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Neurobiology of Aging xxx (2008) xxx–xxx

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**NEUROBIOLOGY  
OF  
AGING**


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## Increased aging in primary muscle cultures of sporadic inclusion-body myositis

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Received 10 June 2008; received in revised form 13 August 2008; accepted 19 August 2008

### Abstract

Ageing is thought to participate to the pathogenesis of sporadic inclusion-body myositis (s-IBM). Although the regenerative potential of s-IBM muscle is reduced in vivo, age-related abnormalities of satellite cells possibly accounting for the decline of muscle repair have not been demonstrated. Here we show that proliferation rate and clonogenicity of s-IBM myoblasts are significantly lower and doubling time is longer than normal age-matched controls, indicating that proliferative capacity of s-IBM muscles becomes exhausted earlier. Telomere shortening is detected in s-IBM cells suggesting premature senescence. Differently from controls, s-IBM myoblasts show increased active  $\beta$ -catenin mainly localized within myonuclei, indicating active Wnt stimulation. After many rounds of muscle growth, only s-IBM myoblasts accumulate congophilic inclusions and immunoreactive  $A\beta_{1-40}$  deposits. Therefore, s-IBM myoblasts seem to have a constitutively impaired regenerative capacity and the intrinsic property, upon sufficient aging in vitro, to accumulate  $A\beta$ . Our results might be valuable in understanding molecular mechanisms associated with muscle aging underlying the defective regeneration of s-IBM muscle and provide new clues for future therapeutic strategies.

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**Keywords:** Aging; Cultured muscle; Satellite cells; s-IBM; Muscle regeneration

### 1. Introduction

Satellite cells represent the main resident stem cells in muscle and, when activated in response to physiological stimuli such as intensive exercise or injury, they show a remarkable proliferative ability and either fuse to form multinucleated myotubes or re-establish a quiescent pool of cells capable of supporting further rounds of regeneration (Shi and

Garry, 2006). Although skeletal muscle has a high capacity for complete regeneration in response to repeated injuries, this ability is not unlimited and satellite cells may finally become exhausted. In progressive muscle diseases characterized by repeated cycles of muscle degeneration–regeneration of fibers, after an initial extensive mobilization of satellite cells, a decline of muscle regenerative ability is usually observed because of replicative senescence of satellite cells that proliferate during muscle repair (Decary et al., 1997, 2000). Aging muscle is characterized by progressive muscle weakness and atrophy (Shavlakadze and Grounds, 2006), increase in fibrous connective tissue and slower regenerative capacity (Mouly et al., 2005; Renault et al., 2002a, b). In aged muscle the number of satellite cells is decreased and cellular senescence, triggered by excessive telomere shortening, limits their proliferative potential (Wright and Shay, 2002). Moreover, satellite cells from aged muscle

*Abbreviations:* s-IBM, sporadic inclusion-body myositis; R, round of mass growth; P, passage; GSK3 $\beta$ , glycogen synthase kinase-3 $\beta$ ; A $\beta$ , amyloid- $\beta$ ; A $\beta$ PP, amyloid- $\beta$  precursor protein; FBS, fetal bovine serum; DT, doubling time; CT, control.

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doi:10.1016/j.neurobiolaging.2008.08.011

Please cite this article in press as: Morosetti, R., et al., Increased aging in primary muscle cultures of sporadic inclusion-body myositis. *Neurobiol Aging* (2008), doi:10.1016/j.neurobiolaging.2008.08.011

display reduced proliferative and fusion capacity and tend also to accumulate fat (Taylor-Jones et al., 2002). Recently, it has been shown that senescence induces deregulation of myogenic specific regulatory factors (Bigot et al., 2008). Defective myoblasts showing a premature proliferative arrest have been characterized from biopsies of patients with genetic diseases such as oculo-pharyngeal muscular dystrophy, typically presenting in late adulthood (Périé et al., 2006).

Sporadic inclusion-body myositis (s-IBM), the most common acquired myopathy in patients above the age of 50 years, presents with slowly progressive atrophy and weakness affecting proximal and distal limb muscles and often also pharyngeal and facial muscles (Needham and Mastaglia, 2007). The cause and the pathogenesis of s-IBM are unknown and its histopathology is characterized by the presence of lymphocytic infiltrates combined with degenerative features such as formation of vacuoles, intracellular inclusions and accumulation of amyloid- $\beta$  protein ( $A\beta$ ) and other related molecules (Engel and Askanas, 2006). s-IBM may be considered an age-related myopathy (Askanas and Engel, 2007) and various muscle changes occurring in normal aging may be exacerbated in s-IBM muscle, thus participating in its pathogenesis. It is known that primary s-IBM myoblasts do not show evident morphological abnormalities, are capable of normally differentiating and can be properly innervated (McFerrin et al., 1999). Although the regenerative potential of s-IBM muscle is apparently greatly reduced in vivo, characteristic age-related abnormalities of satellite cells, that might account for the decline in muscle repair ability, have not been demonstrated so far.

The main aim of our study was to evaluate whether cultured s-IBM myoblasts differ from myoblasts of normal aged individuals in terms of cell proliferation, morphology, differentiation and senescence. In addition, we investigated  $\beta$ -catenin expression which reflects stimulation of the Wnt pathway, which is activated in aged muscle (Brack et al., 2007) and might be involved in the reduction of regenerative potential of s-IBM muscle in vivo.

## 2. Experimental procedures

### 2.1. Patients

Diagnostic muscle biopsies were performed after informed consent at the Neurology Department of Catholic University. We used fresh and fresh-frozen muscle biopsies from 8 sporadic s-IBM (44–83 years, mean  $66.75 \pm 12.84$ ) and 5 different biopsies of age-matched (37–83 years, mean  $62 \pm 20.16$ ) patients free of muscle disease as controls. Diagnosis was based on both clinical evaluation and laboratory studies. None of the patients received either steroids or immunosuppressive therapy before the muscle biopsy. This research was approved by the Ethical Committee of our Institution.

