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Primary human muscle precursor cells obtained from young and old donors produce similar proliferative, differentiation and senescent profiles in culture

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Summary

The myogenic behaviour of primary human muscle precursor cells (MPCs) obtained from young (aged 20–25 years) and elderly people (aged 67–82 years) was studied in culture. Cells were compared in terms of proliferation, DNA damage, time course and extent of myogenic marker expression during differentiation, fusion, size of the formed myotubes, secretion of the myogenic regulatory cytokine TGF-β1 and sensitivity to TGF-β1 treatment. No differences were observed between cells obtained from the young and elderly people. The cell populations were expanded in culture until replicative senescence. Cultures that maintained their initial proportion of myogenic cells (desmin positive) with passaging (n = 5) were studied and compared with cells from the same individuals in the non-senescent state. The senescent cells exhibited a greater number of cells with DNA damage (γ-H2AX positive), showed impaired expression of markers of differentiation, fused less well, formed smaller myotubes and secreted more TGF-β. The data strongly suggest that MPCs from young and elderly people have similar myogenic behaviour.

Key words: aging; cellular senescence; human; sarcopenia; satellite cell; stem cell; skeletal muscle.

Introduction

Skeletal muscle mass is lost with age (sarcopenia) and this loss is hastened by inactivity (Hughes *et al.*, 2001). This process is also associated with an increase in fat and connective tissue (Crane *et al.*, 2010). The mechanisms underlying these changes are not clear. Candidate factors include the loss of alpha motor neurons, reductions in circulating anabolic hormones, inflammation and reduced sensitivity of muscle protein synthesis to feeding and exercise (Degens, 2010). It has also been suggested that there is an age-related impairment in the ability of muscle to repair itself, following exercise-induced damage, which may contribute to sarcopenia (Brooks & Faulkner, 1990).

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Muscle repair and maintenance is facilitated by the action of muscle precursor cells MPCs, (Lepper *et al.*, 2011) which are also referred to as myoblasts, muscle stem cells or satellite cells when they are located in their niche between the basal lamina and the sarcolemma of myofibres (Mauro, 1961). A central question in healthy human aging is thus whether MPCs from old individuals differ in their intrinsic myogenic behaviour from those of young individuals.

The experimental procedure adopted to answer this question was to extract MPCs from muscle biopsy samples obtained from elderly and young people, and compare the responses of the MPCs under standardized conditions *in vitro*. In this study old donor cells were obtained from sedentary, but otherwise clinically healthy subjects differing in age from their young counterparts by at least four decades. We examined the proliferative potential, DNA damage, time course of differentiation marker expression (myogenin and myosin heavy chain), fusion and myotube size. We also examined the secretion of the regulatory cytokine TGF-β1 during differentiation and the ability of young and old cells to respond to, and recover from, TGF-β1 treatment during differentiation. TGF-β1 is a key myogenic regulatory cytokine and studies suggest that its expression may be altered with age (Zentella & Massague, 1992; Carlson *et al.*, 2009a). No significant difference in any of the parameters was observed between young and old donors.

We then passaged cells until replicative senescence to determine if serial passaging rather than chronological age would alter the properties of MPCs. Primary cell culture from muscle biopsy samples produce a mixture of cell types (Yablonka-Reuveni *et al.*, 1988), the two most prevalent of which are MPCs and fibroblasts. The initial proportions of cell types differ between samples and importantly may change with multiple passaging (Schafer *et al.*, 2006). We therefore only studied senescent cultures which maintained their desmin content with time in culture. Senescent MPCs exhibited a distinct phenotype, dissimilar to that of both young and elderly cells obtained from the same individuals prior to expansion in culture.

The results suggest a maintenance of MPC behaviour at least up to the ages studied, and show that the relationship between *in vitro* senescence and *in vivo* aging needs further clarification.

Results

MPC characterization and proliferation

The MPC populations studied were obtained from stocks of early passage primary cultures extracted from biopsies of five young and four elderly subjects. Initial characterization of the cell populations, undertaken within the first 4 days of culture, included analysis of desmin and γ-H2AX marker expression (Table 1 and Fig. 1). The

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Table 1 The percentage of desmin positive cells measured in culture following thawing in young and old subjects

	Age	Gender	% of Desmin^{+ve} cells
Young	23	Female	51
Young	25	Male	86
Young	23	Male	94
Young	24	Male	95
Young	20	Male	89
Old	82	Male	94
Old	80	Female	93
Old	67	Female	50
Old	77	Male	89

latter marker identifies C-Terminally phosphorylated histone 2AX molecules which accumulate close to sites of nascent double stranded DNA breaks (D'adda Di Fagagna, 2008). Figure 1 shows examples of proliferating cultures with a high desmin content and with a mixed population of MPCs and fibroblasts, the latter identified by the marker TE7 (Fig. 1c). Overall the data showed no age-related differences in desmin positive cells in cultures obtained from young and elderly subjects (83 ± 8%, young vs. 82 ± 11%, elderly, Table 1). Analysis of γ-H2AX expression showed only a small proportion (<10%) of cells from each age group positive for this marker and no difference between the two groups of cell donors (Fig. 1d).

The proliferative (Ki67 positive) and myogenic (desmin positive) fractions of cell populations isolated from young and old muscle biopsies were monitored every 48 h throughout the culture period (Fig. 2). Cells were passaged in growth medium until they reached replicative senescence, the point after which no increase in cell numbers was observed. To ensure that replicative senescence was stable, cell numbers were monitored for a further 3 weeks after this point (Di Donna *et al.*, 2003; Bigot *et al.*, 2008). Several populations of cells (two from young subjects and three from elderly subjects) maintained their desmin content (50–94%) over time in culture and underwent 5–12 mean population doublings (MPDs; Fig. 2a,c,e,g). Other populations, however, lost their desmin content over time (50–95% starting, 0% at senescence) and underwent 15–20 MPDs (Fig. 2b,d,f,h). Expression of the proliferation marker Ki67 decreased with time and was absent in senescent cells (Fig. 2e–h). A gradual and continuous decline was more evident when Ki67 expression was monitored only in the MPCs (Fig. 2g,h) as opposed to the mixed population (Fig. 2e,f). Although there was a degree of heterogeneity in behaviour between different cell populations, particularly with regard to Ki67 expression, there was no effect of age of cell donor.

