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Cellular senescence in human myoblasts is overcome by human telomerase reverse transcriptase and cyclin-dependent kinase 4: consequences in aging muscle and therapeutic strategies for muscular dystrophies

Chun-Hong Zhu,¹ Vincent Mouly,² Racquel N. Cooper,² Kamel Mamchaoui,² Anne Bigot,² Jerry W. Shay,¹ James P. Di Santo,³ Gillian S. Butler-Brown² and Woodring E. Wright¹

¹Department of Cell Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX, USA

²UMR S 787 (INSERM & UPMC), Institut de Myologie, Faculté de Médecine Pierre et Marie Curie, 105 bd de l'Hôpital, 75634 Paris Cedex 13, France

³Unité des Cytokines et Développement Lymphoïde, INSERM Unité 668, Institut Pasteur, 25 Rue du Docteur Roux, Cedex 15 Paris, France

Summary

Cultured human myoblasts fail to immortalize following the introduction of telomerase. The availability of an immortalization protocol for normal human myoblasts would allow one to isolate cellular models from various neuromuscular diseases, thus opening the possibility to develop and test novel therapeutic strategies. The parameters limiting the efficacy of myoblast transfer therapy (MTT) could be assessed in such models. Finally, the presence of an unlimited number of cell divisions, and thus the ability to clone cells after experimental manipulations, reduces the risks of insertional mutagenesis by many orders of magnitude. This opportunity for genetic modification provides an approach for creating a universal donor that has been altered to be more therapeutically useful than its normal counterpart. It can be engineered to function under conditions of chronic damage (which are very different than the massive regeneration conditions that recapitulate normal development), and to overcome the biological problems such as cell death and failure to proliferate and migrate that limit current MTT strategies. We describe here the production and characterization of a human myogenic cell line, LHCN-

M2, that has overcome replicative aging due to the expression of telomerase and cyclin-dependent kinase 4. We demonstrate that it functions as well as young myoblasts in xenotransplant experiments in immunocompromised mice under conditions of regeneration following muscle damage.

Key words: hTERT; muscular dystrophy; myopathies; p16; replicative lifespan; replicative senescence; satellite stem cell; telomerase.

Introduction

Aging muscle is characterized by progressive muscle weakness and atrophy (Shavlakadze & Grounds, 2006) as well as by a slower regenerative capacity (Mouly *et al.*, 2005a). At least two factors may be linked to the regenerative capacity of skeletal muscle: (i) the number of progenitor cells, for example satellite cells, which decreases with age in both rodents and humans, and (ii) their proliferative potential, which is limited in humans by cellular senescence, triggered by excessive telomere shortening (Renault *et al.*, 2002; Wright & Shay, 2002). The proliferative potential of human satellite cells decreases during postnatal muscle growth and then is stabilized in adults (Decary *et al.*, 1997), unless degenerative diseases provoke extensive replication of these cells (Decary *et al.*, 2000). The muscular dystrophies are a heterogeneous group of heritable disorders in which progressive muscle degeneration provokes an eventual decrease in muscle mass and extensive fibrosis. Muscle degeneration results in turnover of myonuclei, thus continuously mobilizing satellite cells. Muscle regenerative capacity eventually declines due to the replicative senescence of the satellite cells that proliferate during muscle repair (Decary *et al.*, 2000).

Aging in skeletal muscle can be counteracted by exercise, which increases the number of satellite cells available on muscle fibres (Thornell *et al.*, 2003). However, the consequences of this increase on the future regenerative capacity of skeletal muscle in the elderly population needs further investigation, as it may decrease their proliferative potential (Renault *et al.*, 2002). Numerous mouse models bearing mutations similar to those described in human muscular dystrophies have been employed to develop myoblast transfer therapy (MTT), using satellite cell replacement by either autologous modified myoblasts or allogenic nonmutant myoblasts. Since the pioneering studies of Partridge and co-workers in the mouse, many improvements have been achieved in MTT, thus raising the hopes of patients

Correspondence

Woodring E. Wright, Department of Cell Biology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75390-9039, USA. Tel.: 214 648-2933; fax: 214 648-8694; e-mail: woodring.wright@utsouthwestern.edu

Chun-Hong Zhu and Vincent Mouly contributed equally to this paper.

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suffering from muscular dystrophies (Partridge *et al.*, 1989; Quenneville & Tremblay, 2006). However, clinical trials based on successful animal studies were ineffective, showing little, if any, therapeutic benefit (Karpati *et al.*, 1993; Tremblay *et al.*, 1993; Mendell *et al.*, 1995). In addition to obstacles such as the immune response that limit the success of myoblast transplantation, an important aspect that needs to be considered is the fundamental difference in behavior between mouse and human satellite cells with respect to the proliferative limits of replicative aging.

