

Report Number 12/26

**Age related changes in speed and mechanism of adult skeletal  
muscle stem cell migration**

by

**Henry Collins-Hooper, Thomas E. Woolley, Louise Dyson, Anand  
Patel<sup>1</sup>, Paul Potter, Ruth E. Baker, Eamonn A. Gaffney, Philip K.  
Maini, Philip R. Dash, Ketan Patel**



Oxford Centre for Collaborative Applied Mathematics  
Mathematical Institute  
24 - 29 St Giles'  
Oxford  
OX1 3LB  
England



**Running title: Ageing, Satellite cell bleb-based migration**

**Title: Age related changes in speed and mechanism of adult skeletal muscle stem cell migration**

**Authors: Henry Collins-Hooper<sup>1</sup>, Thomas E. Woolley<sup>2</sup>, Louise Dyson<sup>2</sup>, Anand Patel<sup>1</sup>, Paul Potter<sup>3</sup>, Ruth E. Baker<sup>2</sup>, Eamonn A. Gaffney<sup>2</sup>, Philip K. Maini<sup>2</sup>, Philip R. Dash<sup>1</sup>, Ketan Patel<sup>1\*</sup>**

1 School of Biological Sciences, University of Reading, Reading, UK

2 Centre for Mathematical Biology, Mathematical Institute, University of Oxford, Oxford, UK

3 MRC Harwell, Oxfordshire, UK

**Author contribution**

Henry Collins-Hooper: Collection and/or assembly of data, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Thomas E. Woolley: Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Louise Dyson: Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Anand Patel: Collection and/or assembly of data, Final approval of manuscript

Paul Potter: Provision of study material or patients, Final approval of manuscript

Ruth E. Baker: Data analysis and interpretation, Final approval of manuscript

Eamonn A. Gaffney: Data analysis and interpretation, Final approval of manuscript

Philip K. Maini: Data analysis and interpretation, Final approval of manuscript

Philip R. Dash: Conception and design, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

Ketan Patel: Conception and design, Financial support, Data analysis and interpretation, Manuscript writing, Final approval of manuscript

**\*Corresponding author:**

Professor Ketan Patel

School Biological Sciences

Hopkins Building

University of Reading

Whiteknights

Reading

Berkshire, RG6 6UB

UK

Tel (0044) 0118 378 8079

Email [ketan.patel@reading.ac.uk](mailto:ketan.patel@reading.ac.uk)

**Acknowledgement of grants for research support:** BBSRC BB/E52881X-1, British Heart Foundation FS/08/056.

**Key words:** Ageing, Bleb, migration, satellite, amoeboid, stem, cell, skeletal, muscle.

### **Abstract**

Skeletal muscle undergoes a progressive age-related loss in mass and function. Preservation of muscle mass depends in part on satellite cells, the resident stem cells of skeletal muscle. Reduced satellite cell function may contribute to the age-associated decrease in muscle mass. Here we focused on characterising the effect of age on satellite cell migration. We report that aged satellite cells migrate at less than half the speed of young cells. In addition, aged cells show abnormal membrane extension and retraction characteristics required for amoeboid based cell migration. By deploying a mathematical model approach to investigate mechanism of migration, we have found that young satellite cells move in a random ‘memoryless’ manner whereas old cells demonstrate super-diffusive tendencies. Most importantly, we show that nitric oxide, a key regulator of cell migration, reversed the loss in migration speed and reinstated the unbiased mechanism of movement in aged satellite cells. Our study shows that satellite cell migration, a key component of skeletal muscle regeneration, is compromised during aging. However, we propose

clinically approved drugs could be used to overcome these detrimental changes.

## **Introduction**

The increase in the longevity of individuals in Western societies will present considerable challenges to the healthcare and economic systems of developed countries as they care for people whose body functions undergo age-related deterioration. Changes in the structure and function of skeletal muscle with regards to ageing have been extensively studied not only in animal models but also in humans. Old age is associated with a progressive loss of skeletal muscle mass, a process called sarcopenia. Age-related muscle loss leads to lack of muscle strength, resulting in reduced posture and mobility, increased risk of falls, all of which contribute to a decrease in quality of life . In humans, sarcopenia first becomes evident at middle age. Large population studies have reported that over 20% of 60- to 70-year olds have sarcopenia and that the number reaches 50% in those over the age of 75 years .

Aged skeletal muscle undergoes a progressive reduction in the cross sectional area of muscle fibres , selective loss of fast glycolytic fibres and increase in muscle connective tissue . At the sub-cellular level, aged muscle fibres display an increased level of mitochondrial abnormalities and susceptibility to apoptosis . Myofibres from aged muscle show a reduction in force generation and are more susceptible to contraction-induced injury.

Elegant studies performed by Gutmann and Carlson have shown that aged skeletal muscle can regenerate but the rate of regeneration diminishes over time . The repair and maintenance of skeletal muscle is carried out by a resident stem cell population known as satellite cells that are located beneath the basal lamina that surrounds each myofibre. Satellite cells are maintained in a metabolically quiescent state in undamaged muscle. Upon injury, satellite cells undergo a process of activation which involves extensive changes in gene expression. Activated cells subsequently divide to form a pool of myoblasts which then differentiate to form myotubes, replacing or supplementing existing muscle. Satellite cells from aged muscle display a lag in the initiation of proliferation following their isolation as well as decreased capacity to generate progeny, indicative of compromised activation and proliferation .

A much neglected feature of regeneration is the necessity for satellite cells to migrate to the lesion focus in order to carry out cellular tissue repair. Recent work using high-resolution in-vivo imaging of muscle has shown the directional movement of satellite cells to the site of myofibre damage . Furthermore, matrix metalloproteinase, which promotes muscle cell migration and differentiation, has been shown to significantly increase the size of myoblast engraftment territory in a regenerating model . The importance of satellite cell migration for tissue regeneration is evident from animal models and human trials using myoblast injection aimed at restoring muscle function . The failure of these studies was largely due to the fact that cells rarely moved from the point of injection and did not display the requisite whole muscle dispersal.

Using time-lapse microscopy, we and others examined the movement of satellite cells localised on muscle fibres and have shown a number of unexpected features, including the ability of cells to rapidly change direction, to migrate as clusters of cells and jump from one fibre to another .

Migration has been traditionally explained by the force generated by structures called lamellipodia that form at the leading edge of the cells and the retraction of the cell at the rear. However, a wealth of data has recently emerged showing that cells can move using a novel mechanism called blebbing or amoeboid-based movement mediated by extension of plasma membrane protrusions - or blebs - into the extracellular matrix driven by local hydrodynamic changes in cytosolic pressure . We demonstrated that satellite cells move on their in-vivo substrate using an amoeboid or bleb-dependent mechanism rather than using lamellipodia . In addition, we also showed that the ability of satellite cells to form blebs and migrate was dependent on nitric oxide, a potent signalling molecule that regulates cell movement in numerous biological systems including endothelial, epithelial and trophoblast cells .

In this study, we have compared migration characteristics of satellite cells localised on muscle fibres isolated from young (4 month) and aged mice (24 months) using time-lapse microscopy. We report that satellite cells from aged animals migrate significantly slower than young satellite cells. By comparing statistics from the tracked data to those derived from Fickian diffusion, we characterised the migration of satellite cells along



the fibres from the two age groups. This provided us with evidence that the young cells move with diffusive characteristics while old cells display super-diffusive traits, indicating that satellite cells from old muscle move in a fundamentally different way. Finally, we show that the addition of exogenous NO reverses both the speed deficit of old satellite cells and their movement characteristics so that they move in the manner of young cells.

## **Methods**

### *Single myofibre culture*

Myofibres were isolated from the extensor digitorum longus (EDL) muscle of four male 4-month-old (young) and four male 24-month-old (old) C57Bl6 mice as described previously . Briefly, an undamaged EDL muscle was dissected with both tendons intact, and the single fibres were liberated through digestion with (0.2%) type I Collagenase in DMEM at 37°C and supplemented with 5% CO<sub>2</sub>. Using tapered glass pipettes, single fibres were plated out in floating culture wells containing DMEM supplemented with 10% horse serum and 0.5% chick embryo extract. When required, small molecule inhibitor PAPA (100µM), or Hepatocyte Growth Factor (20-100 ng/ml R & D Systems) were added to myofibre culture media 24 hours after culture initiation.

