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Sera from young and older humans equally sustain proliferation and
differentiation of human myoblasts

by

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Running title: Ageing serum and human satellite cell behaviour

Abstract

Using a human primary muscle cell culture model the behavior of myoblasts (satellite cells) cultured in human serum obtained from either young or elderly individuals was studied. Serum was obtained from a total of 13 young (7 male and 6 females aged, 23-36 years) and 9 elderly (4 male and 5 females aged 69-84 years) subjects and used in a number of experiments. Myoblasts were extracted from human muscle biopsy samples taken from the vastus lateralis. In the first experiment myoblasts were isolated immediately after extraction from the biopsy in media containing human sera to examine its effects on the onset and progression of Ki67 and desmin expression. No effect of the age of the serum was observed at 3, 5 or 7 days of proliferation. In addition, cells that had been expanded initially in optimum myoblast growth medium (GM, containing foetal calf serum and additional growth factors) prior to culture in medium containing 15% human serum were studied. The proportion of proliferating muscle cells co expressing desmin and Ki67 antigens after 46 hours was again similar in the young and old serum conditions. Culturing these myoblasts in media containing 2% human serum to study their fusion and differentiation also resulted in no difference between young and old serum conditions in terms of the percentage of nuclei inside myosin heavy chain positive myotubes. Despite the variability of different samples of myoblasts, the age of the serum has no affect on the expression of any measured index.

1. Introduction

Satellite cells are muscle precursor cells located between the sarcolemma and basal lamina of myofibres (Mauro, 1961). Satellite cells fit the criteria for a stem cell (Zammit et al. 2006) and once detached from their anatomical location are usually referred to as myoblasts. These cells play an integral role in the repair and adaptation of muscle. Upon activation, these cells proliferate, upregulate expression of myogenic markers (e.g. Myf5, MyoD, desmin), then terminally differentiate by withdrawing from the cell cycle and fusing with existing myofibres for repair and adaptation (Zammit et al. 2006).

The loss of skeletal muscle mass with age (sarcopenia) has been attributed, at least in part, to impairments in satellite cell mediated repair (Grounds, 1998). Indeed, there is evidence of a loss of satellite cells in the muscles of older people (Carlson et al. 2009, Kadi et al. 2004, Verdijk et al. 2007), which seems to be specific to type II fibres (Verdijk et al. 2007). In rodents, an age-related impairment of satellite cell mediated muscle repair has been observed in response to severe damage (Conboy et al. 2003). This impairment seemingly resided not with the intrinsic properties of the cells themselves, but within the satellite cell niche, the local environment bathing the satellite cell. A negative effect of the aged systemic milieu was first implied 20 years ago in a study which showed that the mass and function of long term (60 days) transplanted rat muscles was impaired by the age of the host, not by the age of the transplanted muscle (Carlson & Faulkner 1989). Long-term reinnervation deficiencies in the elderly hosts were subsequently implicated in this phenomenon (Carlson et al. 2001) and satellite cell mediated myogenesis was not investigated. More recently, further support for a systemic impairment was shown by the observation of improved

repair and decreased fibrosis in regenerating muscles of old mice that had been exposed to a youthful systemic milieu, by conjoining their circulation with that of a young partner (Conboy et al. 2005). Subsequently, the onset of expression of myogenic and proliferative markers in the first two days of culture has been shown to be impaired when cells are cultured in serum from older compared to young animals (Carlson & Conboy, 2007). This also appears to be the case when human satellite cells are cultured in serum from young and older people (Carlson et al. 2009); although pooled human serum was used in heterochronic experiments (i.e. in which old serum was used on young cells and vice versa), eliminating the possibility for comparison between individual serum donors. Other studies have cultured human myoblasts, but these were all carried out in growth media containing foetal calf serum (Dellavalle et al. 2007, Fulle et al. 2005, Schäfer et al. 2006, Jacquemin et al. 2004, 2007).

In the present study we have sought to examine further the effects of adding human serum to the culture medium on the proliferation and differentiation of myoblasts studied *in vitro*. There were three aims to our study. The first was to compare the effects of serum obtained from young and elderly subjects on the ability of freshly isolated cells obtained from human muscle biopsies to establish proliferating myogenic cultures. The second was to evaluate the ability of young and elderly sera to sustain proliferation of myoblast cultures initially isolated and expanded in an optimum myoblast growth medium (GM), containing foetal calf serum and additional growth factors. The third was to study the ability of these myoblasts to terminally differentiate

in the presence of human serum. Observations on the effectiveness of human serum against commercial GM were also made.

2. Methods

2.1 Ethical Approval

The study was approved by the Research Ethics committee of King's College London and conformed to the standards set by the Declaration of Helsinki. Informed consent was obtained in writing prior to participation in the study.

2.2 Subjects and sample collection

Prior to acceptance in the study, participants completed a health questionnaire and were only included if they fulfilled the "healthy" or "medically stable" criteria (Greig et al. 1994). In total sera was collected from 13 young (7 males and 6 females aged 23-36 years) and 9 elderly (4 males and 5 females, aged 69-84 years) subjects and used as specified in the figure legends.

