AGE (2011) 33:107–118
DOI 10.1007/s11357-010-9166-4

Caloric restriction attenuates the age-associated increase of adipose-derived stem cells but further reduces their proliferative capacity

Eric G. Schmuck · Jacob D. Mulligan · Kurt W. Saupe

Received: 11 March 2010 / Accepted: 23 June 2010 / Published online: 14 July 2010

Abstract White adipose tissue is a promising source of mesenchymal stem cells. Currently, little is known about the effect of age and caloric restriction (CR) on adipose-derived stem cells (ASC). This is important for three reasons: firstly, age and CR cause extensive remodeling of WAT; it is currently unknown how this remodeling affects the resident stem cell population. Secondly, stem cell senescence has been theorized as one of the causes of aging and could reduce the utility of a stem cell as a reagent. Thirdly, the mechanism by which CR extends lifespan is currently not known, one theory postulates that CR maintains the resident stem cell population in youthful "fit" state. For the purpose of this study, we define ASC as lineage negative (lin^-)/$CD34^{+(low)}$/$CD31^-$. We show that aging increases the abundance of ASC and the expression of Cdkn2a 9.8-fold and Isl1 60.6-fold. This would suggest that aging causes an accumulation of non-replicative ASC. CR reduced the percentage of ASC in the lin^- SVF while also reducing colony forming ability. Therefore, CR appears to have anti-proliferative effects on ASC that may be advantageous from the perspective of cancer, but our data raises the possibility that it may be disadvantageous for regenerative medicine applications.

Keywords Stem cells · Aging · Caloric restriction · Adipose tissue · Regeneration · Cancer

E. G. Schmuck
Department of Physiology, University of Wisconsin, Madison, WI 53706, USA

J. D. Mulligan
Department of Medicine, University of Wisconsin, Madison, WI 53706, USA

K. W. Saupe (✉)
Department of Medicine, University of Wisconsin, 1300 University Ave, Madison, WI 53706, USA
e-mail: kws@medicine.wisc.edu

Introduction

Adult or "resident" stem cells are found in most organs/tissues (Alison et al. 2006; Alison and Islam 2009). Their abundance and wide tissue distribution suggests an important role in normal tissue functioning as well as in pathophysiological processes (Pardal et al. 2003; Reya et al. 2001; Sharpless and DePinho 2007). For example, dysfunction of adult stem cells has been implicated in the pathophysiology of specific types of cancers as well as in heart failure and adult onset diabetes (Butler et al. 2003; Chimenti et al. 2003; Krishnamurthy et al. 2006; Pardal et al. 2003; Reya et al. 2001; Rota et al. 2006; Sharpless and DePinho 2007; Torella et al. 2004). Given the link between alterations in adult stem cells and diseases with high morbidity, surprisingly little is known about how these cells are affected by

conditions such as aging and diet that often strongly correlate with disease. The presence of adult stem cells in a large variety of tissues also raises the question of which tissue sources of stem cells are best suited for applications in each of the many diseases where regenerative therapy may be possible. For example, damaged myocardium has been repaired with varying levels of success using satellite cells, bone marrow-derived and adipose-derived mesenchymal stem cells (MSC) (Beitnes et al. 2009; Dill et al. 2009; Hagege et al. 2003; Madonna et al. 2009; Menasche et al. 2001). Adipose-derived MSC have several characteristics which make them well suited for regenerative medicine, they are: (1) abundant, (2) easily harvested, (3) have been shown to be multipotent, (4) possesses a degree of immunoprivilege, and (5) are amenable to good manufacturing practices (Gimble et al. 2007; McIntosh et al. 2006; Zuk et al. 2002).

The present study focuses on a stem/progenitor cell population located within the stromal vascular fraction (SVF) of white adipose tissue (WAT) There have been multiple cell fractions described within the SVF displaying varying degrees of potency; for example, (1) $lin^-/CD29^+/CD34^+/Sca\text{-}1^+/CD24^+$ cells are reported to reconstitute a normal WAT depot in A-Zip lipodystrophic mice (Rodeheffer et al. 2008), (2) $Flk\text{-}1^+$ endothelial progenitor cells cultured from processed lipoaspirate in three-dimensional cell clusters (Martinez-Estrada et al. 2005), (3) $Nestin^+/ABCG2^+/SCF^+/Thy\text{-}1^+$(CD90)/$Isl\text{-}1^+$ cells differentiate into a pancreatic endocrine phenotype (Timper et al. 2006).

In addition to the above-mentioned fractions, one of particular interest is the $CD34^{+\ (low)}/CD31^-$ cell fraction. These cells have been reported to be multipotent having adipogenic, osteogenic, chondrogenic, neurogenic, and angiogenic (endothelial) capabilities (Boquest et al. 2005; Gronthos et al. 2001; Miranville et al. 2004; Planat-Benard et al. 2004; Sengenes et al. 2005; Yoshimura et al. 2006). In the present study, to ensure the cell population is of mesenchymal origin, and not blood derived, a lineage sort to remove any blood-derived cells was carried out. Thus, for the purpose of this study, $lin^-/CD34^{+\ (low)}/CD31^-$ cells will be referred to as adipose-derived stem cells (ASC).

Adipose tissue is not simply a storage depot for excess energy but instead is a labile endocrine organ that when "dysfunctional" plays a causative role in the pathophysiology of multiple diseases including diabetes and heart failure (Butler et al. 2003; Chimenti et al. 2003; Krishnamurthy et al. 2006; Rota et al. 2006; Torella et al. 2004; Trayhurn and Beattie 2001). Based on this, one might suspect that the number and functioning of the stem cell population within adipose tissue might be altered in situations where fat mass changes dramatically. Supporting this prediction is the observation that aging causes substantial changes in the size and cellular composition of WAT (Cartwright et al. 2010; Kirkland et al. 1990, 1994; Kuk et al. 2009). To date, the effects of aging on adipose stem/progenitor cells have only been studied in the non-specific heterogeneous SVF (Cartwright et al. 2010; Kirkland et al. 1990, 1994) and has yet to be described in a more specific ASC population. Of additional interest is a diet that greatly reduces the amount of WAT, a diet chronically restricted in calories (caloric restriction (CR)), extends mean and maximal life span of mammals via its anti-aging effects (Anderson et al. 2009; Mair and Dillin 2008; Weindruch 1996; Weindruch et al. 1986). To date, the effects of CR on ASC are currently not known.

Therefore, the purpose of the present study was to test the hypotheses that: (1) normal aging alters the number and/or "fitness" of ASC, and (2) a CR diet maintains ASC in a youthful "fit" state. To test these hypotheses, epididymal adipose tissue from adult and aged mice (half of each age group receiving a CR diet) were studied. The effects of advanced age and a CR diet on fundamental properties of these cells, such as their abundance, single cell clonality, expression of stem cell associated genes, and enzymatic activities, were then assessed.

