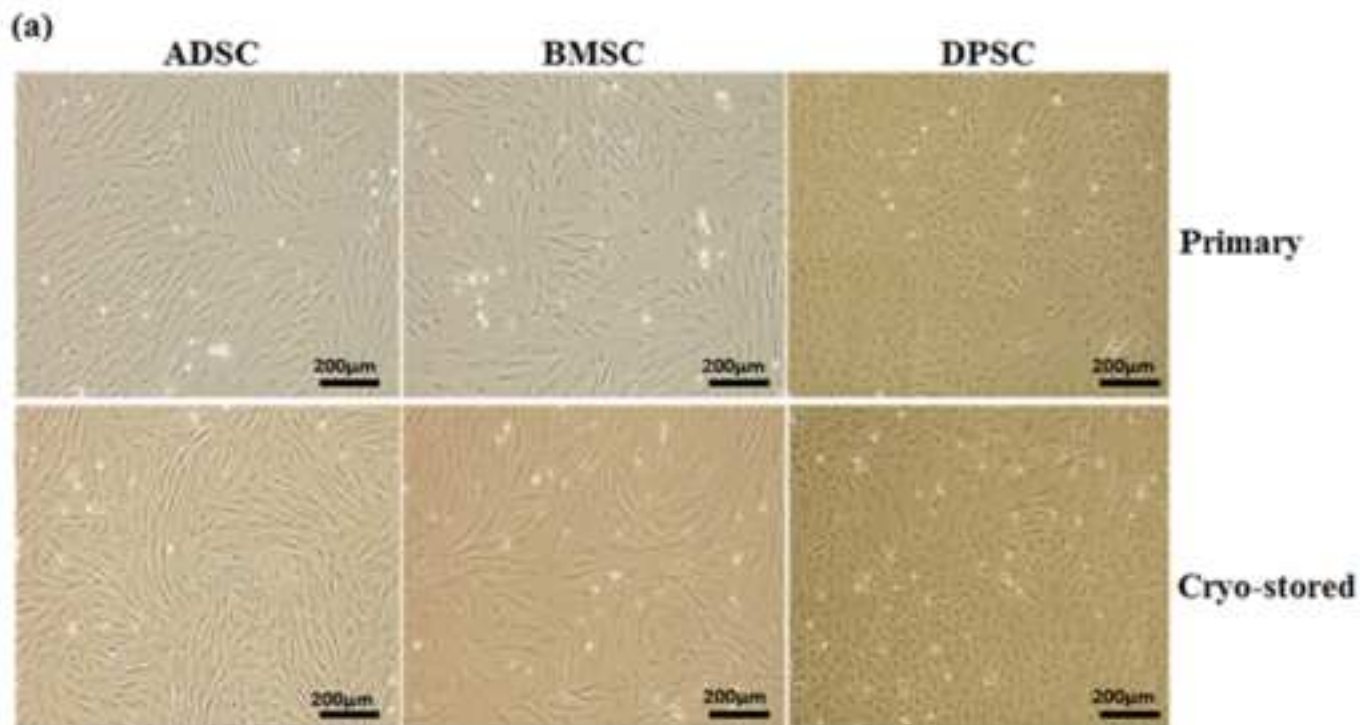
307 **Figure 2.** (a) Representative flow cytometry profiles for cryopreserved and non-cryopreserved ADSC, BMSC
308 and DPSC populations established at passage 2. Y-axis represents the percentage of positive cells within a
309 manually defined area that was selected to exclude dead/clumped cells. (b)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

