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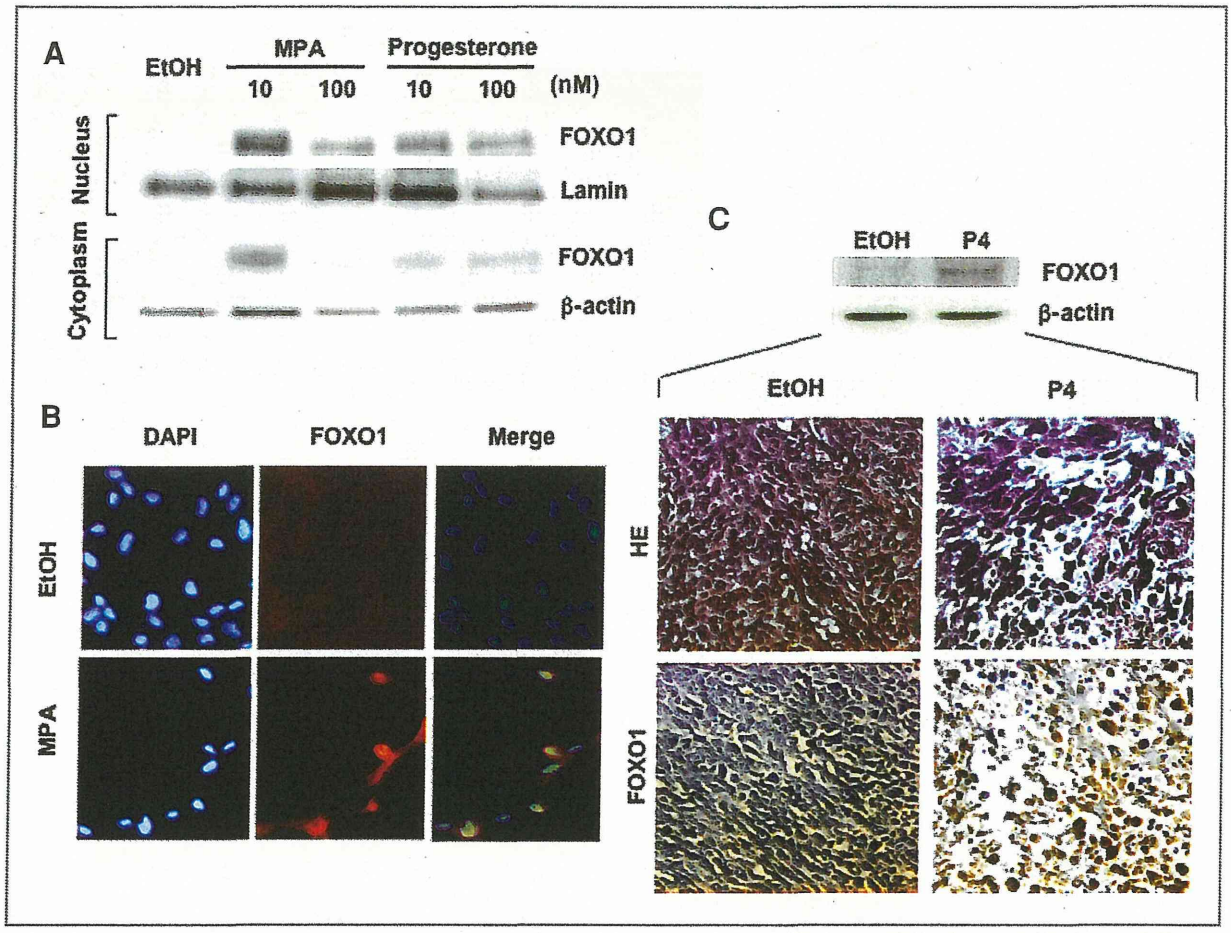