Subjects visited the laboratory on a single occasion following an overnight fast. A venous blood sample was taken from the median antecubital vein into serum collection vials (BD vacutainer ref. 367958). Serum was left to coagulate at room temperature for 30min, spun at 3000g for ten minutes, aliquoted, and stored at -70°C. A fresh aliquot of serum was thawed for each experiment. Muscle biopsies were taken from the vastus lateralis muscle using the needle technique (Bergström, 1965) with applied suction following local anesthesia (2% lignocaine). Biopsy samples were

obtained from a total of six young subjects. The age and gender of the subjects are indicated in Table 1.

2.3 Cell culture

Biopsy tissue was immediately placed on ice in solution A (30.2mM HEPES, 130.1mM NaCl, 3mM KCl, 11 mM D-Glucose, 3.2nM Phenol red, ddH₂O, pH 7.6; Sigma). Under sterile conditions, the biopsy sample was rinsed in fresh solution A and excess fat and connective tissue were removed by dissection. The remaining muscle tissue was minced with a scalpel in a 60mm petri dish containing 5ml ATE solution (solution A + 5mg/ml trypsin EDTA, Invitrogen). The pieces were then incubated three times in 15ml ATE solution for 15min at 37°C with gentle agitation. After each digestion the supernatant was removed and mixed with 15ml DMEM (Gibco) containing 10% foetal calf serum (FCS Gold, PAA Laboratories), and 1% PSG (100units/ml Penicillin, 100µg/ml Streptomycin and 292µg/ml L-Glutamine, Gibco). The three supernatant aliquots were combined and centrifuged for six minutes at 650g.

2.3.1 Freshly Isolated Cultures:

Cells isolated from the muscle biopsy were immediately resuspended in Skeletal Muscle Cell Basal Medium (BM, Promocell, Germany) and plated in each well of 24 well dishes, coated with ECM matrix (8mg/ml, Sigma E6909). An equal volume of medium supplemented with 30% human serum, 2% PSG or 2X GM was then added to each well such that final serum and antibiotic concentrations were halved. Four populations of cells were examined in these experiments from biopsies 1, 2, 3 and 4 (Table 1).

2.3.2 Established Myoblast Cultures:

Cells were re-suspended in Skeletal Muscle Cell Growth medium (Promocell, Germany) supplemented with 10% FCS and 1% PSG (GM) and plated in an uncoated 60mm dish. Established myoblast cultures were passaged with trypsin (5mg/ml) after 4 days, replated, expanded for 5 population doublings (Jacquemin et al. 2004), frozen in serum free medium (Cryo-SFM, Promocell, Germany) and stored in liquid N₂. Cells were thawed, passaged once and plated in 96 well dishes at a density of 1250 cells/well for proliferation experiments and 5000 cells per well for differentiation experiments. After 24 hours the medium was replaced with BM supplemented with 1% PSG and either 15 or 2% human serum for proliferation and differentiation experiments, respectively. Sera from different subjects were analysed individually and not pooled, nor heat inactivated. Differentiation was also examined in serum free BM containing transferrin and insulin (SF; Jacquemin et al. 2004). Two populations of cells were examined in these experiments from biopsies 5 and 6 (Table 1).

For both cells isolated directly in human serum and established myoblasts cultures the media were changed 72 hours after initial isolation and every 48 hours thereafter. Before discarding, old media was centrifuged at 650g for six minutes to harvest any floating myoblasts. The supernatant was discarded and any pelleted material resuspended in fresh medium and added to the appropriate culture wells. Cells were cultured in a humidified incubator at 37°C and 5% CO₂.

2.4 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde, 0.2% Triton and Phosphate buffered saline (PBS, Sigma) for 5 min. Blocking was for 1 hr at room temperature (RT) in blocking solution (1% bovine serum albumin fraction V \geq 96% (BSA; Sigma) in PBS). Cells were then immunostained overnight at RT using primary antibodies for desmin (mouse monoclonal D33, Dako Cytomaton; 1:100 in PBS, 1% BSA), Ki67 (rabbit polyclonal, Biocare Medical; 1:100 in PBS, 1% BSA) and myosin heavy chain (mouse monoclonal MF20, Developmental Studies Hybridoma Bank; 1:40 in PBS, 1% BSA). After washing in blocking solution (3 x 5min) secondary antibodies were for 1hr at RT in Alexa fluor 594 goat anti rabbit and Alexa fluor 488 goat anti mouse (both 1:1000 in PBS, 1% BSA). Washing was for 3 x 5min in blocking solution as before. Nuclei were stained with Hoechst 33258 (10 μ g/ml, Sigma) for 5min at RT, final washes were in PBS (3 x 5min). All samples were coded and blinded to the investigator. Cells were viewed using a Zeiss Axiovert 135 fluorescence microscope and manually counted. At least 250 nuclei were counted for each well from at least 5 randomly selected fields of view, and at least one duplicate well analysed per sample. All nuclei were counted in wells containing less than 250 cells. The mean of these measurements was taken as the sample value. Desmin (muscle) and Ki67 (cell proliferation marker) staining were expressed as percentage of labelled cells relative to total cells in the culture ascertained by counting all Hoechst stained nuclei. Differentiation was expressed by the percentage of nuclei in myosin heavy chain positive cells containing at least two nuclei (fusion index).

