Human primary skeletal muscle-derived myoblasts and fibroblasts reveal different senescent phenotypes

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Abstract

**Background** The age-related loss of muscle mass and quality, sarcopenia, has many contributing factors, one of which may be cellular senescence, but this is not well defined in human skeletal muscle.

**Method** Primary cells were isolated from biopsy samples of the vastus lateralis muscle from healthy adult males (n = 6, 22 ± 1 years), sorted (magnetic activated cell sorting) and chemically induced (doxorubicin, DOX, 0.2 μM) to a senescent state. This allowed the parallel and simultaneous investigation of the two main skeletal muscle-derived cell types: satellite cell-derived CD56+ve/desmin+ve myoblasts (muscle precursor cells) and CD56−ve/TE7+ve fibroblasts (at >95% purity). Both cell types were followed for up to 35 days post DOX treatment with a combination of quantitative immunocytochemistry and qRT-PCR for senescent markers and senescence-associated secretory phenotype (SASP) factors.

**Results** Myoblasts and fibroblasts showed temporal and quantitative differences in many of the senescence markers studied. p16 protein expression increased across the time course (P < 0.0001) with no difference between cell types, whereas at the mRNA level, myoblasts showed increased p16 expression from 4 days post treatment (FC = 3.03 ± 0.99), and in fibroblasts, this appeared later at 10 and 35 days post DOX treatment (FC = 8.09 ± 2.46, P < 0.0001). Both myoblasts (FC = 8.83 ± 1.72) and fibroblasts (FC = 2.33 ± 1.10) showed significant increases in p21 mRNA (P < 0.0001), which remained elevated in the myoblast cell populations across the 35-day time course but returned to baseline in the fibroblasts from 4 days post DOX treatment. Within 35 days post DOX treatment, all cell populations of both myoblasts and fibroblasts had reached 100% SA-β-Gal-positive cells (P < 0.05). γH2aX expression (a marker of DNA damage) increased 1 day after DOX treatment in the myoblasts (FC = 3.3 ± 1.1, P < 0.05) but returned to baseline within 4 days post DOX treatment, whereas fibroblasts showed a similar trend that did not reach statistical significance. Significant reductions in expression of the proliferation marker Ki67 1 day post DOX treatment were seen in both cell types and were maintained throughout the time course (FC = 0.11 ± 0.07, P < 0.0001). Significant changes over the time course were also observed in mRNA expression of selected SASP factors (e.g. PAI-1, MMP3, and IGFBP3, P < 0.05).

**Conclusions** Neither cellular senescence nor sarcopenia is fully understood. The present data on human primary myoblasts and fibroblasts obtained from the same tissue sample show that senescence is a complex, non-linear, and dynamic cellular process which shows intra- and inter-cell variability.

**Keywords** Human; Cellular senescence; Skeletal muscle; Myoblasts; Fibroblasts; Cell culture

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Introduction

A loss of skeletal muscle mass and decline in function are two of the most observable characteristics of the ageing process, and this decline becomes defined as ‘sarcopenic’ when given thresholds of mass and function are crossed. Sarcopenic muscles are characterized by a reduction in contractile tissue mass (motor unit and fibre loss, Type II fibre atrophy) and an infiltration of other cell types (fat and connective tissue), which reduces muscle quality relative to tissue mass. Although there are a number of contributing factors associated with the sarcopenic phenotype, one purported contributory mechanism is the negative effects of cellular senescence.

Originally demonstrated following serial passaging of cultured skin fibroblasts, senescence is a phenomenon whereby cells cease to divide. It is now recognized that senescence can occur in multiple tissues, can be induced by both ageing and different stressors, and is associated with distinctive phenotypic alterations. These include a pro-inflammatory senescence-associated secretory phenotype (SASP), macromolecular damage, and altered metabolism. A range of markers have been used to define the state of senescence, but the senescent phenotypes of different cells within human skeletal muscle are relatively poorly defined.

Senescence in cultured primary human CD56+/desmin+ muscle precursor cells (myoblasts), which originate from satellite cells, has previously been investigated, but the maintenance of myogenic purity with serial passaging to replicative senescence in primary cell culture is a serious confounding factor. This is because primary muscle cultures can become overrun by Collagen VI+/Vermentin+/Fibronectin+/PDGFRα+/TE7+ fibroblasts. Prior cell sorting may alleviate this problem, but even in cultures that have a high (>90%) starting content of myogenic cells, cultures can still become completely overrun by fibroblasts. Despite fibroblasts being commonly viewed as an unwanted contaminant of cell culture, they also play an important role in skeletal muscle regeneration and are known to be important contributors to the increased connective tissue content of the sarcopenic muscle. Fibroblasts from skeletal muscle are therefore worthy of investigation in their own right, and in these particular cells, little is known about the role of senescence.

