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REGULAR ARTICLE

Guided evaluation and standardisation of mesenchymal stem cell culture conditions to generate conditioned medium favourable to cardiac c-kit cell growth

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Abstract

Mesenchymal stem cells (MSCs) are known to secrete cardioprotective paracrine factors that can potentially activate endogenous cardiac c-kit cells (CCs). This study aims to optimise MSC growth conditions and medium formulation for generating the conditioned medium (CdM) to facilitate CC growth and expansion in vitro. The quality of MSC-CdM after optimisation of seeding density during MSC stabilisation and medium formulation used during MSC stimulation including glucose, ascorbic acid, serum and oxygen levels and the effects of treatment concentration and repeated CdM harvesting were assessed based on CC viability in vitro under growth factor- and serum-deprived condition. Our data showed that functional CdM can be produced from MSCs with a density of 20,000 cells/cm^2, which were stimulated using high glucose (25 mM), ascorbic acid supplemented, serum-free medium under normoxic condition. The generated CdM, when applied to growth factor- and serum-deprived medium at 1:1 ratio, improved CC viability, migration and proliferation in vitro. Such an effect could further be augmented by generating CdM concentrates without compromising CC gene and protein expressions, while retaining its capability to undergo differentiation to form endothelial, smooth muscle and cardiomyocytes. Nevertheless, CdM could not be repeatedly harvested from the same MSC culture, as the protein content and its effect on CC viability deteriorated after the first harvest. In conclusion, this study provides a proof-of-concept strategy to standardise the production of CdM from MSCs based on rapid, stepwise assessment of CC viability, thus enabling production of CdM favourable to CC growth for in vitro or clinical applications.

Keywords Conditioned medium · Cardiac cells · Mesenchymal stem cells · Cytoprotective · Paracrine factors

Introduction

The heart was thought to be a terminally differentiated organ with almost no intrinsic regenerative potential to circumvent the impact of myocardial infarction. This dogma was challenged by the presence of a small population of Lin^{Neg} c-kit^{Pos} cells residing in the heart that have the ability to self-renew and differentiate into cardiac cells (Beltrami et al. 2003). These cardiac c-kit cells (CCs) demonstrated the capability to regenerate the infarcted myocardium (Bearzi et al. 2007; Beltrami et al. 2003; Urbanek et al. 2005) and have been selected for testing in the Stem Cell Infusion in Patients with Ischemic cardiOmyopathy (SCIPIO) clinical trial (Bolli et al. 2011). The discovery of CCs offers hope to treat myocardial infarction and prevent its progression to heart failure. However, they present in a relatively low number and extensive expansion ex vivo is unavoidable to obtain a sufficient cell number for therapy.

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Mesenchymal stem cells (MSCs) are multipotent adult stem cells that are commonly isolated from bone marrow, umbilical cord and adipose tissue (Williams and Hare 2011). Evidence has shown that MSCs possess cardioprotective properties and are able to facilitate cardiac repair (Amado et al. 2005; Hatzistergos et al. 2010; Quevedo et al. 2009). Administration of these cells into injured heart was also found to improve heart function, reduce scar size, stimulate angiogenesis (Dai et al. 2007) and protect the heart from ischemic/reperfusion insults (DeSantiago et al. 2013). These observations have also lead to several high profile clinical trials such as POSEIDON (Hare et al. 2012), PROMETHEUS (Karantalis et al. 2014) and TAC-HFT (Heldman et al. 2014).

To date, the exact mechanism underlying the observed improvement in heart function is unclear. Some studies suggest that the observed improvement may attribute to direct contact of MSCs with cardiomyocytes via tunnelling nanotubes (Figeac et al. 2014) or by juxtacrine-mediated notch-1/jagged-1 signalling that triggers cardiomyocyte proliferation (Sassoli et al. 2011). Little data support the notion that MSCs contribute to direct new myocyte formation via transdifferentiation (Martin-Rendon et al. 2008; Siegel et al. 2012) and cell fusion (Noiseux et al. 2006). The most widely accepted mechanism exerted by MSCs in cardiac regeneration is mostly a result of its derivative paracrine factors (Yao et al. 2015) and exosomes (Lai et al. 2010). This hypothesis was also proven when MSC-derived conditioned medium (CdM) alone was found to ameliorate function of the injured heart (Timmers et al. 2008, Timmers et al. 2011; Shabbir et al. 2009). More importantly, recent studies also found interesting crosstalks between MSCs and CCs, among which were the activation of CCs through priming with CdM (Nakanishi et al. 2008) and better cell engraftment, survival and migration in CdM-treated CCs in ischemic hearts after transplantation (Iso et al. 2014). The known factors that possibly drive CC activation are insulin-like growth factor-1 (IGF-1) and hepatocytes growth factor (HGF) (Ellison et al. 2011; Koudstaal et al. 2013), vascular endothelial growth factor (VEGF) (Wang et al. 2006), stromal-derived growth factor-1 (SDF-1) (Tang et al. 2011) and platelet-derived growth factor (PDGF) (Windmolders et al. 2014; Xu et al. 2015). These factors have been identified as part of the secretomes from bone marrow-derived MSCs (Chang et al. 2013; Markel et al. 2008; Yu et al. 2009; Zisa et al. 2009). Therefore, harvesting MSC secretomes from its CdM and either using it as a lone therapeutic without co-administration with stem cells (Yeo et al. 2013) or utilizing it as an alternative supplement to culture CCs, may help to further augment the therapeutic efficacy in cardiac regeneration.

The use of conditioned medium is, however, hampered by the lack of a standardised production method with high consistency. To date, no standardised protocol has been proposed to reproducibly manufacture CdM from MSCs. Hence, optimisation of MSC culture is needed to ensure that the production of paracrine factor rich CdM from MSCs is suitable and effective for use in therapy. Here, we perform step-by-step optimisation of MSC culture conditions based on its effects on CC growth in vitro by testing the parameters that may directly affect the quality of the produced CdM, including the MSC seeding cell density, stabilisation time after passaging prior to stimulation (Eslaminejad and Nadri 2009; Neuhuber et al. 2008; Sotiropoulou et al. 2006), stimulation time for CdM generation, the use of serum or serum replacement, ascorbic acid supplementation and glucose and oxygen level (Jun et al. 2014; Krinner et al. 2009; Rosova et al. 2008) during stimulation.

Materials and methods

Ethical approval

C57/BL6N mice were obtained via Universiti Sains Malaysia Animal Research and Service Centre (ARASC). All C57/BL6N mice were sacrificed at 4–6 weeks old. C-kit cardiac cells and bone marrow cells were isolated in accordance with procedures reviewed and approved by the USM Animal Ethics Committee [USM/Animal Ethics Approval/2011/(74)(387)].