Figure 4. Induction of FOXO1 by progestin. A, EM-E6/E7/TERT/PR cells preincubated in growth media for 24 h were incubated in the serum-depleted phenol red–free DMEM in the absence or presence of MPA or progesterone for 48 h at the indicated concentrations. Nuclear or cytoplasmic extracts were collected from cells and the western blot analysis was performed using FOXO1 antibody. Lamin and β–actin were used as controls for nuclear or cytoplasmic protein loading, respectively. B, immunocytochemistry of FOXO1. EM-E6/E7/TERT/PR cells preincubated in growth media for 24 h were incubated on LAB TEK chamber slides for 24 h in the absence or presence of MPA for 24 h. After fixation, the cells were incubated with primary antibody to FOXO1, followed by fluorescent anti-IgG secondary antibody. The cells were also incubated with DAPI for nuclear staining and observed under a fluorescence microscope. Note that FOXO1 expression is induced preferentially in the nuclei (consistent with DAP staining) by MPA treatment. D, mouse tumors formed with EM-E6/E7/TERT/RAS/PR cells were treated with or without progesterone (P4) via subcutaneous injection of hormone pellets and were collected 4 weeks after the treatment. Then, whole-cell extracts were prepared, followed by western blot analysis for FOXO1. Immunohistochemistry of FOXO1 was also performed with matched samples of the EM-E6/E7/TERT/RAS/PR tumors treated with or without P4. HE staining of the tumor samples and induced FOXO1 expression mainly in the nuclei of the tumor cells are shown.

synthesis. We confirmed that this activation was not obvious in cells lacking PR-overexpression (EM-E6/E7/TERT cells) (Fig. 5A), suggesting that FOXO1 activation by MPA is PR-dependent. We further performed the luciferase reporter assays using *FOXO1* gene promoter spanning 2.0 kb sequences upstream of the first ATG. As shown in Figure 5B, MPA treatment of EM-E6/E7/TERT/PR cells activated the *FOXO1* promoter approximately by 3-fold, while no activation was observed in EM-E6/E7/TERT cells. These findings indicate that MPA directly induces FOXO1 expression via the transcriptional activation of *FOXO1*. To analyze the status of signaling pathway, which regulates subcellular localization of FOXO1, we examined the expression of PTEN and p-AKT as critical components of phosphatidylinositol 3-kinase (PI3K)/AKT pathway. As shown in Figure 5C, activation of PTEN expression or reduction of p-AKT expression, both of which facilitate nuclear retention of FOXO1, was not observed by the treatment with MPA.

FOXO1 mediates progestin to inhibit epithelial cell growth

To investigate the role of FOXO1 in the effect of progestin, a knockdown experiment for *FOXO1* was performed via siRNA inhibition. EM-E6/E7/TERT/PR cells were transfected with siRNA against *FOXO1* and treated

