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Decline in cellular function of aged mouse c-kit$^+$ cardiac progenitor cells

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Key points

- While autologous stem cell-based therapies are currently being tested on elderly patients, there are limited data on the function of aged stem cells and in particular c-kit$^+$ cardiac progenitor cells (CPCs). We isolated c-kit$^+$ cells from young (3 months) and aged (24 months) C57BL/6 mice to compare their biological properties.
- Aged CPCs have increased senescence, decreased stemness and reduced capacity to proliferate or to differentiate following dexamethasone (Dex) treatment *in vitro*, as evidenced by lack of cardiac lineage gene upregulation.
- Aged CPCs fail to activate mitochondrial biogenesis and increase proteins involved in mitochondrial oxidative phosphorylation in response to Dex.
- Aged CPCs fail to upregulate paracrine factors that are potentially important for proliferation, survival and angiogenesis in response to Dex.
- The results highlight marked differences between young and aged CPCs, which may impact future design of autologous stem cell-based therapies.

Abstract Therapeutic use of c-kit$^+$ cardiac progenitor cells (CPCs) is being evaluated for regenerative therapy in older patients with ischaemic heart failure. Our understanding of the biology of these CPCs has, however, largely come from studies of young cells and animal models. In the present study we examined characteristics of CPCs isolated from young (3 months) and aged (24 months) mice that could underlie the diverse outcomes reported for CPC-based therapeutics. We observed morphological differences and altered senescence indicated by increased senescence-associated markers β-galactosidase and p16 mRNA in aged CPCs. The aged CPCs also proliferated more slowly than their young counterparts and expressed lower levels of the stemness marker LIN28. We subsequently treated the cells with dexamethasone (Dex), routinely used to induce commitment in CPCs, for 7 days and analysed expression of cardiac lineage marker genes. While MEF2C, GATA4, GATA6 and PECAM mRNAs were significantly upregulated in response to Dex treatment in young CPCs, their expression was not increased in aged CPCs. Interestingly, Dex treatment of aged CPCs also failed to increase mitochondrial biogenesis and expression of the mitochondrial proteins Complex III and IV, consistent with a defect in mitochondria complex assembly in the aged CPCs. Dex-treated aged CPCs also had impaired ability to upregulate expression of paracrine factor genes and the conditioned media from these cells had reduced ability to induce angiogenesis *in vitro*. These findings could impact the design of future CPC-based therapeutic approaches for the treatment of older patients suffering from cardiac injury.

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Abbreviations CPC, cardiac progenitor cell; Dex, dexamethasone; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GATA4, GATA binding protein 4; GATA6, GATA binding protein 6; MEF2C, myocyte enhancer factor 2c; NDUFAF1, NADH:ubiquinone oxidoreductase complex assembly factor 1; NRF-1, nuclear respiratory factor 1; NS, nucleostemin; OXPHOS, mitochondrial oxidative phosphorylation; PECAM, platelet endothelial cell adhesion molecule; PGC1α, peroxisome proliferator-activated receptor gamma coactivator 1 alpha; PGC1β, peroxisome proliferator-activated receptor gamma coactivator 1 beta; POLG, proofreading-deficient mitochondrial DNA polymerase γ; SAβ-Gal, senescence associated beta-galactosidase; SDF-1, stromal cell-derived factor-1; TGF-β, transforming growth factor-beta; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Introduction

Average life expectancy has increased dramatically over the past century as a consequence of advances in the biomedical field. Currently 11% of the entire human population is composed of elderly individuals (>60 years old) and epidemiological studies project an increase to 22% by 2050 (Kanasi *et al.* 2016). The unprecedented increase in human lifespan over the past years has driven scientific interest in the study of human diseases that arise with ageing. Ageing can be defined as the process by which the physiological integrity of an organism decreases over time, ultimately leading to death. At the cellular level senescence is the degenerative process that takes place due to DNA damage during ageing (Terzi *et al.* 2016). This phenomenon is mainly, but not only, dependent on the organism age and lifespan.

The heart is one of the organs of the body that is most affected by ageing, undergoing more than three billion contraction cycles during the average human lifespan. In addition, the decline in cardiac function with ageing is often accompanied by other age-related risk factors such as diabetes or hypertension that accelerate cardiovascular senescence (Brodsky *et al.* 2004; Kosugi *et al.* 2006). The loss of myocyte turnover (Bergmann *et al.* 2015) and decreased contraction due to impairment in calcium handling are some of the causes underlying the development of heart failure in the aged heart (Lakatta & Levy, 2003; Bergmann *et al.* 2015). Thus, it is not surprising that cardiovascular disease occurs more frequently in the elderly population (Writing Group Members *et al.* 2016).

Strategies to 'rejuvenate' the ageing heart are currently being investigated. Recent studies have identified follistatin like 1 (Masters & Riley, 2014; van Rooij, 2016), Pim-1 (Cottage *et al.* 2010), growth differentiation factor 11 (Olson *et al.* 2015; Rochette *et al.* 2015; van Rooij, 2016) and insulin like growth factor 1 (Leifke *et al.* 2000; Fontana *et al.* 2012) as possible anti-ageing factors capable of inducing endogenous regeneration in the ageing heart. Although it is unclear whether the heart can be made to regenerate, cardiac progenitor cells identified in the adult heart are multipotent and have the ability to differentiate (Beltrami *et al.* 2003; Yellamilli & van Berlo, 2016). A randomized control phase I clinical trial (SCIPIO) investigated whether isolating c-kit$^+$ cardiac progenitor cell (CPCs) from patients and re-introducing them back into the injured heart would be beneficial. The study has produced encouraging results and paved the way for future phase II clinical trials (Bolli *et al.* 2011; Hong & Bolli, 2014). It is clear, however, that a major hindrance for cell therapy is the poor stem cell survival and engraftment in the injured heart. Although studies have suggested that CPCs are capable of differentiating into cardiomyocytes (Beltrami *et al.* 2003; Dawn *et al.* 2005; Hsieh *et al.* 2007; Bolli *et al.* 2013; Ellison *et al.* 2013), the prevailing theory is that the reparative capacity of CPCs may be mediated through paracrine mechanisms that modulate immune responses and promote cell survival and angiogenesis (Stastna *et al.* 2010; Khanabdali *et al.* 2016; Der Sarkissian *et al.* 2017; Sharma *et al.* 2017).

It has been suggested that the decline in regenerative capacity of stem cells contributes to the loss of organ function and tissue homeostasis (Hariharan & Sussman, 2015). Taking advantage of the relatively short mouse lifespan, genetically modified mouse models have been used to study CPC activity during ageing (Cottage *et al.* 2010; Goichberg *et al.* 2011; Toko *et al.* 2014). For example, Hariharan *et al.* (2015) demonstrated that nucleostemin (NS) expression is lower in CPCs isolated from aged compared to young mice. Aged CPCs engineered with NS had preserved 'stemness' properties while deficiency of NS led to myocardial ageing due to telomere shortening. Ageing is also associated with accumulation of mitochondrial DNA mutations that we previously reported to lead to impaired function of CPCs (Orogo *et al.* 2015). Recent proteomics analysis of human CPCs revealed basal age-based differences, which could account for variability in the capacity of stem cells to promote myocardial recovery (Sharma *et al.* 2017). However, there is a lack of information in the literature regarding the cell biology of aged CPCs under conditions that promote differentiation.