### 2.2. Primary muscle cultures

Muscle biopsies were dissected into small fragments and either directly cultured or vitally frozen for subsequent culturing as previously described (Carter and Askanas, 1981). Details are available in [Supplementary Material](#). For muscle cultures, 2 mm<sup>3</sup> muscle fragments were placed on the bottom of a Petri dish, coated with a 1% gelatin solution containing human plasma (Askanas and Engel, 1975, 1992; Broccolini et al., 2006). To allow a high rate of cell proliferation, cultures were maintained in culturing medium with 15% fetal bovine serum (FBS) and growth factors. Myoblasts were then let outgrow from each muscle fragment until 70–80% confluence was achieved in a 60 mm culture dish. Muscle fragments were then mechanically detached from the bottom of the dish and transferred to a new one for another cycle of cell growth. This will be defined as “round of mass growth” hereafter. For simplicity, the set of cells obtained from each round of mass growth was identified with the round number (R, followed by the digit specifying the number of times the muscle fragments had been already transferred from one dish to another for a new round of cell mass outgrowth) and the passage number (P, followed by the digit specifying the number of times each set had been already trypsinized during mass growth). Proliferating myoblasts from each round of mass growth were kept growing up to the 6th passage. Each culture set was evaluated for myogenic purity by calculating the percentage of desmin-positive cells.

Details for skeletal muscle differentiation are provided in [Supplementary Material](#).

### 2.3. Growth curves and clonogenic assays

Proliferating myoblasts from both early (R2) and late (R7) rounds were plated for growth curves. Clonogenic assays were performed for the 8 s-IBM and the 5 controls cultures. Each experiment was performed at least two times. Details are provided in [Supplementary Material](#).

### 2.4. Telomere restriction fragment length assay

Proliferating myoblasts from R2, passage 5, were analyzed for telomere shortening. Details are provided in [Supplementary Material](#).

### 2.5. Immunocytochemistry

Cells were cultured on optical quality plastic m-Dishes (ibidi Integrated Biodiagnostics, Munich, Germany) and fixed in 4% PF. Details are available in [Supplementary Material](#). Samples were analyzed using a TCS SP5 laser scanning confocal microscope (Leica Microsystems, Wetzlar, Germany).

## 2.6. Western blot analysis

Protein expression was analyzed by western blot according to standard methods. Details are available in [Supplementary Material](#).

## 2.7. Statistical analyses

Statistical analyses were performed in all experiments by ANOVA for repeated measures and comparisons between groups was assessed by Student's *t*-test. Significance level was set at  $P \leq 0.05$ . Data are reported as means  $\pm$  S.D. for all groups.

## 3. Results

### 3.1. Primary muscle cultures

Primary muscle cultures were obtained from muscle biopsies of 8 s-IBM patients (44–83 years, mean  $66.75 \pm 12.84$ ) and 5 age-matched normal controls (37–83 years, mean  $62 \pm 20.16$ ) ([Supplementary Table 1](#)). We achieved a satisfactory outgrowth of myoblasts up to R10 for control biopsies

and up to R8 for 6 s-IBM biopsies (2 s-IBM biopsies, namely the IBM1 and IBM3, stopped at R3). However, controls and s-IBM fragments did not differ significantly in terms of time of culture from the day of first plating to R8 ( $38.25 \pm 7.3$  days vs.  $43.33 \pm 5.0$  days, respectively;  $P = 0.112$ ). Of note, early and late rounds of growth of s-IBM cultures did not differ in terms of number of cells isolated ( $3.2 \pm 1.69 \times 10^6$  from R2 and  $2.2 \pm 1.89 \times 10^6$  from R7, respectively;  $P = 0.220$ ). In all culture sets myoblasts accounted for 80–90% of total cells, as evaluated by desmin staining. All s-IBM muscle cells from both early and late rounds (R2, R7) and up to passage 4, did not show any structural cell abnormality compared with the 5 normal controls in identical culture conditions (not shown).

### 3.2. Proliferation ability of s-IBM myoblasts

The proliferation ability and clonogenicity were analyzed in all s-IBM myoblast cultures at an early round of growth (R2-P4) and compared with age-matched normal controls taken at the same round and passage. Both proliferation rate and clonogenic ability of s-IBM myoblasts at R2-P4 were significantly lower than controls ([Fig. 1A and B](#)). In order to verify whether the impairment of regenerative capacity of