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

316

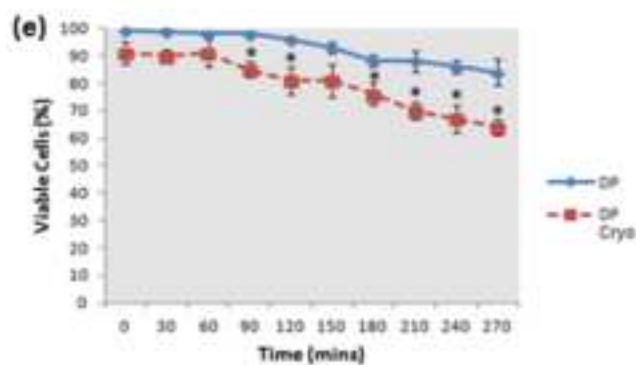
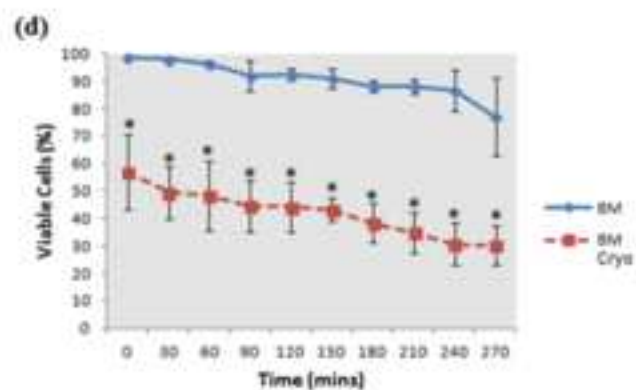
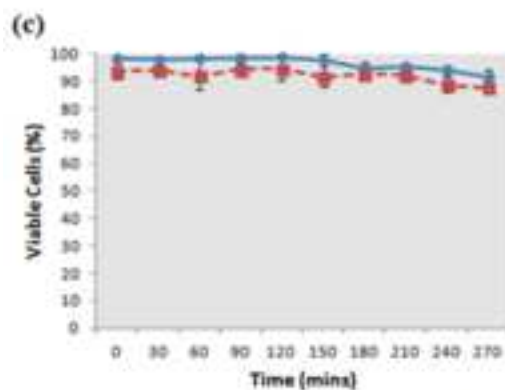
317 **APPENDIX**