Methods

Animals Mice (C57BL/6 males) age 4 months or 21–29 months were purchased from a colony maintained by the National Institute on Aging (NIA) and housed singly in an AAALAC accredited University of Wisconsin Animal Care Facility. Mice were fed either an ad libitum (ad lib) (n=24) diet or subjected to approximately 40% caloric restriction since 16 weeks of age (n=24). Mice in the adult ad lib group consumed an average of 0.55 kcal/day/g body weight while the aged ad lib group consumed an average of

0.70 kcals/day/g body weight of NIH-41 5F diet (3.4 kcals/g). All CR mice were maintained on the NIA feeding schedule of 0.39 kcals/day/g body weight of NIH-41-fortified diet (3.33 kcals/g) to ensure that they received adequate micronutrients. Mice were fed daily and body weights measured weekly. Mice were studied at an average of 9 months for adult ad lib and CR groups. Aged ad lib and CR groups were studied at 27 and 28 months, respectively.

Isolation of lineage negative SVF Mice were sacrificed via cervical dislocation. Epididymal fat pads were excised bilaterally and submerged in ice cold phosphate buffered saline (PBS). Fat pads were minced, added to freshly made digestion solution (2 mg/ml collagenase 1A (Sigma, St. Louis, MO, USA) in PBS with 2% FBS) and incubated for 35 min at 37°C with continuous agitation. Digest was then sieved through a 40 μm cell strainer and centrifuged at 1,000×*g* at 4°C for 10 min. The resultant pellet was subjected to lineage depletion using the Lineage Cell Depletion Kit (Miltenyi, Auburn, CA, USA, no. 130-090-858); cells were incubated with a panel of biotin-conjugated antibodies against blood lineage markers (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119) followed by incubation with anti-biotin-coated magnetic beads. Cells were then passed over a MACS MS column and the lineage-depleted flow-through collected.

Isolation of ASC and Colony forming assay Lineage negative ASC were stained for cell surface markers CD34 and CD31. Cells were analyzed and sorted on a FACSVantage SE instrument with FACSDiVa digital electronics (BD Biosciences, San Jose, CA, USA) at the University of Wisconsin Comprehensive Cancer Center Flow Cytometry Facility. $CD34^{+(low)}/CD31^{-}$ ASC were either sorted singly into 96-well plates containing culture medium (DMEM/F12 with 10 mM HEPES, 10% FBS, and 1% Penicillin/Streptomycin) or collected for real-time polymerase chain reaction (PCR) array analysis. Cells sorted singly into 96-well plates were cultured for 21 days with media replacement every 2–3 days. After 21 days the cells were fixed with 10% formalin and stained with Eosin Y. Wells were then examined for colonies (wells containing more than five cells). Cells sorted for real-time PCR array analysis were washed in PBS and frozen at −80°C.

Telomerase activity Lin^{-} SVF was isolated and washed with PBS; 1×10^{5}–1×10^{6} cells were suspended in 50 μl lysis buffer and incubated on ice for 30 min. The sample was centrifuged at 12,000×*g* for 30 min at 4°C. Supernatant was removed and protein concentration determined by Bradford assay. Quantitative Telomerase Detection Assay (Allied biotech Inc, Vallejo, CA, USA, no. MT3011) was used according to the manufacturer's instructions. Assay was performed with an ABI Prism 7000 (Applied Biosystems, Foster City, CA, USA) quantitative real-time PCR machine.

Senescence associated β-galactosidase assay Each well of a 24-well plate was seeded with 2×10^{3} lin^{-} SVF in growth media (DMEM/F12 with10 mM HEPES, 10% FBS, and 1% penicillin/streptomycin) and cultured for 21 days under standard culture conditions (37°C, 5% CO_2). Senescence Cells Histochemical Staining Kit (Sigma, St. Louis, MO, USA, no. CS00030) was used to stain the cells for senescence associated β-galactosidase activity according to the manufacturer's instructions. Cells were then washed with PBS and counterstained with Eosin Y for each well; 24 mm^2 (12% of the well) of each well was analyzed for both total and senescent cells using an overlaid grid.

Quantitative real-time PCR array RNA was extracted from flow cytometry-sorted $lin^{-}/CD34^{+(low)}/CD31^{-}$ cells with the PicoPure RNA Isolation kit (Arcturus, Sunnyvale, CA, USA, no. KIT0204) according to manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophometer (NanoDrop, Wilmington, DE, USA). RNA integrity number for each sample was determined with a RNA6000 PicoChip (Agilent Santa Clara, CA, USA, no. 5067-1513) run on an Agilent 2100 BioAnalyzer. Custom RT^2 Proflier PCR Arrays (SABiosciences, Frederick, MD, USA, no. CAPM09265) containing 42 genes of interest, four housekeeping genes, and two PCR controls (Table 1), were used according to manufacturer's instructions.

Statistics To assess the effects of diet and age, group comparisons were made using a two-way ANOVA with Bonferroni post-tests when indicated. A p value <0.05 was considered statistically significant. All data are expressed as mean ± SEM.

Table 1 RT-PCR array gene table

Gene	Description
Stem/progenitor	
Abcb1a	ATP-binding cassette B1A/Mdr1
Abcg2	ATP-binding cassette G2
Aldh1a1	Aldehyde dehydrogenese 1A1
lst1	lslet1
Pou5f1	POU domain 5, factor 1/Oct4
Rexo1	RNA exonuclease 1 homolog
Kdr	Kinase insert domain protein reception/Flk1
Cell cycle	
Cona2	Cyclin A2
Cond1	Cyclin D2
Cdkn2a	Cyclin-dependent kinase inhibitor 2A/p161NK4a
Mki67	Ki67
Rb1	Retinoblastoma1
Tert	Telomerase reverse transcriptase
Trp53	Transformation-related protein 53/p53
Cell fate determination	
Hdec1	Histone deacetylase 1
Jag1	Jagged 1
Notch 1	Notch gene homolog 1
Numb	Numb gene homolog
Pard6a	Partitioning defective 6 homolog alpha/Par-6
Axin1	Axin1
Wnt1	Wingless-related MMTV integration site 1
Gja1	Gap junction protein alpha 1/Connexin 43
Differentiation	
Acan	Aggrecan (chondrocyte)
Acta2	Aorta actin alpha 2 (smooth muscle)
Actc1	Cardiac actin alpha 1 (cardiac muscle)
Cd4	CD4 antigen (T cell)
Cdh5	Cadherin 5/(vascular endothelium)
Col1a1	Collagen type 1 alpha 1 (bone)
Cnn1	Calponin 1 (smooth muscle)
Gata4	GATA-binding protein 4 (cardiac muscle)
Myod1	Myogenic differentiation 1 (skeletal muscle)
Nes	Nestin (neuron)
Nkx2-5	Nk2 trascription factor related locus 5 (cardiac muscle)
Pah	Phenylalarine hydroxylase (liver)
Pparg	Peroxisome proliterator-activated receptor gamma (adipose)
Growth factors	
Bmp2	Bone morphogenic protein 2
Cxcl12	Chemolone C-X-C motif ligand 12
Hgf1	Hepalocyte growth factor
Igf1	Insulin-like growth factor 1
Aging	
Csprs	Component of Sp100-rs

