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The age-dependent decrease of mitochondrial complex II activity in human skin fibroblasts

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Responses to Reviewers

We have made the following revisions to the manuscript as requested.

We have removed the speculation that complex II activity could be a future potential biomarker for monitoring the progression of skin aging as advised (Abstract last sentence).
The age-dependent decrease of mitochondrial complex II activity in human skin fibroblasts

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Short title: Skin complex II activity decreases with age

Abbreviations:
ETC, electron transport chain; FACS, fluorescence-activated cell sorting; mtDNA, mitochondrial DNA; ROS, reactive oxygen species; SDHA, succinate dehydrogenase complex subunit A; SDHB, succinate dehydrogenase complex subunit B; SDHC,
succinate dehydrogenase complex subunit C, SDHD, succinate dehydrogenase complex subunit D.
Abstract

The mitochondrial theory of aging remains one of the most widely accepted aging theories and implicates mitochondrial electron transport chain (ETC) dysfunction with subsequent increasing free radical generation. Recently, complex II of the ETC appears to be more important than previously thought in this process, suggested predominantly by non-human studies. We investigated the relationship between complex II and aging using human skin as a model tissue. The rate of complex II activity per unit of mitochondria was determined in fibroblasts and keratinocytes cultured from skin covering a wide age range. Complex II activity significantly decreased with age in fibroblasts \( (P=0.015) \), but not in keratinocytes. This was associated with a significant decline in transcript expression \( (P=0.008 \) and \( P=0.001) \) and protein levels \( (P=0.0006 \) and \( P=0.005) \) of the SDHA and SDHB catalytic subunits of complex II respectively. In addition there was a significant decrease in complex II activity with age \( (P=0.029) \) that was specific to senescent skin cells, our study being the first to investigate these differences with senescence and skin age. There was no decrease in complex IV activity with increasing age, suggesting possible locality to complex II.
Introduction

Over 40 years ago it was first speculated that mitochondria play a key role in the aging process (Harman, 1972), due mainly to the fact that the majority of endogenous reactive oxygen species (ROS) are produced by these dynamic organelles as a by-product of respiration. In addition, higher levels of mitochondrial dysfunction, mitochondrial DNA (mtDNA) damage, and ROS generation are observed with increasing age (Birket and Birch-Machin, 2007; Boffoli et al., 1994; Capel et al., 2005; Hayakawa et al., 1992; Koziel et al., 2011; Short et al., 2005). However, the exact role of mitochondria in the aging process remains unknown.

Mitochondrial complex II is the least studied of the 5 mitochondrial complexes of the electron transport chain (ETC). It is the smallest complex, composed of only 4 subunits (SDHA, SDHB, SDHC, and SDHD), and is entirely nuclear-encoded. Recent observations have implicated a possible role for this complex in the aging process. It has been demonstrated that complex II can generate ROS to a similar extent as complexes I and III in mitochondria isolated from rat skeletal muscle (Quinlellan et al., 2012), and may play a role in ROS production in human skin cells (Anderson et al., 2014). It was also recently observed that complex II activity is lower in the skin of naturally aged mice as compared to younger mice (Velarde et al., 2012). Other work implicating a role for complex II in the aging process include studies showing that mutations in the subunits of complex II can result in accelerated aging in animals; for example, SDHC and SDHB mutations accelerate aging and decrease lifespan in C.
For Review Only

*elegans* (Adachi et al., 1998; Hosokawa et al., 1994; Huang and Lemire, 2009; Ishii et al., 1998; Pfeiffer et al., 2011) and *D. melanogaster* (Tsuda et al., 2007; Walker et al., 2006). In addition, complex II activity has been shown to decrease with increasing age in human muscle (Boffoli et al., 1994; Short et al., 2005); however, the activity of complex II with age in human skin has not been investigated previously, and was therefore chosen to be examined in this study. Skin is the largest organ of the body, acting as a barrier to external insults such as ultraviolet radiation (UVR), infection, toxicity, and mechanical stress (Haake et al., 2001). It is therefore highly important to understand the aging process in this organ, not only to allow maintenance of dermatological health, but as an organ that can be accessed and studied easily with the findings from skin research having profound relevance and application to aging in other body tissues.