In the present study we compared CPCs isolated from 3- and 24-month-old C57Bl/6 mice, ages that roughly correspond to <20 years and >60 years of age in humans (Ferando *et al.* 2016). We have recently demonstrated that young CPCs have the potential to upregulate markers for multiple cardiac cell types in response to dexamethasone (Dex) treatment (Castaldi *et al.* 2016). Here we demonstrate that aged CPCs not only have decreased proliferation and increased senescence compared to young CPCs, but that their ability to respond

to Dex with increased expression of cardiac lineage markers and OXPHOS mitochondrial proteins as well as mitochondrial biogenesis was repressed. In addition, aged CPCs failed to show Dex-induced upregulation of factors involved in survival and proliferation and to secrete factors important for angiogenesis. Thus, our findings demonstrate marked differences in the young and aged CPCs, basally and under conditions of differentiation, that could impact design of future CPC-based therapeutic approaches for the treatment of elderly patients suffering from cardiac injury.

Methods

Ethical approval

All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California San Diego. Male 3- and 24-month-old C57BL/6J mice were used for the isolation of c-kit$^+$ CPCs. Young mice were bred at the University of California San Diego and aged mice were obtained from the National Institute on Aging aged rodent colony (Bethesda, MD, USA).

Cardiac c-kit$^+$ cell isolation and culture

Adult c-kit$^+$ CPCs were isolated from 3 (young)- and 24 (aged)-month-old C57BL/6J mice. Three young and three aged mice were killed with CO_2 followed by cervical dislocation and CPCs were isolated and cultured in growth medium as previously described (Castaldi *et al.* 2016). For isolation, the purity of the CPC population was assessed by fluorescence-activated cell sorting analysis for c-kit+ and being haematopoietic negative at early passages (∼60–80% c-kit$^+$ at early passage and 100% by passage 15). Cells were used for experiments between passages 15 and 20. The data reported here are from independent experiments, each performed in triplicate on three young or three aged cell lines.

Immunofluorescence

CPCs were seeded on six-well tissue culture plates (10 000 cells per well) in growth medium and fixed the following day in paraformaldehyde solution 4% in PBS, permeabilized with 10% Triton X-100, and incubated overnight with LIN28 antibody 1:200 (LSBio, Seattle, WA, USA). The following day, cells were washed and incubated with secondary antibody, Alexa-fluor 488 dye 1:200 (Thermo Fisher Scientific, Waltham, MA, USA) and rhodamine phalloidin 1:200 (Invitrogen, Carlsbad, CA, USA) for 1 h. After washing, the slides were mounted using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA) and images were acquired using a Leica SP5 confocal microscope (40× oil immersion objective). Images were acquired at a focal distance of 0.49 μm and z-stacks were compressed to provide a composite image (ImageJ software analysis, plugin Bio-format). For morphological evaluation, permeabilized CPCs were stained with Phalloidin-488 (Invitrogen) for 1 h. After washing, the slides were mounted using Vectashield with DAPI (Vector Laboratories) and images were acquired using a Leica DMi8 fluorescence microscope (20× objective).

β-Galactosidase staining

CPCs were seeded on six-well tissue culture plates (10 000 cells per well) in growth medium and fixed with a paraformaldehyde solution 4% in PBS for 15 min at room temperature. Senescence-associated β-galactosidase (SA-β-Gal) was detected using the Senescence Detection Kit (Abcam, Cambridge, MA, USA) following the manufacturer's protocol, as previously described (Hariharan *et al.* 2015). Images were acquired using a Leica DMi8 microscope (20× magnification objective). Images from four independent experiments were analysed and the percentage of cells positive for SA-β-Gal was calculated for each (eight fields per experiment).

Glycolysis assay

CPCs were seeded on 12-well tissue culture plates (5000 cells per well) and serum removed the following day. Growth medium complete with 10% embryonic stem cell fetal bovine serum was added back to the cells 24 h after starvation. L-Lactate concentration in the growth medium was measured after 4 days using a glycolysis cell-based assay kit (Cayman Chemical, Ann Harbor, MI, USA) according to the manufacturer's protocol, as previously described (Orogo *et al.* 2015). CPCs were lysed in RIPA buffer and protein concentration was evaluated with Micro BCA (Thermo Fischer Scientific). L-Lactate concentration was normalized to total protein. Colorimetric measurements were made using an INFINTE M200 microplate reader (TECAN, Männedorf, Switzerland) and I-Control 1.6 Software.

ATP assay

CPCs were seeded on 10 cm culture dishes (150 000 cells per dish) and harvested the following day. ATP levels were measured using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's protocol, as previously described (Orogo *et al.* 2015). CPCs (40 000 cells/100 μl) were added to a 96-well black plate. Luminescence measurements were made using an INFINTE M200 microplate reader (TECAN) and I-Control 1.6 Software.

Proliferation assay

CPCs were seeded on a transparent 48-well tissue culture plate (8000 cells per well) cultured in growth medium. Cell growth was measured on day 0 and after 1, 2 and 3 days using a CyQUANT NF Cell Proliferation Assay (Invitrogen). Plates were assayed according to the manufacturer's protocol and as previously described (Castaldi *et al.* 2016). Fluorescence intensity was determined using an INFINTE M200 microplate reader (TECAN) and I-Control 1.6 Software.

Dexamethasone treatment

CPCs were seeded on 10 cm culture dishes (30 000 cells per dish) in growth medium. The following day medium was changed to minimum essential medium Eagle-alpha modification supplemented with 10% fetal bovine serum and Dex (10 nM). Dex was added fresh every 3 days, as previously described (Fischer *et al.* 2011). Cells were harvested on day 7 and gene/protein expression was compared to Time 0 (no Dex).

Western blot analysis

For LIN28 protein expression analysis, CPCs were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.4, 1% NP40, 1% sodium deoxycholate (NaDoc), 0.1% SDS, 2 mM EDTA, 50 mM NaF) with freshly added leupeptin (10 μg), phenylmethylsulfonyl fluoride (PMSF; 1 mM), *p*-nitrophenylphosphate (PNPP; 1 mM), Na_3VO_4 (0.2 mM) and aprotinin (0.6%; Fischer BioReagents). For mitochondrial oxidative phosphorylation proteins (OXPHOS) analysis, CPCs were lysed in lysis buffer as previously described (Orogo *et al.* 2015). In total, 30 μg of lysate was loaded on NuPAGE Bis-Tris Gels (12% for LIN28 and 10% for OXPHOS; Thermo Fisher). The membranes were probed with the following antibodies: MitoProfile Total OXPHOS Rodent WB antibody mixture 1:500 (MitoSciences, Eugene, OR, USA), LIN28 1:1000 (LSBio), Actin and α-Tubulin 1:1000 (Cell Signaling Technologies, Danvers, MA, USA). Membranes were imaged using a MyECL Imager (Thermo Fischer Scientific) and signal quantified using AlphaView SA Software.

