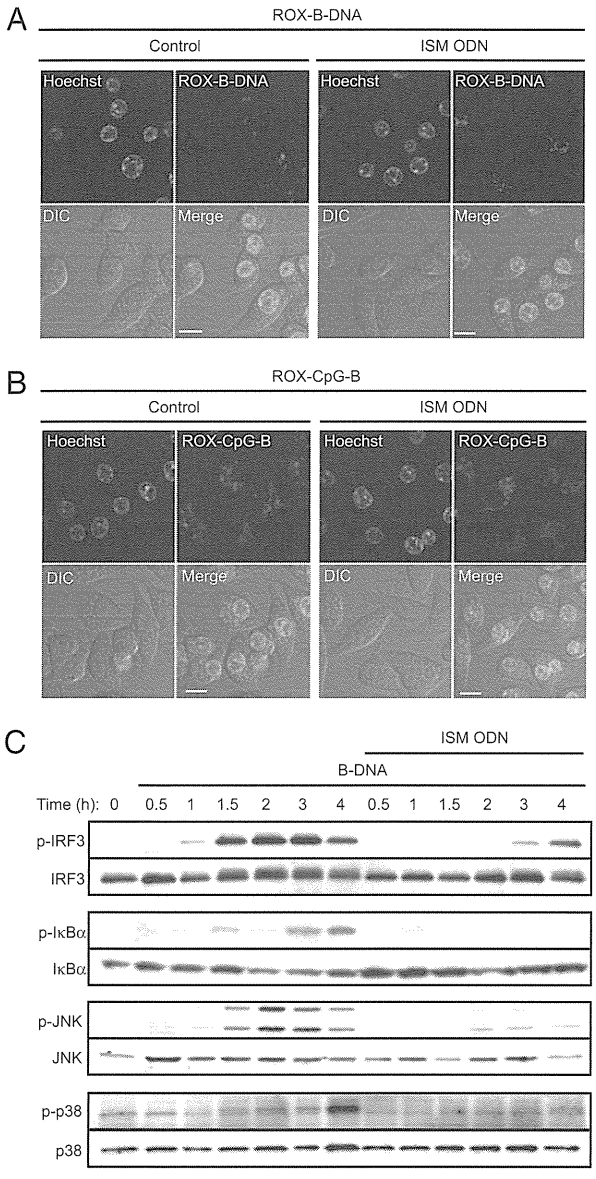

Fig. 3. Suppression of nucleic acid-mediated signaling pathways by ISM ODN. (*A* and *B*) RAW264.7 cells were left untreated (control) (*Left*) or treated with ISM ODN (1 μM) (*Right*) for 1 h, and then stimulated with rhodamine-conjugated B-DNA (1 μg/mL) (ROX-B-DNA; red) (*A*) or rhodamine-conjugated CpG-B ODN (1 μM) (ROX-CpG-B; red) (*B*) for 1 h. Fluorescence images were observed by confocal microscope. The nucleus was stained with Hoechst 33342 (green). (Scale bars, 10 μm.) (*C*) MEFs were left untreated or pretreated with ISM ODN for 1 h, and then stimulated with 10 μg/mL B-DNA for the indicated time periods. The phosphorylation of IRF3, IκB-α, JNK, and p38 was assessed by immunoblot analysis using the indicated antibodies.

essentially similar to that of the TLR9 agonist CpG-B ODN, as demonstrated in previous studies (21, 22), and suggests that the endosomal/lysosomal trafficking of these ODNs occurs similarly. On the other hand, rhodamine-labeled B-DNA merged with dextran but not with LysoTracker (Fig. S3*D*). Thus, taken together with previous reports showing that HMGB1 is also expressed in endosomes (11, 23), these findings suggest that it is the endosomal compartment where ISM ODN exerts its suppressive effect on B-DNA–mediated innate immune signaling by competing with this and other immunogenic nucleic acids for binding with HMGBs (*Discussion*).

To further consolidate the notion that ISM ODN indeed suppresses innate immune signaling, we examined the induction of phosphorylation of IRF3, IκB-α, JNK, and p38 MAPK following cytosolic B-DNA stimulation in MEFs with or without ISM ODN pretreatment. As shown in Fig. 3*C*, the phosphorylation of all was inhibited by ISM ODN pretreatment, which was similarly observed in cells stimulated by poly(I:C) (Fig. S3*E*). Because the sequence of ISM ODN is identical to that of the TLR9 agonist CpG-B ODN aside from one nucleotide, we wished to confirm that the inhibitory effect of ISM ODN is not mediated by TLR9 by any means. When wild-type and *Tlr9*-deficient cDCs were cytosolically stimulated with B-DNA or poly(I:C), we observed that the induction of IL-6 and TNF-α mRNAs was equally suppressed by ISM ODN in cells of both genetic backgrounds, indicating that ISM ODN bound to HMGBs exerts its function independently of TLR9 (Fig. S3*F*). Taken together with these findings, we surmise that ISM ODN does not interfere with the uptake of nucleic acids but rather inhibits the subsequent ligand binding to the PRRs, thereby blocking downstream signal transduction events (*Discussion*).

ISM ODN Suppresses Nucleic Acid-Mediated Immune Responses in Vivo. Nucleic acids potently enhance adaptive immune responses, be they protective or harmful to the host, via the activation of innate immune receptors (24). To investigate the effect of ISM ODN on nucleic acid-mediated adaptive immune responses in vivo, mice were immunized with chicken ovalbumin (OVA) protein using B-DNA or CpG-A ODN as an adjuvant (22) and either with or without pretreatment with ISM ODN, and the induction of $CD8\alpha^+$ cytotoxic T lymphocytes (CTLs) was examined by flow cytometry. As shown in Fig. 4*A*, the induction of OVA-specific CTL responses was strongly suppressed by the pretreatment of mice with ISM ODN. In addition, when OVA-specific IgG1 production was monitored in the sera from mice immunized with OVA and B-DNA, it was found to be suppressed and almost undetectable in ISM ODN-pretreated mice (Fig. S4*A*). Thus, ISM ODN also serves as a potent suppressor of nucleic acid-mediated adaptive immune responses.

Therapeutic Effects of ISM ODN in Immunological Disorder Models. The above findings prompted us to examine whether ISM ODN suppresses inflammation or autoimmune disorders in which the contribution of nucleic acids and/or HMGB1 has been implicated. There is ample evidence that autoantibodies bound to self-DNA or -RNA, or DNA derived from dead cells of inflammatory lesions, can activate nucleic acid-sensing receptors and subsequent immune responses (4, 24–26). A typical example is experimental autoimmune encephalomyelitis (EAE), an animal autoimmune demyelinating disease model of human multiple sclerosis, wherein signaling by nucleic acid-sensing TLRs contributes to the development and pathology of the disease (27–29). Thus, we examined whether ISM ODN can suppress EAE development in mice immunized with myelin oligodendrocyte glycoprotein peptide emulsified in complete Freund's adjuvant. Eight days after immunization, these mice were injected i.v. with ISM ODN or were mock-injected. Remarkably, EAE development, which was observed in the mock-injected mice, was strongly suppressed in the ISM ODN-injected mice (Fig. 4*B*).

We next examined the effect of ISM ODN on an established LPS-induced septic shock model for which an active role for HMGB1 has been reported (30). Interestingly, when mice were i.p. injected with a lethal dose of LPS, those pretreated with ISM ODN showed a significantly high survival rate: As shown in Fig. 4*C*, whereas all mice without ISM ODN pretreatment died within 48 h, 70% of those mice with ODN pretreatment survived even 72 h after LPS injection. It is worth noting that, unlike the control mice, the induction of serum aspartate transaminase (AST) and alanine transaminase (ALT), which are indicators of liver injury, remained strongly suppressed in ISM ODN-pretreated mice 16 h after LPS injection, suggesting that ISM ODN itself has little or no toxicity (Fig. S4*B*). To further examine how ISM ODN suppresses LPS-induced lethality in mice, we analyzed the levels of serum proinflammatory cytokines after LPS injection. As shown in Fig. S4*C*,

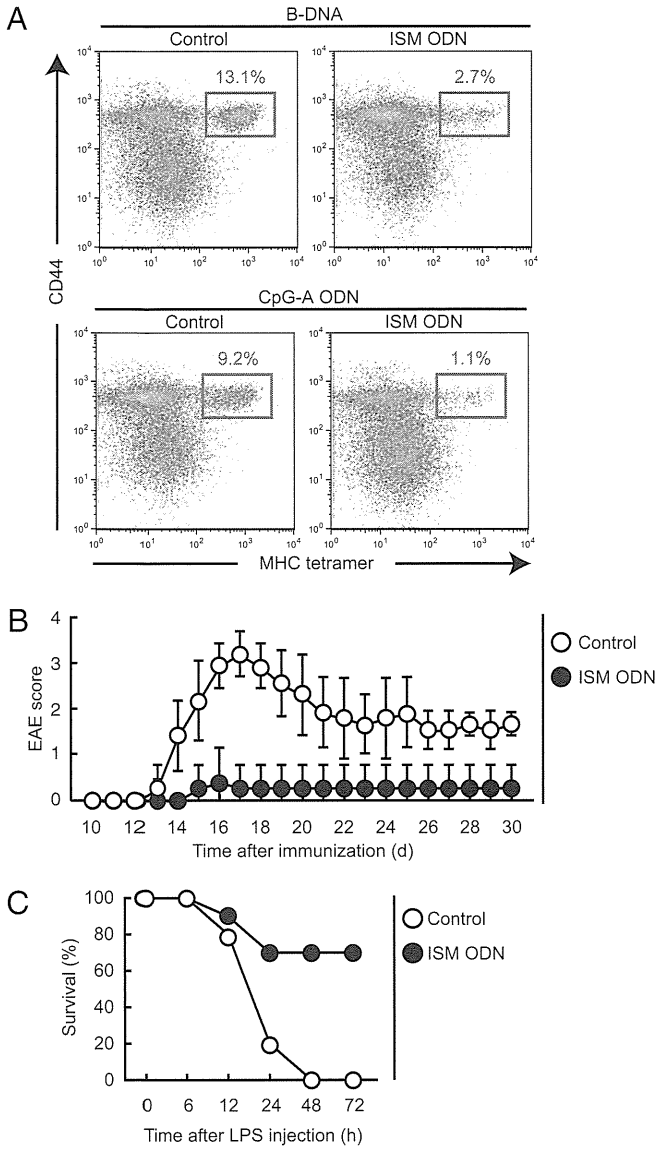

Fig. 4. Effect of ISM ODN on nucleic acid-mediated immune responses and disease models in vivo. (*A*) Mice were immunized with 0.5 mg of OVA and DNA (10 μg of B-DNA or 50 μg of CpG-A ODN) in the presence or absence of 1 mg of ISM ODN. Six days later, splenocytes were isolated and subjected to three-color FACS analysis using an anti-CD8α antibody, anti-CD44 antibody, and OVA-specific MHC class I tetramer. The data shown were gated on CD8α-positive events. The numbers indicate the percentage of tetramer-positive cells relative to the total number of $CD8\alpha^+$ T cells. (*B*) Mean EAE scores ± SD for mice treated with PBS (control) or ISM ODN ($n = 4$ per group) are shown. (*C*) Mice were i.p. injected with 1.25 mg of LPS, with or without (control) 0.1 mg of ISM ODN 1 h before LPS injection. The survival rate of each group was monitored for 72 h ($n = 10$).

IL-6 and TNF-α production levels in sera were found to be the same between ISM ODN-pretreated and mock-pretreated mice 2 h after LPS injection; however, cytokine levels were markedly reduced in the ODN-pretreated mice at later time points. These findings suggest that the early-phase induction of these cytokines via the LPS-TLR4 signaling pathway is not affected by ISM ODN, but rather it is the later-phase induction that is mediated by HMGB1 which is inhibited by the ODN. Indeed, HMGB1 is induced by LPS injection at this later phase (i.e., 8–32 h after injection) (30). It has also been shown that HMGB1, which activates TLR4 and possibly other receptors, is required for a lethal inflammation (12, 13, 16). Thus, these observations raise the interesting possibility that ISM ODN not only suppresses nucleic acid-mediated immune responses but may also affect the activity of HMGB1 as a proinflammatory cytokine (*Discussion*).

