Satellite Cell Proliferation: Starting Point and Key Steps to Regeneration and Cell-Mediated Gene Therapy

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Abstract

Regeneration of muscle fibres following injury requires the activation of quiescent satellite cells, their proliferation and finally their differentiation and fusion into multi-nucleated myotubes which will, after maturation, replace the damaged fibre. Although many reports have been published concerning the differentiation potential of satellite cells in vitro, and the formation of new fibres in vivo, very few studies have looked at their activation and their proliferation. In this study we report that myf-5, a member of the myogenic regulatory factors, is an early marker of the activated satellite cells. During regeneration in human muscle it is important to consider the proliferative capacity of the satellite cells since the proliferation of human somatic cells is limited. Although we show that the limit in proliferation should not interfere with regeneration in normal muscle, it is probably a limiting factor in cases of muscular dystrophy when the muscle will undergo repeated cycles of degeneration and regeneration. Another field where the limit in proliferation is crucial is cell-mediated gene-therapy, since if satellite cells are to be used as vectors they must be amplified before introduction into the host. In this study we give a detailed evaluation of the proliferative capacity of human satellite cells at different ages, and describe a system which has been used to increase this capacity, using T antigen from SV40. The effect of the expression of T antigen in human satellite cells, and the general consequences of the limit in the proliferative capacity on both pathological situations and cell-mediated gene therapy are discussed.

Key words: satellite cell, proliferation, myogenic factors, telomeres.

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Muscle fibres are multinucleated elements responsible for the contractile properties of skeletal muscles. They contain post-mitotic nuclei which are actively transcribing the genes involved in muscle function and maintenance, but which can no longer divide [55]. Whenever a muscle fibre is damaged, its replacement is assumed by a population of cells situated under the basal lamina of the fibres called satellite cells [38, 56]. Satellite cells stay quiescent during the normal function of muscle and can be characterized by the expression of M-cadherin [8]. Satellite cells can be identified morphologically in the human fetus at 14 weeks of development [12] and are responsible for the subsequent pre- and post-natal growth of muscle [45, 46]. Following damage to muscle fibres, satellite cells will be activated, proliferate and then fuse to form new muscle fibres. Satellite cells are the only source of muscle cells in the adult [61], and this process of repair has been called regeneration [5]. Part of the satellite cell population will escape differentiation and restore the pool of quiescent cells under the basal lamina of the newly formed fibres. Although it has been shown that fibroblasts can, with a low probability activate a myogenic program under certain conditions [63, Cossu personal communication], satellite cells are the only cells which have been demonstrated to restore muscle fiber function under normal conditions in vivo.

Different aspects of both proliferation and differentiation of satellite cells have been extensively studied in vitro [21, 57, 65], as well as in vivo [2, 25, 26, 41]. In our laboratory we have shown that all human satellite cells coexpress in vitro fast and slow myosin heavy chains [19]. This result implies that in human skeletal muscle different classes of satellite cells corresponding to their fibre type of origin do not exist, which is in contrast to results obtained on avian [21] and murine [18, 53] muscles.

Participation in regeneration in vivo involves three steps for the satellite cells: (i) quiescent cells become activated, (ii) they proliferate and then (iii) differentiate. Many studies concerning these steps have been carried out in rodents [25, 26, 39]. It is clear that the control of each step during, regeneration must be tight since (i) satellite cells are not normally activated outside regeneration and (ii) some satellite cells are not committed to differentiation and restore the pool of quiescent cells. It has been shown that innervation does not play a crucial role in these early events of muscle regeneration [39].

Proliferation is therefore one of the key-steps in regeneration to provide a sufficient number of nuclei for the future muscle fibre. However, it is now well established that human diploid cells are limited in their proliferative capacity, as shown initially on fibroblasts [32] and more recently on satellite cells [16, 31]. During their lifespan, human cells will gradually replicate more slowly, until they reach the limit in proliferation. Once in this state they can no longer replicate and are described to be "senescent" [24]. One mechanism putatively involved in this process concerns the shortening of telomeric sequences. It has been clearly shown on numerous cell types that there is a regular loss of telomeric DNA at each cell division e.g. [11, 29, 30], due to the assymetric replication of DNA. The replicative lifespan of a human satellite cell population can therefore be followed by measuring its mean telomere length [16].