Gene expression analysis

RNA was isolated from CPCs using Trizol (Invitrogen) following the manufacturer's protocol, cDNA synthesized with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems ABI, Foster City, CA, USA) and real-time quantitative PCR performed with TaqMan Universal Master Mix II, with UNG (Applied Biosystems ABI). IDT TaqMan probes for mouse myocyte enhancer factor 2C (MEF2C), GATA binding protein 4 (GATA4), GATA binding protein 6 (GATA6), platelet endothelial cell adhesion molecule 1 (PECAM), p16, complex IV subunit 4, NADH:ubiquinone oxidoreductase complex assembly factor 1 (NDUFAF1), NADH:ubiquinone oxidoreductase complex assembly factor 1 (NRF-1), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α), peroxisome proliferator-activated receptor gamma coactivator 1-beta (PGC1β), stromal cell derived factor-1 (SDF-1), transforming growth factor-beta (TGF-β), beta-catenin (β-catenin) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used. Relative quantification was calculated using the $\Delta\Delta$Ct method and expressed as fold change.

Endothelial tube formation assay

CPCs (young and aged) were seeded on 24-well plates (8000 cells per well) and treated with Dex as described above for 7 days to generate conditioned medium. Bovine aortic endothelial cells (75 000 cells per well) (Lonza, Walkersville, MD, USA) were seeded on Geltrex (Invitrogen)-coated 24-well dishes with conditioned media from young or aged CPCs. After 6 h, tube formation was visualized and images from six fields per well were captured using a Leica DMi8 microscope (10× magnification). The number of tubes/field was calculated, as previously described (Moc *et al.* 2015).

Statistical analyses

Researchers were blinded to the treatment group during analyses. Data are represented as means ± SEM. Differences at $P < 0.05$ were considered statistically significant and were assessed using unpaired Student's *t* test (for two groups), one-way (for multiple comparisons) or two-way (for multiple comparisons with more than one variable) ANOVA with *post hoc* Tukey analysis using GraphPad Prism software (GraphPad, La Jolla, CA, USA).

Results

c-kit$^+$ CPCs become senescent with ageing

To determine how ageing affects the functional properties of c-kit$^+$ CPCs, CPCs were isolated from 3- and 24-month-old C57BL/6 mice. These are referred to in the present work as young and aged mouse CPCs. Three separate CPC preparations, isolated from animals at either 3 or 24 months, were passaged and analysed.

There were evident morphological differences between young and aged CPCs, the former being elongated and the latter more flat (Fig. 1*A*). Flattened cell morphology can be indicative of senescence (Goichberg *et al.* 2013; Hariharan *et al.* 2015), so we investigated whether aged

CPCs presented a more senescent phenotype. Young and aged mouse CPCs were stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and the SA-β-Gal activity was detected as a blue/dark signal (Fig. 1*B*). As hypothesized, there was a significantly higher percentage of SA-β-Gal-positive cells in the aged mouse CPCs (~60%) compared to young mouse CPCs (~35%; Fig. 1*C*). In addition, aged mouse CPCs expressed higher levels of the senescence marker p16 (Fig. 1*D*). It has been shown that senescence is associated with a metabolic switch from oxidative phosphorylation to glycolysis (Feng *et al.* 2016). Therefore we analysed levels of lactate, a byproduct of glycolysis, in young and aged mouse CPCs cultured for 4 days in growth medium. Lactate content was significantly higher in the aged *versus* young CPCs (Fig. 1*E*), suggesting that cells from older animals at baseline are more glycolytic then their younger counterparts. ATP concentration was modestly but not significantly