Results

Aging and CR decrease the size of epididymal fat pads, but increase the number of SVF cells per milligram WAT

Aging may alter the utility of ASC use in regenerative medicine therapies by altering abundance and/or proliferation capacity of the cells. To what end an anti-aging diet, CR, could reverse the effects of aging on ASC is not known. To investigate this possibility, the SVF and ASC populations were studied. Aging and CR have been reported to have large effects on epididymal fat pad mass and the SVF (Cartwright et al. 2010; Kirkland et al. 1990, 1994; Kuk et al. 2009). Consistent with Kirkland et al.'s (1994) findings in 17- and 27-month-old rats, we found that epididymal fat pad weight was significantly reduced with age ($p \leq 0.0003$) and also CR ($p \leq 0.0001$) (Fig. 1a). Despite differences in the epididymal fat pad mass, body weights of the aged and adult cohorts did not differ significantly, while CR significantly reduced body weights equally in both age groups ($p \leq 0.001$) (Fig. 1b). Lin^- SVF abundance was not altered with aging, while CR significantly reduced this cell population ($p \leq 0.0001$) (Fig. 2a). However, if the number of lin^- SVF cells is expressed as cellular density (lin^- SVF cells per mg epididymal adipose tissue), CR ($p \leq 0.02$) and age ($p \leq 0.002$) both significantly increased cellular density (Fig. 2b) of the epididymal fat pads. Therefore, aging and CR alter not only the mass of the epididymal fat pads, but also the abundance of the lin^- SVF.

CR but not aging reduces the percentage of ASC ($lin^-/CD34^{+\ (Low)}/CD31^-$) in WAT

To determine the effects of aging and CR on the abundance of ASC, flow cytometry was used to

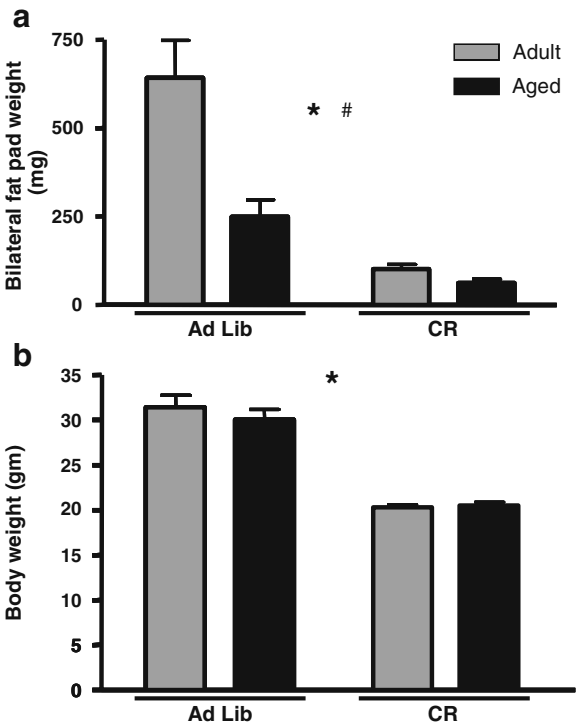

Fig. 1 Effect of age and CR on bilateral Epididymal fat pad weight (**a**) and body weights (**b**) at time of sacrifice. Mean ± SEM for n=8–12 per group. *Asterisk*, significant effect of CR, *Number sign*, significant effect of age

measure the expression of cell surface markers CD34 and CD31 in lineage negative SVF (Fig. 3a, b). CR significantly reduced the percentage of ASC in the lin$^-$ SVF from 22% to 4% ($p \leq 0.0009$) whereas age had no significant affect. The number of ASC per mg WAT was then calculated (percentage × lin$^-$ SVF per mg tissue) (Fig. 4). Although not significant by two-way ANOVA, there is a trend toward an increase in the abundance of ASC with age that is attenuated with CR.

Aging and CR reduce colony formation in ASC

It has been previously reported that aging reduced the proliferative capacity of plastic adherent non-adipocytes (Kirkland et al. 1990). To what extent the resident ASC population is effected is currently not known. To determine how age and CR affect colony formation of freshly isolated ASC, fluorescence-activated cell sorting was used to deposit one freshly isolated ASC per well in 96-well plates (Fig. 5). Colony formation rates were as follows: adult ad lib 11.5% (113 colonies/1152 total wells), aged ad lib 7.7% (89 colonies/1,152 total wells), adult CR 2.7% (15 colonies/559 total wells), aged CR 0.8% (five colonies/621 total wells). CR ($p \leq 0.0001$) and age ($p \leq 0.04$) significantly reduced the rate of spontaneous colony formation. CR reduced colony formation by 81% in the adult groups and 89% in the aged groups. Aging reduced colony formation by 33% in the ad lib and 62% in the CR groups. Therefore aging reduces the single cell clonality (proliferation) of ASC, which is further reduced by CR.

CR has mixed effects on enzymatic activities associated with aging in lin$^-$ SVF

Senescence associated β-galactosidase (SA β-gal) has long been used to identify senescence cells in culture (Dimri et al. 1995; Serrano et al. 1997; van der Loo et al. 1998). To determine how age and CR affect

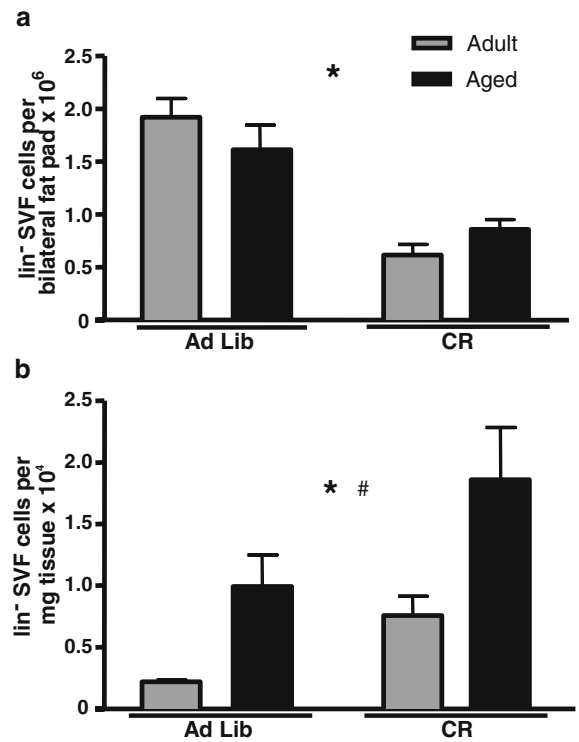

Fig. 2 Effect of age and CR on lin$^-$ SVF cells per bilateral fat pad (**a**) and lin$^-$ cells per mg tissue (**b**). Despite a decrease in total lin$^-$ SVF cells with CR, when adjusted for the total amount of tissue, CR significantly increased the lin$^-$ cellular density of the white adipose tissue. Mean ± SEM for n=8-12 per group. *Asterisk*, significant effect of CR; *number sign*, significant effect of age