The possible extension of muscle cell lifespan by the expression of T antigen (Tag) from SV40 has been studied in rodents [28, 34, 44, 52] as well as in humans [33, 47], using contracts differing mainly in the elements used to control Tag expression. Its effect varies from straight immortalization in rodent cells [52] to limited extension in human fibroblasts [58], subsequently leading to another proliferative arrest, called in this case crisis. Whether senescence and crisis are distinct steps in the proliferative lifespan has been the subject of some controversy [54, 64]. It has been proposed that immortalization, which is the escape to the proliferative limit of human cells, could occur then by mutational events [59]. Such a limit is less conspicuous in the mouse, since the frequency of mutation leading to immortalization is much higher than in man ($\sim 10^4$ fold) [58].

The proliferative limit implies directly a limit for the regenerative capacity of human satellite cells, since once satellite cells are senescent they will not be able to proliferate, even though a signal of activation is triggered by muscle damage. Although the importance of this limit during the normal lifespan of individual is still unknown, it could be critical in situations where regeneration is strongly stimulated. This will be the case for diseases characterized by repeated cycles of degeneration and regeneration of muscle fibres, i.e. muscular dystrophy [17]. Blau et al [7, 62] already reported differences in vitro between the proliferative potential of satellite cells isolated from DMD patients and from normal subjects.

The proliferative limit of human cells will also have a direct consequence on their possible use in cell-mediated gene therapy. Since differentiated myonuclei within muscle fibres are post-mitotic, but can be replaced by nuclei from satellite cells during regeneration, skeletal muscle has been proposed as a target for satellite cell-mediated gene therapy [42, 49, 50]. However, this approach implies the isolation of satellite cells, an eventual modification of their genome, and their amplification before being introduced into the host. If the potential of proliferation of donor cells is not sufficient due either to an extensive amplification and/or to the age of the donor, the result will be the introduction into the host of senescent or nearly senescent cells, and might therefore lead to the failure of the therapy.

In this paper, we present results that we have obtained concerning the activation and proliferative potential of human satellite cells. In vivo, activated satellite cells were identified by the presence of the myogenic regulatory factor Myf5 both in human biopsy material and in a transgenie mouse where the regulatory sequences of the myf5 gene is linked to the lacz reporter gene [60]. Regeneration was induced in the mouse by injection of cardiotoxin. We have also extended their proliferative potential using Tag in a system where it is negatively regulated as differentiation proceeds. The consequences and limitations of such myogenic cell proliferation are discussed with respect to both pathological situations and cell-mediated gene therapy.

Material and Methods

Material

Quadriceps muscle biopsies were obtained after surgery or during autopsy, in accordance with the French legislation on ethical rules. The biopsies of normal muscle (see Table 1) showed no signs of neuromuscular disease, and one biopsy from Duchenne Muscular Dystrophy (DMD) was taken for diagnostic purposes from the quadriceps of a 3.5 year old boy and used for histochemical studies.

Regeneration in mouse muscle

Regeneration was induced in the hindlimb muscles of myf-5/LacZ mice [60] by injection of 750 µl of Cardiotoxin (Latoxan) and muscles were dissected 5 days after injection. The muscles were frozen in Tissue-Tek in cold isopentane. 5 µm sections were cut on a cryostat, and LacZ expression was revealed on mouse sections as described in Current Protocols in Molecular Biology [3].

Human cell cultures

Satellite cell populations were isolated from biopsies (table 1) obtained from young infants (5 days, 5.5 months), young adults (26, 28 years) and older adults (81 and 86 years) as described previously [16, 19]. Three days after the first mononucleated cells migrated out of the explants, explants were removed and the cells were trypsinized (trypsin-EDTA, GIBCO), collected by centrifugation and counted. All cultures were incubated at 37°C in a humid

air atmosphere containing 6% CO_2. Growth medium consister of HAM's F-10 supplemented with 50 μg/ml gentamycin and 20% FCS (GIBCO). At cell isolation, all cell populations were considered to be at 1 mean population doubling (MPD). At each cell passage, this number of MPDs was reevaluated according to the following formula:

$$MPD = \frac{\ln(\text{final number of cells} / \text{initial number of cells})}{\ln 2}$$

Cultures were considered senescent when they failed to reach sub-confluency after 3 weeks of refeeding [15].