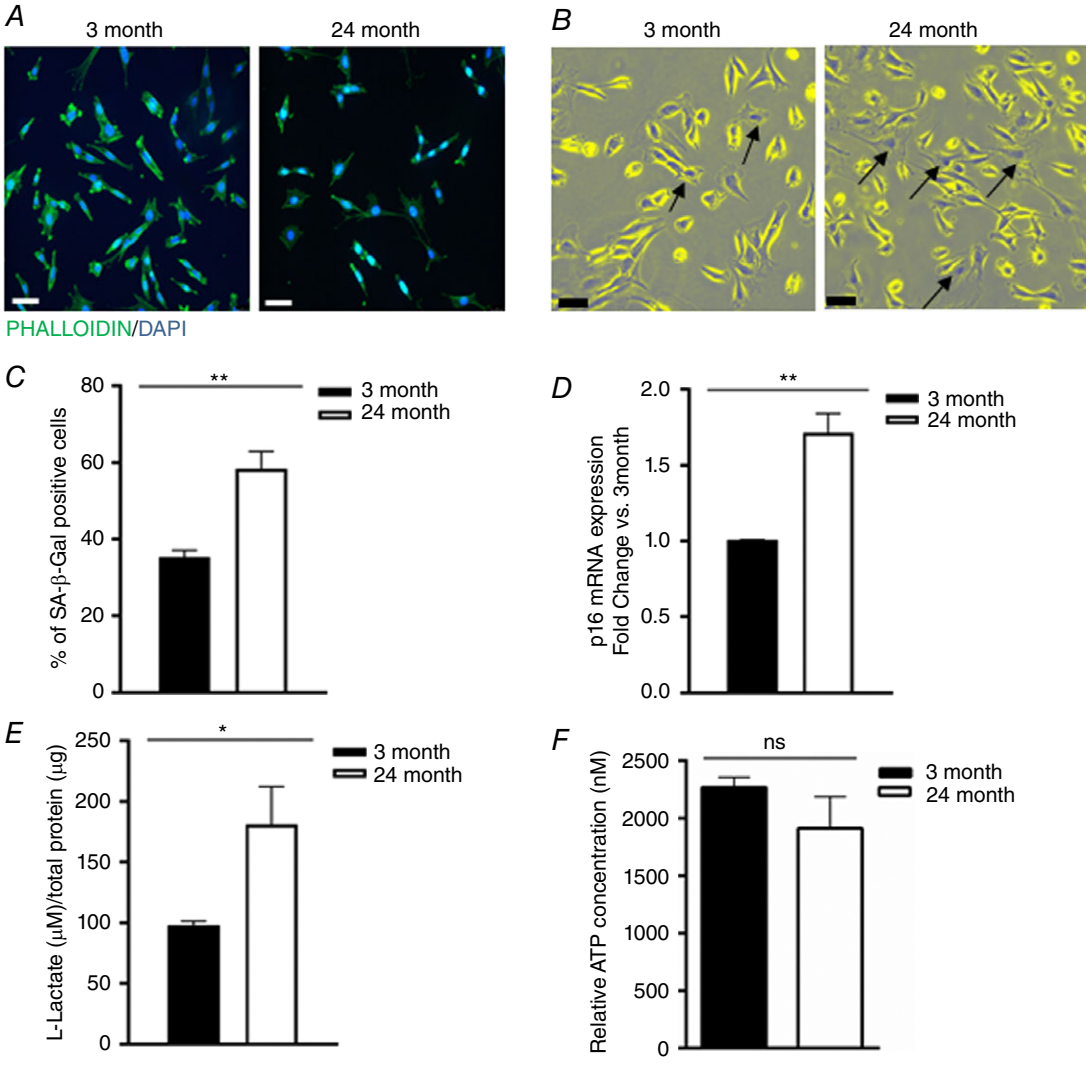

Figure 1. Aged mouse c-kit+ CPCs present a flat, senescent and glycolytic phenotype compared to young mouse CPCs
A, morphological evaluation of young (3 months) and aged (24 months) mouse CPCs: representative immunofluorescence. Cells were stained with Phalloidin (green) and DAPI (blue). Images were acquired on a Leica DMi8 microscope (20× magnification). Scale bar = 50 μm. *B*, senescence-associated expression of β-galactosidase (SA-β-Gal staining): young and aged mouse CPCs were stained with X-gal to detect endogenous β-galactosidase activity. Brightfield images were acquired on a Leica DMi8 microscope (20× magnification), Scale bar = 50 μm. *C*, quantification of the SA-β-Gal staining: eight fields per slide were counted, from three independent experiments; *t* test $^{**}P < 0.01$. *D*, mRNA expression of the senescence marker p16 in aged *vs.* young mouse CPCs; three independent experiments, *t* test $^{*}P < 0.05$. *E*, L-lactate concentration in 4 day conditioned media from young and aged mouse CPCs; three independent experiments, *t* test $^{*}P < 0.05$. *F*, ATP concentration in young and aged CPCs; three independent experiments, *t* test ns = not significant. [Colour figure can be viewed at wileyonlinelibrary.com]

lower in aged compared to young mouse CPCs (Fig. 1*F*). These data suggest that aged CPCs are more senescent and have altered function, presenting an overall phenotype distinct from that of young mouse CPCs.

Ageing impairs proliferative capacity and 'stemness' of c-kit$^+$ CPCs

To further investigate whether the biological function of CPCs is altered with ageing, we compared the proliferative capacity of young and aged CPCs in serum-containing medium. Aged CPCs proliferated at a significantly lower rate, showing a 40% decrease compared to young mouse CPCs after 2 days in serum (Fig. 2*A*). The combined decrease in proliferation and increase in markers of senescence suggested that 'stemness' might be diminished in the aged mouse CPCs. To address this we examined the expression of LIN28, an RNA binding protein involved in pluripotency and tissue repair (Zhong *et al.* 2010; Oshima *et al.* 2016) by both immunofluorescence and western blotting. Immunofluorescence was detectable but considerably lower in the aged compared to the young mouse CPCs (Fig. 2*B*, 3 month left panel, 24 month right panel). Western blotting also revealed a considerably lower level of LIN28 protein expression in aged compared to young mouse CPCs (Fig. 2*C*).

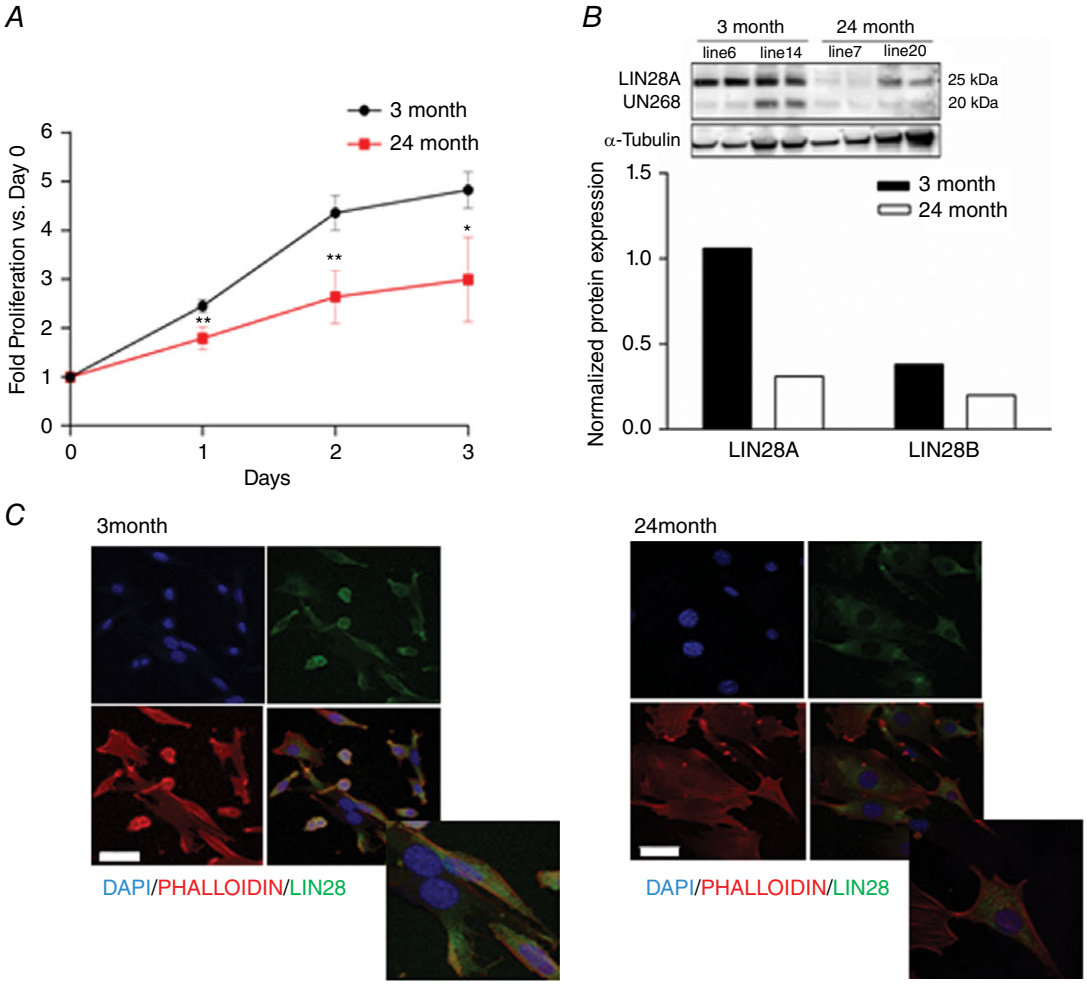

Figure 2. Aged mouse c-kit$^+$ CPCs have slower proliferation and lower levels of the stemness marker LIN28
A, proliferation of young (3 months) and aged (24 months) mouse CPCs in growth media was measured using CyQuant. Three independent experiments, *t* test *vs.* 3 months $^*P < 0.05$ $^{**}P < 0.01$. *B*, western blot analysis of LIN28A and LIN28B protein expression and quantification in young (3 months) and aged (24 months) mice; two independent lines of each were analysed in duplicate. *C*, representative immunofluorescence of young and aged mouse CPCs. Cells were stained with DAPI (blue), Phalloidin (red) and LIN28 antibody (green) and images were acquired using a Leica SP5 confocal microscope. Scale bar = 50 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