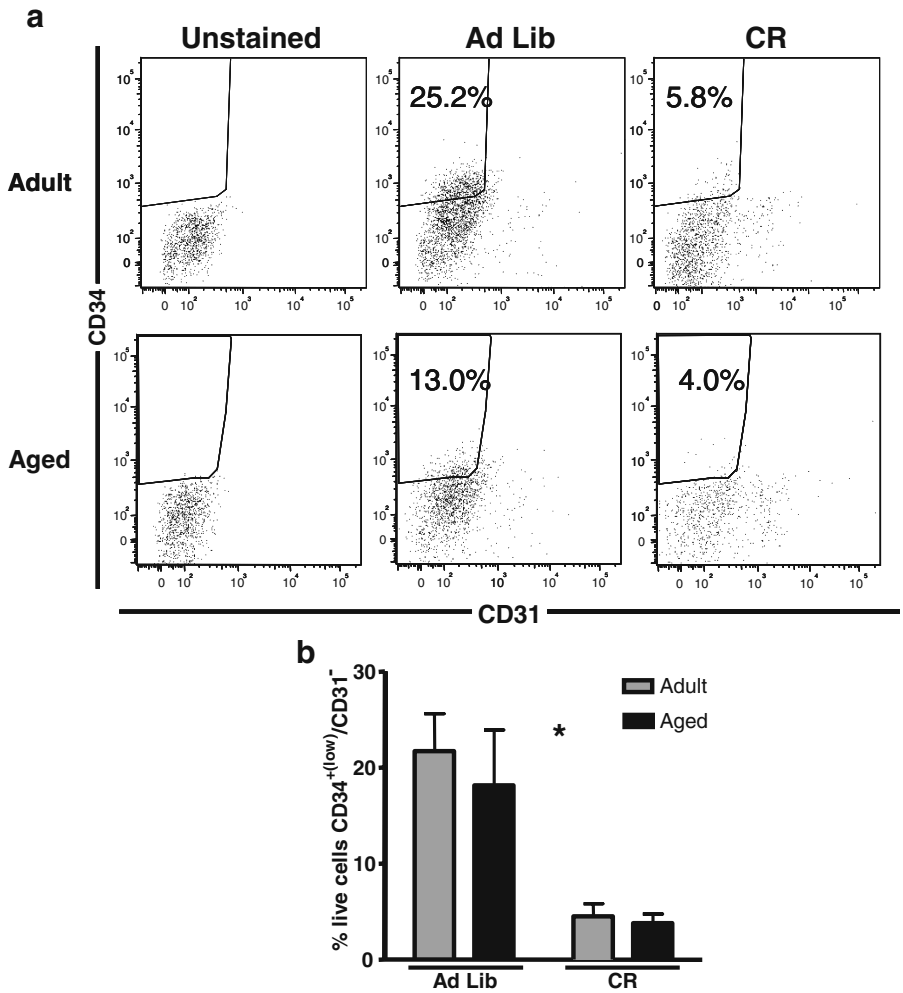

Fig. 3 Effect of age and CR on lin$^-$/CD34$^{+(low)}$/CD31$^-$ cell population. **a** Representative flow cytometry plots demonstrating the effects of age and CR on percentage of ASC in the SVF. Gating was determined by unstained and single stained cell populations. **b** percentage of live lin$^-$ cells expressing the marker CD34 but not CD31. Mean ± SEM for n=5–8 per group. *Asterisk*, significant effect of CR

expression of SA β-gal in lin$^-$ SVF, a SA β-gal assay was performed (Fig. 6a–c). Age significantly increased the percentage of cells expressing SA β-gal ($p \leq 0.01$), while CR did not have an effect.

Telomerase activity, an indicator of replicative capacity, was assessed with real-time quantitative PCR in the lin$^-$ SVF. Compared with the adult ad lib group (Fig. 7), aged ad lib (128-fold), adult CR (10.6-fold), and aged CR (2.6-fold) groups had decreased telomerase activity. In the aged cohort, CR increased telomerase activity by 48.5-fold relative to the ad lib group. These results are consistent with reports that aging decreases the proliferative potential of plastic adherent non-adipocytes from WAT. Additionally, these findings indicate that within the lin$^-$ SVF of the aged cohort, CR is preserving the replicative capacity of some cell population(s).

ASC gene expression does not change greatly with aging or CR

To determine how age and CR affect gene expression associated with key stem cell characteristics such as potency, proliferation and differentiation, custom real-time RT-PCR arrays containing 42 genes were used (Fig. 8a). A volcano plot comparing adult ad lib to aged groups revealed that age (Fig. 8b) caused a 9.77-fold increase in Cdkn2a and a 60.55-fold increase in

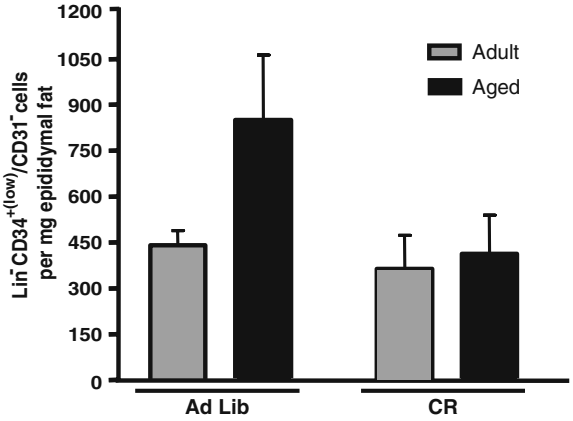

Fig. 4 Effect of age and CR on the number of ASC per mg tissue. ASC numbers were calculated from the SVF cell density and the percentage of ASC in the SVF. Two-way ANOVA did not reveal significance between the groups, but age did cause a trend toward an increase number of ASC

Isl1, Wnt1 was expressed in the aged ad lib but not the adult ad lib group. A volcano plot comparing aged ad lib with aged CR groups revealed that CR (Fig. 8c) did not cause a significant change in gene expression compared to the aged ad lib group. Mki67 and Ccnd1 expression was observed in the aged ad lib group but not in the aged CR group. These results are consistent with findings in plastic adherent non-adipocytes that only a small percentage of genes change with aging (Cartwright et al. 2010).

