# Evidence that satellite cell decrement contributes to preferential decline in nuclear number from large fibres during murine age-related muscle atrophy

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## Summary

**Skeletal muscle fibres are multinucleate syncitial cells that change size during adult life depending on functional demand. The relative contribution of change in nuclear number and/or cell growth to fibre size change is unclear. We report that nuclei/unit length decreases in larger fibres during skeletal muscle ageing. This leads to an increased size of nuclear domain (quantity of cytoplasm/number of nuclei within that cytoplasm). Initially, larger fibres have more satellite cells than small fibres, but this advantage is lost as satellite cells decline with age. These changes are accompanied by an overall decline in fibre size, returning domain size to the normal range. Exacerbated loss of fibre nuclei per unit length during ageing of *myoD*-null mice provides the first experimental support for the hypothesis that a satellite cell defect causes inadequate nuclear replacement. We propose a model in which a decline in satellite cell function and/or number during ageing leads to a loss of nuclei from large fibres and an associated domain size increase that triggers cytoplasmic atrophy through the normal cell-size-regulating machinery.**

Key words: Muscle, Ageing, Fibre, Myoblast, Satellite cell, MyoD

## Introduction

The control of cell size is a fundamental aspect of cell biology that is poorly understood. Most post-mitotic cell types have a precisely controlled size achieved by balancing synthesis and degradation of cellular constituents. Skeletal muscle fibres are among the few cell types that alter cell size during adult life. Exercise promotes an increase in fibre size, whereas inactivity leads to fibre shrinkage. Muscle fibres are multinucleate syncitia, so the concept of nuclear domain size (cytoplasmic volume per nucleus) is useful in considering fibre growth or atrophy. A change in fibre size could arise from alteration in nuclear number, nuclear domain size or both (Allen et al., 1999; Hughes and Schiaffino, 1999).

Muscle fibres undergo atrophy during ageing of humans or animals, but the reasons for this are not entirely clear (Proctor et al., 1998). Studies of fibre atrophy conflict on whether domain size is maintained or not (Allen et al., 1997; Allen et al., 1996; Kasper and Xun, 1996; Manta et al., 1987; Mitchell and Pavlath, 2001; Roy et al., 1999; Wada et al., 2002). One reason for confusion might be that cytoplasmic volume/nucleus differs between fibres of different type and increases with fibre size (Bruusgaard et al., 2003). In rodent fast muscle, where nuclear number is roughly proportional to surface area (Bruusgaard et al., 2003), one might expect a decline in cytoplasmic volume/nucleus as fibres shrink during ageing.

Recent advances show that an acute response to stimuli that trigger atrophy is activation of proteolysis (Sandri et al., 2004). This suggests that, at least in some atrophy models, proteolysis might cause atrophy by decreasing domain size prior to significant change in nuclear number. However, in general, changes in fibre size are accompanied by changes in nuclear number, implicating changes in nuclear turnover in fibre size control (Chen et al., 2002; Dupont-Versteegden et al., 2000; Mitchell and Pavlath, 2001). Thus, it is unclear whether a decline in fibre volume precedes or follows nuclear loss.

Nuclear turnover in muscle fibres probably affects domain size. Nuclei are added to fibres from proliferative myogenic precursor satellite cells (SCs) during both growth and atrophy (Darr and Schultz, 1987). Fusion of a small myoblast to a large fibre would reduce domain size, at least transiently. Yet it is known that fibres with more nuclei are larger, and that larger fibres have a greater cytoplasmic volume/nucleus than small fibres (Roy et al., 1999), so one might expect nuclear addition eventually to lead to domain enlargement. Therefore, domain size probably undergoes dynamic changes as nuclei turn over.

The SC pool from ageing muscle shows various defects compared with younger animals (Bockhold et al., 1998; Chargé et al., 2002; Conboy et al., 2003; Renault et al., 2002), which might contribute to fibre atrophy. Myogenic regulatory transcription factors (MRFs) are central to appropriate SC function. For example, *myoD*-null mice have defective SC differentiation (Cornelison et al., 2000; Sabourin et al., 1999) and poor muscle repair (Megeney et al., 1996). Here, we show that loss of *myoD* function leads to failure to maintain myonuclear density and increased nuclear domain size as fibres age, probably due to SC differentiation defects. During age-

related fibre atrophy, syndecan-4 (Syn4)-expressing SCs associated with large fibres decline preferentially, paralleling a nuclear domain size increase in large fibres. We propose a model whereby failure of nuclear replacement in large fibres leads to an excessive domain size that triggers fibre atrophy.

## Materials and Methods

All animal work was approved by the Home Office (UK). Single fibres were isolated, and fibre size and myonuclear number was measured as described by Wada et al. (Wada et al., 2002). Briefly, skinned hindleg muscle from CBAj mice at 2 months, 12 months, 22-24 months (ageing) and 26-29 months (aged) or homozygous *myoD*$^{m1}$ mice at 2 months and 24 months (Rudnicki et al., 1992) was clamped to minimize fibre length variability and fixed in 4% paraformaldehyde (PFA) for 2 days at room temperature. Small bundles of fibres from the almost pure IIb anterior superficial surface of the tibialis anterior (TA) were dissected, incubated in 40% NaOH for 2 hours and agitated vigorously for 20 minutes. Released fibres were washed in PBS, stained with 10 μM DAPI and images of 25-45 single fibres per animal were captured with a Zeiss Axiophot 10× objective. Fibre diameter was measured at four locations over at least 500 μm length of fibre and all nuclei were counted using Openlab software (Improvision, UK). Fibre surface area/nucleus=fibre segment length × π × mean diameter/nuclei in segment. Volume/nucleus=fibre segment length × π × (radius)$^2$/nuclei. On occasion, fibres were pooled into small (<1500 μm$^2$), medium (1500-2500 μm$^2$) and large (>2500 μm$^2$) categories, ensuring no fewer than 10% fell in any pool at any age. An advantage of calculating the volume/nucleus is that it normalizes for fibre stretching or changes in total fibre length, which would decrease fibre diameter and nuclei per unit length, but not change the number of nuclei per fibre. Therefore, this measurement is constant during fibre elongation.