In the mouse, only a small minority of myoblasts survive following injection (Beauchamp *et al.*, 1999), which then have to proliferate extensively in order to participate in host regeneration. If these results are confirmed with human cells, the limited proliferative capacity of human satellite cells will be an obstacle to their use in MTT. This is particularly important in light of results demonstrating an inverse relationship between the replicative potential *in vitro* of human satellite cells and donor age (Decary *et al.*, 1997). Furthermore, we have recently shown that prolonged amplification of satellite cells in culture and the resulting approach of these cells to the stage of replicative senescence are important factors that may reduce the success of myoblast transplantation (Cooper *et al.*, 2003). Finally, limitation of the proliferative capacity of satellite cells by cellular senescence restricts the development of *in vitro* models to study disease processes and to develop therapeutic strategies. Much effort has been invested into extending the proliferative potential of human satellite cells by overcoming cell senescence in order to (i) increase the success of myoblast transplantation protocols, and (ii) develop *in vitro* models of neuromuscular diseases to test new therapeutic strategies.

Telomeres are specialized structures at the ends of linear eukaryotic chromosomes that help maintain chromosomal integrity (Blackburn, 1990). Progressive telomere shortening, a consequence of the 'end-replication problem', leads to replicative senescence. The regulation or maintenance of telomere length in the germline, in stem cells as well as in immortal cells, involves the action of telomerase. Telomerase is a ribonucleoprotein enzyme complex composed of an RNA component that provides the template for telomere repeat synthesis and a human telomerase reverse transcriptase (hTERT) catalytic subunit (Shay & Wright, 2004). The introduction of the cDNA coding for hTERT leads to telomere elongation and extended lifespan in a number of cell types including fibroblasts, retinal pigment cells and endothelial cells (Bodnar *et al.*, 1998; Vaziri & Benchinol, 1998). Telomerase is not sufficient to immortalize cells that stop dividing due to reasons unrelated to telomere shortening, such as growth conditions that for one reason or another are insufficient to support long-term proliferation (Ramirez *et al.*, 2001), and human myoblasts cultured under current protocols fall into this category (Di Donna *et al.*, 2003). Use of the Simian vacuolating virus 40 (SV40) large T antigen (TAG) can result in myoblasts bypassing some of the checkpoint activities induced by inadequate growth conditions. The introduction of the telomerase gene in Duchenne muscular

dystrophy (DMD) myoblasts previously transformed by SV40 large TAG considerably extends their lifespan (Seigneurin-Venin *et al.*, 2000) but still does not lead to immortalization. The expression of TAG from SV40 produces chromosomal rearrangements, delays and reduces cellular fusion and interferes with expression of the adult myogenic program (Mouly *et al.*, 1996). Recently, it has been demonstrated that human bronchial epithelial cells can be successfully and reproducibly immortalized by the expression of two genes, hTERT and cyclin-dependent kinase 4 (cdk4) (Ramirez *et al.*, 2004). These results indicate that there is another pathway leading to proliferative arrest, which is linked to cellular stress, such as that represented by inadequate culture conditions. The overexpression of cdk4 overcomes the p16-mediated stress response, thereby preventing this premature growth arrest. A similar approach, using a combination of hTERT and Bmi-1 expression, has resulted in the immortalization of human satellite cells (Cudré-Mauroux *et al.*, 2003). However, although this approach resulted in the isolation of an immortalized clone from a DMD biopsy that was able to form myotubes *in vitro*, the immortalized clones isolated from a control biopsy never showed any potential to differentiate, suggesting that immortalization of human myoblasts might still require secondary changes in these conditions.

The aim of this study was to create a reliable model of immortalized normal human myoblasts that exhibit normal differentiation. Such a cell line has never been isolated without the use of oncogenes (Hashimoto *et al.*, 2006), and the possibility of immortalizing human myoblasts would represent a powerful tool to test new therapeutic strategies for a number of skeletal muscle defects. In order to achieve this, we both optimized culture conditions and expressed hTERT in combination with cdk4, avoiding the use of oncoproteins. The isolation of such a cell line, a human equivalent of lines that exist for mouse (C2) and rat (L6), should open new avenues of research in fields as varied as aging, development and diseases, and facilitate the development of therapeutic strategies for skeletal muscle. We describe in this report the characterization of these immortalized cells *in vitro* and their regenerative capacity *in vivo* following injection into an immunocompromized mouse model.

Results

Immortalization of human skeletal myoblasts

The expression of the telomerase catalytic subunit hTERT in normal human myoblasts was sufficient to yield telomerase activity and elongate telomeres, but it failed to produce immortalization (Di Donna *et al.*, 2003). This behaviour is characteristic of cells grown under conditions inadequate for their long-term proliferation, where stress/damage checkpoints are induced before telomere shortening becomes limiting (Sherr & DePinho, 2000; Wright & Shay, 2000; Ramirez *et al.*, 2001; Forsyth *et al.*, 2003). A large number of conditions were examined for their ability to increase growth rates or to extend the lifespan of adult human satellite cells. Negative results were obtained using

myotube-conditioned medium (from either C2C12 mouse cultures or PD6 human myoblasts) (Mezzogiorno *et al.*, 1993), erythropoietin (Ogilvie *et al.*, 2000), leukemia inhibitory factor (LIF) (Kurek *et al.*, 1996; Schoser *et al.*, 1998), nerve growth factor (Seidl *et al.*, 1998), insulin/insulin-like growth factor 1 (Quinn & Haugk, 1996; Barton-Davis *et al.*, 1998; Jacquemin *et al.*, 2004), or media such as Ham's F10 (Blau *et al.*, 1983), MCDB 110 or MCDB 201. Only dexamethasone (Giorgino & Smith, 1995) and hepatocyte growth factor (HGF) (Anastasi *et al.*, 1997; Gal-Levi *et al.*, 1998) gave positive results. A combination of dexamethasone and HGF extended proliferative capacity by about 70% (Fig. 1a).