### *Time lapse Microscopy*

Myofibre cultures were monitored using phase contrast microscope systems that harbour controlled-environment chambers maintained at

37°C, supplemented with 5% CO<sub>2</sub>. Time lapse video was taken at rate of 1 frame every 15 minutes for periods of up to (24 hrs) hrs for satellite cells using a 10X objective, a previously well studied time course of satellite cell progression following skeletal muscle injury . In a separate analysis, high power time lapse microscopy using a 100X objective was carried out, enabling the visualisation of cell surface blebbing on single satellite cells. For such analysis, cells were cultured for 24-48 hrs then selected and subsequently filmed at a rate of 1 frame per 2 seconds.

### *Immunocytochemistry*

Immunocytochemistry of satellite cells on single myofibres was carried out as described previously described . Briefly, fibres were fixed in 4% Paraformaldehyde in PBS (PFA) for 10 minutes and washed 3 times in PBS. Myofibres were permeabilised in a solution of 20 mM HEPES, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl<sub>2</sub> and 0.5% Triton X-100 (pH7) at 4°C for 15 minutes and incubated in blocking wash buffer (5% newborn calf serum in PBS containing 0.01% Triton X-100) for 30 minutes prior to antibody incubation. Antibodies were diluted and pre-blocked in wash buffer for 30 minutes prior to addition to the myofibres. Primary antibodies used were: Polyclonal rabbit anti-Laminin (Sigma L-9393 1:200), monoclonal mouse anti-Pax7 (Developmental Studies Hybridoma Bank (DSHB) 1:1), polyclonal rabbit-anti-MyoD (Santa Cruz biotechnology 1:200) polyclonal rabbit-anti-myogenin (Santa Cruz biotechnology 1:200), eNOS rabbit polyclonal IgG Santa Cruz biotechnology (sc-654) (1:200), anti-integrin alpha 6 rat monoclonal Abcam (ab105669) (1:200), integrin

$\beta$ 1 mouse monoclonal Santa Cruz biotechnology (sc-9970) (1:200) and anti-integrin alpha 7 rabbit, (1:200) (antibody was a gift from Professor Uli Mayer). All primary antibodies were incubated overnight at 4°C. Primary antibodies were visualised using the following secondary antibodies: Alexa Fluor goat anti-mouse 488 (Molecular probes A11029) , Alexa Fluor goat anti-rabbit 633 (Molecular probes A21071) and fluorescein rabbit anti-rat IgG Vector Laboratories (FI-4000) (1:200). Secondary antibodies were used at 1:200 and incubated at room temperature for 45 minutes. All myofibres were mounted in Fluorescent mounting medium (DAKO) containing 5 $\mu$ g/ml DAPI for nuclear visualisation.

#### *Fluorescence microscopy*

Mounted myofibres were analysed using a Zeiss Axioscope fluorescent microscope and images were captured using an Axiocam digital camera system and analysed using Axiovision image analysis software (version 4.7).

#### *Scanning Electron Microscopy (SEM)*

Single myofibres were fixed in 4% PFA for 10 minutes. Myofibres were dehydrated through 30, 50, 70, 80, 90 and 100% Ethanol solutions (15 minutes for each step) and transferred to a critical point drier (Balzers CPD 030 - using liquid carbon dioxide). Dried myofibres were transferred to SEM chucks using micro forceps under a light microscope. Thereafter myofibres were gold-coated using an Edwards S150B sputter-coater and

examined using a FEI 600F scanning electron microscope aided by analysis software for image collection.

### *Image and movie analysis*

All image analysis was carried out using freeware package ImageJ (version 1.4.3). Satellite cells were individually manually tracked using the plugin MTrackJ and bleb dynamics were quantified using ImageJ. Bleb number was quantified manually on SEM images of cells using Adobe Photoshop version CS2, only SEM images of whole cells were used for quantification. Satellite cell differentiation profiles following differing culture conditions were manually assessed using quantification of live images through the Zeiss axioscope and axiovision digital camera system. Statistical analysis was performed using Student t-test unless indicated otherwise with a significance level of  $p < 0.05$ .

### *Mathematical Data Analysis*

Using Matlab (version 7.12.0.635) the data was preconditioned by rotating the tracked data such that the fibre lay horizontally. This was done by extracting two coordinates,  $(x_1, y_1)$  and  $(x_2, y_2)$ , along the straight fibre between the extreme points of cell motion. From this we extracted the angle the fibre made with x-axis,  $\theta_f$ , through

$$\tan \theta_f = \frac{y_2 - y_1}{x_2 - x_1}. \quad (1)$$

The Cartesian form of the tracked points is  $(x(t), y(t))$ , where  $x(t)$  and  $y(t)$  are the  $x$  and  $y$  components of the cell's position at time  $t$ , respectively.

The Cartesian form was then converted to its polar form,  $z(t) = r(t) \exp(i\theta(t))$ , where  $r(t) = \sqrt{x(t)^2 + y(t)^2}$  and  $\tan(\theta(t)) = y(t)/x(t)$ . The tracked cell coordinates were rotated by applying the transformation  $z^*(t) = z(t) \exp(-i\theta_f) = r(t) \exp(i\theta^*)$  and converted back to the Cartesian form, ( $x^*(t) = r(t) \cos(\theta^*)$ ,  $y^*(t) = r(t) \sin(\theta^*)$ ). The new  $x^*(t)$  and  $y^*(t)$  coordinates of the satellite cells' positions were normalised to start from zero at the initial time point, producing trajectory data  $x_0^*(t)$  and  $y_0^*(t)$ .

In order to compare the trajectory data with a diffusive model of movement we approximate the fibre with a cylinder that is infinitely long in the  $x$ -direction and has constant radius  $r = 50\mu\text{m}$  (Supplementary Figure 1A).

Since the fibre is assumed to be unbounded, the MSD along the  $x$ -direction of a diffusing particle can be related to the diffusion coefficient,  $D_x$ , and time,  $t$ , through the linear relation

$$\langle x^2 \rangle = \frac{1}{N} \sum_i x_{i0}^*(t)^2 = 2D_x t, \quad (2)$$

where the summation occurs over all tracks,  $i$ , at a given time point,  $t$ , and  $N$  is the total number of tracks. Thus, we are able to characterise the motion along the fibre as diffusive if the MSD of the processed  $x$  data lies along a straight line. The diffusion coefficient can be derived from the gradient of this line.

However, this can only be used to characterise the  $x$  data. Across the fibre, in the  $y$ -direction, the movement of the cell on the surface of the fibre is bounded by the radius of the fibre. To produce a similar relation relating the MSD in the  $y$ -direction and the corresponding diffusion coefficient,  $D_y$ , we must take into account that the cell moves along a curved cylinder and we are projecting this motion onto the flat, one-dimensional  $y$ -axis. Note that, since the fibre is translucent, we are able to see cell as it travels from back to front or front to back. Thus, instead of modelling the motion of the cell around the entire fibre we are able to restrict the cell's motion to a semicircle with reflective boundary conditions (Supplementary Figure 1B).

Using this idealised set up we consider a particle diffusing along a semicircle with constant diffusion coefficient,  $D_y$ , the evolution equation of the probability of the particle,  $P(\omega, \omega_0, t)$ , making an angle  $\omega$  to the centre of the circle at a time  $t$ , given that the particle started at an angle  $\omega_0$ , can be shown to take the form of an axisymmetric diffusion equation in polar coordinates ,

$$\frac{\partial P}{\partial t} = \frac{D_y}{r^2} \frac{\partial^2 P}{\partial \omega^2}, \quad (3)$$

where the reflective boundaries are given by

$$\frac{\partial P}{\partial \omega}(\omega, \omega_0, t) = 0 \text{ at } \omega = 0 \text{ and } \pi \text{ rads.} \quad (4)$$