As well as investigating complex II in terms of biological aging, we also looked at this complex in terms of cellular senescence. Whereas biological aging describes the functional decline of a whole organism over time leading to an increase in susceptibility to disease and eventually death, cellular senescence describes the transformation of cells from a proliferating to a non-proliferating state as a tumor suppressive mechanism to prevent cells with potentially cancerous DNA mutations from undergoing replication (Campisi and d'Adda di Fagagna, 2007). Senescent cells release ROS (Nelson et al., 2012; Passos et al., 2010), with the number of senescent cells increasing with age (Campisi, 2005). Complex II has recently been linked to the process of senescence, whereby mice with mitochondrial superoxide dismutase (SOD) knocked-out were shown
to have increased ROS production, higher levels of nuclear DNA damage, decreased complex II activity, accelerated aging, and increased senescence levels in the skin, with no change in the other mitochondrial complex studied (complex IV) (Velarde et al., 2012). In addition to investigating the relationship between complex II and aging in human skin (activity and transcript expression/protein levels), we chose to determine whether differences in complex II are evident in senescent cells from older individuals as compared to senescent cells from younger individuals. The findings implicate the potential involvement of complex II in both cellular senescence and in the overall aging process, with human in vivo data from future studies required to determine whether this decline in complex II activity with increasing age has a functional relevance in skin and other tissues.
Results

Complex II activity decreases with age in human skin fibroblasts. Complex II activity was measured in fibroblasts cultured from the skin of 27 donors, ranging in age from 6 to 72 years old, from a sun-protected region of skin, in order to determine whether this specific mitochondrial complex shows a difference in activity with increasing age. Complex II activity was measured using spectrophotometry, with the results normalized to citrate synthase activity, which is a common mitochondrial marker used to determine mitochondrial amount (Birch-Machin and Turnbull, 2001) to allow complex II activity per unit of mitochondria to be determined (CII/CS). It was found that CII/CS activity decreased significantly with increasing age in skin fibroblasts (Figure 1A; $P=0.015$, rho=-0.461). To determine whether this decrease in complex II activity with age was specific to skin fibroblasts, or was also present in another skin cell type, CII/CS activity was determined in skin keratinocytes from 19 donors of differing ages. The skin keratinocytes did not show a significant change in complex II activity with age (Figure 1B; $P=0.726$, rho=-0.086), suggesting that the decrease in complex II activity with age was specific to fibroblasts in the skin.

Complex II activity is approximately two-fold higher in fibroblasts than in keratinocytes. Interestingly, it was noted that CII/CS activity was approximately two-fold higher in the fibroblasts compared to the keratinocytes in this study (Figure 1Ci; $P<0.0001$). This was also confirmed to be the case in dermal and epidermal tissue when compared directly following tissue-grinding (Figure 1Cii; $P=0.0006$), which
excludes any possible interference caused by the different media and culture conditions of the fibroblasts and keratinocytes. Due to the observed decrease in complex II activity in skin fibroblasts with age, and their high overall activity, these cells were chosen to be studied further in order to understand the cause of this decrease in complex II activity with age.

**Complex IV activity does not change with age in human skin fibroblasts.** To determine whether the decrease in mitochondrial complex activity in skin fibroblasts with increasing age was specific to complex II, or was also present in another mitochondrial complex, an additional complex was examined. Complex IV was chosen to be used as a control as this complex is not directly linked to complex II at the ETC via the ubiquinone pool, unlike complexes I and III. Complex IV was measured in 18 fibroblast cell samples cultured from human skin from the same individuals for which complex II was analyzed. Complex IV activities were normalized to citrate synthase activity (CIV/CS). The results showed that CIV/CS activity did not change with age in fibroblasts (Figure 1D; \( P=0.148, \rho=0.355 \)).