We have previously shown that expressing wild-type cdk4 to sequester the increased p16 that often accompanies inadequate growth conditions can permit hTERT to immortalize a variety of cell types (Ramirez *et al.*, 2003, 2004). Expression of hTERT produced a modest extension of lifespan in adult human myoblasts, which was further extended in cells also infected with a retrovirus expressing cdk4 (Fig. 1b). The transduction of cdk4 alone increased the lifespan of the cells by 50%. Telomere length in these cells shortened until they reached 6.1 kb, a value very similar to that usually observed in senescent fibroblasts, while the telomere length in control myoblasts after they stopped dividing was 7.7 kb (Fig. 1d). We did not find significantly increased p16 levels in control myoblasts, while cdk4 expression produced a compensatory increase in p16 as observed previously (Ramirez *et al.*, 2003, 2004) (Fig. 1e, normalized to the expression of the nuclear protein emerin). The increase in lifespan caused by the expression of both hTERT and cdk4 was found to be variable in other strains of human myoblasts, and clones isolated under these conditions displayed little, if any, myogenic differentiation (data not shown). These results are consistent with the failure of hTERT plus Bmi-1 (which represses p16 expression) to immortalize normal myogenic cells (Cudré-Mauroux *et al.*, 2003). However, combining the expression of hTERT and cdk4 with optimized culture conditions (dexamethasone and HGF) resulted in cell proliferation for at least 200 population doublings (PD) (Fig. 1c). Analysis of 20 metaphase spreads at PD 108 showed that the cells maintained a normal 46 XY diploid karyotype (data not shown). No tumors were ever found after injection of these immortalized cells into nude mice (4-month observation).

These adult human myoblasts were designated HSKM5 (Human Skeletal Muscle isolate no. 5). The immortalized cells were renamed LHCN in order to keep track of the factors and selectable markers they contained (lox-hTERT hygromycin + cdk4-neo). They were plated at low density at PD 52, and a clone exhibiting good myotube formation after exposure to differentiating conditions was isolated ('LHCN-M2' for LHCN + myogenic clone no. 2). This clone also exhibited a normal diploid karyotype.

In vitro characterization of immortalized cells

The myogenicity of the mortal parental population, the immortalized population and the immortalized clone as monitored by desmin expression was 47, 53 and 83%, respectively (Fig. 2a,g,m).