Satellite cells were co-transfected as described previously [47] with one plasmid containing the large T antigen sequence (Hu-Pro-Vim), [52] and another containing the neomycin resistance gene directed by the RSV promoter (PRSV-Neo). Transfected cells were selected twice in the presence of 400 μg/ml of G418 (GIBCO).

Immunocytochemistry

Both single and double-immunolabelling were performed as described previously [19] using Vectastain kits (VECTA). Primary antibodies were diluted 1/100 for the anti-Ki67, a marker for proliferating cells expressed during part of G1, and all along G2, S and M phases of cell cycle (monoclonal, Novocastra), 1/800 for the anti myf-5 (polyclonal, a generous gift from M. Buckingham, Paris), 1/20 for the anti-slow myosin heavy chain (MHC), a marker of adult muscle differentiation [19] (monoclonal, Novocastra) or used pure for the antiT antigen (monoclonal) [47] or anti myogenin FD5, (monoclonal, a generous gift from W. Wright, Texas) [47]. The specificity of the anti-myf-5 antibody was confirmed by western blot analysis (data not shown). The mouse sections were counterstained with Hematoxylin.

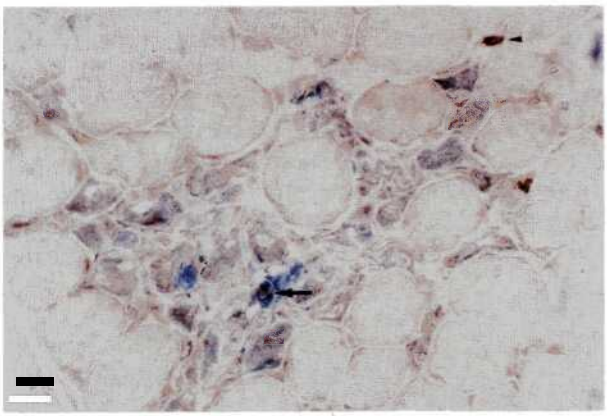
Analysis of telomere length

Telomere length analyses were performed as described previously [15]. Briefly, DNA samples were digested with the restriction enzyme HinfI, and 5 μg of digested genomic DNA was separated in 0.7% agarose gels. The gels were dried, denatured, neutralised and hybridized to a ^{32}P-(TTAGGG)4 probe as described by Allsopp et al 1992 [1]. Autoradiographs exposed within the linear range of the signal response were analysed by a computer-assisted system [16]. The mean length (in kbp) of telomere restriction fragments (TRF) was determined three times for each sample on three different gels.

Results

Myf5 is a marker of activated satellite cells

Activation of satellite cells cannot be easily detected since markers which are expressed in actively proliferating, as opposed to quiescent cells, reveal all proliferating cell types involved in muscle regeneration, including the ones which don't belong to the muscle lineage (e.g. macrophages or fibroblasts). Markers specific for the muscle compartment, such as desmin, will also be expressed in, adjacent myofibres as well as in smooth muscle cells in the blood vessels. We have found that myf-5, one of the myogenic factors, is expressed in activated satellite cells, but not in the nuclei of mature adjacent fibres. Figure 1A shows an example of a regenerating area in a biopsy taken from the quadriceps of a boy suffering from DMD, where myf-5 protein is detected in activated satellite cells, as revealed by an antibody conjugated to peroxidase (brown). The antigen Ki67, a marker of proliferating cells, is revealed using an antibody conjugated to phosphatase (blue). While proliferating cells (Ki67 +) can be observed in the regenerating area, only some of them express also myf-5, and are therefore actively proliferating satellite cells (see arrow on fig. 1A). Myf-5 expression can also be detected in activated satellite cells which are not in direct contact with a regenerating fibre (arrowheads). It should be noted