Fibres were stained after isolation as described (Chargé et al., 2002). Briefly, TA was incubated in 0.2% collagenase (type 1, Sigma) for 80 minutes, triturated in DMEM 10% horse serum (HS), fixed in 4% PFA for 15 minutes and stored in methanol at –20°C for RNA analysis or PBS 0.05% azide at 4°C for immunoreaction with 1:50 mouse monoclonal anti-MyoD (Novacastra) antibody, 1:5 mouse monoclonal Pax7 antibody (Hybridoma Bank) and chicken polyclonal anti-Syn4 antibody (1:1500; gift from B. B. Olwin, University of Colorado, Boulder, CO), detected by 1:1000 goat anti-mouse-Alexa543 antibody or 1:1000 goat anti-chicken-Alexa488 antibody (Molecular Probes) and counterstained with DAPI. In situ hybridization of single fibres used digoxygenin-tagged full-length MyoD1 probe (pVZC11β; gift from A. B. Lassar, Harvard University, Boston, MA). Sections (15 μm) from TA were rehydrated in PBS, blocked in 5% HS, incubated overnight at 4°C in BF-F3 and N2.261 antibody to detect myosin IIb and IIa/I isoforms, respectively, washed in PBS 0.05% Tween 20, blocked (5% HS, 5% goat serum 30 minutes), detected with goat anti-mouse IgM Cy3 (1:100) and horse anti-mouse IgG FITC (1:400) for 1 hour, washed and mounted in antifade (DABCO/gelvatol mix).

Dissected muscles from *MD6.0-lacZ* (Asakura et al., 1995) on a CBAj background were either fixed in 4% PFA for 30 minutes and stained with X-gal or weighed, homogenized in Galacto-light buffer containing 0.2 mM phenylmethylsulphonylfluoride (PMSF), 1 mM dithiothreitol, 5 μg/ml leupeptin, centrifuged to remove insoluble material and stored frozen. Aliquots were analysed for protein (BCA kit; Pierce), DNA (Hoechst 33258) and β-gal activity (Galacto-light kit; Tropix).

Data was tested for normality and compared using either one-way ANOVA and Tukey post-hoc test, or a Kruskall Wallis and Dunn's post-hoc test. Within each genotype and age, animal-animal variability was not significant. Therefore, regression analysis by non-linear least-square was carried out across all individual fibres pooled into groups based on genotype and age. Data are mean±s.e.m. Significance was accepted at $P<0.05$.

## Results

### Fibres shrink and have fewer nuclei per unit length during ageing

Nuclear number and size of single dissociated superficial TA fibres were analysed after chemical removal of all other cells. Young fibres have nuclei with uniform morphology that are generally aligned with the fibre axis (Fig. 1A). By contrast, fibres from old animals have nuclei with a variety of dispositions and shapes. Approximately 22% of fibres from 22-24-month-old mice and 25% from 26-29-month-old mice have longer nuclei than those in the 2-month-old and 1-year-old animals, although sarcomere length is unchanged (Fig. 1B,C and data not shown). Furthermore, 7% of fibres in 26-29-month-old animals have long chains of nuclei (Fig. 1D), despite normal fibre morphology without signs of splitting or necrosis. Other regions of these fibres appear depleted in nuclei (data not shown).

Fibre size varies significantly with age. Aged mice (26-29 month) have a 33% reduction in mean fibre size compared with adults ($P$=0.008, Fig. 1E). Nuclear number per unit length of

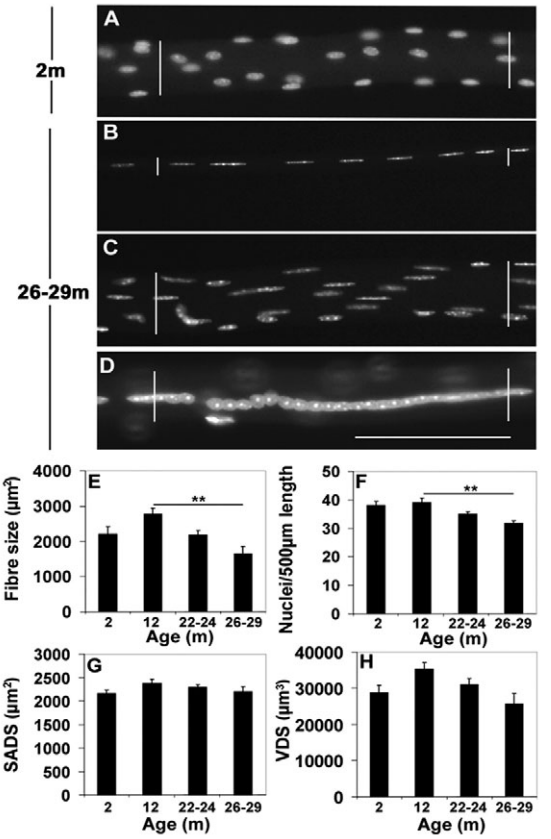


**Fig. 1.** Isolated single TA muscle fibres lose cytoplasm and nuclei during ageing. (A-D) Micrographs of DAPI-stained nuclei in fibres. At 2 months (A), all fibres show well-spaced oval peripheral nuclei aligned with the fibre axis. At 26-29 months, some small (B) and large (C) fibres have elongated or mis-orientated nuclei, or nuclear chains (D). Vertical bars denote fibre diameter. Horizontal bar, 100 μm. Mean fibre cross-sectional area (E), nuclei per unit fibre length (F), surface area domain size (SADS, G) and volume domain size (VDS, H) in wild-type mice at 2 months (number of animals per group, $n$=9), 12 months ($n$=5), 22-24 months ($n$=5) and 26-29 months ($n$=8). **$P<0.01$. Error bars indicate animal to animal s.e.m.

fibre (hereafter termed nuclei/unit length) declines with age ($P$=0.0001, Fig. 1F). Therefore, ageing is associated with atrophy of fast muscle fibres after 2 years of age, and fibres have fewer nuclei/unit length.