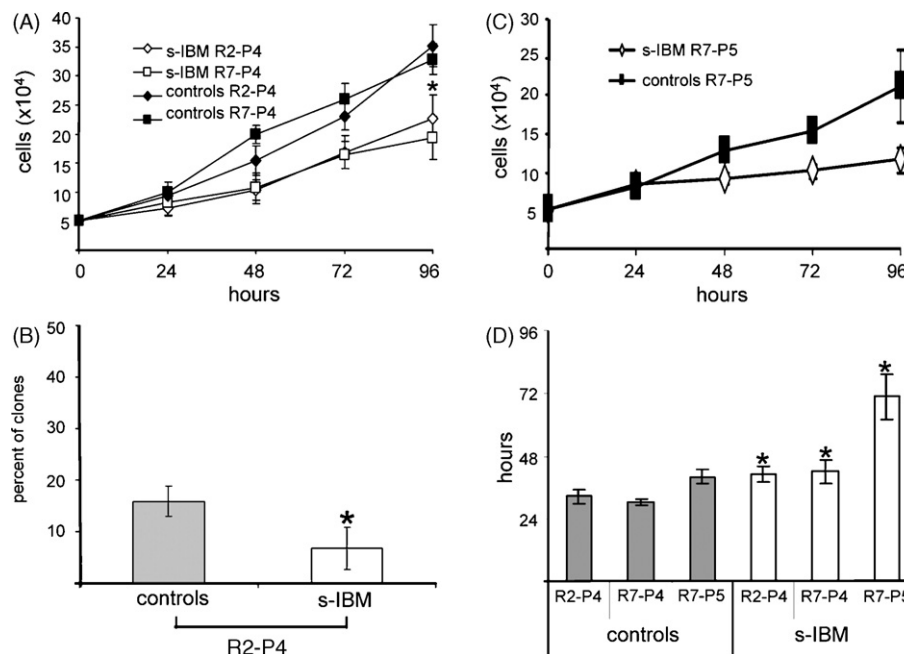


Fig. 1. Proliferation ability of s-IBM myoblasts is lower than age-matched control myoblasts. (A) Growth curves performed in 8 s-IBM muscle cultures compared to 5 normal controls at R2-P4 and in 6 s-IBM muscle cultures compared to 5 normal controls at and from R7-P4. No significant difference was observed in proliferation rate of s-IBM myoblasts from early (R2-P4) or late (R7-P4) rounds of mass growth ( $P = 0.125$ ); by contrast proliferation ability of s-IBM cells was significantly lower than controls, compared at early and late rounds of mass growth ( $*P = 0.015$  and  $0.0002$  at R2P4 and R7P4, respectively). Cell growth was assessed after 24, 48, 72 and 96 h. Results are expressed as absolute counts. Bars represent the mean  $\pm$  S.D. of triplicate samples of one representative experiment out of three. (B) Clonogenic ability of s-IBM myoblasts even from early explants (R2-P4) was significantly lower than controls ( $*P = 0.008$ ). A representative experiment, out of two, performed for the 8 s-IBM and the 5 controls culture sets, plated by limiting dilutions in two 48-well dishes, is shown. (C) s-IBM myoblasts from R7-P5 show a dramatic decrease of their proliferation rate. Bars represent the mean  $\pm$  S.D. of triplicate samples performed in 6 s-IBM muscle cultures compared to 5 normal controls of one representative experiment out of three. (D) Doubling time of s-IBM myoblasts was significantly longer than normal controls. Bars represent the mean  $\pm$  S.D. of triplicate samples of one representative experiment out of three.  $*P = 0.009$ ,  $0.006$ ,  $0.002$ , at R2-P4, R7-P4 and R7-P5, respectively.

s-IBM satellite cells progresses with the age of culture, we also analyzed the proliferation ability of myoblasts from the 6 s-IBM biopsies available at R7-P4 (as stated above, 2 s-IBM biopsies stopped at R3) and 5 normal controls. While s-IBM myoblasts taken at R2 maintained their proliferation ability up to P6, s-IBM cells from late rounds (R7) displayed an exponential growth only up to P4 (Fig. 1A). The proliferation rate of all s-IBM myoblasts at P4 (from both R2 and R7) was overall significantly lower than in normal controls ( $P \leq 0.05$ ), whereas no significant difference was observed between s-IBM myoblasts from either early or late rounds (i.e., R2-P4 and R7-P4, respectively) (Fig. 1A). s-IBM myoblasts from the 6 biopsies that reached R7, when plated for growth curves at P5, showed a dramatic decrease of their proliferation rate compared to control myoblasts at the same round and passage, as this latter group kept an exponential growth up to 96 h (Fig. 1C). Doubling time of s-IBM myoblasts from both early and late rounds was significantly longer than that of normal controls ( $41.11 \pm 2.78$  h in s-IBM at R2-P4, and  $42.06 \pm 4.6$  h in s-IBM at R7-P4, vs.  $32.71 \pm 2.69$  in controls at R2-P4, and  $30.33 \pm 1.18$  in controls at R7-P4;  $P \leq 0.05$ ) (Fig. 1D). No significant difference was observed between s-IBM myoblasts at R2-P4 and those taken at R7-P4, while doubling time of s-IBM myoblasts at R7-P5 was significantly longer than that of cells from an earlier passage of the same round (Fig. 1D). Results were always consistent throughout all experiments. Each experiment for each culture set was performed in triplicate, at least two times.

### 3.3. Telomere shortening in s-IBM myoblasts

The regenerative capacity of satellite cells decreases with age and is accompanied by telomeres shortening. Since an abnormal proliferation rate was observed in all s-IBM muscle cultures, even when obtained from early rounds (R2), we hypothesized a telomere shortening in our myoblasts causing premature senescence of satellite cells. Fig. 2 clearly shows a significant telomere shortening already evident in three different cultures of s-IBM myoblasts from an early round of cell proliferation (R2-P5), indicating that these cells started to undergo senescence earlier than normal age-matched controls. The same experiment was performed for the other 5 sets of s-IBM myoblasts at the same round and passage with consistent results (not shown).

### 3.4. $\beta$ -Catenin expression in s-IBM primary muscle cultures

Wnt signaling with glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ) and its substrate  $\beta$ -catenin have been implicated in the regulation of myogenesis (Polesskaya et al., 2003). Its canonical pathway regulates  $\beta$ -catenin stability and, in the absence of Wnt stimulation,  $\beta$ -catenin is phosphorylated by GSK3 $\beta$  and degraded via the proteasome pathway (Prunier et al., 2004). Binding of Wnt to its receptor Frizzled results in inactivation of GSK3 $\beta$  and inhibition of  $\beta$ -catenin degrada-