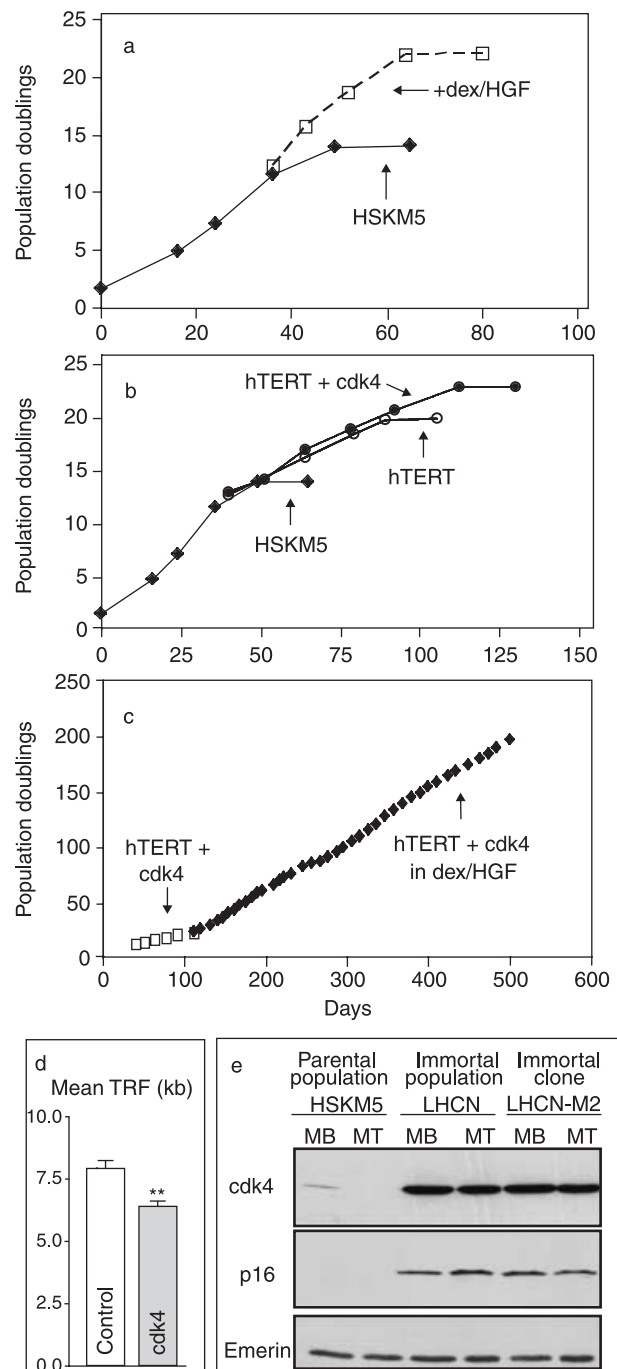


Fig. 1 Proliferative potential, telomere length and p16 expression. (a–c) Lifespan plots of the parental population (a) with or without HGF and dexamethasone, (b) transduced with human telomerase reverse transcriptase (hTERT) and/or cyclin-dependent kinase 4 (cdk4), and (c) transduced with both hTERT and cdk4 with or without HGF and dexamethasone. Immortalization was only obtained by the combination of hTERT, cdk4, HGF and dexamethasone. (d) Mean length of telomere restriction fragments (TRF) measured on cultures at proliferative arrest with or without transduction with cdk4. (e) Western blot analyses of the level of expression of p16 and cdk4 in the parental population (HSKM5, Human Skeletal Muscle isolate no. 5), cells immortalized by transduction with both hTERT and cdk4 (LHCN), and in the immortalized clone selected from LHCN (LHCN-M2). The results are compared to the expression of the nuclear protein emerin.

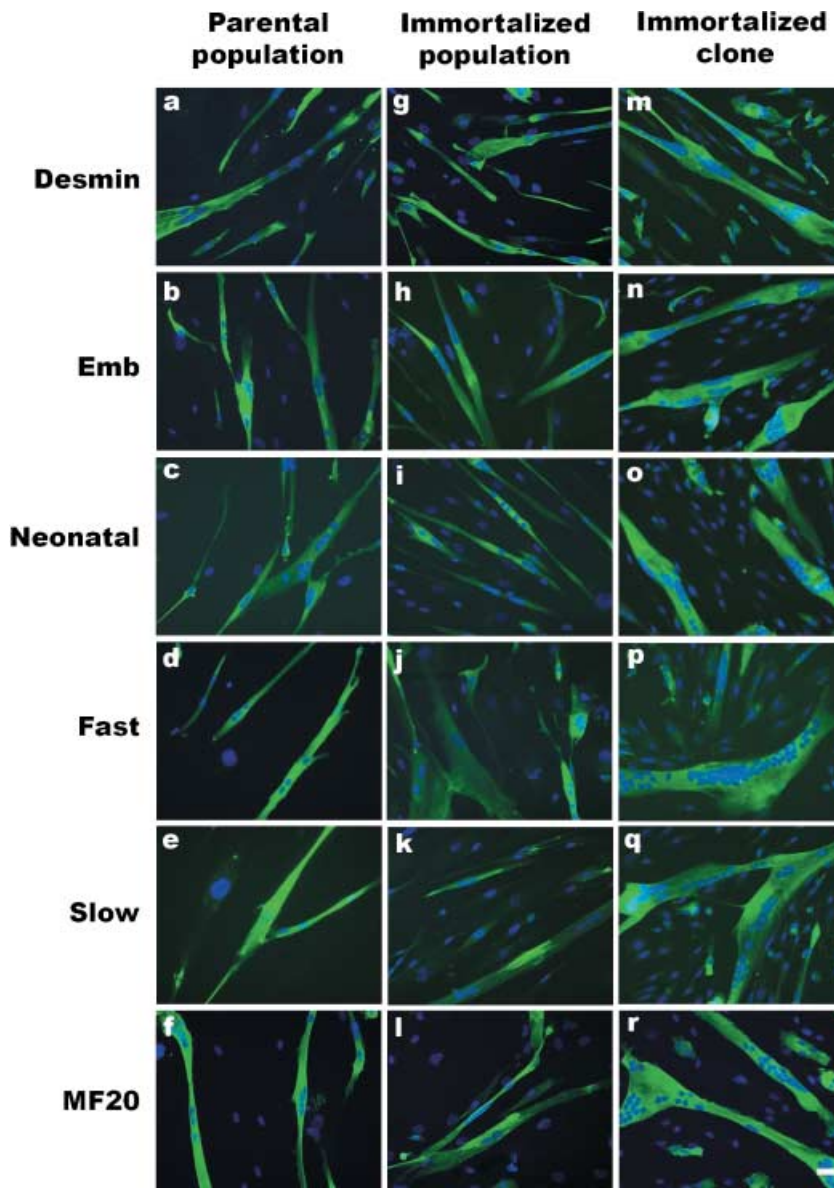


Fig. 2 *In vitro* characterization of parental and immortalized cells. Immunofluorescence of the parental population (a–f; PD 12), the immortalized population (g–l; PD 62) and the immortalized clone (m–r; PD 127) with an antibody directed against desmin (a, g, and m) and antibodies specific for embryonic, neonatal, fast, slow and all skeletal muscle MyHCs (b–r). Specific antibody labeling was revealed using Alexa fluor 488 green fluorochrome; nuclei were visualized with Hoechst (blue). Expression was evaluated on Day 7 of differentiation. Scale bar = 15 μ m.