2.5 Statistics

Data were verified for normality of distribution using normal Q-Q plots. Data were compared using repeated measures ANOVA with post-hoc Bonferroni comparisons or

independent t-tests where appropriate. All values are expressed as individual data points or as mean values \pm SE.

3. Results

3.1 Isolation of myoblasts in GM

Typical staining patterns of desmin and Ki67 are shown in Figure 1. To determine the time course of desmin and Ki67 expression immediately after isolation from muscle biopsies, cells from four biopsies were cultured in GM and monitored after three, five and/or seven days. Expression in biopsy 4 was monitored on days three and five. The time course of desmin expression was very consistent between all biopsies examined with 50-70% of cells staining positive for desmin after three days and 70-85% after seven days (Figure 2a). The time course of Ki67 expression in the entire culture (i.e. both desmin positive and negative cells) showed greater heterogeneity between biopsies (Figure 2b), with one population of cells (biopsy 2) occupying a markedly lower proliferating fraction of the culture. However, the proportion of proliferating muscle cells (Ki67 and desmin positive) was always very high relative to the total number of proliferating cells, as can be seen by the similar profiles of Figure 2b and Figure 2c.

3.2 Effects of human serum on expression of proliferation and myogenic markers of freshly isolated muscle cells. The effects of medium containing human sera on desmin and Ki67 expression were also examined in biopsies 2 and 3 at three, five and seven days (Figure 3a-c). No significant differences were observed between the young and elderly serum conditions at three, five or seven days (compare the closed symbols

(young) with the open symbols (elderly) at each time point for the two cell assays). Figure 3d-f shows the individual values for each serum or for GM. The co-variance values for measurements of desmin, Ki67 and D+, Ki67+ co-stained cells at all time points were $6.3\pm 0.9\%$, $8.1\pm 1.1\%$ and $11.3\pm 1.0\%$ respectively. Human sera were generally more effective at inducing desmin and Ki67 expression than GM. This is particularly evident in biopsy 3 at day three (Figure 3d) where desmin expression in GM is 25% lower than the average for human sera and 15% lower than the least effective human serum condition. Almost all cells expressing desmin also express Ki67 irrespective of whether they are cultured in human serum or GM.

3.3. Effects of human serum on the proliferation and differentiation of established myoblasts.

Given that the human sera examined were more effective than GM at promoting desmin and Ki67 expression in freshly isolated myoblasts, we next examined whether these sera were also effective on myoblasts that had been initially isolated and expanded in GM. Two established myoblasts cultures containing 50% and 97% desmin for biopsies 5, and 6 respectively, were transferred to media containing human serum for 46 hours. The performance of the myoblasts under these conditions was much more variable with some sera leading to both reduced desmin (Figure 4a) and Ki67 (Figure 4b) expression compared to GM. In contrast to the freshly isolated cells, the myoblasts initially isolated and expanded in GM showed increased co-expression of desmin and Ki67 (Figure 4c) in GM compared to human serum. However, as with the freshly isolated cells there was no difference in the ability of young or old human sera to sustain proliferation and desmin expression in these myoblasts. Finally, myoblast

differentiation was examined in cells cultured for six days in medium containing 2% human serum. Fusion indices varied considerably in different sera (Figure 5) and were always less efficient in media containing human sera than in the optimal serum free medium (SF; Figure 5 – red triangles). The inhibition of differentiation by different sera was not related to the ability to sustain proliferation and/or desmin expression in the cultures and as with proliferation, there was no effect of the age of the serum donor on cell behaviour.

4. Discussion

The main finding of the present study was that there was no relationship between the performance of human sera in various *in vitro* human muscle cell assays and the age of the serum donor.

The freshly isolated cell assay examines the early events of myoblast culture, specifically upregulation of myogenic markers and cell cycle entry. We initially studied the time course of freshly isolated cell populations which had been extracted in GM from four different biopsy samples (Figure 2). Cells from the different biopsies showed similarity in response to culture in terms of relative desmin expression, but considerable heterogeneity in Ki67 labelling such that the proportion of cells that were both Ki67 and desmin positive (i.e. proliferating muscle cells) varied, being markedly lower in biopsy 2 compared with the others. Heterogeneity has been reported previously in desmin expression between human myoblast populations regardless of age (Schäfer et al. 2006). However, in all samples, expression of both desmin and Ki67

increased with time in culture and virtually all desmin positive cells eventually expressed Ki67 showing that myogenic commitment and proliferation are closely related at this early stage. At present we cannot determine whether the increase in double labelled cells is due to increased desmin expression in proliferating cells, increased proliferation in desmin positive cells, or more robust proliferation of the double labelled cells.