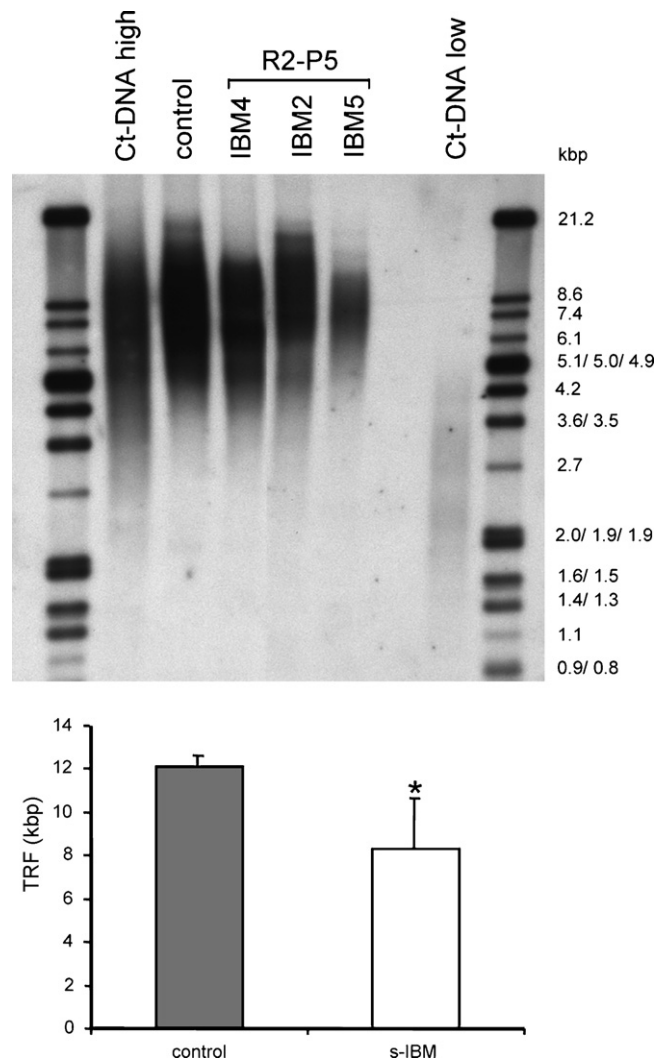


Fig. 2. s-IBM myoblasts undergo senescence earlier than controls. Measurements of the telomeric smear in DNA obtained from normal control and 3 s-IBM patients' myoblasts (R2-P5). TRFs from the s-IBM patients are significantly shorter than normal control smear. \* $P=0.02$ .

tion, thereby  $\beta$ -catenin is accumulated in the nucleus of the myogenic cell (Petropoulos and Skerjanc, 2002). Recently, an impaired regenerative capacity of muscle cells has been associated with activation of the canonical Wnt signaling cascade with an increase of active  $\beta$ -catenin in aged satellite cells (Brack et al., 2007). In all myoblast cultures from the 8 s-IBM patients, even at early rounds (R2), we observed an increased expression of active  $\beta$ -catenin which was almost exclusively localized in the nucleus, while a diffused weak cytoplasmic immunostaining was present in our control cultures that showed only rare positive myonuclei (Fig. 3A). An increased level of active  $\beta$ -catenin in s-IBM cultures and a lower level of p- $\beta$ -catenin compared to controls were confirmed by western blot analysis (Fig. 3B). Accordingly, increased levels of the inactive form of GSK3 $\beta$  (phosphorylated at Ser9) were detected in s-IBM myoblast compared to normal controls (Supplementary Fig. 1).

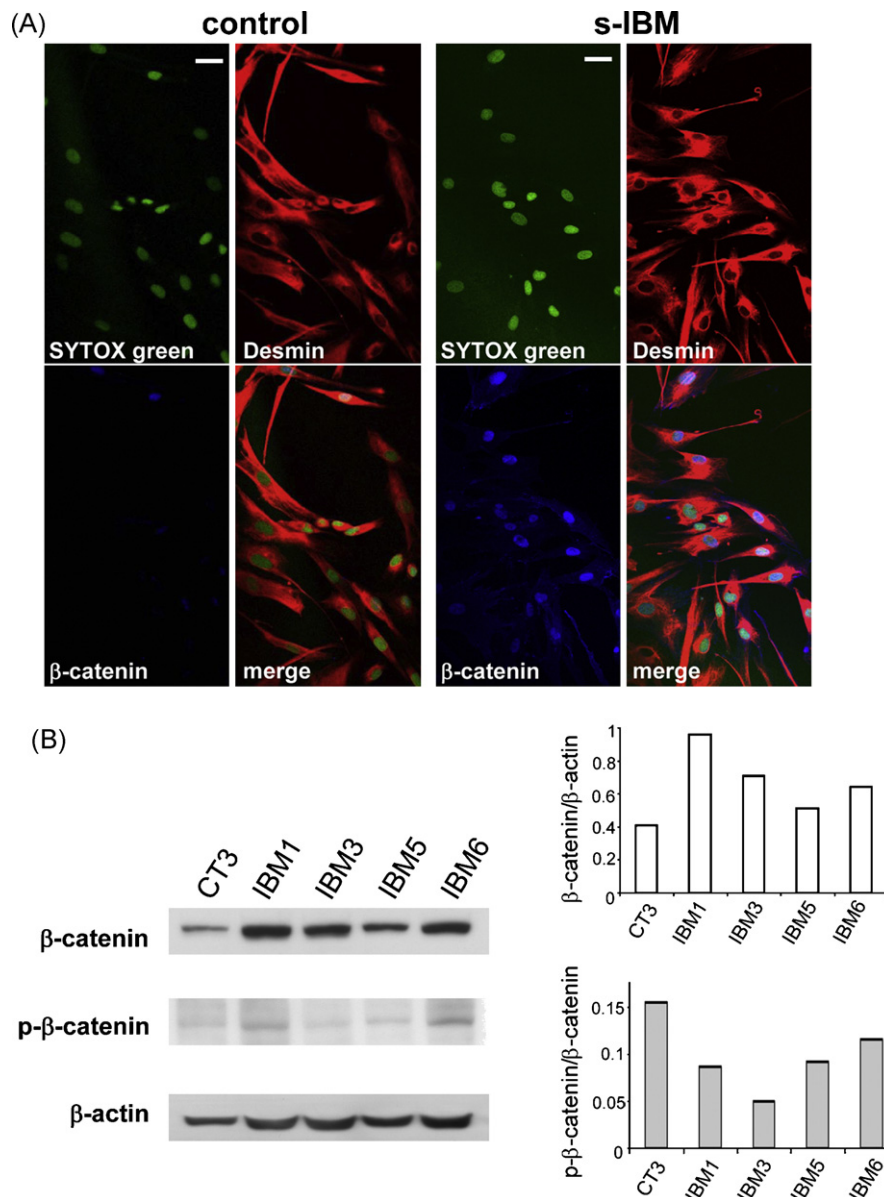


Fig. 3.  $\beta$ -catenin expression is increased in s-IBM myoblasts. (A) In our 8 s-IBM myoblast cultures (each from a different patient) even from early rounds of mass growth (R2) increased expression of active  $\beta$ -catenin (labelled in blue by Alexa Fluor<sup>®</sup> 405-conjugated secondary antibody) almost exclusively localized to the nucleus was detected, while a diffused faint cytoplasmic immunostaining was present in 5 control cultures that showed only rare positive nuclei. Representative cultures of one control and one s-IBM patient are shown. Nuclei are visualized by SYTOX green. Scale bars: 10  $\mu$ m. (B) Western blot analysis shows increased levels of active  $\beta$ -catenin in s-IBM cultures, while p- $\beta$ -catenin protein levels are significantly lower than controls.