**Transcript expression levels of the complex II catalytic subunits decrease with age.** To further elucidate the cause of the observed decrease in complex II activity in skin fibroblasts with increasing age, the transcript expression levels of the 2 catalytic subunits of complex II, SDHA and SDHB (i.e. those directly responsible for the measured enzyme activity), were analyzed in the fibroblasts from individuals of different ages. In addition, 1 of the 2 anchoring subunits, SDHC (which is not directly associated
with enzyme activity), was also measured. SDHA, SDHB, and SDHC expression were studied in fibroblasts from 25 of the donors used previously, and normalized to the internal control β-actin (Li et al., 2011). It was found that SDHA transcript expression decreased significantly with age (Figure 2A; $P=0.008$, rho=-0.516), as did SDHB (Figure 2B; $P=0.001$, rho=-0.613). There was no significant correlation observed with age for SDHC transcript expression (Figure 2C; $P=0.120$, rho=-0.320).

**Protein levels of the complex II catalytic subunits also decrease with age.** As the transcript expression levels of the complex II subunits SDHA and SDHB decreased with age in fibroblasts, the translated protein levels of these subunits were chosen to be investigated to determine whether these also show a decrease with age. SDHA and SDHB protein levels were measured in 14 of the donors used previously, via Western blotting, and normalized to the internal control β-actin. Significant decreases in both the SDHA protein levels (Figure 3A; $P=0.0006$, rho=-0.801) and the SDHB protein levels (Figure 3B; $P=0.005$, rho=-0.704) were observed with increasing donor age. The Western blot immunoblot (Figure 3C) gives an example of this observation in a representative young and old donor.

**Complex II activity decreases with age in senescent skin fibroblast populations, but not in non-senescent populations.** To further investigate the reason behind the observed decrease in complex II activity with age in human skin fibroblasts, fibroblast samples cultured from 15 of the same donors as used previously (aged 6 to 71 years old) were sorted into senescent and non-senescent populations for each individual by
fluorescence-activated cell sorting (FACS), using a previously established technique reported in our group (Birket et al., 2009). This was achieved by selecting cells within the upper and lower 20% of lipofuscin autofluorescence during the cell sorting process, to represent the senescent and non-senescent cell populations respectively, as lipofuscin is present only in senescent cells and is a well-established biomarker of senescence (Birket et al., 2009). Following cell sorting, senescence was confirmed using β-galactosidase (β-gal) (Dimri et al., 1995), which showed that the cells in the lower 20% lipofuscin autofluorescence contained mostly β-gal negative (non-senescent) cells (Figure 4A; \( P<0.0001 \)), and that the cells in the upper 20% lipofuscin autofluorescence contained mostly β-gal positive (senescent) cells (Figure 4B; \( P<0.0001 \)). The lower and upper 20% lipofuscin autofluorescence populations are referred to as non-senescent and senescent populations respectively in future text.

CII/CS activity was determined for the senescent and non-senescent fibroblast populations for the 15 donors. For the senescent cell populations, it was found that there was a significant decrease in CII/CS activity with age (Figure 5A; \( P=0.029, \rho=-0.563 \)). However, for the non-senescent cell populations there was no correlation between CII/CS activity and age (Figure 5B; \( P=0.537, \rho=-0.173 \)). This decrease in mitochondrial complex activity in senescent skin fibroblast populations appeared to be specific to complex II, as no significant change in CIV/CS activity was observed with increasing age for either the senescent cell populations (data not shown; \( P=0.498, \rho=0.321 \)) or the non-senescent cell populations (data not shown; \( P=0.556, \rho=-0.286 \)).
Discussion

Using a previously established method for determining mitochondrial complex II activity, it was found that complex II activity declined with age in human skin fibroblasts, per unit of mitochondria, which to our knowledge has not been demonstrated previously in human skin. The observed decrease in complex II with age is, however, in accordance with some previous studies in various tissues of non-human species. For example, complex II activity is decreased with age in rat heart, muscle, liver, kidney, lung, brain, and lymphocytes (Braidy et al., 2011; Cocco et al., 2005; Kumaran et al., 2004; Sandhu and Kaur, 2003; Tatarkova et al., 2011). There are few studies of complex II activity in skin; however, a recent study found that complex II activity is lower in the skin of naturally-aged older mice compared to younger mice (Velarde et al., 2012). There are a limited number of studies in human tissue; however, previous work using human muscle has demonstrated that complex II activity does appear to decline with age (Boffoli et al., 1994; Short et al., 2005). This correlates with our results; however, studies in other human tissues are required to determine whether this is a phenomenon observed throughout the entire human body. In addition, in a model for older and younger cells, achieved by using human lung fibroblasts and human lung fibroblasts transfected with telomerase respectively (Anderson et al., 2014), complex II activity was found to be much higher in the ‘younger’ cells than in the ‘older’ cells.