Discussion

Nucleic acid-mediated immune responses have become an attractive area of study, given their connection to protective and pathological immunities. In view of our previous finding that HMGBs function as universal sentinels for the innate immune responses activated by nucleic acids (11), we aimed here to demonstrate proof of concept for targeting HMGBs as a means for therapeutic intervention for immunological disorders. To do so, we reasoned that ni-ODNs with a high affinity for HMGBs would interfere with the activation of subsequent nucleic acid-mediated immune responses. Indeed, ISM ODN was found to bind HMGBs with high affinity (Fig. 1 and Figs. S1 *D* and *E* and S2*A*) and to suppress immune responses induced by nucleic acid-sensing cytosolic receptors or TLRs (Fig. 2 and Fig. S2 *C*, *D*, and *F*). Because intracellular uptake/delivery of the immunogenic nucleic acids remained unaffected in the presence of ISM ODN (Fig. 3*A* and *B*) yet downstream signaling events were suppressed (Fig. 3*C* and Fig. S3*E*), it is likely that ISM ODN competes with immunogenic nucleic acids for HMGB binding. On the basis of our observations (Fig. S3 *C* and *D*) and on previous reports showing that HMGB1 is expressed also in endosomes (11, 23), this suppression of HMGB1 function by ISM ODN likely occurs in endosomal vesicles. This view is consistent with our previous notion that the association of ligands with the endosomal membrane and their binding to innate receptors are the common events for the activation of nucleic acid-mediated immune responses (11, 23, 31).

We surmise further that HMGBs function as essential and common components of nucleic acid ligands, without which nucleic acids cannot efficiently bind and activate their cognate receptors, and that the ni-ODNs described here likely interfere with this critical interaction through their binding to HMGBs. Indeed, our data show a correlation between the binding affinity of ODNs with HMGB1 and the magnitude of the suppressive effect of ODNs. Of the structural elements of ODNs that determine binding affinity with HMGBs, our data indicated that a length of more than 15 nucleotides and a phosphorothioate backbone are most critical (Fig. 1 *B* and *C* and Fig. S1*D*). In this regard, previous reports have shown that one HMGB1 molecule covers 15–18 bp of DNA (32, 33), which corroborates our findings that affinity markedly decreased as the ODNs became shorter than 20 nucleotides (Fig. 1*C* and Fig. S1*D*). Our results also show the importance of phosphorothioate modification of a normal phosphodiester backbone for the high-affinity interaction of ni-ODNs with HMGBs (Fig. 1*B*); it is interesting to note that this backbone structure per se also shows binding affinity with other proteins including TLR9 (18, 34). However, it is clear that the nucleotide bases attached to the backbone are also critical for ODN–HMGB interaction. In an attempt to suppress the activation of TLR7- or TLR9-mediated immune responses, relatively short (15- to 25-mer, in most cases) partially or entirely phosphorothioated ODNs have been studied (35, 36). In addition, several classes of "inhibitory ODNs" have been developed, and some of them are reported to ameliorate disease symptoms of animal models of autoimmune diseases and septic shock (36); however, it has been shown that the affinity of inhibitory ODNs with the receptors does not necessarily correlate with their inhibitory activity (37). In light of our present findings, these ODNs may also exert their inhibitory activities on TLR signaling through their interaction with HMGBs.

Nucleic acid-mediated immune responses have been implicated in the pathogenesis of inflammatory and autoimmune diseases (4, 24). Our present study indicates that ISM ODN may serve as an effective compound for the treatment of autoimmunity and sepsis (Fig. 4 *B* and *C*). Notably, ISM ODN treatment did not affect the growth of primary MEFs in vitro (Fig. S3*G*) and did not change AST and ALT levels in sera (Fig. S4*B*), suggesting that ISM ODN treatment has little, if any, toxic effect. Because it has been sug-

gested that TLR9 signaling is related to the exacerbation of EAE symptoms (28, 29), we surmise that ISM ODN may block pathological TLR9 signaling, thereby attenuating the progression of the disease. Moreover, we show ISM ODN suppression of LPS-mediated endotoxin shock, which provides yet another interesting possibility for ODN-based therapy for sepsis and numerous other inflammatory diseases involving HMGB1 (16). Although further study will be required to clarify how ISM ODN inhibits LPS-mediated endotoxin shock, the following possibilities may be considered. It is possible that ISM ODN inhibits the function of HMGB1 as an inflammatory cytokine when released into the extracellular environment by active secretion from stimulated monocytes/macrophages (30) or by passive diffusion from necrotic cells, functioning as a potent proinflammatory cytokine (12) via the activation of TLR4 and other receptors (13). Thus, ODN interaction with secreted HMGB1 may cause the functional inactivation of HMGB1 as an inflammatory cytokine, possibly through the induction of a conformational change. On the other hand, because ISM ODN shows no effect on LPS-mediated response in vitro (Fig. S2 *B* and *G*), HMGB1 may need to interact with an additional molecule for its inflammatory cytokine action in vivo. Because LPS injection into mice induces massive death of hepatocytes and other cells (38), one possibility is the suppression of inflammatory responses by the necrotic cell-derived DNA that may activate the innate immune receptors via interaction with HMGBs. This notion is supported by our observation that IL-6 and TNF-α production induced by necrotic cells is inhibited by treatment with ISM ODN (Fig. S4*D*). Another intriguing possibility is that HMGB1 secreted from cells associates with nucleic acids and that the resulting complex may be the active form, whose function is inhibited by the ODN.

Because of its inflammatory activity, HMGB1 has been targeted for therapy in the treatment of numerous inflammatory diseases. For instance, a previous study has demonstrated that injection of an anti-HMGB1 antibody protects mice from LPS-induced lethal shock (30). In addition, because the protein is often overexpressed in cancer cells, there is much hope for therapeutic strategies based on targeting HMGB1 (39). Thus, ISM ODN or more effective derivatives may lead to the development of alternate therapeutic interventions for these diseases. Further evaluation of the efficacy and safety of ISM ODN and other ODNs, compared with other strategies targeting HMGB1, will be critical for pursuing such possibilities.

Materials and Methods

Poly(dA-dT)·poly(dT-dA) (B-DNA), poly(U), and LPS were purchased from Sigma. Poly(I:C) was purchased from GE Healthcare Biosciences. ODNs for CpG-B (17), CpG-A (GGtgcatcgatgcaGGGGGg) (40), ISM, ISR, PD-ISM (tccatgaggttcctgatgct), poly(dA), and poly(dC) (uppercase, phosphorothioate backbone; lowercase, phosphodiester backbone) with or without biotin, rhodamine, or FITC were purchased from FASMAC. PS (18) was used as described previously (11). Complex formation of B-DNA, poly(I:C), or poly(U) with Lipofectamine 2000 (Invitrogen) and that of CpG-A ODN with *N*-[1-(2,3-dioleoyloxy)propyl]-*N*, *N*, *N*-trimethylammonium methyl-sulfate (DOTAP) (Roche Applied Science) was prepared as described previously (11, 22). Additional information is available in *SI Materials and Methods*.

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ENABLING TECHNOLOGIES

CDK4 and cyclin D1 allow human myogenic cells to recapture growth property without compromising differentiation potential

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In vitro culture systems of human myogenic cells contribute greatly to elucidation of the molecular mechanisms underlying terminal myogenic differentiation and symptoms of neuromuscular diseases. However, human myogenic cells have limited ability to proliferate in culture. We have established an improved immortalization protocol for human myogenic cells derived from healthy and diseased muscles; constitutive expression of mutated cyclin-dependent kinase 4, cyclin D1 and telomerase immortalized human myogenic cells. Normal diploid chromosomes were preserved after immortalization. The immortalized human myogenic cells divided as rapidly as primary human myogenic cells during the early passages, and underwent myogenic, osteogenic and adipogenic differentiation under appropriate culture conditions. The immortalized cells contributed to muscle differentiation upon xenotransplantation to immunodeficient mice under conditions of regeneration following muscle injury. We also succeeded in immortalizing cryopreserved human myogenic cells derived from Leigh disease patients following primary culture. Forced expression of the three genes shortened their cell cycle to <30 h, which is similar to the doubling time of primary cultured human myogenic cells during early passages. The immortalization protocol described here allowed human myogenic cells to recapture high proliferation activity without compromising their differentiation potential and normal diploidy.
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Keywords: muscle satellite cell; CDK4; telomerase; immortalization; replicative senescence; growth arrest

INTRODUCTION

Skeletal muscle stem cells of adult muscle are known as muscle satellite cells because they are located adjacent to the plasma membrane of myofibers beneath the basement membrane. The postnatal growth, repair and maintenance of skeletal muscle rely on muscle satellite cells that proliferate and then fuse together to form myotubes. Actually, phenotypic analysis of Pax7-deficient mice strongly suggests that the loss of satellite cells abolishes the regenerative capacity of skeletal muscle.[1,2] The decrease of regenerative capacity of muscle results in muscle dysfunction during both normal aging and progression of muscle-regenerative diseases, such as muscular dystrophies.

Most of the data on the regulation of proliferation and differentiation of muscle satellite cells and their descendant progenitor cells have been obtained from primary cultured chick myogenic cells or mouse myogenic cell lines.[3–5] However, several previous studies strongly suggest that animal myogenic cells do not always use the same pathways to control proliferation and differentiation as human myogenic cells.[6,7] Although animal cell models certainly contribute to understanding the mechanisms of human myogenesis and muscle diseases, the precise and detailed analysis of human myogenic cells is essential for fundamental and therapeutic investigation. Unfortunately, progressively compromised differentiation potential, as well as proliferation potential, is seen in cultured human myogenic cells.[8,9] The limited proliferation capacity and progressive alterations of characteristics of human myogenic cells do not allow us to carry out both qualitative and quantitative analyses with high reproducibility.

Previous attempts have been made to extend the replication capacity of human myogenic cells using viral oncogenes such as simian virus 40 large T antigen and/or the reverse transcriptase component of human telomerase (hTERT).[10] However, no reliable model of immortalized human myogenic cells that exhibit differentiation potential had been established until our previous study.[9] We previously reported that constitutive expression of hTERT and human papillomavirus type 16 gene E7 immortalizes a primary normal human myogenic cell clone designated Hu5. The immortalized human myogenic cell clone Hu5/E18 largely preserves the myogenic phenotype represented by parental Hu5 cells, but their doubling time is approximately 12 h longer than that of primary human myogenic cells during early passages. E7 is an oncogene that inactivates the retinoblastoma protein pRb,[11] and does not transform human myogenic cells. However, we cannot exclude a possibility that E7 also affects other biological functions, including transformation-related pathways.

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Cellular stress activates a pathway of the cyclin-dependent kinase inhibitor $p16^{INK4a}$, resulting in premature cell cycle arrest before telomere attrition,[12] probably due to the activation of Rb. The forced expression of wild-type cyclin-dependent kinase 4 (CDK4) enabled hTERT to immortalize primary human myogenic cells, presumably because cdk4 sequesters the increased p16 exclusively when stimulated with dexamethasone and hepatocyte growth factor.[13] In addition, the co-expression of hTERT and Bmi-1, which suppresses $p16^{INK4a}$ expression, failed to immortalize human myogenic cells.[9,14] These results indicate that combining the expression of hTERT and sequestration of $p16^{INK4a}$ is insufficient to immortalize human myogenic cells, or that the $p16^{INK4a}$ pathway is incompletely suppressed under these conditions.

In the present study, to block the $p16^{INK4a}$–Rb pathway and enhance cell cycle progression, without the use of oncoprotein E7, expressions of hTERT and both mutant CDK4 (CDK4R24C) and cyclin D1 were induced in human myogenic cells. Combined expression of the three genes efficiently immortalized normal human myogenic cells. The immortalized cells still retained multipotentiality and a doubling time similar to that of primary cultured human myogenic cells. The established normal human myogenic cell clones in the present study are the human equivalents of mouse cell lines such as C2 (ref. 3) and Ric10.[5,15] In addition, we succeeded in immortalization of diseased muscle-derived primary human myogenic cells that showed the prolonged doubling time. The newly established method for immortalization of primary human myogenic cells will open new avenues for mechanistic and therapeutic research on human muscle diseases.

RESULTS

$p16^{INK4a}$–Rb pathway is activated upon growth arrest of primary cultured human myogenic cells

Proliferation capacity of primary cultured human myogenic cells severely declined during serial passages under the present culture condition (Figure 1a). The doubling time of the cells became longer as they were serially succeeded (Supplementary Figure 1). Constant or high level expression of cyclin D1, CDK4, cyclin-dependent kinase inhibitor $p21^{cip1}$ and p53 was observed in primary human myogenic cells even upon growth arrest (Figure 1b). In contrast, the amount of the cell cycle inhibitor $p16^{INK4a}$ increased along with the culture period, whereas the amount of hyperphosphorylated form of Rb declined. The amount of another cell cycle-driving kinase CDK2 decreased following the disappearance of hyperphosphorylated form of Rb. The results indicate that the $p16^{INK4a}$–Rb pathway is activated before growth arrest of primary cultured human myogenic cells, suggesting that their cell cycle arrest is due to the activation of Rb. The disappearance of hyperphosphorylated Rb seems unlikely to depend on the downregulation of either CDK2 or CDK4 that are kinases relevant for phosphorylation of Rb.