Finally, we need to specify an initial condition. Since we are unable to control the initial angle  $\omega_0$  we assume that the blebbing cells are

uniformly placed across the surface of the cylinder at the beginning of the tracked data. Thus, to begin with we give the cell an arbitrary but known initial point  $\omega = \omega_0$ . Whence, the initial condition is  $P(\omega, 0) = \delta(\omega - \omega_0)/r$ , where  $\delta(\omega - \omega_0)$  is a delta function. Essentially, the delta function is zero everywhere except at  $\omega_0 \in [0, \pi]$  and its integral over the interval  $[0, \pi]$  is one. The factor of  $r$  in the initial condition is needed to correct for the cylindrical polar coordinate transformation (Supplementary Figure 1C). Equation (4) can be solved using a separable solution of Fourier modes. Hence, we derive the solution:

$$P(\omega, \omega_0, t) = \frac{1}{r\pi} + \frac{2}{r\pi} \sum_{m=1}^{\infty} \cos(m\omega) \cos(m\omega_0) \exp\left(-D_y t \left(\frac{m}{r}\right)^2\right). \quad (5)$$

The mean square displacement is, thus, given by

$$\langle (y - y_0)^2 \rangle = \int_0^\pi (r \cos(\omega) - r \cos(\omega_0))^2 P(\omega, \omega_0, t) r d\omega. \quad (6)$$

However, this is still conditioned on the initial point being  $y = y_0$  when  $\omega = \omega_0$ . Thus, we average this MSD over the semicircle,

$$\langle y^2 \rangle = \frac{1}{r\pi} \int_0^\pi \int_0^\pi (r \cos(\omega) - r \cos(\omega_0))^2 P(\omega, \omega_0, t) r^2 d\omega d\omega_0. \quad (7)$$

All of these integrals can be done analytically, allowing us to derive

$$\langle y^2 \rangle = r^2 \left( 1 - \exp\left(-\frac{D_y t}{r^2}\right) \right). \quad (8)$$

Rearranging equation (8) shows that the diffusion coefficient,  $D_y$ , and the MSD of  $y$  can be related by,

$$\log\left(1 - \frac{\langle y^2 \rangle}{r^2}\right) = -\frac{D_y t}{r^2}. \quad (9)$$

Hence, if the data, when transformed as in equation (9) lies along a straight line with negative slope then this suggests that the motion along

the  $y$  direction is also diffusive. The diffusion coefficient can then be calculated from the gradient.

## **Results**

### *Activation and emergence is delayed in aged satellite cells*

Satellite cells are maintained in a quiescent state in undamaged muscle. Upon injury they undergo activation resulting in a significant change in their molecular profile including the induction of MyoD expression. Isolation of single fibres from whole muscle mimics processes associated with muscle damage. In the first set of experiments, we examined the behaviour of aged satellite cells immediately after single fibre isolation. We found that the activation of satellite cells from aged fibres was significantly delayed compared to those found on fibres from young mice. Profiling of satellite cells showed that over 96% of young satellite cells had initiated the expression of MyoD 8 hours after single muscle fibre isolation. In contrast, a significantly lower number of old satellite cells (88%) expressed MyoD expression after the same time period (49 young 41 old fibres examined,  $p < 0.05$ , Figure 1A).

We investigated whether the lag in activation of aged satellite cells delayed the emergence of satellite cells from their original sub-basal position. Single muscle fibres were isolated and cultured for differing periods of time and then fixed prior to immunohistological examination for position of the satellite cell (determined by Pax7 expression) in relation to the basal lamina (indicated by laminin expression). We found that after 15



hour culture, the first satellite cells on young fibres had taken a supra-basal position (Figure 1B and 1C). Significantly, at the same time point we were unable to detect any satellite cells in a similar position on aged muscle fibres (32 young 30 old fibres examined,  $p=0.05$  Figure 1B and 1D). Indeed there was a 3 hour delay for the emergence of aged satellite cells, although there were significantly more satellite cells on the surface of the fibre from the young animals compared to the old (26 young and 23 old fibres examined,  $p<0.05$ , Figure 1B). Using SEM we found that aged satellite cells remodelled the EMC to leave a scar on the surface of the muscle fibre and that the satellite cells displayed a smooth surface after emerging onto the myofibre, features also displayed by young satellite cells (Figure 1E). The delay in emergence was transient, as an equal percentage of cells had assumed a supra-basal position 24 hours after single fibre isolation (30 young and 26 old fibres examined,  $p>0.05$ , Figure 1B). We have previously shown that activated satellite cells express eNOS, an enzyme responsible for the production of the potent signalling molecule Nitric Oxide (NO). We compared the abundance of eNOS in satellite cells on young and old fibres at different times after muscle dispersal. We found that satellite cells on young fibres cultured for 24h expressed eNOS in the cytoplasm (Figure 2A-A''). In contrast similarly treated old fibres displayed very little eNOS in the satellite cells (Figure 2B-B''). However after 48h, satellite cells on both old and young fibres displayed abundant levels of eNOS in the cytoplasm (Figure 2C-D''). These experiments therefore showed that activation, emergence and

expression of important signalling associated proteins, three early key phases in the regeneration process, are delayed in aged muscle.

#### *Proliferation defects in satellite cells from aged muscle fibres*

We compared the proliferation and differentiation characteristics of young and aged satellite cells. The number of satellite cells on young fibres was significantly greater than on aged fibres (n= 6.9 young, 4.7 old, 41 young and 36 old fibres examined,  $p < 0.05$ , Figure 3A). The number of satellite cells had more than doubled following the culture of young fibres for a period of 48hrs. In contrast, the number of satellite cells on aged fibres after an identical culture period had not increased compared to the number on freshly isolated 24 hours fibres and maintained the significant differences in cell number between the two cohorts (52 young 48 old fibres examined,  $p < 0.05$ , Figure 3A). In addition, the proliferation rate was also reduced in aged satellite cells since we found that the number increased by 3.2 fold in aged fibres compared to a 3.8 fold increase in young fibres from a period of 48 to 72 hours (60 young and 50 old fibres examined,  $p < 0.05$ , Figure 3A). Furthermore, we examined the localisation of proliferating satellite cells by determining the size and number of clusters on young and aged fibres. Even though young satellite cells proliferated more rapidly compared to older cells, the number of cells per cluster was similar between the two groups at all time points examined ( 1.8 in young and 1.4 in old from an examination of 52 young and 48 old fibres at 48 hours and 4.2 in young and 3.6 in old from an examination of 60 young and 50 old fibres at 72 hours,  $p > 0.05$ , Figure 3B). However, we

found that the young satellite cells formed significantly more clusters compared to aged satellite cells ( 7.1 in young and 4.0 in old from an examination of 52 young and 48 old fibres at 48 hours and 11.1 in young and 4.9 in old from an examination of 60 young and 50 old fibres at 72 hours,  $p>0.05$ , Figure 3C). Next, we examined the molecular profile of cells at 72h to determine the effect of ageing on the process of myogenic commitment to differentiation. Pax7 expression was used as an indicator of cells still in a precursor state whereas expression of myogenin was taken as an indicator of cells committed to myogenic differentiation . We found that the young fibres contained approximately 68% of cells expressing Myogenin and 32% expressing Pax7. In contrast, aged fibres showed a distinct differentiation profile: there were significantly fewer myogenin expressing cells and more Pax7 expressing cells (from a profile of 60 young and 50 old fibres at 72 hours,  $p<0.05$ , Figure 3D). Therefore, aged satellite cells display a delay in their commitment to differentiation.

Finally we examined the distribution of marker proteins associated with maintaining physical contact between satellite cells and the muscle fibres. To that end we characterised the expression of the muscle associated integrins-  $\alpha 6$ ,  $\alpha 7$  and  $\beta 1$  in satellite cells on muscle fibres from young and old animals that had been cultured for 36 hours. A comparable relationship was established between the integrins and satellite cells from the two age groups in that the young satellite cells expressed higher levels of cell adhesion molecules compared to the levels displayed by old satellite cells (Figure 4). This was particularly prominent in the expression of integrin  $\alpha 7$  and  $\beta 1$  (Figure 4C-F''). These results show that ageing

influences the profile of proteins functionally responsible for satellite cell-muscle fibre adhesion.