When cells from two of these biopsies, which represented a range of cell behaviour, were cultured in media containing human sera, no effect of the age of the serum donor was observed (Figure 3). In addition, human serum was generally as effective, if not more effective, than GM in promoting desmin and Ki67 expression in these muscle cells when measured over this time course.

Whilst the freshly isolated cell assay examined the early events of myoblast culture, the expanded myoblast assay is an established culture containing committed myoblasts. Here we once again treated the cells populations with young and elderly serum. Again we observed heterogeneity in the response between two different cell populations, but once more no effect of the age of the serum donor was observed. The somewhat superior Ki67 expression of myoblasts treated with GM relative to human serum in the expanded cultures (Figure 4c) compared to the freshly isolated cultures (Figure 3d-f) suggests that the initial expansion and culture in GM prior to the experiment may have had a conditioning effect on the cells. A less likely and untested possibility, is that the freezing of the cells for storage has some effect.

Our findings showing no age related impairment in myoblast behaviour in freshly isolated cells are in contrast with results from studies in mice (Carlson et al.2007, Brack et al. 2007) and humans (Carlson et al. 2009) where serum from aged donors decreases the ability of myoblasts from both young and old donors to incorporate BrdU and express the muscle markers, Pax7, Myf5, MyoD, and desmin (Carlson et al. 2007, Brack et al. 2007). The difference between these findings and our own is difficult to explain with certainty. Our human myoblast isolation procedures are comparable with those of many laboratories (Schafer et al. 2006, Dellavalle et al. 2007) and our cells show an inhibition of desmin expression for at least five days when TGF- β 1 is added to the culture medium (containing either foetal calf or human serum), even at low concentrations (1 and 2ng/ml, unpublished observations). There are however, other methodological differences between the two studies on human cells including the use of different enzymes for tissue digestion (trypsin as opposed to collagenase), the use of different markers to measure cell proliferation (Ki67 as opposed to BrdU), different time points for initial measurements (72 versus 48 hours), different serum concentrations (15% versus 10%) and the fact that the sera were not pooled by age group in any of the present assays. The fact that our cells remain sensitive to TGF- β 1 suggests that the choice of digestion enzyme, proliferation marker, or time point of the assay does not affect the ability of the assay to effectively measure interactions of the cells with their environment. We have also directly compared proliferating cells containing 10% and 15% serum in the culture medium and found no difference in the percentages of desmin and Ki67 positive cells between the two conditions. Pooling of serum may represent the major source of difference in observations presented here and in Carlson et al. (2009). Inclusion of sera in which the cells show an impaired

response to a pool of serum could skew the data for the whole batch to suggest that there is a difference between young and old subjects when in fact only one or two outliers may show a diminished response. Further work is needed to clarify what effects these methodological differences may have. However, our results would suggest that the source of any age-related inhibition of satellite cell response is not serum borne.

As we have alluded to, there are good data showing impairments in the activation and initial myogenic marker expression in rodents *in vivo* e.g. Conboy et al. 2005, Brack et al., 2007, 2008, Carlson and Conboy 2007), but the results of other studies suggest that this has no long term consequences for muscle repair. For example, in a study by Smythe et al. (2008), EDL muscle grafts were cross-transplanted between young and old BALB/c mice and although there was a slight delay in the early inflammatory response and in the appearance of new myotubes, there was no effect of age on the overall formation of new muscle. A more recent study by Shavlakadze et al. (2010) similarly showed a delay in the onset of regeneration after autografting muscle into a geriatric mouse host, though again the long-term formation of new muscle was unaffected and there was no effect of the geriatric host on regenerating muscle. Other animal studies which have used a variety of different models including chronic low-frequency electrical stimulation (Putman et al. 2001), denervation (Dedkov et al 2003) as well cross-transplantation in mdx mice (Boldrin et al. 2009) have similarly observed no age related impairment to muscle repair. Taking the above into account, it is also possible to speculate that there is a slower activation of young cells cultured in old serum that might be detectable before day three of culture, but the data presented

show that even if this were the case, these initial differences have no effect on later myogenic responses.

In humans, there are also somewhat conflicting reports as to age related changes in satellite cell behaviour. There are several reports comparing the terminal differentiation (i.e. myotube formation and myosin heavy chain expression) in human myoblasts from young and old donors (Jacquemin et al. 2004, Lorrenzon et al. 2004, Beccaficco et al., 2007) that suggest impaired or delayed differentiation in old myoblasts. However, when comparing individual data from more than one young and one old donor these differences are less clear, with cells from many old individuals showing no reduction in myotube formation (Fulle et al., 2005, Mouly et al., 2005, Schäfer et al., 2006). In vivo, satellite cells can be identified in muscle cryo-sections, by using specific markers (e.g. NCAM, Pax7), or through electron microscopy. The results of a number of training studies suggest that in the long term, satellite cell number can be successfully increased in older individuals (Roth et al. 2001, Mackey et al. 2007, Verdijk et al. 2009, Verney et al. 2008) with no reported age related impairments (Roth et al. 2001, Verdijk et al. 2009). Furthermore, in an analysis of young and old male and female subjects who underwent sixteen weeks of strength training exercise (Petrella et al 2006) and who were subsequently clustered into cohorts of responders and non-responders (Petrella et al. 2009), the ability of responders to increase myofibre cross-sectional area was correlated with increased satellite cell and myonuclear number, independently of age. In the short term however, there is some evidence that the exercise mediated increase in satellite cell number may be blunted in older subjects either following a bout of acute resistance exercise (Dreyer et al. 2006) or a short

