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Age-Dependent Decrease of Mitochondrial Complex II Activity in Human Skin Fibroblasts

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The mitochondrial theory of aging remains one of the most widely accepted aging theories and implicates mitochondrial electron transport chain dysfunction with subsequent increasing free radical generation. Recently, complex II of the electron transport chain appears to be more important than previously thought in this process, suggested predominantly by nonhuman 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 succinate dehydrogenase complex subunit A and subunit B 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. There was no decrease in complex IV activity with increasing age, suggesting possible locality to complex II.

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INTRODUCTION

It was first speculated more than 40 years ago that mitochondria play a key role in the aging process (Harman, 1972), mainly because the majority of endogenous reactive oxygen species (ROS) are produced by these dynamic organelles as a byproduct of respiration. In addition, higher levels of mitochondrial dysfunction, mitochondrial DNA 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 five mitochondrial complexes of the electron transport chain. It is the smallest complex, composed of only four succinate dehydrogenase complex subunits A through D (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 (Quinlan 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 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 *Caenorhabditis elegans* (Adachi et al., 1998; Hosokawa et al., 1994; Huang and Lemire, 2009; Ishii et al., 1998; Pfeiffer et al., 2011) and *Drosophila 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, 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 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 nonproliferating state as a tumor suppressive mechanism to prevent cells with potentially cancerous DNA

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Abbreviations: β-gal, β-galactosidase; CII/CS, complex II activity per unit of citrate synthase activity; CIV/CS, complex IV activity per unit of citrate synthase activity; FACS, fluorescence-activated cell sorting; 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

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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 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 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. Human in vivo data from future studies are 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, 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 complex II activity per unit of citrate synthase activity (CII/CS). It was found that CII/CS activity decreased significantly with increasing age in skin fibroblasts ($P = 0.015$, rho $= -0.461$; Figure 1a). 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 ($P = 0.726$, rho $= -0.086$; Figure 1b), suggesting that the decrease in complex II activity with age was specific to fibroblasts in the skin.

Complex II activity is approximately twofold higher in fibroblasts than in keratinocytes

Interestingly, it was noted that CII/CS activity was approximately twofold higher in the fibroblasts compared to the keratinocytes in this study ($P < 0.0001$; Figure 1c, i). This was also confirmed to be the case in dermal and epidermal tissue when compared directly after tissue grinding ($P = 0.0006$; Figure 1c, ii), which excludes any possible interference caused by the different media and culture conditions of the fibroblasts and keratinocytes. Because of 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 determine 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