The decline in both nuclei/unit length and fibre size suggests a particular nuclear domain size might be maintained during ageing. Following others (Bruusgaard et al., 2003; Roy et al., 1999), we distinguished changes in fibre volume per nucleus and surface area per nucleus, which we refer to as volume domain size (VDS) and surface area domain size (SADS), respectively. Neither mean SADS nor mean VDS changes significantly at any age (Fig. 1G,H), although VDS varied more, as expected due to the exponential relationship of surface area and volume of a cylinder. Hereafter, we employ SADS to analyse control of domain size because, as shown below, SADS varies linearly with fibre size.

At first sight, one might explain decline in nuclei/unit length and fibre size by stretching of muscle fibres with age, without overall change in nuclear number. However, this seems unlikely because VDS shows, if anything, a decline with age, whereas it would remain constant if a fibre were passively stretched. Similarly, SADS remains constant with age, yet would be predicted to rise after a stretch (Fig. 1G,H).

## Nuclear domain size increases with fibre size

Given the apparent constancy of SADS during age-related atrophy (Fig. 1G), we investigated the relationship of SADS and fibre size in more detail. In adult muscle, although large fibres have more nuclei/unit length, a positive relationship between SADS and fibre size is observed ($P$<0.05; Fig. 2A,B). A comparison between 2-month-old (during growth) and 1-year-old (after the major growth phase) mice shows the linear relationship between SADS and fibre size is maintained when growth is occurring (shift rightward in Fig. 2A,B). Therefore, in adult mice, nuclei of large fibres have to support more cytoplasm and surface area than those of small fibres.

## Nuclear domain size of large fibres increases with age

The constant mean SADS across all ages tested, despite a reduction in mean fibre size during ageing, is surprising in the light of the lower SADS in small adult fibres. Two opposing trends occurring during ageing explain this phenomenon. First, nuclei/unit length decline to a greater extent in large (>2500 μm$^2$) compared with small (<1500 μm$^2$) fibres of older animals (Fig. 2C,G), and this leads to a progressive rise in SADS in large fibres with age (Fig. 2D,H). Second, fibres are on average smaller in older muscle and the relationship

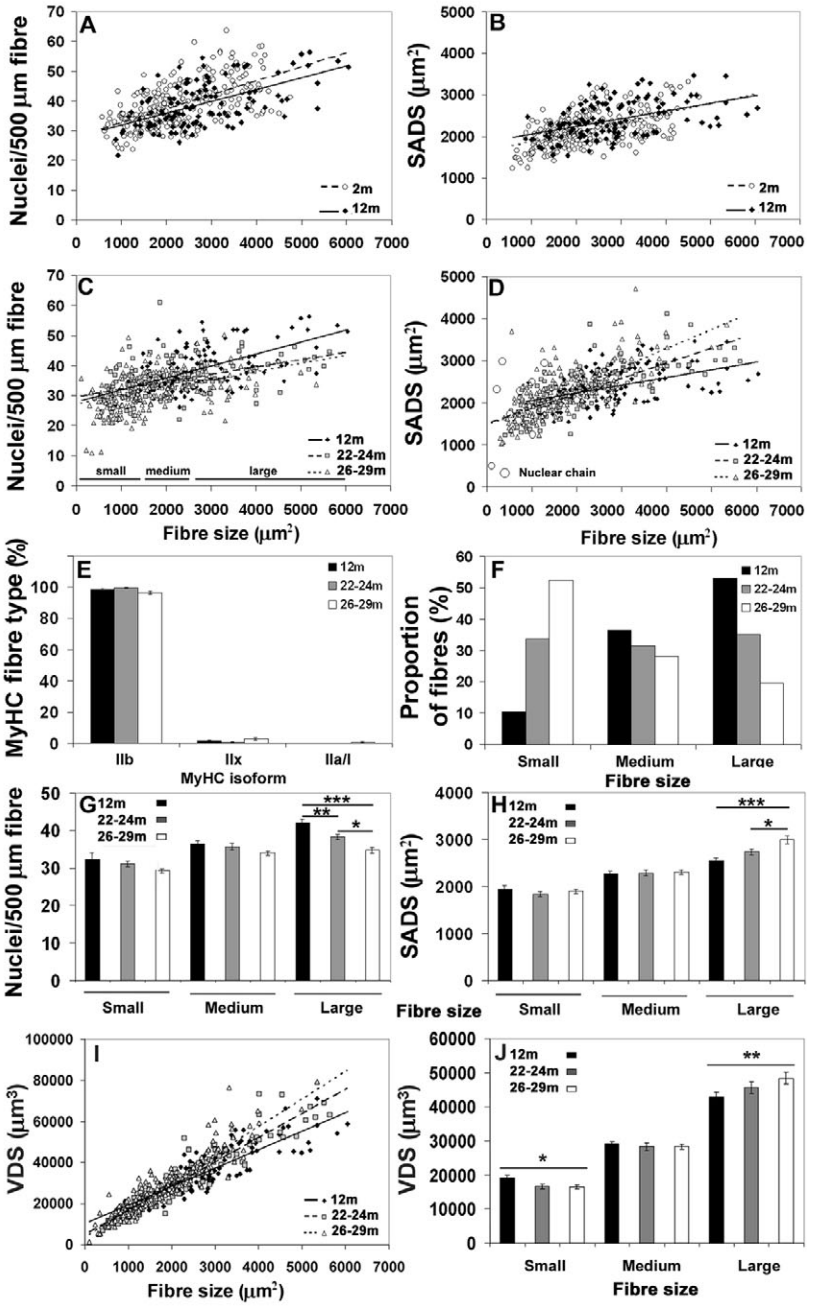


**Fig. 2.** Nuclear density decreases with age preferentially in large fibres with high domain size. Relationship of nuclei/unit fibre length (A,C,G), SADS (B,D,H) and VDS (I,J) to fibre size in young/adult (A,B) and adult/old (C-J) animals. (A,B) Larger fibres have more nuclei/unit length and each nucleus has a larger nuclear domain than small fibres during adult life ($n$=253 and 75 fibres in 2-month-old and 12-month-old mice, respectively). (C,D) With ageing, nuclei/unit length decrease and SADS increases in large fibres. Small fibres show no significant change. (E) Fibre type frequency is not significantly altered in superficial TA with age. (F-J) Fibres pooled into small (<1500 μm$^2$), medium (1500-2500 μm$^2$) and large (>2500 μm$^2$) groups emphasize that the fibre population becomes smaller during ageing (F). Number of fibres in small-sized ($n$=27, 54, 100) at 12 months, 22-24 months and 26-29 months, respectively, in medium-sized ($n$=37, 37, 45) and large-sized ($n$=37, 29, 40) fibres. Large fibres have more nuclei per unit length, but lose this advantage with age (G). This change is reflected by an increase in domain size in large fibres (H-J). *$P$<0.05, **$P$<0.01, ***$P$<0.001. Error bars indicate fibre to fibre s.e.m.

