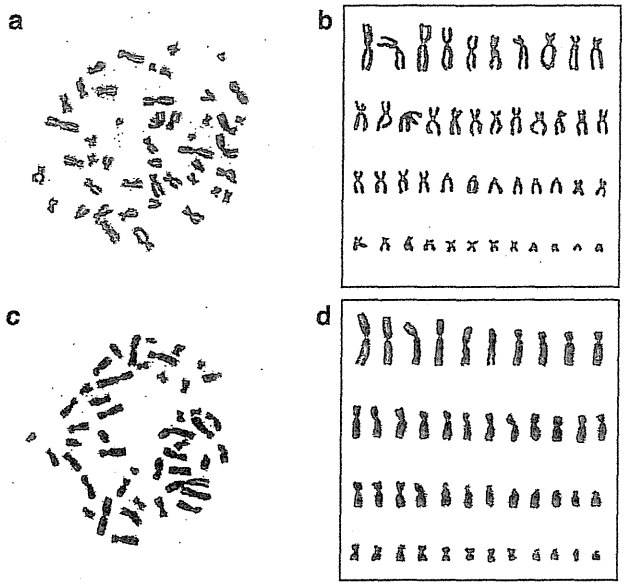

e

	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.

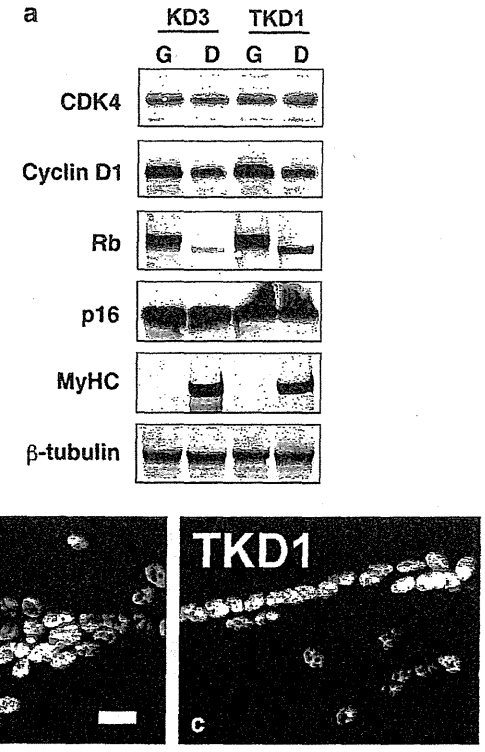

Figure 5 Expression patterns of growth- and differentiation-related proteins in immortalized human myogenic cells. (**a**) KD3 and TKD 1 cells were cultured in pmGM (g) or in primary cultured myocyte differentiation medium for 5 days (d). Fifteen micrograms of total proteins were subjected to immunobloting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

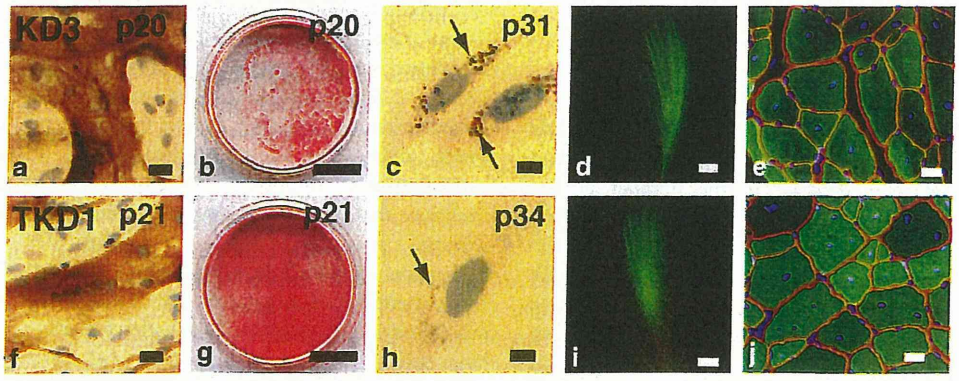

Figure 6 Multipotentiality of immortalized human myogenic cell clones KD3 and TKD1. KD3 (**a–e**) and TKD1 (**f–j**) were induced to undergo myogenic, osteogenic and adipogenic differentiation. (**a, f**) Cells were cultured for 5 days in primary cultured myocyte differentiation medium. Myosin heavy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

