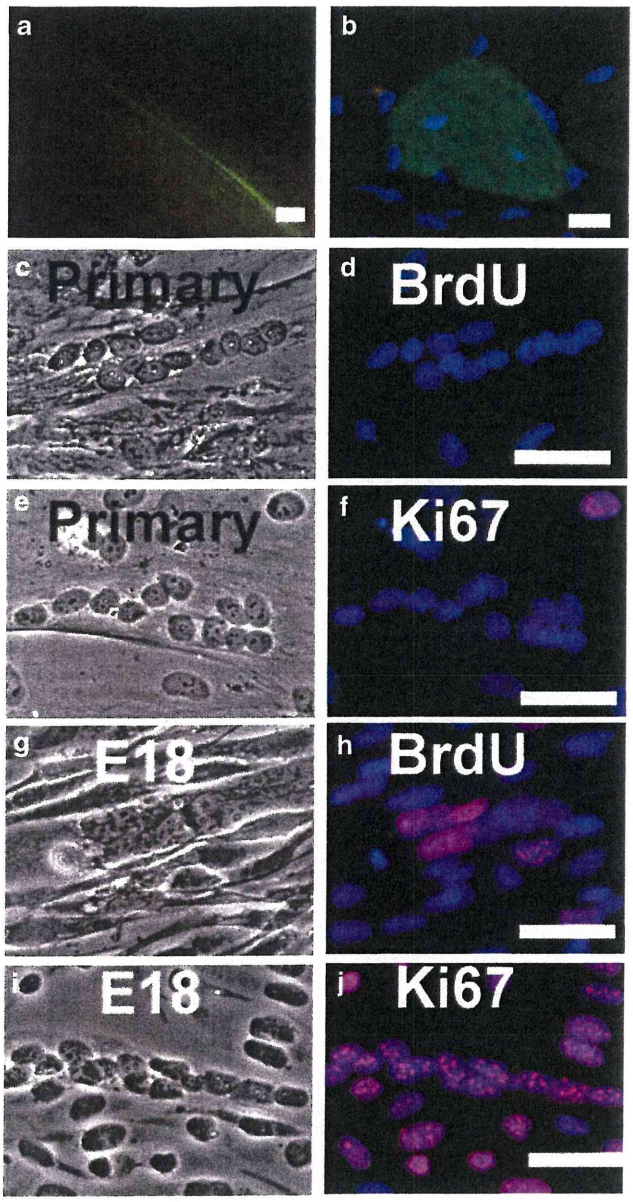


Figure 2 Nuclear progression in terminally differentiated immortalized human myogenic cells expressing telomerase and E7. (**a**, **b**) E18 cells were labeled with modified green fluorescent protein, and then 2.5×10^6 cells were transplanted into the TA muscles of NOD/Scid mice. (**a**) Whole TA muscles 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

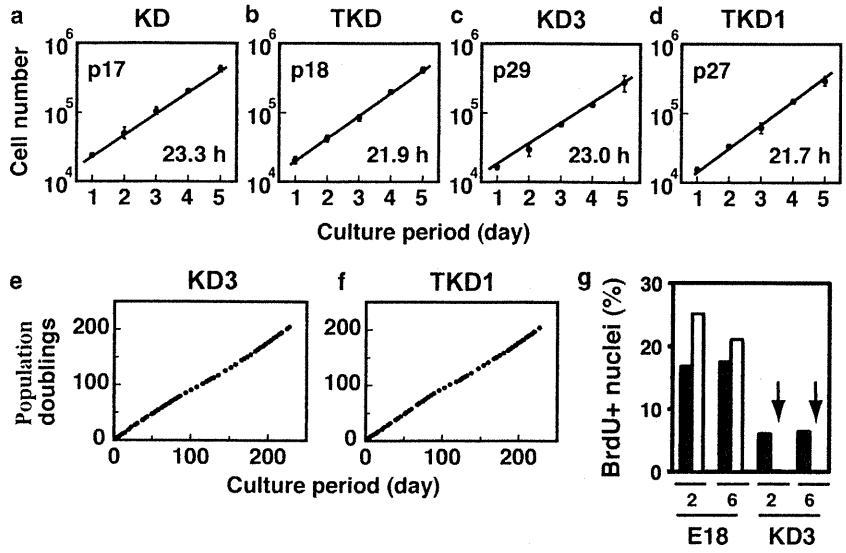


Figure 3 Proliferation of immortalized human myogenic cells. (**a–d**) Growth properties of a multiclonal population named KD, expressing hTERT, CDK4R24C and cyclin D1 under the control of a cytomegalovirus promoter (**a**), a multiclonal population named TKD, expressing hTERT, CDK4R24C and cyclin D1 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.

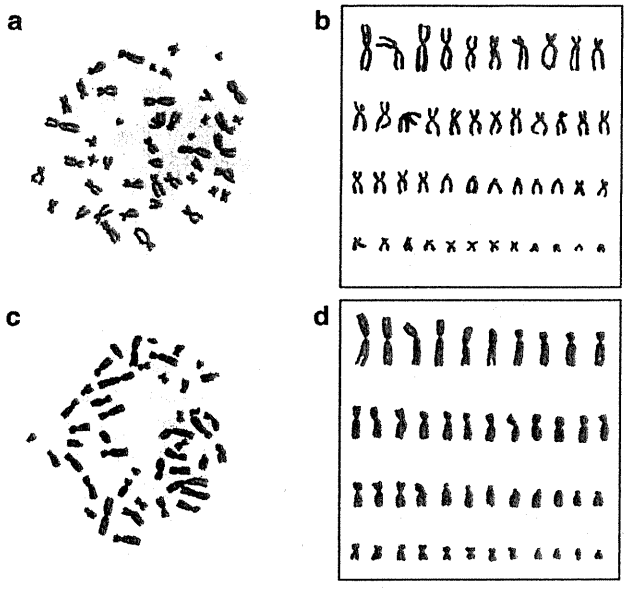
10.2 ± 9.1% of total TA myofibers, respectively) (Figures 6d and i). The relatively large s.d. in the present results was because of the low numbers of positive myofibers in the two specimens, probably due to leakage of the transferred cells to the injected TA muscle. Venus-positive myofibers were regenerated 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.

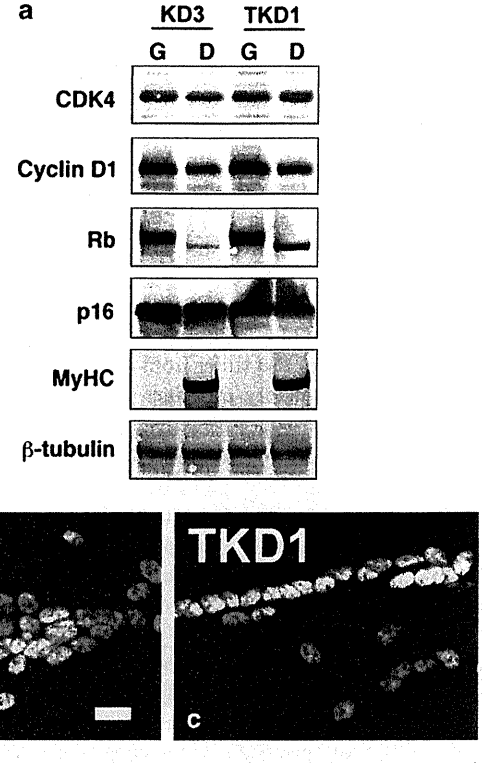


Figure 5 Expression patterns of growth- and differentiation-related proteins in immortalized human myogenic cells. (**a**) KD3 and TKD 1 cells were cultured in pmGM (g) or in primary cultured myocyte differentiation medium for 5 days (d). Fifteen micrograms of total proteins were subjected to immunobloting 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).