period (4 weeks) of rehabilitation immediately following a 2 week period of cast immobilization (Carlson et al. 2009). It is important to note that satellite cell activation measured following resistance exercise is a different model to that used in most animal studies (muscle damage, cross transplantation) and may involve quite different signals to those that we are able to study in vitro. With the exception of Carlson et al. (2009) none of the studies cited compared the behaviour of satellite cells from the same individuals in vivo and in vitro and therefore caution must be employed when comparing studies. There is clearly more research needed to elucidate any age related impairments to satellite cell behaviour and whether these are intrinsic to the cell, the niche, or the more general systemic aged milieu. Furthermore, the model to be used, to confirm whether effects due to ageing or combination of ageing and deleterious factors to health, such as inactivity, needs to be carefully considered (Larazus and Harridge 2010). Such variability may account for the heterogenous behaviour of myoblasts in our study.

In summary, our data suggest that the ability of freshly isolated or expanded human myoblasts to proliferate, differentiate and fuse is not impaired by culture in an aged milieu and that human myoblasts can be very successfully isolated and expanded in human serum.

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Table 1. Subject and cell characteristics for each biopsy sample.

Biopsy	Subject age	Subject Gender	Myoblast yield* (%)	Fusion index** (%)
1	38	Female	96	nd
2	25	Male	78	nd
3	27	Male	80	nd
4	29	Male	89	-
5	23	Female	50	63
6	24	Male	99	96

*determined up to ten days after biopsy in GM; ** determined after six days of differentiation in serum free media; nd: not determined

Figure 1:

Micrographs of human (a) myoblasts and (b) myotubes. (a) Cells from biopsy 5 were stained for (a) Ki67 (red), desmin (green) and Hoechst (blue) after 46 hours culture in 15% human serum (in this case serum was from a male subject aged 81 years). (b) The same cells stained for myosin heavy chain (green) and Hoechst after six days in 2% human serum (serum from the same 81 year old male subject). Scale bar = 100 μ m.

Figure 2:

Time course of (a) desmin (b) Ki67 and (c) desmin and Ki67 expression in cells freshly isolated in growth medium (GM containing 15% foetal calf serum and additional growth factors). Cells obtained from biopsies 1,2, 3 and 4 were cultured in GM for up to 7 days. Cells were fixed at 3, 5 and 7 days and immunostained”

Figure 3:

Time course of (a) desmin, (b) Ki67 and (c) desmin and Ki67 expression in cells freshly isolated in human serum. Cells were obtained from biopsies 2 (triangles) and 3 (squares) and cultured in media containing serum obtained from young (closed symbols, solid lines, n=6, age 23-29 years) or elderly (open symbols, dashed lines, n=7 age 69-82) subjects. Values are mean \pm SE. No difference was observed between young and old sera conditions. Day 3 is significantly different from days 5 and 7 (* p<0.01, ** p<0.001) in biopsy 2 and significantly different from day 7 ($^{\circ}$ p<0.05) in biopsy 3 for both serum conditions. Individual data points for culture in young serum (YS) or old serum (OS) are shown for desmin and Ki67 and positive cells for the two biopsies at days 3 (d), 5 (e) and 7 (f), red squares indicate responses in GM. Individual serum and

gender ages are denoted by the following symbols: Elderly: F82◆; F69□; M80▲;

M84●; F77△; M82✕; M74 X. Young: F27▲; F24 X; M23○; M24◆; F25●, M25 ✕.

M=male, F=female and numbers indicate age of subjects in years.

Figure 4:

Proportion of (a) desmin, (b) Ki67 and (c) desmin and Ki67 expressing cells in

expanded cells. Cells were isolated and expanded in GM, prior to culture for 46 hours in 15% serum obtained from either young (n=11, aged 23-36 years 7 male 4 female, YS) or elderly (n=8, aged 73-84 years 4 male and 4 female, OS) subjects. Cells were obtained from biopsies 5 and 6. Data are expressed as individual points labelled with the sex and age of each subject, red squares indicate responses in GM. No difference was observed between culture in young or old serum conditions. Individual serum and gender ages are denoted by the following symbols: Elderly: F82◆; F69□; M80▲; M84●; F77△; M82 ✕; F82 +; M74X; F73 —. Young: F27▲; F24 X; M23○; M24◆; F25●; M24 —; M24—; F36□; M24 +; F23◇; M25✕. M=male, F=female and numbers indicate age of subjects in years.