between SADS and size is preserved in small fibres during ageing (Fig. 2D,F). Thus, a general lowering of SADS as fibres shrink during ageing is offset by an unexpected rise in mean SADS in large fibres of older mice. Together, these two trends explain why, on average across the entire fibre population, SADS does not change with age (Fig. 1G). Consistent with this view, there is a significantly steeper slope ($P<0.05$) in the relationship between SADS and fibre size of aged fibres compared with adult (Fig. 2D). These counter-intuitive results show that an increase in domain size in large fibres accompanies atrophy.

We next asked whether the changes in fibre size, nuclei/unit length or SADS could be accounted for by changes in fibre type. In the anterior superficial region, which we chose to analyse to avoid the confounding effects of fibre type variation, fibre type is essentially pure IIb at 2 months, 12 months and 22-24 months, and <5% of fibres have become IIx at 26-29 months (Fig. 2E). The IIx fibres were generally smaller than IIb fibres (data not shown). Analysis of the proportions of fibres in small (<1500 $\mu m^2$), medium (1500-2500 $\mu m^2$) and large (>2500 $\mu m^2$) size groups showed that large fibres decline from 50% to 20% of total, whereas small fibres increase from 10% to 50% of total during ageing (Fig. 2F). This shift is significantly larger than can be accounted for by fibre type conversion. Thus, although TA muscle does become slower with age, the declining fibre size, loss of nuclei/unit length and rise in SADS in large fibres cannot be accounted for by fibre type transition. Indeed, slower fibres types generally have a smaller SADS and therefore could not account for increase in SADS (Bruusgaard et al., 2003; Roy et al., 1999).

## Nuclear loss drives ageing-related SADS increase

Increased SADS could arise through overall lengthening of the muscle, nuclear loss or fibre size increase. Two different ages were considered, 22-24 months and 26-29 months, which enabled a temporal relationship to be established between changes in nuclear number and atrophy. A significant decline in nuclei/unit length of large-sized (>2500 $\mu m^2$), but not small-sized (<1500 $\mu m^2$) or medium-sized (1500-2500 $\mu m^2$), fibres occurs in 22-24 month mice (Fig. 2G). Stretching of muscle would be expected to decrease nuclei/unit length equally in all fibre sizes and therefore cannot account for this result. Although a shift in fibre size is observed during ageing (Fig. 2F), the mean size of large fibres does not change significantly between 12 months and 22-24 months (3572±119 $\mu m^2$ and 3462±126 $\mu m^2$). Thus, during age-related atrophy, the first significant change we detect is a loss of nuclei/unit length preferentially in larger fibres. Subsequently, by 26-29 months, further significant loss of nuclei in large fibres leads to increased SADS (Fig. 2H). This change accompanies the general shift of fibres to smaller sizes (Fig. 2F). By contrast, stretching of the muscle would be expected to increase SADS equally in all fibre size classes, which is not observed (Fig. 2D,H). We also considered the possibility of transient fibre size increase during atrophy and eliminated it for three reasons. First, there are fewer fibres in the large pool early in ageing at 22-24 months (Fig. 2F). Second, maximal fibre size does not increase during ageing (Fig. 2C,D). Third, fibre necrosis, which can be accompanied by swelling, is not significant during ageing (histological analysis of cryosections, data not shown). In conclusion, the data indicate that a loss of nuclei/unit length in large fibres is the first significant ageing-related change detected to account for the increased domain size.

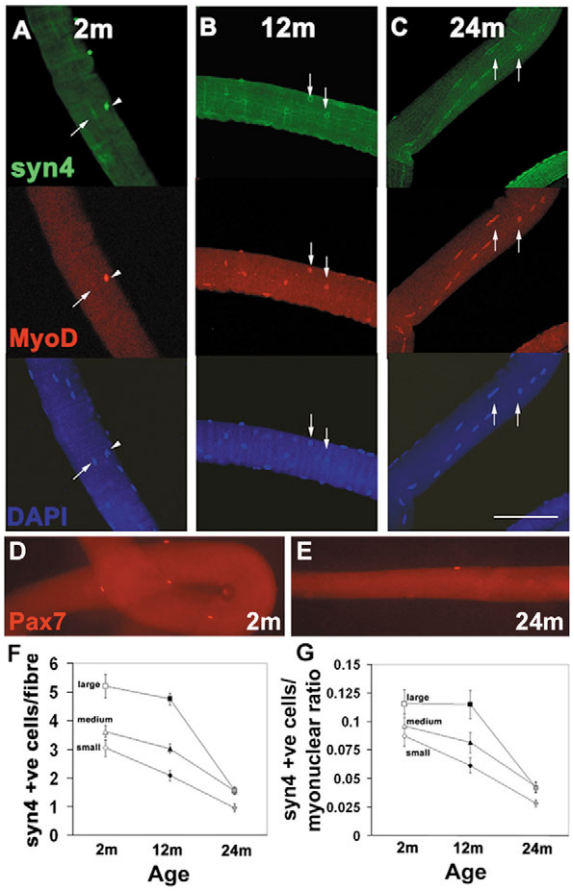


**Fig. 3.** SCs decline with age. (A-C) Syndecan-4 (Syn4, green), MyoD (red) and DAPI (blue) staining of freshly-isolated superficial TA fibres from 2-month-old (A), 12-month-old (B) and 24-month-old (C) mice. Arrows, myonuclei; arrowhead, SCs. Bar, 100 $\mu m$. (D,E) Pax7 staining (red) of fibres from 2-month-old (D) and 24-month-old mice (E). (F) $Syn4^+$ SCs/fibre in large (>2500 $\mu m^2$), medium (1500-2500 $\mu m^2$) and small (<1500 $\mu m^2$) fibres reveal that large adult fibres have more SCs and that the age-related decline in SCs is greatest on large fibres. (G) Comparison of the ratio of $Syn4^+$ SCs to myonuclei (calculated as [$Syn4^+$ cells.$fibre^{-1}$]/[$DAPI^+$ nuclei/500 $\mu m$]), showing that large fibres lose their advantage by 24 months. Number of fibres counted are 20, 30 and 27 for small, medium and large fibres at 2 months, and 23, 37 and 38 at 1 year, and 45, 70 and 74 at 24 months.