Discussion

Adipose tissue is a promising source of MSC for use in autologous and allogeneic regenerative therapy (McIntosh et al. 2006; Nakagami et al. 2006). Reasons for this include the observations that MSC derived from adipose tissue are abundant, easily, harvested, are multipotent, possess a degree and immunoprivilege, and are amenable to good manufacturing practices (Gimble et al. 2007; McIntosh et al. 2006; Zuk et al. 2002). However, as is the case with any tissue from which MSC are extracted, the effects of factors that "remodel" the tissue need to be evaluated. This is particularly true for MSC derived from adipose tissue since changes in the anatomy, histology, cellular composition, and endocrine output occur with routine biological events such aging and changes in diet (Anderson et al. 2009; Kuk et al. 2009; Torella et al. 2004; Zhu et al. 2007). Accordingly, our goal was to determine if aging alters fundamental characteristics of ASC that would be expected to impact their clinical utility, and if so, could the age-associated effects of aging be attenuated by an anti-aging diet.

Aging

Consistent with previous reports in plastic adherent non-adipocytes (Kirkland et al. 1994; Wu et al. 2007), our data demonstrate an age-associated increase in the density of the lin$^-$ SVF (number of cells/g epididymal fat) within white adipose tissue. However, our study extends these results to a specific ASC population. Although not significant by two-way ANOVA, our results indicate that aging causes a trend toward an increase in ASC density. The biological significance of a higher density of ASC, i.e., whether it is adaptive or maladaptive, can only be inferred by examining the cells in more detail. To this end, we found that single cell (ASC) clonality was decreased 33% with aging. The finding of a decrease in proliferation with aging is consistent with reports in plastic adherent non-adipocytes as well as several other progenitor cell populations (Djian et al. 1983; Kirkland et al. 1990).

Recent reports by de Girolamo et al. and Zhu et al. investigating the effects of age on human ASC (hASC)

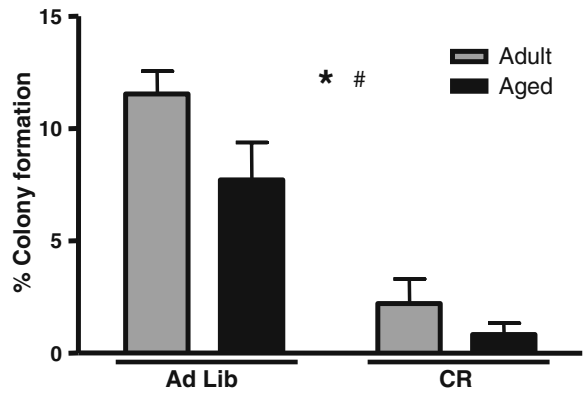

Fig. 5 Effect of age and CR on colony formation. Lin$^-$/CD34$^{+(low)}$/CD31$^-$ cells were plated at one cell per well using a FACSVantage SE instrument and allowed to grow culture for 21 days before staining with eosin Y, 93–288 individual wells per mouse were analyzed depending on the number of cells recovered. A significant reduction in colony formation was observed with both age ($p \leq 0.04$) and CR ($p \leq 0.0001$). Mean ± SEM for $n=5$–8 mice per group. *Asterisk*, significant effect of CR; *number sign*, significant effect of age

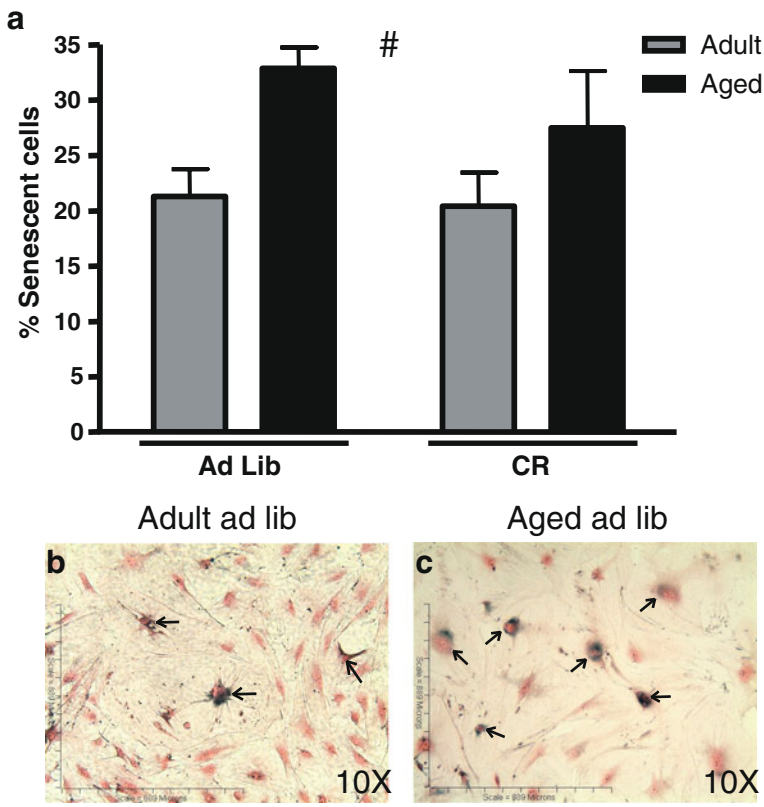

Fig. 6 Effect of age and CR on expression of senescence associated -β-galactosidase. **a** A 2×10^3 lin^- SVF cells were plated into each well of a 24-well plate and grown in culture for 21 days. Senescence Cells Histochemical Staining Kit was used to probe for SA-β-galactosidase activity and 24 mm^2 of each well analyzed for expression. A significant increase in SA-β-galactosidase activity was observed with age ($p\leq$ 0.01). CR did not significantly affect the expression of SA-β-galactosidase activity. **b**–**c** Representative examples of SA-β-galactosidase staining. *Arrows* denote positive staining for SA-β-galactosidase. Mean ± SEM for n=5–8 mice per group. *Number sign*, significant effect of age

(plastic adherent non-adipocytes) have yielded consistent data with ours regarding an increase in ASC cell density (de Girolamo et al. 2009). Additionally, these studies demonstrate a reduction in the multipotential of hASC with age. Specifically, hASC have a reduced capacity for osteogenic lineage differentiation, while maintaining adipogenic potential (de Girolamo et al. 2009; Zhu et al. 2009). The mechanism for the reduction in osteogenic lineage differentiation is unknown, but appears not to be a reduction in the number of osteoprogenitors.

Gene expression analysis on 42 genes related to potency, proliferation and differentiation indicated that only a small percentage of genes (two genes) reached our criteria of a change greater than 2-fold and a p value <0.05 to be considered significantly altered with aging. This result was consistent with a report by Cartwright et al. investigating gene expression in preadipocytes (plastic adherent non-adipocytes) (Cartwright et al. 2010). Cdkn2a, which induces cell cycle arrest and Isl1, a mesenchymal stem cell marker, were increased 9.8- and 60.6-fold, respectively, with age. The up regulation of Cdkn2a is consistent with the decrease in clonality, while the increase in Isl1, which is a transcription factor shown to confer multipotential to mesenchymal stem cells (Bu et al. 2009; Eberhardt et al. 2006; Lin et al. 2006), is difficulat to interpret. We speculate that the up regulation of Isl1 could correlate with the increase in lin^- SVF and ASC, or it could indicate increased multipotential or differentiation of the ASC population. Taken together with our data from the lin^- SVF which demonstrated an increase in the biochemical marker of senescence, SA-β galactosidase, and a decrease in telomerase activity (a marker of cellular youth and proliferative capacity), it would seem more likely that aging causes an accumulation of what are likely non-replicative ASC in the epididymal fat pad.