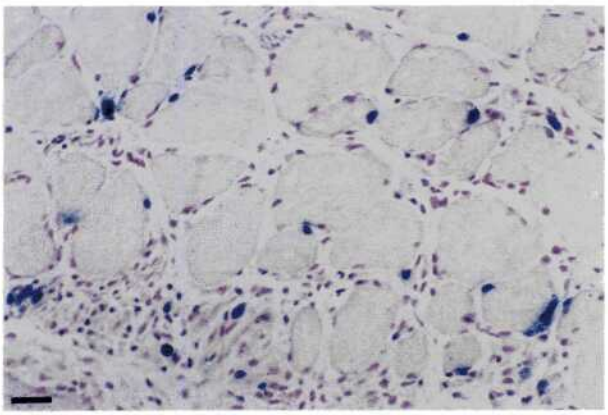


Figure 1. Detection of myf-5 in regenerating muscle using a specific polyclonal antibody. A: Section from a biopsy of a 3.5 year old DMD boy. Myf-5 is revealed by peroxidase (brown), while Ki67, a marker of cell proliferation, is revealed using phosphatase (blue). Arrow indicates a nuclei positive for both markers, while arrowhead indicates a nuclei positive for myf-5 only. bar = 10 μm. B: Section of a regenerating muscle from a 2 month old myf5/LacZ mouse 5 days after injeetion with cardiotoxin. Activation of the myf5 gene is revealed by the activity of β-galactosidase (blue). Nuclei are counterstained (pink) with hematoxylin. bar = 10 μm.

that these labelled cells do not always express Ki67. The expression of myf-5 in activated satellite cells was also confirmed in the mouse, using transgenic animals where one copy of the myf-5 gene has been replaced by LacZ. Fig. 1B shows the detection of myf-5 expression in the leg muscles of such mice after regeneration has been induced for 5 days, and where activated cells expressing the transgene can be seen at a distance from the degenerating area, situated at the bottom of the picture.

Lifespan in vitro of human satellite cells

The number of divisions that human satellite cells can make as a function of time was studied in culture. Figure 2 shows examples of this analysis: lifespan curves were obtained on three populations of satellite cells isolated from biopsies of respectively a child 5 days after birth, a 5.5 months old infant and an adult (28 year old). In all three cultures, the cell population divides rapidly in the initial phase. The division rate decreases slowly during the lifespan. Finally, the division rate reaches a plateau which represents the step when no more divisions are observed for each population. At this point of proliferative arrest, the populations have reached senescence. This is further confirmed since these populations could be maintained in vitro for several weeks without any cell division or cell death. Therefore, the nearer to senescence the cells are, the slower they will divide.

Although the overall shape of the three curves is similar, it is evident that the proliferation rate decreases more rapidly in the population isolated from the older subject, and that senescence is reached earlier, i.e. after having completed fewer cell divisions.

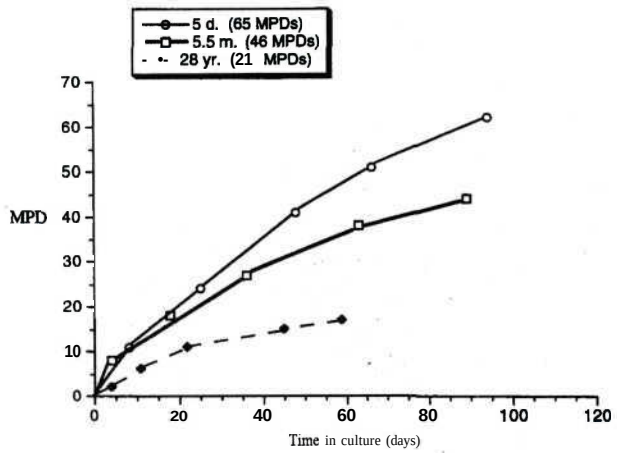


Figure 2. Lifespan curves of satellite cell populations isolated from human donors aged respectively 5 day post-natal (5d), 5.5 months (5.5 m) and 28 years (28 yr). The maximum number of divisions (as mean population doubling, MPD) made in culture for each age is indicated in brackets. The culture was considered to have reached senescence when no replication was observed during three weeks.

Proliferative potential of human satellite cells in vitro

Lifespan curves were established for human satellite cell populations isolated at different ages from normal subjects and the number of divisions at which proliferative arrest is reached was determined. Table 1 gives examples of the total number of divisions carried out by satellite cell populations from their isolation to the proliferative arrest which represents the mean number of divisions (mean population doubling or MPD) that these populations can achieve in vitro. From table 1, two phases in the proliferative potential of human satellite cells can be defined: the number of MPDs decreases rapidly during an initial phase, from birth to 26 years (see Table 1), and it then reaches a stable value at later ages.