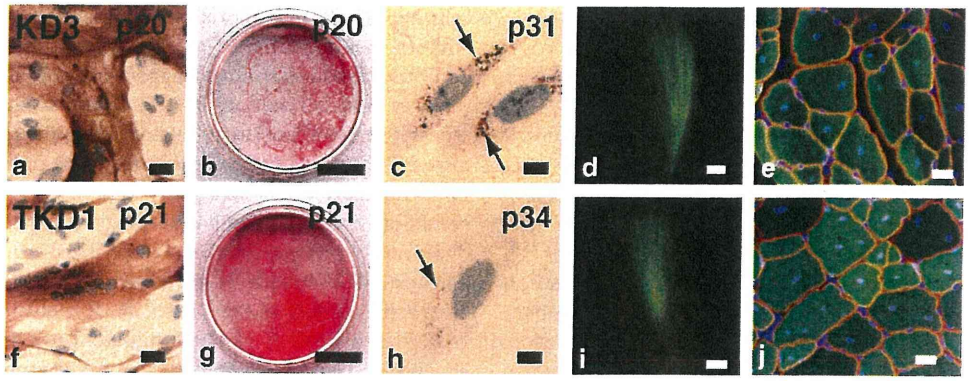


Figure 6 Multipotentiality of immortalized human myogenic cell clones KD3 and TKD1. KD3 (**a–e**) and TKD1 (**f–j**) were induced to undergo myogenic, osteogenic and adipogenic differentiation. (**a**, **f**) Cells were cultured for 5 days in primary cultured myocyte differentiation medium. Myosin heavy 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.

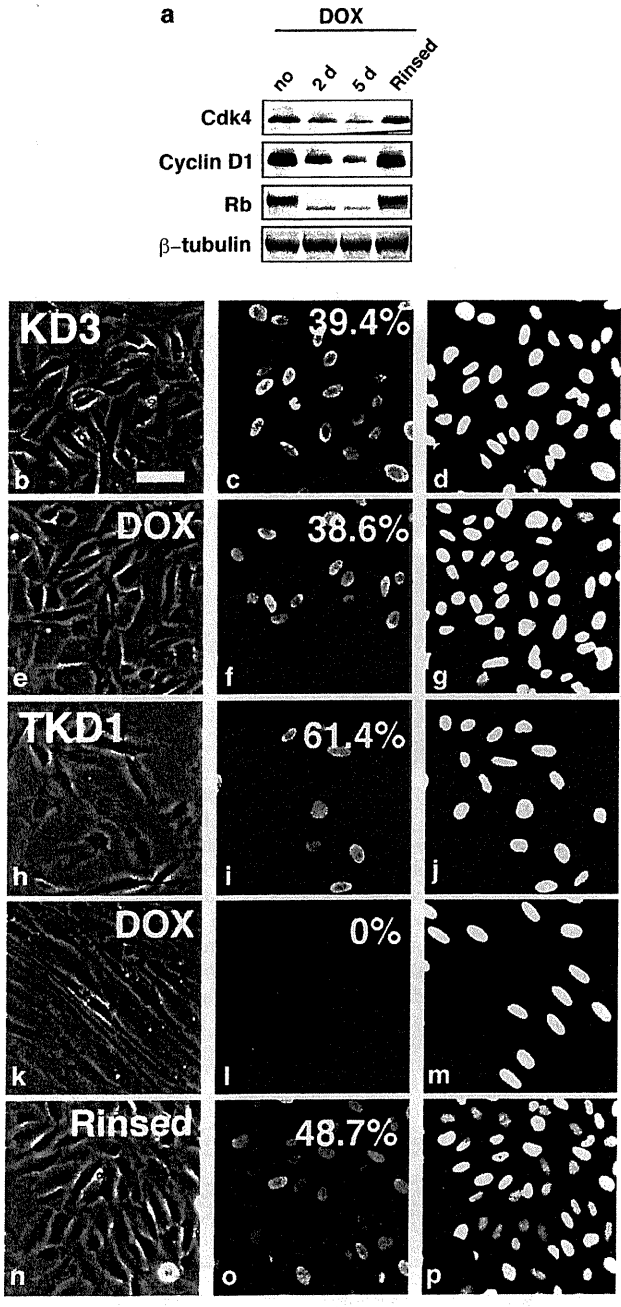


Figure 7 Reversible and precocious growth arrest induced by doxycycline in TKD1. (**a**) Fifteen micrograms of total proteins were subjected to immunobloting analysis with antibodies against CDK4, cyclin D1, Rb and β-tubulin. TKD1 cells were cultured for 2 days in medium containing 0.1% ethanol (vehicle) (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

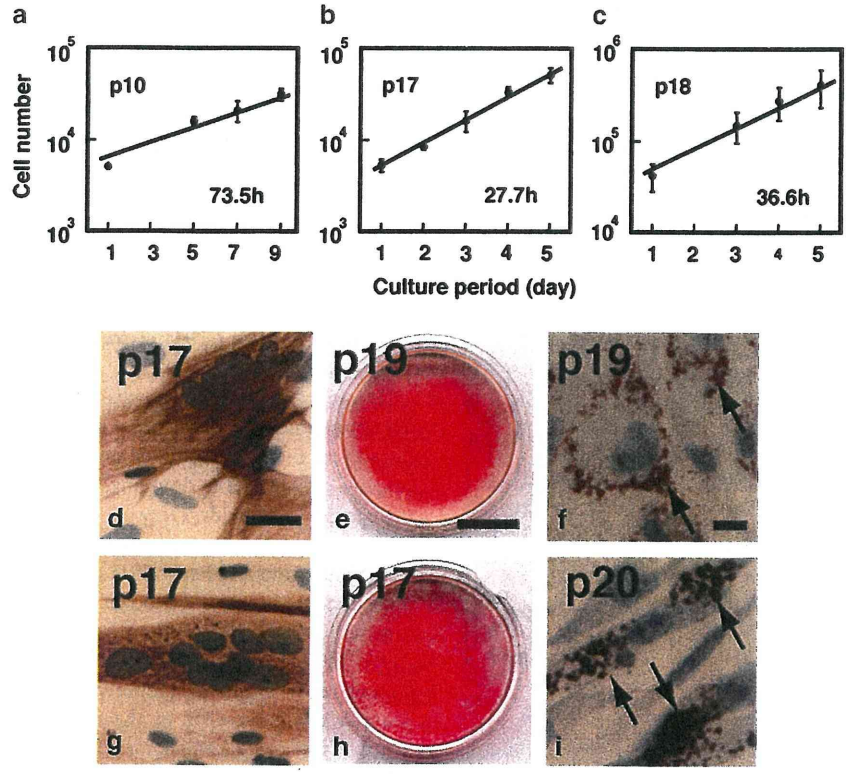


Figure 8 Recapture of proliferation capacity by myogenic cells derived from human muscle diseases. (**a–c**) Growth properties of primary cultured human myogenic cell clone HM2-5 obtained from muscle of a Leigh disease patient (**a**), immortalized clone HM255 derived from HM2-5 established by transduction 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.

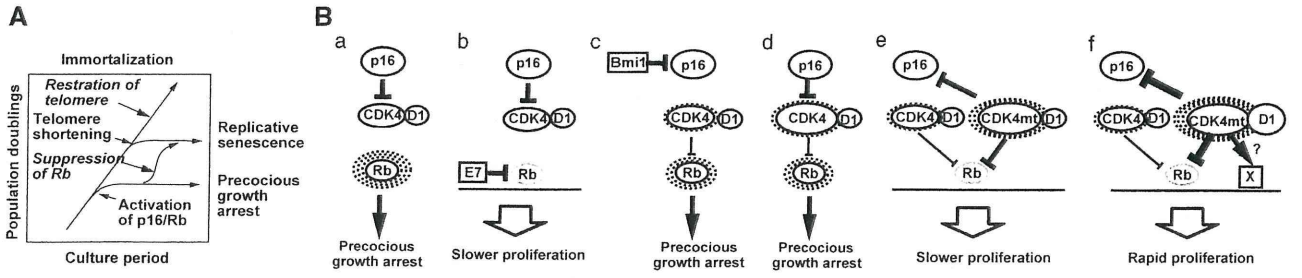


Figure 9 Premature growth arrest and replicative senescence of human myogenic cells. (**A**) Putative stress-induced activation of the $p16^{INK4a}$–Rb pathway triggers precocious growth arrest, independent of telomere shortening. Human myogenic cells under this state are able to recapture proliferation 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.

ACKNOWLEDGEMENTS

We thank H Miyoshi for providing lentivirus vectors. This study was supported by grants to NH and TK from the Ministry of Health, Labor and Welfare of Japan.

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

DOI:10.1158/1078-0432.CCR-10-2291

Clinical Cancer Research

Activation of NF-κB Is a Novel Target of *KRAS*-Induced Endometrial Carcinogenesis

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Abstract

Purpose: Although the *KRAS* mutation is one of critical genetic alterations in endometrial carcinogenesis, the downstream targets are not known.

Experimental Design: In this study, we investigated the molecular targets of *KRAS* signals, using tumorigenic cells with oncogenic *KRAS* mutation established from telomerase reverse transcriptase (*TERT*)-immortalized endometrial epithelial cells.