### 3.5. s-IBM myoblasts differentiation

All s-IBM myoblasts obtained from early and late rounds up to R7-P4, fully differentiated into multinucleated myosin-positive myotubes (fusion index  $0.75 \pm 0.06$ ) (Fig. 4A), as confirmed also by normal activation of skeletal muscle differentiation signaling pathways. Western blot analysis showed an up-regulation of myogenin expression during differentiation. In addition, neprilysin expression increased with induction of differentiation at day 2 and declined thereafter coincident with the appearance of myosin, as previously reported during differentiation of normal myoblasts (Fig. 4B)

(Broccolini et al., 2006). By BrdU incorporation virtually all myotubes were terminally differentiated with no BrdU-positive nuclei (not shown).

### 3.6. Phenotype of aged s-IBM cultures

s-IBM myoblasts from the 6 biopsies who reached R7 showed a dramatic reduction of their growth rate at P5 compared to control myoblasts of the same age (Figs. 1C and 5A). Therefore, s-IBM cells were plated at R7-P5 and then either fixed after 72 h or 96 h of culture for morphological analysis, or shifted to differentiation medium to study their

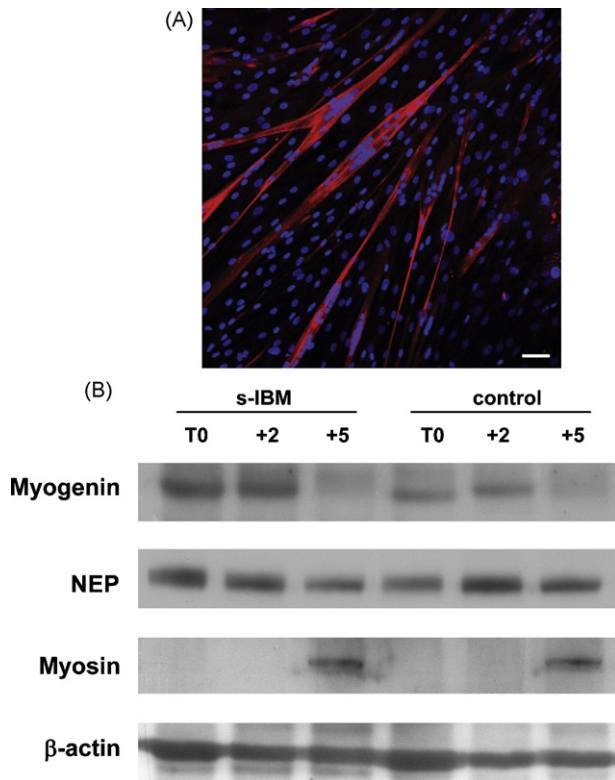


Fig. 4. s-IBM myoblasts fully differentiate into multinucleated myosin-positive myotubes. (A) Immunofluorescence for myosin heavy chain (red). Scale bar: 40 μm. (B) Western blot analysis of myogenin, neprilysin (NEP) and myosin expression during differentiation of s-IBM and control myoblasts. An up-regulation of myogenin expression can be observed in both s-IBM and control cells. NEP expression increases with the induction of differentiation at day 2 and declines thereafter coincident with the appearance of myosin.

differentiation ability. Cultures utilizing myoblasts from the same patient, taken at an earlier round and passage (R2-P5), were used as controls in this experiment. Desmin staining of s-IBM myoblasts from late rounds (R7-P5) showed mild cytoplasmic abnormalities with moderately increased vacuolization and focal areas of desmin accumulation (Fig. 5B right panel). It is well known that abnormal s-IBM vacuolated muscle fibers are characterized by an accumulation of amyloid-β precursor protein (AβPP) and Aβ (Askanas et al., 1993a, b). In addition, the overexpression of the AβPP gene into well-differentiated cultured normal myotubes recapitulates several aspects of the s-IBM phenotype of mature muscle fibers (Askanas et al., 1997). Interestingly, our s-IBM myoblast cultures at R7-P5 showed strongly positive immunostaining for Aβ<sub>1-40</sub> mainly in the form of granular cytoplasmic inclusions, differently from myoblasts from the same patient at R2-P5 (Fig. 5B). In addition, Congo Red staining showed the presence of congophilic inclusions only in late s-IBM myoblasts (Fig. 5C). We then asked whether the presence of intracellular Aβ<sub>1-40</sub> was due, at least in part, to up-regulation of its precursor protein. Thus we performed western blot analysis for AβPP and observed that basal level of expression of the protein was similar in s-IBM and aged

controls and did not show a significant modulation over time (Fig. 6). After 5 days in differentiation medium both early and late s-IBM myotubes were fixed for morphological analysis. Myotubes from late s-IBM cells were hypernucleated, often presented cytoplasmic microvacuolation with some larger subsarcolemmal vacuoles and showed strong intracellular accumulation of Aβ<sub>1-40</sub>, much more abundant than what observed in their precursor cells (Fig. 5D and E). By contrast, myotubes obtained from myoblasts at an earlier round did not show significant abnormalities (Fig. 5D and E). Aβ<sub>1-40</sub> increased expression was also confirmed by western blot analysis that showed higher levels of the Aβ<sub>1-40</sub> peptide in late (R8-P5) s-IBM myotubes compared to age-matched control at the same passage (Fig. 5F).