The proliferative potential of human satellite cells can be extended

To artificially extend the lifespan of human proliferating satellite cells a construct was used which contained Tag, excluding small t, and placed under the control of the human vimentin promoter, since this promoter will be down-regulated once differentiation is triggered [51]. This construct was introduced by stable transfection into two distinct populations of human satellite cells, using a system of co-transfection with a neomysin resistance gene. One result of the effect of such a co-transfection is illustrated in Figure 3: the lifespan of a population of satellite cells isolated from a 5.5 month old infant was extended after transfection with Tag by 23 divisions. The replacement of growth factors contained in the serum by insulin and transferrin led to the differentiation of such transfected human satellite cells. The process of differentiation, although generally delayed by 48-72 hrs, occured with a similar time-course as in control cells, as could be illustrated using an antibody (FD5) specific for the myogenic regulatory factor myogenin. Figure 4 represents the percentage of nuclei positive for myogenin as a function of time in differentiation conditions. Note that the maximum percentage of fusion, i.e. the ratio of myogenin positive nuclei included in myotubes, was always slightly lower (45-50%) than in control experiments (60-65%). The decrease observed after the maximum corresponds to the down-regulation of myogenin after myotube formation.

Tag expression does not perturb the expression of embryonic and fetal isoforms of MHC in differentiated cells [data not shown, 47], but has a deleterious effect on adult

Table 1. Lifespan of human satellite cells isolated from biopsies of donors of different ages, ranging from 5 days (post-natal) to 86 years.

Donor age	5 d	5.5 m	26 yr	28 yr	81 yr	86 yr
n divisions	68	46	19	21	15	15

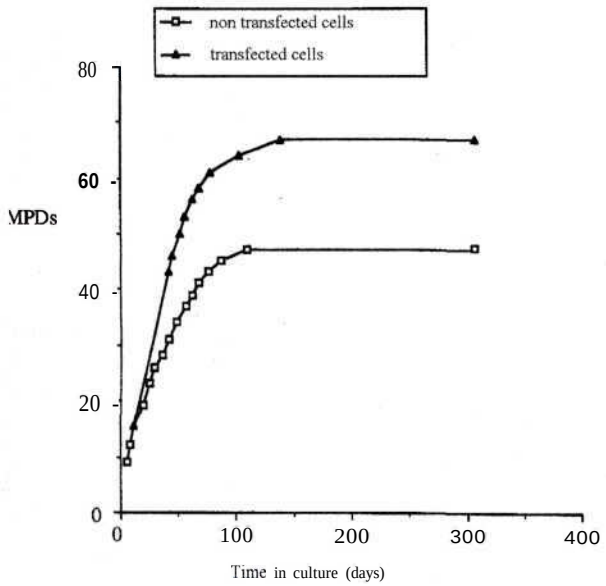


Figure 3. Lifespan curves (i.e. number of divisions made as a function of time in culture) of normal human satellite cells isolated from a biopsy of muscle of a 5.5 month old donor, as compared to the same population transfected with the Tag construct.

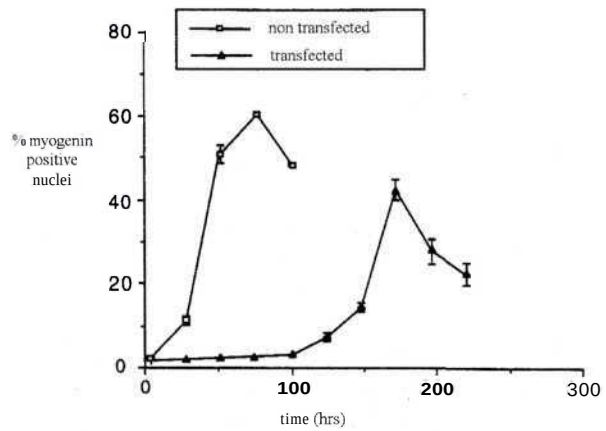


Figure 4. Percentage of myogenin positive nuclei during differentiation of satellite cells with or without transfection of Tag as a function of time of culture in differentiation conditions. The initial time-point (0) represents the time when medium was changed to trigger differentiation.

Myosin Heavy Chain isoform expression, as illustrated in Figure 5. Adult slow isoform is detected only in myotubes containing nuclei where Tag was no longer detectable, although most of the nuclei detected in figure 5 are myonuclei differentiated and included in myotubes, as can be revealed by the expression of embryonic and fetal MHC (data not shown). The detection of Tag in nuclei of multinucleated myotubes, which is evident on Figure 5, indicates clearly that Tag does not block muscle differentiation and fusion.