Results: We first confirmed that the RAF-ERK pathway, but not the PI3K-Akt pathway, was activated in *KRAS* tumorigenic cells. However, the introduction of constitutively active MAP/ERK kinase into immortalized cells to mimic RAF-ERK activation failed to obtain tumorigenic phenotypes, indicating the existence of other carcinogenic pathways triggered by *KRAS*. Recent evidence suggestive of linkage with *KRAS* signals prompted us to examine the involvement of NF-κB in endometrial carcinogenesis. We found that the DNA-binding activity of NF-κB was markedly elevated in *KRAS* tumorigenic cells compared with *TERT*-immortalized cells. Furthermore, the ability of NF-κB to activate the target gene promoters significantly increased in *KRAS* tumorigenic cells. Introduction of a mutant IκB that is resistant to degradation and thereby enhances the inhibitory effect on NF-κB largely abrogated the transformed phenotypes of *KRAS* tumorigenic cells. Thus, oncogenic *KRAS* signals contributed to the tumorigenic phenotypes of endometrial cells by activating the transcription function of NF-κB.

Conclusions: These findings clearly show that NF-κB activation is a novel target of oncogenic *KRAS* in endometrial carcinogenesis, implying the potential utility of NF-κB inhibitors for endometrial cancer chemoprevention, especially with *KRAS* mutation. *Clin Cancer Res; 17(6); 1341–50. ©2011 AACR.*

Introduction

The genetic alterations frequently observed in endometrial cancer involve microsatellite instability and mutations in *PTEN*, *PIK3CA*, *β-catenin*, and *KRAS*, whereas a relatively small percentage of endometrial cancers have *p53* mutations (1, 2). Because some of these gene mutations, including *KRAS* mutation, were detected in precursor lesions, they are thought to be early events in endometrial carcinogenesis (1–4). Ras signals activate various effector pathways in a species- or tissue-specific manner (5). However, the Ras downstream signals essential for endometrial carcinogenesis remain unclear.

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doi: 10.1158/1078-0432.CCR-10-2291

The study of human tumor specimens has provided much of our current understanding of the molecular basis of carcinogenesis. However, most human cancers harbor complex karyotypes and multiple genetic mutations, so the specific types and mechanisms of genetic alterations contributing to carcinogenesis remain unclear. One potential way to overcome these issues is to develop a carcinogenesis model, using defined genetic elements. We have previously created an *in vitro* model of endometrial carcinogenesis in which purified endometrial epithelial cells were immortalized by stably introducing HPV16 *E6* and *E7* and the catalytic subunit of telomerase (*hTERT*; resulting in EM-E6/E7/TERT cells; ref. 6), followed by the additive introduction of oncogenic *KRAS* alleles to obtain tumorigenic cells with anchorage-independent growth and tumorigenicity on nude mice (EM-E6/E7/TERT/RAS cells; ref. 7). One of the most important characteristics of the EM-E6/E7/TERT/RAS cells is their genetic purity with intact chromosomes. Therefore, these immortalized and tumorigenic endometrial epithelial cell lines created with defined genetic rearrangements are advantageous and available for analyzing the oncogenic pathways of endometrial carcinogenesis.

NF-κB has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory

DOI:10.1158/1078-0432.CCR-10-2291

Translational Relevance

The signal transduction or oncogenic pathways in endometrial carcinogenesis remain unclear, although some genetic factors, including *PTEN* and *KRAS* mutations and microsatellite instability, have been identified to play etiologic roles in the development of this tumor type. Most researchers believed that KRAS-ERK1/2 pathway plays central roles in it, but few studies have directly proved it. In this study, we for the first time found that the conventional KRAS-ERK1/2 pathway is insufficient for endometrial carcinogenesis and that NF-κB is a critical target of KRAS-induced endometrial carcinogenesis. This information implies the novel molecular mechanisms of endometrial carcinogenesis and the future therapeutic direction for cancer prevention by suppressing this novel pathway, such as with NF-κB inhibitors.

responses. Accumulating evidence supports a key role of the constitutive activation of NF-κB in controlling the initiation and progression of human cancer (8). NF-κB has also been documented both to be activated downstream of oncogenic Ras signals in some types of human cancers and to participate in the transformation of rodent cells (9–11). However, the role of NF-κB in endometrial carcinogenesis remains unclear. In this study, we show for the first time that NF-κB activation plays a central role in *KRAS*-mediated endometrial carcinogenesis.

Materials and Methods

Electrophoretic mobility shift assay

The nuclear extracts were prepared as previously described (12). A consensus oligonucleotide containing the NF-κB binding site (Promega) was end labeled with the kit (MEGALABEL; Takara Bio Inc.). For the assay, 50 μg of nuclear protein extract was incubated for 30 minutes at room temperature in a final volume of 25 μL containing 10,000 cpm of labeled oligonucleotides, 1 μg of poly (dI-dC), 0.5 mmol/L of phenylmethylsulfonylfluoride, 1 mmol/L of dithiothreitol, 10% glycerol, 25 mmol/L of HEPES (pH 7.9), and 50 mmol/L of KCl. DNA–protein complexes were then separated from free probes by electrophoresis on a 5% polyacrylamide gel. For competition assays, 100-fold molar excess of unlabeled consensus oligonucleotides for AP2, SP1, or NF-κB were used as competitors. For supershift assays, the nuclear extracts were incubated with specific antibodies against NF-κB for 30 minutes before addition of the labeled oligonucleotides. Antibodies against p65 (sc-109X) and p50 (sc-114X) were purchased from Santa Cruz Biotechnology, Inc.

Luciferase reporter assay

Cells were cultured in 24-well culture plates and transfected with 0.4 μg of luciferase reporter plasmid driven by NF-κB–responsive elements (Panomics, Inc.), using Lipofectamine Plus (Invitrogen Corp.), according to the manufacturer's protocol. After 48 hours of incubation, the cells were harvested in passive lysis buffer (Promega) and luciferase assays were carried out. To examine the role of IKK (IκB kinase complex) pathways in promoter activation, 5 μmol/L of IKK inhibitor X (Calbiochem) was added to the medium after the reporter transfection. All experiments were carried out at least 3 times, and the results represent average relative luciferase activity.

Establishment of stable transfectants

The plasmid encoding a constitutively active mutant of *MEK1* (HA-MEK1DD; ref. 13) was kindly provided by Dr. S. Meloche (Université de Montréal, Quebec, Canada). HA-MEK1DD and the mutant IκBα cDNA-encoding superrepressor (IκBα-SR) harboring S32A and S36A mutations (Clontech; catalogue no. 6319233) were cloned and recombined into retroviral vectors to generate pCMSCVpuro-HA-MEK1DD and pCMSCVbsd-IκBα (Ser32/36Ala) as described previously (14). The production and infection of recombinant retroviruses have been described previously (6). These retroviruses and backbone vectors were infected into EM-E6/E7/TERT/RAS cells. The infected cells were selected in the presence of 1 mg/mL of puromycin and 8 mg/mL of blasticidin S.

Immunoblot and immunoprecipitation

Whole-cell extracts were prepared as previously described (12), with specific antibodies against phospho-p44/42MAPK (Thr202/Tyr204), Akt, phospho-Akt (Ser473), phospho-NF-κB p65 (Ser536), phospho-NF-κB p65 (Ser276), IκBα (Cell Signaling Technology), IκBε (Abcam), IκBβ (Delta Biolabs), NF-κB p65 phospho-Ser529 (Millipore), NF-κB p52, NF-κB p50, and actin (Santa Cruz Biotechnology). The LAS3000 CCD-Imaging System (Fujifilm Co. Ltd.) was used for the detection and quantification of proteins visualized by ECL Plus Western blotting detection reagents (GE Healthcare UK Ltd.).

Immunoprecipitation was done using Dynabeads Protein G kit (Invitrogen) with antibodies against p65 (sc-8008; Santa Cruz Biotechnology) or normal mouse IgG (sc-2025; Santa Cruz Biotechnology), according to the manufacturer's protocol. Immunoprecipitated lysates were subjected to the Western blot analysis with antibodies against p65 (sc109; Santa Cruz Biotechnology) or IκB (Cell Signaling Technology).

