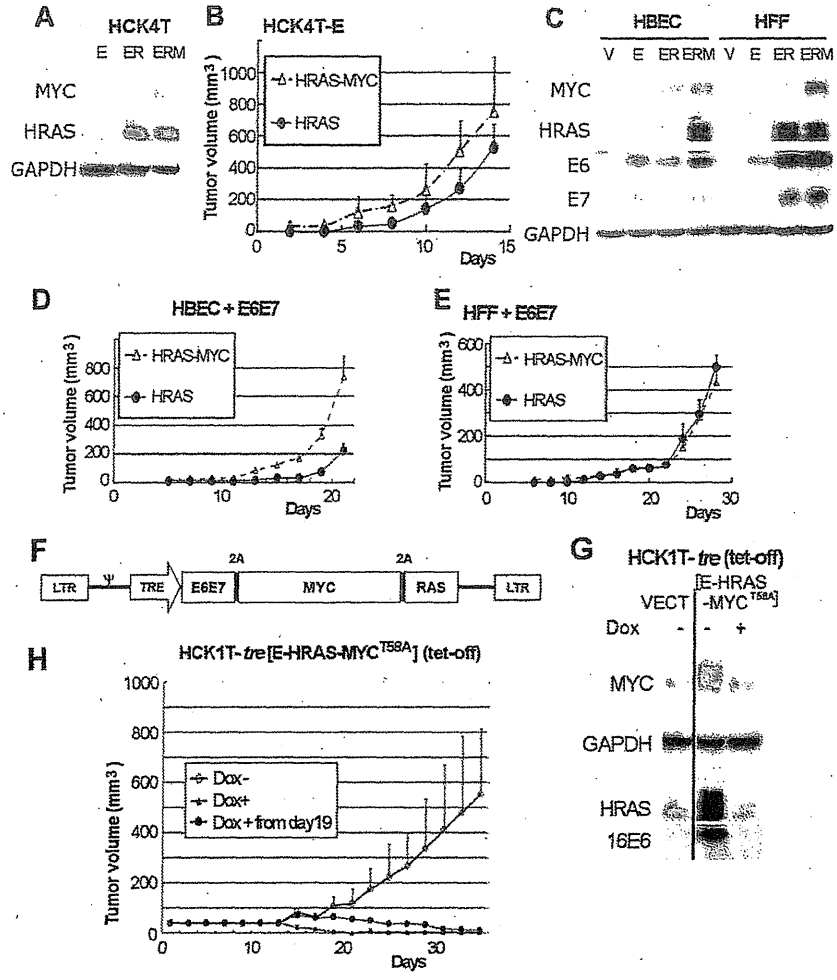

Fig. 1. HRAS with increased endogenous MYC expression is sufficient for full transformation of normal human cells expressing HPV16 E6E7. (**A**) Endogenous MYC levels in the presence of HRAS (ER) were compared with exogenous expression (ERM) by western blotting (ER for E6E7–HRAS and ERM for E6E7–HRAS–MYC). (**B**) *In vivo* tumor-forming ability of HCK4T-E cells with HRAS alone or HRAS–MYC. Cells were subcutaneously injected into nude mice (1×10^6 cells) and tumor size was measured every other day. The tumor volume (mm^3) was calculated as $L \times W^2 \times 0.52$, where L is the longest diameter and W is the shortest diameter. Each point is the mean of data for four to six samples ± SD. (**C**) Transgene products and MYC levels in human HBEC and HFF were determined by western blotting. (**D** and **E**) Tumorigenic ability of HBEC (D) and HFF (E) expressing HPV16 E6E7 and hTERT with HRAS alone or HRAS–MYC was determined as in (B). (**F**) Schematic of a single polycistronic virus in which expression of E6E7, MYC and HRAS are regulated by doxycyclin (Tet-off). These genes were separated by the sequences encoding the autonomous self-cleaving 2A peptides derived from foot-and-mouse disease virus (7). (**G**) The expression of the transgenes was determined by western blotting. (**H**) HCK1T cells transduced with this vector together with a Tet-off vector were transplanted into nude mice. When tumors had started to grow (the volume of the tumor exceeded 100 mm^3), the gene expression was terminated by adding doxycyclin in the drinking water.