Isolation and characterisation of endogenous cardiac cells

Endogenous cardiac cells were isolated from the mouse heart according to published protocols (Smith et al. 2014). Briefly, the whole heart was extracted and minced into small pieces. The tissues were then subjected to enzyme digestion using Collagenase A (Sigma, USA) at 37 °C for 2 h. The digested tissues were grinded and filtered through a 40-μm nylon cell strainer. The filtrate was centrifuged at 300×*g* for 3 min at room temperature. The cell pellet was re-suspended and sorted for c-kit using the EasySep Mouse CD117 Selection Cocktail Kit (Stem Cell Technologies, Canada) according to the manufacturer's instructions. The selected c-kit positive cells were cultured on a 1.5% (*w*/*v*) gelatin (Sigma, USA) coated surface in cardiac cell complete growth medium (CGM) (see supplementary information). Cells were clonogenically amplified from single cells in a 96-well plate to obtain highly proliferative, homogenous CCs. Clonogenic CCs of passage 10–15 were used in all described experiments. The differentiation ability of CCs was tested by culturing CCs in cardiogenic medium (see supplementary information) for 7 days and assessed by immunofluorescence staining.

Isolation, characterisation and differentiation of bone marrow-derived mesenchymal stem cells

Bone marrow-derived mesenchymal stem cells were isolated from 4- to 6-week-old mice (Anjos-Afonso and Bonnet 2008). Briefly, the femur and tibia were extracted from the mice. The marrow was flushed out of the bone once epiphyses were cut opened. Alternatively, the marrow was collected after crushing the bone using a mortar and pestle. The collected marrow was centrifuged at 300×*g*, brake set at 7, at 4 °C. The isolated marrow cells were re-suspended and seeded at a density of 1×10^6 cells/cm^2 in complete MSC growth medium (see supplementary information). The confluent adherent cells at passage 3 were passaged and depleted for unwanted committed blood cells using the EasySep Mouse Haematopoietic Progenitor Cell Enrichment Kit (StemCell Technologies, Canada) according to the manufacturer's protocols. The lineage negative cells were expanded and cryopreserved till use. The isolated MSCs were characterised by their ability to differentiate into adipocytes, osteocytes and chondrocytes. MSCs were also characterised by flow cytometry using antibodies and dilutions listed in Table 1. Trilineage differentiation assay was performed using StemPro adipogenesis, osteogenesis and chondrogenesis differentiation medium (Gibco, USA). The differentiation medium was replenished every 3–4 days, for 14 days. Then, MSCs were stained with Oil Red O and alkaline phosphatase to detect successful adipocytes and osteocytes differentiation, respectively. Alcian blue was used to stain chondrocytes in differentiated MSCs.

Immunofluorescence staining

The CCs or differentiated CCs were fixed with 4% paraformaldehyde on ice for 20 min and washed three times with PBS. For intracellular protein staining, the cells were permeabilised with 0.1% (*v*/*v*) Triton X100 for 10 min, followed by washing thrice with PBS with 0.1% (*v*/*v*) Tween20, prior to blocking with 10% (*v*/*v*) donkey serum for 30 min. Then, the cells were labelled with primary antibodies listed in Table 1 overnight at 4 °C and counter-labelled with secondary antibodies conjugated with Alexa Fluor−488/−568 at dilution 1:500 for 1 h at 37 °C. Nuclei were counterstained with DAPI and visualised using Olympus IX71 fluorescent microscope.

Flow cytometry

For flow cytometry, a total of 2×10^5 expanded MSCs were labelled with FITC- or PE- conjugated primary antibodies (Table 2) and incubated for 1 h at 4 °C. The labelled samples were analysed using a BD FACS Canto II machine (BD Bioscience, USA). The unstained cells served as the control.

Optimisation of culture conditions for generating MSC conditioned medium

We performed the optimisation of MSC growth conditions during (i) MSC stabilisation and the (ii) MSC stimulation phase. During the stabilisation phase, MSCs could regain their optimal growth and were ready for CdM production after trypsinisation, including the stabilisation time and MSC seeding density (10,000, 15,000, or 20,000 cells/cm^2). No collection of CdM was performed at this stage. During the stimulation phase, optimisation for MSC-CdM production was performed by assessing the glucose concentration (5 mM or 25 mM), ascorbic acid supplementation, choice of serum/serum replacement, oxygen level (2%, 21%) and number of harvest. All MSCs were cultured in complete MSC growth medium during the stabilisation phase and replaced with stimulation medium consisting of standard DMEM supplemented with 1× penicillin-streptomycin, unless stated otherwise. MSC-CdM generated according to the specified parameters were harvested, pooled and stored in small aliquots at − 80 °C prior to use.

Table 1 List of antibodies for immunostaining

Antibody	Dilution factor	Manufacturer
Rabbit anti-CD117	1:50	Santa Cruz Biotechnology, Germany
Rabbit anti-GATA-4	1:50	Santa Cruz Biotechnology, Germany
Rabbit anti-NKX2.5	1:50	Santa Cruz Biotechnology, Germany
Goat anti-Tryptase	1:50	Santa Cruz Biotechnology, Germany
Rabbit anti-troponin I	1:50	Santa Cruz Biotechnology, Germany
Mouse anti-smooth muscle actinin	1:400	Sigma-Aldrich, St Louis, MO
Rabbit anti-von Willebrand Factor	1:400	Dako, CA, USA
Donkey anti-rabbit AF488	1:500	Molecular Probes, CA
Donkey anti-goat AF488	1:500	Molecular Probes, CA
Donkey anti-mouse AF488	1:500	Molecular Probes, CA

Conditioned medium concentrates and protein quantitation

Conditioned medium was concentrated using the Amicon ultra-centrifuge unit according to the manufacturer's protocols (3000 MWCO, Merck Milipore, USA). Briefly, crude conditioned medium was centrifuged for 1 h at 5000×*g*. The samples were desalted twice with DPBS, followed by centrifugation at the same speed for 1 h. The final concentrates were collected and made into aliquots and stored frozen at − 80 °C before use. The protein level of concentrated conditioned medium was quantified using a MicroBCA Protein Assay Kit (Thermo Fisher Scientific, USA) according to the manufacturer's protocol.

Cell viability assay

The effects of MSC-CdM were tested on CC viability. The CCs were seeded at 500 cells/well onto a gelatin-coated plate and allowed to settle for 4 h prior to treatment with MSC-CdM diluted with fresh DMEM/F12 medium at a ratio of 1:1. After 72 h, the medium was replaced with 10% Presto Blue solution (Gibco, USA) and incubated for 1 h prior to reading using a FLUOstar Omega multimode microplate reader (BMG Labtech, USA) at an excitation wavelength of 570 nm and emission wavelength of 590 ± 10 nm. Controls were CCs without CdM supplementation. The fluorescence intensity was normalised to the intensity of the control. CC viability was expressed in percentage using the formula below:

$$\text{Cell Viability (\%)} = \frac{\text{Normalised fluorescent intensity of tested sample}}{\text{Normalised fluorescent intensity of control}} \times 100$$

Scratch migration assay

Approximately 60,000 cells/cm^2 were seeded onto the gelatin-coated plate with CC complete growth medium and allowed to grow to full confluency under standard culture conditions. CCs were pre-treated with 10 μg/ml mitomycin C for 2 h at 37 °C, followed by scratching using 200 μl yellow pipette tips to create a wound site for cell migration. The medium was then replaced by DMEM/F12 supplemented with or without CdM. At least 10 images were acquired for each sample at baseline and at 8 h. These images were analysed using ImageJ software for the difference in gap area at 8 h after CdM treatment with the baseline using the formula below:

Formula 1: $\text{Gap}_{\text{difference}} = (\text{Gap area}_{\text{baseline}} - \text{Gap area}_{8\text{h}})$

Formula 2: $\text{Gap area}_{\text{baseline}}$ = Average gap size in percentage at baseline

Formula 3: $\text{Gap area}_{8\text{h}}$ = Average of gap size in percentage after 8 h

Quantitative real-time polymerase chain reaction (qPCR)

QPCR was used to examine CC gene expression related to its stemness after being treated with CdM. RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and reversed-transcribed to generate cDNA using the Quantitect Reverse Transcriptase Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocols. The gene expression was quantified using Quantinova SYBR PCR Mix (Qiagen, Hilden, Germany) using StepOnePlus™ Real-Time PCR Systems (Applied Biosytems) according to the manufacturer's protocols. The primers used in qPCR are listed in Table 3.