### 3.7. Inhibition of GSK3β in early s-IBM myoblasts

In order to evaluate whether GSK3β inhibition might play a role in the premature aging of s-IBM muscle cultures, we exposed myoblasts at early round of muscle growth (R1-P4) to LiCl, a well-characterized inhibitor of GSK3β. Inhibition of active GSK3β, as indicated by up-regulation of p-GSK3β (Supplementary Fig. 2A), was already observed after 24 h of treatment. This was accompanied by a significant inhibition of cell proliferation ( $P = 0.0024$ ) (Supplementary Fig. 2B). Interestingly, LiCl induced also morphological changes and formation of very small Aβ<sub>1-40</sub> immunoreactive aggregates in both myoblasts and myotubes (Supplementary Fig. 2C). These effects of LiCl on early s-IBM muscle cells, partly resembling the changes spontaneously observed only in very late rounds of muscle growth, raise the possibility that GSK3β dysregulation may be one of the mechanisms involved in the premature aging of s-IBM cultured muscle.

## 4. Discussion

s-IBM is the most common acquired muscle disease of older persons and, because of its generally unsatisfactory response to antidysimmune treatments, has a progressive course leading to severe disability. Ageing is considered a crucial aspect contributing to the pathogenesis of s-IBM and to the development of its characteristic molecular phenotype. In s-IBM muscle the presence of degenerative features, such as vacuolated fibers containing Aβ and amyloid-related proteins (Askanas and Engel, 2006), reflects a complex multifactorial pathogenesis involving misfolded and unfolded proteins and increased oxidative stress in the context of a cellular “aged” milieu which act in concert with chronic inflammation (Askanas and Engel, 2003). Degenerating muscle fibers display progressive vacuolization, atrophy and accumulation of multiprotein aggregates accompanied by proteasome inhibition and endoplasmic reticulum stress (Askanas and Engel, 2007). In particular, the increased intracellular expression of AβPP and its toxic proteolytic fragment

A $\beta$  is thought to play a key upstream role in the pathogenic cascade leading to muscle degeneration (Askanas and Engel, 2006). It has also been postulated that the aging environment of s-IBM with its characteristic muscle degenerative changes may elicit a T-cell inflammatory reaction facilitating the establishment of the chronic lymphocytic inflammation (Askanas and Engel, 2007). Satellite cell-dependent regeneration occurs also in s-IBM muscle wherein multiple metabolic pathways normally involved in muscle development are activated (Broccolini et al., 2004, 2006). However, in s-IBM muscle, despite the activation of potentially repairing mechanisms and an apparently normal representation of satellite cells (M. Mirabella, personal observation), regenerating muscle fibers are scarce in comparison to other myopathies and, overall regeneration is insufficient to counterbalance the ongoing muscle fibers degeneration. Our study demonstrates that it is possible to isolate myoblasts from s-IBM muscle biopsies to a lesser extent compared to age-matched controls. In fact, two of the s-IBM biopsies were not able to produce myoblasts beyond R3, whereas in the other six biopsies, myoblast proliferation was steady, although quantitatively less pronounced compared with normal aged controls, up to R7 and then abruptly stopped at R8. Proliferation rate and clonogenicity of s-IBM myoblasts were significantly lower and doubling time significantly longer than normal controls, even from young cultures obtained from R2, indicating that these findings do not simply reflect replicative senescence due to extended proliferation in culture and suggesting that the regenerative capacity of s-IBM satellite cells might be constitutively impaired. These observations were also supported by the detection of telomere shortening in s-IBM samples already at R2-P5, thus indicating premature senescence even in “young” s-IBM cultures. Our results may be valuable in understanding the molecular mechanisms underlying the defective regeneration of muscle fibers observed in s-IBM muscle. As a matter of fact, we do believe that promoting in culture the outgrowth of myoblasts directly from the muscle explant, rather than obtaining them through direct dissociation of the specimen, more closely resembles the physiologic mechanism of myoblast proliferation that takes place in muscle. This probably because the myogenic cells, that are resident in the muscle fragment and give rise to the outgrowing muscle cells, remain in their tissue micro-environment when experimentally promoted to replicate. In addition, in the cultured monolayer, muscle cells coexist with fibroblasts that also emerge from the muscle fragments, thus resembling more the *in vivo* situation, where muscle fibers are surrounded by non-muscle cells (Askanas and Engel, 1992). Using this experimental approach, we have shown aging-related phenotypic abnormalities that seem exclusive of s-IBM cultures and would have been missed if the observation was conducted only on myoblasts obtained right after the initial plating of the muscle fragment in the culturing dish. Overall, our evidence strongly argues in favor of the fact that the proliferation capacity of s-IBM muscles becomes exhausted

at an earlier stage compared with their age-matched normal counterparts.

Increasing evidence has demonstrated that age-related myopathies, such as sarcopenia, are characterized by impaired muscle regeneration and numerous regenerative defects due to the macro- and micro-environment possibly associated with the aged muscle (Coggan et al., 1992; Chakravarthy et al., 2000). In animal models the impaired proliferation of satellite cells of aged muscle has been shown to be, at least in part, due to perturbed Notch signaling and exposure to yet unknown factors circulating in young mice can enhance regeneration of the aged muscle (Conboy et al., 2005). Therefore both local and systemic influences that change with age appears to be important in modulating aged satellite cells (Brack and Rando, 2007).

Activation of the canonical Wnt pathway has been recently demonstrated in satellite cells from aged mice that, along with impairment of regenerative potential, show conversion to a fibrogenic lineage, thus suggesting a critical role for Wnt signaling in tissue-specific stem cells aging and increased fibrosis with age (Brack et al., 2007). This prompted us to investigate whether Wnt/ $\beta$ -catenin pathway is increasingly activated in s-IBM muscle cultures in comparison with normal myoblasts from aged individuals. We showed that cultured s-IBM myoblasts expressed increased levels of active  $\beta$ -catenin and p-GSK3 $\beta$  compared to myoblasts from aged-matched controls, while the level of p- $\beta$ -catenin, normally targeted for ubiquitination and subsequent proteasomal degradation, was reduced. By immunofluorescence a large proportion of s-IBM myoblasts, differently from normal aged controls, showed detectable stabilized non-phosphorylated  $\beta$ -catenin in the nucleus where it is translocated upon Wnt stimulation, and presumably interacts with transcription factors, such as T-cell factor, to activate target genes. Both changes regarding the levels and the subcellular localization of  $\beta$ -catenin observed in s-IBM myoblasts are indicative of activation of Wnt signaling (Hagen et al., 2002). The observation of increased levels of p-GSK3 $\beta$  in s-IBM along with the effects of LiCl on early muscle cultures lend support to the hypothesis that dysregulation of GSK3 $\beta$  may play a role in the premature aging of s-IBM and warrants further studies. Although Wnts proteins have pleiotropic effects and may also promote myogenic lineage progression during development and post-natally (Nusse, 2005), increased Wnt signaling in murine aged myogenic progenitors *in vivo* has been demonstrated to promote an aging phenotype impairing muscle regeneration and enhancing a fibrotic response (Brack et al., 2007). Our observation suggests that the modulation of Wnt signaling pathway may be relevant in designing therapeutic strategies directed to ameliorate the impairment of muscle regeneration in s-IBM and to reduce the effects of the aged environment on myogenic progenitor cells fate.