Thus, to achieve the aim of characterizing the senescent phenotype of both myoblasts and fibroblasts from human skeletal muscle, a different approach was taken. Cells were isolated from muscle biopsy samples taken from healthy young adults, subjected to magnetic activated cell sorting (MACS) sorting and at early passage, induced to become senescent through the use of the chemotherapeutic drug, doxorubicin (DOX). This approach allowed cells to be followed for up to 35 days whilst maintaining their starting cell type purity. Cells were then investigated with multiple senescence markers and SASP factors using immunocytochemistry and qRT-PCR. In addition, parallel experiments on the same sorted myoblast and fibroblasts were performed, whereby cells were passaged in order to elicit replicative senescence. These latter experiments confirmed the challenges of replicative senescence studies in both maintaining purity of myogenic cultures on the one hand and being able to induce senescence in fibroblasts in a reasonable timescale on the other. By contrast, DOX treatment induced senescence in both cell types. Myoblasts and fibroblasts maintained their distinct cell populations over time in culture but exhibited differences in senescence marker and SASP factor expression.

Methods

Participants and cell isolation

Cell isolation was performed as described in Agley et al. Briefly, muscle samples were obtained from young volunteers using the needle biopsy technique with additional suction from the vastus lateralis of healthy young adult volunteers (age 21 ± 1 years) who gave written informed consent to participate in this study (n = 6). All experiments were performed with UK NHS Ethics Committee approval (London Research Ethics Committee; reference: 16/LO/1707) and in accordance with the Human Tissue Act and Declaration of Helsinki. The muscle biopsy had visible adipose and connective tissue removed, and they were then minced into small pieces in basal medium (C-23060; PromoCell, Heidelberg, Germany) containing collagenase D (2 mg/mL, Roche, Germany) and Dispase II (2 mg/mL, Sigma). The minced samples were incubated for 1 h at 37°C with trituration every 15 min. Enzymatic dissociation was terminated by addition of proliferation medium (15% FBS, C-23060; PromoCell, Heidelberg, Germany) containing collagenase D (2 mg/mL, Roche, Germany) and Dispase II (2 mg/mL, Sigma). The minced samples were incubated for 1 h at 37°C with trituration every 15 min. Enzymatic dissociation was terminated by addition of proliferation medium (15% FBS, C-23060; PromoCell, Heidelberg, Germany), and the cell suspension passed through a 100-μm filter (BD Falcon). The filtered cell solution was then centrifuged at 657 g for 6 min at 20°C.

Cell culture

The cell pellet was resuspended in a small volume of skeletal muscle proliferation medium and transferred to a T-25 tissue culture vessel (Nunc, Germany). Cells were maintained in a humidified incubator at 37°C and 6% CO2 for 7 days, allowing most cells to attach. The medium was replaced every 48 h, and cells from the first change were collected by centrifugation, resuspended in fresh medium and returned to their original culture vessel.
**Immuno-magnetic cell sorting procedure**

At Day 7 post-biopsy, the cell monolayer was rinsed with sterile phosphate-buffered saline (PBS) and trypsinized (0.04% Trypsin, Gibco). Primary skeletal muscle-derived myoblasts and fibroblasts were purified by immuno-magnetic cell sorting (MACS) using CD56\(^{\text{ve}}\) Magnetic beads (Miltenyi Biotec) to separate CD56\(^{\text{ve}}\) (enriched for myoblasts) and CD56\(^{\text{ve}}\) (enriched for fibroblasts) fractions, as described previously.\(^{21}\)

**Replicative senescence**

All cell populations were passaged every 72 h starting after the MACS sort on Day 7 post-biopsy. Passaging of both CD56 sort fractions continued until the CD56\(^{\text{ve}}\) cell population either reached replicative senescence, as determined by zero increases in cumulative population doublings, (Population doublings = \log_2{(N_1/N_0)}/\log_2), or at a time point when it was assumed that the population has lost myogenic purity. Purity was deemed to be lost when a slowing of doubling rate across two passages was followed by an observable increase in doubling rate.

**Doxorubicin treatment**

Early passage myoblasts and skeletal muscle origin fibroblasts (Day 22 post-biopsy) were incubated with DOX (0.2 \(\mu\)M, #15007 Cayman Chemical) for 24 h. After 24 h, DOX was removed, and cultures were thoroughly washed (PBS ×5) before fresh growth media was added. Cell populations were characterized at 1, 4, 7, 10, 14, 21, 28, and 35 days after DOX treatment.