*Attenuated migration speeds, bleb number and bleb dynamics in aged satellite cells*

The reduction in cluster number displayed by aged satellite cells could be explained by a defect in migration that reduces cluster splitting and separation. We examined migration characteristics directly using time-lapse microscopy and found that satellite cells from aged fibres migrated significantly slower (18.0 $\mu$ m/h from 12 tracked cells) than young satellite cells (40 $\mu$ m/h from 17 tracked cells,  $p < 0.05$ , Figure 5A and Supplementary Movie 1). We have previously shown that migrating satellite cells display dynamically extending and retracting membrane blebs. We performed a detailed study to characterise blebbing on the surface of young and aged satellite cells. Quantification of bleb number revealed that young satellite cells displayed over twice the number of blebs compared to aged satellite cells (27.4 blebs per cell on young satellite cells ( $n=12$ ) versus 11.4 blebs per cell on old satellite cells ( $n=12$ ),  $p < 0.05$ , Figure 5B, 5F and 5G). However the average size of the blebs and shape (ratio of length/width) was similar between the two cohorts (0.07 $\mu$ m for young satellite cells ( $n=9$ ) versus 0.06 $\mu$ m for old satellite cells ( $n=10$ ),  $p > 0.05$ , Figure 5C and 5H). We performed a detailed analysis of bleb dynamics between the two ages and found that the life span of a bleb (comprising phases of bleb extension, maintenance and retraction) was greater in aged cells (30.25 seconds for young satellite

cells (n=16) versus 36.93 seconds for old satellite cells (n=60),  $p < 0.05$ , Figure 5D). Next we investigated bleb dynamics by analysing extension, stabilisation and retraction properties in young and aged cells. We found that there was a significant increase in the duration of bleb extension in aged cells (4.00 seconds for young satellite cells (n=16) versus 4.93 seconds for old satellite cells (n=60),  $p < 0.05$ , Figure 5E). On the other hand, stabilisation time of aged cells compared to their younger counterparts was not significantly different (7.00 seconds for young satellite cells (n=16) versus 7.63 seconds for old satellite cells (n=60),  $p > 0.05$ , Figure 5E). However, there was a significant increase in the duration of time it took aged satellite cells to retract the membrane extension compared to younger cells (19.25 seconds for young satellite cells (n=16) versus 24.37 seconds for old satellite cells (n=60),  $p < 0.05$ , Figure 5E). Despite the changes in the dynamics of the bleb life cycle with ageing, there was no significant change in the overall bleb shape in the two age cohorts as determined by examining the ratio of bleb length versus bleb diameter (Figure 5H).

#### *Mathematical modelling of young and aged satellite cell migration*

We modelled the muscle fibre as a cylinder with infinite length and radius  $r = 50 \mu\text{m}$ . Blebbing cells are capable of moving along the fibre (x-axis) as well as along its curved surface. This curved surface was then projected on the flat plane allowing us to use the y-axis coordinates. Since the fibre is translucent and the cell can be tracked even if it travels behind the fibre we model these boundaries as reflective (Supplementary figures 1B and

1C). It is a standard result that for an unconstrained diffusion process, the mean squared displacement (MSD) increases linearly in proportion to time. Hence if it is possible to fit a straight line through the MSD in the x direction, then it can be said that the cells move in a manner consistent with Fickian diffusion. The gradient of this line can be used to find the diffusion coefficient for the movement, and an  $R^2$  value can be found to give a measure of the goodness of fit (where  $R^2 = 1$  indicates a perfect fit). This is not true for the bounded y-axis and in the methods section we have derived the corresponding relationship between the MSD in the y direction and the diffusion coefficient.

Analysis of movement of young cells along the length of the fibres (x-axis) gives an  $R^2$  value of 0.903 at a gradient of  $26.3 \mu\text{m}^2/\text{min}$ , implying a diffusion rate of  $13.2 \mu\text{m}^2/\text{min}$  (Figure 6A). These data indicate near diffusive movement along the fibre with relatively large movements during each time period. We found that the young cells also seem to move in a diffusive manner along the width of the fibres ( $R^2=0.819$ ) but with smaller movements during each time period compared to those along the fibre (gradient= $-4.78 \times 10^{-4} \mu\text{m}^2/\text{min}$ , diffusion rate= $1.19 \mu\text{m}^2/\text{min}$ , Figure 6B). A completely different profile was discovered following the analysis of aged satellite cell migration. The best fit curve for these data was a power-law plot, with  $\text{MSD} = 1.45 \times 10^{-11} t^{5.62}$  (Figure 6C), giving  $R^2=0.983$ . We were able to calculate an  $R^2$  value of 0.663 for migration along the width of the fibre (y-axis) with a gradient of only  $-8.56 \times 10^{-5} \mu\text{m}^2/\text{min}$ , giving a diffusion rate of  $0.214 \mu\text{m}^2/\text{min}$  (Figure 6D).

### *Nitric Oxide rescues migration deficit of aged satellite cells*

We have previously shown that inhibition of nitric oxide production results in reduction in migration speeds of young satellite cells . In this study, we found that aged satellite cells had slower migration rates (18.0  $\mu\text{m}/\text{h}$ ) compared to younger cells (40  $\mu\text{m}/\text{h}$ ) (Figure 5A) and therefore we investigated whether provision of nitric oxide could overcome this deficit. We isolated single fibres from aged muscle and supplemented the media with the potent NO donor (Z)-1-[N-(3-aminopropyl)-N-(n-propyl)amino]diazen-1-ium-1,2-diolate (also known as PAPA-NONOate-100 $\mu\text{M}$ ). Addition of PAPA-NONOate resulted in aged satellite cells migrating at over twice the speed and at rates comparable to young satellite cells (44.2 $\mu\text{m}/\text{h}$  from 20 tracks,  $p < 0.05$  Figure 5I and Supplementary Movie 1). Therefore, addition of nitric oxide significantly reverses the deficit in migration speed displayed by aged satellite cells. Interestingly, addition of PAPA-NONOate to young muscle fibres had no significant effect on satellite cells migration speed (Figure 5J).

We next examined the effect of PAPA-NONOate on the directionality of movement of aged satellite cells by calculating the MSD along and across the fibre. In contrast to the nonlinear MSD profile of untreated aged satellite cells, the same cells treated with PAPA-NONOate displayed a linear profile ( $R^2 = 0.593$ ) with a gradient of  $-1.7 \times 10^{-3} \mu\text{m}^2/\text{min}$  (Figure 6E). PAPA-NONOate reduced the  $R^2$  value for displacement along the width of the fibre although it increased the diffusion rate from 0.214  $\mu\text{m}^2/\text{min}$  to 4.31  $\mu\text{m}^2/\text{min}$  (Figure 6F). Therefore, NO not only restores the speed of

migration in aged satellite cells but also the diffusive characteristics displayed by young cells, particularly in the x direction, along the fibre.

### Hepatocyte Growth Factor restores aged satellite cells migration speed but not directionality of movement

Hepatocyte growth factor (HGF) has been shown to promote satellite cell activation and has been proposed to induce migration and therefore has functional properties akin to those of NO. We carried out a suite of experiments to determine whether HGF mimicked the action of NO. We found that HGF was able to promote the activation of not only young but also old satellite cells in a concentration dependent manner (Figure 7A). We quantified the effect of supplementing culture medium with HGF on the emergence of satellite cells from the sub-laminal position and found that it accelerated the rate at which cells assumed a supra-basal localisation (Figure 7B). A similar effect was induced on old muscle fibres by PAPA-NONOate (Figure 7B). We next determined the effect of HGF on satellite cell migration speed. We found that HGF caused a significant increase in the rate of movement of old satellite cells (from 25.6 to 41.5  $\mu\text{m/hr}$  Figure 7C) but had no significant effect on young satellite cell (Figure 7D). Having found that HGF altered the rate of aged satellite cell movement we next determined its effect on bleb development. Measurement of bleb dimensions revealed that HGF did not alter the size of blebs on old satellite cells (Figure 7E). However it induced a greater number of blebs (Figure 7F, 7H and 7J). HGF had no significant effect on the size or number of blebs on young satellite cells (Figure 7E, 7F, &G and



7I). Finally we next determined the impact of HGF on the directionality of movement of aged satellite cells by calculating the MSD along and across the fibre. The best fit curve for these data not a linear relationship but a power-law plot, with  $MSD = XXX \text{ Thomas}$  (Figure 6G), giving  $R^2=XXX \text{ Thomas}$ . We were able to calculate an  $R^2$  value of  $XXX \text{ Thomas}$  for migration along the width of the fibre (y-axis) with a gradient of  $XXX \text{ Thomas } \mu\text{m}^2/\text{min}$ , giving a diffusion rate of  $XXX \text{ Thomas } \mu\text{m}^2/\text{min}$  (Figure 6H). These results show that while HGF is able to accelerate the rate of aged satellite cell activation, emergence and migration speed it does not re-establish the memoryless character of satellite cell movement along the fibre displayed by young cell.