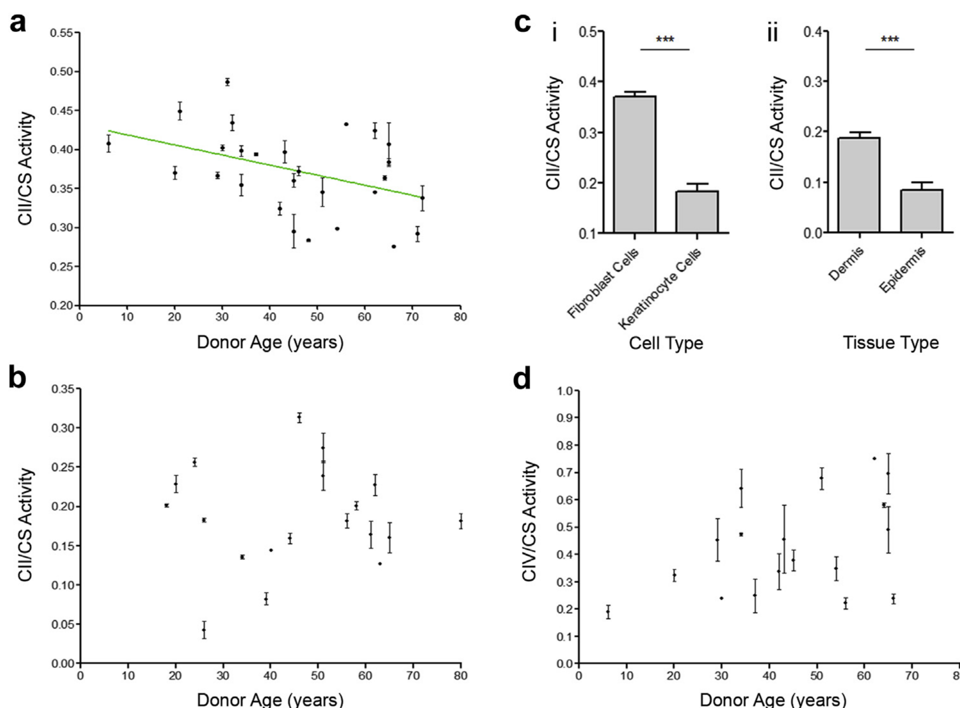

Figure 1. Mitochondrial complex activity in human skin cells from donors of different ages. (**a**) Complex II activity was normalized to citrate synthase activity (CII/CS) in fibroblasts from 27 donors. There was a significant decrease in CII/CS activity with age ($P = 0.015$, rho $= -0.461$; n $= 3 \pm$ standard error of the mean [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 $= 3 \pm$ SEM). (**c, i**) There was approximately twofold higher CII/CS activity in fibroblasts compared to keratinocytes using data from Figure 1a and b (n $= 27 \pm$ SEM). ***$P < 0.0001$. (**c, ii**) There was approximately twofold higher CII/CS activity in dermal tissue compared to epidermal tissue from five donors (n $= 5 \pm$ SEM). ***$P = 0.0006$. (**d**) There was no significant change in CIV/CS activity with age in fibroblasts from 18 donors ($P = 0.148$, rho $= 0.355$; n $= 3 \pm$ SEM). Error bars for **a**, **b**, and **d** represent variation within a single donor. Error bars for **c** represent variation between donors.