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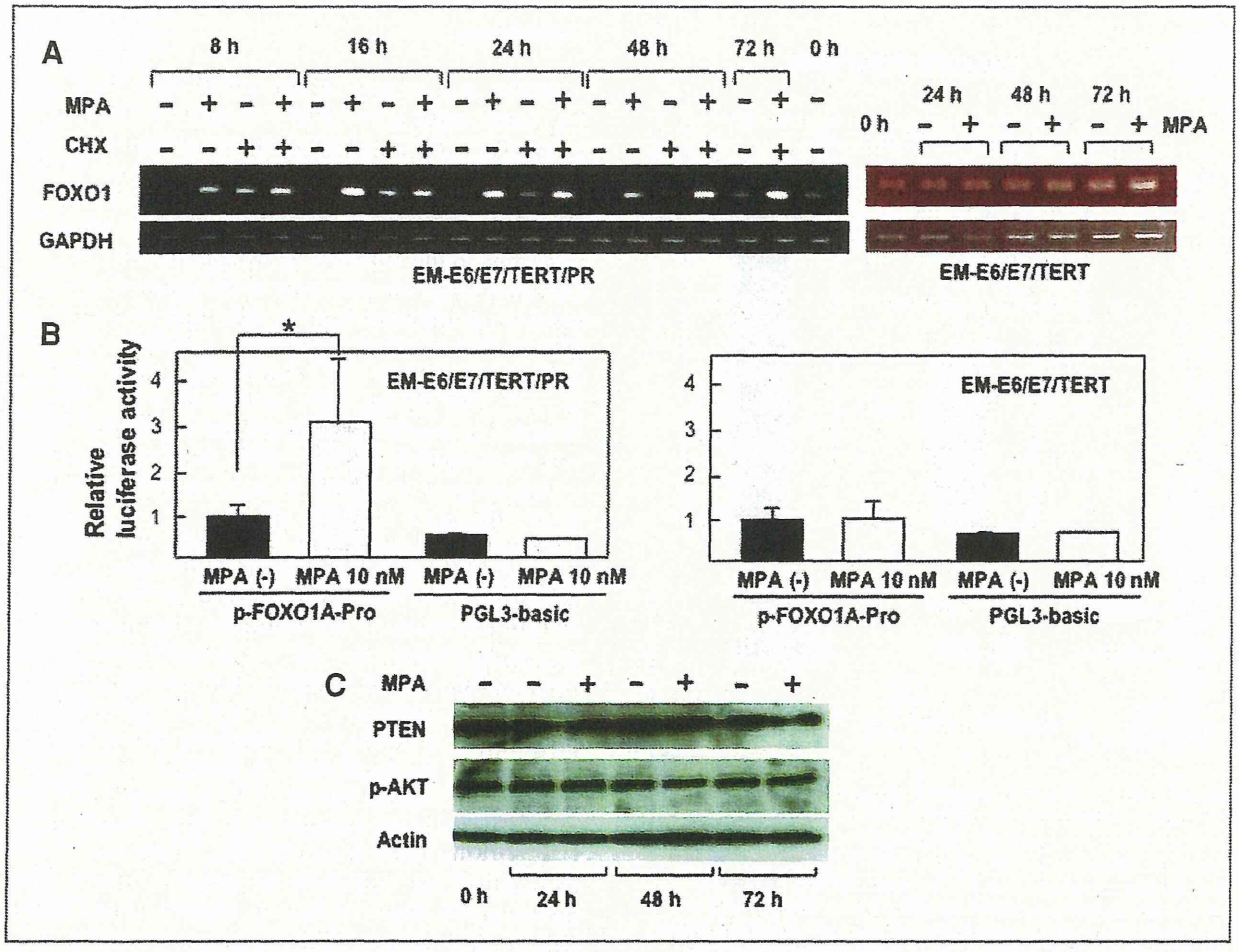

Figure 5. Mechanisms of FOXO induction by progestin. A, RT-PCR analysis of FOXO1 in EM-E6/E7/TERT/PR or EM-E6/E7/TERT cells. The cells were cultured with or without MPA in the presence or absence of cycloheximide (CHX) for different time periods and RNAs were collected and subjected to the RT-PCR for FOXO1. B, luciferase reporter assay using FOXO promoter. EM-E6/E7/TERT or EM-E6/E7/TERT/PR cells were transfected with luciferase reporter plasmid containing 2.0 kb of FOXO promoter or with blank reporter plasmid (pGL3-basic), followed by treatment with or without MPA. Seventy-two hours after treatment, cells were recovered and luciferase assays were performed. Relative luciferase activity is shown in each reporter plasmid. Data are presented as mean ± SD of the three independent experiments. *$P < 0.05$. C, EM-E6/E7/TERT/PR cells were treated with or without MPA at 10 nM and incubated at different time periods, followed by the western blot analysis for PTEN or p-AKT.

with or without MPA at 10 nM, followed by monitoring cell growth. Western blot analysis confirmed that knockdown was successful, exhibiting apparently decreased FOXO1 expression. These cells showed only minimally inducible FOXO1 expression on treatment with MPA (Fig. 6). In the absence of MPA, cells with knocked-down *FOXO1* had increased growth rate compared with those with control siRNA, indicating that endogenous FOXO1 plays some role in cell proliferation. Treatment with MPA significantly inhibited the growth of cells transfected with control siRNA, while the inhibition was largely abrogated in those with knocked-down *FOXO1*. Thus, the effect of MPA was attenuated via knockdown of *FOXO1*, supporting the role of FOXO1 in progestin action. We sought to confirm whether similar effect was observed in other endometrial cancer cell lines as well. *FOXO1* knockdown was performed in Ishikawa cells in the same way and MPA effect was examined (Supplementary Fig. 3). Ishikawa cells exhibited growth inhibition as well as FOXO1 induction by the treatment with MPA, but with lesser extent, probably due to very low levels of PR expression. FOXO1 knockdown effectively cancelled growth inhibition by MPA.