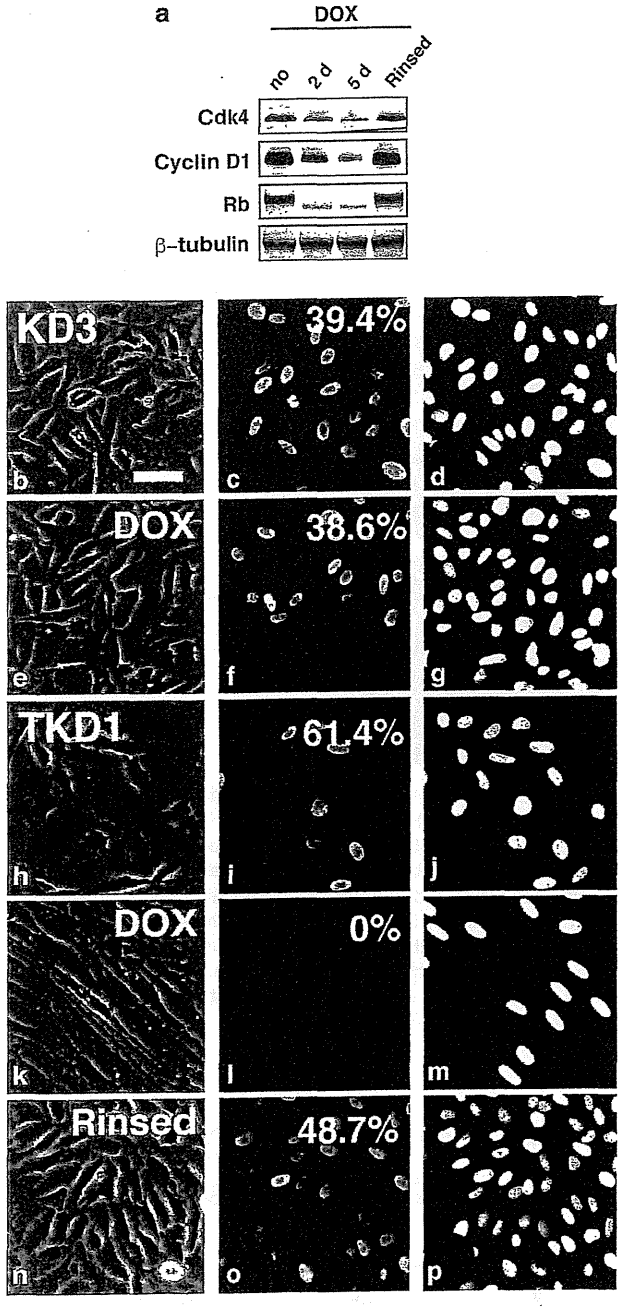

Figure 7 Reversible and precocious growth arrest induced by doxycycline in TKD1. (**a**) Fifteen micrograms of total proteins were subjected to immunobloting analysis with antibodies against CDK4, cyclin D1, Rb and β-tubulin. TKD1 cells were cultured for 2 days in medium containing 0.1% ethanol (vehicle)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

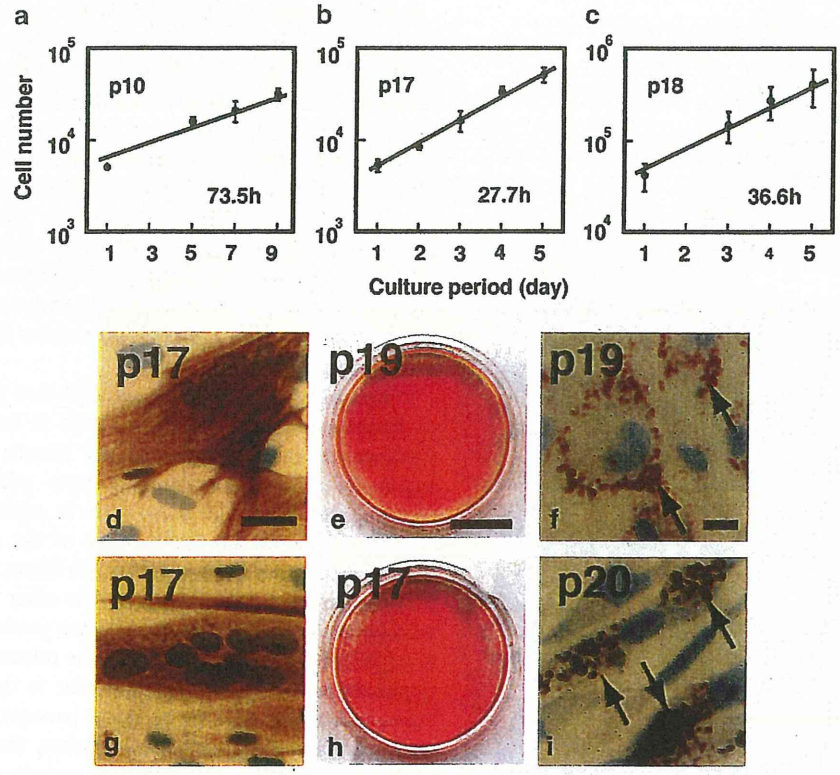

Figure 8 Recapture of proliferation capacity by myogenic cells derived from human muscle diseases. (**a–c**) Growth properties of primary cultured human myogenic cell clone HM2-5 obtained from muscle of a Leigh disease patient (**a**), immortalized clone HM255 derived from HM2-5 established by transduction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