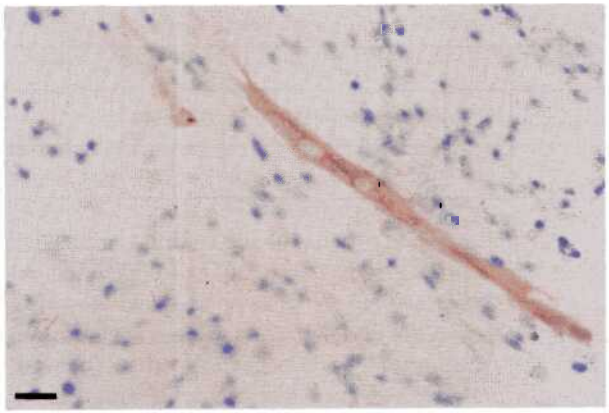


Figure 5. Double-immunolabelling of adult slow myosin heavy chain, revealed eroxidase (brown), and Tag expression revealed by phosphatase (blue). Although most of blue nuclei die within myotubes, note the absence of blue nuclei in the myotube positive for adult slow MHC. bar = 10 µm.

Telomere length analysis

Cellular DNA was prepared regularly during the lifespan of the population of satellite cells used for the transfection described in the former paragraph. The length of the telomeric restriction fragments (TRFs) was calculated using a probe specific for the telomeric repeats (see Material and Methods and fig. 6A). Since selection of neomycin resistant cells following low efficiency (0.5%) transfection implied further divisions to reach a critical number for DNA preparation, no DNA preparation could be made from transfected cells before their lifespan reached 44 divisions. It is clear from Figure 6 that TRF length decreases regularly (figure 6B) during the lifespan of an untransfected cell population, as we have previously shown [16]. However, the data presented in figure 6A show that the TRF length continues to decrease after Tag transfection. One should note that since transfection efficiency was low (0.5%), a lifespan of 44 divisions in culture was required before sufficient cells could be removed for DNA preparation without endangering the transfected populations.

Discussion

Muscle regeneration, like skeletal muscle development, is a complex process requiring both positive and negative regulation of a large number of genes. The repair, or regeneration, following damage of the muscle fibre is carried out by the satellite cells which will need to proceed through three distinct steps: activation which represents an exit from quiescence, proliferation to produce enough nuclei for the new fibre and differentiation by fusion of mononucleated cells to form myotubes and then myofibres. Extensive in vitro and in vivo studies, mainly in birds and rodents, have investigated the terminal events, while proliferation has been studied mainly in rodents [25, 26, 41, 57].

A

MPD	13	16	21	30	33	40	44	56	62
TRF normal	12190	11825	11060	10465	9550	9125	7850		
TRF transf.	ND	ND	ND	ND	ND	to	7510	7460	7160

B

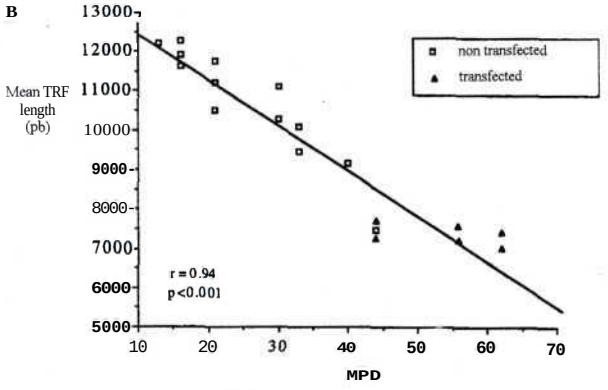


Figure 6. Analysis of Telomere Restriction Fragments (TRF) length in normal human satellite cells isolated from a biopsy of muscle of a 5.5 months old donor, as compared to the same population transfected with the Tag construct. A. Size in base pair of the TRF as a function of the number of Mean Population Doubling (MPD) made by the cells. B. Graphic representation of the change in TRF length as a function of the divisione made. The populations presented a total lifespan of 46 divisions (control non transfected) and 69 divisions (Tag transfected).