Cell culture and *in vitro* growth assay

Establishment of immortalized (EM-E6/E7/TERT) and tumorigenic (EM-E6/E7/TERT/RAS) endometrial epithelial cells has been described elsewhere (6, 7). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and penicillin–streptomycin in an atmosphere of 5% CO_2 at 37°C. Growth activity of EM-E6/E7/TERT/RAS cells with overexpressed mutant IκBα (IκBαM) or with control vectors was evaluated in normal serum (10% FBS) or low serum (0.5% FBS) conditions by

DOI:10.1158/1078-0432.CCR-10-2291

counting cell number on days 3, 4, 5, and 6 after the seeding of 5×10^4 cells in 6-cm dishes.

Anchorage independence of growth

A total of 1×10^4 cells was seeded in 60-mm dishes containing a top layer of 0.33% noble agar in DMEM supplemented with 10% FBS and a bottom layer of 0.5% base agar in DMEM supplemented with 10% FBS as described elsewhere (7). The number of colonies larger than 0.05 mm in size after 4 weeks of incubation was counted under a microscope.

Nude mouse xenograft experiments

Cells were resuspended in growth media (10^7 cells/mL) and injected (0.1 mL) subcutaneously at the base of the trunk of female BALB/c *nu/nu* mice (age range, 7–9 weeks; Japan SCL). Tumor size, if any, was monitored weekly for 8 weeks.

Matrigel invasion assay

The invasive ability of cells was assayed *in vitro* using a BioCoat Matrigel Invasion Chamber (Becton Dickinson Biosciences), as described elsewhere (15). Cells were suspended in the upper wells of Matrigel chambers at 2.5×10^4 cells/chamber in DMEM containing 0.1% bovine serum albumin. Chambers were set into 24-well plates with DMEM containing 10% FBS. After 22 hours of incubation, cells on the upper surface of the membrane were removed by wiping with cotton swabs and cells that had migrated through the membrane containing Matrigel to the lower surface were fixed with methanol and stained with Mayer's hematoxylin. The cells on the lower surface of the membrane were counted microscopically as the invasion index. Chemotaxis assays were conducted in the same manner as for chemoinvasion, except that the filters were not coated with Matrigel, and the number of cells on the lower surface of the membrane was counted as the migration index. The invasive ability of cells was described as the relative value of invasion index versus migration index.

Statistical analysis

The data from the anchorage-independent growth assay and Matrigel invasion assay were presented as the mean ±SD of triplicate assays per group. Differences between groups were evaluated using Student's *t* test. The value of $P < 0.05$ was considered to be statistically significant.

Results

RAF-ERK and PI3K-Akt pathways do not play major roles in *KRAS*-induced endometrial carcinogenesis

Numerous effector pathways have been shown downstream of oncogenic *KRAS* signals, including the RAF-ERK and PI3K-Akt pathways. Activation of ERK upregulates the transcription of genes associated with cell proliferation, whereas the activation of Akt leads to the induction of antiapoptotic genes; thus, both ERK and Akt play crucial roles in cancer initiation. We first examined whether either or both pathways were activated by oncogenic *KRAS* signals during endometrial carcinogenesis. As shown in Figure 1, the expression of phosphorylated ERK apparently increased in tumorigenic EM-E6/E7/TERT/RAS cells compared with immortalized control EM-E6/E7/TERT cells whereas phosphorylated Akt expression was not detected in both cell types, suggesting that the RAF-ERK pathway, but not the PI3K-Akt pathway, was activated by oncogenic *KRAS* signals in endometrial cancer cells.

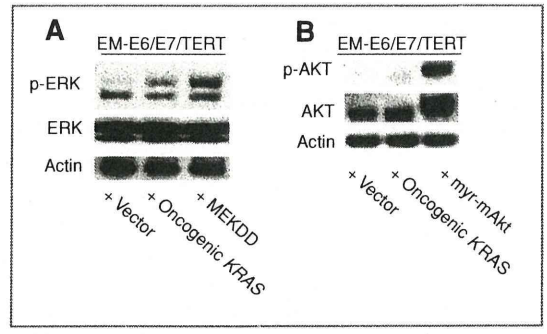

Figure 1. The RAF-ERK pathway, but not the PI3K-Akt pathway, is activated in endometrial epithelial cells transformed by oncogenic *KRAS*. Immortalized endometrial epithelial (EM-E6/E7/TERT) cells were stably transfected with oncogenic *KRAS*, constitutively activated *MEK* (MEKDD), or constitutively activated *Akt* (myr-mAkt) alleles and the expressions of phosphorylated ERK (A) and phosphorylated Akt (B) were examined by Western blot analysis. Cells with constitutively activated *Akt* alleles were used for the positive control of the activated PI3K-Akt pathway.

We next examined the biological roles of RAF-ERK activation in *KRAS*-induced endometrial carcinogenesis. To clarify the role of RAF-ERK signaling, a constitutively active form of the *MEK1* (MEK1 S218D/S222D) allele (16) was retrovirally introduced into EM-E6/E7/TERT cells; these cells were confirmed to express stable and strong levels of p-ERK comparable with those of EM-E6/E7/TERT/RAS cells (Fig. 1A). Then, the phenotypic changes were observed. However, these cells completely lacked anchorage-independent growth or tumorigenicity in mice (Table 1). Thus, ERK activation is not a critical factor to induce transformed phenotypes on oncogenic *KRAS* signals in endometrial epithelial cells.

Table 1. Change in transformed phenotypes of endometrial epithelial cell lines by introducing defined genetic elements

EM-E6/E7/TERT cells	Anchorage-independent growth	Tumorigenicity (BALBc *nu/nu*)
+ Vector	No	No
+ Oncogenic *KRAS*	Yes (100%)	Yes (100%)
+ Active MEK	No	No

DOI:10.1158/1078-0432.CCR-10-2291

Oncogenic *KRAS* enhances the transcriptional function of NF-κB in endometrial cancer cells

On the basis of these results, we sought other candidate factors involved in *KRAS*-induced endometrial carcinogenesis. One such factor is NF-κB, based on emerging evidence that NF-κB is one of the putative effectors of Ras-mediated cellular transformation in rodent cells (9–11). Therefore, we investigated whether the introduction of oncogenic *KRAS* regulates NF-κB activity in EM-E6/E7/TERT cells. First, we examined the change in the DNA-binding activity of NF-κB by electrophoretic mobility shift assay (EMSA), using consensus oligonucleotides for NF-κB and nuclear extracts prepared from EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells. As shown in Figure 2A, binding complexes were clearly observed in extracts of EM-E6/E7/TERT/vec cells. These bands were apparently intensified in extracts of EM-E6/E7/TERT/RAS cells. However, these bands were completely inhibited in competition assays by the addition of excess amounts of NF-κB consensus oligonucleotides but not by unrelated SP1 or AP2 oligonucleotides. Furthermore, they were supershifted by the addition of antibodies against NF-κB p50 or p65 subunits (Fig. 2B). We confirmed that endogenous expression levels of NF-κB were equivalent in EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. These findings indicate that oncogenic *KRAS* facilitates the DNA binding of NF-κB to its target sequences in endometrial cancer cells.

Next, we examined the change in the ability of NF-κB to transactivate the target promoters by oncogenic *KRAS*. Both EM-E6/E7/TERT/KRAS and EM-E6/E7/TERT/vec cells were transfected with the luciferase reporter plasmid containing the NF-κB–responsive elements (pNFκB-luc), and the relative luciferase activities of cell lysates were measured 48 hours after transfection. As shown in Figure 2C, the luciferase activity significantly increased (up to 5-fold) in EM-E6/E7/TERT/RAS cells compared with EM-E6/E7/TERT/vec cells, showing that oncogenic *KRAS* enhances the ability of NF-κB to transactivate the target gene promoter in endometrial cancer cells. Interestingly, this upregulation of NF-κB transcriptional activity was not cancelled by the addition of the MAP/ERK kinase (MEK)-inhibitor U0126, indicating that the RAF-ERK pathway is not involved in this activation. Taken together, we concluded that oncogenic *KRAS* functionally activates NF-κB in endometrial epithelial cells in a RAF-ERK pathway–independent manner.