To confirm that tumorigenicity is readily induced by expression of E6E7, HRAS and MYC (endogenous/exogenous) without further genetic changes and is reversible on cessation of such gene expression, E6E7, MYC^{T58A} and HRAS were cloned into a single lentiviral vector in which expression of transgenes was regulated by doxycyclin (Tet-off) (Figure 1F, G and H). HCK1T cells transduced with this vector together with a Tet-off (tTA) vector were transplanted into nude mice. When tumors had started to grow (the volume of the tumor exceeded 100 mm^3), the gene expression was terminated by adding doxycyclin to the drinking water and this resulted in halted tumor growth followed by complete regression (Figure 1H). These data support the idea that E6E7, HRAS and MYC are sufficient for tumor-forming ability of human cells without additional genetic alterations.

MYC stabilization by HRAS

We have reported that endogenous as well as exogenous MYC protein stability is increased in the presence of HRAS in exponentially growing HCK1T cells adapted to calcium and serum (grown in DMEM) (4). Because most of the data in this report were prepared with cells kept in KGM, which does not contain serum and high calcium, endogenous MYC protein stability was determined using HCK1T-E cells with a vector, AKT (myr-AKT1), MEK1DD [constitutively active form of MEK1which activates the extracellular signal-regulated kinase (ERK) pathway (10,11)] or HRAS in KGM. Endogenous MYC protein levels were increased in the order of the genes listed above (vector, AKT, MEK1DD and HRAS) (Figure 2A). In parallel with the MYC levels, Survivin (12), phosphorylated 4EBP1 (13) and phosphorylated p70S6K levels were increased and TSC2 levels were decreased (14). Increased ERK phosphorylation was observed in MEK1DD- and HRAS-expressing cells. Although similar trends were observed in subconfluent culture (data not shown), they were more evident under post-confluent culture conditions (Figure 2A).

Furthermore, increased MYC protein stability was found in the presence of HRAS (Figure 2C, also with post-confluent cells; data not shown) without significant increase in MYC messenger RNA

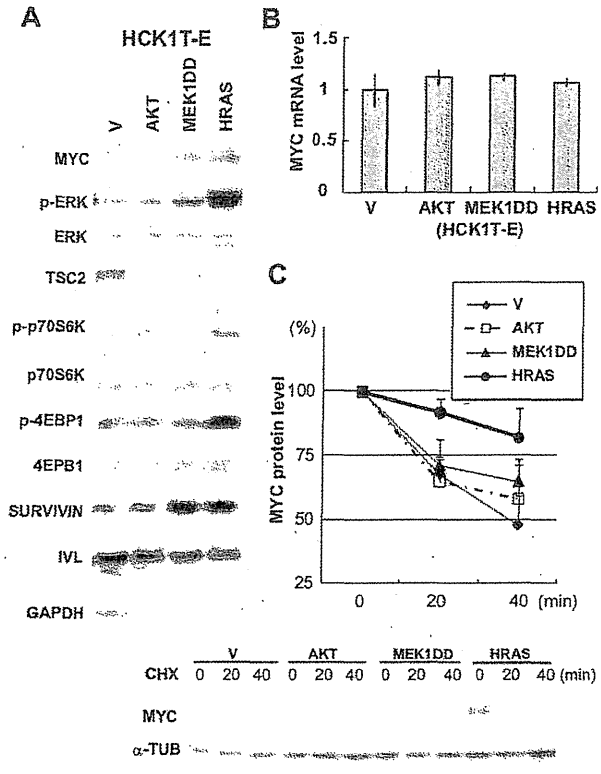

Fig. 2. Increased MYC protein stability in HRAS-positive cells. (**A**) Endogenous MYC protein levels in HCK1T-E with vector, AKT (myr-AKT1), MEK1DD (constitutively active form of MEK1) and HRAS from post-confluent (3d) cells were determined by western blotting. (**B**) Quantitative reverse transcription–PCR of MYC transcript levels from exponentially growing cells. Experiments were performed in triplicate and results were normalized to beta-2-microglobulin and presented as mean ± SD. (**C**) Exponentially growing cells were treated with 25 μg/ml cycloheximide (*CHX*) for the indicated time periods and the MYC degradation rate was assessed by western blotting. Results of quantitation in three experiments are shown with ± SD (**D**).

levels (Figure 2B). The levels of phosphorylated MYC, which has been reported to correlate with its function (15,16), were increased in the presence of HRAS (Supplementary Figure 2 is available at *Carcinogenesis* Online). From these observations, it is suggested that in HRAS-expressing cells, endogenous MYC expression is maintained at a higher level than in other cells.