Cell proliferation assay

CCs were seeded at 20,000 cells per well in a gelatin-coated 24-well plate using CGM. Immediately after 4 h of incubation, culture medium was removed and replaced with MSC-CdM diluted in growth factor- and serum-free DMEM/F12 medium at a ratio of 1:1. Test medium was high-glucose DMEM diluted in DMEM/F12 at 1:1 ratio without any supplementation. After 3 days, CCs were passaged with TrypLE Express dissociation solution (Gibco, US) and sub-cultured at 1:2 split ratio and allowed to grow up to day 7. CCs after trypsinization were reseeded with complete CGM and replaced with test medium

Table 2 List of fluorochrome-conjugated antibodies used in the flow cytometry

Antibody	Dilution factor	Manufacturer
Mouse anti-CD34-FITC, Clone RAM34	1:50	BD Bioscience, USA
Mouse anti-CD90.1-FITC, Clone His51	1:10	Miltenyi Biotec, Germany
Mouse anti-CD90.2-FITC,Clone 53–2.1	1:50	BD Bioscience, USA
Mouse anti-Sca-1-FITC, Clone D7	1:10	Miltenyi Biotec, Germany
Mouse anti-CD44-FITC, Clone IM7.8.1	1:50	Miltenyi Biotec, Germany
Mouse anti-CD29-FITC, Clone HMβ1–1	1:10	Miltenyi Biotec, Germany
Mouse anti-CD105-PE, Clone MJ7/18	1:10	Miltenyi Biotec, Germany

Table 3 Primer list used in this study

Gene/accession number	Primer sequence (5′-3′)
c-kitNM_022264.1	Forward: GAAAGGGAGGCCCTAATGTC
	Reverse: CGTTTGAGCTGTCACAGGAA
GATA4	Forward: TCTCTGCATGTCCCATACCA
NM_008092.3	Reverse: TGTGTGTGAAGGGGTGAAAA
Nkx2.5	Forward: GCTACAAGTGCAAGCGACAG
NM_008700.2	Reverse: GGGTAGGCGTTGTAGCCATA
Sox2	Forward: GCGGAGTGGAAACTTTTGTCC
NM_011443.3	Reverse: CGGGAAGCGTGTACTTATCCTT
GAPDH	Forward: ACCCAGAAGACTGTGGATGG
NM_008084.2	Reverse: CACATTGGGGGTAGGAACAC

after 4 h. Numbers of CCs at each timepoint (day 0, 3 and 7) were manually counted using a haemocytometer.

Statistical analysis

The experiments were performed in triplicate unless stated otherwise. All statistical analyses were performed using SPSS and the differences were analysed using independent sample student *t* test and analysis of variance (ANOVA) with Tukey post-hoc test. The data were presented as mean ± standard error of the mean (SEM). Difference between groups was significant when $p < 0.05$.

Results

Bone marrow-derived mesenchymal stem cells can be isolated from C57/BL6N mice

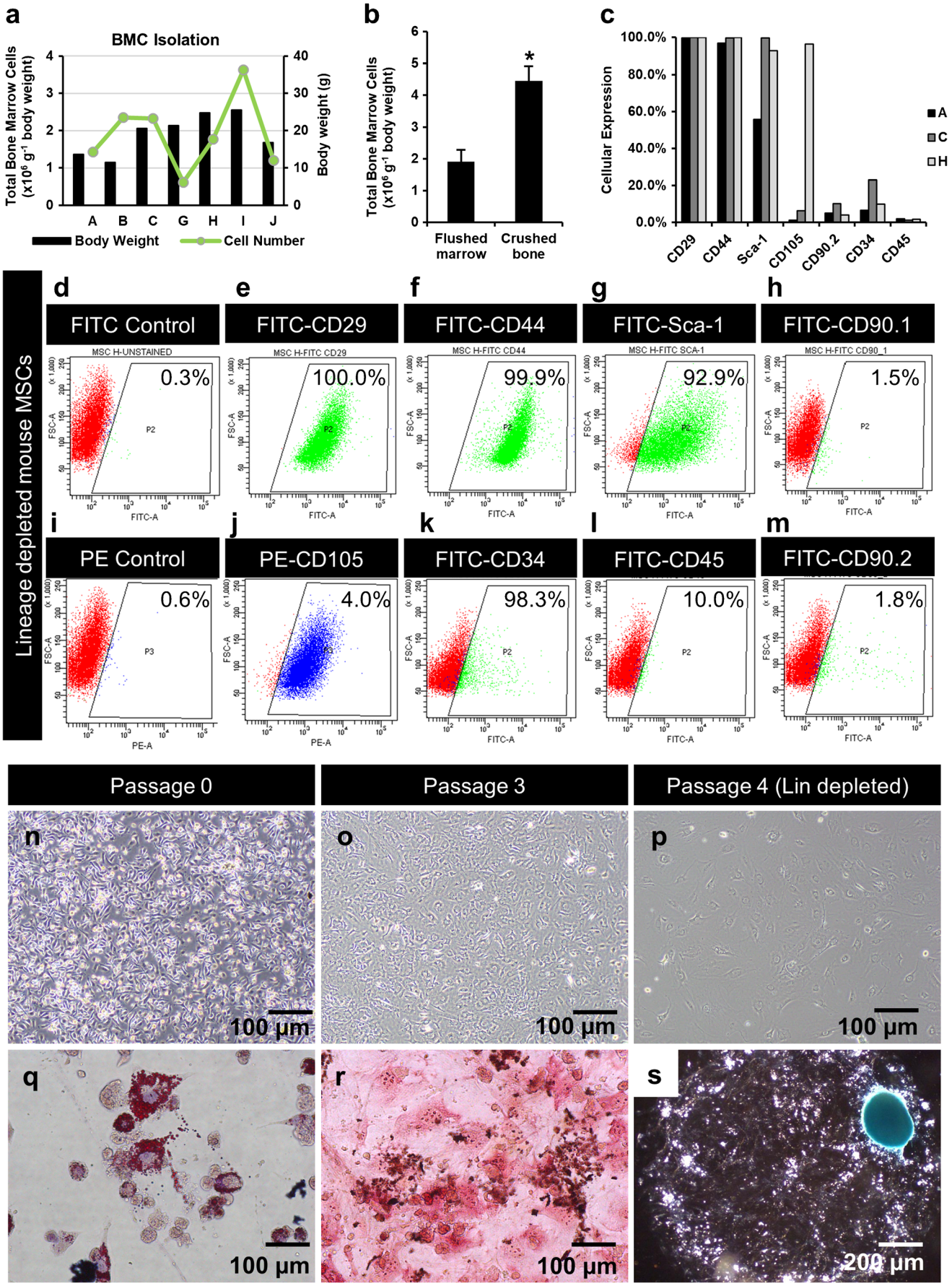
In order to obtain bone marrow MSCs from C57/BL6N mice, marrow cells were flushed out from femur and tibia marrow taken from seven mice (A, B, C, G, H, I and J), as previously described (Baustian et al. 2015). However, the total bone marrow cells (BMCs) yield varied from batch-to-batch (Fig. 1a), with an average cell number of 2.3×10^6 cells g^{-1} of body weight. To increase the yield, BMCs were also isolated from crushed bones with a mortar and pestle. Expectedly, the total number of BMCs from the crushed bone was significantly higher (8.3×10^6 cells g^{-1}) than the cells from flushed marrow ($p < 0.05$) (Fig. 1b). However, the total plastic adherent cells from crush bones were very low and could not be processed further with haematopoietic lineage depletion, thus were excluded from this study. Only three of the most proliferative BMCs isolated from flushed marrow (A, C and H) were selected for further depletion based on CD5, CD11b, CD19, CD45R, Ly6G/C and TER119 after 3–4 passages. These cells were then characterised for CD29, CD44, Sca-1, CD90, CD105, CD34 and CD45 with flow cytometry after expansion for at least 10 passages. Our data show that the expression of CD29 (> 99%), CD44 (> 99%), CD90 (10%) and CD45 (< 2%) were similar in all three lineage-depleted bone marrow-derived mesenchymal stem cells (Fig. 1c). However, high Sca-1 expression was only observed in MSCs from C and H (> 92%) but not A (< 60%). Additionally, high CD105 expression was only observed in H (> 96%), while low in A and C (< 10%). The representative flow cytometric dot plot shows that MSCs from H were highly positive for CD29, CD44, Sca-1 and CD105 but lack the expression of CD90, CD45 and CD34 at passage 10 (Fig. 1d–m).