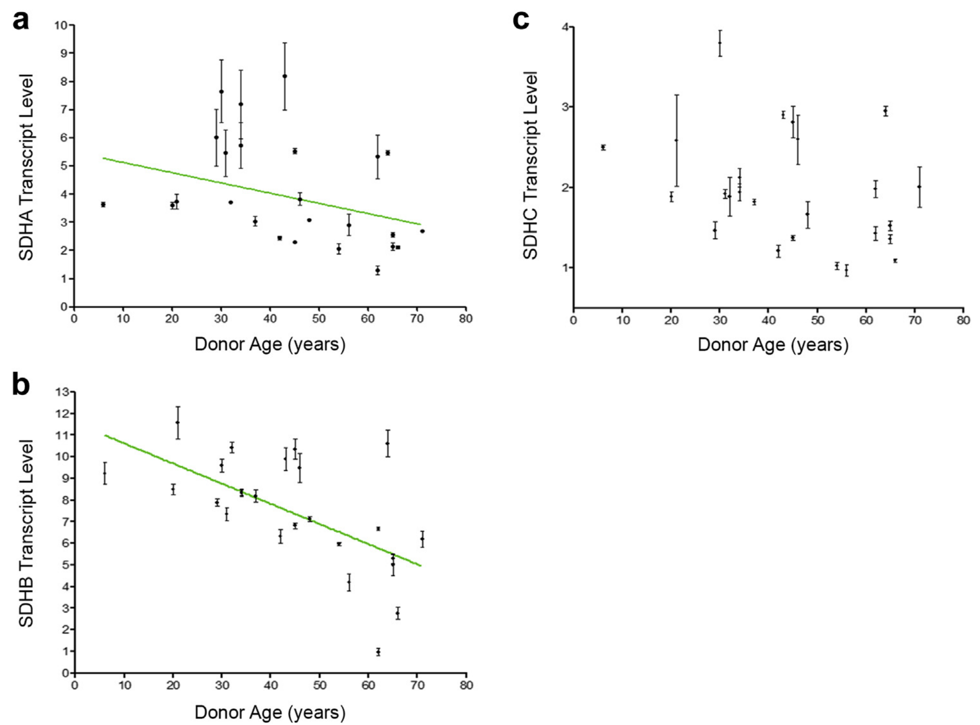

Figure 2. Mitochondrial complex II subunit transcript expression in skin fibroblasts from donors of different ages. (**a**) Succinate dehydrogenase complex subunit A (SDHA) expression normalized to β-actin expression in fibroblasts from the skin of 25 donors of different ages, relative to the sample with the lowest expression level. There was a significant decrease in SDHA expression with age ($P =$ 0.008, rho = −0.516; n = 6 ± standard error of the mean [SEM]). (**b**) Succinate dehydrogenase complex subunit B (SDHB) expression in fibroblasts from donors of different ages. There was a significant decrease in SDHB expression with age ($P =$ 0.001, rho = −0.613; n = 6 ± SEM). (**c**) Succinate dehydrogenase complex subunit C (SDHC) expression in fibroblasts from donors of different ages. 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.

mitochondrial complex, an additional complex was examined. Complex IV was chosen to be used as a control because this complex is not directly linked to complex II at the electron transport chain 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 whom 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 ($P = 0.148$, rho = 0.355; Figure 1d).

Transcript expression levels of 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 two 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, one of the two 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 ($P = 0.008$, rho = −0.516; Figure 2a), as did SDHB ($P =$ 0.001, rho = −0.613; Figure 2b). There was no significant correlation observed with age for SDHC transcript expression ($P = 0.120$, rho = −0.320; Figure 2c).

Protein levels of complex II catalytic subunits also decrease with age

Because 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 SDHA protein levels ($P =$ 0.0006, rho = −0.801; Figure 3a) and SDHB protein levels ($P = 0.005$, rho = −0.704; Figure 3b) were observed with increasing donor age. The western blot immunoblot gives an example of this observation in representative young and old donors (Figure 3c).

Complex II activity decreases with age in senescent skin fibroblast populations but not in nonsenescent 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 (age 6−71 years) were sorted into senescent and nonsenescent 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 nonsenescent cell populations, respectively, as lipofuscin is present only in senescent cells and is a well-established biomarker of senescence (Birket et al., 2009). After 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 (nonsenescent) cells ($P < 0.0001$; Figure 4a), and that the cells in the upper 20% lipofuscin autofluorescence contained mostly β-gal−positive (senescent) cells ($P < 0.0001$; Figure 4b). The lower and upper 20% lipofuscin autofluorescence populations are referred to as nonsenescent and senescent populations, respectively, in this text.