It could be speculated that the activity of complex II declined in an age-related manner as a consequence of factors such as an increase in ROS abundance caused by lower
cellular defenses. An increase in ROS levels may cause damage to the nuclear DNA-encoded subunits of complex II as well as the complex II protein directly, resulting in the expression of dysfunctional mitochondrial subunits, and potentially further ROS production as proposed in the vicious cycle theory of aging (Bandy and Davison, 1990). A decrease in complex II activity could also play a direct causal role in the aging process, as a decrease in activity may result in an increase in ROS leakage (Quinlan et al., 2012) leading to damage of cellular components and a decrease in tissue function. However, further in vivo work in a future study would be required to elucidate whether complex II plays a causal role in aging. It has been suggested previously that the reason for complex II being entirely nuclear-encoded (and therefore within the protection of the nuclear-repair mechanisms and exposed to lower ROS levels), is because complex II dysfunction is so detrimental that protective measures to prevent its dysfunction have been implemented by cells (Wojtovich et al., 2013).

We found that the age-related decline in complex II activity in the skin is specific to fibroblasts, as a decline was not observed in keratinocytes. It was speculated that this was due to the epidermal skin cells being replaced on a regular basis due to normal skin turnover (Iizuka, 1994). In agreement with this, the level of the age-related mitochondrial 3895 bp deletion does not accumulate as readily in the epidermis as in the dermis (Harbottle and Birch-Machin, 2006), human dermal fibroblasts are more sensitive to mtDNA damage than human dermal keratinocytes (Latimer et al., 2015), and mtDNA damage generally accumulates more readily in cells which undergo slower turnover (Cortopassi et al., 1992). Interestingly, the fibroblasts analyzed in this study
showed an approximately two-fold higher level of complex II activity than the keratinocytes which was confirmed in dermal and epidermal tissue directly. Hornig-Do et al., (2007) suggested that under normal conditions in vivo, fibroblasts have the potential for faster respiration, as keratinocytes are required to use their ETC for both energy and superoxide production for differentiation (Hornig-Do et al., 2007), and are therefore likely to lack the reserve functional capacity in the ETC for increased activity when ATP production is needed unlike fibroblasts.

Our study suggests that the decline in mitochondrial complex activity with age in human skin is specific to complex II (a unique observation), as complex IV showed no detectable change in activity with age in the skin fibroblasts which argues against a general reduction in overall respiratory chain activity and protein subunit levels. However, the activities of the remaining multi-subunit complexes would have to be determined in a wide-ranging program of study to confirm this speculation.

A possible reason for the decrease in complex II activity with age could be that the actual activity of complex II is lower in older individuals per unit of mitochondria, or that the amount of complex II present per unit of mitochondria is decreased. It was found that the gene expression levels of SDHA and SDHB both decreased in an age-dependent manner in the fibroblasts, which was not found to be the case for SDHC. The translated protein levels of SDHA and SDHB were also found to decrease significantly with increasing age. Unfortunately, due to limited sample amounts it was not possible to test this for SDHC. These observations suggest that the amount of complex II per unit of
mitochondria is decreased with age. Although SDHC expression did not show a reduction with age, the level of complex II activity is likely to be affected by a decrease in the catalytic subunits SDHA and SDHB, which are vital for activity.

It has been shown previously that increased ROS levels (generated by SOD-knockout) can result in a decrease in SDHB protein expression and in complex II activity in mouse heart (Morten et al., 2006) and skin (Velarde et al., 2012), which could also be occurring with natural age as demonstrated in our work. It has been demonstrated previously that the overexpression of SDHA/SDHB can restore complex II activity in neuronal cells from the brains of Huntington’s disease patients (Benchoua et al., 2006), which could suggest a possible future therapeutic potential for the treatment of aging.