Despite differences in surface marker expressions, all three cultures showed non-adherent phase bright cells residing on top of the stromal cells in freshly isolated bone marrow cells at passage 0 and the non-adherent cells were gradually removed after 3 passages (Fig. 1 n–p). After lineage depletion, bone marrow-derived mesenchymal stem cells acquired a spindle-like shape and retained the differentiation plasticity to form adipocytes, osteocytes and chondrocytes, confirming their mesenchymal stem/stromal cell characteristics (Fig. 1 q–s). In our experience, the primary MSCs from C57/BL6N mice took an average of 5–6 months to enter the exponential growth phase and acquired a proliferative capacity with a population doubling time of 24.3 ± 0.3 h.

MSC culture conditions and the effects of generated CdM on CC viability

CdM optimisation was performed at three phases; MSC stabilisation (sb), stimulation (st) and testing phase. To establish default culture conditions and enable systematic modifications to be made in the subsequent experiments, multiple parameters, including the MSC seeding density, ascorbic acid supplementation and FBS and glucose concentration, were tested concurrently. CdM generated from 10,000, 15,000 and 20,000 cells/cm^2 MSC culture did not significantly affect the overall CC growth, measured by relative CC viability (Fig. 2a). However, higher CC viability was observed when $serum^{st}$ concentration was increased from 2 to 5% ($p < 0.01$ at 10,000 cells/cm^2 and $p < 0.05$ at 15,000 and 20,000 cells/cm^2). The improvement in CC viability was markedly augmented by 97% in all groups with an increased $glucose^{st}$ level to 25 mM. This effect remained unaltered when the serum level was decreased from 5 to 2% in groups with lower MSC numbers but not in the group with 20,000 MSCs of which the CC viability declined by 17.6% ($p < 0.01$). As we observed better MSC confluency and morphology with 20,000 cells/cm^2 and a high number of MSCs may mean a higher level of secreted factors, we used a MSC seeding density of 20,000 cells/cm^2 for subsequent experiments.

As supplementation of fetal bovine serum (FBS) during CdM production may introduce batch-to-batch variability


a
BMC Isolation
Total Bone Marrow Cells (x10^6 g^-1 body weight)
Body weight (g)
A B C G H I J
Body Weight
Cell Number
b
Total Bone Marrow Cells (x10^6 g^-1 body weight)
*
Flushed marrow
Crushed bone
c
Cellular Expression
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
A
C
H
CD29
CD44
Sca-1
CD105
CD90.2
CD34
CD45
Lineage depleted mouse MSCs
d
FITC Control
MSC H-UNSTAINED
0.3%
e
FITC-CD29
MSC H-FITC CD29
100.0%
f
FITC-CD44
MSC H-FITC CD44
99.9%
g
FITC-Sca-1
MSC H-FITC SCA-1
92.9%
h
FITC-CD90.1
MSC H-FITC CD90_1
1.5%
i
PE Control
0.6%
j
PE-CD105
4.0%
k
FITC-CD34
98.3%
l
FITC-CD45
10.0%
m
FITC-CD90.2
1.8%
FSC-A
FITC-A
PE-A
P2
P3
Passage 0
Passage 3
Passage 4 (Lin depleted)
n
o
p
100 μm
q
r
s
200 μm

◀ **Fig. 1** Isolation and characterisation of bone marrow-derived mesenchymal stem cells. (a) Total bone marrow-derived cell yield from flushed marrows per mice body weight from seven batches of isolation, namely A, B, C, G, H, I and J. (b) Mean bone marrow cell yield from flushed marrow and crushed bone. (c) Flow cytometric analysis of MSC surface markers. (d–m) Representative flow cytometric dot plot marker expression by MSCs (isolated from batch H). (n–p) Representative images of MSC at early passage (P0), passage 3 (P3) and passage 4 (P4) after lineage depletion. (q–s) The isolated MSCs were capable of forming adipocytes, osteocytes and chondrocytes in vitro. Scale bar 100 μm. Scale bar for adipogenesis and osteogenesis 50 μm. Scale bar for chondrogenesis 200 μm. *$p < 0.05$ compared to BMCs from flushed marrow

and minimising the use of FBS is always preferable for clinical applications, we evaluated the effects of CdM generated from 20,000 cells/cm^2 serum starved MSC (CdM^{ss}) during stimulation on CC viability (Fig. 2b). Our data showed that the viability of CdM^{ss}-treated CC was increased by 63.2% when compared to serum-free non CdM-treated control. In contrast, CdM from MSCs cultured with 2% FBSst did not significantly promote CC viability when compared to the 2% FBS supplemented fresh medium treated control but was found lower than CdM^{ss}-treated CCs ($p < 0.05$), suggesting that serum-starvation is better in generating MSC-derived CdM.