Aged CPCs fail to upregulate lineage markers in response to Dex treatment

Protocols to induce the expression of cardiac lineage markers in c-kit$^+$ CPCs in culture are well established, the most routinely used being Dex treatment (Fischer *et al.* 2011; Castaldi *et al.* 2016). We subjected young and aged CPCs to 7 day treatment with Dex and evaluated mRNA expression of cardiac lineage markers MEF2C and GATA4, as well as the vascular smooth muscle marker GATA6 and endothelial marker PECAM by quantitative PCR analysis. As demonstrated in Fig. 3, Dex increased mRNA levels for MEF2C, GATA4, GATA6 and PECAM in young CPCs by ∼3-, ∼4-, ∼7- and ∼8-fold, respectively. In contrast, aged CPCs failed to upregulate any of these lineage markers in response to Dex. There did not appear to be a generalized defect in transcriptional activity in the aged mouse CPCs, however, as tunicamycin induced the endoplasmic reticulum stress marker Grp78 to the same extent as in young mouse CPCs (data not shown).

Aged CPCs fail to increase oxidative phosphorylation and mitochondrial biogenesis in response to Dex treatment

We have previously shown that Dex treatment increases OXPHOS and mitochondrial biogenesis in CPCs (Orogo *et al.* 2015). Here, using the young mouse CPCs isolated from C57Bl/6 mice, we confirmed that OXPHOS protein levels increased following 3 and 7 days of Dex treatment (Fig. 4*A*, left panel and Fig. 4*B*, black bars). Aged mouse CPCs, by contrast, completely failed to increase expression of subunits in respiratory Complex III and Complex IV in response to Dex treatment (Fig. 4*A*, right panel and Fig. 4*B*, white bars). We also observed upregulation of mRNA for Complex IV Subunit 4 and of the mRNA for NDUFAF1 following Dex in young CPCs, responses that were markedly attenuated in aged CPCs (Fig. 4*C*). These data suggest that the aged CPCs are impaired in their capacity for transcriptional activation of genes involved in OXPHOS. Consistent with these observations, aged mouse CPCs displayed impairment in mitochondrial

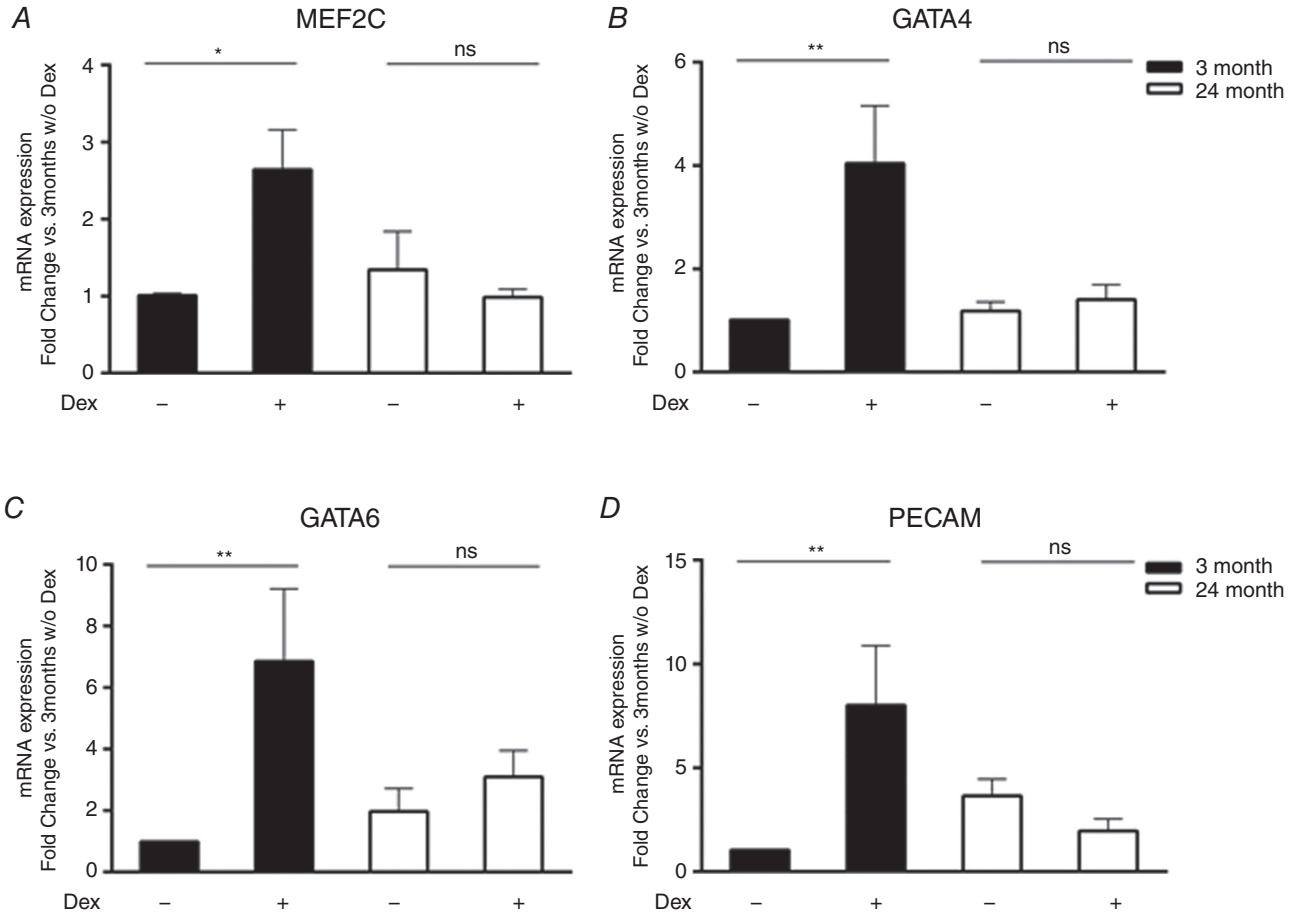

Figure 3. Cardiac lineage markers are not induced by dexamethasone (Dex) in aged mouse CPCs
Quantitative PCR analysis of cardiac lineage genes MEF2C (*A*) and GATA4 (B), smooth muscle lineage gene GATA6 (*C*) and endothelial lineage gene PECAM (*D*) in young and aged mouse CPCs treated with Dex (10 nM) for 7 days. Six independent experiments, two-way ANOVA $^*P < 0.05$, $^{**}P < 0.01$, ns = not significant.

biogenesis, as indicated by failure to upregulate NRF-1, PGC1α and PGC1β mRNAs in response to Dex treatment (Fig. 4*D*). Taken together, our findings demonstrate that aged CPCs exhibit an overall impairment in the induction of cardiac lineage commitment and a failure to induce mitochondrial biogenesis and upregulation of OXPHOS proteins following Dex.