Akt signaling limits progestin action on endometrial epithelial cell growth

FOXO family members are direct downstream targets of the PI3K/Akt signal transduction pathway. Activation of PI3K/Akt signals phosphorylates FOXO proteins, resulting in cytoplasmic retention and inhibiting their transcriptional activity. Therefore, we speculated that Akt signaling might affect the action of progestin. To investigate this possibility, a special cell line, named EM-E7/E7/TERT/PR/DN-AKT, was established with an introduced dominant

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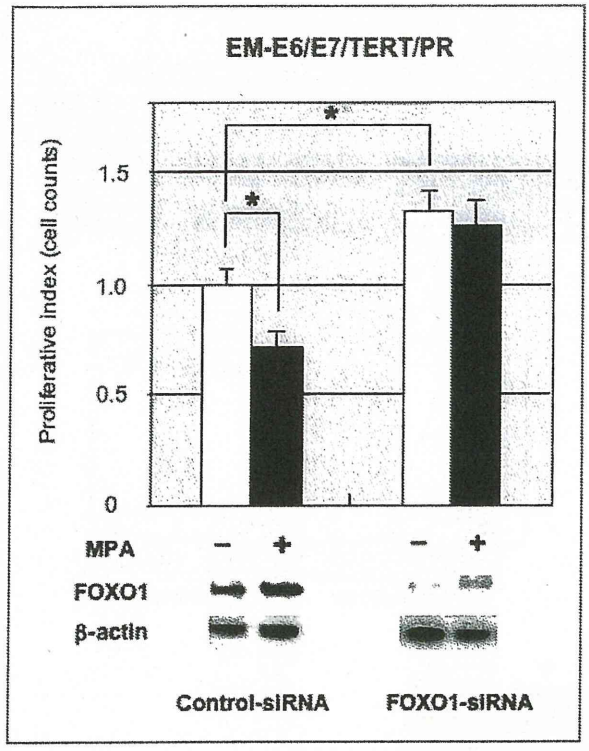

Figure 6. Role of FOXO1 in the action of progestin. EM-E6/E7/TERT/PR cells were transfected with non-specific scramble $siRNA_{control}$ or FOXO1-specific siRNA. Forty-eight hours after transfection, the cells were incubated with or without MPA at 10 nM for 72 h. Then, western blot analysis was performed to confirm the levels of FOXO1 expression. Simultaneously, cell number was counted in paired samples and shown as the relative value (proliferation index) in each sample to evaluate the effects of MPA. Data are presented as mean ± SD of the three independent experiments. *$P < 0.05$.

negative allele of *Akt* gene from EM-E7/E7/TERT/PR cells. Introduction of the dominant negative allele of *Akt* gene has been confirmed to inhibit Akt function in endometrial epithelial cell lines (12). Both cells exhibited similar growth rate in the absence of MPA (Fig. 7A). We then compared the effects of MPA on these cells. Treatment with MPA at 10 nM led to 35% growth inhibition of EM-E7/E7/TERT/PR/vector cells on day 3 and 46% on day 6 (Fig. 7A). The same treatment in EM-E7/E7/TERT/PR/DN-AKT cells resulted in 56% growth inhibition on day 3 and 66% on day 6. Thus, introducing DN-AKT caused enhanced growth inhibition by MPA.