Previous studies on cultured human muscle have demonstrated that overexpression of A $\beta$ PP by gene transfer in normal muscle reproduces most of the typical phenotypic abnormalities of s-IBM muscle fibers (Askanas et

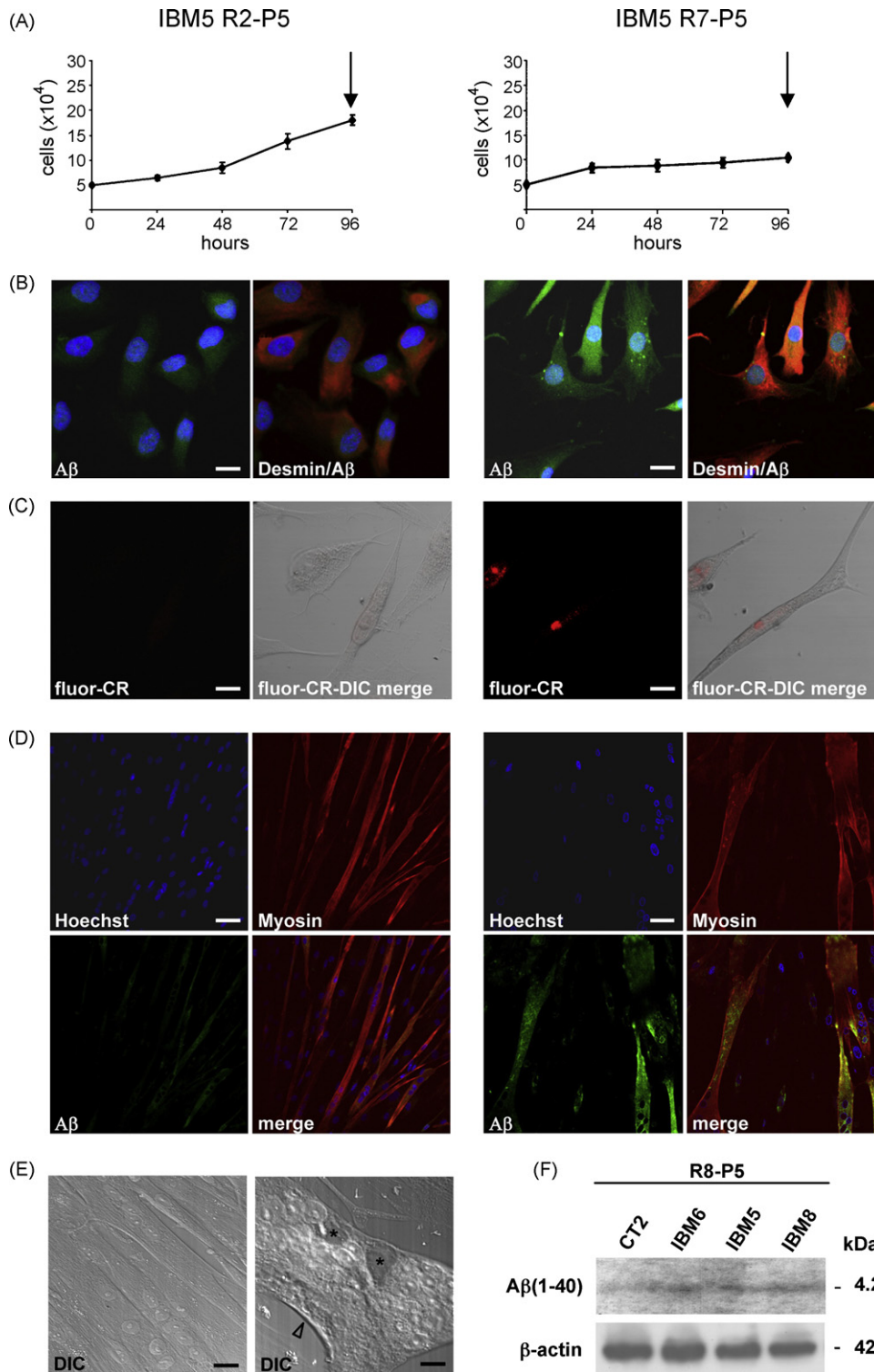


Fig. 5. (A) Growth curve of s-IBM myoblasts from IBM5. s-IBM myoblasts from R7 show a dramatic reduction of their growth rate at passage 5 compared to cultures of myoblasts from the same patient obtained from R2 at the same passage. After 96 h of culture cells were fixed for immunocytochemistry or shifted to differentiation medium (arrow). For growth curve, cells were plated in triplicate. A representative experiment from patient IBM5 is shown. (B) Immunofluorescence for Desmin (red) and Aβ<sub>1-40</sub> (green) of s-IBM myoblasts from IBM5. Desmin staining of late s-IBM myoblasts showed some mild cytoplasmic alterations with focal areas of desmin accumulation. s-IBM myoblast cultures from R7-P5 showed strongly positive immunostaining for Aβ<sub>1-40</sub> compared to sister cultures of myoblasts from R2-P5 from the same patient. Scale bar: 5 μm (C) Congo Red staining viewed under fluorescent light using Texas-Red filter (fluor-CR) showed the presence of congophilic inclusions only in late s-IBM myoblasts. (DIC, differential interference contrast trough Nomarski optics). Scale bar: 5 μm Immunofluorescence for Myosin (red) and Aβ<sub>1-40</sub> (green) of s-IBM myotubes from IBM5 (D and E). Myotubes from late s-IBM myoblasts were hypernucleated, showed strong intracellular accumulation of Aβ, alike observed in their precursors but more abundant and in a larger number