The myogenic lineage markers MyoD and Pax7 were expressed equivalently in all three groups (data not shown). In order to establish whether the expression of telomerase affected the myogenic differentiation program, as was observed for TAG, we stained differentiated cultures with antibodies specific for different myosin heavy chain (MyHC) isoforms. MF20, an antibody recognizing all skeletal muscle MyHCs, showed that all myotubes express MyHCs (Fig. 2f,l,r). Embryonic, neonatal, fast and slow isoforms were all expressed in the differentiated myotubes of immortalized cells (Fig. 2h–k,n–q). The pattern of expression was very similar to that found in the mortal parental population (Fig. 2b–e). Although the pattern of expression was identical in all cell groups, myotubes from the immortalized clone specifically selected for efficient differentiation were bigger and contained more nuclei than the parental or immortalized populations.

In vivo muscle formation using immortalized cells

To determine the *in vivo* regenerative capacity of these immortalized cells, they were injected into cryodamaged tibialis anterior (TA) muscles of RAG2^{-/-} γ C^{-/-}JC5^{-/-} mice as described previously (Cooper *et al.*, 2001, 2003). Transplanted cells were identified by antibodies specific for human lamin A/C (expressed in all human nuclei) and human spectrin (expressed in differentiated fibers). Figure 3 shows the presence of large mature muscle fibers containing human spectrin in grafts from both the immortalized population and the immortalized clone (Fig. 3a). The total number and distribution of fibers positive for human spectrin was determined as a function of the distance from the proximal end of the injected TA. The mortal population produced significantly (30 ± 9 , $P < 0.01$) fewer fibers expressing human spectrin than the immortalized cells at 6 weeks post-

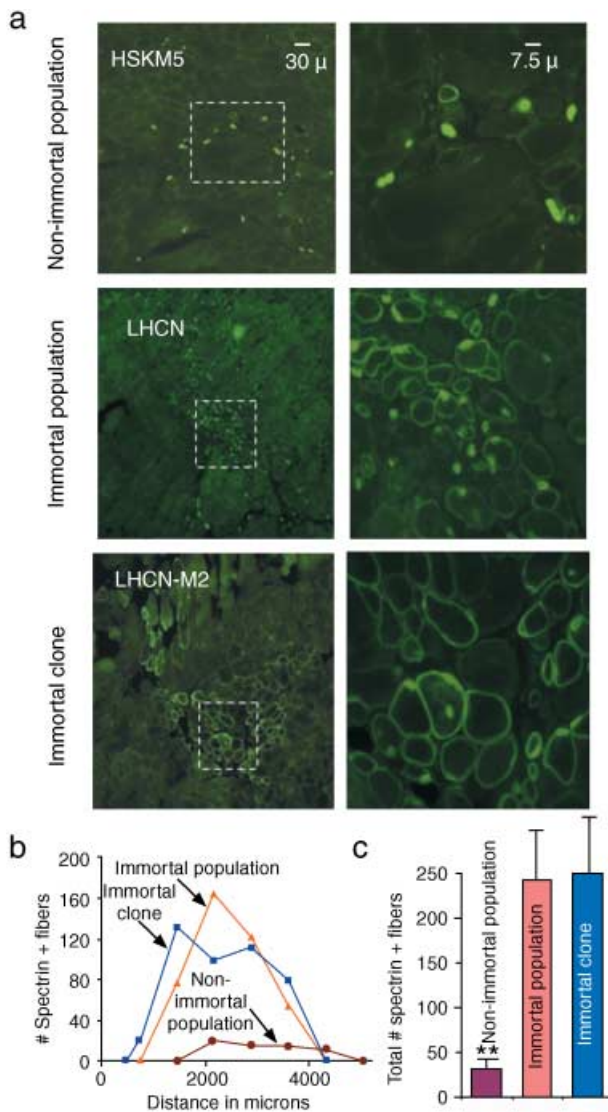


Fig. 3 *In vivo* muscle formation using immortalized cells. Tibialis anterior (TA) muscles containing implanted myoblasts (the parental population HSKM5 was injected at PD 14, the immortal population LHCN at PD 63 and the immortalized clone LHCN-M2 at PD 114) were dissected at 6 weeks post-implantation. Double immunofluorescence labeling with human-specific antibodies against spectrin and lamin A/C was performed on 5 μm transverse sections. These are both mouse monoclonal antibodies but because of their different cellular localization they could both be revealed using the same Alexa fluor 488 green fluorochrome. Pictures in the right-hand column represent higher magnifications of the outlined areas in pictures from the left hand column. Scale bars = 30 μm (left column) and 10 μm (right column). The graph (b) demonstrates the distribution of injected cells in the host muscles 6 weeks after injection. The histogram (c) shows the number of spectrin-positive fibers 6 weeks after myoblast transplantation. Values represent the mean ± SEM. $n = 6$ for each cell type. $**P < 0.01$.