**SA-\(\beta\)-Gal assay**

Cultured cells were plated onto a six-well plate at a density of 2500 cells/cm\(^2\). The DOX treatment was applied as above, and at each time point, the SA-\(\beta\)-Gal staining kit was used as per manufacturer’s instructions (#9860 Cell Signalling Technologies).

**Immunocytochemistry**

Cultured cells were either cytopun on to poly-L-lysine microscopy slides or grown on collagen-coated coverslips (0.5 mg/\(\mu\)L) at a density of 2500 cells/cm\(^2\) for proliferation and 7000 cells/cm\(^2\) for differentiation for immunocytochemical analysis. Myogenic differentiation was induced by replacing proliferation medium with basal medium (0% FBS, PromoCell, Heidelberg, Germany) for 96 h with a refeed after 48 h. Cells were fixed with 4% paraformaldehyde in PBS for 10 min. Cells were blocked for 1 h with 1% BSA in PBS and then probed with the relevant primary antibodies. For intracellular antigens, cells were permeabilized after fixation by addition of 0.2% Triton X-100 in PBS with 1% BSA for 10 min and then blocked and incubated with primary antibodies (Table S1). Unlabelled primary antibody binding was followed by incubation with species-specific fluorescently labelled secondary antibodies (Alexa Fluor, Invitrogen) and 10-min incubation in DAPI before mounting in Dako ‘antifade gold’ medium (Dako UK Ltd, Cambridgeshire, UK).

**Image capture and analysis**

Immunofluorescent probes were illuminated by epifluorescence, and signals were visualized through red (Texas red), green (FITC), and blue (DAPI) bandpass filters (Carl Zeiss, Cambridge, UK) on an Axioplan microscope with AxioVision software (Carl Zeiss). To ensure comparability, all time points from the same experiments, stained with the same label of interest, were photographed at identical exposures and in the same microscopy session. Image analysis occurred in Fiji using custom-built image analysis macros. Expression of protein was determined by mean fluorescence intensity of pixels within each individual nuclear ROI defined by DAPI staining in 10 random fields of view per sample. To be able to compare between different participants that were imaged on different days, mean fluorescence intensity of all times points were expressed as fold change from pre-DOX treatment expression for each individual.

**RT q-PCR**

In preparation for mRNA extraction, cells were washed twice in their wells before addition of lysis buffer (350 \(\mu\)L buffer rlt). mRNA was extracted using Qiagen RNeasy Plus Mini Kits as per manufacturer’s protocol and quality was measured using a nanodrop. For cDNA amplification, 1 \(\mu\)g of total mRNA was reverse transcribed using an Invitrogen reverse transcription kit. Transcription reaction protocol: 10 min at 25°C, 30 min at 48°C, 5 min at 95°C. RT q-PCR was run using iQ SYBR green dye (Bio-Rad) and primer pairs (Table S2) on a Bio-Rad CFX Connect real-time system with Bio-Rad CFX Manager 3.1 software following protocol of 5 min at 95°C to denature the cDNA, followed by 40 cycles of 15-s denaturation at 95°C, 30-s annealing at 60°C, and 30-s elongation at 72°C. After these cycles, the plate was either cooled and stored at 4°C or went through a melt curve protocol to check PCR specificity of 81 cycles at 55–95°C for 30 s. Data were exported into Microsoft Excel where fold expression values were calculated using the 2-\(\Delta\Delta\)CT method using GAPDH and \(\beta\)-actin as reference genes.

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Statistical analysis

The statistical test used for each analysis is stated in the relevant figure legends. However, in general unpaired t-tests or Mann–Whitney U tests (when data were non-normally distributed) were used to compare differences between groups. For analysis of more than two groups, because repeated measures ANOVA cannot handle missing values, we analysed the data instead by fitting a mixed model using the Geisser–Greenhouse correction and post hoc Dunnett’s adjustments as implemented in GraphPad Prism 8.0. This mixed model uses a compound symmetry covariance matrix and is fit using restricted maximum likelihood (REML). In the absence of missing values, this method gives the same P values and multiple comparisons tests as repeated measures ANOVA. In the presence of missing values (missing completely at random), the results can be interpreted like repeated measures ANOVA. To ensure normality of residuals, an inverse transformation was done. In all cases, N numbers are given for experiments, and an alpha value of P < 0.05 was accepted as statistically significant. All statistical analysis was completed in GraphPad Software (San Diego, California USA).