Differentiation of early passage, proliferating cells

In early passages, non-senescent cells showed myogenin expression within 24 h of a change from proliferation to differentiation

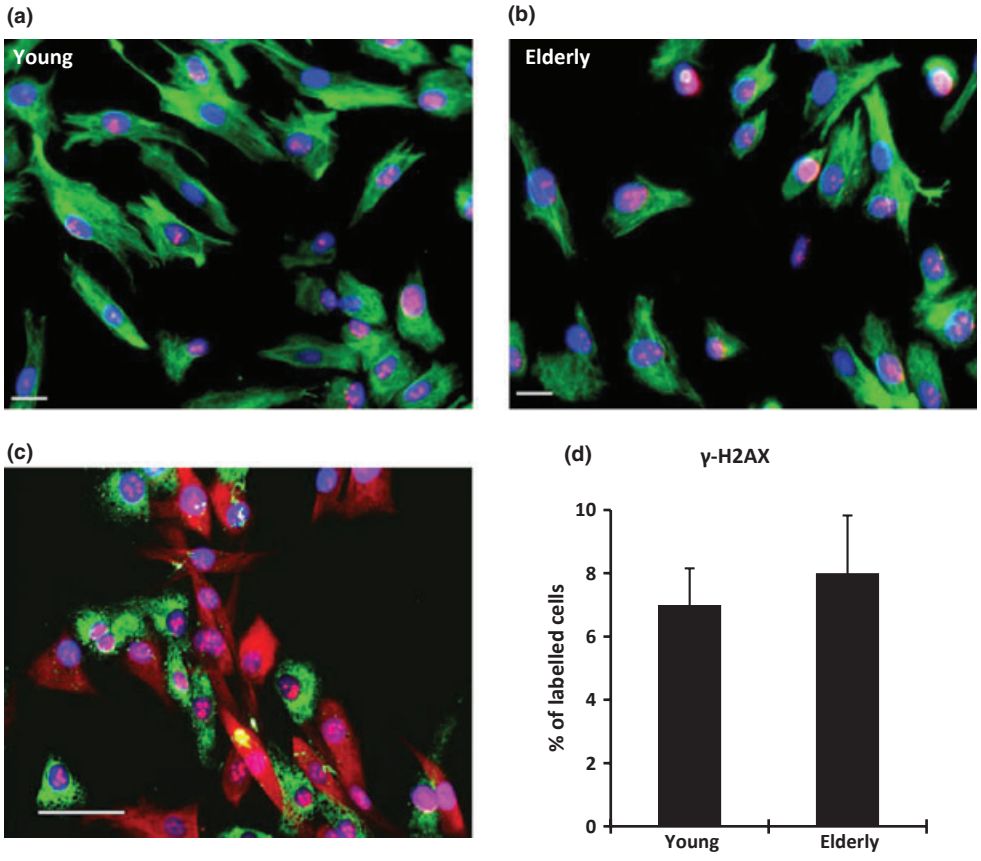

Fig. 1 Images of cells taken from a young male subject aged 20 years (a) and from an elderly subject aged 77 years (b). Cells were stained for desmin (green), Ki67 (red) and Hoechst (blue) after 24 h in growth medium. Scale bar = 20 μm. (c) Typical mixed population of human primary muscle precursors (Desmin+/red) and muscle derived fibroblasts (TE7+ /green) 7 days post isolation. Freshly isolated cells of both lineages are highly proliferative as evidenced by strong and organized nuclear Ki67 expression. Scale bar = 50 μm. (d) DNA damage in human MPCs obtained from young (n = 5) and elderly (n = 4) subjects as determined by γ-H2AX staining (see Fig. 4 for representative images). Data are mean values ±SD. No significant difference was observed between young and old MPCs.

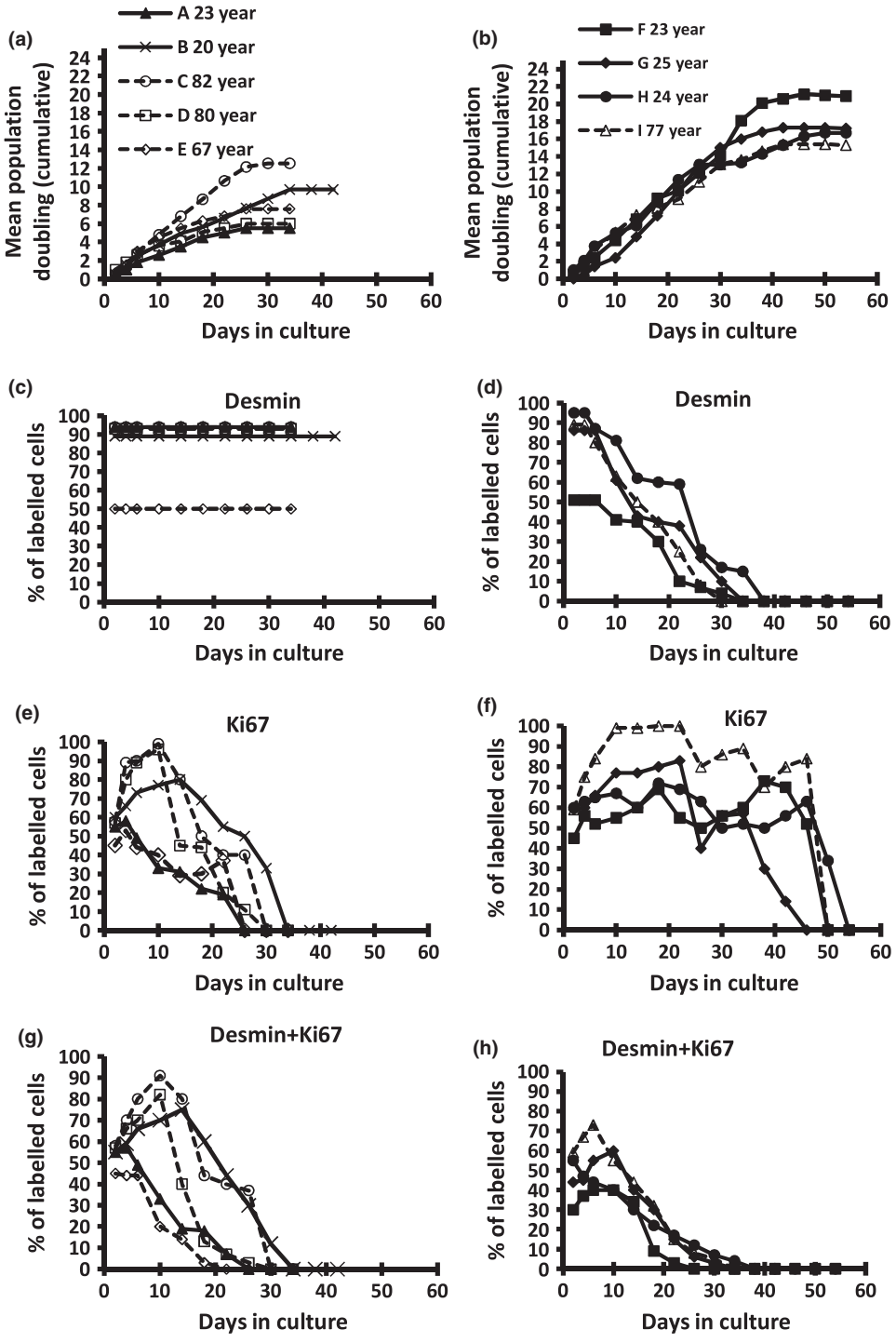

Fig. 2 *In vitro* expansion of human MPCs isolated from subjects of different ages. The number of population doubling at every passage was calculated as PD = log (N_1/N_0)/log2 where N_1 is the final cell number and N_0 is the initial number of cells seeded. (a) cells that maintained desmin content (b) cells that lost desmin content with time in culture. (c), (e) and (g) Time course of desmin, Ki67 and desmin+Ki67 expression for cells that maintained desmin, in human MPCs obtained from young (n = 2) and elderly (n = 3) subjects over time in culture. (d), (f) and (h). Time course of desmin, Ki67 and desmin+Ki67 expression for populations that lost desmin. Cells were obtained from young (n = 3) and elderly (n = 1) subjects. A, B, C, G, H & I = Male, D, E & F = Female.

medium (Fig. 3a,b). At this time point, an average of 36 ± 4% of young and 34 ± 3% of old cells expressed myogenin rising to 66 ± 7% of young and 65 ± 7% of old cells on day 7. Expression of myosin heavy chain (MHC, marker of terminal differentiation) was not detected until 48 h of differentiation (Fig. 3c,d) when an average of 35 ± 6% of young and 33 ± 8% of old cells expressed the marker rising to 71 ± 4% of young and 70 ± 3% of old cells by day 7. The transcription factor MyoD is essential for myogenic differentiation and was expressed in all nuclei within MHC positive cells (Fig. 3e,f). By day 3 of differentiation, all MyoD-labelled nuclei were found within MHC-labelled myotubes (Figure S1). No significant differences were observed in the percentage of nuclei labelled with MyoD, Myogenin or Ki67, or within MHC labeled cells between young and elderly MPCs at any point during differentiation.