In view of the possibility of high cellular oxidative stress-induced by an elevated glucose level, we examined if addition of ascorbic acid (AA) as an antioxidant to offset the detrimental oxidative stress is necessary for CdM generation. Our data showed that CdM from AAst-MSCs did not show significant improvement in CC viability (Fig. 2d). However, 80% of AA-treated MSCs remained viable following H_2O_2 treatment as compared to the untreated control (~61%) (Fig. 2c). These data suggest that AAst is necessary to improve MSC resistance to oxidative stress and cell viability during CdM production but may not significantly alter the content of the secretion factors that would affect CC viability.

MSC recovery from trypsinisation is important to ensure its optimal paracrine performance and viability prior to stimulation phase. Hence, we tested 4-h and 24-h stabilisation time. Interestingly, no additional beneficial effect from CdM was

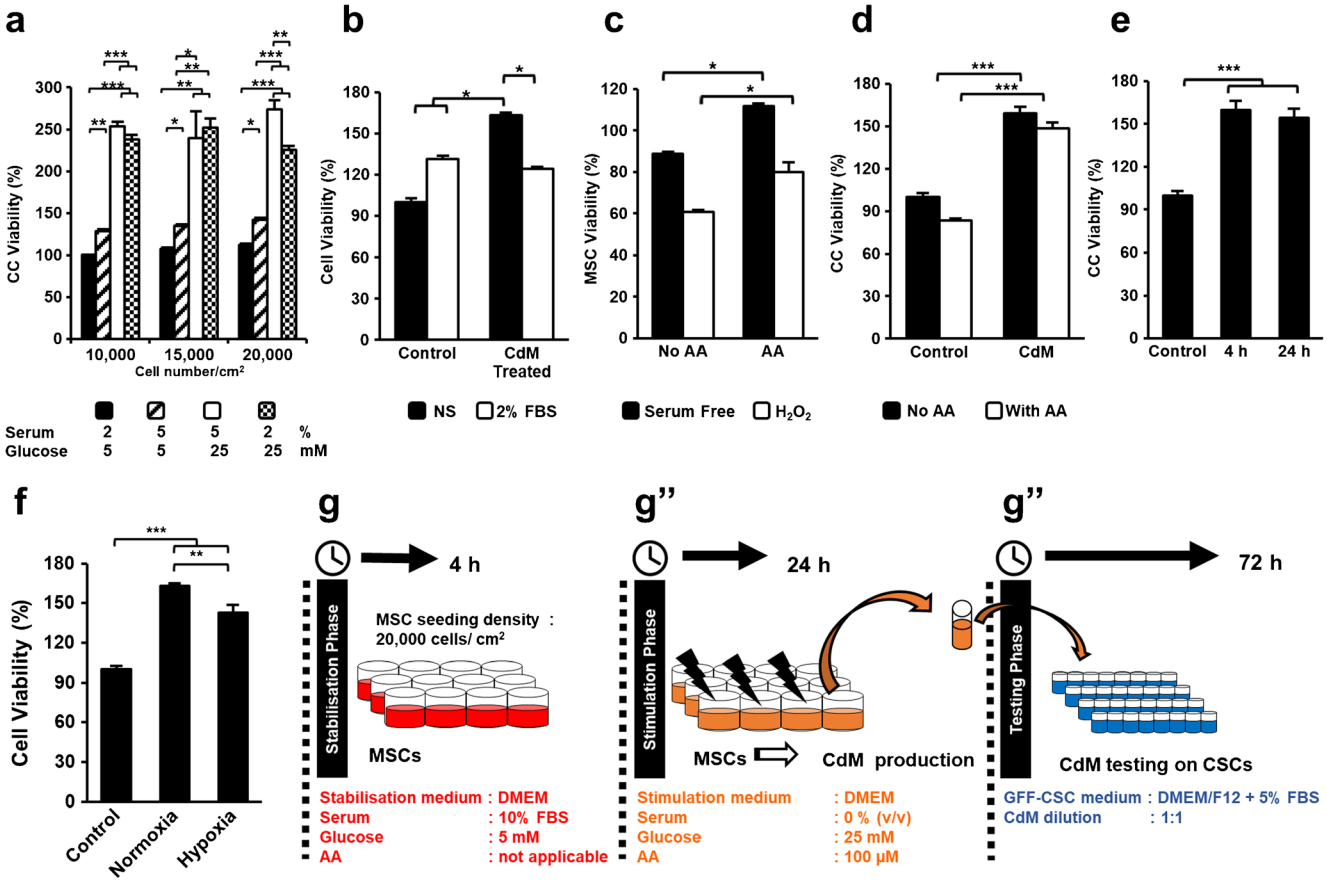


Fig. 2 The effects of cell seeding density, glucose concentration, FBS, ascorbic acid, stabilisation time and oxygen levels on the CdM cytoprotective properties in maintaining CC growth. (a) CC viability in response to CdM generated using different cell density, serum concentration and glucose concentration (b) The effect of CdM^{ss} on CC viability. (c) The effect of ascorbic acid in salvaging H_2O_2-treated MSCs. CC viability in response to CdM generated by (d) ascorbic acid supplementation, (e) 4 h and 24 h MSC stabilisation time, (f) normoxic and hypoxic conditions. (g–g'') Schematic diagram for CdM generation from stabilisation phase, stimulation phase and testing phase. SS indicate serum starved; GFF indicate growth factor free. All data are expressed in mean ± SEM. *$p < 0.05$;**$p < 0.01$; ***$p < 0.001$

observed on CC viability (159.7 ± 6.5% vs. 154.1 ± 6.6%, $p > 0.05$) when MSCs were stabilised for 4 h or 24 h prior to stimulation phase (Fig. 2e). Next, we also demonstrated that CdM generated under normoxiast contributed to the highest CC growth (163.2 ± 1.7%) as compared to hypoxic-CdM (142.7 ± 5.9%, $p < 0.01$) (Fig. 2f). A summary of culture conditions used in all these phases during MSC culture and CdM generation is shown in Fig. 2(g–g"), unless modifications for experimentation purposes were stated otherwise.

Treatment concentration and its effect on CC viability and migration

CdM generated based on previously determined culture conditions (Fig. 2g–g") was examined for dilutions at 25% and 50% for CC treatment (Fig. 3a). CCs treated with 50% CdM showed significantly greater CC viability as compared to 25% CdM (154.1 ± 6.6% vs. 132.5 ± 5.6%, $p < 0.01$) (Fig. 3a). We then investigated the effects of CdM on CC migration, tested using the scratch migration assay. Again, MSC-derived CdM consistently improved CC migration after 8 h, regardless of treatment concentration tested at 25% and 50% (*v*/*v*) (Fig. 3b, e, f'). To exclude CC proliferative effects that may complicate the interpretation of CC migration results, CCs were treated with 10 μg/ml mitomycin C (MtC) prior to CdM treatment. MtC treatment significantly attenuated CC proliferation over 7 days (Fig. 3c). We repeated the experiment and again showed that the gap closure of CdM-treated CC was similar with or without prior MtC treatment (Fig. 3d), confirming the effect of CdM on CCs migration.