Caloric restriction

Currently, the only known non-genetic manipulation capable of extending maximal lifespan across a large range of species is CR, restriction of caloric intake without malnutrition (Weindruch 1996; Weindruch et

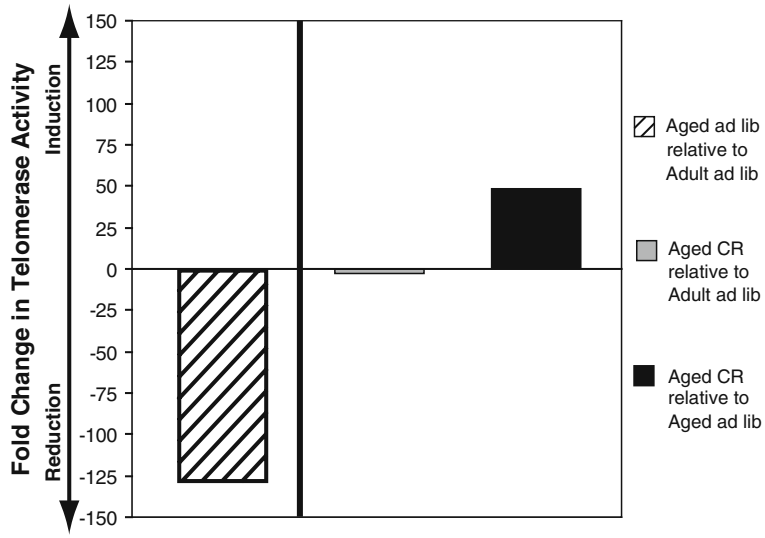

Fig. 7 Effect of age and CR on telomerase activity. Telomerase activity in the lin$^-$ SVF was detected using real-time quantitative PCR. Aging caused a 128-fold reduction in telomerase activity in mice fed ad lib. CR almost completely eliminated this effect of age. CR caused a 48.5-fold induction of telomerase activity in the aged CR relative to the aged ad lib group

al. 1986). While the exact mechanism(s) by which this lifespan extension occurs remain unclear, it has been established that CR causes not only a potent anti-cancer effect, but also a specific anti-aging effect that can be seen on both cellular and transcriptional levels. These mechanism(s) may involve resident stem cell populations. For example, CR could preserve the resident adult stem cell population in a "youthful state" allowing them to maintain proper tissue homeostasis for a longer period of time and thus extend lifespan. Alternatively, the effects of CR may reduce stem cell proliferation, effectively keeping them in a prolonged quiescent state, thus contributing to the potent anti-cancer effect of CR. Given the dramatic remodeling of white adipose tissue induced by CR from the anatomical to molecular levels we hypothesized that a CR diet would maintain the stem cells in a youthful "fit", state. In fact the effects of CR on ASC were more complex than anticipated. Specifically, CR increased the density of lin$^-$ SVF while attenuating the trend of an age-associated increase in ADCS abundance. This attenuation coincided with a decrease in clonality to 10% and 7% of levels in adult and aged ad lib groups respectively. These results would appear to be consistent with the stem cell population being maintained in a quiescent state. We speculate that this is further supported by gene expression analysis indicating that genes involved in cell cycle regulation, specifically, Mki67 and Ccnd1 were not expressed at detectable levels in the aged CR mice. Mki67 is expressed during all phases of cell proliferation while Ccnd1 is responsible for the transition from G1 to S phase of the cell cycle (Blagosklonny and Pardee 2002). This could indicate that the cells are maintained in a quiescent state. Coupled with our finding in lin$^-$ SVF showing that telomerase activity, a marker of cellular youth, is at near adult ad lib levels in the aged CR group, it would seem most likely that CR maintains the ASC population in a quiescent state. This effect would be consistent with studies demonstrating that CR decreases the proliferation rates of dividing non-stem cells such as keratinocytes, mammary epithelial cells and T cells (Hsieh et al. 2005).

The idea that CR maintains stem cell population in a quiescent state is consistent with the idea that CR reduces the rate of cellular turnover. By reducing stem cell cycling (cell division), CR reduces the possibility of acquiring errors during replication, thus contributing to the potent anti-cancer effects. Additionally, this data raises the possibility that CR may be disadvantageous for regenerative medicine applications.

There are limitations of this study that merit mention. Specifically, when adequate numbers of ASC could be obtained, these cells were studied; at other times lin$^-$ SVF was studied when larger cell numbers were needed. Additionally, studies were conducted in freshly isolated or rapidly frozen primary cells except the colony forming assay and the SA-β-galactosidase assays which required 21 days in standardized culture (non-native milieu) conditions following primary isolation (frozen cells were never

a

	Fold Regulation	
	Adult ad lib vs Aged ad lib	Aged ad lib vs Aged CR
Abcb1a	1.92	1.24
Abcg2	No Expresion	No Expresion
Acan	No Expresion	No Expresion
Acta2	2.34	-1.93
Actc1	No Expresion	No Expresion
Aldh1a1	1.74	3.36
Axin1	1.47	1.87
Bmp2	2.31	1.10
Ccna2	1.32	-1.86
Ccnd1	-1.29	No Expresion-Aged CR
Cd4	-1.14	4.00
Cdh5	-1.35	2.76
Cdkn2a	9.77	-1.30
Cnn1	3.03	-1.16
Col1a1	-1.33	1.07
Csprs	1.48	-2.03
Cxcl12	2.13	1.28
Gata4	5.97	-2.70
Gja1	1.32	-1.11
Hdac1	1.03	-1.24
Hgf	3.78	-1.01
Igf1	2.92	-1.40
Isl1	60.55	2.23
Jag1	1.01	2.17
Kdr	1.29	5.94
Mki67	-1.09	No Expresion-Aged CR
Myod1	No Expresion	No Expresion
Notch1	1.71	2.04
Nes	-2.40	2.78
Nkx2-5	No Expresion	No Expresion
Numb	-1.21	1.85
Pah	-1.26	2.51
Pard6a	1.18	6.63
Pou5f1	-2.47	1.33
Pparg	3.34	2.09
Prom1	No Expresion	No Expresion
Rb1	1.29	1.54
Rexo1	No Expresion	No Expresion
Sox2	No Expresion	No Expresion
Tert	1.16	-1.47
Trp53	-1.28	1.10
Wnt1	No Expresion-adult ad lib	No Expresion