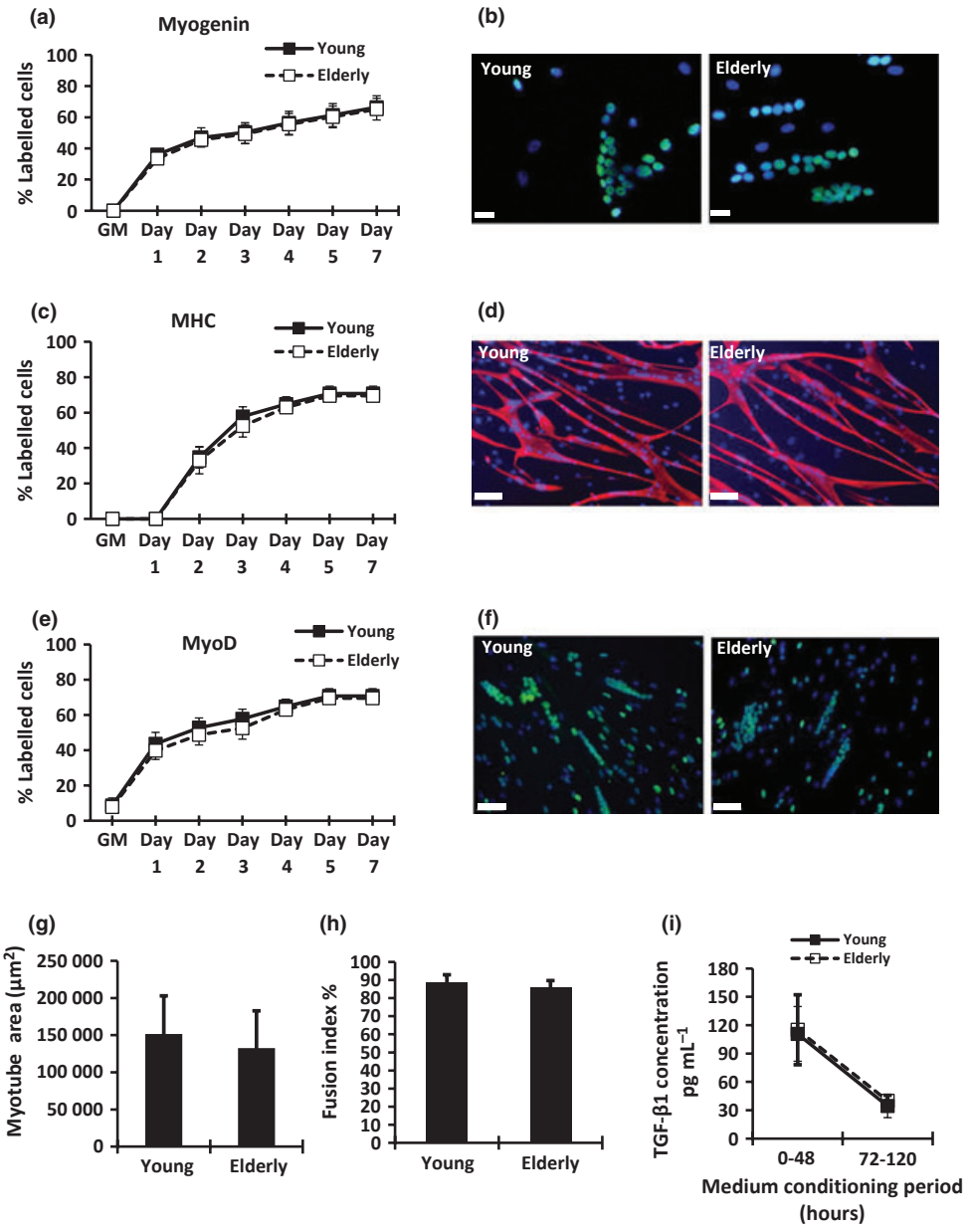

Fig. 3 Time course of (a) myogenin, (c) MHC and (e) MyoD expression, in human cells obtained from young (n = 5) and elderly (n = 4) subjects. Images taken of young (from a male subject aged 25 years) and elderly (from a male subject aged 80 years) cells following 7 days in differentiation medium. Cells stained for (b) myogenin (green) and Hoechst (blue). Scale bar = 20 μm. (d) Cells stained for MHC (red) and Hoechst (blue). Scale bar = 100 μm. (f) Cells stained for MyoD (green) and Hoechst (blue). Scale bar = 100 μm. (g) Measurement of myotube total area and (h) fusion index, in MPCs obtained from young (n = 5) and elderly (n = 4) subjects. (i) TGF-β1 levels in the conditioned media of young and old cells during differentiation. Data are mean values ± SD.

Fusion of cells, as determined by percentage of nuclei in MHC-labelled myotubes, was measured on day 7, as was myotube size (Fig. 3g,h). No difference in either parameter was observed between cells obtained from the young and elderly donors. The concentration of TGF-β1 was measured in culture media after conditioning for a period of 48 h at early (day 1–2 or 0–48 h) and late (days 3–4 or 72–120 h) time points of differentiation. This regulatory cytokine was shown to have a concentration with a mean value of 111 ± 29 pg ml^{-1} for young and 115 ± 37 pg ml^{-1} for old cells in the early and 34 ± 12 pg ml^{-1} for young and 40 ± 6 pg ml^{-1} for old cells in the late period (Fig. 3i). There was no difference in the levels secreted by the cells obtained from the two donor populations at either time point.

Characterization of marker expression and differentiation in senescent cells

As no difference in any characteristic could be observed between cells obtained from young and elderly people, the data from the senescent cell populations that maintained desmin expression throughout time in culture (n = 3 young, n = 2 old) were combined so that their behaviour at senescence could be compared directly with their behaviour in the first 4 days of culture, when the same cell populations were at early passages. At senescence, the desmin-labelled cells demonstrated an altered morphology consistent with that previously described for human satellite cells at senescence (Fig. 4; Renault *et al.*, 2000). The marker, γ-H2AX, was again used to study DNA damage and this analysis showed that, in contrast to