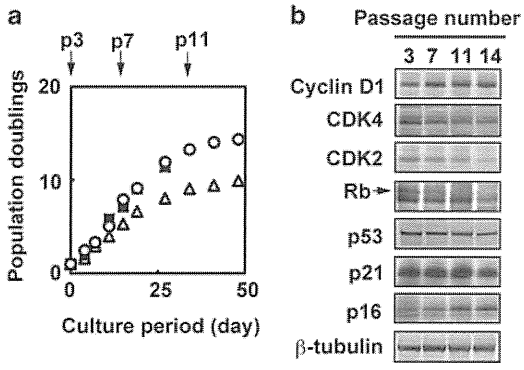


Figure 1 Growth properties of primary cultured human myogenic cells. (**a**) Life span plots of primary cultured human myogenic cells Hu20 (filled square), Hu23 (triangle) and Hu26 (circle) between passages 3 and 13. Arrows show the timing of passages 3, 7 and 11. Day 0 of culture period represents the day when the cells were plated for passage 3. (**b**) Expression patterns of growth-related proteins in primary cultured human myogenic cell H23 during serial passages. Fifteen micrograms of total proteins were subjected to immunoblotting analysis with antibodies against proteins shown in the left panels. Similar expression patterns of the proteins were obtained in Hu20 and Hu26. An arrow represents the position of hyperphosphorylated Rb protein.

E7 promotes nuclear progression in terminally differentiated myotubes

The primary human myogenic progenitor cell clone Hu5 was obtained from a healthy muscle of a non-dystrophic woman.[4] Hu5 cells have limited ability to proliferate but can be immortalized by constitutive expression of both telomerase and the *E7* gene from human papillomavirus type 16.[9] E7 inactivates Rb but is also suspected to affect other cellular functions. To determine whether constitutive expression of E7 transforms human myogenic cells, the Hu5-derived myogenic cell clone Hu5/E18 (ref. 9), immortalized by constitutive expression of both hTERT and E7, was transplanted into cardiotoxin-injected TA muscles of immunodeficient NOD/Scid mice (Figures 2a and b). Before transplantation, E18 cells were infected with a lentivirus vector encoding green fluorescent protein Venus (kindly provided by Dr Miyoshi). Transplanted cells were identified by the fluorescence of Venus and antibodies specific for green fluorescent protein. Transplanted E18 cells (2.5×10^6 cells per TA) gave rise to myofibers labeled with green fluorescence. No tumor was formed in the transplanted TA muscles. Soft agar assays also showed that E18 was unable to grow in an anchorage-independent way (Supplementary Figure 2). The results indicate that E18 cells did not show any oncogenic potential either *in vivo* or *in vitro*.

In the next experiment, effects of the immortalization on cell cycle exit during terminal muscle differentiation were analyzed *in vitro*. E18 cells undergo myogenic terminal differentiation under the myogenic differentiation-inducing condition.[9] Primary cultured human myogenic progenitor cells exited the cell cycle and gave rise to terminally differentiated myotubes (Figures 2c–f). In contrast, the nuclei of E18 myotubes synthesized DNA and also contained the proliferation marker protein Ki-67, although neither nuclear nor cellular division was observed in the myotubes (Figures 2g–j). The results suggest that E7 promotes nuclear progression in terminally differentiated myotubes that have lost mitogenic potential. In addition, the doubling time of the Hu5-derived immortalized cells is approximately 35 h,[9] whereas primary cultured human myogenic cells divided at 20–29 h intervals (Supplementary Figure 3). Taken together with the results here, the expression of hTERT and E7 immortalizes human myogenic cells without the loss of their differentiation potential but also affects their cell cycle properties during the terminal myogenic differentiation.

Cell cycle drivers efficiently immortalize primary cultured human myogenic cells

E7 promotes nuclear progression in myotubes, perhaps, because it accelerates the degradation of Rb family proteins including Rb, p130 and p107. To inactivate Rb directly and avoid unusual promotion of nuclear progression in myotubes, Hu5 cells were infected with recombinant lentiviruses encoding hTERT, CDK4R24C and cyclin D1.

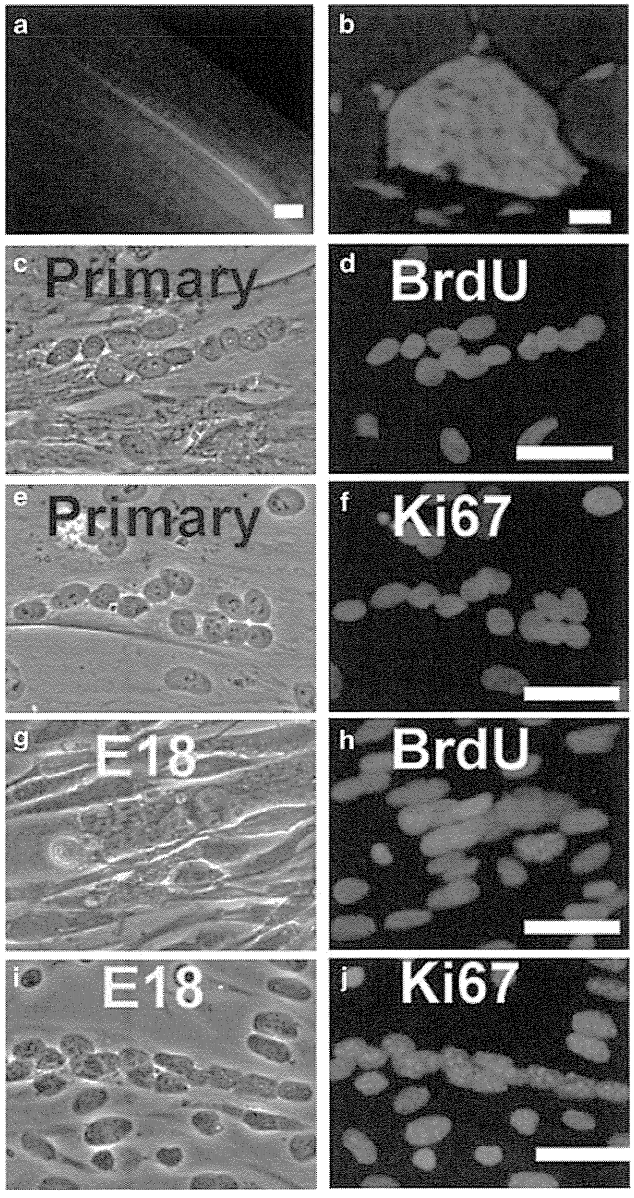


Figure 2 Nuclear progression in terminally differentiated immortalized human myogenic cells expressing telomerase and E7. (**a**, **b**) E18 cells were labeled with modified green fluorescent protein, and then 2.5×10^6 cells were transplanted into the TA muscles of NOD/Scid mice. (**a**) Whole TA muscles were recovered at 4 week after transplantation. Scale bar, 1 mm. (**b**) Pathological view of a TA muscle. Modified green fluorescent protein (green) was detected by immunofluorescence. Nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate. Scale bar, 50 μm. (**c–j**) Primary cultured human myogenic cell Hu26 (**c–f**) and immortalized human myogenic cell clone E18 expressing telomerase and E7 (**g–j**) were cultured for up to 78 h in primary cultured myocyte differentiation medium. For the detection of DNA synthesis, cells were incubated with 10 μM 5-bromo-2′-deoxyuridine for the last 6 h of a 78-h differentiation culture (**c**, **d**, **g**, **h**). Phase contrast images (**c**, **e**, **g**, **i**) and immunofluorescence analysis with anti-5-bromo-2′-deoxyuridine antibody (red in **d**, **h**), or Ki67 (red in **f**, **j**) of the same fields are shown in each row. Nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (blue in **d**, **f**, **h**, **j**). Scale bars, 50 μm.

The single amino acid change in CDK4 prevented a cyclin-dependent kinase inhibitor, $p16^{INK4a}$, from inhibiting kinase activity of CDK4. Forced expression of CDK4R24C, cyclin D1 and hTERT efficiently expanded the lifespan of Hu5 cells and virtually immortalized Hu5 cells. Immortalized Hu5 derivatives expressing CDK4R24C and cyclin D1 under control of the human cytomegalovirus immediate early promoter were designated as Hu5/KD, whereas the cells expressing them under the control of the Tet-Off system were designated as Hu5/TKD. The pooled populations, Hu5/KD and Hu5/TKD, and their derivative clones, KD3 and TKD1, divided rapidly at a similar interval as primary myogenic cells did (Figures 3a–d). The expression of hTERT, CDK4R24C and cyclin D1 culminated in continuous cell proliferation for more than 200 population doublings (Figures 3e and f). In contrast to E7, the cell cycle drivers did not promote nuclear progression in terminally differentiated myotubes nor interfere with the cell cycle exit of myogenic progenitor cells under the differentiation-inducing condition (Figures 3g–k; Supplementary Figure 4). Hu5 derivatives transduced with recombinant lentiviruses encoding hTERT and CDK4R24C proliferated continuously but relatively slowly. Forced expression of hTERT and cyclin D1 did not immortalize Hu5 cells. We therefore concluded that the combined expression of the three genes immortalized human myogenic progenitor cells, resulting in restoration of their growth properties similar to that of primary cultured human myogenic cells.

Immortalized human myogenic cells preserve myogenic phenotype

To determine the karyotype of immortalized human myogenic cells at passages 18–30, about 22–32 metaphase spreads of each cell type were analyzed. The results show the cells maintained a normal 46XX diploid karyotype in both the immortalized populations and the immortalized clones (Figure 4).

High-level expression of CDK4 and cyclin D1 was observed in the immortalized cells (Figure 5a). pRb was highly phosphorylated under the growing condition. The cell cycle inhibitor $p16^{INK4a}$ remained at an extremely high level in the immortalized cells (Figure 5a). However, hypophosphorylated form of pRb was accumulated under the myogenic differentiation-inducing condition. Both the immortalized populations and the immortalized clones fused together and gave rise to myotubes. In addition, MyoD was highly expressed in the nuclei of myotubes (Figures 5b and c). The results here indicate that the immortalized clones KD3 and TKD1 preserved the myogenic phenotype represented by the previously immortalized Hu5 derivatives.[9]

Immortalized human myogenic cells retain differentiation potential both *in vivo* and *in vitro*

The cells immortalized by the forced expression of hTERT and E7 preserved the phenotypic characteristics of their parental Hu5 cells, including multipotentiality; one of the E7-expressing immortalized Hu5 cell clones, E18, retained the ability to undergo myogenic, osteogenic and adipogenic terminal differentiation.[7,9] The CDK4R24C and cyclin D1-expressing immortalized clones, KD3 and TKD1, also underwent myogenic, osteogenic and adipogenic terminal differentiation under the appropriate culture conditions (Figures 6a–c and f–h), although adipogenic differentiation was induced at relatively low efficiency.

To determine whether the immortalized human myogenic cells contributed to muscle regeneration *in vivo*, KD3 and TKD1 cells were transplanted into cardiotoxin-injected TA muscles of immunodeficient NOD/Scid mice. Before transplantation, KD3 and TKD1 cells were infected with a lentivirus vector encoding green fluorescent protein Venus. Transplanted cells were identified by the fluorescence of Venus and antibodies specific for green fluorescent protein. Transplanted KD3 and TKD1 cells (1×10^6 cells per TA) gave rise to many myofibers labeled with strong green fluorescence (8.6 ± 4.3 and

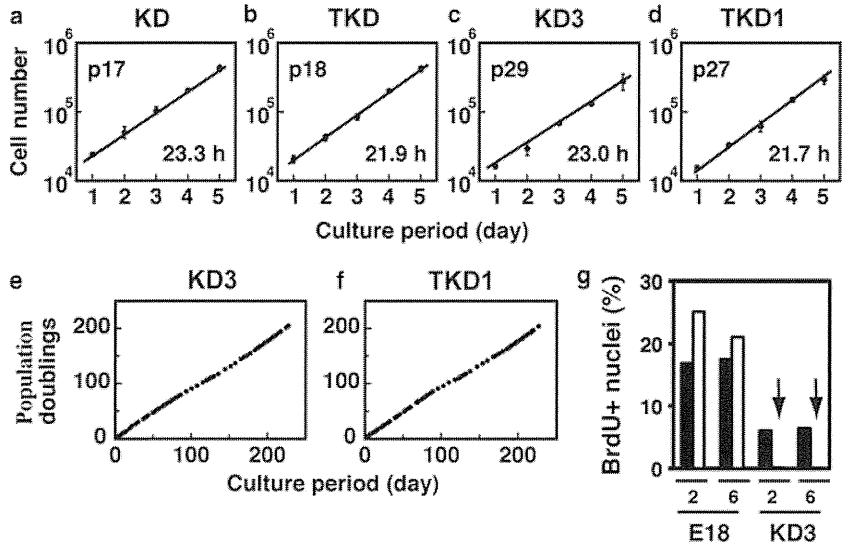


Figure 3 Proliferation of immortalized human myogenic cells. (**a**–**d**) Growth properties of a multiclonal population named KD, expressing hTERT, CDK4R24C and cyclin D1 under the control of a cytomegalovirus promoter (**a**), a multiclonal population named TKD, expressing hTERT, CDK4R24C and cyclin D1 under the control of a Tet-off system (**b**), a clone named KD3 isolated from KD (**c**) and a clone named TKD1 isolated from TKD (**d**). Passage numbers and doubling times are shown in the panels. (**e**, **f**) Life span plots of immortalized clones KD3 (**e**) and TKD1 (**f**). (**g**) E18 and KD3 cells were incubated with 10 μM 5-bromo-2′-deoxyuridine for the last 2 or 6 h of a 78-h culture in primary cultured myocyte differentiation medium. Ratios of 5-bromo-2′-deoxyuridine-positive nuclei in mononucleated progenitors (filled column) and myotubes (open column) were estimated from 1466–3196 nuclei of mononucleated progenitors and 404–1223 nuclei of myotubes, respectively. Numbers under the column represent the incubation time with 5-bromo-2′-deoxyuridine. Arrows represent the positions of open columns.