The myogenic factors were discovered a decade ago, and have been defined as a family of transcription factors comprising MyoD, Myf5, myogenin and MRF4 involved in regulating distinct steps during the embryonic development of skeletal muscle [9]. However little is known about the role of these myogenic factors during regeneration, and how their expression relates to that observed during development. Previous studies looking at MyoD and myogenin expression have confirmed that these factors were present both in replicating satellite cells and in newly regenerated muscle fibres [23, 27]. In this study, we have shown that myf-5, which is the earliest factor detected during development [48, reviewed in 37], is also the earliest factor to be expressed during regeneration. Our result show that Myf 5, in addition to being detected in proliferating satellite cells as shown by its co-localization with the proliferation marker Ki67, is also detected in satellite cells both on the edges of muscle fibres and in regenerating areas which are negative for Ki67. Ki67 is expressed throughout the cell cycle, and is absent from cells in the Go phase. We hypothesize that myf-5 is expressed in activated satellite cells, even prior to proliferation, a finding that is confirmed by our observation on the regenerating muscles of myf-5 transgenic mice. Myf-5 would then be a marker of activation of satellite cells. This is in agreement with the fact that myf-5 is the earliest myogenic marker during development [37]. It should be noted that this gene is transiently expressed during development in cells isolated from the neural tube and therefore located outside the muscle, compartment. Myf5 is then negatively regulated as soon as these cells become committed to a non-muscle fate [60], which argues in favour of an expression even before cells are irreversibly determined.

The next step in regeneration for an activated satellite cell is proliferation. Many autoradiographic studies have concerned the onset of proliferation in rodents [25, 26, 39]. We have isolated and evaluated in vitro the proliferative potential of human satellite cells by measuring the number of divisions these cells could make before proliferative arrest was reached. Although this technique does not necessarily reflect exactly what would actually happen in vivo, it can be analysed in comparison for a large number of populations. Since all cultures are maintained and passaged in exactly the same conditions, the comparison of the mean number of divisions we measure in vitro reflects differences in the proliferative potential of the initial populations. A model which has been widely studied since the first finding of Hayflick [32] is that all human cells have a putative number of divisions, they can make before reaching senescence, which is represented by their proliferative potential. Since it has also been shown in fibroblasts that there is a regular loss of telomeric DNA at each cell division [1], which can be measured by analysing the decrease in size of the TRFs during in vitro proliferation until senescence, this measurement provides an additional way to follow the proliferative lifespan of a cell population. It should be pointed out that the onset of proliferation during regeneration in mice has been shown to be delayed and reduced in older animals [40, 41], although some changes in the environment probably participate to the degradation of the regenerative potential, as proposed in rats [10].

When this approach was applied to the study of human satellite cells, we did not observe a regular loss of proliferative potential with age, as has been reported for skin fibroblasts [32]. Instead we found that there was a rapid loss of proliferative capacity during the first two decades (i.e. from 68 divisions at birth to 19 divisions at 26 years, see table 1), but that after this age the number of cell divisions remained relatively constant with all of the cultures isolated from adult muscle, even from the older subjects. During the first two decades, this corresponds to a massive growth of muscle, during which satellite cells will proliferate extensively to provide additional nuclei to the growing muscle. These results extend and confirm the initial observation made by Haushka [31]. The fact that the proliferative potential does not change in the adult can be explained by the fact that regeneration is probably a rare and locally restricted event. Since we are measuring a mean number of divisions in a population isolated from a small

biopsy, our method probably does not allow us to detect the small amount of divisions caused by eventual local regeneration in human. However, it shows a massive loss in the proliferative potential of satellite cells during muscle growth. This analysis also points out that the proliferative potential, which is directly linked to the capacity to regenerate, remains relatively constant even in the older subjects.

We can also predict that the situation will be different if regeneration is strongly stimulated, such as in muscular dystrophies. If regeneration gives rise to fibres which will later degenerate, as in DMD, satellite cells will be constantly stimulated to proliferate. One can then predict that the proliferative potential of satellite cells will rapidly decrease. Figure 2 clearly shows that the more divisions the cell has gone through, the longer the cell will take to divide. Therefore, DMD satellite cells will take longer and longer to divide as they approach senescence, will fuse less well (data not shown), and therefore a direct consequence of this will be the occurence of an abortive regeneration which will become more frequent with age. This prediction corresponds actually with what has been described by clinicians [17], a fact that could not be deduced from the lack of dystrophin, which is the genetic cause of the disease [43]. It also corresponds to observations of defective myoblasts in DMD [7, 62]. It should be noted that the failure of regeneration would leave a space where the degenerated fibre was. When satellite cells become senescent and are no longer able to proliferate and fill this space with regenerated fibres, it will probably be filled by fibrous tissue. Therefore fibrosis could be a direct consequence of abortive regeneration.