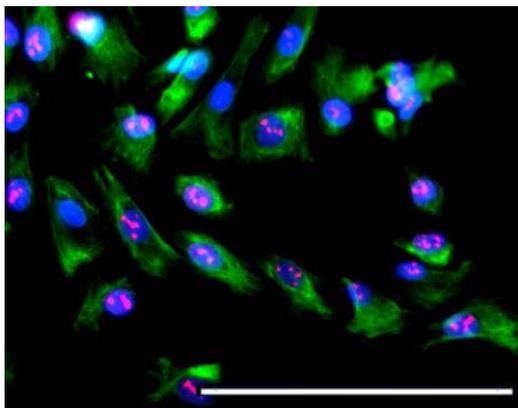
Figure 5:

Fusion indices of human muscle cells cultured in serum obtained from young or

elderly subjects. (a) Fusion indices of cells cultured in medium containing 2% human serum for 6 days or in a serum free differentiation medium containing insulin and transferrin (red triangles). (b) The same data normalised to the desmin content of the cultures immediately prior to the addition of differentiation medium (50% and 97% for

cells from biopsies 5 and 6 respectively). Sera from 8 young (aged 23-26, YS) and 7 elderly (aged 80-84, OS) subjects were examined. Data are expressed as individual points labelled with the sex and age of each subject. No significant difference was observed between the means of young and elderly serum conditions. Individual serum and gender ages are denoted by the following symbols: : Elderly: F82◆; F69□; M80▲; M84●; M82 ; F82 + ; M74 X. Young: F27▲; F24X; M23○; M24 —; M24—; F36□; F23◇, M25 .

a)



b)