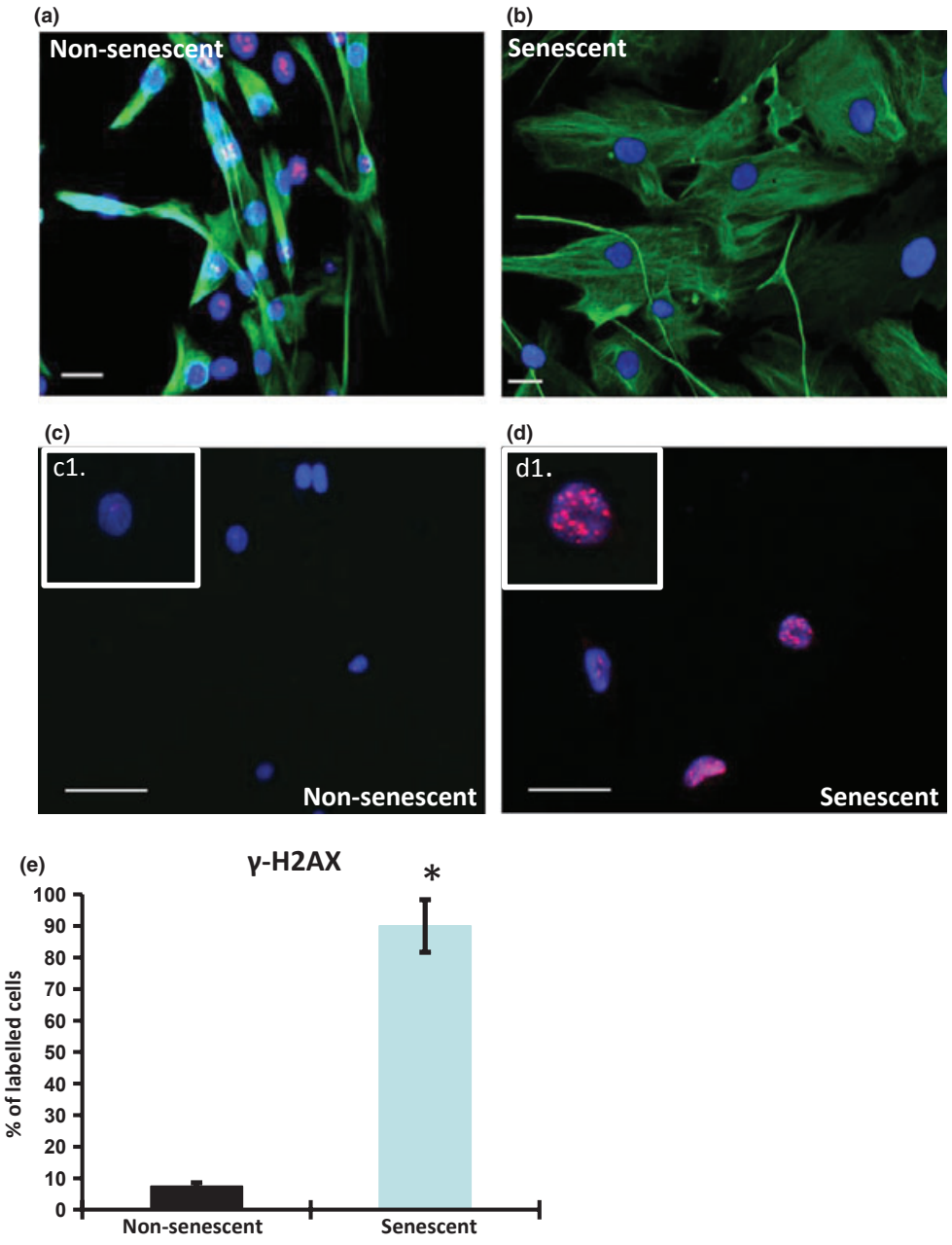

Fig. 4 (a) and (b) Images taken from non-senescent and senescent MPCs and stained for desmin (green), Ki67 (red) and Hoechst (blue) 24 h after plating in growth medium. Scale bar = 20 μm. (c) and (d) DNA damage in human MPCs at early passage and after replicative senescence *in vitro*. Senescent nuclei exhibit characteristic enlarged morphology and express numerous punctate γ-H2AX foci (stained red) indicating the presence of double stranded DNA rupture. Inserts c1 and d1 show expanded views of γ-H2AX expression in typical control and senescent nuclei respectively. Scale bar = 50 μm. (e) DNA damage in senescent cells (n = 5) vs. non-senescent cells (n = 5). Data are mean values ± SD. *, $P < 0.0005$ statistically significant difference from non-senescent cells.

the non-senescent cells, the vast majority (90%) of MPCs exhibited DNA damage. When compared with the non-senescent cells, the senescent cells exhibited a delay in the onset of the differentiation markers myogenin (Fig. 5a) and MHC (Fig. 5b). Despite being plated at the same density, the cells fused less well (Fig. 5h) and produced smaller myotubes (Fig. 5g). The secretion of TGF-β1 was also shown to be markedly higher at the early time point 280 ± 31 pg ml^{-1} compared with the non-senescent cells 75 ± 25 pg ml^{-1} (Fig. 4i). By the late time point, this had fallen dramatically approaching the value of the early passage, non-senescent cells and corresponding to the time when myogenin expression increased in the senescent cells.

A further analysis of cells triggered to differentiate prior to full senescence (after 16 and 28 days of passaging) revealed a progressive reduction in the expression of both myogenin (Fig. 5a) and MHC (Fig. 5c). This was associated with a progressive decline in the percentage of cells positive for MyoD (Fig. 5 and S2).

Response to exogenous TGF-β1

Informed by the TGF-β1 concentration in the conditioned media of the senescent cells at the early time point (300 pg ml^{-1}, associated with inhibition of myogenin expression) and the late time point (100 pg ml^{-1}, associated with the onset of myogenin expression) of differentiation, a further experiment was undertaken to determine if there was an age-related change in the ability of early passage, non-senescent cells from older people to respond to TGF-β1