Aged CPCs fail to upregulate paracrine factors and generate angiogenic factors in response to Dex treatment

While the cardiogenic potential of CPCs is highly debated there is a growing consensus that CPCs have the potential to secrete trophic factors involved in survival, repair or angiogenesis (van Berlo *et al.* 2014; Khanabdali *et al.* 2016; Cai & Molkentin, 2017; Der Sarkissian *et al.* 2017; Sharma *et al.* 2017). Age-dependent changes in secreted proteins from human CPCs were recently reported (Sharma *et al.* 2017), but the ability of young and aged CPCs to upregulate paracrine signalling in response to Dex-induced differentiation has not been explored. We observed a significant increase in β-catenin, TGF-β and SDF-1 mRNA expression following Dex treatment in young CPCs, which was not recapitulated in aged CPCs (Fig. 5). To determine if functionally different factors were secreted by young and aged CPCs we tested conditioned medium in an endothelial tube formation assay. Conditioned media from Dex-treated aged CPCs elicited significantly less tube formation than that from

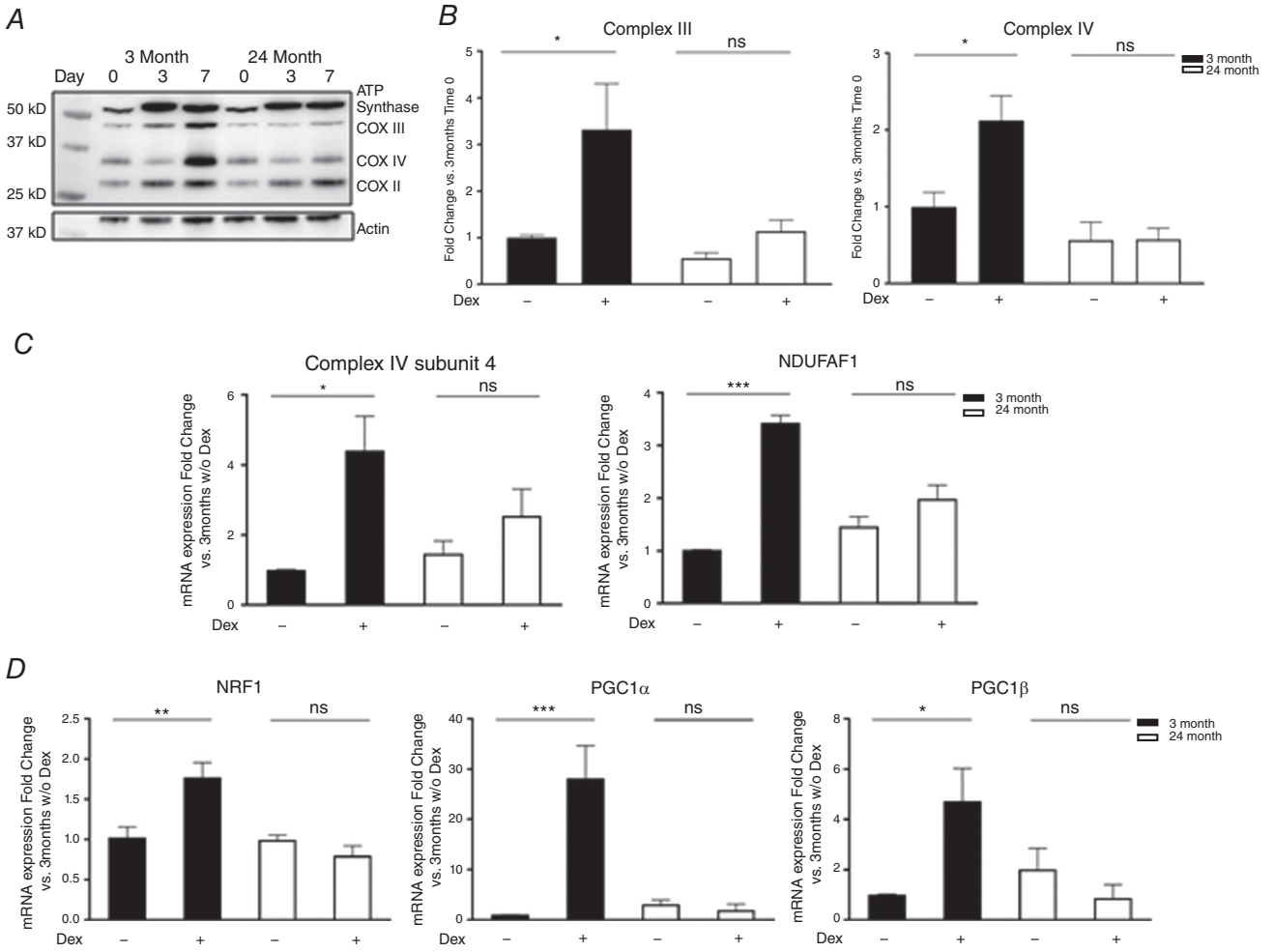

Figure 4. Aged CPCs do not increase mitochondrial OXPHOS and mitochondrial biogenesis in response to dexamethasone (Dex) treatment
A, OXPHOS protein expression in young and aged mouse CPCs treated with Dex for 3 and 7 days. Representative western blot analysis. *B*, quantification of Complex III and Complex IV protein level at time 0 and 7 days after Dex. *C* and *D*, mRNA expression levels of the complex IV subunit 4 and complex assembly factor NDUFAF1 (*C*) and of the mitochondria biogenesis markers NRF-1, PGC1α and β (*D*) by quantitative PCR in aged (24 months) *vs.* young cells (3 months), 7 days after Dex treatment. Three independent experiments; two-way ANOVA $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$.

young CPCs (Fig. 6). Thus, treatment of CPCs with Dex induces factors that are potentially important for survival and proliferation and that promote angiogenesis *in vitro,* and these responses become impaired with ageing.

Discussion

Perhaps the greatest controversy in the field of cardiac research over the past decade has been the existence and the functional role of adult resident c-kit$^+$ CPCs. Since their discovery more than 10 years ago (Beltrami *et al.* 2003), multiple research studies have been carried out to understand the potential role of these cells in cardiac regeneration following injury. CPCs isolated from rodents and porcine models as well as humans have been expanded *in vitro* and re-introduced into the injured heart with beneficial outcomes (Tang *et al.* 2010; Bolli *et al.* 2011, 2013; Li *et al.* 2011; McCall *et al.* 2012). In addition, mouse models have been used to surgically impose cardiac injury and demonstrate recruitment of endogenous stem cells along the border zone area (Williams *et al.* 2013; Quijada *et al.* 2015; Zhang *et al.* 2016). A major bias in these studies, both *in vitro* and *in vivo*, has been the use of relatively young animals. In contrast, the portion of the human population that suffers from cardiac disease and would be most likely candidates for stem cell-based therapy are mainly the elderly. Studies have been carried out using CPCs isolated from human heart biopsies from heart failure patients (Samse *et al.* 2015), but the heterogeneity between samples and the lack of a direct younger control make these less than ideal for clarifying the effect of ageing on the properties of CPCs.