In order to further elucidate the reasons behind the observed decrease in complex II activity with age, the activity of complex II was examined in senescent and non-senescent skin fibroblast cell populations, as senescent cells are thought to play a prominent role in the aging process, potentially via mitochondrial dysfunction (Passos et al., 2010; Velarde et al., 2012). The present study demonstrated that complex II activity decreases in an age-dependent manner in FACS-sorted senescent cells, but not in FACS-sorted non-senescent cells. This could suggest that the overall decrease in complex II activity observed in skin fibroblasts with age demonstrated in this study was due to the senescent cells only. To our knowledge, there has been no previous work investigating the differences in mitochondrial complex activity between senescent cells from older and senescent cells from younger individuals. This study therefore provides
the first evidence that senescent cells from older individuals are less efficient in terms of mitochondrial complex II activity than senescent cells from younger individuals, which could have implications in terms of deciphering the causes of the overall decrease in cellular efficiency observed with age. Higher levels of ROS generation are present in senescent compared to non-senescent cells (Passos et al., 2007), potentially resulting in increased mtDNA and nuclear DNA damage and mitochondrial dysfunction (Passos et al., 2007) and a possible decrease in complex II activity if damage becomes sufficiently high. Damage to senescent cells may be higher in the skin of older individuals due to the lower levels of antioxidants observed with age (Micallef et al., 2007), as well as the age-related decline of senescent cell removal systems such as the immune system (Rodier and Campisi, 2011) and the autophagy/lysosomal pathway (Dutta et al., 2012). These factors could result in a lower complex II activity in senescent cells of older individuals. In conclusion, the rate of complex II activity within human skin fibroblasts was shown to be lower in older individuals in this study, specifically in their senescent cells. It is likely that a decrease in complex II activity, whether causal or consequential in terms of the aging process, is likely to be exacerbating mitochondrial dysfunction with age. Human in vivo data (from future studies) will be required to confirm the suggested role of decreased complex II activity in skin aging. Indeed there is some in vivo data showing a decrease in complex II activity with increasing age in mouse skin (Velarde et al., 2012). By staining skin sections from young, middle-aged, and old mice for succinate dehydrogenase (i.e. SDH, which is a measure of mitochondrial complex II activity) they found that the proportion of sections with high SDH activity decreased with increasing age of the mice. Even though we do not have
similar information from our present study (in fact SDH staining is very problematic in human skin (Durham et al., 2002)) we do show that CII/CS activity in the fibroblasts compared to the keratinocytes (Figure 1Ci) was mirrored in the original dermal and epidermal biopsy tissue from the same individual (Figure 1Cii) thereby confirming in a very small number of cases the in vivo relevance of our in vitro findings. All this provides background evidence that a future in vivo human study would not only help to clarify whether complex II plays a causative role or a purely consequential role in aging, it would help to highlight the potential importance of complex II in other roles, such as the recently speculated role in the hyperpigmentation protective response (Boulton and Birch-Machin, 2015).
Materials and Methods

Primary cell culture. Primary skin fibroblasts and keratinocytes were cultured from foreskin samples obtained from donors from the Royal Victoria Infirmary (Newcastle upon Tyne, UK). Fibroblasts were grown in DMEM containing 10% fetal calf serum and penicillin/streptomycin, and keratinocytes were grown in Epilife Medium containing 10% fetal calf serum and penicillin/streptomycin supplemented with human keratinocyte growth supplement, both in a humidified atmosphere with 5% CO\textsubscript{2} at 37 °C.

Spectrophotometric analysis of mitochondrial complex activity. The levels of citrate synthase activity, complex II activity, and complex IV activity, were determined via spectrophotometric methods as described previously (Birch-Machin et al., 1994).