Inhibition of NF-κB activity abrogates the transformed phenotypes of endometrial cancer cells

Regulation of NF-κB activity is controlled mainly by the inhibitory function of the IκB family, including IκBα. Phosphoryation of IκBα at 2 serine residues (Ser32 and

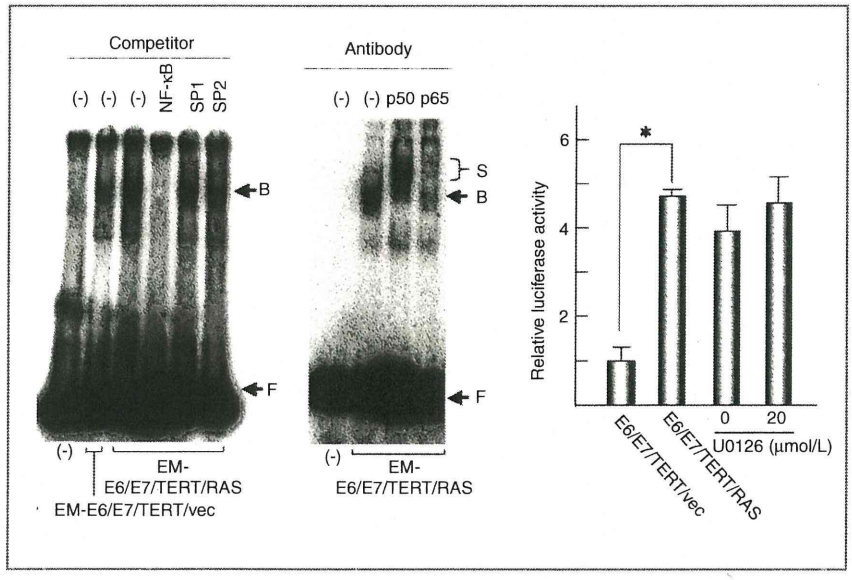

Figure 2. Oncogenic *KRAS* activates NF-κB in endometrial epithelial cells. A and B, EMSA to examine the DNA-binding activity of NF-κB. Nuclear extracts prepared from EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells were incubated with γ^{32}-P–labeled consensus oligonucleotides containing the NF-κB–responsive elements, followed by electrophoresis. For competition assays, 100-fold molar excess of unlabeled consensus oligonucleotides for NF-κB, AP2, and SP1 were used as competitors (A). For the supershift analysis, specific antibodies against NF-κB subunit p50 or p65 were added in the reactions. B, binding complexes; F, labeled free probes; S, supershifted complexes. C, luciferase reporter assays to examine the ability of NF-κB to activate the target promoters. EM-E6/E7/TERT/RAS cells and the vector control EM-E6/E7/TERT/vec cells were transfected with luciferase reporter plasmids containing the NF-κB–responsive elements. Plates were harvested 48 hours after transfection and luciferase assays were conducted. For inhibition of the RAF-ERK pathway, 20 μmol/L of U0126 or dimethyl sulfoxide was added to the medium 6 hours after transfection. The results are presented as relative luciferase activity in which the activity from EM-E6/E7/TERT/vec cells was normalized to 1.0. Values are represented as the means of 3 independent experiments. Bars, SD. *, $P < 0.05$.

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Ser36) leads to ubiquitination of IκB and subsequent proteasome-mediated degradation in the canonical NF-κB induction pathway (17). A dominant-negative mutant of IκBα, named IκBαM, has been engineered to be protected from phosphorylation and degradation. The introduction of this mutant form tightly represses the nuclear translocation and DNA binding of NF-κB (18). To elucidate the role of NF-κB in *KRAS*-induced endometrial carcinogenesis, we established cell lines with disabled NF-κB function by introducing IκBαM into EM-E6/E7/TERT/RAS cells. We first confirmed the inhibitory effect of this mutant, using the luciferase reporter assay. As shown in Figure 3A, the introduction of IκBαM significantly repressed the ability of NF-κB to activate the target promoters. We also confirmed that ERK activity was not affected by the introduction of IκBαM by Western blot analyses (Fig. 3B). Transformed phenotypes of this transfectant were evaluated by cell growth *in vitro* and the soft agar colony formation assay, tumor formation assay in nude mice, and Matrigel invasion assay. Under normal serum conditions, there was no significant increase in exponential growth rate by the introduction of IκBαM (data not shown). However, in low-serum conditions with 0.5% FBS, cells with overexpressed IκBαM exhibited decreased growth rate (Fig. 4A). We also observed that anchorage-independent growth in soft agar was almost completely abolished in these mutant cells (Fig. 4B). Furthermore, these cells completely lost their tumorigenic potential in mice (Fig. 4C). Similarly, their invasive ability significantly decreased, as evaluated by the Matrigel invasion assay (Fig. 4D). These findings indicate the crucial roles of NF-κB in *KRAS*-mediated endometrial carcinogenesis.

NF-κB activation by oncogenic *KRAS* is IKK dependent but independent of p65 phosphorylation or IκBα degradation/dissociation

We next sought to identify the molecular mechanisms of NF-κB activation by oncogenic *KRAS*. We first tested whether IKK signaling involves this activation. EM-E6/E7/TERT/RAS cells were treated with or without the IKK inhibitor X, the molecule known to inactivate IKKβ and IKKα. As shown in Figure 5A, the addition of IKK inhibitors largely inhibited the activity of NF-κB–responsive promoter in *KRAS*-introduced cells but not vector cells, indicating that this activation was IKK dependent.

Recent studies have focused on IκB subtype regulation (19) or p65 nuclear modification which can affect DNA binding and interactions with coactivators and corepressors (20, 21), Thus, we compared the basal expression levels of IκB subtypes or p65 modification between EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. As shown in Figure 5B, the expression levels of IκBα, β, ε, and p105 and those of phospho-p65 at Ser276, 529, and 536 were basically equivalent in both cells except p100. These findings suggest that IκB subtype regulation or p65 nuclear modification does not significantly contribute to *KRAS*-induced NF-κB activation during endometrial carcinogenesis.

One potential mechanism of NF-κB activation includes the degradation of IκB by its ubiquitination. Therefore, we further evaluated the change in degradation rate of IκBα by Western blot analyses, using the protein synthesis inhibitor emetine. As expected, the treatment of cells with emetine resulted in the decreased expression of IκBα in both EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells but not in

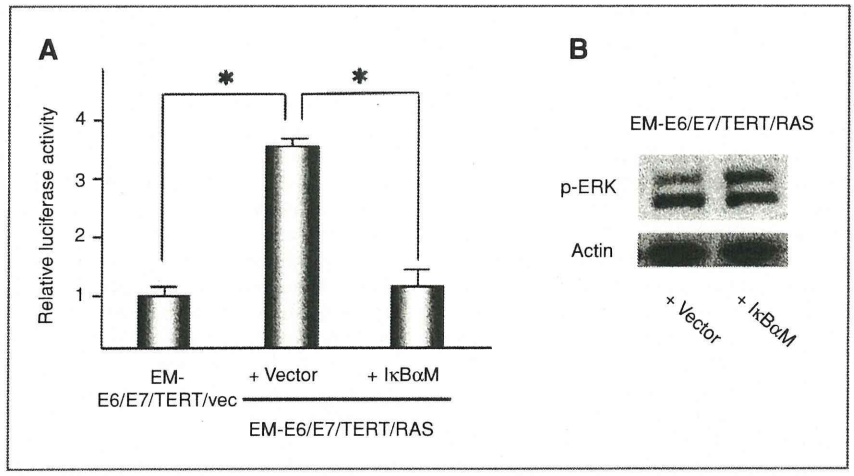

Figure 3. Introduction of IκBαM effectively inhibits NF-κB activity in transformed endometrial epithelial cells. EM-E6/E7/TERT/RAS cells were stably overexpressed with IκBαM lacking phosphorylation sites essential for its degradation, thereby becoming stable against degradation signals. Eventually, these transfectants were expected to have disabled NF-κB by IκB activity. To confirm the *in vivo* function of NF-κB in these cells, luciferase assays were done, in which EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells were transfected with reporter plasmids containing the NF-κB–responsive elements and the luciferase assays were conducted. A, relative luciferase activities are shown as the mean values of 3 independent experiments, in which those of E6/E7/TERT/vec cells were normalized to 1.0. Bars, SD. *, $P < 0.05$. B, states of the RAF-ERK pathway were compared between EM-E6/E7/TERT/RAS cells transfected with IκBαM and the vector control cells, examining the phosphorylated ERK expression by Western blot analysis.