Results

Doxorubicin induces different senescent phenotypes in skeletal muscle myoblasts and fibroblasts

To investigate senescence within two different cell types from the same tissue, human primary skeletal muscle derived cells were sorted using MACS selecting for CD56 expression. This method enables the separation of CD56<sup>−ve</sup> myogenic precursor cells from the CD56<sup>−ve</sup> cell populations, which are predominantly skeletal muscle origin, TE7<sup>−ve</sup> fibroblasts. The pre-sort cell populations had a CD56<sup>−ve</sup> cell proportion of 67.3 ± 6.4% (Figure S1). Following MACS sorting, the starting purities were 96.1 ± 2.8% (CD56<sup>−ve</sup>) and 94.3 ± 6.5% (CD56<sup>−ve</sup>) for the myoblasts and fibroblasts, respectively (Figure S1). Early passage (Day 22 post-biopsy) populations of both cell types were simultaneously treated with the chemotherapeutic drug doxorubicin (DOX, 0.2 μM) for 24 h to induce cellular senescence. To determine when the cells had entered a senescent state, a panel of commonly used markers of senescence were assessed and quantified on a cell-by-cell basis using immunofluorescence (Figure S2). Protein expression of p16, SA-β-Gal, and DNA double-strand break marker γH2aX and lack of proliferation marker Ki67 were determined. Additionally, mRNA expression of the senescence-associated cell cycle inhibitors p16 and p21 was also investigated, as well as expression of common SASP factors (a schematic of the experimental protocol is summarized in Figure 1).

DOX induced cellular senescence by causing DNA damage in the myoblasts, which was observed by a significant main interaction effect for γH2aX expression (P < 0.0001; Figure 2A). γH2aX expression increased 1 day after DOX treatment in the myoblasts (P < 0.05) but returned to baseline within 4 days post DOX treatment. There were no significant changes in γH2aX expression in the fibroblasts.

The senescence-associated cell cycle inhibitor downstream of the DNA damage response pathway is p21. A significant interaction effect was seen for p21 mRNA (P < 0.0001; Figure 2F). Both cell types showed significant increases in p21 mRNA expression 1 day post DOX treatment. This remained elevated in the myoblast cell populations across the 35-day time course, but, by contrast, returned to baseline levels in the fibroblasts from 4 days post DOX treatment. This increase in DNA damage-associated cell cycle inhibition was corroborated by significantly reduced expression of the proliferation marker Ki67 after 1 day post DOX treatment in both cell types. This further decreased across the time course indicative of non-proliferating cells (P < 0.0001; Figure 2B).

Expression of the other most common senescence-associated cell cycle inhibitor, p16, was analysed at both protein (Figure 2C) and mRNA (Figure 2E) levels. At the protein level, there was a main effect of time showing an increase across the time course (P < 0.0001) with no difference between cell types and no interaction effect (Figure 2C). However, at the mRNA level, there was a significant interaction effect where myoblasts showed increased p16 mRNA expression from 4 days after DOX treatment and fibroblasts later at 10 and 35 days post DOX treatment. Both mRNA and protein expression of p16 showed a large degree of heterogeneity between cells obtained from the different donor populations (Figure 2C and 2E).

Further confirmation of a senescence state was shown in the myoblasts in a functional assay. Myoblasts at Day 4 post-DOX treatment were subject to serum withdrawal to induce fusion and differentiation into myosin heavy chain expressing mature myotubes. DOX treatment caused an altered morphology, and the fusion index was reduced from 84.8 ± 4.0 to 8.9 ± 3.9% (Figure S1C).

The cessation of proliferation and increased senescence-associated cell cycle inhibitor expression were also accompanied by significant increases in the enzymatic senescence marker, SA-β-Gal. Within 4 days post DOX treatment, 100% of myoblasts were positive for SA-β-Gal (P < 0.05). However, only one of the fibroblast populations exhibited 100% senescence cells at 4 days, but by 35 days post-DOX treatment, all cell populations of both myoblasts and fibroblasts had reached 100% SA-β-Gal positive cells (P < 0.05; Figure 2D). When taken together, these results show that DOX induces a senescent state in both myoblasts and fibroblasts extracted from skeletal muscle. However,
depending on which markers were being used, senescence could be deemed to have been entered at different time points in different cell types.

**SASP factor expression differs in myoblasts and fibroblasts**

One of the main features of senescent cells is their altered secretory phenotype (SASP), which is usually pro-inflammatory. A panel of nine commonly identified SASP factors were measured at the mRNA level using qRT-PCR across the DOX treatment time course in both cell types. Overall, six of the nine SASP factors showed a time and cell type interaction effect (only IGFBP7, IL-6, and TNF-α did not) with MMP3 and IL-8 showing the largest fold changes in expression in myoblasts and IGFBP3 in fibroblasts; seven of the nine SASP factors showed a significant main effect of time with only IL-6 and TNF-α not significantly changing over time. Four of the nine SASP factors showed a significant time × cell type interaction across the study (MMP3, IL-8, IGFBP3, and IL-6), whereas a differential effect was observed in some factors with PAI-1 and CXCL-5 being consistently higher in the fibroblasts compared with myoblasts and the reverse was the case for TNF-α and IL-6. Again, there was variability in SASP factor expression for a given cell type obtained from different individuals at any given time point as highlighted by the large standard deviation of IL-6 in myoblasts and IGFBP3 in fibroblasts.