We next examined the extent of FOXO1 induction by MPA in the nuclei of both cells (Fig. 7B). The western blot analysis revealed the enhanced FOXO1 induction in EM-E7/E7/TERT/PR/DN-AKT compared with EM-E7/E7/TERT/PR/vector cells on days 3 and 6. Especially, most prominent induction of FOXO1 was observed in EM-E7/E7/TERT/PR/DN-AKT on day 6, when the maximal growth inhibition was confirmed in Figure 7A. These findings proved that Akt signaling is a critical factor that limits the progestin action to endometrial epithelial cells.

Discussion

FOXO1 is a member of the FOXO subfamily of the Forkhead/winged helix family of transcription factors that is involved in cell cycle regulation and apoptosis (17): the subfamily contains the mammalian members FOXO1 (Fkhr), FOXO3a (Fkhr-l1), and FOXO4 (Afx) (18, 19). The role of FOXO1 in endometrial biology has been known in relation to the process of decidualization (17, 20, 21). FOXO1 induces the expression of decidualization-specific genes of endometrial stromal cells, such as insulin-like growth factor binding protein 1 (*IGFBP1*), decorin (*DCN*), and prolactin (*PRL*): this is enhanced by the action of cyclic AMP (camp). Here, we focused on the roles of FOXO1 in progestin action on endometrial epithelial cells and clearly showed it to be a novel target of progestin to inhibit the growth of both non-tumorigenic and tumorigenic endometrial epithelial cells.

The canonical pathway of FOXO1 regulation has been thought to be on the PI3K pathway. Growth-factor–dependent activation of the PI3K pathway blocks the function of all FOXO members by Akt-dependent phosphorylation of their three conserved residues, which leads to inhibition of DNA binding, nuclear exclusion, and subsequent sequestration in the cytoplasm (22–24). Recently, a unique role of progestin in the survival of endometrial stromal cells has been reported by Labied and colleagues (17). According to their results, progestin treatment of stromal cells enhanced the expression of phosphorylated FOXO1, which, because it is strictly localized to the cytoplasm, is considered to be an inactive form. Withdrawal of progestin induced rapid nuclear translocation of FOXO1, which activated expression of *BIM*, a known FOXO target gene encoding for a proapoptotic Bcl-2 homology 3 domain-only protein (25). This unique role of FOXO1 in response to progestin withdrawal was demonstrated in differentiating stromal cells of the endometrium. On the other side, we found that progestin induced FOXO1 expression mainly in the nuclei upon progestin stimulation in endometrial epithelial cells. RT-PCR assay clearly showed that FOXO1 mRNA is upregulated approximately 4–8 h after treatment with MPA, even in the presence of cycloheximide. Luciferase reporter assays demonstrated that MPA upregulated the transcriptional activity of *FOXO* promoter. Therefore, our results support the direct transcriptional activation of *FOXO1* gene by progestin. Computer-assisted homology search found potential PR-binding sites that have a homology with the glucocorticoid receptor-responsive element on the *FOXO1* promoter (data not shown). We are currently confirming the specific interaction of PR with such sites on the *FOXO* promoter. What is the molecular mechanism of the nuclear FOXO1 accumulation upon progestin stimulation? The most probable scenario might be that MPA inhibits PI3K/AKT signaling pathway, leading to the nuclear FOXO1 translocation. However, we confirmed that PI3K/AKT pathway was not inhibited by MPA in endometrial epithelial cells (Fig. 5D). Alternatively, it is known that FOXO1 binds to PR in the nuclei (26) and this physical