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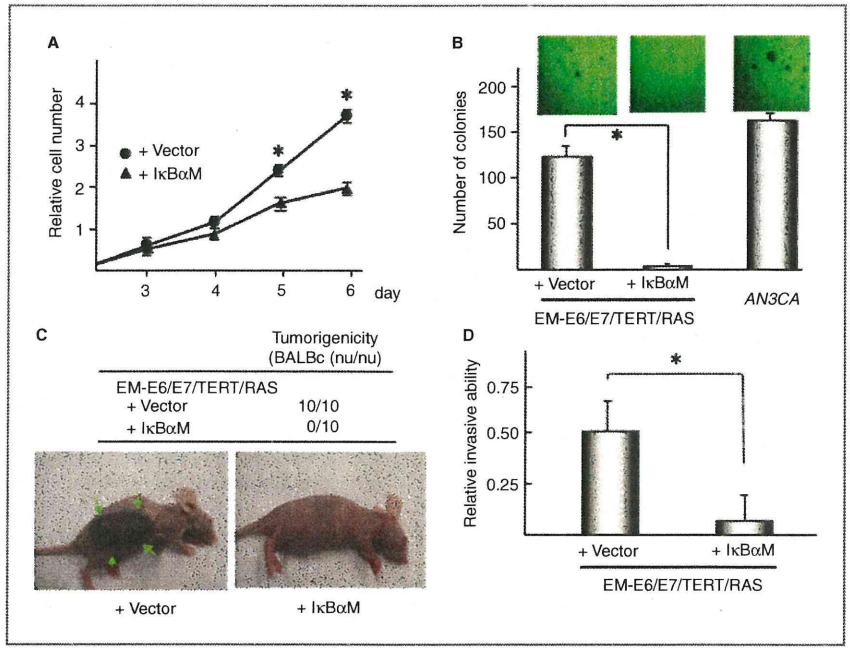

Figure 4. Transforming activity of oncogenic *KRAS* is disturbed by inhibiting NF-κB activity in endometrial epithelial cells. Transformed phenotypes of EM-E6/E7/TERT/RAS cells overexpressed with IκBαM were evaluated by various experiments. A, cell growth assay in a low-serum condition. The growth curve is shown in which EM-E6/E7/TERT/RAS cells transfected with or without IκBαM were cultured at a low-serum concentration with 0.5% FBS. Bars, SD. *, statistically significant decrease ($P < 0.05$) in the number of EM-E6/E7/TERT/RAS cells overexpressed with IκBαM compared with the number of vector control cells on days 5 and 6. B, soft agar colony formation assay. A total of 10,000 cells were seeded on soft agar in 6-cm dishes and colonies with a diameter of more than 0.05 mm 4 weeks after seeding were counted. Bars, SD. *, $P < 0.01$. C, nude mice xenograft experiment. A total of 10^7 cells of EM-E6/E7/TERT/RAS with IκBαM or control vector were inoculated subcutaneously into the right trunk of immunodeficient mice. Tumor formation was monitored for 8 weeks after inoculation. Breast cancer AN3CA cells were used as a positive control for colony formation. D, Matrigel invasion assay. EM-E6/E7/TERT/RAS cells with IκBαM or control vector were suspended in the upper wells of Matrigel chambers at 250,000 cells/chamber. After incubation, cells on the upper surface of the membrane were removed, and the cells that had migrated through the membrane to the lower surface were counted microscopically. The numbers are shown as relative invasive ability. Bars, SD. *, $P < 0.05$.

cells with overexpressed IκBαM lacking phosphorylation sites responsible for degradation (Fig. 5C). However, the degradation ratio was equivalent in EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. These results show that the activation of NF-κB by oncogenic *KRAS* is not due to accelerated degradation of IκBα.

The remaining possibility of activation mechanism might be an enhanced dissociation of p65 with IκBα (22). We tested this possibility by immunoprecipitation with p65 antibody, followed by the Western blot analysis with IκBα antibody, using extracts from EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells. As shown in Figure 5D, the ratio of IκBα associated with p65 was similar between EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells, denying the involvement of enhanced dissociation of p65 with IκBα.

Discussion

Using an *in vitro* carcinogenesis model with human endometrial epithelial cells, we first investigated the status of 2 major signaling pathways, the RAF-MEK-ERK and PI3K-Akt pathways, downstream of Ras. As expected, phosphorylated ERK expression significantly increased in EM-E6/E7/TERT/RAS cells (Fig. 1A). In contrast, phosphorylated Akt expression was not detected in both EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells (Fig. 1B). However, the introduction of a constitutively active form of MEK, mimicking the activated RAF-MEK-ERK pathway, failed to show transformed phenotypes. Thus, activation of ERK alone was not sufficient to transform EM-E6/E7/TERT cells. There are several reports showing that constitutive activation of MEK successfully transformed rodent epithelial cells (23–26). In contrast, Boehm and colleagues showed that the introduction of a constitutively active form of MEK failed to transform immortalized human embryonic kidney epithelial cells (27). These results together with our results suggest that activation of the MEK-ERK pathway alone may not be sufficient to transform human epithelial cells and that the activation of other oncogenic pathways is required. Thus, we sought novel effectors involved in KRAS-mediated endometrial carcinogenesis.