**The effects of serial passaging of myoblasts and fibroblasts**

In parallel to the DOX treatment experiments, cell populations of post-MACS sorted myoblasts and fibroblasts were serially passaged (replicative senescence) to investigate their proliferative differences and senescent marker expression. As described previously, the starting CD56+ve cell populations were highly enriched with desmin +ve myoblasts (97.6 ± 1.7%; Figure 4A), and the CD56-ve cell populations were more heterogeneous but were predominantly TE-7+ve fibroblasts (86.7 ± 9.7%; Figure 4C). This starting purity differs slightly from the starting purity of the DOX-treated cell populations due to the extra passages needed to allow for sufficient expansion of the cell populations before DOX treatment.

With serial passaging, only three of the myoblast cell populations maintained their initial post-sort myogenic purity (Figure 4A), with the other myoblast populations starting to lose cell type purity (decrease in the percentage of desmin+ve cells) from roughly 28 days in culture. Re-sorting the cells...
using CD56 was trialled at late passage to try and maintain myogenicity, but this proved to be unsuccessful; indeed, many Desmin+ve cells were found to be present in the CD56/C0 sort population (Figure S3). The overrunning cell populations were confirmed to be TE7+ve fibroblasts (Figure 4A). As a result, only two of the initial six myoblast cell populations were deemed to have met the criteria for a replicative senescence population of myoblasts (maintenance of cell purity and cessation of division; Figure 4B). At this point, the cells were less able to fuse (Figure 4F), whereas p16 varied between these two cell populations obtained from different donors, with one showing a fourfold increase and the other a 1.2-fold increase (Figure 4E). Interestingly, the population that maintained myogenicity (Y05) but did not reach replicative senescence contained cells co-expressing desmin and TE-7 at the later time point (Figure 4A), a phenomenon not observed in other replicative or DOX-induced experiments.

The CD56/C0 fibroblast cell experiments were terminated after the same time in culture as the myoblasts, at which point(s) all fibroblast cell populations were found to still be proliferative, with Ki67 expression reducing only to 0.69 ± 0.15-fold change from early passage expression. The fibroblast cell populations had also maintained or increased their TE-7 expression with 97.8 ± 1.1% cells being TE-7+ve at late passage. Although their initial population doublings times were similar, the proliferative capacity of myoblasts seems to be much shorter than fibroblasts from the same tissue (Figure 4G).
As only two myoblast populations reached replicative senescence, no statistical comparison could be used to determine if expression levels of SASP factors had significantly changed. The time course of SASP marker expression over serial passaging for the two individual cell populations are shown in Figure S4.

Discussion

Senescence is a process by which mitotically competent cells permanently arrest proliferation in response to a variety of physiological signals and pathological stresses and has been implicated in the aetiology of sarcopenia. Senescence is an important tumour-suppressive mechanism as growth arrest prevents the propagation of stressed or damaged cells, but because senescent cells accumulate with age, they can cause or contribute to several degenerative diseases associated with ageing. Recent experiments, where p16−ve senescent cells have been knocked out, have been shown to ameliorate many of the features of physiological decline in ageing mice. In subsequent studies, the injection of senescent cells into young mice was shown to impair muscle function (running speed, hanging endurance, and grip strength), an effect that could be rescued through treatment with a senolytic cocktail (dasatinib and quercetin), which pharmacologically disables or eliminates senescent cells. It is unclear if cellular senescence is an inherent problem within muscle cells or whether muscle simply responds to changes in the surrounding milieu caused by inflammatory cytokines, chemokines, proteases, and growth factors (i.e. the SASP) released from other senescent cell types (e.g. adipocytes). The finding that Pax 7−ve/CD56−ve myogenic cells taken from muscle samples from a 96-year-old male, 17 days post-mortem, are able to form myotubes in a culture environment suggests a high degree of inherent robustness in these cell types. Furthermore, it has been known for many years from both

Figure 3 SASP factor expression in DOX treated myoblasts and fibroblasts. Time course of common SASP factors mRNA expression in DOX-treated myoblasts and fibroblasts expressed as fold change relative to each individual cell population’s pre-DOX-treated expression levels. Data presented as mean ± SD compared using mixed-effects model, P < 0.05. Main effects of statistical comparisons presented underneath each SASP factor graph. No post hoc tests were undertaken.