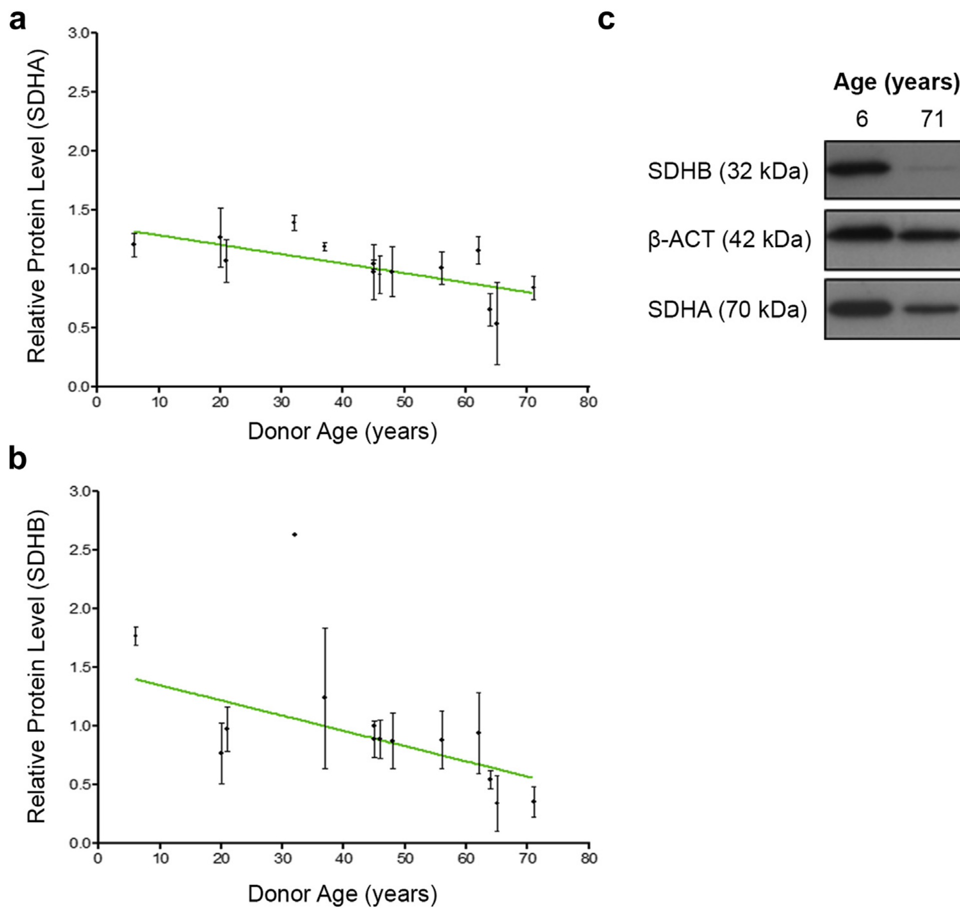

Figure 3. Mitochondrial complex II subunit protein levels in skin fibroblasts from donors of different ages. (**a**) Succinate dehydrogenase complex subunit A (SDHA) protein level normalized to β-actin in fibroblasts from the skin of 14 donors of different ages, relative to a control sample. SDHA protein levels decreased significantly with age ($P = 0.0006$, rho $= -0.801$; n $= 4 \pm$ standard error of the mean [SEM]). (**b**) Succinate dehydrogenase complex subunit B (SDHB) protein levels in fibroblasts from the same 14 donors of different ages. SDHB protein levels decreased significantly with age ($P = 0.005$, rho $= -0.704$l n $= 4 \pm$ SEM). Error bars represent variation within a single donor. (**c**) Western blot immunoblot results for two representative fibroblast samples (one young and one old), with the ages of donors (in years) provided. Protein levels of the complex II subunits SDHA (70 kDa) and SDHB (32 kDa) and the control protein β-act (42kDa) are shown.

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

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 has not been previously reported for human skin. The observed decrease in complex II with age is, however, in accordance with some previous studies in various tissues of nonhuman 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