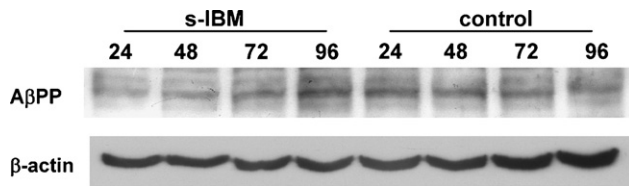


Fig. 6. A $\beta$ PP protein is expressed at comparable levels in s-IBM and control myoblasts. Cells obtained from R7-P4 were plated in triplicate and harvested at different time points for protein extraction. No significant differences between s-IBM and controls were detected, nor a significant modulation of protein expression could be observed over time, in each culture set. A representative western blot is shown.

al., 1997). However, aneurally cultured muscle cells from s-IBM patients, similar to normal muscle, did not show A $\beta$ PP increased immunoreactivity suggesting that they do not spontaneously accumulate A $\beta$ PP (McFerrin et al., 1999). These findings have been interpreted considering that the factors responsible for A $\beta$ PP/A $\beta$  accumulation in mature s-IBM fibers may not be functioning in the short-term, thus outlining the importance of aging cellular environment. Our study confirms that relatively short-term cultures of s-IBM appear morphologically indistinguishable from normal muscle cultures and do not show A $\beta$  accumulation, congophilic inclusions or structural abnormalities. It is known that prolonged amplification of satellite cells acts as a factor promoting replicative senescence (Cooper et al., 2003). However, in our experimental setting aimed at examining more aged muscle cells after many rounds of muscle growth, surprisingly we found that only s-IBM, unlike normal aged muscle, showed congophilic inclusions and immunoreactive A $\beta$ <sub>1–40</sub> deposits in myoblasts and, after fusion, a much stronger A $\beta$ <sub>1–40</sub> accumulation often accompanied by the appearance of vacuolar changes. Interestingly, this A $\beta$ <sub>1–40</sub> accumulation was not due to an increased expression of A $\beta$ PP. Therefore, we can speculate that, in our *in vitro* system, the abnormal metabolic changes caused by the aging process of myoblasts in culture lead to an impairment of the cellular A $\beta$  clearing system, thus promoting the accumulation of A $\beta$ <sub>1–40</sub> despite a normal level of expression of its precursor. Based on the results of our study, the possibility exists that, in s-IBM muscle, an impairment of the A $\beta$  clearing system, due to abnormal mechanisms of increased aging, is indeed primarily responsible for the initial deposition of A $\beta$  within muscle fibers. This would possibly activate a “vicious-circle” scenario where the increased accumulation of A $\beta$  leads to increased oxidative stress, which in turn causes over-expression of specific transcription factors capable of increasing the A $\beta$ PP production, thus creating a self-perpetuating cellular destructive mechanism. However, further studies are necessary to clarify this issue.

In our opinion these data bear two important consequences. First, satellite cells of s-IBM from which cultured myoblasts are derived, have the intrinsic property, upon sufficient aging *in vitro*, to accumulate A $\beta$ , manifesting at least some of the aspect of the phenotype of mature muscle fibers. Obviously, because aneural muscle cultures remain quite immature, it is not expected that they would display the whole phenotype characteristic of the mature s-IBM muscle fiber, even by using this experimental setting that may somehow amplify the effects of aging. Nevertheless, our results clearly outline the importance of aging in the pathogenesis of s-IBM and, in particular, in the defective muscle regeneration observed *in vivo*. However, we do believe that aging is necessary but not “*per se*” sufficient to produce the muscle s-IBM phenotypic changes because cultured muscle from normal aged people in identical culture setting do not show such abnormalities. The second main consideration, which is a direct consequence of this line of evidence, is that s-IBM cultured cells have an increased susceptibility to undergo degenerative changes in response to various stressors and the occurrence of spontaneous accumulation of A $\beta$  in aged s-IBM cultures indicates that this is indeed a primary aging-related mechanism contributing to s-IBM muscle degeneration *in vivo*.

The control of systemic and metabolic factors known to influence *in vivo* the development of tissue changes related to aging, such as increased mitochondrial DNA deletions, oxidative stress and interleukin 1- and 6-mediated inflammatory tissue burden (possibly achievable with caloric restriction, blood sugar and cholesterol levels lowering, administration of non steroid anti-inflammatory drugs and statins) has been suggested (Finch, 2006) and may prove to be beneficial to also reduce the regenerative decline of s-IBM muscle.

*In vivo* also of possible future application of myogenic stem cells-based therapies, it is of paramount importance to look for pharmacological or molecular strategies trying to protect transplanted cells from the pathogenic factors promoting excessive aging which operate in the s-IBM muscle environment *in vivo* and that may also affect them, thus greatly reducing their muscle regenerative potential and clinical usefulness.

Therefore a better understanding of molecular mechanisms associated with muscle fiber aging may provide new perspectives towards s-IBM therapy.

#### Acknowledgements

We thank Manuela Papacci for her precious technical assistance. This study was supported by grants from Catholic

of cells, and often presented cytoplasmic microvacuolation with some larger subsarcolemmal vacuoles (right panels). By contrast myotubes from early sister cultures of the same patient did not show significant structural abnormalities or A $\beta$ <sub>1–40</sub> accumulation (left panels). Nuclei are visualized in blue by Hoechst 33258 staining. Scale bars: 40  $\mu$ m in (D), 7  $\mu$ m in (E) (left), 5  $\mu$ m in (E) (right). Representative cultures from the same patient (IBM5) are shown. (F) Western blot analysis showed increased levels of the A $\beta$ <sub>1–40</sub> peptide in late (R8-P5) s-IBM myotubes compared to age-matched control at the same passage.

University, Fondazione Don Carlo Gnocchi and Istituto Superiore di Sanità to Massimiliano Mirabella. The authors have no conflicts of interest to disclose.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neurobiolaging.2008.08.011.

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