Gene expression analysis of complex II subunits. RNA was extracted from cells using an RNeasy Mini Kit (Qiagen, Manchester, UK), and used to generate complimentary DNA (cDNA) via reverse transcription. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Paisley, UK) as per the manufacturers guidelines. Using the generated cDNA, real-time quantitative PCR (qPCR) was performed to determine the relative expression levels of the complex II subunits SDHA, SDHB, and SDHC. Primers to amplify a 70 bp region of the SDHA gene (on chromosome 5), a 77 bp region of the SDHB gene (on chromosome 1), and an 86 bp region of the SDHC gene (on chromosome 1) (Applied Biosystems, Paisley, UK) were chosen for analysis. For the qPCR reaction, the following
components were assembled to a final volume of 25 µl per well: deionized H$_2$O, 1x TaqMan Gene Expression Master Mix (Applied Biosystems, Paisley, UK), 1x TaqMan Gene Expression Assay primer/probe set (Applied Biosystems, Paisley, UK), and 20 ng template cDNA. Reactions were performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Paisley, UK) with the results viewed using StepOne Software V2.1 (Applied Biosystems, Paisley, UK). The following conditions were used for the reaction: 50°C for 2 minutes; 95°C for 10 minutes; and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

The SDHA, SDHB, and SDHC transcript levels were normalized to β-actin, a housekeeping gene transcript at equal levels in all cell types. Relative expression levels were normalized as determined by the 2$^{-\Delta\Delta C_{T}}$ method (Livak and Schmittgen, 2001).

**Protein concentrations of complex II subunits.** To determine the amount of complex II subunit protein present per sample, Western blotting was performed as described previously (Boulton and Birch-Machin, 2015). The mouse monoclonal primary antibodies used were SDHA (clone number 2E3GC12FB2AE2, Abcam, Cambridge, UK), SDHB (clone number 21A11AE7, Abcam, Cambridge, UK), and the positive control β-actin (clone number mAbcam 8226, Abcam, Cambridge, UK), at a concentration of 1 in 5000 in BSA. The secondary antibody, peroxidase-labelled anti-mouse IgG (Vector Laboratories Inc., Peterborough, UK), was added at a concentration of 1 in 5000 in BSA for SDHA and SDHB, and 1 in 10,000 in 5% skimed milk (with 0.1% sodium azide, in TBS-tween) for β-actin. Films were analyzed using a FluorChem
FC2 Imaging System with reflective white light and results viewed using AlphaEase FluorChem Software (Alpha Innotech, San Leandro, California). AlphaEase FluorChem Software was also used to quantify proteins via densitometry, during which the band was selected and the background subtracted to determine overall intensity for each band.

**FACS sorting into senescent and non-senescent populations.** Fluorescence-activated cell sorting (FACS) was used to separate human skin fibroblasts into senescent and non-senescent populations based on lipofuscin autofluorescence according to previously described procedures (Birket *et al.*, 2009). Following FACS, senescence-associated β-gal staining was performed using a Senescence Cells Histochemical Staining Kit (Sigma-Aldrich, Poole, UK). This is based on the observation that β-gal is only present in senescent cells (Dimri *et al.*, 1995). Following staining, the number of blue cells (senescent cells) and the number of non-blue cells (non-senescent cells) were counted under a light microscope for 500-1000 cells (Birket *et al.*, 2009).

**Statistical analysis.** When comparing correlations between data sets, non-parametric Spearman correlation was used to determine statistical significance. When comparing differences between groups, the unpaired *t*-test was used. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc., San Diego, California).
Conflict of Interest

The authors state no conflict of interest.

ACKNOWLEDGEMENTS

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References


Figure Legends

**Figure 1.** Mitochondrial complex activity in human skin cells from differently-aged donors. A) Complex II activity was normalized to citrate synthase activity (CII/CS) in fibroblasts from 27 donors. There was a significant decrease in CII/CS activity with age ($P=0.015$, $\rho=-0.461$) (n=3 ±SEM). B) There was no significant change in CII/CS activity with age in keratinocytes from 19 donors ($P=0.726$, $\rho=-0.086$) (n=3 ±SEM). Ci) There was approximately two-fold higher CII/CS activity in fibroblasts compared to keratinocytes ($P<0.0001^{***}$), using data from Figures 1A and 1B (n=27 ±SEM). Cii) There was approximately two-fold higher CII/CS activity in dermal tissue compared to epidermal tissue from 5 donors ($P=0.0006^{***}$) (n=5 ±SEM). D) There was no significant change in CIV/CS activity with age in fibroblasts from 18 donors ($P=0.148$, $\rho=0.355$) (n=3 ±SEM). Error bars for A, B, and D represent variation within a single donor, error bars for C represent variation between donors.