## **Discussion**

Sarcopenia is defined as the degenerative loss of skeletal muscle mass and strength associated with the ageing process. Previous work has shown that skeletal muscle undergoes numerous changes with age including: reduction in myofibre cross section area ; a shift towards an oxidative metabolic profile ; an increase in mitochondrial proliferation and accumulation of mitochondrial mutations ; an increase in interstitial connective tissue deposition ; a decrease in satellite cell number and a decrease in the efficiency of skeletal muscle regeneration . Results from this study extend the list of age related changes in skeletal muscle to include a reduction in satellite cell migration speed and altered mechanism of migration along the muscle fibre. We have previously shown that in the single fibre culture model, satellite cells become

activated after the dispersal process and then remodel the basal lamina to develop an opening through which they move to assume a position on the surface of the myofibres (Otto et al, 2011). In the present study, we found that both the time of activation and time for the satellite cells to assume a supra-basal laminal position were delayed in aged muscle. The delay in activation may be explained by the differing levels of satellite cell quiescence in the two age groups. Although satellite cells at both time points are in a mitotically arrested state it has been previously shown that young muscle stem cells exist at a more metabolically active level than older muscle stem cells (in particular those required for protein synthesis and protein processing) compared to older cells . Therefore, the delay in the activation of aged satellite cells could be due to the increase in time required for the translation of proteins used as markers associated with this process. We suggest that the activation process of old satellite cells is not only hindered by greater metabolic inactivity but also potentially by the age related development of the basal lamina between the stem cells and the myofibre . We propose that a basal lamina between the satellite cell and myofibre would impede the diffusion of activation signals that arise from the myofibre.

In addition, the delay in the emergence period highlights changes in the extra cellular environment that take place in muscle during ageing. It is well documented that ageing leads to an increase in the thickness of the endomyosium on top of the satellite cell and therefore it would be more of a challenge for cells to create an opening through which to emerge .

The key finding of our study is the reduced migration speed of aged satellite cells along the muscle fibre. We found that the young cells move at twice the rate of old cells (40µm/hr versus 18.5µm/hr). It is important to point out that both sets of fibres were treated in an identical manner during their preparation and cultured in the same medium. Therefore, the difference in migration speed indicates the existence of an inherent difference between young and old satellite cells with regards to movement. It is generally accepted that adhesions need to be established between a cell and its substratum in order to generate traction forces required for movement. Integrins are a large family of heterodimeric glycoproteins that serve as cell-extracellular matrix adhesion molecules . Previous work has shown that embryonic and adult skeletal muscles intergrin  $\alpha6\beta1$  and  $\alpha7\beta1$  both of which have a high affinity for laminin a major constituent of muscle fibre basal lamina . We have found a reduction in the expression of the three major muscle intergrins in aged satellite cells. These results suggest that altered intergrin expression by satellite cells is a feature of ageing which could impact on migration. This notion is supported by elegant work performed by Siegel and colleagues who showed that the deployment neutralising antibodies directed against integrins  $\alpha7$  or  $\beta1$  significantly decreased the speed of primanry satellite cells . The high migration speeds displayed by young satellite cells can be attributed to the blebbing mechanism that is used for migration . Importantly, our time lapse studies showed that even though old satellites move at relatively low speed they undergo blebbing and do not revert to the slower lamellipodia based migration mechanism. It is worth

highlighting that rate of satellite cell movement does not seem to be related to myogenic differentiation. We have previously shown that the early on-set of differentiation in satellite cells (signified by myogenin expression) is not accompanied by a significant change in migration speed . This point is further accentuated by the findings in this study that show that aged satellite cells have a delay in their myogenic differentiation programme yet move slower than young cells which are further down their commitment programme. However, the dynamics of blebbing between the two age groups differ significantly and this rather than the differentiation status seems to be a major factor in controlling migration speeds. We found that the size of blebs between the two cohorts is similar but there were significant differences in the number of blebs as well as the time taken for bleb extension and retraction. These results give an insight into the importance of features of blebbing in relation to movement suggesting that the number of blebs and their rapid extension and retraction support high migration speeds. This conclusion is supported by the data from the two manipulations of old satellite cells that increased the migration speed of aged satellite cells,: both PAPA-NONOate and HGF treatments resulted in an increase in the number and size of blebs. Importantly, these parameters can be manipulated by altering properties of the cytoskeleton and future work will examine the significance of each particular feature of blebbing on the migration speed of satellite cells .

Mathematical analysis of the migration trajectories of satellite cells revealed a number of important features. We showed that young satellite

cells move in a diffusive manner both along and across the fibre. This suggests that the blebbing cells undergo a “memoryless” random walk with equal probability of moving left or right at each time point. Additionally, data analysis of the gradients from the mean squared displacement over time plots implies that young satellite cells take bigger steps along the fibre than across its width in a given time frame. In contrast, aged satellite cells appear to move along the fibre in a super-diffusive manner, so that the MSD is higher than a linear dependence on time would suggest. This indicates a more active movement than the usual Fickian diffusion, and is most often associated with directed active transport processes .

NO is a potent regulator of migration in numerous cell types . Here we showed that the speed deficit and migration anomalies of aged satellite cells can be reversed through the action of NO donors. These results are consistent with our previous finding that showed that small compound inhibitors of NO production decreased the rate of satellite cell migration . These results imply that inherent age related changes in the myofibre and satellite cells can be overcome, at least as far as migration is concerned by delivering NO.

In addition our work reveals that migration is a multifaceted mechanism and involves parameters other than speed of movement. We have shown that aged satellite cells treated with HGF respond by increasing their rate of movement to levels displayed by young cells. However HGF did not correct the Fickian diffusion characteristics of aged satellite cells. Previous

work has shown in the context of satellite cell activation that NO acts upstream of HGF . Our data is congruent with this relationship. We suggest that NO promotes the rate of satellite cell activation and movement possible via HGF but also another important property, direction of movement, but that this process is independent of HGF mediated signalling. We are currently dissecting the divergence of NO mediated signalling from HGF action to gain a comprehensive understanding of the role played by these molecules in controlling satellite cell behaviour.

We have shown that aged satellite cells move more slowly compared to young cells and by a different mechanism. One simple hypothesis encompassing our major finding is that NO production is decreased during ageing. If such a deficit exists we do not know whether it is due to compromised NO production by the myofibres and/or satellite cells as both are capable of its production .The evidence for decreased age related NO activity in muscle is controversial with some studies showing such a trend whereas others propose that NO levels actually increase . Nevertheless, there is an extensive body of work that shows that NO promotes skeletal muscle repair, a process attributed to satellite cell activation . We have shown that the activation of eNOS expression by aged satellite cells is delayed following fibre dispersal. We propose that the lag in NO production by aged satellite cells could be as detrimental to the regenerative process as not producing it in the first place by accepting that robust regeneration occurs only during a narrow time window limited by the action of other often antagonistic cellular events e.g. macrophage infiltration and fibrosis . In our experiments we introduced the NO donor

after the activation period, when cells were moving on the surface of the fibre, and therefore we believe that this signalling molecule plays roles at multiple stages during regeneration. NO production can be increased by a number of well characterised drugs. For example, the synthesis of NO from nitric oxide synthases (NOS) can be stimulated through the addition of L-arginine (the main substrate for NOS) and through the use of growth factors such as HGF and EGF, both of which act to increase NO production through the Akt-mediated phosphorylation of eNOS or through increased transcription of iNOS. In addition to simply increasing NO synthesis, other therapeutic options include the ability to modulate downstream NO signalling to improve satellite cell migration in older muscle. Treatment with phosphodiesterase type 5 inhibitors, which are routinely used clinically, is known to increase the persistence of cGMP signalling thus amplifying endogenous NO signalling. Importantly a number of these treatments have undergone efficacy and safety trials in humans and are routinely prescribed. In future work we will examine whether drugs that promote NO production or modulate NO signalling improve the regeneration of aged muscle.

Future work will determine whether ageing affects the movement of human satellite cells in a manner discovered in this study in mice. More immediately we will investigate whether activation of the nitric oxide signalling pathway in whole muscle can overcome the age-related increase in regeneration time through its ability to promote satellite cell migration in animal models.