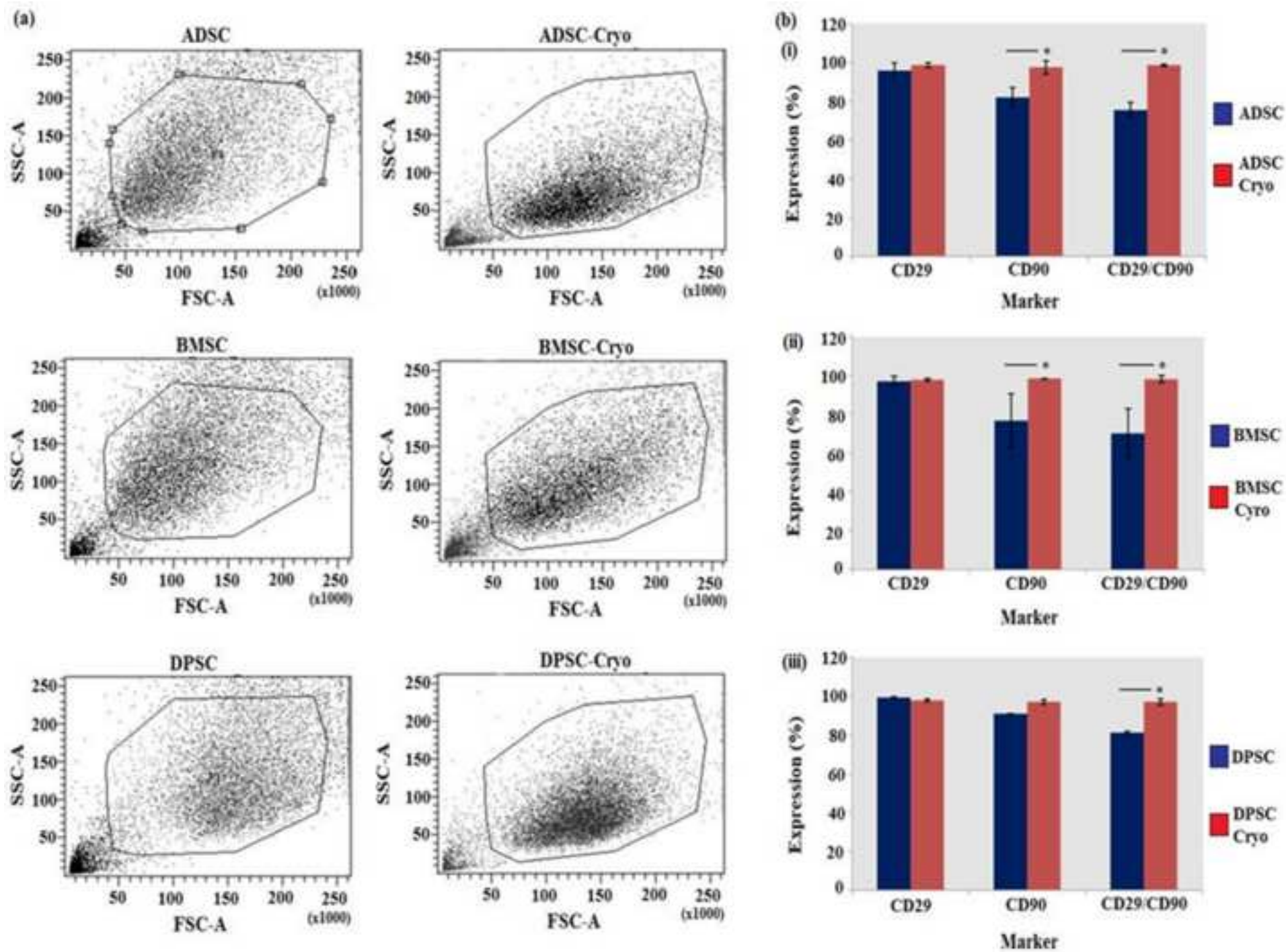
318 **Table. 1** DNA sequences, annealing temperatures, and cycle numbers for primers used in the sqRT-PCR
319 reaction. All primers were designed using Primer Blast software (<http://ncbi.nlm.nih.gov/tools/primer-blast/>)
320 and manufactured by Invitrogen, UK.