transplantation, with no significant difference between the immortalized population and the immortalized clone (239.8 ± 46.40 and 245.7 ± 59.13 , respectively) (Fig. 3b,c). The number of fibers obtained with either the immortalized population LHCN or the clone LHCN-M2 is similar to that obtained previously in separate experiments with myoblasts isolated from a

newborn control subject (Cooper *et al.*, 2003). Again, no tumors were ever observed in this immunodeficient environment. The ability of the immortalized cells to proliferate and fuse efficiently to regenerate mature muscle in an *in vivo* environment without forming tumors suggests that they represent an excellent model system for the study of human muscle cells.

Discussion

Rodent muscle cell lines, such as C2 or L6, have an unlimited proliferative potential and have been a useful tool for the study of the cellular and molecular mechanisms involved in myogenesis. Mouse models have also been used to assess various therapeutic strategies, including MTT. However, the encouraging results obtained by grafting murine cells into the mdx mutant mouse (Partridge *et al.*, 1989), which carries a mutation in the dystrophin gene and is employed as a model for DMD, led to several unsuccessful MTT clinical trials in children with DMD (Karpati *et al.*, 1993; Tremblay *et al.*, 1993; Mendell *et al.*, 1995). One reason for this difference is that most muscles in the mdx mouse undergo only a limited number of cycles of degeneration and regeneration that do not lead to extensive fibrosis as in the human disease. In addition, most of the mouse studies have tested the ability of myoblasts to participate in muscle regeneration following the induction of massive damage to the host muscle. This destruction provokes a regenerative response that recapitulates normal development, and it is not surprising that cells that have been evolutionarily selected to function during development perform well under these conditions. However, it is extremely unlikely that a therapeutic strategy in dystrophic patients would involve the deliberate destruction of the surviving muscle fibers, and it is also unclear whether such destruction in the context of a fibrotic muscle would produce the conditions that recapitulate a developmental regenerative response. The success achieved with injections every 1–2 mm suggests that some regeneration can occur in fibrotic muscle following extensive local damage from multiple needle tracks (Skuk *et al.*, 2006, 2004, 2007).

This report describes the production and characterization of an immortal human myoblast cell line. We demonstrate that it functions as well as the best human primary cultures in the standard assay following the induction of muscle damage in the mouse model. This will prove to be a valuable reagent that can be shared between laboratories, and this approach can be applied to the production of immortal lines from a variety of different human myopathies. However, we believe that the most important implications of immortalizing human myoblasts that retain a normal karyotype and differentiate normally will lie in the ability to genetically engineer them to function therapeutically in a human disease setting.

Insertional mutagenesis is a major concern following the use of retroviral or lentiviral vectors. Even if the frequency of adverse events is low, for example one in a million, risks are significant if one infects 100 million cells. An underappreciated fact, however, is that the simple isolation of a single clone following

infection suffices to reduce the number of insertions to one, thus reducing the risk by a factor of 10^6 – 10^8 . An immortalized normal muscle cell strain with an unlimited proliferative capacity provides enough divisions to isolate such a clone. Furthermore, polymerase chain reaction can be used to isolate short sequences flanking the insertion site to verify an innocuous location, and the clone can be thoroughly characterized prior to therapeutic use. The immortal nature of the cells also allows a series of engineering events to be layered together, as the risks from even ten insertion sites are low and the cells can be cloned successively or cloned after the manipulations have been carried out and characterized. Conditional proliferation-dependent suicide agents like herpes virus thymidine kinase can also be introduced as an additional safety factor in the unlikely event that adverse oncogenic events occur.

Many challenges have already been identified in MTT, including the poor survival of the implanted cells within the first few days of injection and their failure to migrate from the site of the injection. Although they can participate in the initial myogenesis provoked by damage produced by the needle track, they largely fail to proliferate thereafter (Skuk & Tremblay, 2003; Mouly *et al.*, 2005b). These issues can be addressed in immortalized cells in order to create a cellular reagent that is more therapeutically useful for the effective treatment of human disease than its normal primary culture counterpart.