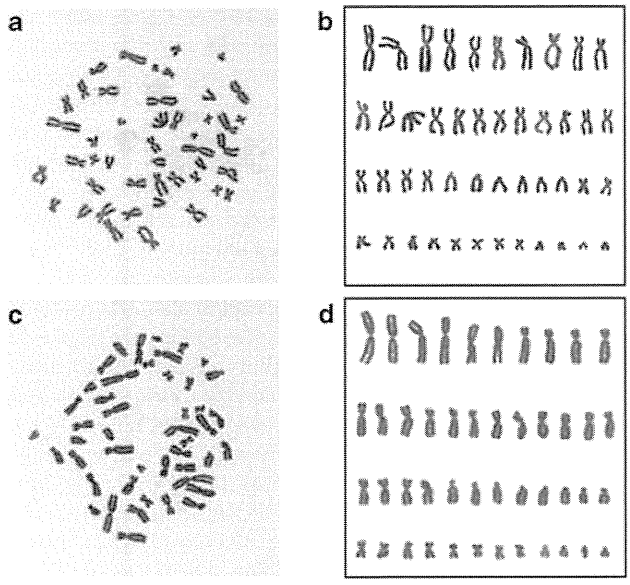
10.2 ± 9.1% of total TA myofibers, respectively) (Figures 6d and i). The relatively large s.d. in the present results was because of the low numbers of positive myofibers in the two specimens, probably due to leakage of the transferred cells to the injected TA muscle. Venus-positive myofibers were regenerated myofibers because they contained central nuclei (Figures 6e and j). No tumor was observed in the transplanted TA muscles. *In vitro* soft agar assay also showed that KD3 cells did not grow in an anchorage-independent way (Supplementary Figure 2). The results suggest that KD3 cells do not possess oncogenic potential. The ability of the immortalized human myogenic cells to regenerate muscle *in vivo* indicates that the immortalized cells established here represent a good model cell system for the fundamental and therapeutic study of human muscle development and disease.

Human myogenic cells recaptured proliferation capacity in cell-cycle driver-dependent manner

Both CDK4R24C and cyclin D1 were expressed under the control of the Tet-Off system in TKD1 cells. To determine the role of cell cycle drivers in the continuous proliferation of human myogenic cells, the expressions of CDK4R24C and cyclin D1 were suppressed by administration of doxycycline. Expression levels of CDK4 and cyclin D1 in TKD1 cells markedly declined during 5 days of incubation with doxcycline (Figure 7a). Doxcycline itself impaired neither the protein levels of either CDK4 or cyclin D1 in KD3 cells (Supplementary Figure 5) nor their DNA synthesis (Figures 7b–g). The number of proliferating TKD1 cells reduced following the decline in CDK4 and cyclin D1 proteins (Figures 7i, l). The morphology of doxycycline-treated TKD1 cells also became more flattened like senescent cells, and the nuclei looked thin during the cessation of proliferation (Figures 7h, j, k, m). In contrast, when doxycycline was removed from the culture, CDK4 and cyclin D1 were restored, and the proliferation capacity was completely recaptured by TKD1 cells (Figures 7a lane 4 and n–p). The results suggest that the proliferation capacity of human myogenic cells expressing hTERT is fully dependent on CDK4R24C and cyclin D1, and that before cellular senescence accompanied by telomeric attrition, human myogenic cells are capable of recapturing proliferation capacity.

Cryopreserved human myogenic cells derived from a disease muscle recapture proliferating activity by immortalization

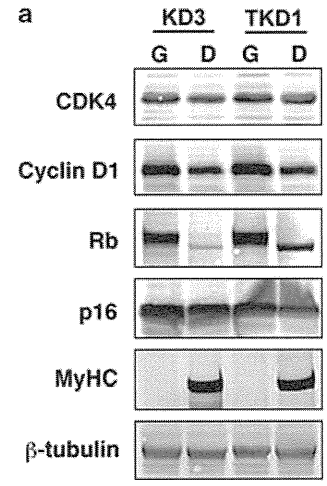
Primary cultured human myogenic cells lose the ability to proliferate by degrees during culture *in vitro*. Cryopreserved primary cultured human myogenic cells obtained from Leigh disease muscle suffered from growth impairment accompanied by a prolonged cell cycle. One of the mortal cell clones from the primary cultured Leigh disease myogenic cells, HM2-5, which had a cell cycle of 73.5 h at passage 10 (Figure 8a), was infected with recombinant lentiviruses. Forced expression of hTERT, CDK4R24C and cyclin D1 had the cells dividing rapidly with a doubling time of 27.7 h (HM255, Figure 8b). A combination of hTERT and E7 also rescued the cells from growth impairment, but their doubling time (36.6 h) (HM253, Figure 8c) was longer than that of the clone immortalized by hTERT, CDK4R24C and cyclin D1. Both immortalized multiclonal populations HM253 and HM255 retained the ability to undergo terminal myogenic, osteogenic and adipogenic differentiation (Figures 8d–i). A cryopreserved mortal cell clone from muscle of another Leigh disease patient also recaptured its proliferation capacity and multipotentiality through immortalization by the combined expression of hTERT, CDK4R24C and cyclin D1 (Supplementary Figure 6). These results suggest that transduction of the three genes renders growth-impaired human myogenic cells proliferative and immortalized without loss of their differentiation potentialities.



e

	Diploid / Total	% Diploid
KD (p.18) (multiclonal)	30 / 32	93.8
KD3 (p.27) (monoclonal)	19 / 22	86.4
TKD (p.19) (multiclonal)	25 / 28	89.3
TKD1 (p.30) (monoclonal)	29 / 32	90.6

Figure 4 Karyotype analysis of immortalized human myogenic cells. Cells were treated with colcemid (2 μM) for 9 h. Metaphase chromosomes were visualized by Giemsa staining (**a**, **c**) and then aligned (**b**, **d**). Immortalized clones, KD3 (**a**, **b**) and TKD1 (**c**, **d**) and multiclones, KD and TKD (**e**), were analyzed.



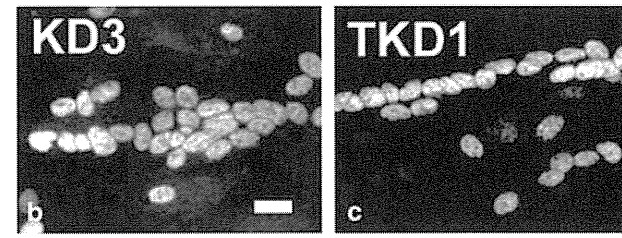


Figure 5 Expression patterns of growth- and differentiation-related proteins in immortalized human myogenic cells. (**a**) KD3 and TKD 1 cells were cultured in pmGM (g) or in primary cultured myocyte differentiation medium for 5 days (d). Fifteen micrograms of total proteins were subjected to immunobloting analysis with antibodies against CDK4, cyclin D1, Rb, $p16^{INK4a}$, myosin heavy chain and β-tubulin. (**b**, **c**) KD3 (**b**) and TKD1 (**c**) were cultured for 6 days in primary cultured myocyte differentiation medium and then subjected to immunofluorescence analysis with antibodies to MyoD. Scale bar, 20 μm.

DISCUSSION

Sarcopenia is an age-related loss of muscle mass leading to muscle weakness and atrophy. The slower regenerative capacity of aging muscle may be attributed to a decrease in the number and/or proliferation and differentiation capacities of muscle satellite cells. Actually, the number of satellite cells declines with age in humans.[16,17] In addition, the proliferation potential of human muscle satellite cells is limited by cellular senescence induced by progressive telomere shortening.[16,18] When the telomere length becomes less than about 5 kb, the Rb and p53 pathways are activated and culminate in irreversible growth arrest.[11,19,20] Cells also enter a state designated as stress or aberrant signal-induced senescence [18,20] (STASIS) or stress-induced premature senescence[21] (SIPS) that closely resembles replicative senescence when subjected to sub-lethal stress or oncogenic signals. The major characteristics of cells undergoing STASIS/SIPS are similar to those of replicatively senescent cells: the Rb and/or p53 pathways are activated and the cells stop proliferation. STASIS/SIPS can be induced in a telomere-independent way in human epithelial cells[11] and even in human fibroblasts,[12] although acceleration of telomere shortening is associated with STASIS/SIPS. Under conventional culture conditions, many types of human cells are likely to undergo precocious growth arrest before replicative senescence induced by telomere shortening,[22] though some types of human cells appear to be immortalized by the expression of hTERT alone without transformation of cell properties.[11,23,24] In fact, our previous and present studies strongly suggest that both inactivation of the Rb pathway and restoration of telomerase activity are required for efficient immortalization of human myogenic cells (Figure 9A). The growth arrest of primary cultured human myogenic cells may be attributable to an inadequate cellular context including culture conditions that stimulate the stress signaling pathway.[25]

Several previous studies emphasized that the age-related dysfunction of muscle is attributed to the age-related changes in environmental factors that attenuate the potential of muscle satellite cells. Transplantation of whole muscles between old and young rats shows that the regenerative capacity of aged muscle is enhanced when grafted into young muscle.[26] The decrease of circulating growth factors[27] and the number of motor units[28] are candidates for the responsible environmental factors or age-related changes in skeletal muscle. In addition, primary cultured human myogenic cells derived from skeletal muscles of aged persons (>75 years old) show growth properties similar to those of the myogenic cells obtained from younger persons under the appropriate culture conditions (Supplementary Figure 3A) (Hashimoto and Okamura, unpublished data). On the other hand, a previous study showed that myogenic cells from

aged muscle demonstrated less ability to proliferate in primary cultures.[6] Given that myogenic cells derived from an aged human are fragile and likely to lose proliferation potential under inappropriate culture conditions, these different results under different culture conditions are plausible. Actually, we have found that the proliferation capacity of human and mouse primary myogenic cells maintained in a medium containing DMEM is higher than that of the cells maintained in a medium containing Ham's F10, even though an F10-based medium was used to isolate and culture primary myogenic cells in many studies.[6,29]

Muscle-degenerative diseases such as muscular dystrophies provoke extensive replication of human muscle satellite cells.[30] Satellite cells in regenerating muscles also suffer from cellular stresses including those induced by inflammatory cytokines. Therefore, precocious growth arrest, as well as the replicative senescence of satellite cells, is likely to cause the loss of muscle-regenerative capacity in muscle-degenerative diseases. Results obtained by previous and present studies indicate a possibility of a new therapeutic strategy for sarcopenia and muscular dystrophy that overcomes the precocious growth arrest triggered by the Rb pathway. Human myogenic cells are vulnerable to cellular stresses and more likely to undergo premature growth arrest than human foreskin fibroblasts because primary cultured human fibroblasts undergo precocious growth arrest/STASIS/SIPS exclusively when exposed to stress inducers such as H_2O_2 and ultraviolet light.[21] From this point of view, the Rb pathway in human myogenic cells will be an attractive target of therapeutic intervention in muscle-degenerative diseases. The present study also shows that the total amount of pRb declined during growth arrest in primary human myogenic cells at later passages, immortalized human myogenic cells undergoing myogenesis and TKD1 cells stimulated with doxycycline. Therefore, we should consider both quantitative and qualitative control of pRb during precocious growth arrest.