## **Acknowledgements**

We are indebted to the BBSRC and Systems Biology Laboratory (SBL) for generous funding that has enabled this work to be performed. In particular we are grateful to Dr Mike Fischer of SBL for his continued support for academic based research focusing on improving human health. We thank Drs S. Jaffer and M. Tindall for assistance in the preparation of this work. PKM was partially supported by a Royal Society Wolfson Research Merit Award. TEW and LD would like to thank the EPSRC for support. This publication was based on work supported in part by Award No KUK-C1-013-04, made by King Abdullah University of Science and Technology (KAUST). PRD would like to thank the British Heart Foundation (Grant FS/08/056) for support. We also thank Dr Silvia Torelli, Dr Francesco Conti and Professor Uli Mayer for providing information and antibodies for integrin profiling of satellite cells.

## **References**

1. Cruz-Jentoft AJ, Baeyens JP, Bauer JM, Boirie Y, Cederholm T, et al. (2010) Sarcopenia: European consensus on definition and diagnosis: Report of the European Working Group on Sarcopenia in Older People. *Age Ageing* 39: 412-423.
2. Berger MJ, Doherty TJ (2010) Sarcopenia: prevalence, mechanisms, and functional consequences. *Interdiscip Top Gerontol* 37: 94-114.
3. Faulkner JA, Brooks SV, Zerba E (1995) Muscle atrophy and weakness with aging: contraction-induced injury as an underlying mechanism. *J Gerontol A Biol Sci Med Sci* 50 Spec No: 124-129.
4. Alnaqeeb MA, Goldspink G (1987) Changes in fibre type, number and diameter in developing and ageing skeletal muscle. *J Anat* 153: 31-45.
5. Marshall PA, Williams PE, Goldspink G (1989) Accumulation of collagen and altered fiber-type ratios as indicators of abnormal muscle gene expression in the mdx dystrophic mouse. *Muscle Nerve* 12: 528-537.
6. Aiken J, Bua E, Cao Z, Lopez M, Wanagat J, et al. (2002) Mitochondrial DNA deletion mutations and sarcopenia. *Ann N Y Acad Sci* 959: 412-423.
7. Radak Z, Chung HY, Goto S (2008) Systemic adaptation to oxidative challenge induced by regular exercise. *Free Radic Biol Med* 44: 153-159.



8. Gutmann E, Carlson BM (1976) Regeneration and transplantation of muscles in old rats and between young and old rats. *Life Sci* 18: 109-114.
9. Schultz E, Lipton BH (1982) Skeletal muscle satellite cells: changes in proliferation potential as a function of age. *Mech Ageing Dev* 20: 377-383.
10. Ishido M, Kasuga N (2011) In situ real-time imaging of the satellite cells in rat intact and injured soleus muscles using quantum dots. *Histochem Cell Biol* 135: 21-26.
11. Wang W, Pan H, Murray K, Jefferson BS, Li Y (2009) Matrix metalloproteinase-1 promotes muscle cell migration and differentiation. *Am J Pathol* 174: 541-549.
12. Lafreniere JF, Mills P, Tremblay JP, El Fahime E (2004) Growth factors improve the in vivo migration of human skeletal myoblasts by modulating their endogenous proteolytic activity. *Transplantation* 77: 1741-1747.
13. Satoh A, Huard J, Labrecque C, Tremblay JP (1993) Use of fluorescent latex microspheres (FLMs) to follow the fate of transplanted myoblasts. *J Histochem Cytochem* 41: 1579-1582.
14. Peault B, Rudnicki M, Torrente Y, Cossu G, Tremblay JP, et al. (2007) Stem and progenitor cells in skeletal muscle development, maintenance, and therapy. *Mol Ther* 15: 867-877.
15. Otto A, Collins-Hooper H, Patel A, Dash PR, Patel K (2011) Adult skeletal muscle stem cell migration is mediated by a blebbing/amoeboid mechanism. *Rejuvenation Res* 14: 249-260.
16. Siegel AL, Atchison K, Fisher KE, Davis GE, Cornelison DD (2009) 3D timelapse analysis of muscle satellite cell motility. *Stem Cells* 27: 2527-2538.
17. Charras GT, Yarrow JC, Horton MA, Mahadevan L, Mitchison TJ (2005) Non-equilibration of hydrostatic pressure in blebbing cells. *Nature* 435: 365-369.
18. Charras G, Paluch E (2008) Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* 9: 730-736.
19. Otto A, Schmidt C, Luke G, Allen S, Valasek P, et al. (2008) Canonical Wnt signalling induces satellite-cell proliferation during adult skeletal muscle regeneration. *J Cell Sci* 121: 2939-2950.
20. Zammit PS, Golding JP, Nagata Y, Hudon V, Partridge TA, et al. (2004) Muscle satellite cells adopt divergent fates: a mechanism for self-renewal? *J Cell Biol* 166: 347-357.
21. Kärger J (1992) Straightforward derivation of the long-time limit of the mean-square displacement in one-dimensional diffusion. *Physical Review A* 45: 4173.
22. van Kampen NG (2007) *Stochastic Processes in Physics and Chemistry*. Amsterdam: North Holland.
23. Collins RE (1999) *Mathematical Methods for Physicists and Engineers*. Mineola: Dover Publications.
24. Kreyszig E (2007) *Advanced Engineering Mathematics*. New Dehli: Wiley-India.
25. Tatsumi R, Sheehan SM, Iwasaki H, Hattori A, Allen RE (2001) Mechanical stretch induces activation of skeletal muscle satellite cells in vitro. *Exp Cell Res* 267: 107-114.
26. Anderson J, Pilipowicz O (2002) Activation of muscle satellite cells in single-fiber cultures. *Nitric Oxide* 7: 36-41.
27. Bua EA, McKiernan SH, Wanagat J, McKenzie D, Aiken JM (2002) Mitochondrial abnormalities are more frequent in muscles undergoing sarcopenia. *J Appl Physiol* 92: 2617-2624.

28. Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z (2006) Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. *Dev Biol* 294: 50-66.
29. Snow MH (1977) The effects of aging on satellite cells in skeletal muscles of mice and rats. *Cell Tissue Res* 185: 399-408.
30. Spence HJ, Chen YJ, Winder SJ (2002) Muscular dystrophies, the cytoskeleton and cell adhesion. *Bioessays* 24: 542-552.
31. Mayer U (2003) Integrins: redundant or important players in skeletal muscle? *J Biol Chem* 278: 14587-14590.
32. Hagmann J, Burger MM, Dagan D (1999) Regulation of plasma membrane blebbing by the cytoskeleton. *J Cell Biochem* 73: 488-499.
33. Kumar N, Harbola U, Lindenberg K (2010) Memory-induced anomalous dynamics: Emergence of diffusion, subdiffusion, and superdiffusion from a single random walk model. *Physical Review E* 82: 021101.
34. Cartwright JE, Tse WK, Whitley GS (2002) Hepatocyte growth factor induced human trophoblast motility involves phosphatidylinositol-3-kinase, mitogen-activated protein kinase, and inducible nitric oxide synthase. *Exp Cell Res* 279: 219-226.
35. Kawasaki K, Smith RS, Jr., Hsieh CM, Sun J, Chao J, et al. (2003) Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis. *Mol Cell Biol* 23: 5726-5737.
36. Noiri E, Peresleni T, Srivastava N, Weber P, Bahou WF, et al. (1996) Nitric oxide is necessary for a switch from stationary to locomoting phenotype in epithelial cells. *Am J Physiol* 270: C794-802.
37. Planitzer G, Baum O, Gossrau R (2000) Skeletal muscle fibres show NADPH diaphorase activity associated with mitochondria, the sarcoplasmic reticulum and the NOS-1-containing sarcolemma. *Histochem J* 32: 303-312.
38. Richmonds CR, Boonyapisit K, Kusner LL, Kaminski HJ (1999) Nitric oxide synthase in aging rat skeletal muscle. *Mech Ageing Dev* 109: 177-189.
39. Capanni C, Squarzone S, Petrini S, Villanova M, Muscari C, et al. (1998) Increase of neuronal nitric oxide synthase in rat skeletal muscle during ageing. *Biochem Biophys Res Commun* 245: 216-219.
40. Filippin LI, Cuevas MJ, Lima E, Marroni NP, Gonzalez-Gallego J, et al. (2011) Nitric oxide regulates the repair of injured skeletal muscle. *Nitric Oxide* 24: 43-49.
41. Grounds MD, Davies MJ (1996) Chemotaxis in myogenesis. *Basic Applied Myology* 6: 469- 483.
42. Tsao PS, McEvoy LM, Drexler H, Butcher EC, Cooke JP (1994) Enhanced endothelial adhesiveness in hypercholesterolemia is attenuated by L-arginine. *Circulation* 89: 2176-2182.
43. Makondo K, Kimura K, Kitamura N, Kitamura T, Yamaji D, et al. (2003) Hepatocyte growth factor activates endothelial nitric oxide synthase by Ca(2+)- and phosphoinositide 3-kinase/Akt-dependent phosphorylation in aortic endothelial cells. *Biochem J* 374: 63-69.
44. Dash PR, Whitley GS, Ayling LJ, Johnstone AP, Cartwright JE (2005) Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin mediated up-regulation of inducible nitric oxide synthase. *Cell Signal* 17: 571-580.
45. Montani D, Chamaus MC, Savale L, Natali D, Price LC, et al. (2009) Phosphodiesterase type 5 inhibitors in pulmonary arterial hypertension. *Adv Ther* 26: 813-825.