Another important consequence of immortalized normal human myoblasts is the potential for producing a universal donor. Current cell therapy approaches involve the isolation of autologous myogenic precursors such as mesoangioblasts (Sampaolesi *et al.*, 2003, 2006) from each patient or a histocompatible donor, expanding them briefly in culture to avoid senescence and then giving them back to the patient. These approaches may produce dramatic therapeutic responses without immortalized cells. However, a realistic assessment of the expense of doing this for each patient strongly suggests that this will never become a widespread therapy. Techniques for producing somatic cell knockouts of genes in human cells have been advancing rapidly (Porteus & Baltimore, 2003). Although one would need to verify that major and minor histocompatibility antigens do not participate in myogenesis, there is no evidence to date suggesting their involvement. As discussed above, immortalized normal human myoblasts have a sufficient number of divisions to enable the successive elimination of the major transplantation antigens, and the inclusion of a variety of safety control mechanisms should largely avoid problems of the immune system's failure to recognize these cells if they malfunction. Nonclassical class I molecules such as human leucocyte antigen (HLA) E or G can be expressed that block natural killer cell action and induce tolerance (Carosella *et al.*, 2001; Wiendl *et al.*, 2003). We believe that the most profound consequence of the present study lies in the creation of a normal human myoblast for the development of a universal donor engineered to function therapeutically in human patients.

Isolating an immortal human myoblast cell line with as few perturbations as possible has been the goal of many research

programs. T antigen, an oncogene encoded by the SV40 virus, has been extensively used under the control of various promoters, including those downregulated by differentiation such as the vimentin promoter (Mouly *et al.*, 1996). However, TAG has been shown to profoundly perturb muscle differentiation, for example by inhibiting the expression of adult MyHC. Thus, even though TAG can block the checkpoint responses induced by inadequate culture conditions, immortalizing such cells by the addition of telomerase would not produce a cell with normal function.

In this report, we show that the transduction of cdk4 to overcome inadequate culture conditions results in an increase in lifespan and a decrease in telomere length from 7.7 to 6.1 kb. This strongly suggests that human myoblasts cultivated *in vitro* stop dividing prematurely, that is before reaching critical telomere shortening. However, it is clear that not all adverse aspects of the initial culture conditions were overcome by cdk4, as telomerase still failed to immortalize these cells. Immortalization was only observed when telomerase and cdk4 expression was combined with HGF and dexamethasone.

The double transduction of cells in the absence of dexamethasone and HGF produced rare clones that were immortalized, did express myogenic lineage markers such as desmin, but rarely exhibited complete differentiation (unpublished data). We believe that the inadequate culture conditions that caused an eventual growth arrest in the parental cells led to the selection of variant clones that are usually unable to differentiate. This may reflect the heterogeneity of myoblasts isolated from human skeletal muscle. The heterogeneity of muscle satellite cells has been widely studied, and recent reports describe functional heterogeneity of satellite cells regarding their potential to differentiate and generate myofibers (e.g. Rouger *et al.*, 2004; Collins *et al.*, 2005). Trono and co-workers have reported the immortalization of myoblasts isolated from normal and DMD subjects after transduction with both Bmi-1, which represses p16 expression, and hTERT (Cudré-Mauroux *et al.*, 2003). However, myoblasts immortalized from control subjects failed to differentiate, while clones of myoblasts retaining the capacity to differentiate could be isolated from transduced DMD cell populations. The results from Cudré-Mauroux *et al.* (2003) could be explained either by chance or if cells prone to proliferate regardless of their potential to differentiate are less frequent in DMD cultures. This might result from the extensive proliferation of precursors that occurs during the evolution of the disease, as shown by their excessive telomere erosion (Decary *et al.*, 2000). The exhaustion of myogenic cells with a 'stem-cell-like' potential has been described in the mdx mice, a model for DMD (Heslop *et al.*, 2000). We propose that the more adequate culture conditions described in this report combined with cdk4 and telomerase allow the maintenance of cells that retain a high potential to differentiate while being expanded and amplified.

The ability to immortalize human myoblasts using a combination of optimized culture conditions and the transduction of both hTERT and cdk4 raises the possibility of creating human

cellular models of various neuromuscular diseases. The rarity of biopsies and the limited proliferation of human myoblasts, particularly when isolated from muscular dystrophies (Decary *et al.*, 2000), has made it difficult to create such models. Immortal cell lines from control subjects of various ages and different neuromuscular disorders are currently being established in our laboratory, and will be made available to the scientific community as soon as they are characterized.

Significant telomere shortening has been observed in a variety of age-related diseases (Meeker *et al.*, 2002; O'Sullivan *et al.*, 2002; Finley *et al.*, 2006) and may contribute to other pathologies. The principles developed here for the muscular dystrophies should be directly applicable to the development of immortalized cells for the study and therapy of a variety of age-related pathologies.

The immortalized myoblasts we describe in this report provide the immediate advantage of a well-characterized cell line that can be shared between different laboratories. However, we believe that the most important implications of this work will be the ability to employ them as the starting material for genetically engineering myoblasts that can overcome the many obstacles of cell therapy thus becoming more therapeutically useful than their normal unmodified counterparts.