The present results suggest that suppression of the Rb signaling pathway is required for immortalization of human myogenic cells in addition to telomere restoration (Figures 9Ba–f). Either Bmi-1 (ref. 9) or wild-type CDK4 (ref. 13) was coexpressed with hTERT in primary cultured human myogenic cells to block the $p16^{INK4a}$ signaling pathway, but the cells did not undergo immortalization. The results indicate that neither Bmi-1 nor the wild-type CDK4 alone allows hTERT to immortalize human myogenic cells, and that immortalization of human myogenic cells still requires secondary changes under these conditions. In fact, the combined expression of wild-type CDK4 and hTERT or Bmi-1 and hTERT results in immortalization of human myogenic cells exclusively under the optimized culture conditions supplemented with dexamethasone and growth factors,[13,14] although the role of those supplements has been unknown. It is conceivable that CDK4 kinase activity released from the inhibition by $p16^{INK4a}$ is not high enough to hyperphosphorylate Rb (Figures 9Bc and d). In contrast, CDK4R24C allows hTERT to promote slow, but continuous, proliferation in primary cultured human myogenic cells (Figure 9Be). CDK4R24C contributes to hyperphosphorylation of Rb, whereas the contribution of forced expression of wild-type CDK4 is quite limited because $p16^{INK4a}$ inhibits the kinase activity of wild-type CDK4. Our previous study indicated that E7 prevents Rb independently of $p16^{INK4a}$ and leads to immortalization of hTERT-expressing human myogenic cells[9] (Figure 9Bb). Given that the suppression of Rb, but not $p16^{INK4a}$, is quite effective in immortalization of human myogenic cells, we concluded that complete inhibition of both Rb activation and telomere shortening is necessary and sufficient for immortalization of human myogenic cells.

Combined expression of CDK4R24C, cyclin D1 and hTERT successfully and reproducibly immortalized human myogenic cells derived from normal and disease muscles, resulting in rapid proliferation without compromising differentiation potential. Cyclin D1 has a crucial role as a limiting factor of CDK4 kinase activity. Forced expression of cyclin D1 increases CDK4R24C kinase activity to an extent that is relevant for hyperphosphorylation of Rb, which then results in rapid proliferation, possibly due to the potent inhibition of Rb function (Figure 9Bf). The slower cycling of human myogenic cells immortalized by either E7 or CDK4R24C and hTERT also implies that higher CDK4 activity is required for rapid proliferation (Figures 9Bb and e). However, we cannot exclude a possibility that extraordinarily high activity of the CDK4R24C/cyclin D1 complex results in the phosphorylation of putative off-target substrates that have an essential role in the cell cycle progression and are usually phosphorylated by another member of the CDK family (Figure 9Bf).

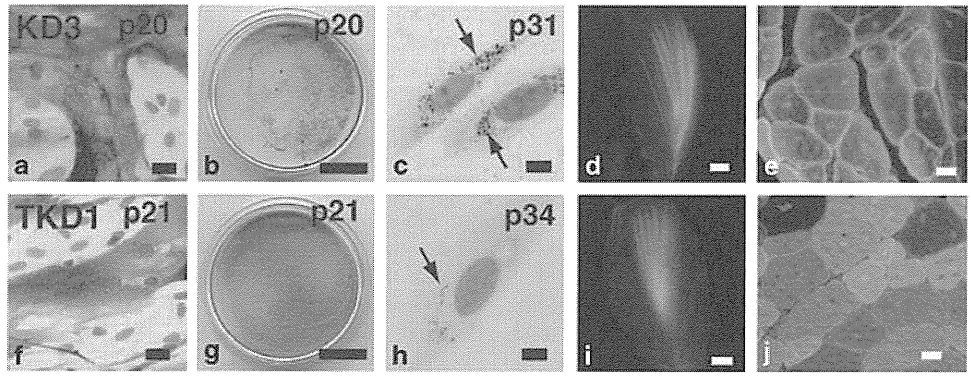


Figure 6 Multipotentiality of immortalized human myogenic cell clones KD3 and TKD1. KD3 (**a–e**) and TKD1 (**f–j**) were induced to undergo myogenic, osteogenic and adipogenic differentiation. (**a**, **f**) Cells were cultured for 5 days in primary cultured myocyte differentiation medium. Myosin heavy chain was detected by immunostaining with a horseradish peroxidase reaction. Nuclei were detected with staining with hematoxylin (blue). Scale bar, 50 μm. (**b**, **g**) The cells were cultured for 9 days in serum-containing medium supplemented with β-GP (10 mM). The cells were then stained with Alizarin Red S. Whole 35-mm dishes are shown. Scale bar, 10 mm. (**c**, **h**) The cells were cultured for 5 days in serum-containing medium supplemented with γ-linolenic acid (100 μM). Numerous lipid droplets (arrows) were stained with Oil Red O. Nuclei were detected by staining with hematoxylin (blue). Scale bar, 10 μm. (**d**, **e**, **i**, **j**) KD3 (**d**, **e**) and TKD1 (**i**, **j**) cells were labeled with modified green fluorescent protein and then 1×10^6 cells were transplanted into the TA muscle of NOD/Scid mice. (**d**, **i**) Whole TA muscles were recovered at 4 weeks after transplantation. Scale bars, 1 mm. (**e**, **j**) Pathological views (**d**, **i**). Modified green fluorescent protein (green) and laminin α2 (red) were detected by immunofluorescence. Nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate. Passage numbers of cells are shown in (**a–c** and **f–h**). Scale bar, 20 μm.

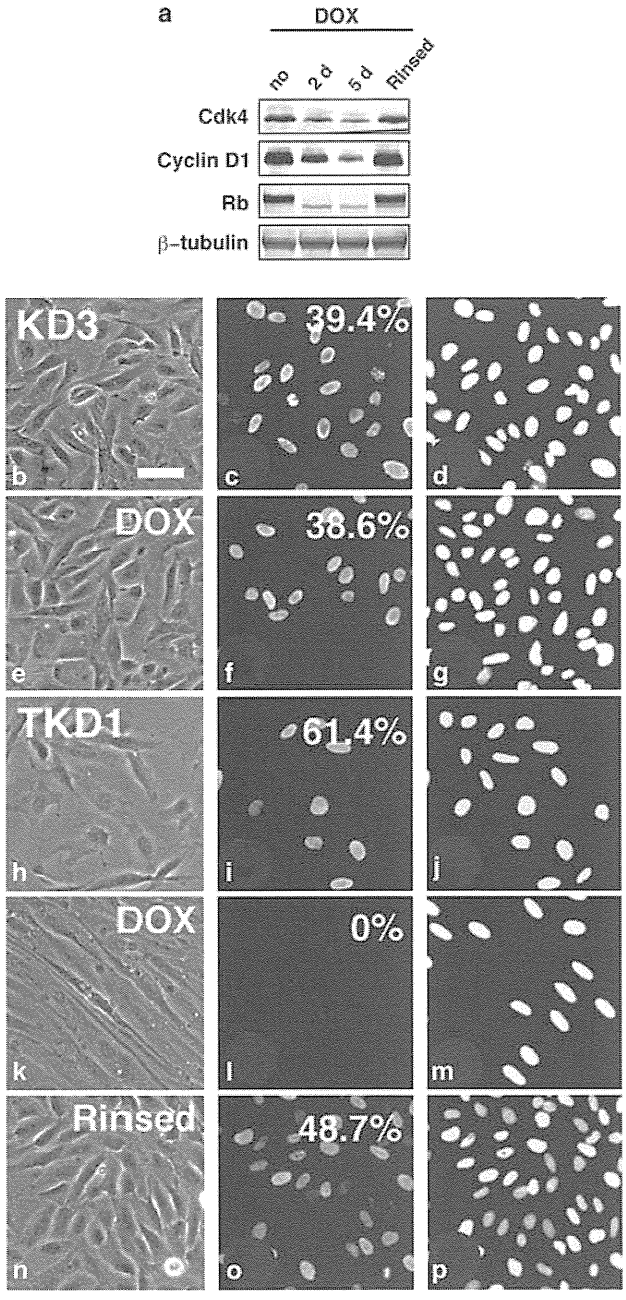


Figure 7 Reversible and precocious growth arrest induced by doxycycline in TKD1. (**a**) Fifteen micrograms of total proteins were subjected to immunobloting analysis with antibodies against CDK4, cyclin D1, Rb and β-tubulin. TKD1 cells were cultured for 2 days in medium containing 0.1% ethanol (vehicle) (lane 1), and for 2 days (lane 2) or 5 days (lane 3) in pmGM containing 250 nM doxycycline. For the recovery of CDK4 and cyclin D1, doxycycline was removed from the TKD1 culture after 5 days doxycyline treatment (lane 4). (**b–g**) KD3 cells were cultured for 2 days in medium containing 0.1% ethanol (vehicle) (**b–d**) or for 2 days in medium containing 250 nM doxycycline (**e–g**). TKD1 cells were cultured for 2 days in medium containing 0.1% ethanol (**h–j**), for 5 days in medium containing 250 nM doxycycline (**k–m**) or for 4 days in doxycycline-free medium following 5 days of culture in medium containing doxycycline (**n–p**). The cells were incubated for the last 6 h of culture in medium containing 10 nM 5-bromo-2′-deoxyuridine. The percentage of 5-bromo-2′-deoxyuridine-positive nuclei/total nuclei is shown in the panels (**c**, **f**, **i**, **l**, **o**). Phase contrast images (**b**, **e**, **h**, **k**, **n**), immunofluorescence analysis with anti-5-bromo-2′-deoxyuridine antibody (**c**, **f**, **i**, **l**, **o**), and nuclear staining with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (**d**, **g**, **j**, **m**, **p**) of the same fields are shown in each row. Scale bar, 50 μm.

Forced expression of CDK4R24C and cyclin D1 did not affect the differentiation potential of human myogenic cells, although forced expression of cyclin D1 alone inhibits myogenesis of the mouse myoblastic cell line C2C12.[31,32] Rb was completely dephosphorylated during the differentiation culture, even though CDK4R24C and cyclin D1 still remained at high levels in the immortalized human myogenic cells. CDK inhibitors $p21^{cip1}$ and $p27^{kip1}$ are unlikely to be involved in the suppression of CDK4R24C activity during terminal muscle differentiation because the amount of the inhibitors does not increase in human myogenic cells (Shiomi and Hashimoto, unpublished data). Therefore, the present results imply another novel pathway leading to the suppression of CDK4/cyclin D1 activity at the post-translational level in human myogenic cells.

Immortalized human myogenic cells that preserve normal differentiation potential have been reported in two previous studies.[9,13] However, the previously established human myogenic cell clones require 36–48 h for doubling, whereas primary cultured human myogenic cells divide every 20–30 h. In addition, one of them also required additional supplementation of the multifunctional steroid dexamethasone and hepatocyte growth factor, whose roles in immortalization process are unknown.[13] The other was established in our previous study with the use of oncogene product E7 for immortalization.[9] In contrast to previous ones, the present human myogenic cell clones retain a growth property similar to that of primary cultured human myogenic cells in the early passages, multipotentiality and normal diploid chromosomes. Therefore, the immortalized normal myogenic cells established in the present study are the human equivalent to mouse myogenic cell lines, and will contribute to fundamental and therapeutic studies.

The novel immortalization method established in the present study is more reliable and reproducible than the previously reported methods. We have succeeded in immortalization of several primary cultured human myogenic cells independently obtained from normal and diseased muscles including Duchenne muscular dystrophy and Fukuyama congenital muscular dystrophy (Hashimoto, unpublished data). Immortalized human myogenic cells from different neuromuscular diseases are currently being established in our laboratories and those of our collaborators. Human cell models of various neuromuscular diseases will contribute to causal analysis of symptoms and therapeutic approaches of rare diseases.

MATERIALS AND METHODS

Isolation and culture of human myogenic cells

The human myogenic cell clone Hu5 was isolated from normal subcutaneous muscle tissue of a 42-year-old woman,[4] and other human myogenic cells were obtained from normal abdominal muscle tissues of a 75-year-old man (Hu20, passage 2; A), a 50-year-old man (Hu21), a 69-year-old man (Hu23) and a 65-year-old man (Hu26). To prepare primary cultured human myogenic cells, muscle fragments were minced, digested with TrypLE Express (Invitrogen, Carlsbad, CA, USA) and then a small amount of cells obtained from 20–40 mg muscle were plated on a 90-mm dish coated with type I collagen (Sumilon, Tokyo, Japan). The cells were maintained at 37°C under 10% CO_2 in dishes coated with type I collagen and containing primary cultured myocyte growth medium (pmGM) consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 2% Ultroser G (Biosepra, Cedex-Saint-Christophe, France) and glucose (4.5 mg ml^{-1}). Cells were plated at 2×10^5 per 90-mm dish and cultured in pmGM. For induction of myogenic differentiation, the medium was changed to primary cultured myocyte differentiation medium after 48 h of culture; it consists of the chemically defined medium TIS[33,34] supplemented with 2% FBS.