The effect of CdM on CC viability after protein enrichment

Next, we sought to test if CdM could be repeatedly harvested from MSCs under the same defined conditions, be further enriched and remain favourable to CC viability. MSCs were cultured with stimulation medium for 24, 48 and 72 h after which the CdM was repeatedly harvested and replaced with fresh medium after the same stimulation time, for three times. CdM harvested from the first stimulation interval is known as first isolation, with the second and third CdM isolation from the subsequent intervals. We measured the total protein level

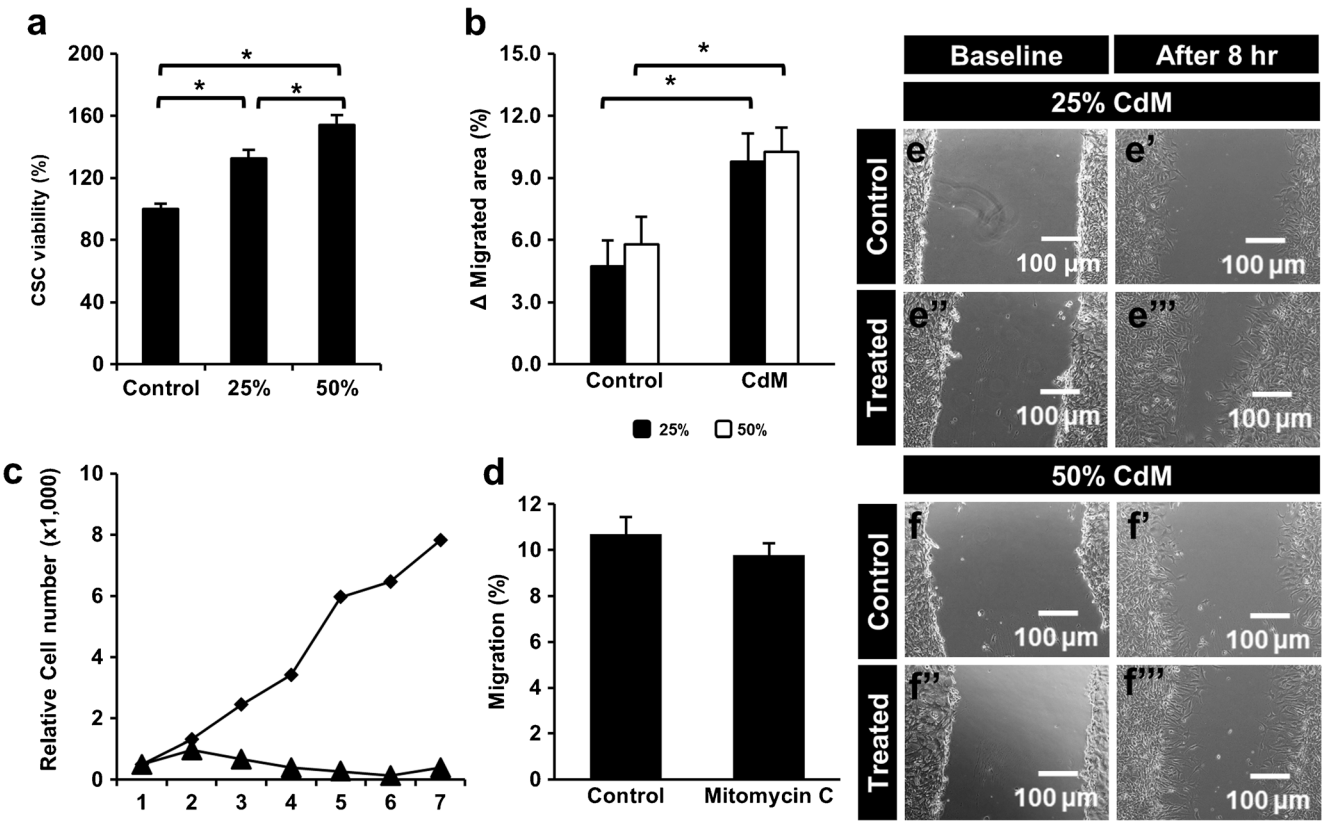


Fig. 3 The effects of treatment concentration on CdM cytoprotective properties in maintaining CC viability and migration stimulating potential. (a) CdM treatment concentration at 25% (*v*/*v*) and 50% (*v*/*v*). (b) CC migration following CdM treatment at 25% (*v*/*v*) and 50% (*v*/*v*) after 8 h. (c) Growth curve of CCs over the course of 6 days after pre-treated with 10 μg/ml mitomycin C. (d) Migration of CC with or without mitomycin C treatment. (e–e"') Representative phase contrast pictures showing the degree of CC migration following 25% and (f–f"') 50% CdM treatment. * indicates significance levels $p < 0.05$

in CdM based on the protein vs. absorbance standard curve (Fig. 4a). The MSC stimulation time and the number of CdM isolations were also evaluated. Our data demonstrated that the enriched CdM concentrate showed a strong correlation between its protein level and CC viability ($R^2 = 0.855$, Fig. 4b). The optimal CdM concentrate was generated from MSC during the first isolation after 24 h of stimulation increased CC viability to 227% (Fig. 4c). We also observed that the effects of CdM on CC viability, regardless of crude or concentrate, significantly deteriorated to a level comparable to the untreated control as the stimulation time and the number of isolations increased. This finding was supported by high protein concentration detected in enriched CdM, which was harvested during the first isolation as compared to the second and third isolations (Fig. 4d).

The effect of CdM on CC stemness, cardiomyocyte differentiation and proliferation

MSCs are known to be beneficial to CC functions. Here, CC stemness gene expressions were tested after being treated with CdM. Our data suggest comparable c-kit, GATA4, NKX2.5 and Sox2 mRNA expressions as compared to the untreated CC group (Fig. 5a–a"'). Immunofluorescence staining showed CCs treated with CdM remained positive for c-kit, GATA4, NKX2.5 and OCT3/4 and negative for CD31 endothelial marker, suggesting that the generated CdM does not compromise CC stemness and identity (Fig. 5b–b"'). Furthermore, the CdM treatment did not affect the CC trilineage differentiation capacity, as evident by the presence of cardiomyocytes, smooth muscle and endothelial cells 7 days after induction of differentiation (Fig. 5c–c").

PrestoBlue assay measures cell metabolism that correlates with cell viability. To determine whether the increased relative fluorescence reading observed in CdM-treated CCs using PrestoBlue assay also coupled with increased cell number, the CC number was counted using both the PrestoBlue reading vs cell number standard curve (Fig. 5d) and the haemocytometer, after being treated with CdM for 3 days. Both methods showed a significant increase in CC number in the CdM-treated group as compared to the untreated control at day 3 (Fig. 5e).

To confirm the proliferative effect of CdM on CCs, CCs were cultured and counted at day 0, 3 and 7. Consistent with our previous observations, CdM-treated CCs were capable of proliferation and reached full confluency on day 7 after being subcultured at day 3 (Fig. 6a–a'). The number of CCs treated with CdM increased from 20,000 cells to 32,760 ± 5980 cells ($p < 0.005$ vs control) at day 3 and 62,417 ± 17,498 cells ($p < 0.001$ vs control) at day 7 under growth factors and serum-free condition, while untreated control CCs showed minimal or reduced growth within the same time frame (Fig. 6b). These observations support that generated CdM can stimulate CC proliferation under growth factors and serum-deprived conditions.