It is not possible to measure total fibre length reliably in isolated fibres, although no obvious changes with age were observed (data not shown). Any stretching of fibres with age, perhaps through shortening of tendons, might contribute to the increase in SADS at large fibre sizes. However, VDS also increases in large fibres in our analysis, which a passive change in length would not predict (Fig. 2I,J). Moreover, whole muscle stretching would increase SADS in small fibres to the same extent as in large fibres, which is not observed. To explain our observations by stretching would require a preferential addition of nuclei to small fibres during ageing, thereby permitting maintenance of nuclei/unit length in these fibres. In any event, therefore, loss of nuclei/unit length appears to drive an increase in domain size with age.

## Additional SCs on large fibres are lost during ageing

The loss of nuclei/unit length in ageing muscle fibres might reflect inadequate replacement by SCs. Reduction in SC numbers occurs in ageing muscle and could contribute to failure of nuclear maintenance (Bockhold et al., 1998; Renault et al., 2002). Using anti-Syn4 antibody to mark SCs on acutely dissociated fibres (Cornelison et al., 2001), we confirm that SCs decrease between 12 months and 22-24 months, both in terms of SCs/fibre and SCs/myonucleus ($P$<0.001; Fig. 3A-G). Pax7 is another marker of SCs from single fibres (Zammit et al., 2004). Pax7$^+$ cells on dissociated fibres decline between 2 months and 24 months from 5.7±0.4 to 2.7±0.2 per fibre, confirming the decline detected with anti-Syn4 antibody. At 2 months and 12 months, all fibres examined had some Syn4-expressing cells, but 23% (42/185) of 22-24 month fibres lacked such cells. During ageing, the number of Syn4$^+$ cells per fibre declines in association with large, medium and small fibres (Fig. 3F; $P$<0.001). Strikingly, in adult animals, large fibres have more SCs/fibre nucleus, but this potential repair advantage is lost during ageing (Fig. 3G). In conclusion, the loss of Syn4$^+$ SCs during ageing may have more severe consequences in large fibres.

## Fewer nuclei in ageing fibres in the absence of *myoD*

To test the hypothesis that the decline of SCs/myonucleus could lead to inadequate nuclear replacement and hence increase in domain size, we examined ageing in *myoD*-null ($D^{-/-}$) mice, which have known SC differentiation defects (Cornelison et al., 2000; Sabourin et al., 1999). Mean fibre size and nuclei/unit length are not different between $D^{-/-}$ and littermate controls at 2 months (Fig. 4A,B). In 22-24-month-old $D^{-/-}$, the nuclei/unit length decreases significantly compared to both 2-month-old $D^{-/-}$ and 22-24-month-old controls (Fig. 4B). Therefore, the loss of nuclei/unit length that occurs during ageing is exacerbated in fibres from $D^{-/-}$ mice. No evidence of change in overall muscle or tendon length was observed (data not shown). Although the mean fibre size appears lower in 22-24-month-old $D^{-/-}$ compared with age-matched controls, this result did not achieve significance. Detailed analysis reveals that nuclei/unit length is significantly reduced in small, medium and large fibres of 22-24-month-old $D^{-/-}$ mice compared with controls (Fig. 4E,G), leading to an increase in SADS at all fibre sizes (Fig. 4F,H). Thus, the increase in nuclear domain size observed only in larger fibres of ageing wild-type mice is exacerbated in mice lacking *myoD*.