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grafting\textsuperscript{39} and parabiotic experiments\textsuperscript{40} in rodents that it is the age of the host rather than age of the muscle that seems to determine effectiveness of tissue repair, implying that the properties of the local environment are the key factor.

There have been limited published investigations about the characteristics of senescence in human skeletal muscle tissue, and most studies do not find significant differences in common senescent markers in skeletal muscle samples comparing young and old people.\textsuperscript{9,10} Though some previous cell culture investigations have compared primary myoblasts obtained from young and older humans,\textsuperscript{17–19,25} there is no clear consensus as to whether cells obtained from older individuals display significant evidence of senescence. Thus, for the purposes of characterizing the senescent phenotype, it makes sense to take early passage cells from healthy young people, induce them to become senescence simultaneously (overcoming stochastic senescence entry), and then follow their senescent progression over time as a more homogeneous population, as we have done in the present study. Furthermore, there is a lack of data on the senescent characteristics of muscle-derived fibroblasts. These cells are important in skeletal muscle regeneration where they interact with myoblasts for effective repair,\textsuperscript{23,41} although an infiltration of connective tissue is a key characteristic of sarcopenic muscle.\textsuperscript{24}

The present study thus aimed to gain a more complete understanding of the senescent characteristics of both human skeletal muscle myoblasts and fibroblasts by inducing primary human skeletal muscle origin cells to become senescent and assessing their marker expression and SASP composition \textit{in vitro}. To do this, we had to overcome a number of experimental difficulties that arose when we were using replicative senescence as an end point. The main inherent experimental problems were as follows: (1) skeletal muscle contains mixed

\textbf{Figure 4} Myoblasts and fibroblasts from the same tissue have different proliferative characteristics. Myoblasts and fibroblasts extracted and sorted from the same muscle tissues samples were serially passaged under culture conditions until myogenic replicative senescence or loss of desmin purity. The number of population doubling at every passage was calculated as $PD = \log(N1/N0)/\log2$ where $N1$ is the final cell number and $N0$ is the initial number of cells seeded. (A) The number of desmin-positive cells in cell populations was maintained in three of the six samples. (B) Two of the six myoblast populations reached replicative senescence whilst maintaining high level of desmin expression cells. (C) TE7 expressing fibroblasts overrun the CD56\textsuperscript{–} cell population with serial passaging. (D) Six fibroblast cell populations were highly proliferative at the point at which myoblast populations had reached replicative senescence or become overrun. (E) Characterization of the two myoblast populations that reached replicative senescence mean cell expression across the time course for yH2aX, Ki67, and p16. (F) Fusion index of myoblasts cell populations that maintained myogenic purity at early passage and at replicative senescence. (G) There was no difference in the peak proliferative rate between the two cell types ($N = 6$, unpaired $t$-test $P = 0.41$). All image scale bars are 100 $\mu$m.

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cell populations; (2) the main cell types have different maximal proliferative capacities and enter replicative senescence many days apart producing logistical problems in relation to culturing and harvest times; (3) these different replicative capacities may allow fibroblasts to outgrow myoblasts as they can relatively easily overrun the myoblasts even when seeded at less than 5% contamination (Figure 3). To overcome these methodological concerns, DOX treatment was used as a method of induction of senescence as DOX has previously been shown to induce and synchronize entry into a senescent state in human primary cells of both cardiac and smooth muscle origins at early passage and was thus a logical candidate for use in the study of senescence in skeletal muscle-derived cells.

DOX-induced senescence of myoblasts and fibroblasts

The results of the present study show that senescent myoblasts and fibroblasts both express common senescent markers p16 and SA-β-Gal in vitro. This is important, as human studies thus far have found little evidence of these markers in aged skeletal muscle, leading to the suggestion that different senescent pathways might be in operation in skeletal muscle.10

By inducing a chemical senescent state on the cell populations, a synchronization of senescence was targeted, with the aim of aligning senescence processes in myoblasts and fibroblasts. There was no noticeable loss of cell density across the DOX treatment time course. In addition, across the time course, only adherent viable cells were analysed. The results show that although DOX successfully induces senescence in both muscle-derived cell populations, they do not do so in an identical manner, as evidenced by significant differences in senescent marker and SASP factor expression between cell types and over time (Figures 2 and 3). Therefore, studies based on one cell type at a single time point and measuring only one marker do not portray the dynamic processes underlying senescence.