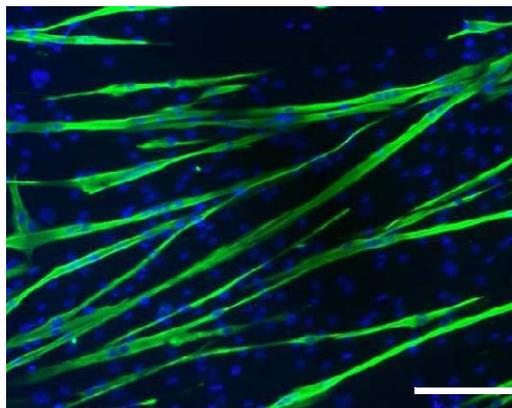


Figure 1

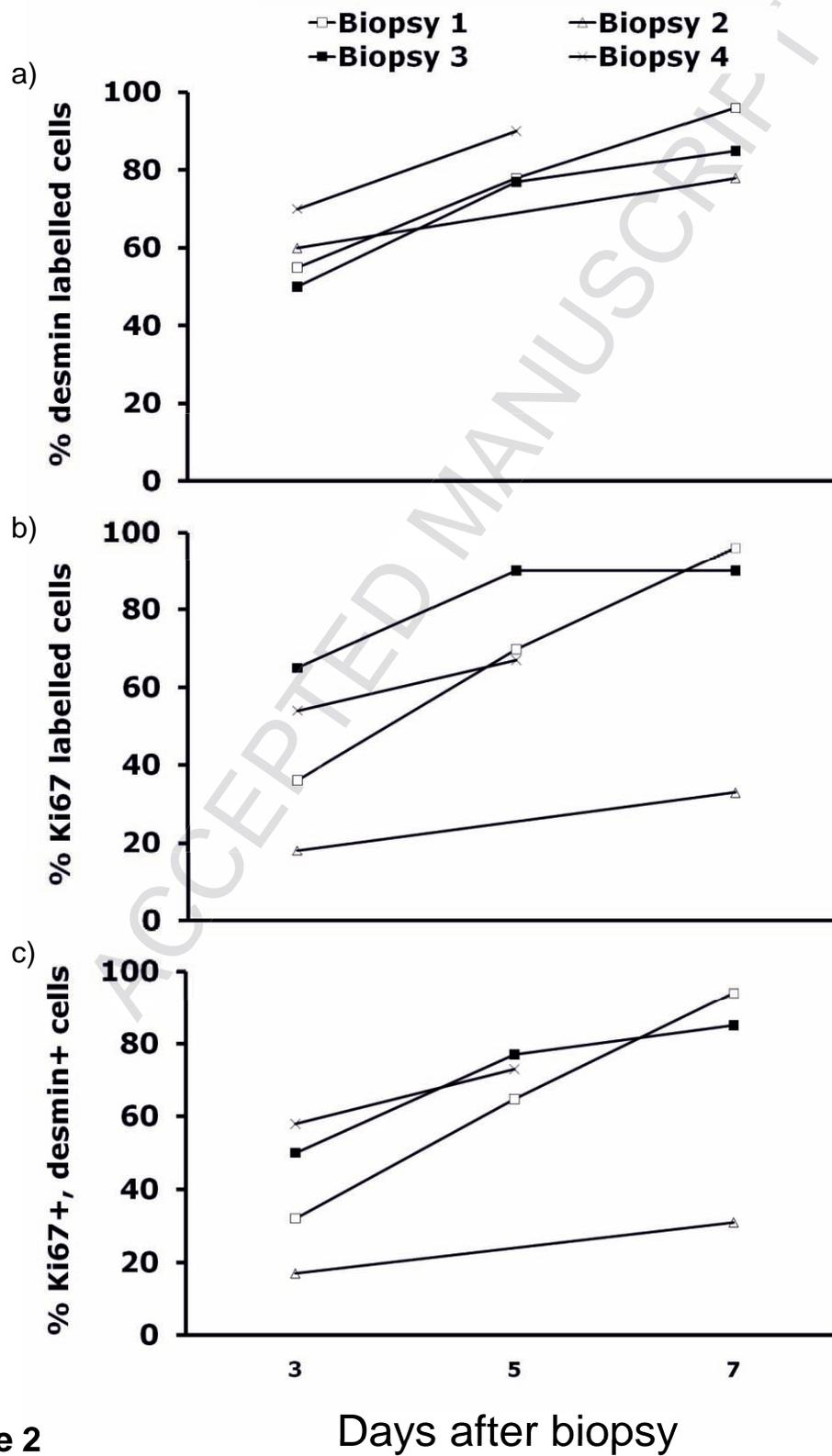


Figure 2

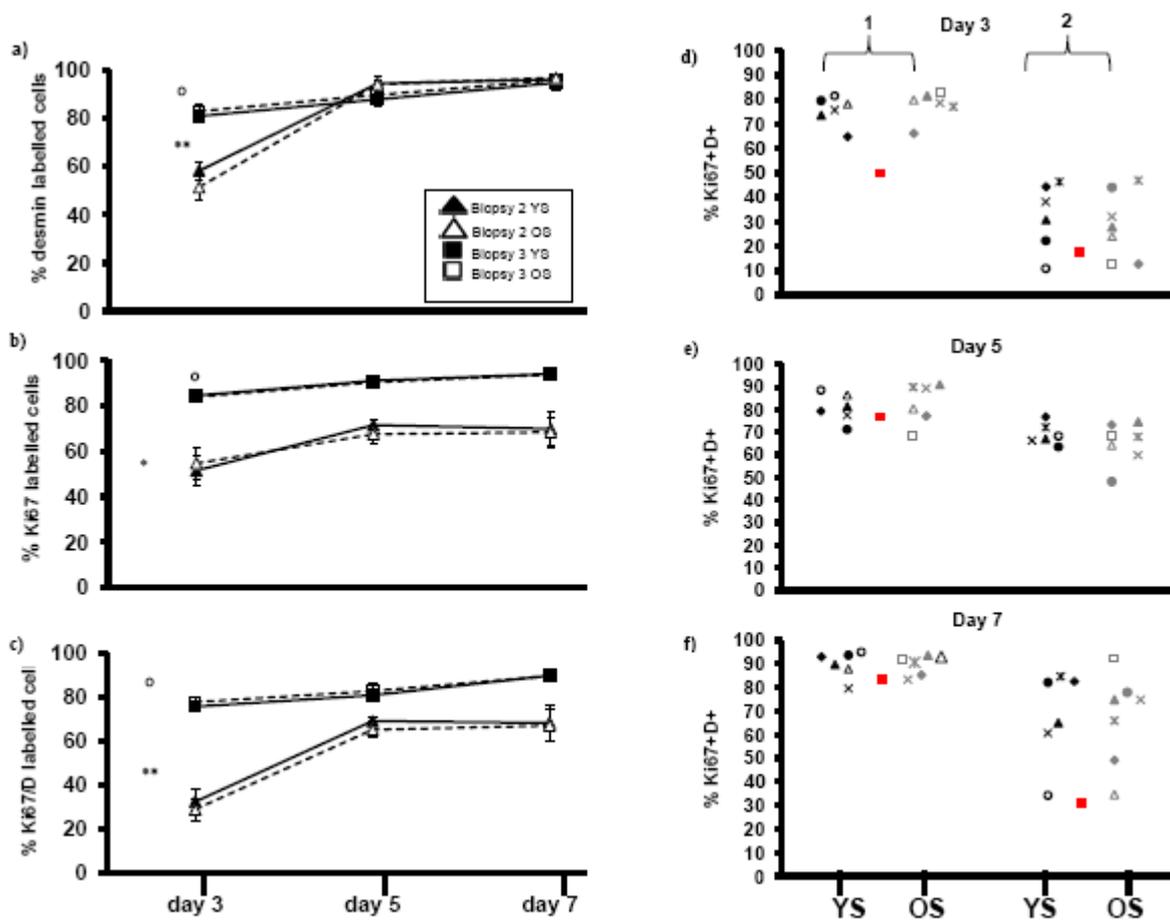


Figure 3