DOI:10.1158/1078-0432.CCR-10-2291

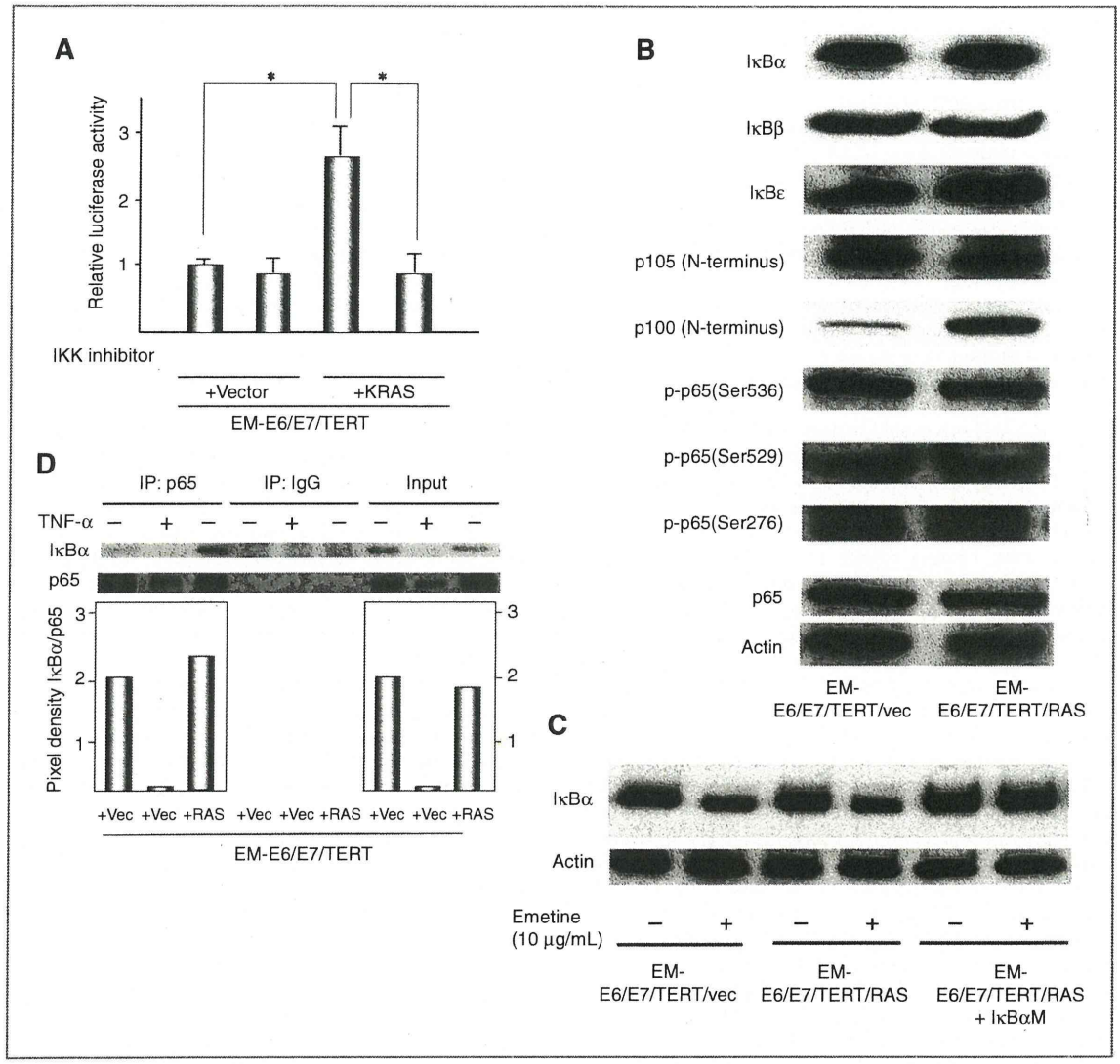

Figure 5. *KRAS*-induced activation of NF-κB in endometrial carcinogenesis is IKK dependent but not on known canonical pathways. A, IKK dependence of NF-κB activation. EM-E6/E7/TERT/vec cells or EM-E6/E7/TERT/RAS cells were transfected with reporter plasmids containing the NF-κB–responsive elements and were incubated with or without 5 μmol/L of IKK inhibitor X. Luciferase assays were carried out after 48 hours of incubation. Relative luciferase activities are shown as the mean values of 3 independent experiments, in which those of E6/E7/TERT/vec cells were normalized to 1.0. Bars, SD. *, $P < 0.05$. B, expression levels of IκB family proteins and phosphorylated p65 in *KRAS*-introduced cells. Whole-cell extracts of EM-E6/E7/TERT/vec cells or EM-E6/E7/TERT/RAS cells were subjected to the Western blot analysis, and the levels of expression in each factor are compared. C, change in IκBα turnover rate was compared between cells with or without oncogenic *KRAS*. EM-E6/E7/TERT/RAS cells or EM-E6/E7/TERT/vec cells were treated with or without 10 μg/mL of emetine and the whole-cell extracts were subjected to the Western blot analysis for IκBαM. As a control, EM-E6/E7/TERT cells with overexpressed IκBαM were used, in which IκBα level is stable even in the presence of emetine due to the lack of the specific phosphorylation site essential for degradation. D, change in dissociation rate of p65 with IκBα was compared between cells with or without oncogenic *KRAS*. Immunoprecipitation (IP) was carried out with antibodies against p65 or control IgG, using whole-cell lysates from EM-E6/E7/TERT/vec or EM-E6/E7/TERT/RAS cells. TNF-α (20 ng/mL for 5 minutes) stimulation was carried out in vector (Vec) cells to facilitate the degradation of IκBα, used as a positive control of degradation status. Western blot analysis was subsequently carried out on immunoprecipitants with antibodies to p65 or IκBα. The pixel densities of IκBα and p65 Western blots were quantified using NIH Scion software. Graph represents the relative pixel density of IκBα normalized to p65 levels in each sample.

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NF-κB transcriptional factor is a putative effector of Ras-mediated transformation (8–10). We showed that oncogenic *KRAS* enhanced the NF-κB binding to its responsive elements and facilitated the transactivation of the target promoters. The introduction of IκBαM successfully inhibited transactivation of NF-κB without affecting ERK activity, and we found that such inhibition completely abrogated the anchorage-independent growth and tumor-forming ability of EM-E6/E7/TERT/RAS cells, suggesting a major contribution of NF-κB activity to *KRAS*-induced carcinogenesis of endometrial epithelial cells.

What is the molecular mechanism of NF-κB activation in *KRAS*-induced endometrial carcinogenesis? The transactivation of promoters by NF-κB is directly controlled by its nuclear translocation and its modification in the nucleus. In most cell types, NF-κB dimers are sequestered in the cytoplasm and inactivated by IκB proteins, which bind to the NF-κB and mask the nuclear localization signal (28). The phosphorylation of a specific serine residue, Ser32/36, in IκBα by the upstream regulators such as IKK results in polyubiquitination and subsequent degradation by 26 S proteasomes, causing release of the NF-κB dimer and promoting its translocation to the nucleus, activating various κB-responsive gene expressions (29, 30). Thus, we first tested the involvement of IKK in the activation. The treatment of EM-E6/E7/TERT/RAS cells with the IKK inhibitor X significantly suppressed NF-κB transcriptional activity, confirming that *KRAS*-induced NF-κB activation was IKK dependent. We further examined the basal expression levels of IκB family proteins in the presence or absence of oncogenic *KRAS* and found that the expression levels of IκB proteins, including IκBα, β, ε, and p105, were not affected by oncogenic *KRAS*. Expression of p100 protein, which is a member of the IκB protein family and a precursor of NF-κB subunit p52 (31), increased in EM-E6/E7/TERT/RAS cells. This is probably because the p100 promoter contains a κB site (32). We do not consider that this phenomenon is involved in NF-κB activation, because the increased expression of p100 may inhibit NF-κB activity but never activates NF-κB. In addition, the expression of p52 was unchanged (data not shown). Therefore, we speculate that the p100/p52 subunit is not likely to participate in NF-κB activation by oncogenic *KRAS*. Furthermore, we focused on the modification of the NF-κB subunit itself. Nuclear NF-κB modification, especially p65/RelA subunit modification, has been investigated and found to affect DNA binding and interactions with coactivators and corepressors and the termination of the NF-κB response (33). These modifications include phosphorylation of Ser536 and Ser529 in the C-terminal transactivation domains and Ser276 in the Rel homology domain (17, 34–36). However, the expression of phosphorylated p65 was not elevated in the *KRAS*-introduced cells. We further confirmed the possibility of increased IκB degradation that might result in NF-κB activation. The IκB turnover assay with protein synthesis inhibitor revealed that the turnover was not accelerated by oncogenic *KRAS*, again denying the possibility as an activation mechanism. Recently proven additional mechanisms of NF-κB activation is a dissociation of IκB from p65 in the absence of IκB degradation (22, 37). We examined the interaction of IκBα and p65 by immunoprecipitation. The expression levels of IκBα attached to p65 was, however, equivalent between EM-E6/E7/TERT/vec and EM-E6/E7/TERT/RAS cells, showing that such dissociation is not involved in *KRAS*-mediated NF-κB activation. Thus, we concluded that *KRAS*-induced activation of NF-κB during endometrial carcinogenesis is IKK dependent but not on known canonical pathways. Identification of such unknown mechanisms is needed using our model for understanding not only of the activation mechanisms of NF-κB but also of the carcinogenesis of endometrium.

So far, only one study has addressed *KRAS* mutation and NF-κB activation in endometrial cancer (38). This report examined surgically resected cancer tissues and reported the high frequency of nuclear location of NF-κB families. However, no correlation was found between the nuclear immunostaining of NF-κB and *KRAS* mutation. These findings do not conflict with our results, because their analyses were carried out using specimens of progressive cancers and not samples at the stage of cancer initiation or development, in which network of etiologic factors might be modified because of acquired genetic alterations during the late stage of cancer development.

This study may provide a clinical implication for NF-κB as a novel molecular target for cancer chemoprevention of the endometrium. Accumulating evidence has clarified chemopreventive effects of anti-inflammatory agents such as aspirin or other nonsteroidal anti-inflammatory drugs on various cancer types partially via inhibition of NF-κB (39, 40). As for endometrial cancer, Moysich and colleagues reported the risk reduction by regular use of aspirin among obese women (41). Interestingly, multiple signaling pathways, including PTEN-PI3K-Akt pathway, are known to activate NF-κB in endometrial cancer cells (42). Loss of function mutation in *PTEN* and activating mutation in *PIK3CA* are putative activator of NF-κB through Akt expression in endometrial cancer and in the precursor lesions (43). Therefore, it is possible that NF-κB plays a role in endometrial carcinogenesis via various pathways other than *KRAS*-driven pathways, giving light to the potential role of NF-κB inhibitors in preventing endometrial carcinogenesis.

In summary, we for the first time show that the activation of NF-κB is a novel target of oncogenic *KRAS* in endometrial carcinogenesis. Blockade of NF-κB activity led to effective inhibition of transformed phenotypes of endometrial cells. These findings may add the novel information on the molecular pathway of endometrial carcinogenesis, implying the potential utility of NF-κB inhibitors for endometrial cancer chemoprevention, especi'ally with *KRAS* mutation.

Disclosure of Potential Conflicts of Interest

The authors declare no conflict of interest.

DOI:10.1158/1078-0432.CCR-10-2291

Grant Support

This study was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the Megumi Medical Foundation of Kanazawa University.

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Received August 26, 2010; revised December 17, 2010; accepted December 29, 2010; published online March 16, 2011.