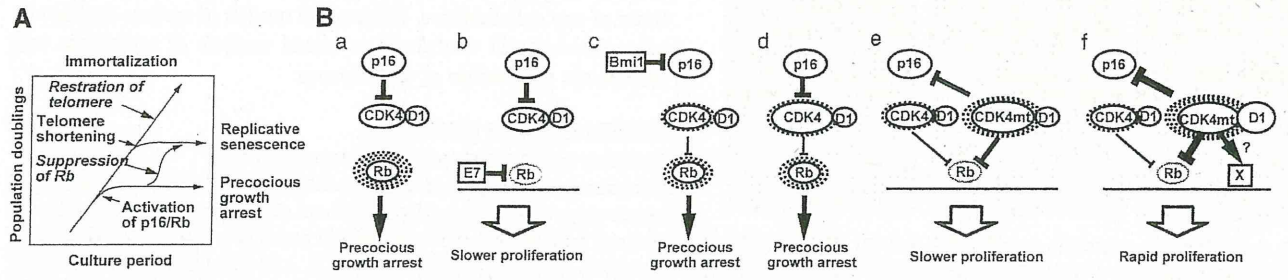

Figure 9 Premature growth arrest and replicative senescence of human myogenic cells. (**A**) Putative stress-induced activation of the $p16^{INK4a}$–Rb pathway triggers precocious growth arrest, independent of telomere shortening. Human myogenic cells under this state are able to recapture proliferation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

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Clinical Cancer Research

Human Cancer Biology

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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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

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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

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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.

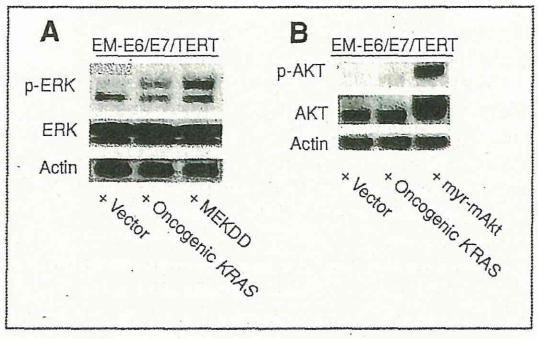

Figure 1. The RAF-ERK pathway, but not the PI3K-Akt pathway, is activated in endometrial epithelial cells transformed by oncogenic *KRAS*. Immortalized endometrial epithelial (EM-E6/E7/TERT) cells were stably transfected with oncogenic *KRAS*, constitutively activated *MEK* (MEKDD), or constitutively 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

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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

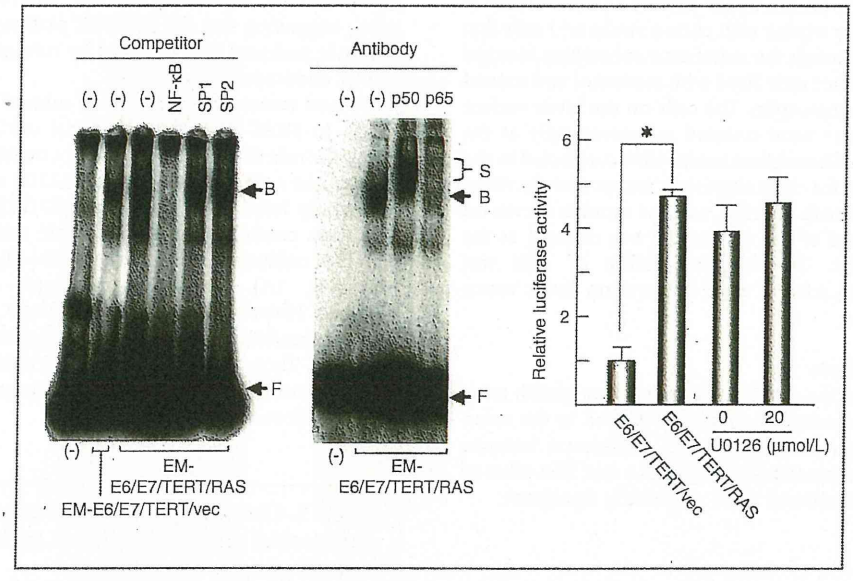

Figure 2. Oncogenic *KRAS* activates NF-κB in endometrial epithelial cells. A and B, EMSA to examine the DNA-binding activity of NF-κB. Nuclear extracts prepared from EM-E6/E7/TERT/RAS cells or the vector control EM-E6/E7/TERT/vec cells were incubated with γ^{32}-P–labeled consensus oligonucleotides 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. *, $P < 0.05$.