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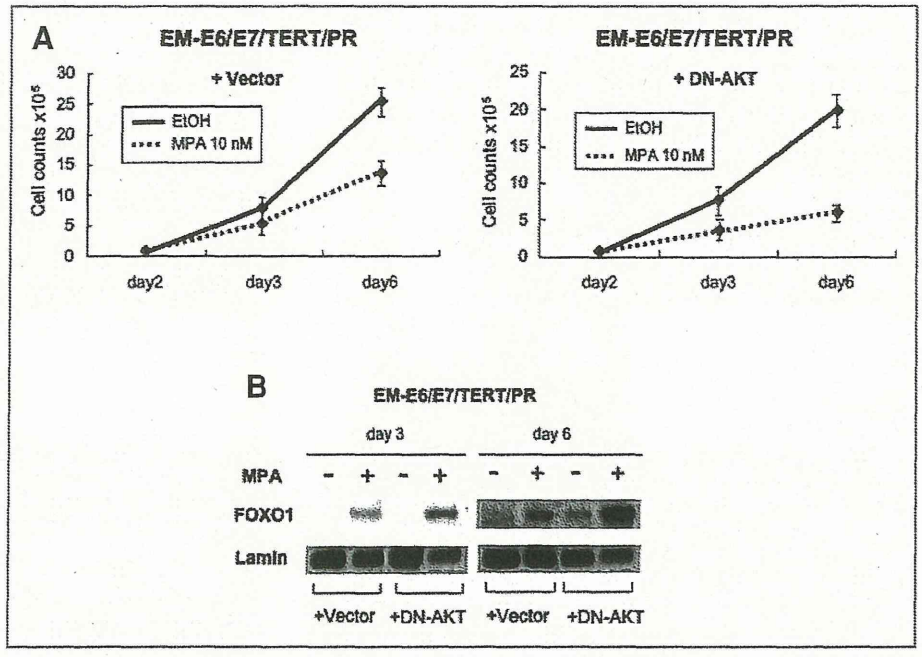

Figure 7. Akt activity limits the MPA effect. A, EM-E6/E7/TERT/PR/DN-AKT or EM-E6/E7/TERT/PR/vector cells were established from EM-E6/E7/TERT/PR cells by retroviral transfection of dominant negative *Akt* gene or blank vector, respectively. These cells were treated with or without MPA for different periods, and the cell number was counted to evaluate the proliferative activity. Data are presented as mean ± SD of the three independent experiments. B, western blot analysis was performed using nuclear extracts of EM-E6/E7/TERT/PR/DN-AKT or EM-E6/E7/TERT/PR/vector cells in the absence or presence of MPA on day 3 or day 6 to compare the levels of nuclear FOXO1 induction. Data are presented as mean ± SD of the three independent experiments.

interaction may account for the nuclear accumulation. Further mechanistic study will be needed to clearly dissect molecular mechanisms of nuclear accumulation of FOXO1 by progestin in endometrial epithelial cells.

A role for FOXO1 in inhibiting cell growth has recently been reported using endometrial cancer cell lines *in vitro* (27). Overexpression of a gain-of-function mutant of the FOXO family inhibited the growth of Ishikawa cells that constitutively express low levels of FOXO1, while siRNA inhibition of the *FOXO* gene in HEC-1® cells that express high levels of FOXO1 enhanced their growth. Furthermore, Ward and colleagues also demonstrated that progestins increased FOXO1 protein levels in endometrial cancer cell lines, specifically through PRB (28), supporting our data. A growth inhibitory effect of FOXO family members has been proposed in other cell types, in particular, in vascular cells (29, 30). More recently, a role of the FOXO family as a tumor suppressor has been proposed (31). To circumvent embryonic lethality, Paik and colleagues used an inducible Cre-lox system to knock out the FOXO family: the widespread somatic deletion of these genes caused thymic lymphomas and hemangiomas, which were associated with increased cell proliferation and survival in these lineages (31). What are the downstream targets of FOXO for inhibiting cell growth? Recently, sprouty (Spry2), a negative regulator of receptor tyrosine kinases, was validated as a direct FOXO target to inhibit cell cycle progression and induce apoptosis (31). Several other forkhead-responsive genes have been reported, including Insulin-like growth factor-binding protein-1 (*IGBP-1*), glucose-6-phosphatase, FasL, Trail, and Bim (20, 25, 32–35). However, in DNA microarray and RT-PCR analyses, we failed to observe upregulation of these candidate genes upon MPA stimulation (data not shown). Therefore, at present, it remains unclear how cell cycle arrest at G0/G1 is conferred by FOXO1 in endometrial epithelial cells.