Another field where the proliferative potential of satellite cells needs to be taken into account is cell mediated gene therapy [42, 49, 50]. Skeletal muscle has been proposed as a target for reintroducing missing genes since, once the donor satellite cells are injected, they will either be included in host muscle fibres which are stable, or become quiescent [66]. In practice two alternatives can be considered: Strategy 1: the missing gene is reintroduced via the satellite cells from the patient. In this case satellite cells must first be isolated, the missing gene has to be reintroduced and, after selection, the recombinant cells are injected back in the muscles. This strategy implies two steps of amplification of the population: the first one after isolation and the second one during selection. Strategy 2: Satellite cells are isolated from a normal donor, and are injected in the muscles of the patient to provide copies of the missing gene. There is then only one stage of amplification after isolation. However, donor's age has to be considered. Our results imply that satellite cells must have a sufficient proliferative potential to go through all these steps of amplification without becoming senescent. In a recent trial when normal myoblasts were transplanted into muscles of DMD boys [35], using strategy 2, most of the injected donor cells were isolated from muscle biopsies taken from adult parents. This trial gave only negative results, but we calculated by comparing our data to those given by the authors that the injected cells were either senescent or very near to it. The only case where some dystrophin was detected was when the injected cells were isolated from a young infant [35]. Moreover it was recently shown that during injections of myoblasts in the muscles of mdxmice, there was a massive cell death in the injected population, which they estimated to concern about 99% of the population [4, 20]. It was shown that the remaining cells proliferate in vivo to repopulate the regenerating muscle of the host [Beauchamp, London, personal communication]. Although irradiation could somewhat emphasize the cell-death after injection, our general conclusion is that the injected donor cells must retain a sufficient proliferative potential in order to improve the efficiency of such cell-mediated gene therapy.

The proliferative potential of human cells can be extended by the expression of Tag [33, 47]. We have used the human vimentin promoter to control Tag expression, since vimentin is down-regulated as differentiation proceeds [51]. Our prediction was that if Tag does not block muscle differentiation it will be gradually down-regulated. Indeed, our results show that Tag does not block differentiation, since we could detect Tag in nuclei situated in myotubes. We also observed that Tag expression was repressed while differentiation proceeded in our cultures [47]. Tag has a negative effect on adult MHC expression, since adult fast and slow MHCs were not expressed in vitro if myotubes contained Tag positive nuclei (see figure 5 and ref 47], but in vivo maturation of the regenerated fibre containing transfected nuclei should enhance the down-regulation of the vimentin promoter driving Tag expression, since vimentin is absent in adult mature muscle.

Although Tag increases the proliferative potential by 23 divisions in our experiments, it does not immortalise human satellite cells. This is in contrast to immortalisation observed with murine cells [52], but is in good agreement with what has been previously observed on other human cell types [58]. Immortalisation of human cells is thought to arise by mutational events [59] which would appear with a low frequency ($1.5\ 10^{-7}$) whereas the frequency of mutation is much higher in the laboratory mice (estimated 10^{-4}) [58]. The telomere analysis confirms that these human cells were not immortalised since telomeres continue to shorten in transfected cells [1, unpublished results from our group]. It has been proposed recently that immortalisation results in the expression of an enzyme, called telomerase, which lengthens telomeres [13, 14, 36]. Cells which are immortalised have telomeres with a constant length, the telomerase adding back DNA at each cell division. Expression of the telomerase would be favoured by short telomeric length. It has also been shown that the repression of telomerase in the immortal human cell line HeLa resulted in its senescence after few divisions [22]. We conclude from our experiments that Tag will not by itself be sufficient to produce immortalised human muscle cell lines. Moreover since immortalisation happens, even with

a low frequency, once telomeres are shortened, one should be aware of the possible effects of such a process. Telomeric sequences have several putative roles, one of which is to prevent chromosomic rearrangments such as fusion. Shortened telomeres could therefore lead to immortalisation but also to chromosomal abnormalities. These problems should be seriously taken into account before using immortalised muscle cells in experimental or therapeutic trials, in addition of course to the problem of immunogenicity of the donor cells.

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