Discussion

Cardiac cell therapy holds promise to regenerate injured heart. Although the recent outcome and meta-analyses from multiple

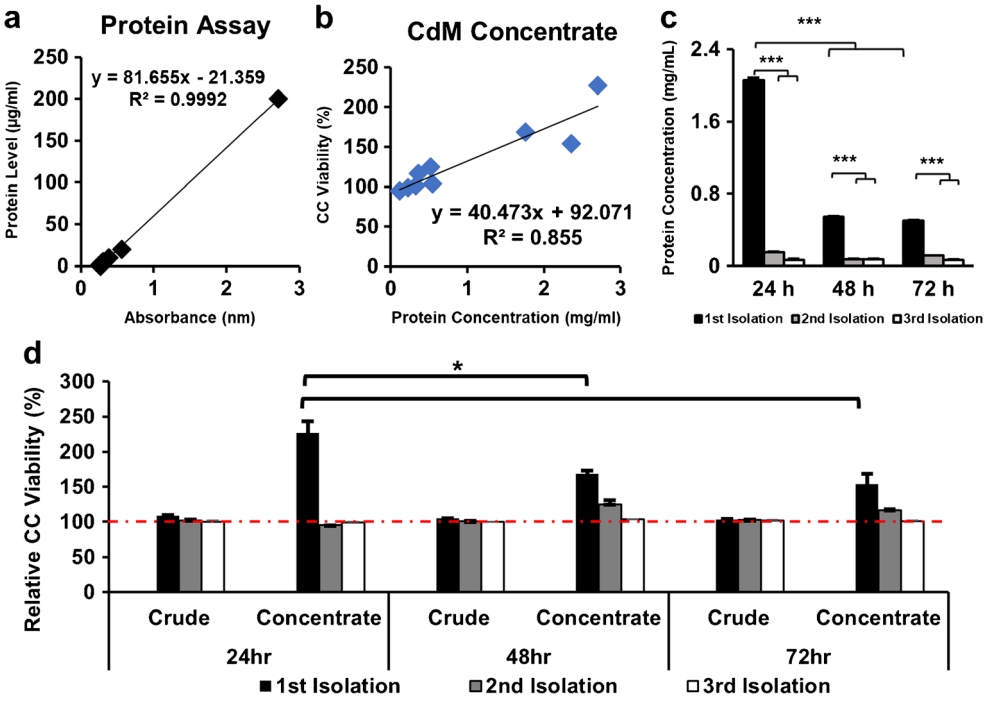


Fig. 4 The effects of repeated harvest and CdM enrichment on CdM cytoprotective properties in CC culture. **a** BSA standard curve. **b** Correlations between CC viability and protein content in CdM after enrichment. **c** The total protein content of enriched CdM from MSCs after first, second and third repeated isolations with stimulation time intervals of 24, 48 and 72 h. **d** The cytoprotective effect of the CdM concentrate harvested at different isolations and different durations of repeated harvest on CC. Red dotted line indicates relative CC viability of the control (untreated) group. All data are expressed in mean ± SEM. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

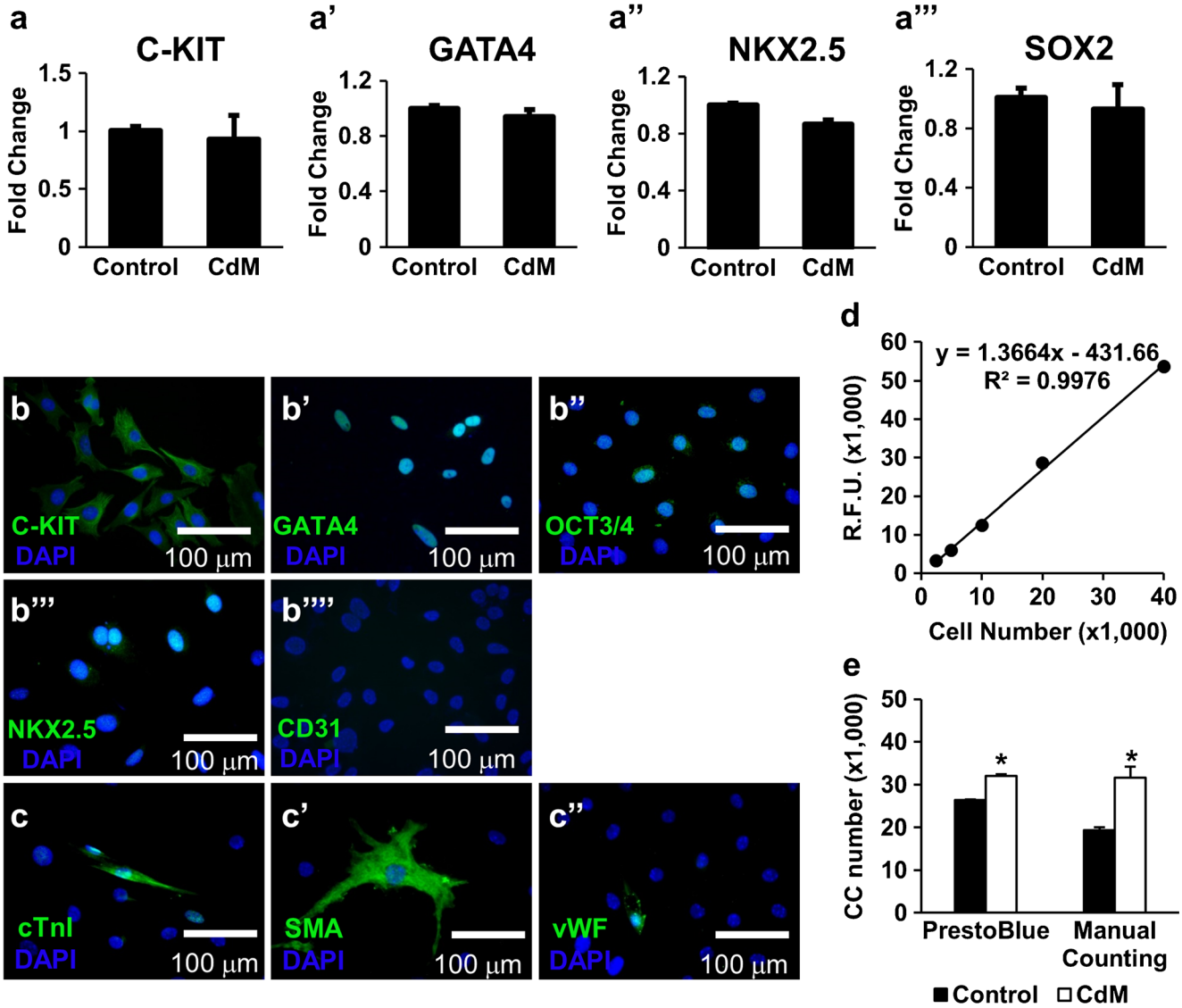


Fig. 5 Functional assessment of c-kit CCs post-CdM treatment. (a) Stemness gene expression of CCs following CdM treatment for 3 days as assessed by QPCR. (b–b'''') No changes in c-kit, GATA4, NKX2.5 and OCT3/4 expression were observed on CCs after being treated with CdM. (c–c'') C-kit-expressing cells could differentiate into cardiomyocyte, smooth muscle and endothelial cells as assessed by detection of cardiac troponin I, smooth muscle actinin and von Willebrand factor, respectively. (d) Standard curve with relative fluorescence unit of PrestoBlue vs cell number was used to calculate relative cell number in CC proliferation assay. (e) Relative CC number obtained from PrestoBlue reading was significantly higher after being treated with CdM as compared to untreated control. This is consistent with absolute CC number obtained by haemocytometer from the same samples. All data are expressed in mean ± SEM. * $p < 0.05$ vs control

human trials with bone marrow cells question the therapeutic benefit and cast doubts about the approach and further investment of resources (Fisher et al. 2015), paracrine effects exerted by the administrated cells remain the most promising