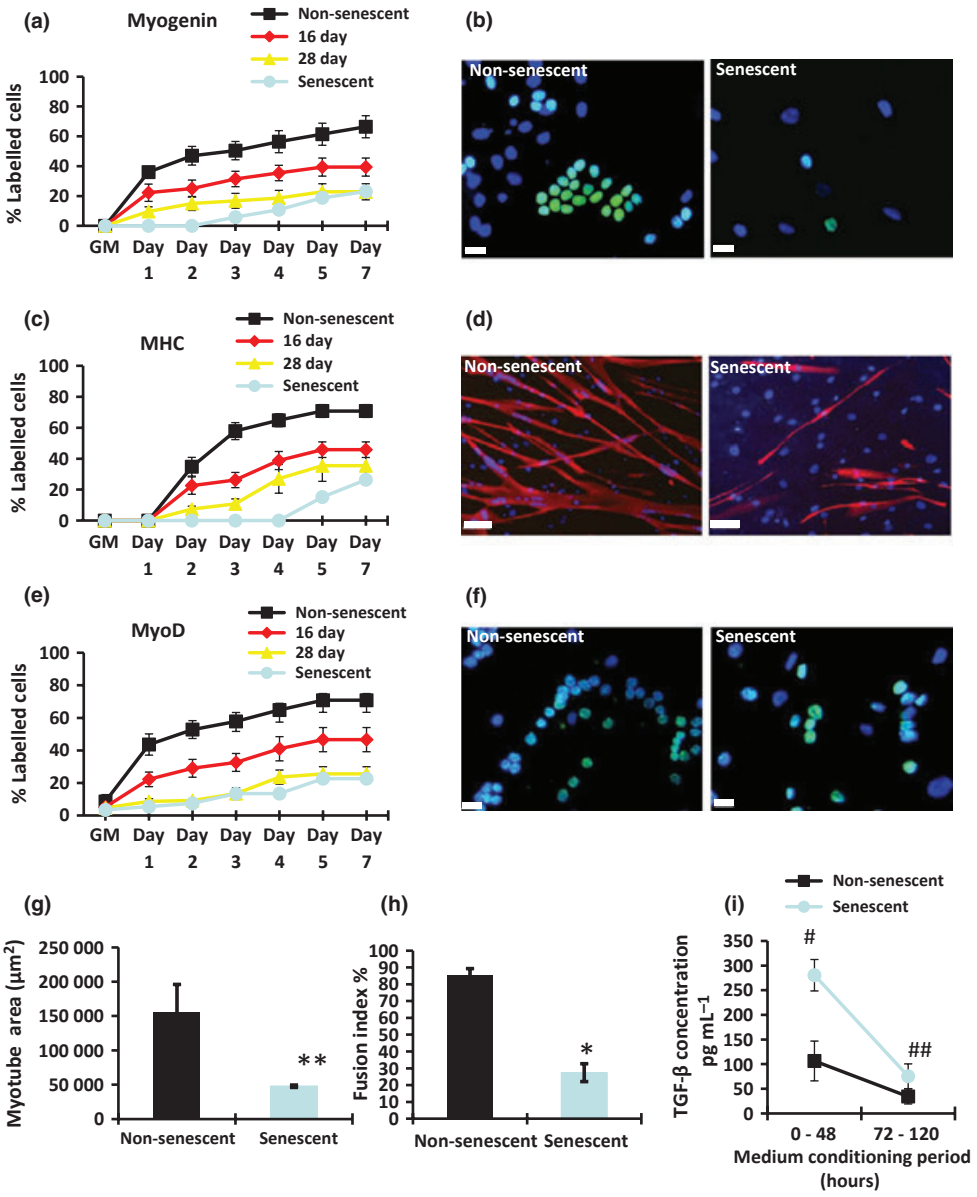

Fig. 5 Time course of (a) myogenin, (c) MHC and (e) MyoD expression, in senescent cells (n = 5) vs. non-senescent cells (n = 5). Cells were either differentiated at early passage or at several time points during the culture period (16 and 28 days, n = 5) until they reached replicative senescence. Images taken from non-senescent and senescent cells after 7 days in differentiation medium. (b) Cells stained for myogenin (green) and Hoechst (blue). Scale bar = 20 μm. (d) Cells stained for MHC (red) and Hoechst (blue). Scale bar = 100 μm. (f) Cells stained for MyoD (green) and Hoechst (blue). Scale bar = 20 μm. (g) Measurement of myotube total area and (h) fusion index, in senescent cells (n = 5) vs. non-senescent cells (n = 5). *, $P < 0.0005$ & **, $P = 0.005$ statistically significant difference from non-senescent cells. (i) TGF-β1 levels for non-senescent and senescent cells during differentiation. #, $P < 0.0005$ & ##, $P = 0.008$ statistically significant difference from non-senescent cells. Data are mean values ± SD.

treatment compared with young cells. The data in Fig. 6 show that 300 pg ml^{-1} inhibited expression of myogenin and MHC in all cells. Lowering the concentration to 100 pg ml^{-1} at Day 3 appeared to remove this inhibition and allowed all cells to progress through the process of differentiation. There was no difference in the response of cells obtained from young or elderly people.

Discussion

The main finding of this study is that the age of donor does not influence the *in vitro* behaviour of human MPCs. This was the case when these cells were examined under a wide variety of experimental conditions, which included proliferation, differentiation, passaging and progression to senescence. With the exception of the data shown in Figs 2 and 5a,c,e, cells were examined at one of two points. The first was within 4 days of culture at which point cells were termed early passage, non-senescent and were proliferating as shown by labelling with Ki67 and subsequent increase in cell number. The second was when the cells reached replicative senescence after which point the cell number did not increase and no Ki67 labelling was observed. Whilst not a primary objective of the study, there were no observable effects of gender on the behaviour of early passage cells.

The senescent cells showed altered morphology and increased DNA damage relative to the early passage, non-senescent cells, as evidenced by γ-H2AX antibody staining, irrespective of the age of the donor. In differentiation medium, senescent cells were still able to express markers of differentiation, but the cultures showed both a delay and a decreased magnitude of this response. The cells fused less well and generated smaller myotubes. The regulatory cytokine TGF-β1 was studied both with regard to its release by the cells and the sensitivity of cells to exogenous TGF-β1 treatment. Here, as with

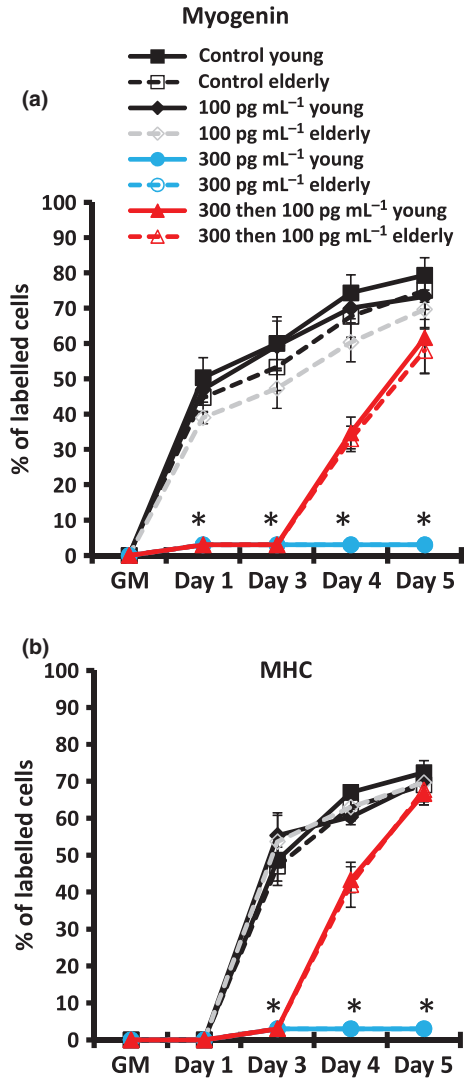

Fig. 6 Time course of (a) myogenin and (b) MHC expression in the presence or absence of TGF-β1. MPCs from young (n = 3) and elderly (n = 3) subjects were first cultured in growth medium for 24 h and subsequently transferred to differentiation medium in the presence or absence of human recombinant TGF-β1 at 100 or 300 pg ml^{-1}. Cells were cultured for a further 5 days in differentiation medium. In some wells, 300 pg ml^{-1} TGF-β1 was substituted with 100 pg ml^{-1} after 3 days and the cells were cultured for an additional 2 days under this condition. Data are mean values ± SD. *, $P < 0.0005$ statistically significant difference from control.

the other parameters studied, no differences were observed between the cells from young and elderly donors. However, during the early phase of differentiation, senescent cells secreted markedly more of the cytokine.