## **Figure legends**

### **Figure 1**

Delayed activation and emergence of aged satellite cells.

A. Significantly fewer satellite cells from aged animals expressed MyoD (88%), a marker of activation, 8 hours after fibre isolation compared to those on young muscle fibres (96%) (\* $P < 0.001$ ). B. Emergence of satellite cells (identified through expression of Pax7 taking a position on top of basal lamina containing laminin) from young and aged fibres. The number of emerging satellite cells was first calculated as a proportion of satellite cells in supra-basal position relative to all satellite cells. The percentage at each time point was set to 100 for the young fibres and the aged cell number compared to this figure. Young fibres contained significantly more satellite cells in supra-basal positions after 15h and 18h compared to aged fibres (\* $P < 0.001$ ). C. Immunocytochemical staining of a single young myofibre using antibodies against Laminin and Pax7 to show the supra-basal position of the satellite cell (arrowhead) at 15h. D. Pax7 expressing aged satellite cell (arrowhead) is still under the basal lamina in old muscle fibres. E. Scanning electron micrograph of old muscle fibre 30 hours after culture. The satellite cell emerges with a smooth surface leaving a scar in the basal lamina (arrowhead). F. Scanning electron micrograph of young muscle fibres 30 hours after culture. The satellite cell displays numerous cytoplasmic protusions or blebs (arrowhead).

### **Figure 2**

### Delayed expression of eNOS in aged satellite cells.

A. Cytoplasmic expression of eNOS in satellite cells on young fibres following 24h of culture (yellow arrowhead). A'. Pax7 expression in eNOS expressing cell shown in A. A''. Cytoplasmic localisation of eNOS (green) and nuclear expression of Pax7 (red) in young fibre satellite cells. B-B''. Old fibres cultured for 24h display very little eNOS expression in satellite cells. C. Young fibres cultured for 48h express eNOS located at the contact point between satellite cells and muscle fibre (yellow arrowheads). C'. MyoD expression in eNOS expressing cell shown in C. C''. Cytoplasmic localisation of eNOS (green) and nuclear expression of MyoD (red) in young satellite cells. D. Old fibres cultured for 48h express eNOS located at the contact point between satellite cells and muscle fibre (yellow arrowheads). D'. MyoD expression in eNOS expressing cell shown in D. D''. Cytoplasmic localisation of eNOS (green) and nuclear expression of Pax7 (red) in satellite cells on old muscle fibres. Scale bar 10  $\mu$ m.

### Figure 3

Aged satellite cells showed decreased rate of proliferation, decreased rate of cluster formation and a delay in their differentiation programme.

A. Young muscle fibres contained significantly more satellite cells identified through expression of Pax7 (6.9) than old muscle fibres (4.7) prior to culture (\* $P < 0.001$ ). Young fibres contained significantly more

myoblasts, identified through expression of Pax7 compared to old fibres at 48h (\*P<0.001). Likewise at 72h, young fibres significantly contained more committed (myogenin) and non-committed cells compared to old fibres (\*P<0.001). B. The number of cells per cluster defined as the number of cells per grouping is not significantly different in cultured young and old muscle fibres. C. The number of clusters per fibre is significantly greater on young fibres compared to the number of clusters on aged fibres at all time points examined (\*P<0.001). D. The number of myogenic precursors (expressing Pax7) on young fibres, 72h after culture, is significantly lower (33%) than those on aged muscle fibres (48.7%) (\*P<0.001). The number of committed myogenic cells (expressing myogenin) on young fibres, 72h after culture, is significantly higher (66.9%) than those on aged muscle fibres (51%) (\*P<0.001).

#### Figure 4

##### Profiling of integrin expression in young and old satellite cells.

A. Expression of integrin  $\alpha 6$  was present at the interface between young satellite cell and the muscle fibre that had been cultured for 36h (yellow arrowhead). A'. Pax7 expression permits the identification of the satellite cell shown in A. A''. Co-visualisation of integrin  $\alpha 6$  (yellow arrowhead) and Pax7 (blue arrow) in young satellite cell. B-B''. Satellite cells from old muscles cultured for 36h expressed very little integrin  $\alpha 6$ . C. Integrin  $\alpha 7$  was detected between the satellite cells and muscle fibre in young muscle samples. C'. Pax7 marks the position of satellite cells in sample shown in C. C'' Co-visualisation of integrin  $\alpha 7$  (yellow arrowhead) and Pax7 (blue

arrow) in young satellite cell. D-D''. Satellite cells from old muscles cultured for 36h expressed less integrin  $\alpha 7$  compared to young cells. E. High levels of integrin  $\beta 1$  localised between the satellite cells and myofibre from young cultured muscle. E'. MyoD marks the position of satellite cell shown in E. E''. Co-visualisation of integrin  $\beta 1$  (yellow arrowhead) and MyoD (blue arrow) in young satellite cell. F. Punctate expression of integrin  $\beta 1$  in an old satellite cell. F'. MyoD marks the position of satellite cell shown in F'. F''. Co-visualisation of integrin  $\beta 1$  and MyoD in an old satellite cell. Scale bar 10  $\mu\text{m}$ .

### Figure 5

Aged satellite cells migrate slower and have abnormal bleb number and blebbing characteristics.

A. Satellite cells on young fibres move significantly faster (40 $\mu\text{m}/\text{h}$  from 17 tracked cells) compared to satellite cells on aged fibres (18.0 $\mu\text{m}/\text{h}$  from 12 tracked cell) (\* $P < 0.001$ ). B. The number of blebs on young satellite cells following 48h of culture is significantly greater (26) than those on aged fibres (12) (\* $P < 0.001$ ). C. The average maximum size of blebs on young and old satellite cells on fibres cultured for 48h did not differ significantly. D. The total lifespan of a bleb was longer in aged satellite cells (36.9 seconds) found on muscle fibres that were cultured for 48h than that on similarly developed fibres from young animals (30.3 seconds, \* $P < 0.05$ ). E. The increase in total bleb lifespan found in aged satellite cells was due to a significant increase in the extension (aged = 4.9 seconds, young = 4 seconds) and retraction (aged = 24.4 seconds, young = 19.3 seconds)

periods (\* $P < 0.05$ ). F. Scanning electron micrograph shows two satellite cells on the surface of young muscle fibres following 48h of culture displaying numerous blebs of similar size (arrowhead). G. Scanning electron micrograph shows a satellite cell on the surface of an old muscle fibre following 48h of culture displaying a few blebs of varying size (arrowheads). H. Bleb shape developed on satellite cells on fibres cultured for 48h calculated as the ratio of maximal length/diameter was not significantly different between young and aged muscles. I. The low migration of aged satellite cells (18 $\mu\text{m/h}$ ) is significantly increased following the introduction of 100  $\mu\text{M}$  PAPA (44.2  $\mu\text{m/hr}$ , 20 cells tracked, \* $P < 0.001$ ). J. The migration speed of young satellite cells (40.0  $\mu\text{m/hr}$ ) is not significantly changed following the introduction of 100  $\mu\text{M}$  PAPA (32.2  $\mu\text{m/hr}$ , XX cells tracked, \*) ( $P > 0.05$ ).