**Figure 2.** Mitochondrial complex II subunit transcript expression in skin fibroblasts from donors of different ages. A) SDHA expression normalized to β-actin expression in fibroblasts from the skin of 25 donors of different ages, relative to the sample with the lowest expression level. There was a significant decrease in SDHA expression with age ($P=0.008$, $\rho=-0.516$) (n=6 ±SEM). B) SDHB expression in fibroblasts from differently-aged donors. There was a significant decrease in SDHB expression with age ($P=0.001$, $\rho=-0.613$) (n=6 ±SEM). C) SDHC expression in fibroblasts from differently-aged donors. There was no significant change in SDHC expression with age ($P=0.120$, $\rho=-0.320$) (n=6 ±SEM). Error bars represent variation within a single donor.
Figure 3. Mitochondrial complex II subunit protein levels in skin fibroblasts from donors of different ages. A) SDHA protein level normalized to β-actin in fibroblasts from the skin of 14 donors of different ages, relative to a control sample. SDHA protein levels decreased significantly with age ($P=0.0006$, $\rho=-0.801$) ($n=4 \pm \text{SEM}$). B) SDHB protein levels in fibroblasts from the same 14 differently-aged donors. SDHB protein levels decreased significantly with age ($P=0.005$, $\rho=-0.704$) ($n=4 \pm \text{SEM}$). Error bars represent variation within a single donor. C) Western blot immunoblot results for 2 representative fibroblast samples (1 young and 1 old), with the ages of donors (in years) provided. Protein levels of the complex II subunits SDHA (70kDa) and SDHB (32kDa), and the control protein β-act (42kDa), are shown.

Figure 4. Senescence-associated β-gal staining following FACS sorting based on lipofuscin autofluorescence. A) The quantity of senescent cells was determined by β-gal staining in 5 of the donors for cells from the lower 20% of lipofuscin autofluorescence, as separated by FACS. There was a significantly lower number of β-gal positive cells as compared to negative cells in the lower 20% lipofuscin population ($P<0.0001^{***}$) ($n=5 \pm \text{SEM}$). B) There was a significantly higher number of β-gal positive cells as compared to negative cells in the upper 20% lipofuscin population ($P<0.0001^{***}$) ($n=5 \pm \text{SEM}$). Error bars represent variation between donors.

Figure 5. Mitochondrial complex II activity in senescent and non-senescent FACS-sorted human fibroblasts. Complex II activity was normalized to citrate synthase activity
in fibroblasts following FACS-sorting into senescent and non-senescent populations based on lipofuscin autofluorescence. A) CII/CS activity for the FACS-sorted senescent populations of fibroblasts from 15 donors. There was a significant decrease in CII/CS activity with age ($P=0.029$, rho=-0.563) (n=2 ±SEM). B) CII/CS activity for the FACS-sorted non-senescent populations of fibroblasts from 15 donors. There was no significant change in CII/CS activity with age ($P=0.537$, rho=-0.173) (n=2 ±SEM). Error bars represent variation within a single donor.
Figure 1

A) CIICS Activity vs. Donor Age (years)

B) CIICS Activity vs. Donor Age (years)

C) i) CII/CS Activity for Fibroblast Cells vs. Keratinocyte Cells
   ii) CII/CS Activity for Dermis vs. Epidermis

D) CIICS Activity vs. Donor Age (years) for different Tissue Types
Figure 2

A

Donor Age (years)

SDHA Transcript Level

B

Donor Age (years)

SDHB Transcript Level

C

Donor Age (years)

SDHC Transcript Level
Figure 3

A

Donor Age (years)

Relative Protein Level (SDHA)

B

Donor Age (years)

Relative Protein Level (SDHB)

C

Age (years)
6 71

SDHB (32 kDa)

β-ACT (42 kDa)

SDHA (70 kDa)
Figure 4

A  
**Lower 20% Autofluorescence**

- β-gal Negative
- β-gal Positive

Cell Status

B  
**Upper 20% Autofluorescence**

- β-gal Negative
- β-gal Positive

Cell Status