Proliferation of early passage, non-senescent cells

This study showed that there was no relationship between the initial myogenic purity (proportion of desmin positive cells) and the age of the donor. This is in agreement with the findings of others (Schafer *et al.*, 2006; Beccafico *et al.*, 2007; Pietrangelo *et al.*, 2009). Of the nine samples studied, five maintained the proportion of desmin expression marker with time in culture (Fig. 1a–d), while the other four progressively lost their desmin content (Fig. 1e–h) and were overgrown by fibroblasts. The finding that some human MPC populations are able to maintain constant desmin expression over a large number of cell divisions (40 days in culture), and others are not has been reported previously (Schafer *et al.*, 2006). These authors also showed that desmin expression varied from donor to donor with values ranging from 10 to 90%. There are a number of possibilities why the change in the proportion of the different cell types may have occurred with time in culture. For example, fibroblast growth may outstrip that of MPCs. The balance of fast proliferating to slow proliferating cells in culture should ultimately determine which cell type will be dominant. Interestingly, two different cell populations had an initial desmin content of ~50%. In one sample, the percentage of desmin remained constant, but in the other desmin content was reduced to zero at the point of senescence. A constant one to one ratio of two cell types undergoing division can only be explained by both cell types having the same doubling times. The biochemical interactions that may be governing these proliferative responses are not known and likely to be complex. All samples were treated in the same way and we are unable to give a definitive answer as to why muscle precursor cells were not overgrown by fibroblasts in some cultures, but were in others. Importantly, there was no clear association between the number of MPD and donor age, with a replicative lifespan of 5–20 MPD being observed for both young and elderly MPCs. This is consistent with previous estimates of the replicative capacity for human MPCs from adults (Decary *et al.*, 1997; Pietrangelo *et al.*, 2009).

Aging and replicative senescence are characterized by increased DNA damage and a shortening of telomere length (Decary *et al.*, 1997; Sedelnikova *et al.*, 2004; Rube *et al.*, 2011), but the precise mechanisms that contribute to cellular senescence have not been defined (Mouly *et al.*, 2005). To investigate whether the DNA damage response might differ between old and young MPCs before and after replicative senescence, γ-H2AX antibody staining was used to label DNA double strand breaks (Rogakou *et al.*, 1998). This marker identifies the histone protein H2AX which is both ubiquitously expressed and dispersed throughout the genome (Takahashi & Ohnishi, 2005). Aside from the potential difference in the level of DNA damage between young and elderly cells at early passages, we anticipated that erosion of the telomeres as cells approached replicative senescence would expose the uncapped double stranded DNA ends and induce subsequent phosphorylated H2AX foci (Takai *et al.*, 2003; Bonner *et al.*, 2008; D'adda Di Fagagna, 2008). Importantly, in the young and elderly early passage, non-senescent MPCs less than 10% of cells expressed this marker and there was no difference between the two populations (Fig. 3). The increased number of cells expressing γ-H2AX following senescence is discussed further below.

Differentiation of early passage and senescent cells

All nine samples were initially examined in the first 4 days of culture, prior to undergoing multiple passages, with regard to their ability to differentiate (Fig. 3). Cells were placed in differentiation medium and marker expression, fusion and myotube size were followed over a

7 day period. MyoD is a myogenic regulatory factor that is required for myogenic commitment and differentiation (Cooper *et al.*, 1999; Buckingham *et al.*, 2003; Berkes & Tapscott, 2005). The data in Fig. 3c show no difference in MyoD expression in cells obtained from young and elderly people. Furthermore, all samples showed remarkably similar behaviour in culture. This was also the case for myogenin and the marker of terminal differentiation, MHC. The latter showed the expected delay in expression relative to myogenin. At day 7, the percentage of nuclei in MHC positive cells was similar between the young and elderly cells indicating similar abilities to fuse. The size of the formed myotubes was also not affected by age.

Taken together, the proliferation and differentiation data indicate no age-related inherent impairments in myogenic behaviour. These findings are in general agreement with previous work. For example, there is evidence to suggest that MPCs obtained from elderly animals can proliferate and differentiate similarly to that of young animals (Dumke & Lees, 2011). Single fibre grafting experiments have shown that satellite cells from old animals possess an equivalent *in vivo* myogenic and self-renewal potential to that of young animals (Collins *et al.*, 2007). Whole muscle grafting experiments have shown that although there is a slight delay in myotube formation, no difference is observed between elderly and young muscle grafted into elderly or young animals (Smythe *et al.*, 2008) or new muscle formation in very elderly animals (Shavlakadze *et al.*, 2010) even though the start of myogenesis was initially delayed. Furthermore, it has been shown that human MPCs from an elderly donor can contribute myonuclei very efficiently to regenerating mouse muscle *in vivo* (Schafer *et al.*, 2006). These studies also argue against a role for reduced motility reported for satellite cells from elderly animals as having a role in impairing muscle regeneration *in vivo* (Collins-Hooper *et al.*, 2012). Reconciling data from *in vitro* and *in vivo* studies in animal models and humans is critical to the interpretation of the findings regarding satellite cell activation, proliferation, motility and differentiation. As discussed previously (George *et al.*, 2010), many human studies show good satellite cell activation in elderly muscle following a physiologically relevant stimulus such as exercise, suggesting that the signalling pathways for activation are intact although other aspects of the satellite cell response *in vivo* remain to be investigated.

In contrast to the present results, other studies have found age associated differences when comparing differentiation of primary human MPCs from donors of different ages. However, some of these reports are based on the comparison of one old subject with one young subject (Jacquemin *et al.*, 2004; Lorenzon *et al.*, 2004; Fulle *et al.*, 2005). Another concern is that some studies compare populations of MPCs that do not have equivalent proportions of cells expressing desmin (Beccafico *et al.*, 2007; Pietrangelo *et al.*, 2009). In human populations, desmin content reflects the proportion of CD56 positive, myogenic cells in the culture, with desmin negative cells being labelled by TE7 expression (Agley, Rowlerson, Velloso, Lazarus and Harridge, unpublished observations) and therefore determines differentiation potential of a cell population independently of age (Pietrangelo *et al.*, 2009).

In the next series of experiments, the time course and extent of differentiation were studied in cell cultures from young and elderly donors that were able to maintain desmin expression through expansion in growth medium to senescence ($n = 5$). Compared with the same cell populations in the non-senescent state, there was a progressive decrease in the ability of cells to express markers of differentiation with increasing time in culture (Fig. 5). Interestingly, whilst no effects of donor age were observed between young and old non-senescent cells, senescent cells showed a delay in expressing markers of differentiation with time in culture. Senescent cells did not start to express myogenin until day 2 and by day 7 only 23 $\pm$ 2% cells had expressed this marker. A similar delayed and impaired response in MHC expression was observed, but was in keeping with the delay between the two markers in the non-senescent cells. As might be predicted from its regulatory role, MyoD expression was also lower in the senescent MPCs. Further experiments are required to determine if this represents a phase shift where marker expression would approach that of the non-senescent cells if analysed beyond 7 days, or whether it is the small percentage of cells not expressing γ-H2AX which are responding to the stimulus to differentiate. The former would raise interesting questions about DNA damage and either its possible repair during differentiation, or a relative lack of importance for differentiation. Interestingly, despite extensive accumulation of DNA damage, no evidence of increased cell death was observed between early passage and senescent cells, although no specific assays were performed in the present study. The interrelationship between the expression of this DNA damage marker and the ability of the senescent MPCs to differentiate needs further examination.

After 7 days of differentiation, the senescent cells were studied with regard to their ability to fuse and form myotubes. In agreement with Bigot *et al.* (2008), the senescent cells exhibited a lower fusion index and a reduced myotube area compared with early passage, non-senescent cells from both young and elderly donors (Fig. 5g,h). A recent study demonstrated that changes in senescence-associated gene expression are not related to *in vivo* aging in human mesenchymal stromal cells (MSC) and human hematopoietic progenitor cells (HPC) (Wagner *et al.*, 2009). The present results show that senescent cells are capable of differentiating and showing not only the expression of appropriate markers, but that these markers follow their predicted time course for appearance, albeit with a delayed onset. Caution is needed when comparing data from cells aged '*in vitro*' to aging of cells *in vivo*.