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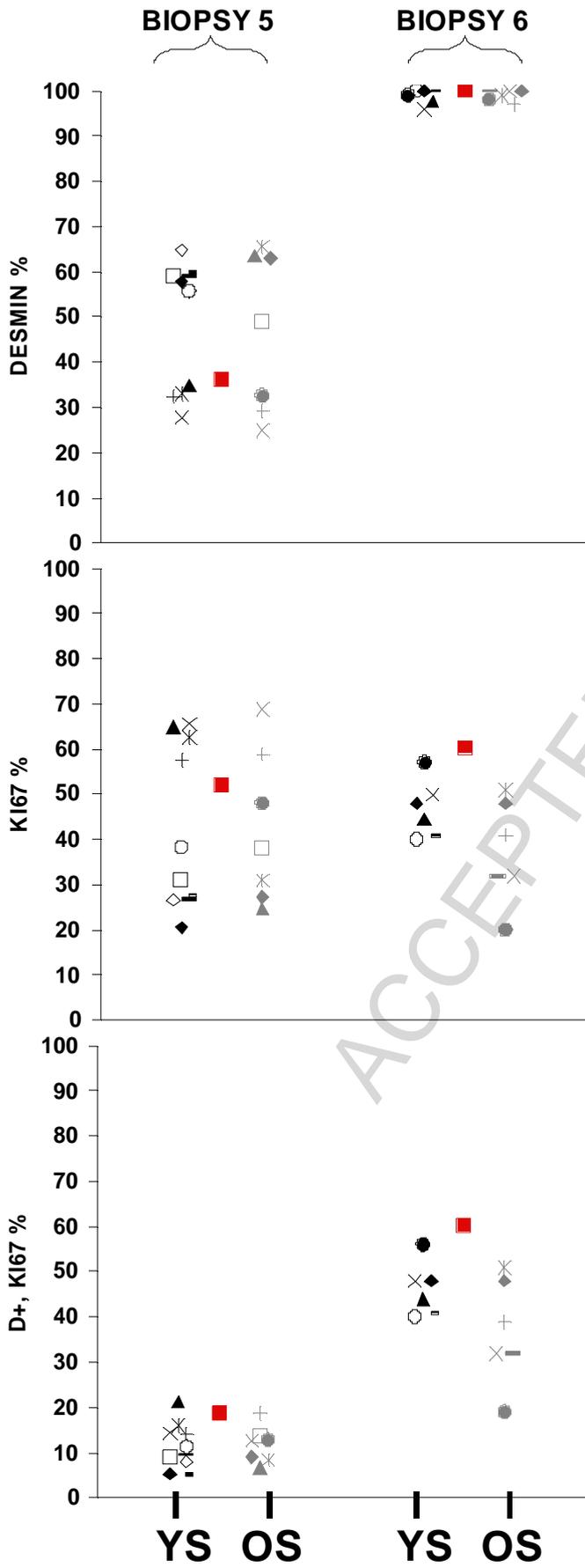


Figure 4.

SIX DAYS FUSION

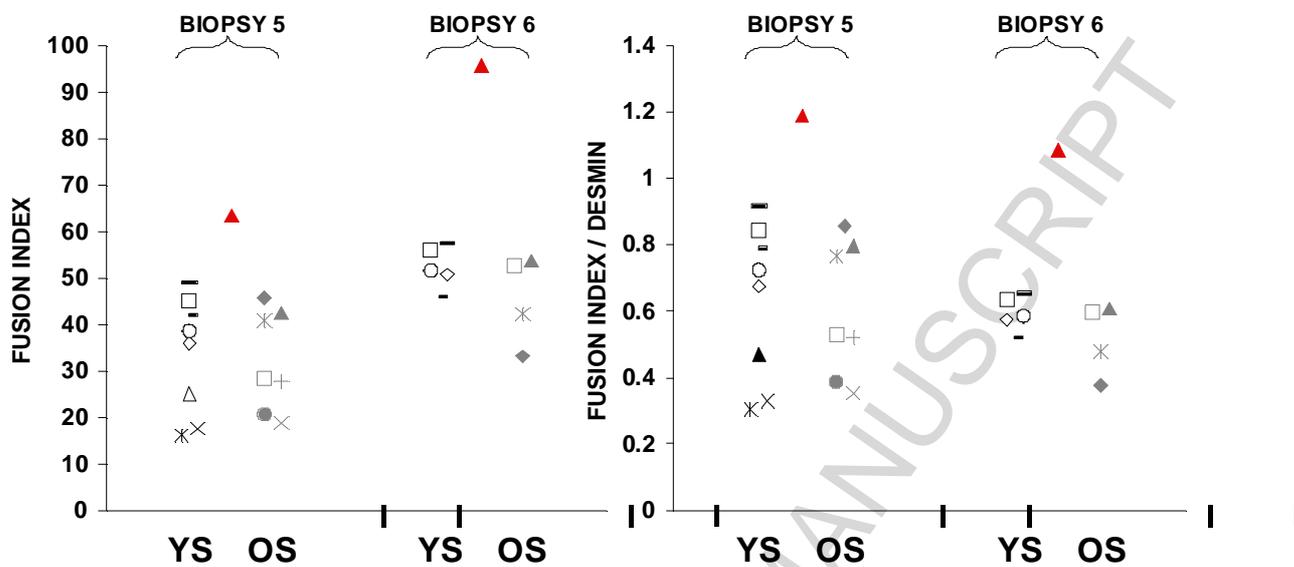


Figure 5.