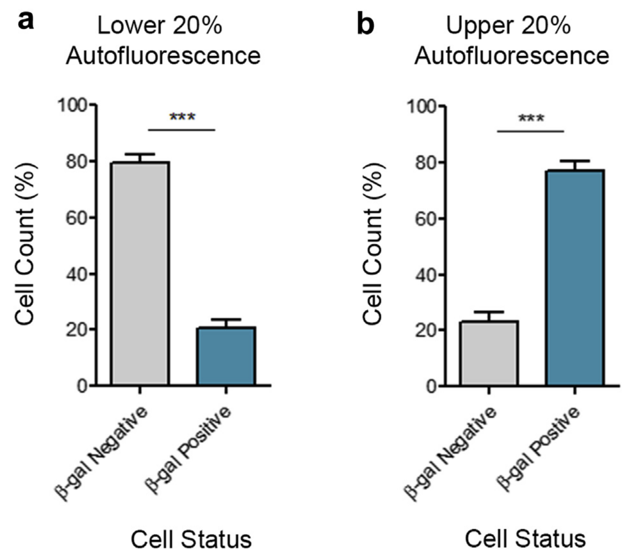

Figure 4. Senescence-associated β-galactosidase (β-gal) staining after fluorescence-activated cell sorting (FACS) based on lipofuscin autofluorescence. (**a**) The quantity of senescent cells was determined by β-gal staining in five of the donors for cells from the lower 20% of lipofuscin autofluorescence, as separated by FACS. There was a significantly lower number of β-gal−positive cells compared to negative cells in the lower 20% lipofuscin population. (n $= 5 \pm$ standard error of the mean [SEM]). $***P < 0.0001$. (**b**) There was a significantly higher number of β-gal−positive cells compared to negative cells in the upper 20% lipofuscin population (n $= 5 \pm$ SEM). $***P < 0.0001$. Error bars represent variation between donors.

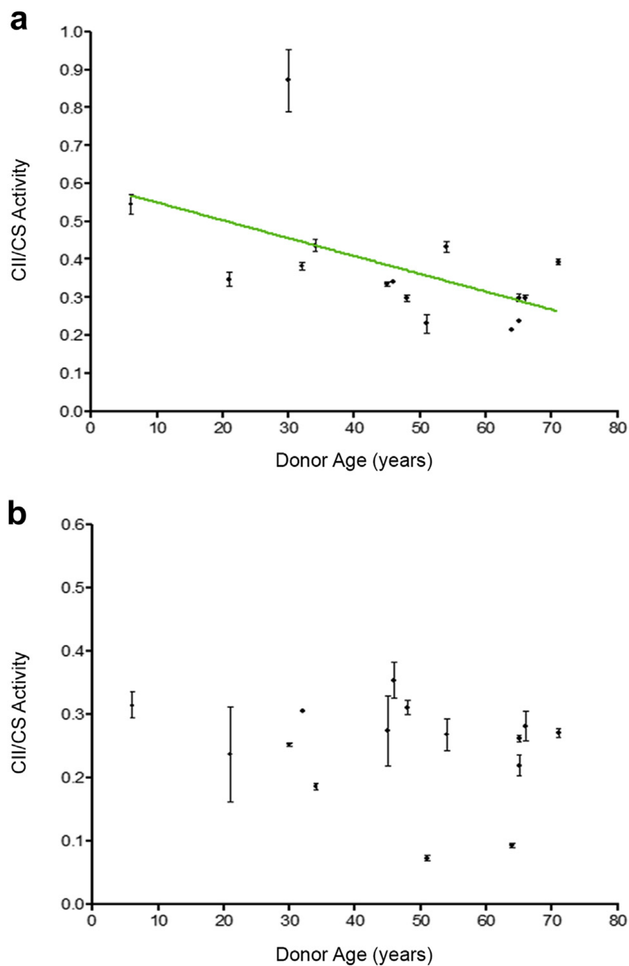

Figure 5. Mitochondrial complex II activity in senescent and nonsenescent fluorescence-activated cell sorting (FACS)-sorted human fibroblasts. Complex II activity was normalized to citrate synthase activity (CII/CS) in fibroblasts after FACS into senescent and nonsenescent populations based on lipofuscin 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 ± standard error of the mean [SEM]). (**b**) CII/CS activity for the FACS-sorted nonsenescent 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.