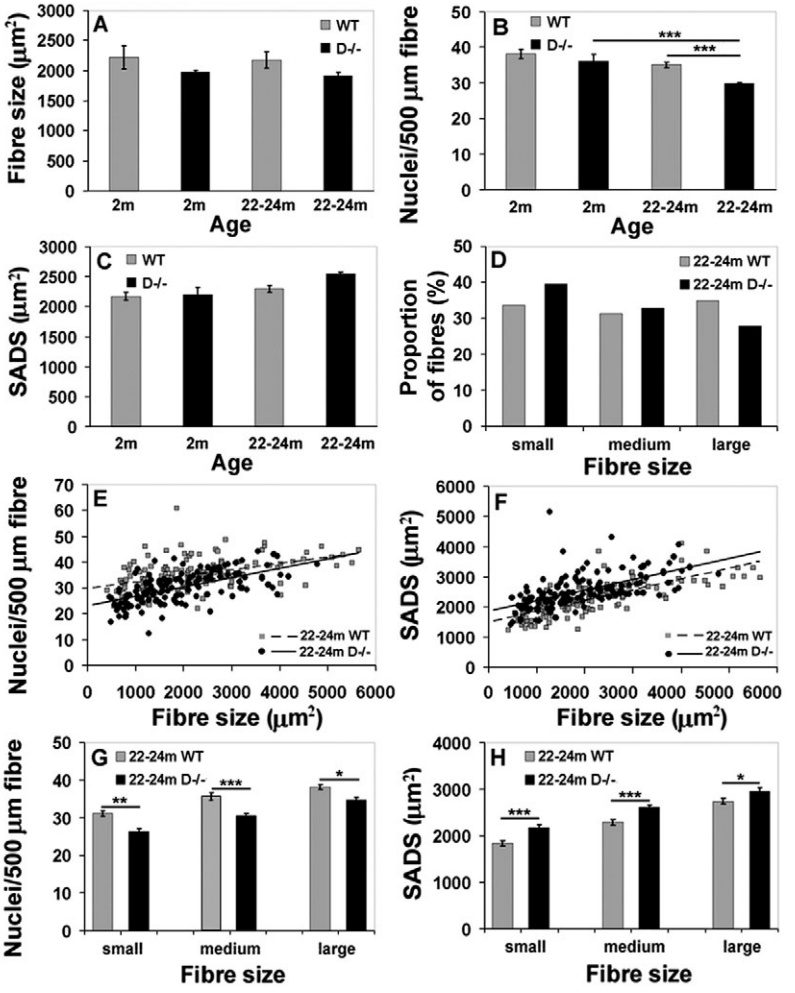


**Fig. 4.** Nuclear density declines at all fibre sizes in the absence of *myoD*. Mean fibre cross-sectional area (A), nuclei per unit fibre length (B), surface area domain size (SADS, C) in wild-type (number of animals per group, $n$=9, $n$=5) and *myoD*-null ($D^{-/-}$) mice ($n$=4, $n$=5) at 2 months and 22-24 months, respectively. **$P$<0.01. Error bars indicate animal to animal s.e.m. (D) Fibres pooled into small (<1500 μm$^2$), medium (1500-2500 μm$^2$) and large (>2500 μm$^2$) groups show the fibre size is not lower in ageing $D^{-/-}$ than wild-type mice. (E,F) Relationship of nuclei/unit fibre length (E) and SADS (F) to fibre size in 22-24-month-old $D^{-/-}$ and age-matched controls. (G,H) Nuclei/unit fibre length decreases (G) and SADS increases (H) in 22-24-month-old $D^{-/-}$ mice in all fibre size ranges compared with both age-matched controls. Number of fibres in 22-24-month-old $D^{-/-}$ mice are 60, 23, 30 for small, medium and large fibres and 54, 37, 29 for 22-24-month-old controls. *$P$<0.05, **$P$<0.01, ***$P$<0.001.

## MyoD is increased during fibre maturation

Although a SC differentiation defect provides a simple explanation for the loss of nuclei in ageing $myoD^{-/-}$ muscle via inadequate nuclear replacement, it is not the only possible reason because *myoD* is also expressed in adult fibres (Hughes et al., 1997). As muscles age, expression of *myoD* has been reported to increase or decrease (Alway et al., 2002b; Dedkov et al., 2003; Tamaki et al., 2000). MyoD is expressed in SCs, which activate *myoD* as they repair muscle (Grounds et al., 1992; Koishi et al., 1995). To distinguish between fibre nuclei and SC expression, first we examined MyoD protein and mRNA levels in satellite myoblasts associated with freshly isolated fibres during ageing (Figs 3A-C, 5A,B). Despite the reduced number of SCs detected on single fibres from 22-24-month-old mice, the proportion containing immunodetectable MyoD protein did not differ between 2 months (34.1±7.1%),

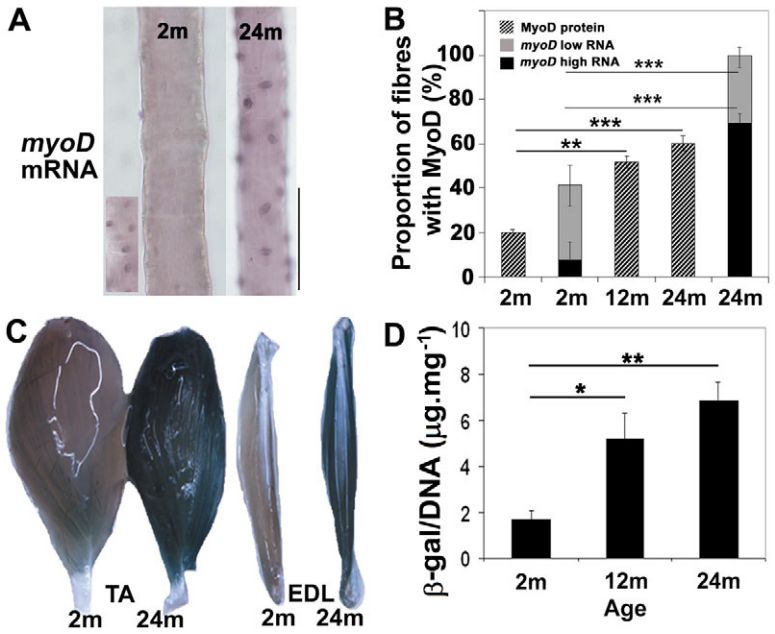


**Fig. 5.** MyoD expression rises with age. (A) In situ mRNA hybridization shows *myoD* mRNA is lower in 2 month (inset shows a rare *myoD*$^+$ fibre region) than 24 month superficial TA fibres. Bar, 100 μm. (B) Both MyoD protein and mRNA increase in myonuclei with fibre maturation. (C) X-gal reaction of wholemount TA and EDL muscle from *MD6.0-lacZ myoD* reporter mice is stronger at 24 months than 2 months. (D) Quantified β-gal activity in TA muscle homogenates increases between 2 months and 12 months of age. **P*<0.05, ***P*<0.01, ****P*<0.001.

12 months (39.0±3.1%) and 22-24 months (37.3±4.1%). Thus, we found no evidence of a change in MyoD within SCs during ageing, although a decline in total satellite-cell-derived MyoD from a fibre is expected on the basis of the lower number of SCs.

We next examined expression of MyoD in fibres themselves. The number of acutely isolated fibres with MyoD-positive myonuclei increased significantly between 2 months and 12 months, but not after 12 months (Fig. 5B, hatched bars). In situ mRNA hybridization revealed fewer and weaker *myoD* mRNA-expressing fibres at 2 months than at 24 months (Fig. 5A,B, solid bars). Next, we used an independent method to quantitate *myoD* expression within fibres. We have recently shown that the *MD6.0-lacZ* transgenic reporter, which contains the proximal 6 kb of *myoD* 5′ genomic sequence driving *lacZ* expression, is solely expressed inside fibres (S. B. Chargé, A.S.B., S. Bayol and S.M.H., unpublished observation). Reporter expression is greater in TA and EDL muscle at 22-24 months compared with 2 months (Fig. 5C,D; $P<0.01$). However, the only significant increase occurs between 2 months and 12 months. In conclusion, MyoD expression in SCs appears independent of age, whereas MyoD increases in muscle fibres with maturity, not specifically during ageing.