### Figure 6

Characteristics of satellite cell motion. See the methods section for more details. In each case, only the first 345 minutes of data were used. The dots denote the data points and the blue curve is the curve of best fit, which is linear in all images except C.

A. Mean square displacement of young satellite cells along the x-axis. Since the points lie along an approximately straight line in both cases ( $R^2 = 0.903$ ) this suggests that young satellite cells move diffusely along and across the fibre with diffusive rate constant  $D_x = 13.1 \mu\text{m}^2/\text{min}$ . B. Logarithmic transformed y-axis displacement data. The negative gradient ( $R^2 = 0.819$ ) can be related back to the diffusion coefficient giving  $D_y = 1.19$

$\mu\text{m}^2/\text{s}$ . C. Mean square displacement of old satellite cells along the x-axis. These points do not lie along a straight line, instead a power law ( $a t^m$ ) is fitted to the data ( $R^2=0.981$ ,  $m=5.6$ ,  $a=1.45 \times 10^{-11}$ ). This suggests that the old cells are not diffusive along the x-axis. D. Logarithmic transformed y-axis displacement data. The negative gradient ( $R^2=0.663$ ) suggests that the particles are diffusive across the fibre with diffusion rate  $D_y=0.214 \mu\text{m}^2/\text{min}$ . E. and F. Mean square displacement and logarithmic transformed mean square displacement of PAPA treated old satellite cells along the x-axis and y-axis, respectively. Since the points lie along an approximately straight line ( $R^2=0.901$  and  $0.593$ , respectively) this suggests that old PAPA-treated satellite cells move diffusely along and across the fibre with diffusive rate constants  $D_x=12.1 \mu\text{m}^2/\text{min}$  and  $D_y=4.31 \mu\text{m}^2/\text{min}$ , respectively. G. Mean square displacement of old satellite cells treated with HGF along the x-axis. These points do not lie along a straight line, instead a power law ( $a t^m$ ) is fitted to the data ( $R^2=\text{XXX THOMAS}$ ,  $m=\text{XX THOMAS}$ ,  $a=\text{XXX Thomas}$ ). This suggests that the old cells are not diffusive along the x-axis. H. Logarithmic transformed y-axis displacement data for old satellite cells treated with HGF. The negative gradient ( $R^2=\text{XXX Thomas}$ ) suggests that the particles are diffusive across the fibre with diffusion rate  $D_y=\text{XXX Thomas} \mu\text{m}^2/\text{min}$ .

Figure 7

HGF accelerates activation, emergence and speed of migration of aged satellite cells.



A. Activation of satellite cells, gauged by expression of MyoD was quantified at 3 hours after fibre dispersal in the presence of HGF. 20ng/ml and 100ng/ml HGF induced greater activation of both old and young cells (\* $P < 0.05$ ). Only significance for old cells is shown for the ease of graphic representation. B. Emergence gauged by the supra-basal lamina position of Pax7 expressing aged satellite cells 18hrs after fibre dispersal was accelerated by 20ng/ml HGF and 100  $\mu$ M PAPA (\* $P < 0.05$ ). C. Satellite cells on old fibres treated with 20ng/ml HGF move significantly faster ( 41.5  $\mu$ m/hr from 5 tracked cells) compared to untreated satellite cells on aged fibres ( 25.6  $\mu$ m/hr from 5 tracked cells) (\* $P < 0.05$ ). D. Satellite cells on young fibres treated with 20ng/ml HGF did not move significantly faster ( 44.4  $\mu$ m/hr from 4 tracked cells) compared to untreated satellite cells on young fibres ( 40.5  $\mu$ m/hr from 22 tracked cells) (\*  $P > 0.05$ ). E. The average maximum size of blebs on young and old satellite cells on fibres cultured for 48h and treated with 20ng/ml HGF did not differ significantly from untreated cells. F. The number of blebs on aged satellite cells following 48h of culture in the presence of 20ng/ml HGF is significantly greater ( 15.7) than those on untreated aged fibres (9.2) (\* $P < 0.05$ ). G. Scanning electron micrograph of young untreated satellite cell decorated with blebs (arrowheads). H. Scanning electron micrograph of old untreated satellite cell showing fewer blebs (arrowheads) compared to untreated young cell in G. I. Scanning electron micrograph of young 20ng/ml HGF treated satellite cell decorated with blebs (arrowheads). J. Scanning electron micrograph of old 20ng/ml HGF treated satellite cell decorated with blebs (arrowheads).

### Supplementary Figure 1

(A) Mathematical approximation of a blebbing cell travelling along a fibre. The fibre is assumed to be a cylinder with radius  $r=50\mu\text{m}$  and infinite along the x-axis. The blebbing cell is constrained to be on the surface of this cylinder. (B) As we are able to track the cell when it moves behind the fibre, we identify this position behind the fibre, A, with the equivalent position on top of the fibre, A'. Physically, it is as though we have reflected the motion in the grey dashed line. This identification allows us to restrict the motion of the cells to a semicircle which has reflecting boundary conditions. (C) Schematic diagram of the curved surface we are solving the motion on and how it is transformed to the  $y$  coordinates. The boundaries at 0 and  $\pi$  radians are defined to be reflective as discussed in Supplementary Figure 1B.



## RECENT REPORTS

03/12	The Fourier transform of tubular densities	Prior Goriely
04/12	Numerical studies of homogenization under a fast cellular flow.	Iyer Zygalakis
05/12	Solute transport within porous biofilms: diffusion or dispersion?	Davit Byrne Osborne Pitt-Francis Gavaghan Quintard
65/11	Adaptive Finite Element Method Assisted by Stochastic Simulation of Chemical Systems	Cotter Vejchodsky Erban
06/12	Effects of intrinsic stochasticity on delayed reaction-diffusion patterning systems	Woolley Baker Gaffney Maini Seirin-Lee
07/12	Axial Dispersion via Shear-enhanced Diffusion in Colloidal Suspensions	Griffiths Stone
08/12	Qualitative Analysis of an Integro-Differential Equation Model of Periodic Chemotherapy	Jaina Byrne
09/12	Modeling Stem/Progenitor Cell-Induced Neovascularization and Oxygenation	Jain Moldovan Byrne
10/12	Allee Effects May Slow the Spread of Parasites in a Coastal Marine Ecosystem	Krkošek Connors Lewis Poulin
11/12	Parasite spill-back from domestic hosts may induce an Allee effect in wildlife hosts	Krkošek Ashander Lewis
12/12	Modelling temperature-dependent larval development and subsequent demographic Allee effects in adult populations of the alpine butterfly <i>Parnassius smintheus</i>	Wheeler Bampfylde Lewis
13/12	Putting “space” back into spatial ecology	Fortin Peres-Neto Lewis
14/12	Wildlife disease elimination and density dependence	Potapova Merrill Lewis
15/12	Spreading Speed, Traveling Waves, and Minimal Domain Size in Impulsive Reaction-diffusion Models	Lewis Li
16/12	MCMC methods for functions modifying old algorithms to make them faster	Cotter Roberts

17/12	Weyl Geometry and the Nonlinear Mechanics of Distributed Point Defects	Yavari Goriely
18/12	A note on oblique water entry	Moore Howison Ockendon Oliver
19/12	Calculus on surfaces with general closest point functions	März Macdonald
20/12	Multiple equilibria in a simple elastocapillary system	Taroni Vella
21/12	Multiphase modelling of vascular tumour growth in two spatial dimensions	Hubbard Byrne
22/12	Chebfun and Numerical Quadrature	Hale Trefethen
23/12	Moment-based formulation of NavierMaxwell slip boundary conditions for lattice Boltzmann simulations of rarefied flows in microchannels	Reis Dellar
24/12	Correspondence between one- and two-equation models for solute transport in two-region heterogeneous porous media	Davit Wood Debenest Quintard
25/12	Rolie-Poly fluid flowing through constrictions: Two distinct instabilities	Reis Wilson

**Copies of these, and any other OCCAM reports can be obtained from:**

**Oxford Centre for Collaborative Applied Mathematics  
Mathematical Institute  
24 - 29 St Giles'  
Oxford  
OX1 3LB  
England  
[www.maths.ox.ac.uk/occam](http://www.maths.ox.ac.uk/occam)**