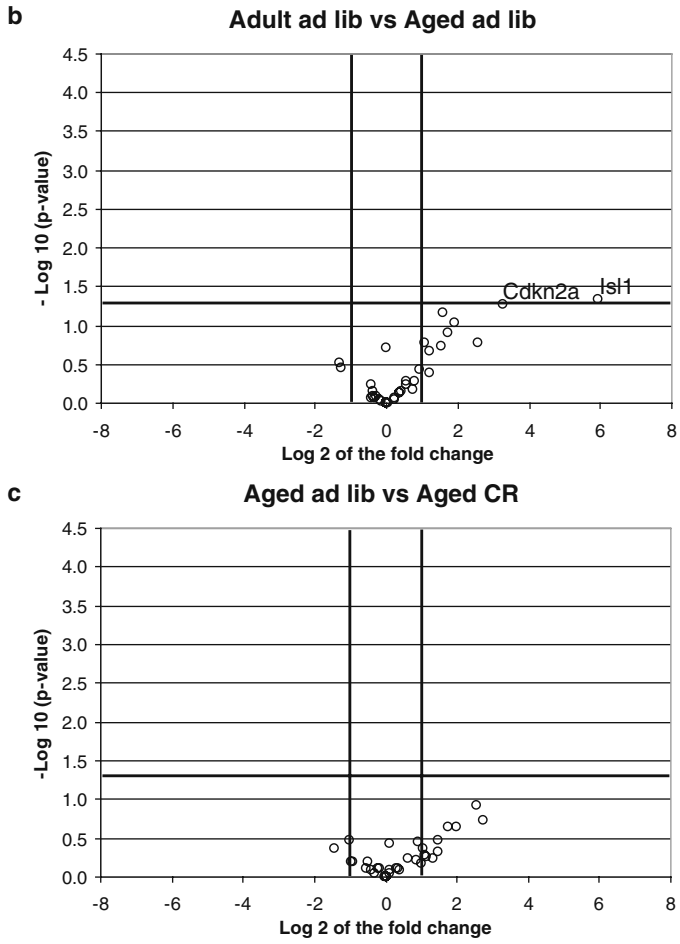

Fig. 8 qRT-PCR gene arrays. **a** Fold change of the 42 genes tested. **b**–**c** Volcano plots detected that two genes were significantly up regulated with age, Cdkn2a and Isl1, while Wnt1 was expressed in the aged ad lib, but not the adult ad lib group. Additionally, Mki67 and Ccnd1 where expressed in the aged ad lib group, but not the aged CR (n=4 per group). The bold horizontal line represents a p=0.05, while the *bold vertical lines* represent a 2-fold change in gene expression

used for culture). Therefore the possibility exists that some of the aging or CR phenotype could have been lost when cells were cultured for extended periods of time. One additional limitation of note should be mentioned here. It has been shown that there are differences between visceral and subcutaneous adipose tissue deposits (Cartwright et al. 2010; Kirkland et al. 1990, 1994). Although subcutaneous adipose tissue may be the most likely source of ASC in clinical uses, CR reduces subcutaneous fat mass to levels that are technically challenging to study. Thus we chose to study the visceral epididymal fat pad, which although significantly remodeled yielded adequate cells to conduct our experiments.

Conclusions/Summary

While white adipose tissue is a promising source of MSC the effects of white adipose tissue remodeling factors such as aging and diet on these cells are unknown. We found that aging causes accumulation

of non-replicative ASC. CR attenuated the age-associated increase in ASC abundance, but decreased clonality to 10% and 7% of levels in adult and aged ad lib groups respectively. Therefore, CR appears to have anti-proliferative effects on ASC that may be advantageous from the perspective of cancer, but our data raises the possibility that it may be disadvantageous for regenerative medicine applications.

Acknowledgments This study was supported by the National Heart, Lung, and Blood Institute grant number 1R21HL092477 and the National Institutes of Health, under Ruth L. Kirschstein National Research Service Award T32 HL 07936 from the National Heart Lung and Blood Institute to the University of Wisconsin-Madison Cardiovascular Research Center.

References

Alison MR, Islam S (2009) Attributes of adult stem cells. J Pathol 217:144–160

Alison MR, Brittan M, Lovell MJ, Wright NA (2006) Markers of adult tissue-based stem cells. Handb Exp Pharmacol 174:185–227

Anderson RM, Shanmuganayagam D, Weindruch R (2009) Caloric restriction and aging: studies in mice and monkeys. Toxicol Pathol 37:47–51

Beitnes JO, Hopp E, Lunde K, Solheim S, Arnesen H, Brinchmann JE, Forfang K, Aakhus S (2009) Long-term results after intracoronary injection of autologous mononuclear bone marrow cells in acute myocardial infarction: the ASTAMI randomised, controlled study. Heart 95:1983–1989

Blagosklonny MV, Pardee AB (2002) The restriction point of the cell cycle. Cell Cycle 1:103–110

Boquest AC, Shahdadfar A, Fronsdal K, Sigurjonsson O, Tunheim SH, Collas P, Brinchmann JE (2005) Isolation and transcription profiling of purified uncultured human stromal stem cells: alteration of gene expression after in vitro cell culture. Mol Biol Cell 16:1131–1141

Bu L, Jiang X, Martin-Puig S, Caron L, Zhu S, Shao Y, Roberts DJ, Huang PL, Domian IJ, Chien KR (2009) Human ISL1 heart progenitors generate diverse multipotent cardiovascular cell lineages. Nature 460:113–117

Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC (2003) Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. Diabetes 52:102–110

Cartwright MJ, Schlauch K, Lenburg ME, Tchkonia T, Pirtskhalava T, Cartwright A, Thomou T, Kirkland JL (2010) Aging, depot origin, and preadipocyte gene expression. J Gerontol A Biol Sci Med Sci 65A:242–251

Chimenti C, Kajstura J, Torella D, Urbanek K, Heleniak H, Colussi C, Di Meglio F, Nadal-Ginard B, Frustaci A, Leri A et al (2003) Senescence and death of primitive cells and myocytes lead to premature cardiac aging and heart failure. Circ Res 93:604–613

de Girolamo L, Lopa S, Arrigoni E, Sartori MF, Baruffaldi Preis FW, Brini AT (2009) Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during in vitro osteoblastic differentiation. Cytotherapy 11:793–803

Dill T, Schachinger V, Rolf A, Mollmann S, Thiele H, Tillmanns H, Assmus B, Dimmeler S, Zeiher AM, Hamm C (2009) Intracoronary administration of bone marrow-derived progenitor cells improves left ventricular function in patients at risk for adverse remodeling after acute ST-segment elevation myocardial infarction: results of the Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction study (REPAIR-AMI) cardiac magnetic resonance imaging substudy. Am Heart J 157:541–547

Dimri GP, Lee X, Basile G, Acosta M, Scott G, Roskelley C, Medrano EE, Linskens M, Rubelj I, Pereira-Smith O et al (1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc Natl Acad Sci USA 92:9363–9367

Djian P, Roncari AK, Hollenberg CH (1983) Influence of anatomic site and age on the replication and differentiation of rat adipocyte precursors in culture. J Clin Invest 72:1200–1208