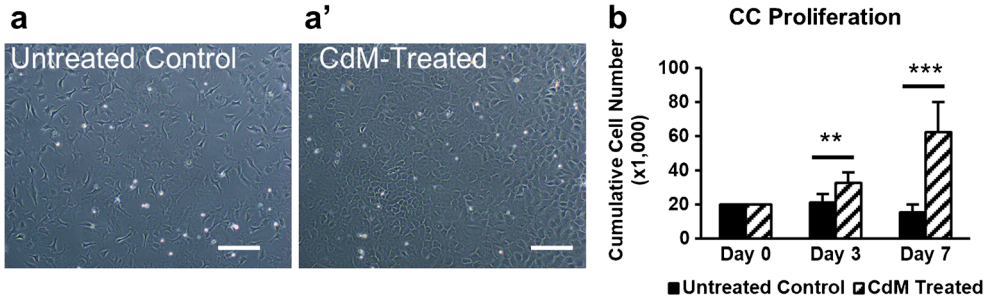


Fig. 6 The effect of CdM on CC proliferation. (a) Bright field morphology view of control and (a') CdM-treated CCs at day 7. Scale bar = 1 mm. (b) Number of CCs at day 0, 3 and 7 with/without CdM treatment. ** $p < 0.005$; *** $P < 0.001$

regenerative mechanism to explain most, if not all, reported functional improvement in damaged myocardium. MSCs are known to secrete multiple cardioprotective factors and treatment using the extracted secretome from CdM also showed a positive outcome in vivo (Timmers et al. 2011). Nonetheless, the production of CdM is challenged by the MSC isolation method and growth conditions, which affect the cell characteristics, homogeneity and the quality of the CdM. Therefore, the standardisation of such a protocol is important to generate clinically compliant MSC-derived conditioned medium. This is a proof-of-concept experiment to demonstrate stepwise optimisation of the MSC growth condition to produce functional CdM of which its effectiveness is rapidly assessed based on CC viability under the growth factors-deprived condition.

Although isolating bone marrow cells from flushed marrow is the most widely used methods (Anjos-Afonso et al. 2004; Gnecchi and Melo 2009; Suire et al. 2012), we showed that the crushed bone method, with a short sacrifice-to-culture time fewer than 30 min, successfully increased the total isolated primary marrow cells. However, the plastic adherent cells from this inbred C57BL/6 mice was relatively low (Phinney et al. 1999). We did not pool the MSCs from multiple mice to increase the total cell number to enable early characterisation and sorting (Obara et al. 2016). Instead, each isolation was kept separate to assess batch-to-batch variability. This study design resulted in a low cell count and thus prohibited early cell characterisation prior to extensive expansion with flow cytometry. Although we do not exclude the possibility that extensive expansion in vitro might have altered the MSC phenotype (Baustian et al. 2015) and presented low CD90 and heterogeneous CD105 expression, previous studies reported a similar observation and attributed it to the animal strain, as they found MSCs from inbred C57BL/6 mice exhibited low CD90 (Peister et al. 2004) and heterogeneous CD105 (Anderson et al. 2013). Regardless, the expanded MSCs were proven proliferative and capable of undergoing trilineage differentiation prior to use in the described experiments.

Studies have shown the MSC-derived factors that stimulate activation of CCs (Hatzistergos et al. 2010; Loffredo et al. 2011). Our data also showed that CdM activated CC proliferation despite deprivation of growth factors and serum in the culture medium up to 7 days. This finding confirms the presence of pro-proliferative factors in the generated CdM. However, we also noticed that CdM alone are not sufficient to promote long-term expansion of CCs beyond day 7 under the same culture conditions (data not shown). Thus, future study to identify a new CC medium formula with further CdM content characterisation, refinement and enrichment is required to enable extended CC culture in vitro.

The identity of endogenous CCs in cardiac regeneration has recently been challenged. Lineage tracing studies using transgenic mice suggests that c-kit cells are not cardiac stem cells and minimally contribute to cardiomyocytes (van Berlo et al. 2014) but are more committed toward endothelial lineage (Sultana et al. 2015). However, whether minimal contribution represents minimal cardiomyogenic potential is still elusive. This question prompted a study that showed that CCs are cardiac neural crest progenitors with full potential to differentiate into cardiomyocytes but the differentiation during heart development is limited by changes in bone morphogenic protein signalling within the niche (Hatzistergos et al. 2015). This suggests CCs are cardiac progenitors but its presence is limited by the environment during development. Furthermore, recent evidence showed that the true c-kit cardiac cells are lineage negative CD45 negative and constitute only 1–2% of the total c-kit myocardial cells, of which only 10% of those can robustly form cardiomyocytes (Vicinanza et al. 2017). Nonetheless, synergistic relationships between c-kit cardiac cells and MSCs in repairing injured myocardium have been demonstrated (Williams et al. 2013) and such interactions should be harnessed to achieve greater myocardial regeneration, including the use of MSC paracrine secretome or exosome on CCs during in vitro preparation and culture, or use as an injectable cell-free, xenogenic-free therapeutic product for stimulating endogenous CC-mediated repair in vivo.

In conclusion, this study recommends a standardised production protocol for generating functional CdM from MSCs. Here, functional CdM were produced from MSCs seeded at a density of 20,000 cells/cm^2, which were stimulated using DMEM with high glucose (25 mM), ascorbic acid supplemented, serum-free medium under normoxic condition. The generated CdM, when applied to growth factor-deprived DMEM/F12 medium at 1:1 ratio, improved CC viability, migration and proliferation in vitro. Although this work was performed using cells isolated from mice, it provides a proof-of-concept experimental design to optimise the growth conditions of human MSCs for generating functional conditioned medium based on rapid, stepwise viability assessments on cardiac c-kit cells. Further in-depth study to evaluate the proteomes and exosomes in CdM and their performance, may be required to realise the production of an ‘off-the-shelf’ therapeutic agent for human therapy.

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Compliance with ethical standards

C57/BL6N mice were obtained via Universiti Sains Malaysia Animal Research and Service Centre (ARASC). All C57/BL6N mice were sacrificed at 4–6 weeks old. C-kit cardiac cells and bone marrow cells were isolated in accordance with procedures reviewed and approved by the USM Animal Ethics Committee [USM/Animal Ethics Approval/2011/(74)(387)].

Conflict of interest JJT received a research grant from CryoCord Sdn Bhd. All funders have no role in conceiving, designing experiments or analysing data from this project. Others declare no conflict of interest.

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