In order to capture the dynamic processing underlying senescence, a number of techniques and markers are needed to determine the differences that are occurring between cells of the same cell type and between different cell types from the same tissue. Our immunocytochemical imaging approach allowed us to quantify at a single cell level the heterogeneity within a cell population for a given senescence marker creating a picture of the variation of expression not captured in previous studies using western blotting. The variability and change in distribution of expression is exemplified in Figure 2S, which shows the changes in the distribution of γH2aX, Ki67, and p16 in both myoblasts and fibroblasts from a single biopsy over time. The cell type differences in senescence marker expression highlight an important consideration when defining cells as being senescent and support the concept that a panel of markers are required to confirm a senescent state. This is because the different markers target different components of the senescence process, for example, γH2aX marks DNA damage, p16 and p21 target cell cycle inhibition, whereas SA-β-Gal marks a lysosomal response associated with senescence.

In addition to the high variability in marker expression between cells, there is likely to be cell-to-cell variability in SASP factor expression as well. This study did not investigate the individual cellular level of SASP factor expression, although individual cell SASP has been investigated recently in other fibroblast cell populations.43 Here, marked differences in expression of key SASP factors such as within the CXCL and interleukin families were seen between cells.

The results of the present study also show the importance of selecting multiple time points to investigate the SASP of a cell population. Both myoblasts and fibroblasts could be considered senescent at multiple time points across the time course (depending on which senescence markers are used), and the SASP profile would also be different depending on which markers and which time points are chosen. For example, IL-8 expression in myoblasts started to become elevated between 10 and 14 days post DOX treatment, which would not be observed if only 4 days post DOX treatment when expression of SA-β-Gal, p16, and p21 would have suggested that the population was senescent (Figure 3). Furthermore, gene expression of SASP factors may not correlate directly with protein level, so the potential variability in protein expression must also be considered. Overall, conclusions about senescence processes from a SASP profile of a cell population at a single time point should be interpreted with caution.

DOX is a pharmaceutical agent that slows or stops the growth of cancer cells by blocking the enzyme topoisomerase II and induces senescence by causing DNA damage.44 The transient increase (P < 0.05) in double-strand breaks, as measured by γH2aX (Figure 2), seen in the myoblasts in the present study, shows that DOX treatment induced DNA damage in these cells, but there was only a tendency in the fibroblasts. The immediate upregulation of p21 (Figure 2) also supports this mechanism, as p21 is the downstream cell cycle inhibitor of the DNA damage response.33 Interestingly, γH2aX expression returned to baseline within 4 days post DOX treatment, suggesting that the DNA damage has been repaired and the double-strand breaks are no longer present.

This DNA damage repair process was not evident in a previous study where γH2aX expression was increased (although not quantitated on a cell-by-cell basis) in replicative senescent human myoblasts.17 The expression profiles of the senescence associated cell cycle inhibitors within myoblasts and fibroblasts would support the suggestion that p21 is more likely to be involved in the initiation of DOX induced senescence, whereas p16 is more likely involved in the maintenance of the senescent state once induced.45
**Replicative senescence in myoblasts and fibroblasts**

Having induced senescence in early passage cells with DOX, we also ran parallel experiments with cells isolated from the same tissue samples that were not subject to chemical DOX treatment but were serially passaged in an attempt to reach replicative senescence. The results of these experiments supported us taking the DOX treatment approach, as only two of the six populations of myoblasts reached the criteria for replicative senescence in that they maintained their myogenic cell proportion and ceased to divide, whereas all the fibroblast populations showed no evidence of ceasing replication.

The two myoblast populations both displayed impaired fusion but differed markedly in expression of p16 (Figure 4). The mechanism for why these two myoblast samples were successful in reaching these criteria is not clear as three of the other myoblast populations became overgrown by a small original fibroblast population. Interestingly, one of these populations contained a few cells that co-expressed desmin and TE7 (Figure 4). It was proposed by Alsharidah et al.\(^\text{17}\) that fibroblasts may outgrow myoblasts by having a shorter division time. However, here we have shown that when these cell populations are in isolation, their peak population doubling times are not significantly different (Figure 4). What we have shown is that when myoblasts are becoming senescent, fibroblasts are still highly proliferative after the same time and number of divisions in culture. Therefore, the data suggest that fibroblasts have a longer proliferative capacity than myoblasts, which could account for their overrunning of myoblasts cultures and might partly explain the increases in connective tissue observed in a sarcopenic muscle.\(^\text{46}\) The mechanisms of these different cell population outcomes are not known, but they highlight that primary cultures containing mixed populations of cells have complex interaction effects in addition to being in various cell states and on different trajectories towards senescence.\(^\text{47,48}\) As regards the fibroblast populations, and in contrast to the myoblast cultures, these cell populations increased their fibrogenicity over time in culture. At the time of termination, these cell populations had not reached the criteria for a senescence fibroblast population, as evidenced by continued cell division and maintained expression of Ki67 (Figure 4). These results highlight the marked difference between the two cell types in terms of their proliferative capacities and reaffirm the challenges of undertaking primary cell culture and maintaining cell purity in replicative senescence studies.