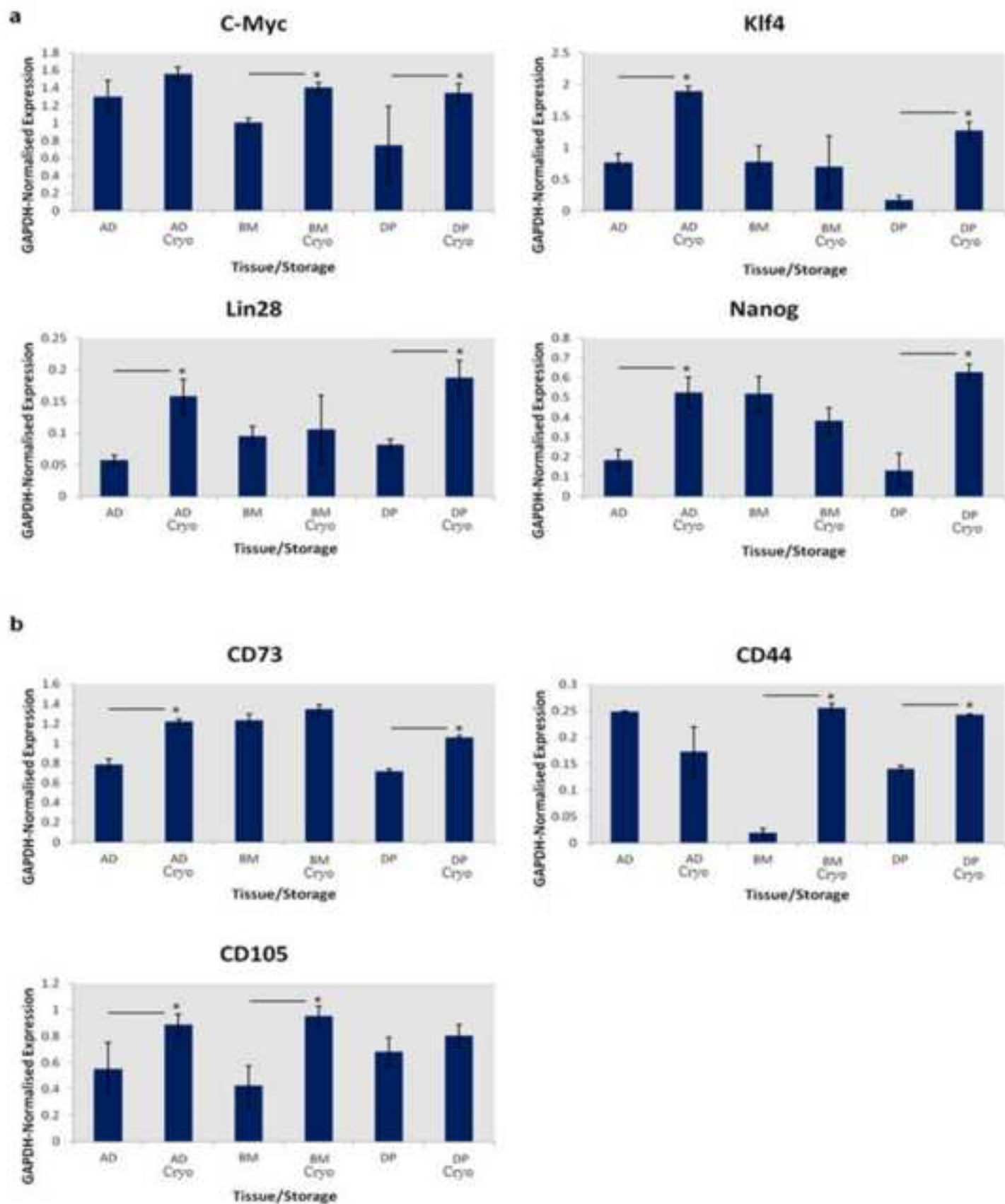


(b)

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







Gene Name	Sequence (5' → 3')	Annealing Temperature (°C)	Cycle Number	Accession Number
Normalisation				
GAPDH	F-CCCATCACCATCTTCCAGGAGC; R-CCAGTGAGCTTCCCCTTCAGC	60.5	21-27	NM_017008
Pluripotent Markers				
Klf4	F-ATCATGGTCAAGTTCCAGC; R-ACCAAGGACCATCGTTTAGG	60.5	35	NM_052713
C-myc	F-CTTACTGAGGAAACGGCGAG; R-GCCCTATGTACACCGGAAGA	60.5	35	BC091699
Nanog	F-TATCGTTTGGAGGGTGAGG; R-CAGCTGGCACTGGTTTATCA	60.5	35	NM_001100781
Lin28	F-TTCTTGTTTCCCCAAATG; R-AGAGGGGCTGGTTGTAAGGT	60.5	35	NM_001109269
SOX-2	F-ATACAAGGGAATTGGGAGGG; R-AAACCCAGCAAGAACCCTTT	60.5	25	NM_001109181
Multipotent Markers				
CD44	F-TGGGTTTACCCAGCTGAATC; R-CTTGCGAAAGCATCAACAAA	60.5	33-37	NM_012924.02
CD105	F-TTCAGCTTTCTCCTCCGTGT; R-TGTGGTTGGTACTGCTGCTC	60.5	41-45	NM_001010968
CD73	F-GGACTGATTGATCCCCTCCT; R-TTGTCCTGGATTTGAGAGG	60.5	25	NM_002526
CD29	F-AATGGAGTGAATGGGACAGG; R-TCTGTGAAGCCAGAGTTT	60.5	25	NM_017022.2
CD90	F-AGCTCTTTGATCTGCCGTGT; R-CTGCAGGCAATCCAATTTT	60.5	26	NM_012673