## Discussion

The present study was carried out to clarify how fibre nuclear density and domain size change during age-related atrophy. We observed that a loss of nuclei per unit length during ageing is exacerbated in large fibres, leading to an increased domain size in these fibres. Loss of nuclei is more marked than the shrinkage of cytoplasm during the early stages of muscle ageing. The finding of fewer nuclei/unit length in ageing *myoD*-null mice compared with age-matched controls provides the first experimental evidence that efficient SC differentiation is required to maintain nuclear number/fibre in ageing muscle fibres. Normally, in adult animals, large fibres have more SCs/myonucleus than small fibres. This advantage is lost early during ageing, which we propose contributes to the decline in nuclear number in big fibres from ageing muscle, triggering a shift to smaller fibre size.

### Fibre nuclear number declines during ageing

In adult mice, although bigger TA fibres have more nuclei/unit length than small fibres, this increase does not completely account for the enlargement of the fibres: both SADS and VDS increase with fibre size. These results confirm that VDS increases with fibre size in some muscles (Bruusgaard et al., 2003; Kasper and Xun, 1996; Ohira et al., 1999) and that SADS varies less (Bruusgaard et al., 2003). Taken together, the data imply that domain size is tightly regulated in the adult. Although a mechanism is not immediately apparent, our working hypothesis is that activity-dependent factors control the set point of domain size (Wada et al., 2002) by interacting with basal-cell-size-regulating machinery.

We show that murine TA fibres lose nuclei/unit length during ageing. Several cryosection studies of ageing human muscle did not observe decrease in myonuclear profiles in ageing, although the extent of fibre atrophy was not reported (Manta et al., 1987; Renault et al., 2002). By contrast, our results on significantly atrophied old muscle reveal a decline in nuclei/unit length. Strikingly, scoring of nuclear profiles within IIb fibres in cryosections of the contralateral muscles to those reported here revealed an apparent increase in nuclei/unit length and decrease in SADS with age (A.S.B., H.B. and S.M.H., unpublished observations). This is probably owing to the age-dependent nuclear elongation we describe, which causes significant over-counting of nuclei in cryosections of aged muscle (Fig. 1). For example, calculation shows that a 50% elongation of nuclei from 10 to 15 μm analysed on 15 μm sections would give a ~30% increase in apparent nuclear number. As ~25% of aged fibres show nuclear elongation, this could potentially negate the nuclei/unit length decline we observe during ageing (Figs 1, 2). Caution is therefore required when interpreting nuclear counts from cryosections. Another potential confounding factor for analysis of nuclear turnover in muscle is overall stretching of muscle fibres, without any change in nuclear number. Muscle fibre length has been reported to decrease in rodent TA muscle during ageing, whereas the resting sarcomere length remained unchanged (Hooper, 1981). An increase in sarcomere length would be expected if the fibres were stretching. We did not observe an increase in sarcomere length from aged muscle. On the contrary, a small decrease in fibre length cannot be definitively excluded (Fig. 5C). Moreover, stretching would lead to a fibre-

size-independent decrease in nuclei/unit length and increase in SADS without change in VDS. Interestingly, nuclei/unit length decline is the first significant change detected prior to significant muscle atrophy, but only in large fibres. This cannot be accounted for by stretching, which would lengthen all fibres equally. Decline in nuclei/unit length is accompanied by an increase in both VDS and SADS, arguing for a genuine loss of nuclei, rather than stretching. Subsequently, a general atrophy of muscle is accompanied by an apparent loss of nuclei across all fibre sizes, although nuclear loss is still most marked in larger fibres. Either excessive loss or inadequate replacement could account for the decline in nuclei in old muscle.

Our data confirm that fibre nuclei/unit length correlates with fibre size and that changes in nuclear number usually parallel changes in fibre size (Chen et al., 2002; Dupont-Versteegden et al., 2000; Mitchell and Pavlath, 2001). In general, how changes in domain size and nuclear number are coordinated during fibre growth or atrophy is unknown. The discussion below suggests a possible mechanism.

## SC decline in ageing muscle is associated with large fibres

SCs are the major source of new myonuclei. We find that SCs (as detected by Syn4 and Pax7 immunoreactivity) decline during ageing. We examined ageing in *myoD*-null mice ($D^{-/-}$), which have defective SCs (Sabourin et al., 1999; Yablonka-Reuveni et al., 1999a; Cornelison et al., 2000; Schuierer et al., 2005). As reported previously, fibre size (Chargé et al., 2002) and nuclei/unit length (Cornelison et al., 2000) was not affected in young $D^{-/-}$ mice. However $D^{-/-}$ fibres show more-severe loss of nuclei during ageing. We think this explains why $D^{-/-}$ mice do not atrophy early in life – the rate of nuclear replacement, even if slowed, is high enough. However, we propose that, when SC numbers also decline, the slower rate of nuclear replacement by *myoD*-null cells is insufficient to keep up with nuclear loss and a domain size increase results. Although the function of MyoD in myonuclei has not been determined, the data suggest that efficient SC function is required to maintain nuclear number/fibre during ageing.

Various markers have been used to detect SCs. On isolated single fibres, the number of SCs/fibre ranges from ~4.5 to 7.5 in fast EDL muscle depending on the marker used for detecting such cells (Cooper et al., 1999; Beauchamp et al., 2000; Zammit et al., 2002; Zammit et al., 2004). Our value of ~5 cells/fibre detected using anti-Syn4 and anti-Pax7 antibodies is at the lower end of this range, although numbers of SCs on single TA fibres have not been reported and muscle-specific variation cannot be excluded. Our data confirm the loss of fibre-associated SCs during ageing (Gibson and Schultz, 1983; Bockhold et al., 1998; Renault et al., 2002). Our data contrast with results obtained using FACS-sorted $CD34^+$ cells (Conboy et al., 2003). As CD34 is also expressed on other cell types (Asahara and Kawamoto, 2004), it is possible that other myogenic or non-myogenic cells not closely associated with fibres may have been included in the FACS analysis. The $Syn4^+$ and $Pax7^+$ cells counted in the present study were attached to the muscle fibre. Perhaps some SCs lose their intimate association with fibres during ageing. Significantly, the number of $Syn4^+$ cells/myonucleus and $Pax7^+$ cells/fibre declines by over 50% and this correlates with the decline in nuclear number. Therefore, it is likely that the reduction in SCs/fibre in ageing muscle limits the replacement of nuclei during ageing. Impaired SC differentiation or proliferation (Chargé et al., 2002; Conboy et al., 2003; Webster and Blau, 1990) might also hinder the replacement of myonuclei. In agreement with human studies (Sinha-Hikim et al., 2003), we find that there are more SCs on larger adult IIb fibres. Moreover, the number of SCs/myonucleus is greater on larger fibres. During ageing, SC frequency/fibre declines on all sizes of IIb fibre. This is seen at 22-24 months, prior to significant atrophy. Strikingly, however, the greater number of SC/myonucleus in large adult fibres is lost early during ageing (Fig. 3). This relative SC depletion might lead to poor nuclear replacement in large fibres, perhaps because SCs would have to migrate further to reach damaged regions. Delayed nuclear replacement would cause domain size increase and further damage in aged animals (Fig. 6).