Eberhardt M, Salmon P, von Mach MA, Hengstler JG, Brulport M, Linscheid P, Seboek D, Oberholzer J, Barbero A, Martin I et al (2006) Multipotential nestin and Isl-1 positive mesenchymal stem cells isolated from human pancreatic islets. Biochem Biophys Res Commun 345:1167–1176

Gimble JM, Katz AJ, Bunnell BA (2007) Adipose-derived stem cells for regenerative medicine. Circ Res 100:1249–1260

Gronthos S, Franklin DM, Leddy HA, Robey PG, Storms RW, Gimble JM (2001) Surface protein characterization of human adipose tissue-derived stromal cells. J Cell Physiol 189:54–63

Hagege AA, Carrion C, Menasche P, Vilquin JT, Duboc D, Marolleau JP, Desnos M, Bruneval P (2003) Viability and differentiation of autologous skeletal myoblast grafts in ischaemic cardiomyopathy. Lancet 361:491–492

Hsieh EA, Chai CM, Hellerstein MK (2005) Effects of caloric restriction on cell proliferation in several tissues in mice: role of intermittent feeding. Am J Physiol Endocrinol Metab 288:E965–E972

Kirkland JL, Hollenberg CH, Gillon WS (1990) Age, anatomic site, and the replication and differentiation of adipocyte precursors. Am J Physiol 258:C206–C210

Kirkland JL, Hollenberg CH, Kindler S, Gillon WS (1994) Effects of age and anatomic site on preadipocyte number in rat fat depots. J Gerontol 49:B31–B35

Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. Nature 443:453–457

Kuk JL, Saunders TJ, Davidson LE, Ross R (2009) Age-related changes in total and regional fat distribution. Ageing Res Rev 8:339–348

Lin L, Bu L, Cai CL, Zhang X, Evans S (2006) Isl1 is upstream of sonic hedgehog in a pathway required for cardiac morphogenesis. Dev Biol 295:756–763

Madonna R, Geng YJ, De Caterina R (2009) Adipose tissue-derived stem cells: characterization and potential for

cardiovascular repair. Arterioscler Thromb Vasc Biol 29:1723–1729

Mair W, Dillin A (2008) Aging and survival: the genetics of life span extension by dietary restriction. Annu Rev Biochem 77:727–754

Martinez-Estrada OM, Munoz-Santos Y, Julve J, Reina M, Vilaro S (2005) Human adipose tissue as a source of Flk-1 + cells: new method of differentiation and expansion. Cardiovasc Res 65:328–333

McIntosh K, Zvonic S, Garrett S, Mitchell JB, Floyd ZE, Hammill L, Kloster A, Di Halvorsen Y, Ting JP, Storms RW et al (2006) The immunogenicity of human adipose-derived cells: temporal changes in vitro. Stem Cells 24:1246–1253

Menasche P, Hagege AA, Scorsin M, Pouzet B, Desnos M, Duboc D, Schwartz K, Vilquin JT, Marolleau JP (2001) Myoblast transplantation for heart failure. Lancet 357:279–280

Miranville A, Heeschen C, Sengenes C, Curat CA, Busse R, Bouloumie A (2004) Improvement of postnatal neovascularization by human adipose tissue-derived stem cells. Circulation 110:349–355

Nakagami H, Morishita R, Maeda K, Kikuchi Y, Ogihara T, Kaneda Y (2006) Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy. J Atheroscler Thromb 13:77–81

Pardal R, Clarke MF, Morrison SJ (2003) Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3:895–902

Planat-Benard V, Silvestre JS, Cousin B, Andre M, Nibbelink M, Tamarat R, Clergue M, Manneville C, Saillan-Barreau C, Duriez M et al (2004) Plasticity of human adipose lineage cells toward endothelial cells: physiological and therapeutic perspectives. Circulation 109:656–663

Reya T, Morrison SJ, Clarke MF, Weissman IL (2001) Stem cells, cancer, and cancer stem cells. Nature 414:105–111

Rodeheffer MS, Birsoy K, Friedman JM (2008) Identification of white adipocyte progenitor cells in vivo. Cell 135:240–249

Rota M, LeCapitaine N, Hosoda T, Boni A, De Angelis A, Padin-Iruegas ME, Esposito G, Vitale S, Urbanek K, Casarsa C et al (2006) Diabetes promotes cardiac stem cell aging and heart failure, which are prevented by deletion of the p66shc gene. Circ Res 99:42–52

Sengenes C, Lolmede K, Zakaroff-Girard A, Busse R, Bouloumie A (2005) Preadipocytes in the human subcutaneous adipose tissue display distinct features from the adult mesenchymal and hematopoietic stem cells. J Cell Physiol 205:114–122

Serrano M, Lin AW, McCurrach ME, Beach D, Lowe SW (1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. Cell 88:593–602

Sharpless NE, DePinho RA (2007) How stem cells age and why this makes us grow old. Nat Rev Mol Cell Biol 8:703–713

Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Muller B, Zulewski H (2006) Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. Biochem Biophys Res Commun 341:1135–1140

Torella D, Rota M, Nurzynska D, Musso E, Monsen A, Shiraishi I, Zias E, Walsh K, Rosenzweig A, Sussman MA et al (2004) Cardiac stem cell and myocyte aging, heart failure, and insulin-like growth factor-1 overexpression. Circ Res 94:514–524

Trayhurn P, Beattie JH (2001) Physiological role of adipose tissue: white adipose tissue as an endocrine and secretory organ. Proc Nutr Soc 60:329–339

van der Loo B, Fenton MJ, Erusalimsky JD (1998) Cytochemical detection of a senescence-associated beta-galactosidase in endothelial and smooth muscle cells from human and rabbit blood vessels. Exp Cell Res 241:309–315

Weindruch R (1996) The retardation of aging by caloric restriction: studies in rodents and primates. Toxicol Pathol 24:742–745

Weindruch R, Walford RL, Fligiel S, Guthrie D (1986) The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J Nutr 116:641–654

Wu D, Ren Z, Pae M, Guo W, Cui X, Merrill AH, Meydani SN (2007) Aging up-regulates expression of inflammatory mediators in mouse adipose tissue. J Immunol 179:4829–4839

Yoshimura K, Shigeura T, Matsumoto D, Sato T, Takaki Y, Aiba-Kojima E, Sato K, Inoue K, Nagase T, Koshima I, Gonda K (2006) Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. J Cell Physiol 208:64–76

Zhu M, Lee GD, Ding L, Hu J, Qiu G, de Cabo R, Bernier M, Ingram DK, Zou S (2007) Adipogenic signaling in rat white adipose tissue: modulation by aging and calorie restriction. Exp Gerontol 42:733–744

Zhu M, Kohan E, Bradley J, Hedrick M, Benhaim P, Zuk P (2009) The effect of age on osteogenic, adipogenic and proliferative potential of female adipose-derived stem cells. J Tissue Eng Regen Med 3:290–301

Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295