TGF-β1 production in early passage and senescent cells

TGF-β1 is an important cytokine involved in regulating skeletal muscle myogenesis (Zentella & Massague, 1992; Carlson *et al.*, 2009a). The ability of TGF-β1 to inhibit myoblast differentiation and to depress proliferation has been documented (Massague *et al.*, 1986; Allen & Boxhorn, 1987; Greene & Allen, 1991). More recently, human MPCs were cultured in the presence of 25 ng ml^{-1} human recombinant TGF-β1 (Carlson *et al.*, 2009b). It was found that myoblast proliferation and differentiation were dramatically reduced by TGF-β1. In conditioned media, we measured TGF-β1 secretion at two time points, early (0–48 h) and late (72–120 h), of differentiation. In the early passage cells, a higher level of secretion was observed at the early time point compared with the later, but

we were once again unable to distinguish between the cells obtained from old and young people. However, when compared with the early passage cells there was a strikingly greater TGF-β1 secretion in the senescent cells, particularly during the early time point. The decline in TGF-β1 secretion in the senescent cells coincided with onset of myogenin expression. To investigate further the possible regulatory role of TGF-β1, we took the concentration of TGF-β1 observed in the conditioned medium during the early phase of differentiation in the senescent cells (300 pg ml^{-1}) and used this concentration of TGF-β1 to study the behaviour of early passage cells from young and elderly donors as they underwent differentiation. We did this for 3 days and then lowered the concentration to that experienced in the later period (100 pg ml^{-1}) when senescent cells appeared to show recovery and began to differentiate. Figure 5 shows that treatment with 300 pg ml^{-1} inhibited the expression of the markers of differentiation and lowering TGF-β1 to 100 pg ml^{-1} initiated recovery, effectively mimicking the behaviour of the senescent cells. We are unable to explain the change in TGF-β1 concentrations, but the key finding as regards the focus of this paper is that the cells from young and older people showed the same sensitivity to TGF-β1 treatment.

Conclusion

Our study reports the following findings. Firstly, the behaviour of MPCs obtained from donors born four decades apart, cannot be distinguished in terms of their proliferative and differentiation characteristics. They fuse in a similar manner develop similarly sized myotubes, exhibit similar low levels of DNA damage and secrete similar amount of the cytokine TGF-β1. Secondly, the expansion of primary cultures of cells obtained from tissue samples can result in dramatic alterations in culture composition, with in some instances MPCs being completely overrun by fibroblasts. The monitoring of population characteristics throughout culture is thus essential in MPC studies. Thirdly, in those cultures that maintained their desmin content a phenotype of senescence was observed where cells had altered morphology, significant amounts of DNA damage, had a delayed time course of differentiation, fused less well, developed smaller myotubes and secreted higher levels of TGF-β1. Again the age of the cell donor was not a factor in determining the behaviour of the senescent cells, but time in culture was, re-emphasizing the importance of studying cells with comparable culture characteristics. Finally, a regulatory role for TGF-β1 on differentiation of non-senescent cells was revealed, this role was again found to be independent of donor age. In conclusion, we are unable to differentiate between young and old MPCs despite subjecting them to a wide variety of incubation conditions judged sufficient to have revealed any discrepancies in their behaviour.

Experimental procedures

Subjects

Prior to acceptance in the study, participants completed a health questionnaire and were only included if they fulfilled the 'healthy' or 'medically stable' criteria (Greig *et al.*, 1994). The muscle biopsies were collected from 5 young (aged 23–25 years, 4 male and 1 female) and 4 elderly (aged 67–82 years, 2 male and 2 female) subjects and used as specified in the figure legends. Ethical approval was given by the Local Research Ethics Committee and all experimental procedures were performed in a Human Tissue Authority licensed laboratory in accordance with the Human tissue Act (2004) and the Declaration of Helsinki.

Muscle biopsy procedure

Biopsies were carried out as previously described (George *et al.*, 2010). Muscle samples were obtained from the vastus lateralis muscle using the needle muscle biopsy technique (Bergström, 1962). Briefly, subjects rested in a supine position. The skin overlaying the area to be sampled was shaved, if required, and thoroughly cleaned with chlorhexidine solution and then anaesthetized (2% lidocaine). After allowing a short time for the anaesthetic to take effect, a small scalpel blade was used to make a 0.5 cm incision through the skin and muscle fascia. A biopsy needle was then inserted through the incision into the muscle belly, and suction applied via a 50 ml syringe. Each biopsy provided 100–300 mg of muscle tissue and was immediately placed in ice cold Solution A (7.2 mg ml^{-1} HEPES, 7.6 mg ml^{-1} NaCl, 224 μg ml^{-1} KCl, 1.98 mg ml^{-1} D-Glucose, 1.134 μg ml^{-1} Phenol red, dd H_2O, pH 7.6; Sigma Aldrich, Poole, UK).

Satellite cell extraction

The muscle biopsy material was moved into the sterile tissue culture hood and washed with Solution A to remove any surface blood. It was then stripped of visible connective and fat tissue using sterile scalpels and the remaining tissue was cut into pieces less than one millimetre in diameter on a Petri dish containing 5 ml ATE solution (solution A + 5 mg ml^{-1} Trypsin EDTA, Invitrogen, Paisley, UK). A wide bore pipette (25 ml) was then used to transfer the enzyme solution containing minced muscle, to a sterile 35 ml Wheaton flask containing a magnetic stirrer. The dish was washed twice with 5 ml ATE solution to collect any muscle fragments which were not aspirated on the first attempt, and placed into the flask making the total volume up to 15 ml. The muscle fragment suspension was heated in a stirring water bath at 37°C for 15 min with gentle agitation to dissociate muscle cells. The supernatant containing suspended cells was aspirated and transferred to a sterile 50 ml falcon tube (BD Bioscience, Oxford, UK) containing 15 ml DMEM (Invitrogen), 10% fetal calf serum (FCS) Gold (PAA) and 1% PSG (100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 292 μg ml^{-1} L-glutamine, Gibco, Paisley, UK). Fresh ATE solution (15 ml) was added to the remaining muscle and the entire procedure repeated twice. The three supernatants were centrifuged for 6 min at 650 g. Following centrifugation, the supernatant containing enzymes was discarded and the cell pellet was re-suspended in skeletal muscle cell growth medium (GM, Promocell, Germany; containing 50 μg ml^{-1} Fetal Calf Serum (FCS), 50 μg ml^{-1} Fetuin (bovine), 10 ng ml^{-1} Epidermal Growth Factor, 1 ng ml^{-1} Basic Fibroblast Growth Factor, 10 μg ml^{-1} insulin and

0.4 μg ml^{-1} dexamethasone) supplemented with 10% FCS and 1% PSG.

Expansion of freshly isolated cells

Freshly isolated cells re-suspended in GM were transferred to sterile T75 flask. The medium was refreshed on the third day after the extraction procedure and subsequently every 48 h. Cells were passaged every 48–96 h depending on when they reached 50% confluence. The cells were expanded for 1 week and frozen in liquid N_2. After thawing the cells were cultured in GM and the medium was changed every 48 h. After a total of 48–92 h, they were passaged once and divided for experimental procedures as outlined below.