The findings presented here demonstrate marked differences in CPCs isolated from hearts of young (3 months) and aged (24 months) mice. Based on a variety of factors such as sexual maturity, deterioration of locomotion and endurance, social behaviour and cognitive

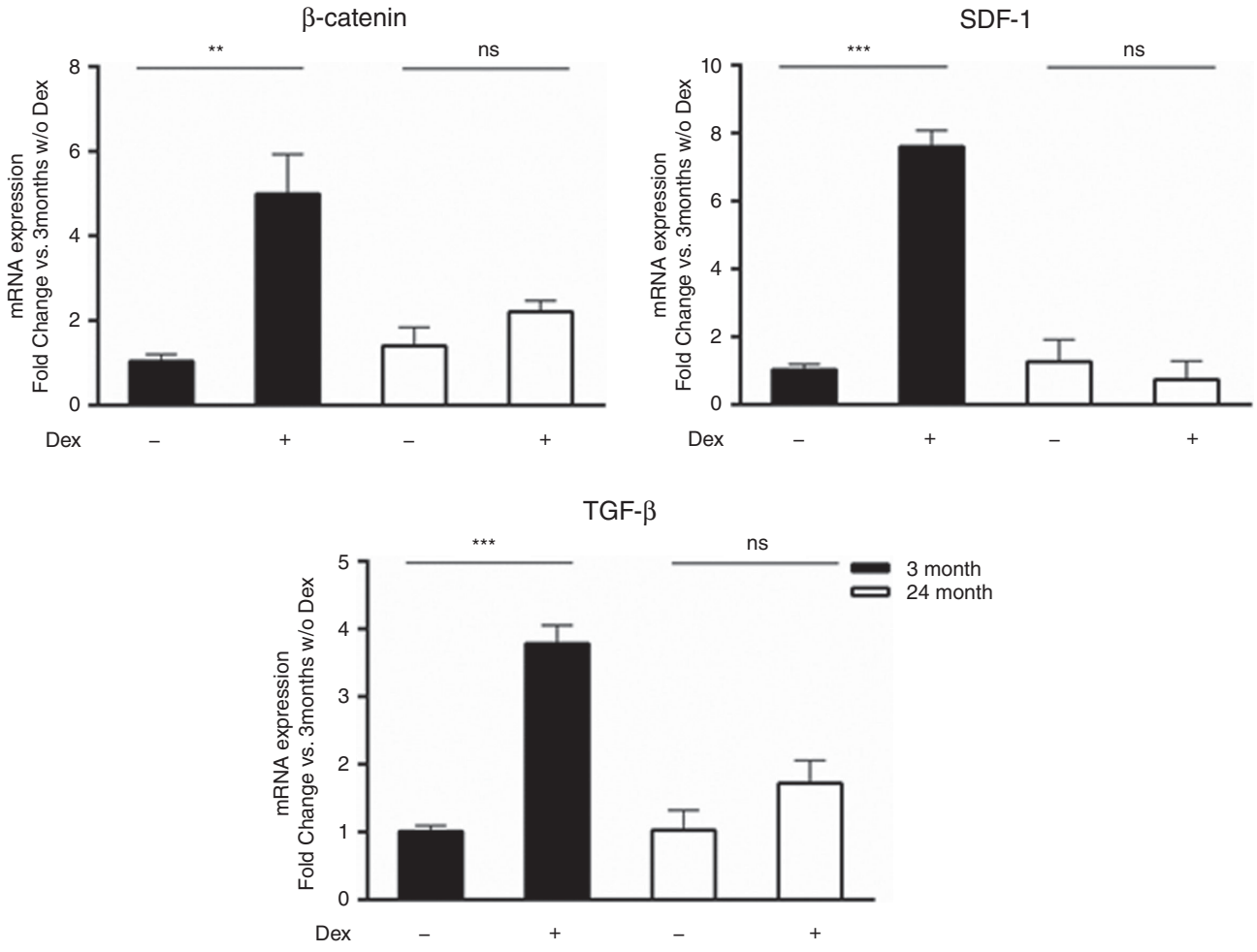

Figure 5. Paracrine factors are not induced by dexamethasone (Dex) in aged mouse CPCs
Quantitative PCR analysis of paracrine factors (β-catenin, SDF-1 and TGF-β) in young and aged mouse CPCs treated with Dex (10 nM) for 7 days. Four independent experiments, two-way ANOVA $^{**}P < 0.01$, $^{***}P < 0.005$, ns = not significant.

development, 24-month-old mice are considered the equivalent of an elderly human (Ferando *et al.* 2016). The present study is, to our knowledge, the first to explore differences between young and aged CPCs isolated from the same strain of mice and grown under identical conditions. Our data demonstrate that CPCs isolated from aged mice differ from those of young mice in (a) morphology and expression of molecular markers of senescence, (b) diminished proliferation rate and 'stemness', (c) failure to respond to Dex by induction of cardiac lineage genes or increased oxidative metabolism and (d) diminished induction of paracrine and release of angiogenic factors following Dex.

Aged mouse CPCs present a flattened morphology accompanied by increased SA-β-Gal and expression of p16 mRNA. This is in line with a previous study in which it was reported that CPCs isolated from 13-month FVBN mice presented flattened morphology and greater SA-β-Gal expression than a younger FVBN cohort (Hariharan *et al.* 2015). It has also been shown that p16 accumulates in various stem/progenitor cells including the brain, bone marrow, pancreas and heart in aged rodents (Krishnamurthy *et al.* 2004; Torella *et al.* 2004; Janzen *et al.* 2006; Molofsky *et al.* 2006; Rota *et al.* 2015). Skeletal muscle stem cells in aged mice also have elevated expression of p16; this contributes to a switch from quiescent to senescent and their lack of activation upon injury (Sousa-Victor *et al.* 2014). In human CPCs, p16 expression has been observed to increase during culture expansion (Goichberg *et al.* 2013).