To address the overrunning of myoblast cell populations with fibroblasts, late passage sorting of the myoblasts cultures was trialled, but even though the CD56\(^{+}\) populations were highly enriched for myoblasts, there were also many desmin-positive cells seen in the CD56\(^{-}\) sort population (Figure S3). CD56 expression has been shown to fluctuate across serial passaging of myoblasts,\(^\text{49}\) which brings potential cell selection bias if resorting of the population occurred during passaging. Recently, the cell surface marker CD82 has been found to positively sort myoblasts from mixed cultures, but it is yet to have been investigated if CD82 expression is more stable across serial passaging.\(^\text{50}\)

**Implications for sarcopenia**

Sarcopenia is a highly complex process involving multiple factors that include loss of motor units, anabolic resistance to exercise and feeding, inflammation and impaired response to damage.\(^\text{51–54}\) The sarcopenic muscle is also characterized by having an infiltration of fat and connective tissue reducing contractile and metabolic quality.\(^\text{55}\) At present there are no effective pharmaceuticals for the treatment of sarcopenia with current recommendations stressing the importance of exercise.\(^\text{56}\) However, senolytics have been shown to improve muscle function in older animals\(^\text{37}\) and human clinical trials using these senolytics are currently ongoing. Additionally, a recent study has shown that older people who undertook an exercise training programme reduced circulating expression of markers of senescence and SASP factors.\(^\text{57}\) Further to this, a systematic review of exercise as a senolytic concludes that there is sufficient evidence to support further work into the possibility of exercise as a senolytic medicine.\(^\text{58}\) Thus, future studies of senescence within skeletal muscle should investigate the interaction between physical activity, senescence, and sarcopenia. How all the contributing factors to sarcopenia interacting with one another and where senescence interacts with them remains to be determined.

The present study has characterized, in a controlled experimental model, the senescent phenotype of human primary myoblasts and fibroblasts. The data thus far on older populations remain inconclusive as to the extent to which senescence is directly affecting skeletal muscle. Although this study has not evaluated the muscle of older individuals, our experimental approach has provided the basis upon which to investigate further senescence processes within human skeletal muscle. The applicability of our in vitro findings to in vivo muscle needs further clarification.

**Conclusions**

The main findings of the current study were that chemical induction of senescence through DOX treatment induced both quantitative and temporal differences in the responses of myoblast and fibroblasts in regard to both markers of cellular senescence and SASP factor expression. The data highlight the complexity and dynamism of the senescent state within
different cell types derived from the same human skeletal muscle and the need to use multiple markers when investigating senescence. It is still to be determined the extent to which senescent cells are present in aged human skeletal muscle and the extent to which they may contribute to sarcopenia.

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Ethics statement

The present study was conducted in accordance with the ethical standards of the Helsinki Declaration and had NHS HRA ethical approval (REC reference: 16/LO/1707).

Conflict of interest

The authors declare that they have no conflict of interest.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Myoblast and fibroblast cell type purity is maintained after DOX treatment and myoblast fusion is impaired. A) Percentage of CD56<sup>ve</sup> and CD56<sup>ve</sup> cell before MACS sorting B) Percentage of desmin positive cells in myoblast, and TE7 positive cells in fibroblast, cell populations across the time course after DOX treatment. Data presented as mean±SD compared using mixed-effects model, *P < 0.05.

Main effects of statistical comparisons presented underneath each graph. Where main interaction effect was significant Dunnetts post hoc tests were used to determine which time points were significantly different from pre DOX treatment. * indicates myoblasts statistically significant from myoblasts pre. C) Fusion index of myoblasts cell populations before DOX treatment and four days after DOX treatment. EP N = 6, DOX D4 N = 5. Data compared using an unpaired two tailed t-test *P < 0.05 * indicates significant between EP and DOX D4. All image scale bars are 100 μm.

Figure S2. Examples of the cell by cell analysis undertaken to determine protein expression of senescent markers of individual cells with myoblast and fibroblast cell populations extracted from the same muscle biopsy sample. Mean fluorescence intensity of γH2aX (left), Ki67 (middle) and p16 (right) within the individual nuclei of Myoblast (A) and Fibroblast (B) cell populations across the DOX treatment time course.

Figure S3. Example images of late passage CD56 MACS sorting resulting in high percentage of Desmin positive myoblasts in the CD56<sup>ve</sup> sort population.

Figure S4. Differences in SASP factor expression of myoblasts and fibroblasts extracted from two individuals which myoblast populations maintained desmin purity to replicative senescence. Fold change in expression of nine common SASP factors relative to cell type early passage expression levels across serial passaging.

Table S1. Supporting Information.
Table S2. Supporting Information.
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