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 can 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 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 future studies are 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 because the epidermal skin cells were being replaced on a regular basis as a result of 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 mitochondrial DNA damage than human dermal keratinocytes (Latimer et al., 2015), and mitochondrial DNA damage generally accumulates more readily in cells that undergo slower turnover (Cortopassi et al., 1992). Interestingly, the fibroblasts analyzed in this study showed an approximately twofold 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 electron transport chain for both energy and superoxide production for differentiation and are therefore likely to lack the reserve functional capacity in the electron transport chain for increased activity when adenosine triphosphate 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, 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 multisubunit 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, because of the 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 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 superoxide dismutase 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 overexpression of SDHA/SDHB can restore complex II activity in neuronal cells from the brains of Huntington 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 nonsenescent 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). This study demonstrated that complex II activity decreases in an age-dependent manner in FACS-sorted senescent cells but not in FACS-sorted nonsenescent 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. Work investigating the differences in mitochondrial complex activity between senescent cells from older and senescent cells from younger individuals has not been previously reported. This study provides 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 nonsenescent cells (Passos et al., 2007), potentially resulting in increased mitochondrial DNA 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 because of 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 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, some in vivo data show 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 (which is a measure of mitochondrial complex II activity), they found that the proportion of sections with high succinate dehydrogenase activity decreased with increasing age of the mice. Even though we do not have similar information from our present study (in fact, succinate dehydrogenase staining is problematic in human skin (Durham et al., 2002)), we do show that CII/CS activity in the fibroblasts compared to the keratinocytes (Figure 1c, i) was mirrored in the original dermal and epidermal biopsy tissue from the same individual (Figure 1c, ii) thereby confirming in a small number of cases the in vivo relevance of our in vitro findings. This provides background evidence that a future in vivo human study would help to clarify not only whether complex II plays a causative role or has a purely consequential role in aging, it also 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, United Kingdom). Ethical approval for this work was granted by the Newcastle and North Tyneside Research Ethics Committee (Ref 08/H0906/95), and the research use of the samples was in accordance with the terms of the written, informed patient consent. The study was performed in accordance with the principles of the Declaration of Helsinki. Fibroblasts were grown in DMEM containing 10% fetal calf serum and penicillin/streptomycin, and keratinocytes were grown in Epilife medium (Thermo Fisher Scientific, Paisley, UK) containing 10% fetal calf serum and penicillin/streptomycin supplemented with human keratinocyte growth supplement, both in a humidified atmosphere with 5% CO_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, United Kingdom) and used to generate complementary DNA (cDNA) via reverse transcription. Reverse transcription was performed using a high-capacity complementary DNA reverse transcription kit (Applied Biosystems, Paisley, United Kingdom) per the manufacturer's guidelines. Using the generated complementary DNA, real-time quantitative PCR 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) were chosen for analysis. For the quantitative PCR reaction, the following components were assembled to a final volume of 25 μl per well: deionized H_2O, 1× TaqMan Gene Expression Master Mix (Applied Biosystems), 1× TaqMan

Gene Expression Assay primer/probe set (Applied Biosystems), and 20-ng template complementary DNA. Reactions were performed on a StepOnePlus real-time PCR system (Applied Biosystems) and the results viewed using StepOne Software version 2.1 (Applied Biosystems). 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.

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 $2^{-\Delta\Delta Ct}$ 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, United Kingdom), SDHB (clone number 21A11AE7, Abcam), and the positive control β-actin (clone number mAbcam 8226, Abcam), at a concentration of 1:5,000 in BSA. The secondary antibody, peroxidase-labeled anti-mouse IgG (Vector Laboratories, Peterborough, United Kingdom), was added at a concentration of 1:5,000 in BSA for SDHA and SDHB, and 1:10,000 in 5% skimmed 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, CA). 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 nonsenescent populations

FACS was used to separate human skin fibroblasts into senescent and nonsenescent populations based on lipofuscin autofluorescence according to previously described procedures (Birket et al., 2009). After FACS, senescence-associated β-gal staining was performed using a senescence cells histochemical staining kit (Sigma-Aldrich, Poole, United Kingdom). This is based on the observation that β-gal is only present in senescent cells (Dimri et al., 1995). After staining, the number of blue cells (senescent cells) and the number of non-blue cells (nonsenescent cells) were counted under a light microscope for 500−1000 cells (Birket et al., 2009).

Statistical analysis

When comparing correlations between data sets, nonparametric 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, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Disclaimer

The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, or the Department of Health.

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