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ORIGINAL ARTICLE

Carrier cell-based delivery of replication-competent HSV-1 mutants enhances antitumor effect for ovarian cancer

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Oncolytic viruses capable of tumor-selective replication and cytolysis have shown early promise as cancer therapeutics. We have developed replication-competent attenuated herpes simplex virus type 1 (HSV-1) mutants, named HF10 and Hh101, which have been evaluated for their oncolytic activities. However, the host immune system remains a significant obstacle to effective intraperitoneal administration of these viruses in the clinical setting. In this study, we investigated the use of these HSV-1 mutants as oncolytic agents against ovarian cancer and the use of human peritoneal mesothelial cells (MCs) as carrier cells for intraperitoneal therapy. MCs were efficiently infected with HSV-1 mutants, and MCs loaded with HSV-1 mutants caused cell killing adequately when cocultured with cancer cells in the presence or absence of HSV antibodies. In a mouse xenograft model of ovarian cancer, the injection of infected carrier cells led to a significant reduction of tumor volume and prolonged survival in comparison with the injection of virus alone. Our results indicate that replication-competent attenuated HSV-1 exerts a potent oncolytic effect on ovarian cancer, which may be further enhanced by the utilization of a carrier cell delivery system, based on amplification of viral load and possibly on avoidance of neutralizing antibodies.
Cancer Gene Therapy (2011) **18,** 77–86; doi:10.1038/cgt.2010.53; published online 1 October 2010

Keywords: oncolytic virotherapy; ovarian cancer; cell carriers; herpes simplex virus

Introduction

In Japan, 8000 cases of ovarian cancer are newly diagnosed and more than 4000 women die of this disease every year.[1] Ovarian cancer has a high fatality rate because of the lack of effective screening strategies and the absence of symptoms during the early stage of disease. Thus, most patients with ovarian cancer present with advanced-stage disease in conjunction with intraperitoneal carcinoma. Advanced epithelial ovarian cancer (EOC) is a highly chemosensitive solid tumor with good response rates to first-line chemotherapy. However, the majority of patients eventually relapse, and ultimately die of recurrent chemoresistant disease. Therefore, novel therapeutic approaches are required. EOC remains localized within the peritoneal cavity in a large proportion of patients, causing local morbidity and lethal complications.[2] Owing to its localized nature, EOC lends itself to intraperitoneal approaches to therapy, including gene therapy.

Oncolytic virotherapy is a promising anticancer therapy because efficient transduction and cancer cell-specific viral replication can boost therapeutic efficacy.[3–7] Therefore, oncolytic viral therapy is viewed as a new strategy for the treatment of advanced cancers. Many published reports describe the effectiveness of genetically engineered herpes simplex virus type 1 (HSV-1). HSV-1 has many advantages over other viruses for cancer gene therapy: (1) it has a broad host range and high efficiency of infection; (2) it has a large genomic capacity and can be engineered to deliver therapeutic transgenes;[8,9] and (3) it can be controlled by anti-herpetic drugs. Unlike retroviruses, the HSV genome does not integrate into the host genome, eliminating concerns of insertional mutagenesis. Clinical trials with several of these agents have been completed, with some efficacy. However, as the majority of those studies have relied on direct administration into target tissue, effective systemic viral delivery is required.

A major theoretical impediment to systemic application of HSV is pre-existing antiviral immunity. Almost all

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Received 22 November 2009; revised 23 April 2010; accepted 9 June 2010; published online 1 October 2010

individuals >30 years (about 80%) have circulating anti-HSV-1 antibodies in Japan.[10] Virus particles injected into the peritoneal cavity are vulnerable to inactivation by complement proteins, uptake by the reticuloendothelial system and neutralization by circulating antibodies.[11] Of these host defenses, antibodies are likely to be the most restrictive barrier to therapy, as they mediate a long-lasting state of immunity to repeated infections.[12] Several widely differing approaches aimed at protecting viral particles within the circulation and ensuring tumor delivery are currently the focus of intense research. For example, one possibility is modification of the viral coat,[13,14] although such technologies are technically challenging. In addition, cellular carriers could be used as Trojan Horse vehicles to shield oncolytic virus (OV) from neutralization following intraperitoneal administration, and act as *in situ* virus factories once arriving at the tumor site.[15,16]

In this study, we demonstrate that the molecular engineering of cellular carriers can increase their ability to support viral replication, promote direct cell-to-cell viral infection of the tumor, and shield oncolytic virus from neutralizing antibodies during delivery *in vitro*. Furthermore, we show the suitability of human peritoneal mesothelial cells (MCs) as a carrier system for delivery of HF10 and Hh101 to maximize the efficacy of oncolytic virus *in vivo*.

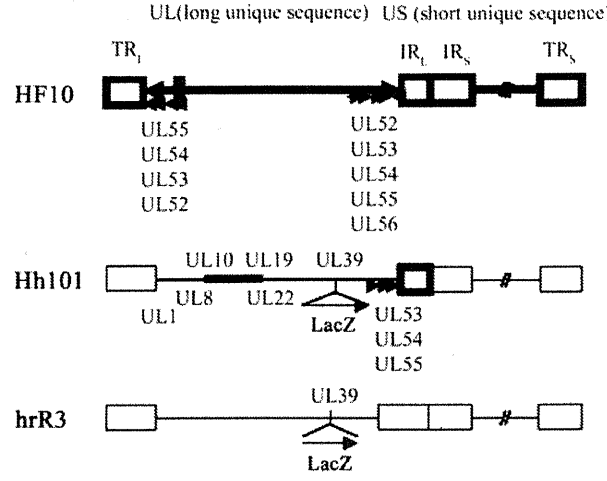

Figure 1 Models of the structures of herpes simplex virus type 1 (HSV-1) mutants. A schematic representation of the structure of the Hh101 and HF10 genomes. The locations of deletions and insertions in the genome of HF10 are shown. Expansions indicate the positions of genes within the deletion and insertion regions. Arrows indicate the position and orientation of genes within the expansions.

Materials and methods

Cell lines and viruses

Human ovarian cancer (SKOV3) cells were generously donated by Memorial Sloan-Kettering Cancer Research Laboratory. African green monkey kidney (Vero) cells were obtained from the Riken Cell Bank (Tsukuba, Ibaragi, Japan). SKOV3 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and penicillin–streptomycin. Vero cells were grown in Eagle's minimal essential medium containing 10% calf serum and 1% penicillin–streptomycin. These cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

hrR3, a ribonucleotide reductase (UL39)-deficient HSV-1 mutant, derived from the parental wild-type strain KOS, was kindly provided by Sandra K Weller (University of Connecticut Health Center, Farmington, CT). HF10 is a non-selected clone derived from HSV-1 strain HF and causes extensive cell membrane fusion in infected cells. Hh101 is a recombinant virus clone isolated from Vero cells co-infected with HF10 and hrR3 (Figure 1). The phenotypes of these viruses have been previously described.[17–19]

To visualize viruses *in vitro* and *in vivo*, the green fluorescent protein (*GFP*) gene was inserted into HF10 under control of the cytomegalovirus (CMV) promoter, in which UL43 was deleted. We named this virus HF-GFP.

Establishment and characterization of immortalized MCs

Human peritoneal MCs were isolated from surgical specimens of human omentum after obtaining consent from each patient, as described previously.[20] Briefly, small pieces of omentum were surgically resected under sterile conditions and were trypsinized at 37 °C for 30 min. The suspension was then passed through a 200-μm pore nylon mesh to remove undigested fragments, and centrifuged at 2000 r.p.m. for 5 min. The collected cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. In the subsequent experiments, cells were used during the second or third passage after primary culture.

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen, Carlsbad, CA). Briefly, hTERT, human cyclin D1 and human mutant Cdk4 (Cdk4R24C: an inhibitor resistant form of Cdk4) were first recombined into entry vectors by BP reaction (Invitrogen). Then these segments were recombined with a lentiviral vector, CSII-CMV-RfA, by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclin D1 and -hCDK4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously.[21] Following the addition of recombinant viral fluid to MCs in the presence of 4 μg ml^{-1} polybrene, infected cells were selected in the presence of 250 μg ml^{-1} of G418, 0.5 μg ml^{-1} of puromycin, 3 μg ml^{-1} of blasticidin-S or 50 μg ml^{-1} of hygromycin-B. These cells are named human omentum mesothelial cells (HOmMCs). The study to establish immortalized MCs was approved by the local ethics committee and institutional review board of our hospital.

Anti-HSV-1 serum

Anti-HSV-1 serum was obtained from mice or guinea pigs by intravenous injection of HSV-1 grown in Vero cells. The neutralizing capacity of antiserum was determined by mixing about 100 plaque-forming units (PFU) of HSV-1 with serial dilutions of antiserum. The serum titer was