In clinical practice of cancer treatment, we have no reliable parameter to predict the efficacy of MPA therapy. We found that the Akt signal, an upstream inhibitory factor of FOXO family, limits the effect of progestin. As we cannot predict the activation of FOXO by MPA before the treatment, the status of Akt activation could be an alternative predictor of MPA therapy. Our preliminary data show that patients responsive to MPA therapy have decreased p-AKT expression confirmed by immunohistochemistry in pretreated samples. It is well known that RAS signalings lead to AKT activation in various cancers via cross-talk signalings (36). It is therefore possible that activated RAS signalings (such as via *RAS* mutation) disturb MPA responsiveness and is an additional predictor of MPA therapy. In the present study, EM-E6/E7/TERT/RAS/PR cells with oncogenic *KRAS* mutation well responded to MPA (Figs. 2 and 3). These cells exhibit only weak levels of p-AKT expression, lacking AKT activation even with *KRAS* mutation (data not shown). Furthermore, in clinical samples, *KRAS* mutation is not always associated with AKT activation in endometrial cancer (37). Therefore, the status of *RAS* does not appear to be a strong predictor of MPA response, but this point requires further investigation.

In summary, our *in vitro* and *in vivo* treatment model has, for the first time, revealed that progestin targets FOXO via transcriptional activation to inhibit the growth of both non-transformed and transformed endometrial

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epithelial cells without p21/WAF-1 induction. Further investigations of the FOXO1 target genes as well as of AKT signaling as a predictor of MPA efficacy are required to fully understand the molecular mechanisms of progestin effects and help define patient selection for progestin therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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ENABLING TECHNOLOGIES

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

K Shiomi[1], T Kiyono[2], K Okamura[3], M Uezumi[1], Y Goto[4], S Yasumoto[5], S Shimizu[6] and N Hashimoto[1]

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

INTRODUCTION

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

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

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

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

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

RESULTS

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

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

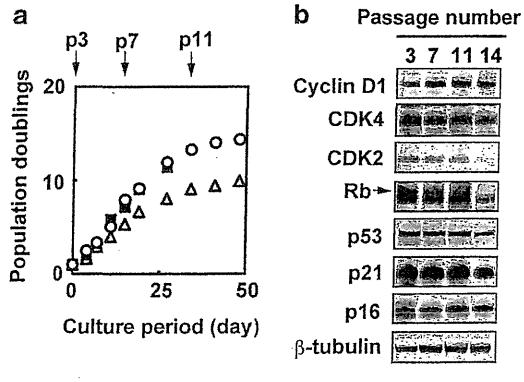

Figure 1 Growth properties of primary cultured human myogenic cells. (a) Life span plots of primary cultured human myogenic cells Hu20 (filled square), Hu23 (triangle) and Hu26 (circle) between passages 3 and 13. Arrows show the timing of passages 3, 7 and 11. Day 0 of culture period represents the day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