Our data do not address the causes of SC loss. However, physical stresses and activity differ with fibre size, with large IIb fibres suggested to experience more damage than smaller fibres (Barton and Morris, 2003). Increased apoptosis during ageing might also increase nuclear turnover (Dirks and Leeuwenburgh, 2002). However, we have been unable to detect changes in rapid (~15 minutes) apoptotic events with age (A.S.B., unpublished observation). Whether apoptosis varies

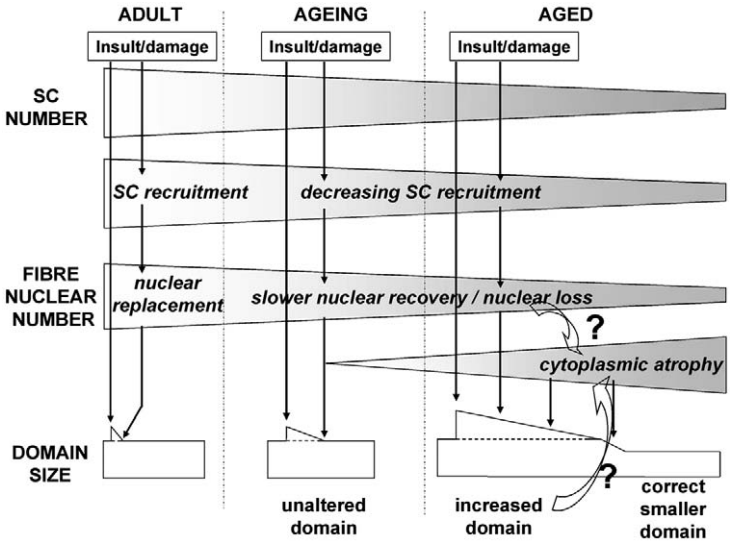


**Fig. 6.** Model of damage/repair cycle changes during ageing. In adult muscle, nuclei lost through damage or exercise may lead to transient domain size increase but are rapidly replaced from myogenic cells, returning domain size to the set point, without significant alteration in fibre size. In ageing muscle, reduced SC function might lead to delayed nuclear replacement and the start of domain size increase. In aged muscle, poor SC function, particularly in large fibres, exacerbates the cycle of decline. Over time, the inability of nuclei to support an excessive domain size leads to cytoplasmic atrophy, returning domain size to the normal set point typical of smaller fibres, which have fewer nuclei and smaller domain sizes than large fibres in healthy adults.

with fibre size is unknown. We speculate that differential requirements for repair throughout life, perhaps combined with reduced myoblast mobility with age, might lead to altered distribution of SCs in ageing muscle.

### MyoD function in ageing muscle

As *myoD* is required for maintenance of ageing muscle, one can ask how *myoD* is involved in the ageing process. MyoD mRNA and protein have been reported to increase and decrease in aged muscle but the significance is unclear (Lowe and Alway, 1999; Alway et al., 2002a; Dedkov et al., 2003). We find that, in isolated single ageing fibres, *myoD* mRNA and protein is increased compared with 2-month-old fibres. Our data suggest that the general increase in *myoD* inside fibres is not specifically related to ageing because (1) most of the increase in MyoD protein occurs between 2 and 12 months and (2) the *MD6.0-lacZ* adult fibre *myoD* reporter indicates that a major increase in *myoD* expression within fibres is related to growth between 2 months and 12 months, not to ageing (Fig. 5). This reporter indicates that the increase is not due to SC activation, consistent with the high frequency of MyoD-immunoreactive nuclei in isolated ageing fibres. Furthermore, the proportion of SCs expressing detectable MyoD was not altered during ageing. Although MyoD is clearly required for efficient muscle repair in ageing, as yet there is little evidence that changes in MyoD are a significant aspect of muscle ageing.

### Domain-size regulation and ageing

Reduced nuclei/unit length leads to an increased domain size of big fibres in aged muscle. The change in domain size only becomes significant in 26-29-month-old muscle, not 22-24-month-old muscle, just when fibre size begins to show a significant decrease. An alternative explanation, that decline in nuclei/unit length and increase in SADS are due to muscle lengthening with age, is not supported by our data both because VDS also increases and because small fibres do not show similar changes. We speculate that muscle atrophy occurs to restore the domain size of a fibre to the set-point after a transient increase triggered by inadequate nuclear replacement. Our observations are not compatible with the idea that reduction in nuclear number follows shrinkage of fibres, as this would lead to a decrease in domain size, at least temporarily.

As larger fibres have greater domain sizes, myonuclei in large fibres have to ‘work’ harder. We propose a model in which fibres require repair during life, leading to SC recruitment and loss of SCs (Fig. 6). The SC decline preferentially hinders efficient nuclear replacement in large fibres, causing domain size increase. This would trigger (1) further damage as nuclei might be unable to maintain fibre structure and (2) cytoplasmic loss and hence atrophy to return domain size to a set point. Perhaps the bigger fibres, in which each nucleus supports more cytoplasm, are the first to suffer when repair rates decline during ageing. Strategies to improve proliferation, migration and/or differentiation of ageing SCs (Chakravarthy et al., 2000; Conboy et al., 2003; Yablonka-Reuveni et al., 1999b) may, therefore, restore muscle fibre nuclear number and prevent age-related atrophy.

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