Population doubling time

The cells were cultured in growth medium as described above. Media was replaced with fresh GM every 2 days. Cells were passaged depending on when they reached 50% confluence. For population doubling experiments, cells were plated at a density of 2.5×10^3 cells cm^{-2} in 35 mm culture dishes in triplicate. The number of population doubling at every passage was calculated as PD = log (N_1/N_0)/log2 where N_1 is the final cell number and N_0 is the initial number of cells seeded. Replicative senescence was defined at the point after which no increase in cell number was observed for three consecutive weeks.

Proliferation and differentiation assays

Cells were plated in 96-well dishes at a density of 1000 cells/well. Cells were fixed after 24 h and different antigens were examined by immunocytochemistry. For differentiation assays, cells were plated at a density of 7000 cells/well. Media were replaced after 24 h with differentiation medium (DM), containing basal medium supplemented with 10 μg ml^{-1} insulin (Sigma), 10 μg ml^{-1} transferin (Sigma) and 1% PS (100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin). Cells were cultured for a further 7 days in differentiation medium, with aliquots of cells being fixed every 24 h.

DNA damage staining

Aliquots of cells (1000 cells) from non-senescent and senescent populations were plated into 96-well dishes in growth medium. The cells were fixed after 24 h and stained for γ-H2AX.

Collection of conditioned medium

At early passage (young and old cells) and late passage (senescent cells), aliquots of 7000 cells were plated into a 96-well dishes in growth medium. After 24 h, differentiation was induced by replacing GM with DM. Conditioned media (CM) were collected after two 48 h incubation periods after placing the cells in DM: The period between day 0 and day 2 of differentiation (i.e., the first 48 h in DM) and between days 3 and 5 of differentiation (i.e., 72–120 h). CM was immediately stored at −70°C.

Immunocytochemistry

Fixation procedure

At the end of all experiments, cells were fixed in their wells by the addition of an equal amount of 2X PFA solution (7.4% paraformaldehyde ± 0.4% Triton X-100) to the experimental culture medium for 10 mins. The fixative and medium were then aspirated off and the wells were washed two times for 5 min with PBS/0.01% NaN_3 to remove any excess paraformaldehyde and Triton and stored at 4°C for up to 1 week.

Immunocytochemistry procedure

After fixation, cells were incubated in blocking solution, 1% bovine serum albumin fraction V ≥ 96% (BSA; sigma) in PBS, 0.01% NaN_3 for 1 h at room temperature. The primary antibodies used and their working dilutions are described in detail in table S1. Primary antibodies were diluted in 1% BSA and incubated overnight at room temperature. Wells were washed two times for 5 min in PBS/NaN_3, and then incubated for 1 h at room temperature in appropriate secondary antibody (diluted 1:1000 in 1% BSA). Wells were washed two times for 5 min in PBS, 0.01% NaN_3. Nuclei were stained by incubating for 10 min in 1 μg ml^{-1} Hoechst 33342 solution (Sigma). Secondary antibodies were Alexa fluor 594 goat anti-rabbit, Alexa flour 594 goat anti-mouse and Alexa fluor 488 goat anti-mouse (Invitrogen).

All samples were blinded and coded to the investigator. Images of at least five randomly selected fields of view were selected and photographed using a digital camera mounted to a Zeiss Axiovert 135 (Cambridge, UK) inverted fluorescence microscope at 10 or 32× objectives. A grid system was used to avoid re-sampling of a counted area. Two wells were studied per condition and the mean value used for analysis. At least 250 nuclei within each well were counted and analysed.

Luminex-based assays

The assay was prepared and used according to the manufacturer's protocol using High Sensitivity Human Cytokine Magnetic Bead (cat. # HSCYTMAG-60SK, Millipore, Billerica, MA, USA) and TGF-β1 Single Plex (cat. # TGF-β-64K-01, Millipore, Billerica, MA, USA). In brief, an aliquot of culture supernatant was thawed prior to the experiments. A volume of 50 μl of each sample or, the manufacturer provided positive and negative controls and standard curve samples were added to wells of the plate containing 50 μl of assay buffer and 50 μl of diluted beads (1:2.5). The plate was sealed, covered with aluminium foil to protect it from light and incubated with agitation on a plate shaker overnight at 4°C. The sample was then removed and washed two times with washing buffer provided in the kit. A volume of 50 μl of detection antibodies was added to each well and incubated for 1 h at room temperature to bind the bead-cytokine complexes. Then Streptavidin–Phycoerythrin was added and incubated for 30 mins at room temperature. The sample was then removed and washed two times with washing buffer. One hundred microlitres of sheath fluid was then added to each well and the plate was read in a Luminex plate reader, where

a red laser excited the fluorochromes in the beads and, the identity of the bead and corresponding antigen were determined. At the same time, a green laser excited the phycoerythrin-coupled antibodies to determine the amount of cytokine associated with the specific bead. Samples were tested in duplicate and the average concentration for each sample was taken.

Fusion index

Fusion index was measured after 7 days and expressed as the percentage of nuclei in myosin heavy chain positive cells containing at least two nuclei.

Measurement of myotube area

Myotubes were defined and measured using Adobe Photoshop Cs version 8 as described by Agley *et al.* (2012). In brief, digital images of MHC-stained cells were taken at the same exposure from five nonoverlapping fields per well using Zeiss Axiovert (Cambridge, UK) 135 inverted fluorescence microscope at 10× objective. A representative colour range selection mask was then created based on MHC staining and applied to all fields of view to select and measure myotube surface area.

Statistics

Statistical comparisons were made using SPSS 19 software (IBM, Portsmouth, UK), and P values ≤ 0.05 were considered significant. Statistical significance for interactions between groups and time was determined using mixed two-way repeated measure ANOVA with *post-hoc* Bonferroni. One-way analysis of variance (ANOVA) with *post-hoc* Dunnett comparisons to control was conducted to confirm statistical significance between variables of interest, e.g., between treated and nontreated cells as the Dunnett test is the most appropriate to compare each of a number of treatments with a single control. Independent *T*-tests were used when comparing two different groups with a single condition. All values are shown as mean values ± SD.

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Author contributions: Conceived and designed the experiments: MA, CV, NL and SH. Performed the experiments, statistical analysis and analysed the data: MA. Contributed γ-H2AX idea and analysis tools for myotube area: CA. Isolated human MPCs: TG. Wrote the paper: MA, NL, CA, CV and SH.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 (a) Time course of MyoD + MHC expression, in human MPCs obtained from young (n = 5) and elderly (n = 4) subjects. (b) Time course of MyoD + MHC expression, in senescent cells (n = 5) vs. non-senescent cells (n = 5). Cells were either differentiated at early passage or at several time points during the culture period (16 and 28 days, n = 5) until they reached replicative senescence. Data are mean value ± SD.

Fig. S2 Images of cells taken from (a) young (from a male subject aged 25), (b) elderly (from a male subject aged 80) and (c) senescent MPCs stained for MyoD (green) + MHC (red) and Hoechst (blue) after 7 days in differentiation medium. Scale bar = 20 μm. Young and elderly cells were similar in terms of MHC and MyoD expression. There was a decrease in the onset of MHC and MyoD expression when cells reached senescence.

Table S1 Primary antibodies used.