For induction of terminal osteogenic differentiation, cells were cultured in DMEM supplemented with 10% FBS, glucose (4.5 mg ml^{-1}) and 10 mM β-glycerophosphate (β-GP) (Sigma, St Louis, MO, USA) alone. The cells were

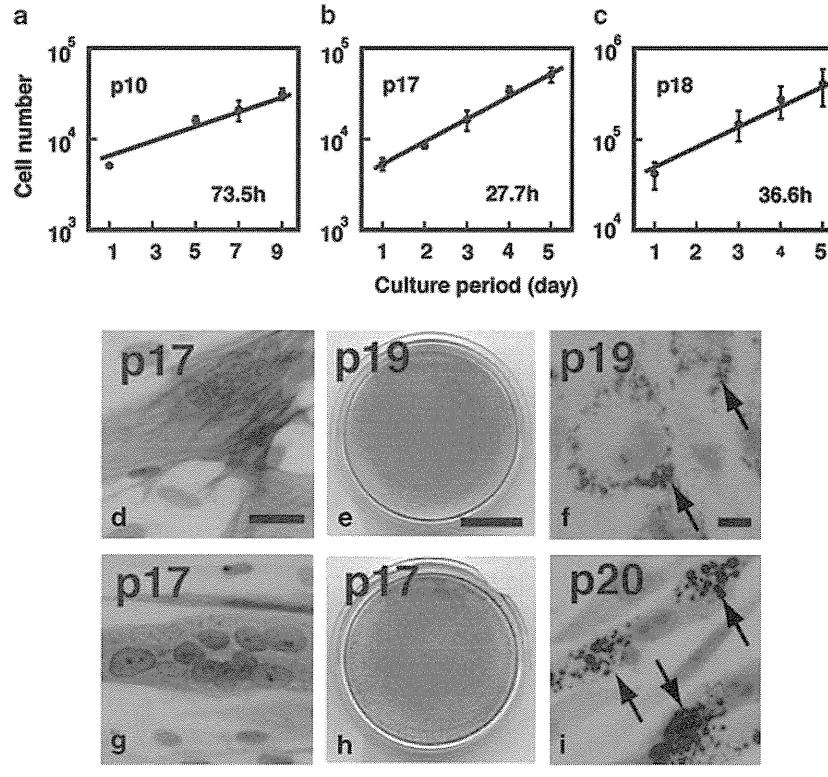


Figure 8 Recapture of proliferation capacity by myogenic cells derived from human muscle diseases. (**a–c**) Growth properties of primary cultured human myogenic cell clone HM2-5 obtained from muscle of a Leigh disease patient (**a**), immortalized clone HM255 derived from HM2-5 established by transduction with hTERT, CDK4R24C and Cyclin D1 (**b**), and immortalized clone HM253 derived from HM2-5 established by transduction with hTERT and E7 (**c**). Passage numbers and the doubling time were shown in the panels. (**d–i**) Multipotentiality of immortalized human myogenic cell clones derived from Leigh disease patients. HM255 (**d–f**) and HM253 (**g–i**) were induced to undergo myogenic, osteogenic and adipogenic differentiation. (**d**, **g**) Cells were cultured for 5 days in primary cultured myocyte differentiation medium. Myosin heavy chain was detected by immunostaining with a horseradish peroxidase reaction product. Nuclei were detected with staining with hematoxylin. Scale bar, 50 μm. (**e**, **h**) Cells were cultured for 9 days in serum-containing medium supplemented with β-GP (10 mM). Cells were then stained with Alizarin Red S. Whole 35-mm dishes are shown. Scale bar, 10 mm. (**f**, **i**) The cells were cultured for 5 days in serum-containing medium supplemented with γ-linolenic acid (100 μM). Numerous lipid droplets (arrows) were stained with Oil Red O. Nuclei were detected with staining with hematoxylin. Passage numbers of cells were shown in (**d–i**). Scale bar, 10 μm.

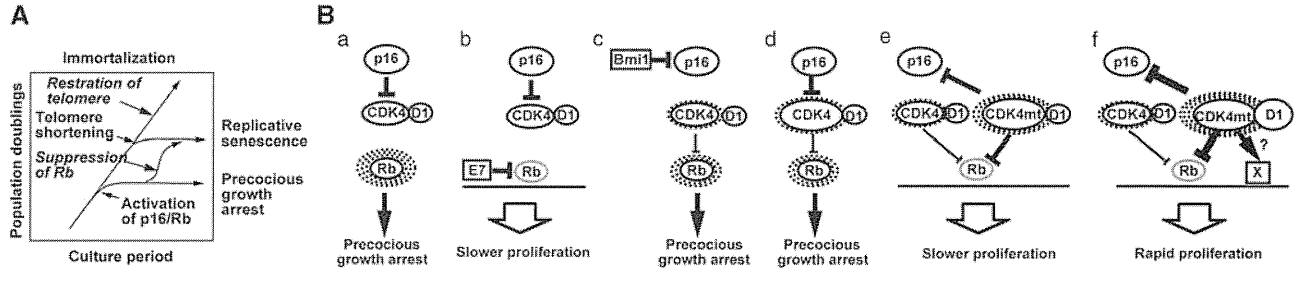


Figure 9 Premature growth arrest and replicative senescence of human myogenic cells. (**A**) Putative stress-induced activation of the $p16^{INK4a}$–Rb pathway triggers precocious growth arrest, independent of telomere shortening. Human myogenic cells under this state are able to recapture proliferation capacity by suppression of the Rb pathway. Telomere shortening also triggers activation of the Rb pathway and leads the cells to enter the irreversible growth arrest called replicative senescence. (**B**) Mechanistic scheme of suppression of precocious growth arrest by mutant CDK4 (CDK4R24C) and cyclin D1. (a) Putative stress-induced activation of $p16^{INK4a}$ inhibits endogenous CDK4, resulting in precocious growth arrest. (b) Papillomavirus type 16 gene *E7* suppresses Rb independently of p16. (c) Bmi-1 inhibits p16 expression. Endogenous CDK4 does not completely suppress Rb. (d) Forced expression of wild-type CDK4 sequesters p16, but does not completely suppress Rb because its kinase activity is inhibited by p16. (e) Forced expression of CDK4R24C sequesters p16, severely suppresses Rb, and allows human myogenic cells to proliferate slowly because CDK4R24C is not inhibited by p16. (f) Combined expression of CDK4R24C and cyclinD1 sequesters p16, induces hyperphosphorylation of Rb and allow human myogenic cells to proliferate rapidly, because the amount of cyclin D1 limits CDK4 kinase activity. A possibility that extraordinarily high activity of the CDK4R24C/cyclin D1 complex results in the phosphorylation of putative off-target substrates (represented as 'X') cannot be excluded. A putative action of the CDK4R24C/Cyclin D1 is represented as '?'. Dotted circles represent functional activity of Rb and CDK4.

stained with the calcium dye Alizarin Red S (2%, Sigma).[4] Images of stained dishes were obtained with a digital scanner (GT-9700F; Epson, Osaka, Japan) and then post-processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA). To induce adipogenic differentiation, we cultured myogenic cells in DMEM supplemented with 10% FBS, glucose (4.5 mg ml^{-1}) and 100 μM γ-linolenic acid (Sigma) for up to 5 days. The cells were stained with 0.3% Oil Red O (Sigma).[4]

Multiclonal populations of primary cultured myogenic cells HM1 and HM2, which were originally registered as M06-736 and M07-635, were obtained from biceps brachii muscles of Leigh disease patients, who were 3-month- and 5-year-old males, at the National Center of Neurology and Psychiatry (Kodaira, Japan). The mortal clones HM1-8 and HM2-5 were isolated from HM1 and HM2, respectively, at the National Center for Geriatrics and Gerontology. HM1 and HM2 had been cultured at 37°C under 5% CO_2 in non-coated standard tissue culture dishes containing DMEM/Ham's F12=1:1 supplemented with 20% FBS and glucose (4.5 mg ml^{-1}) alone, and cryopreserved at the National Center of Neurology and Psychiatry. The cells were cultured under the same conditions as Hu5 in the present study.

Viral vector construction and viral transduction

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen). Briefly, the EF1a promoter in CSII-EF-RfA (a gift from Dr H Miyoshi, RIKEN) was replaced with a tetracyclin-inducible promoter, TRE-Tight, from pTRE-Tight (Clontech, Mountain View, CA, USA) to generate CSII-TRE-Tight-RfA. Human cyclin D1 and human mutant CDK4 (CDK4R24C: an INK4a resistant form of CDK4, generously provided by Dr E Hara) were first recombined into entry vectors by a BP reaction (Invitrogen). Then these segments were recombined with CSII-TRE-Tight-RfA by an LR reaction (Invitrogen) to generate CSII-TRE-Tight-cyclin D1 and -CDK4R24C. The rtTA segment from pTet-Off Advanced (Clontech) was amplified by PCR and first recombined with the donor vector pDONR221 by BP reaction (Invitrogen) to generate pENTR221-TetOff, and then recombined with a lentiviral vector, CSII-CMV-RfA, by LR reaction (Invitrogen) to generate CSII-CMV-TetOff. Construction of CSII-CMV-cyclin D1, -CDK4R24C and -hTERT was described previously.[35] The recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein were produced as described previously.[36] The recombinant retroviruses encoding hTERT and E7 were produced as described previously.[9,37]

Hu5, HM1-8 and HM2-5 cells were transduced with recombinant lentiviruses and retroviruses as described.[9,11,35] Following inoculation with viruses, the continuously proliferating cells were selected without drug treatment.

For single-cell cloning, transfected Hu5 cells were suspended at 5 cells per ml, and then 100 µl of the cell suspension was dispensed to each well of a 96-well plate coated with collagen, so that each well contained zero or one cell. Single-cell-derived clones were isolated and expanded for experimentation. The immortalized human myogenic cell clone KD3 will be available from RIKEN BioResorce Center (http://www.brc.riken.go.jp).

Analysis on growth properties

In total, 2000 cells were plated per well of a 12-well plate coated with type I collagen. Cells were collected and cell numbers were counted every 24 h between days 3 and 8 of culture in pmGM. Averages and s.d.'s of cell numbers per well from three independent wells were estimated.

To detect synthesizing DNA, cells were incubated with 10 µM 5-bromo-2′-deoxyuridine (Sigma) for the last 6 h of each culture, fixed in paraformaldehyde for 10 min and then subjected to immunofluorescence analysis after denaturation of DNA with 2 M HCl and neutralization with 0.1 M $Na_2B_4O_7$ according to the manufacturer's instructions (Roche Diagnostics, Indianapolis, IN, USA).

Karyotyping

After incubation in pmGM supplemented with 2 µM colcemid at 37°C for 6 h, cells were trypsinized and incubated in 0.5 ml of 1% sodium citrate for 15 min. This was followed by addition of 0.5 ml of Carnoy's fixative (methanol/acetic acid, 3:1 by volume). The fixed cells were then spun down and resuspended in 0.5 ml of Carnoy's fixative. Metaphase chromosomes were stained with 10% Giemsa solution (Wako Pure Chem., Osaka, Japan) for 10 min.

Immunobloting analysis

Sample preparation and immunoblot analysis were performed as previously described.[33,34,38] Immune complexes were detected by colorimetry with a BCIP/NBT detection kit (Nacalai, Kyoto, Japan) or an ECL kit (GE Healthcare, Piscataway, NJ, USA). Primary antibodies included mouse monoclonal antibodies to chicken sarcomeric myosin heavy chain (MF20, undiluted culture supernatant),[39] $p16^{INK4a}$ (BD Bioscience, Franklin Lakes, NJ, USA), $p21^{cip1}$ (Merk KGaA, Darmstadt, Germany), p53 (Merk), Rb (BD Bioscience), CDK2 (8A12, Medical Biological Laboratory, Nagoya, Japan), cyclin D1 (BD Bioscience) and β-tubulin (GE Healthcare), and a rabbit polyclonal antibody to CDK4 (Hashimoto, unpublished). Secondary antibodies included alkaline phosphatase (DAKO, Carpinteria, CA, USA)—or horseradish peroxidase (GE Healthcare)—labeled antibodies to mouse or rabbit immunoglobulin G. Immune complexes on the PVDF membranes (Fluoro Trans W; Pall, Port Washington, NY, USA) were scanned with a digital scanner (GT-9700F; Epson) or LAS-4000 IR multicolor (Fujifilm, Tokyo, Japan) and then post-processed using Adobe Photoshop (Adobe Systems).

Transplantation of human myogenic cells

Immortalized human myogenic cells were labeled with modified Venus green fluorescent protein by transduction with a lentivirus vector, CSII-CMV-MCS-IRES2-Venus (kindly provided by Dr Miyoshi). Tibialis anterior (TA) muscles of 10-week-old female NOD/Scid mice were injected with 20 µl of 10 µM cardiotoxin (Wako Pure Chem.).[40] On the next day, 1×10^6 of the Venus-labeled cells suspended in 30 µl of L-15 (Sigma) were transplanted into the regenerating TA muscle. At 4 weeks after transplantation, the TA muscles were removed and quickly frozen in isopentane cooled with liquid nitrogen and processed for preparation of cryosections as described.[41] Muscle specimens were sectioned at a thickness of 7 µm with a cryostat.