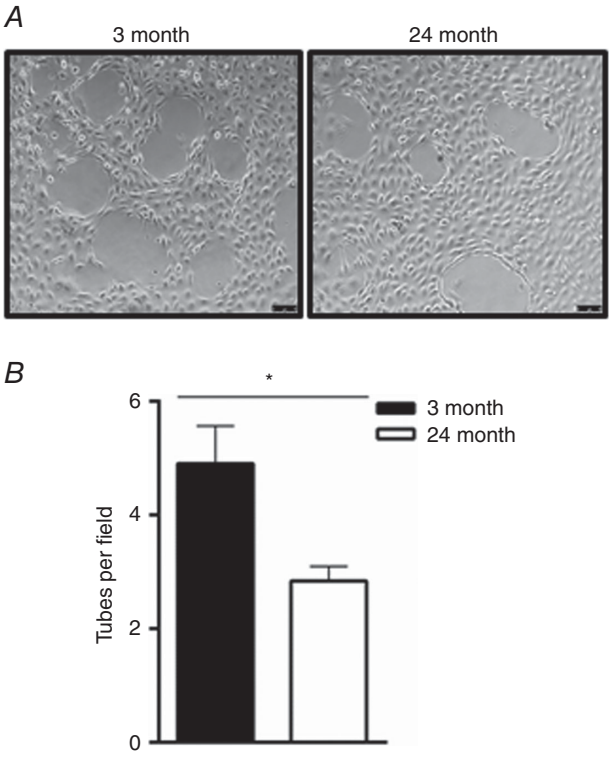

Figure 6. Conditioned medium from aged CPCs has impaired ability to induce endothelial tube formation
A, bovine aortic endothelial cells were plated in conditioned media from young (3 months) or aged (24 months) CPCs for 6 h and tube formation was visualized using a Leica DMi8 microscope (10× magnification). Scale bar = 75 μm. *B*, quantification of the number of tubes per field. Three independent experiments, *t* test $^*P < 0.05$.

From a metabolic point of view, mammalian organs such as liver, skeletal muscle and brain shift toward increased glycolysis with ageing (Feng *et al.* 2016). We report here that aged CPCs also appear to rely more on glycolysis than oxidative phosphorylation for energy production, as indicated by the increased release of lactate into the medium. Stem cells have been shown to decrease their replicative capacity during *in vitro* expansion (Wong *et al.* 2015). Here, we report a reduction in proliferative capacity that could extrapolate to donor age, as CPCs from aged mice proliferate at a rate that is 40% slower than that of young CPCs. Similar observations have been made with human mesenchymal stem cells and CPCs isolated from elderly compared with paediatric donors (Baxter *et al.* 2004; Sharma *et al.* 2017). Interestingly, we also observed a concomitant and dramatic downregulation of the 'stemness' marker LIN28 in aged CPCs, a change that may influence their overall function.

Multipotent stem cells have the potential to differentiate into various tissue-specific cell lineages. At present there is no evidence that CPCs are capable of differentiating into mature cardiac myocytes *in vitro*. They have, however, been shown to upregulate cardiac lineage markers in response to Dex treatment (Fischer *et al.* 2011; Castaldi *et al.* 2016). In this study comparing the response of young and aged CPCs to Dex treatment we show that the aged CPCs failed to upregulate cardiac lineage markers. We also discovered that aged CPCs failed to activate mitochondrial biogenesis and increase proteins involved in OXPHOS in response to Dex treatment. We have previously found that mitochondrial biogenesis and OXPHOS proteins are upregulated in young CPCs during differentiation when the differentiating cells are switching to mitochondrial respiration for more efficient ATP production (Orogo *et al.* 2015). Also, pluripotent stem cells typically switch their metabolism from glycolytic to oxidative in the transition from a quiescent to activated state (Xu *et al.* 2013; Ito & Suda, 2014). Thus, our findings in the present study suggest that the mitochondrial biogenesis response is impaired in aged CPCs. This is in agreement with our previous study on CPCs isolated from mice expressing a proofreading-deficient mitochondrial DNA polymerase γ (POLG) (Orogo *et al.* 2015). Ageing is associated with accumulation of mitochondrial DNA mutations, which may explain in part why aged CPCs resemble those from POLG mice in failing to activate mitochondrial biogenesis and increase OXPHOS proteins in response to Dex treatment.

Recently accumulated evidence suggests that the limited but documented cardiac repair observed with autologous therapy results from release of cytokines and growth factors from stem cells including CPCs (Torella *et al.* 2004; Rota *et al.* 2015; Khanabdali *et al.* 2016). SDF-1, otherwise known as CXCL12, is a chemokine that mediates recruitment and trafficking of both haematopoietic and non-haematopoietic stem cells (Abbott *et al.* 2004; Bromage *et al.* 2014). SDF-1 has been found to be secreted at high levels from patient-derived cardiosphere-derived stem cells *in vitro* (Cheng *et al.* 2014), although adult human CPCs secrete much lower levels than do neonatal cells (Sharma *et al.* 2017). TGF-β is another factor examined in various stem cell cultures in which upregulation of TGF-β has been demonstrated to promote differentiation and the Wnt/β-catenin pathway to promote expansion and survival (Goumans *et al.* 2007; Cohen *et al.* 2008; Khanabdali *et al.* 2016; Der Sarkissian *et al.* 2017). We show here that β-catenin, SDF-1 and TGF-β gene expression can be upregulated in young CPCs in response to Dex treatment whereas these responses are completely absent in aged cells. While *in vitro* observations such as those made in our studies might not mimic the physiological environment, it is important to consider that protocols for use of CPCs in clinical trials require cell isolation and expansion *in vitro* prior to re-injection into patients at the site of injury. The inability of aged CPCs to respond to proliferative and differentiation cues, or generate and release trophic factors could account for the limited regenerative potential of aged cells.

While CPCs have limited capacity to differentiate into cardiomyocytes, it has been recently theorized that these cells may be more endothelial-like (van Berlo *et al.* 2014; Cai & Molkentin, 2017) and capable of producing paracrine factors (Cai & Molkentin, 2017; Sharma *et al.* 2017). Recent studies have shown that media from human CPCs (Sharma *et al.* 2017) as well as human cardiospheres (Chimenti *et al.* 2010) can promote tube formation *in vitro*. Our study revealed that in response to differentiation signalling, young CPCs generated factors that were able to stimulate endothelial tube formation, a response that was significantly impaired in the aged CPCs. While the ability of aged CPC conditioned media to promote endothelial tube formation is clearly impaired, the limited angiogenic effect provided by aged CPCs could nonetheless afford partial benefit following ischaemic damage to the heart.

In conclusion, there are multiple previously undescribed and unanticipated differences in c-kit$^+$ CPCs isolated from young and aged mouse hearts. The more accommodating behaviour of the commonly studied young CPCs belies the more limited proliferative capacity, increased senescence and refractoriness to 'differentiation' and paracrine signalling of the aged mouse CPCs. Attempts to understand and perhaps shift the deficits seen in the aged cells may at least enhance their ability to expand *in vitro* and potentially improve their ability to express unknown factors that contribute to their reported ability to improve the failing heart.

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Additional information

Competing interests

The authors have no competing interests.

Author contributions

Experiments were performed in the Department of Pharmacology and in the Skaggs School of Pharmacy and Pharmaceutical Sciences at the University of California San Diego. JHB, NHP and ÅBG contributed to the experimental design of the work. JHB, NHP, ÅBG, AC, RMD, AMO, CMZ and RHN contributed to the acquisition, analysis or interpretation of the data. JHB, NHP and AC wrote the paper. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work and for ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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