Experimental procedures

Cell culture

Human satellite cells were derived from the pectoralis major muscle of a 41-year-old male Caucasian heart-transplant donor, in accordance with ethical legislation. Minced muscle fragments were incubated in a 100-mm dish containing 4 mL of medium, sufficient to cover the bottom but preventing floating of the fragments thus removing their contact with the dish. Cultures were initiated in medium [four parts Dulbecco's modified Eagle's medium (4.5 mg mL⁻¹ glucose) to one part medium 199] supplemented with 20% fetal bovine serum, 30 mM HEPES and 5 ng mL⁻¹ HGF (R&D systems, Minneapolis, MN, USA), on dishes coated with 0.1% pigskin gelatin (Sigma-Aldrich, St. Louis, MO, USA). Outgrowth of satellite cells was more robust when cultured in a 2% oxygen chamber than in room oxygen (20%). Medium was increased to 15 mL after 4 days. The initial confluent culture was designated as PD 0. Cells were subcultivated at 125 000 cells per T25 flask (3125 cells cm⁻²) when just confluent (7–10 days). The final culture conditions (see Results) consisted of the above basal medium supplemented with 1 μM dexamethasone, 1.4 mg L⁻¹ zinc sulphate (ZnSO₄) and 0.03 mg L⁻¹ vitamin B₁₂. Strains were designated as HSKM5 (the parental myoblasts), LHCN (the immortalized population of HSKM5) and LHCN-M2 (a selected clone of LHCN).

Retroviruses

A variety of cDNAs were subcloned into the pBabe retroviral backbones (Morgenstern & Land, 1990) containing different

modifications or selectable markers. The telomerase (hTERT) cDNA containing the endogenous 5' UTR but lacking the 3'UTR was cloned into pBabeHygro in which lox sites had been placed internal to both 5' and 3' LTRs. Expression of Cre recombinase can then excise the entire cassette leaving just the LTRs behind (Steinert *et al.*, 2000). Following infection, myoblasts were selected in 40 μg mL⁻¹ hygromycin for 14 days. Cdk4 was cloned into pBABEneo (Ramirez *et al.*, 2003).

Assay for telomere length

Mean telomere lengths were determined by telomeric restriction fragment length analysis using the restriction enzyme *HinfI* as described (Di Donna *et al.*, 2003).

In vitro characterization

The myogenic purity of cell preparations was determined by counting the number of desmin-positive cells as a percentage of the total number of nuclei. Immunocytochemistry was performed using an antibody specific for desmin diluted at 1 : 50 (clone D33; DAKO, Trappes, France). The myogenic program expressed by the cultures after 7 days of differentiation was revealed by immunofluorescence analyses using specific antibodies directed against embryonic (clone 2B6; pure, a gift from J. A. Kelly), neonatal (1/5, NCL-MHCn; Novacastra, Newcastle upon Tyne, UK), adult fast (1/5, NCL-MHCf; Novacastra), adult slow (1/5, NCL-MHCs; Novacastra) and MF20 (pure, detects all sarcomeric myosins, ATCC) myosin heavy chains. Specific antibody binding was revealed with Alexa Fluor 488 coupled secondary antibody (Molecular Probes, Montluçon, France). To visualize nuclei, cells were mounted in medium (Mowiol, Calbiochem-Novabiochem, San Diego, CA, USA) containing bis-benzimide (0.0001% w/v, Hoechst, no. 33258; Sigma-Aldrich). All images were digitized using a Photometrics and MetaView image analysis system.

In vivo muscle formation

Cells (5 × 10⁵) were injected into the TA muscle of RAG2^{-/-}/γC^{-/-}/C5^{-/-} mice. Prior to myoblast implantation, TA muscles were subjected to freezing lesions in order to provoke regeneration. The three different cell types (the parental strain HSKM5, the immortalized derivative LHCN and the immortalized clone LHCN-M2) were injected into a total of 12 mice. In the TA of six mice, HSKM5 was injected into one hindlimb whereas the other hindlimb received no cells. Six other mice were injected with LHCN-M2 in one hindlimb and LHCN in the contralateral limb. The injection protocol employed was as previously described (Cooper *et al.*, 2003) except that a single injection was performed in the mid belly of the TA. Mice were sacrificed 6 weeks postimplantation and the muscles analyzed for the presence of human fibers. Four mice were evaluated 12 weeks postimplantation to verify the absence of long-term tumors.

Double immunofluorescence analyses were performed using human-specific mouse monoclonal antibodies against spectrin (1/50, NCL-Spec1; Novacastra) and lamin A/C (1/400, NCL-Lam-A/C; Novacastra) as described (Cooper *et al.*, 2003). All images were digitized as described.

Analysis of injections *in vivo*

Five micrometers sections were cut along the entire length of the muscle. At distances corresponding to the distal, mid belly and proximal levels, sections were collected and stained for human lamin and spectrin. The number of positive profiles in a total of 300 sections per cell type was counted and compared. Significance between population means was tested using the unpaired *t*-test with Welch's correction. In all histograms, the columns represent the mean \pm standard error of the mean. Statistical analysis was performed using GraphPad Prism software (version 4.0b, GraphPad Software Inc., San Diego, CA, USA) with *P* < 0.05 being considered significant.

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