Immunofluorescence analysis

The frozen sections and cultured cells were fixed with 4% paraformaldehyde at 4°C for 30 or 10 min, respectively, and then incubated with primary antibodies. Primary antibodies included those to mouse monoclonal antibodies to mouse MyoD (5.8A, 1:10 dilution, Novocastra, Newcastle, UK), myosin heavy chain (undiluted supernatant), laminin α2 (1:100 dilution, Enzo Life Science, Farmingdale, NY, USA), 5-bromo-2′-deoxyuridine (1:50 dilution, Roche Diagnostics) and rabbit polyclonal antibodies to green fluorescent protein (1:500 dilution, Medical Biological Laboratory) and Ki-67 (1:2 dilution, YLEM, Rome, Italy). Secondary antibodies were biotinylated Alexa 488 or Cy3-labeled antibodies to mouse, rat (Jackson ImmunoResearch Laboratory, Bar Harbor, ME, USA) or rabbit (Molecular Probes, Eugene, OR, USA). The biotinylated antibodies were detected with streptavidin-conjugated horseradish peroxidase. The peroxidase reaction was performed with 3,3-diaminobenzidine (Sigma). Cell nuclei were stained with 2,4-diamidino-2-phenylindole dihydrochloride *n*-hydrate (1.0 µg ml^{-1}, Sigma) or hematoxylin (Wako). Samples were visualized using an upright microscope (model BX50; Olympus, Tokyo, Japan) and a CCD camera (DP70; Olympus), or an inverted microscope (model IX71; Olympus) and a CCD camera (DP70; Olympus). Images were post-processed using Adobe Photoshop (Adobe Systems).

Suppression and induction of gene expression using Tet-Off system

TKD1 cells (5×10^4 cells per 35-mm dish) were cultured for 2 days in pmGM and then the medium was changed to pmGM supplemented with 250 nM doxycycline (Sigma). To remove doxycyline from the culture, the cells were replated twice and cultured in pmGM (Roche Diagnostics) according to the manufacturer's instructions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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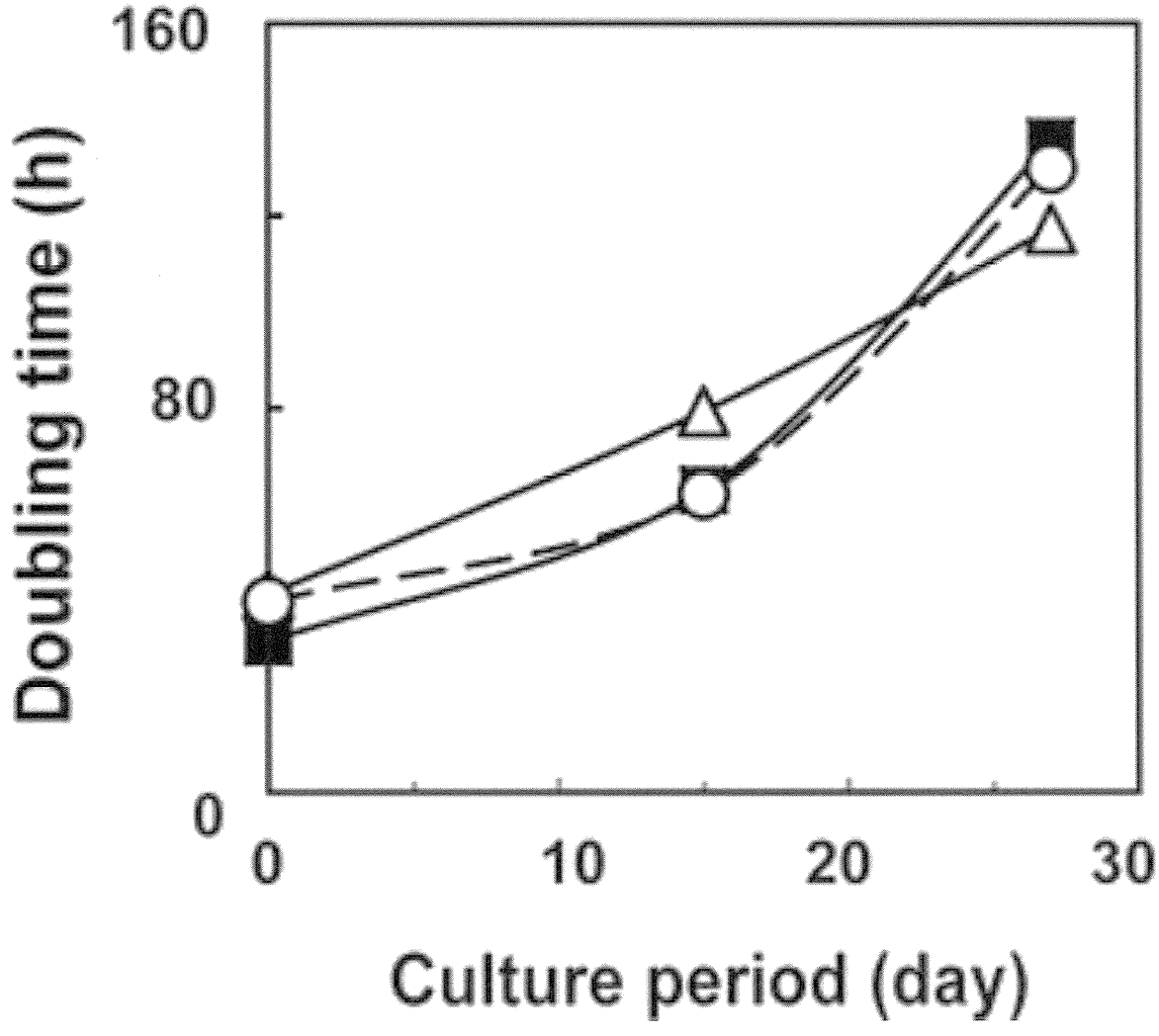
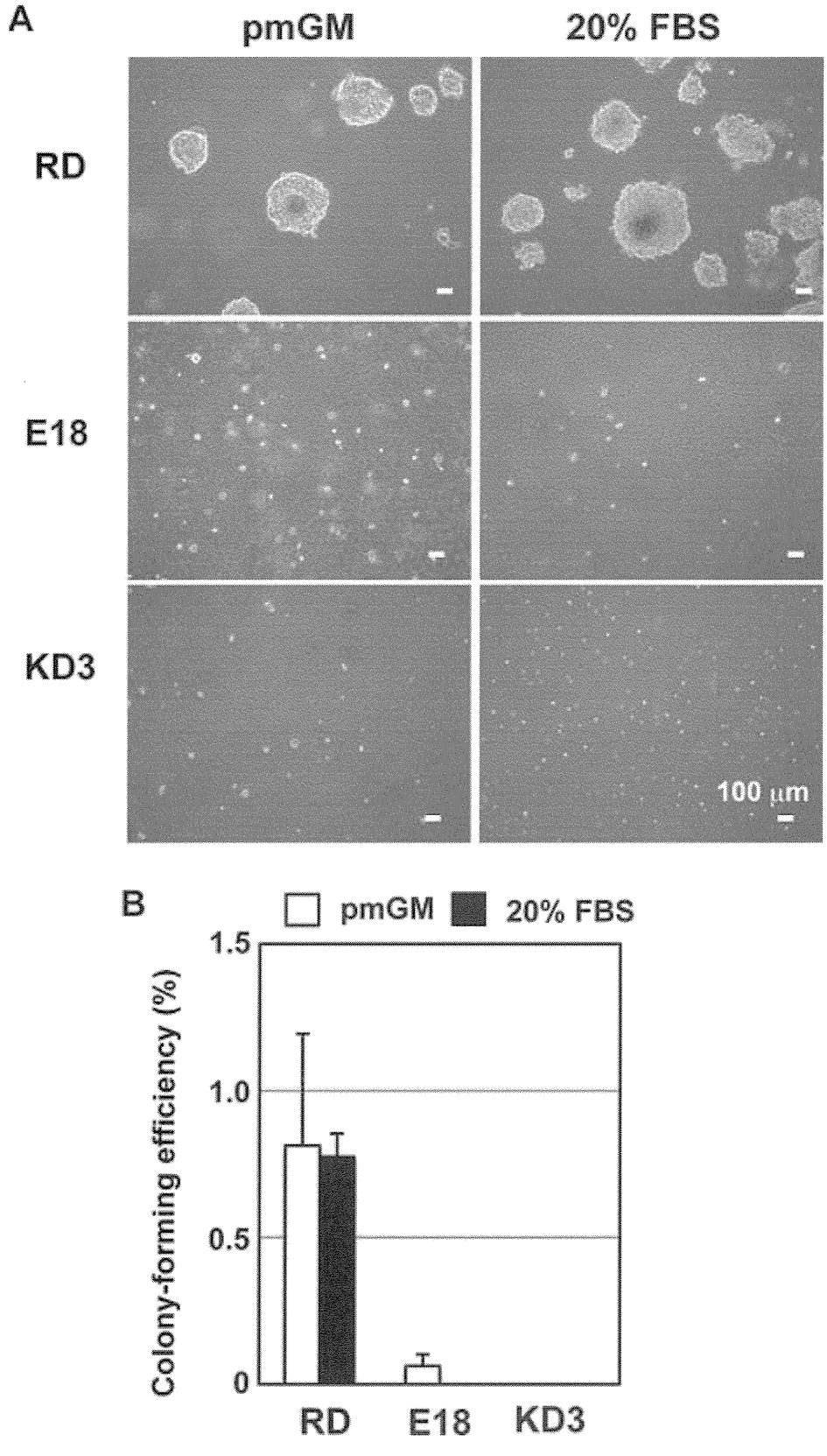
Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)