E7 promotes nuclear progression in terminally differentiated myotubes

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

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

Cell cycle drivers efficiently immortalize primary cultured human myogenic cells

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

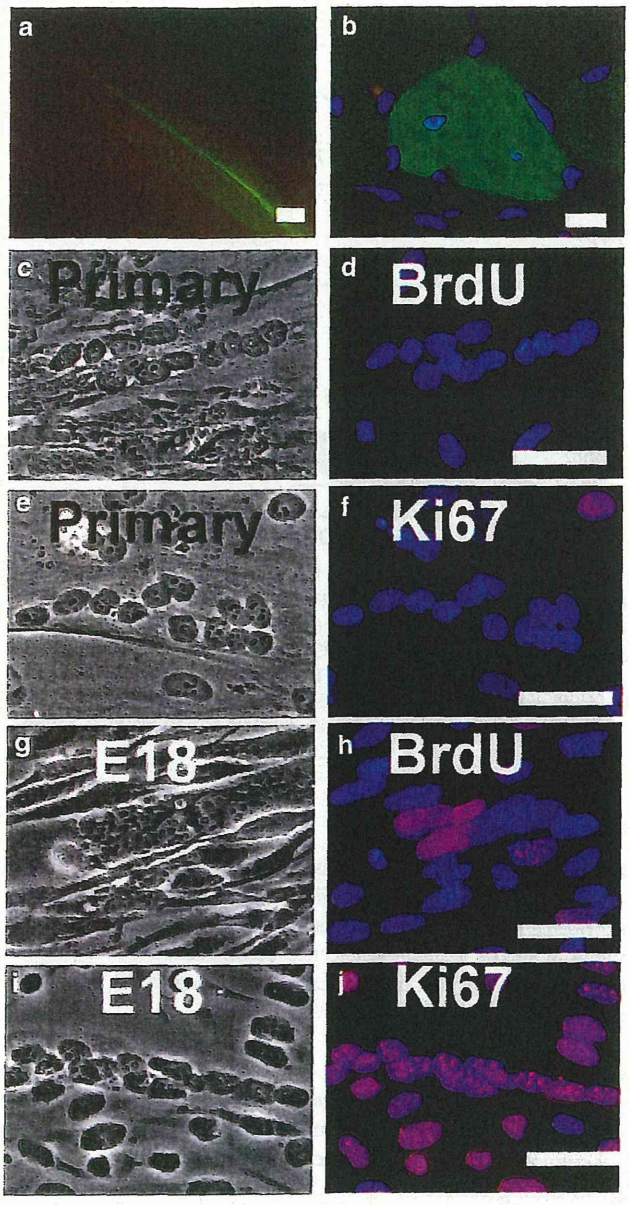

Figure 2 Nuclear progression in terminally differentiated immortalized human myogenic cells expressing telomerase and E7. (**a**, **b**) E18 cells were labeled with modified green fluorescent protein, and then 2.5×10^6 cells were transplanted into the TA muscles of NOD/Scid mice. (**a**) Whole TA muscles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

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

Immortalized human myogenic cells preserve myogenic phenotype

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

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

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

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

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

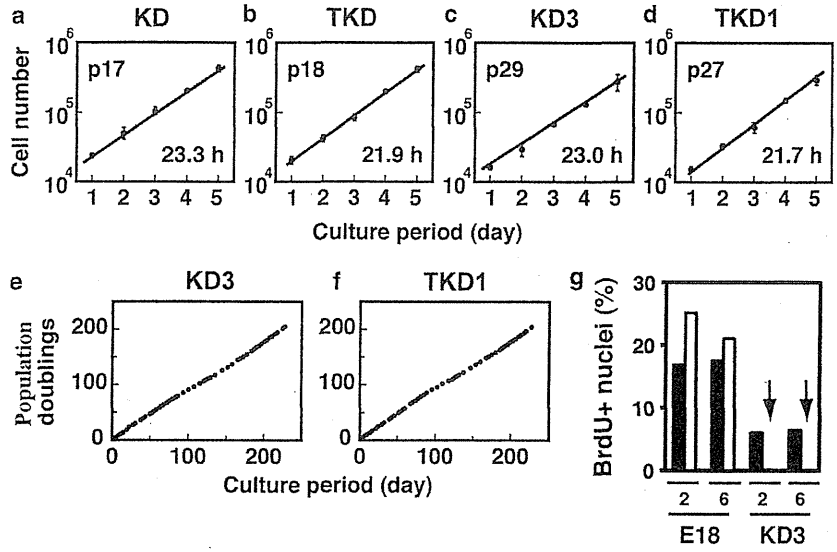

Figure 3 Proliferation of immortalized human myogenic cells. (**a–d**) Growth properties of a multiclonal population named KD, expressing hTERT, CDK4R24C and cyclin D1 under the control of a cytomegalovirus promoter (**a**), a multiclonal population named TKD, expressing hTERT, CDK4R24C and cyclin D1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

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

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

Both CDK4R24C and cyclin D1 were expressed under the control of the Tet-Off system in TKD1 cells. To determine the role of cell cycle drivers in the continuous proliferation of human myogenic cells, the expressions of CDK4R24C and cyclin D1 were suppressed by administration of doxycycline. Expression levels of CDK4 and cyclin D1 in TKD1 cells markedly declined during 5 days of incubation with doxycycline (Figure 7a). Doxycycline 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.