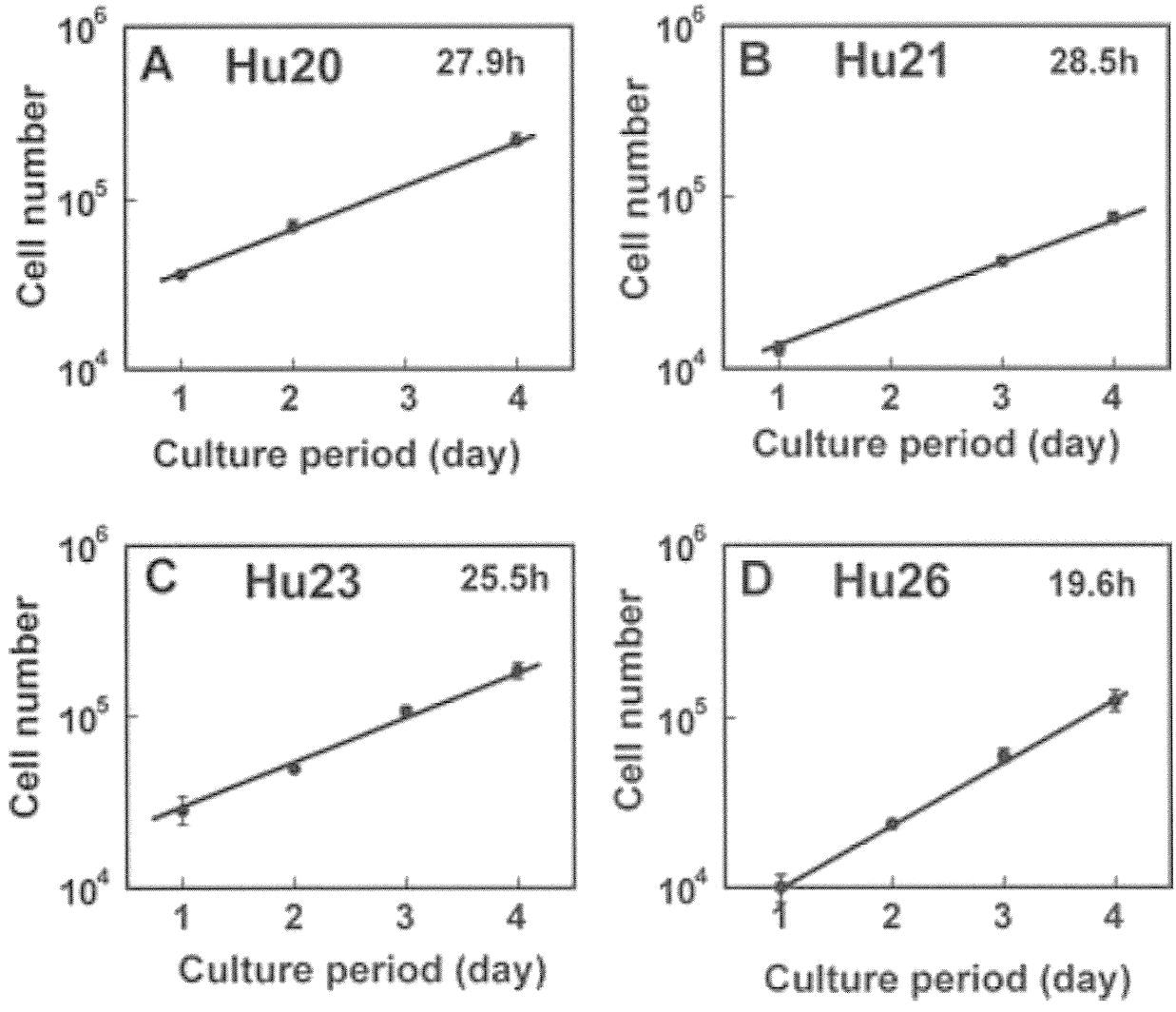
Supplementary Figure 1 *Shi*



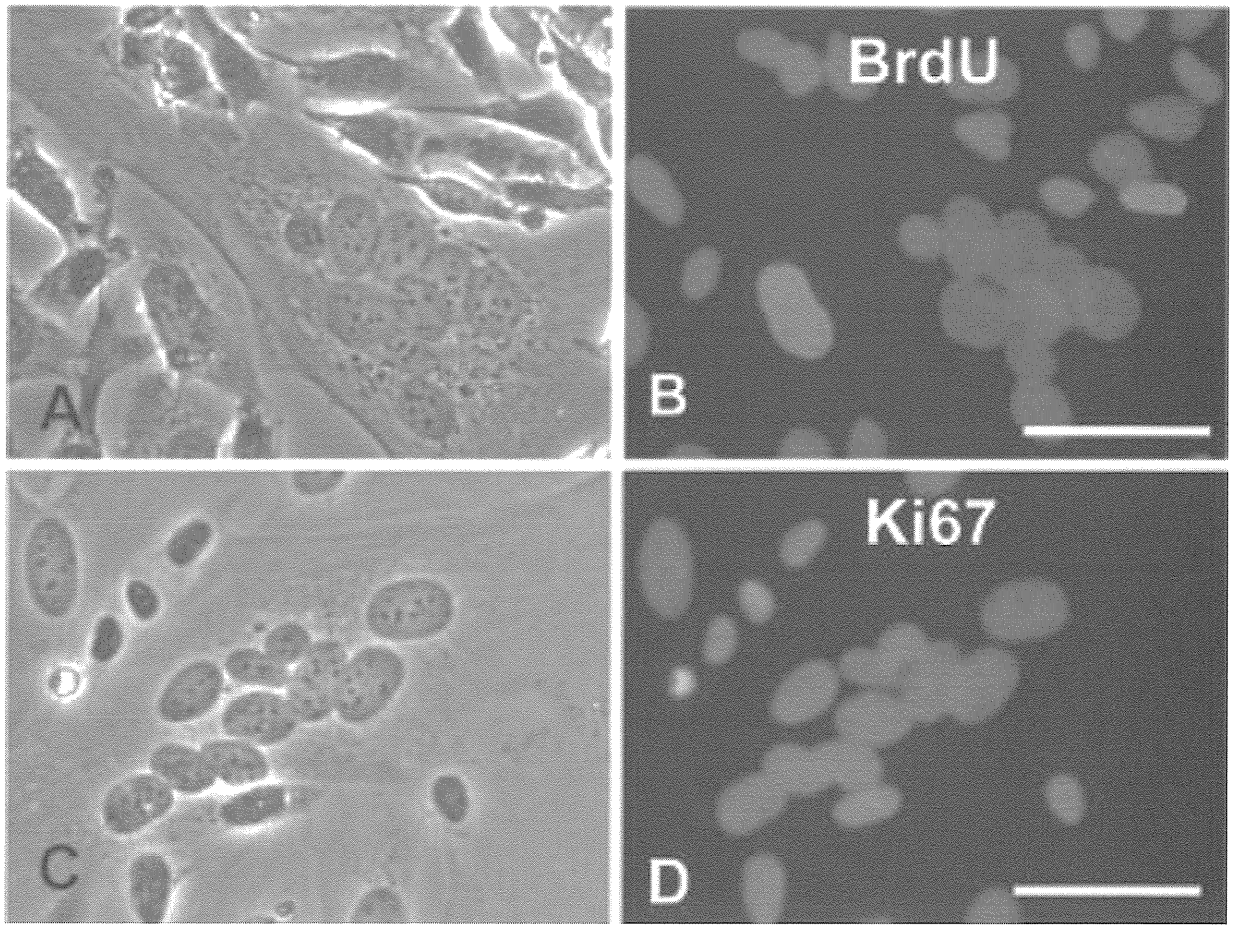
Supplementary Figure 2



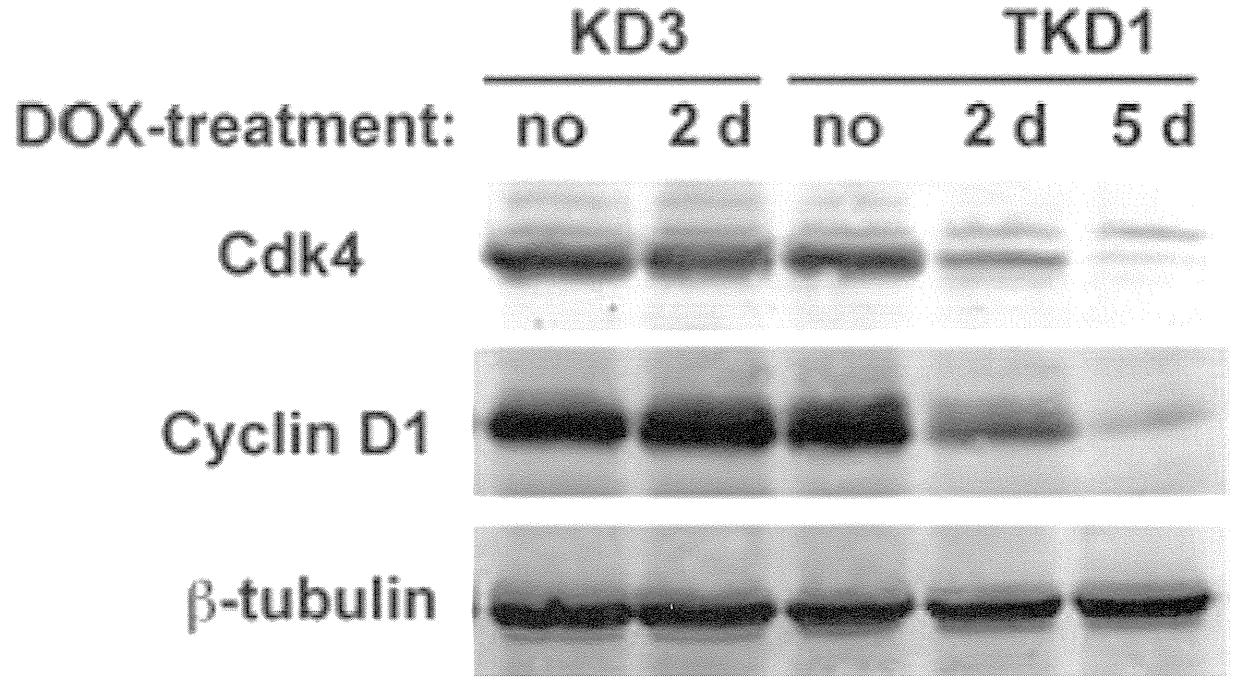
Supplementary Figure 3



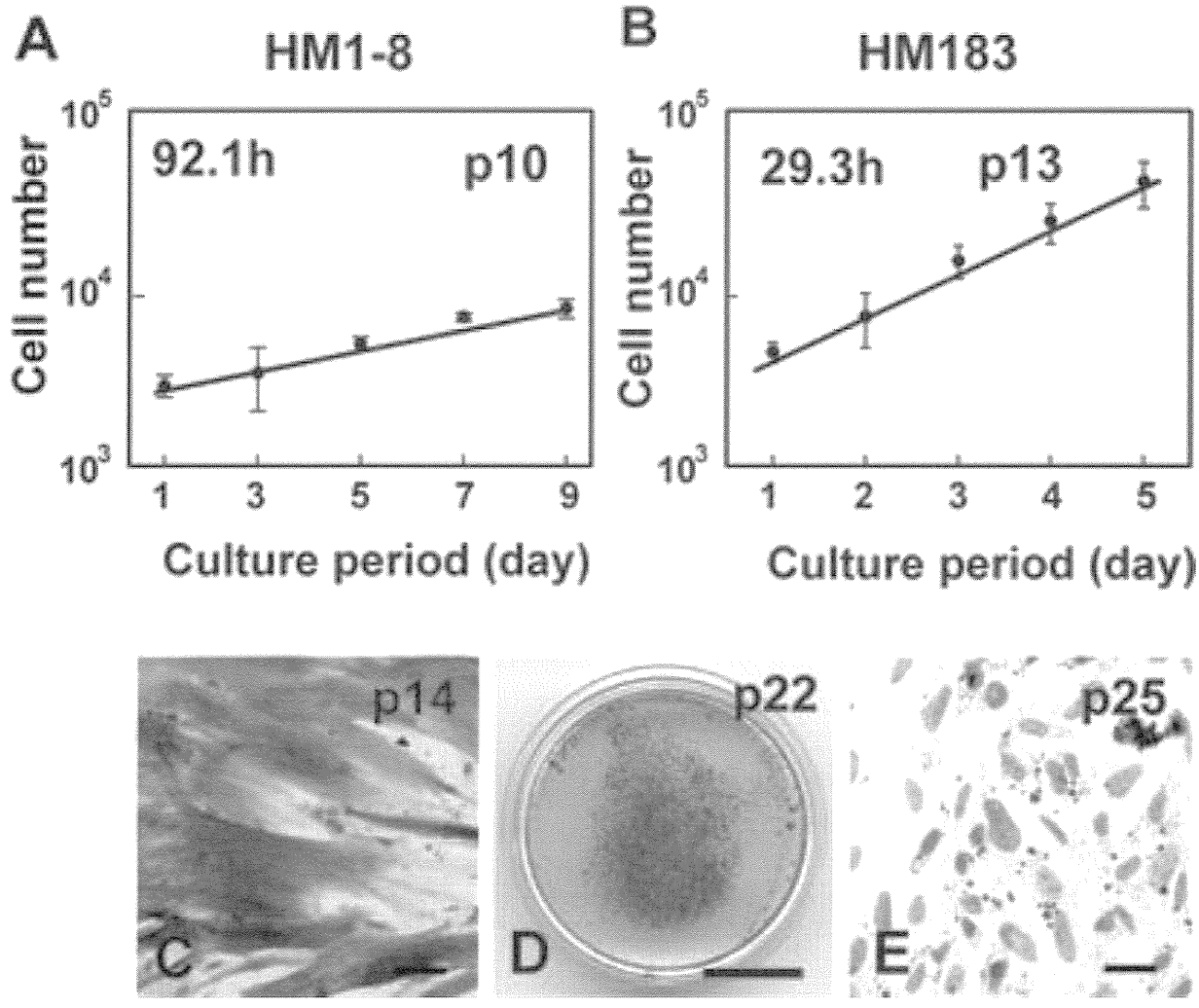
Supplementary Figure 4



Supplementary Figure 5



Supplementary Figure 6



Explanation of Supplementary Figures

Supplementary Figure 1 Doubling time of primary cultured human myogenic cells Hu20 (filled square), Hu23 (triangle) and Hu26 (circle) was determined at passage 3, 7, and 10. Estimates of doubling time were based on cell numbers.

Supplementary Figure 2 Immortalized human myogenic cells did not grow in soft agar. E18, KD3, and rhabdomyosarcoma cell line RD were seeded at 5 x 10^4 pr 35-mm plate in an appropriate medium (pmGM or DMEM supplemented with 20% FBS alone) with 0.4% agarose and a 0.7% agarose underlay as described (Narisawa-Saito et al., Oncogene 26, 2988-96, 2007). Colonies over 150 μm in diameter were photographed (A) and counted (B) after 11 days. The experiments were performed in triplicate. Colony-forming efficiency represents [colony number]/[seeded cell number] (%).

Supplementary Figure 3 Growth properties of primary cultured human myogenic cells. Primary cultured human myogenic cells were obtained from abdominal muscles of 75-year-old man (Hu20, passage 2; A), 50-year-old man (Hu21, passage 2; B), 69-year-old man (Hu23, passage 2; C) and 65-year-old man (Hu26, passage 2; D), and then cultured in pmGM. Estimates of doubling time were based on cell numbers.

Supplementary Figure 4 Cell cycle exit of KD3 during terminal differentiation. Immortalized human myogenic cell clone KD3 was cultured for up to 78 h in pmDM. To detect DNA synthesis, cells were incubated with 10 μM BrdU for the last 6 h of a