Dissection of HRAS signaling pathways in tumor formation

In order to evaluate the importance of stabilized MYC for tumorigenic potential of HCK1T-E, we examined biological effects with MYC^{T58A}. Although we speculated that increased MYC expression is crucial for HRAS-induced tumorigenic potential, HCK1T-E with induction of MYC^{T58A} alone did not give rise to tumors. Though MYC^{T58A} mutant is reported to have reduced activity to induce apoptosis than MYC (16,17), it increased cleavage of caspase 3 in HCK1T-E cells (Figure 3A). To reduce the negative effect of MYC^{T58A}, BCL2 was introduced to HCK1T-E cells (Figure 3A). HCK1T-E with BCL2 cells showed weak tumor-forming ability upon MYC^{T58A} induction (Table I, C), but 4–5 months were necessary for palpable tumor masses, indicating a possible requirement for additional genetic and/or epigenetic alterations. MYC^{T58A} induction together with expression of either active AKT or MEK1DD conferred tumorigenic potential on HCK1T-E cells (Figure 3B and C). Although without induction of MYC^{T58A} they eventually started to form small tumors at the end of observation period (7–9 weeks), this might have been due to leakage of MYC^{T58A}

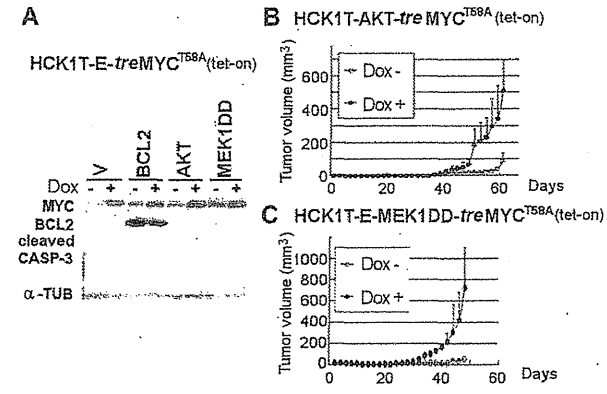

Fig. 3. Stabilized MYC expression is required for the tumorigenic potential of HCK1T-E cells with activated AKT or ERK. (**A**) MYC^{T58A} expression with the Tet-regulated expression system was determined by western blotting. Dox +; cells were treated with 1 μg/ml doxycycline (Dox) for 5 days. *In vivo* tumor-forming ability of AKT (**B**) and ERK (**C**) activated HCK1T-E cells with induction of MYC^{T58A} were determined as for Figure 1B. Mice were treated with doxycycline (1 μg/ml in their drinking water) or the vehicle (ethanol).

from the Tet-regulated expression system because HCK1T-E with AKT or MEK1DD alone did not form tumors within 20 weeks (Table I, C). Since tumorigenic potential was less than with HCK1T-E-RAS cells, it is evident that multiple RAS signaling pathways other than simply MYC stabilization are cooperatively involved in tumorigenic transformation of HCK1T-E cells.

MYC confers resistance to calcium- and serum-induced terminal differentiation and activates the mTOR pathway in HCK cells

Then, the biological effects of MYC on HCK were examined. Upon induction of MYC^{T58A} in HCK1T cells with BCL2, the expression levels of carbamoyl phosphate synthase/aspartate transcarbamoylase/dihydroorotase, a bona fide MYC target gene (18) and survivin (12) were increased, whereas the levels of a differentiation marker, involucrin, and a key inducer of keratinocyte differentiation, NOTCH1 (19), were decreased (Figure 4A). Furthermore, repression of TSC2 accompanied by activation of the mammalian target of rapamycin (mTOR) pathway was observed upon MYC^{T58A} induction. Activation of NOTCH1 and accumulation of involucrin induced by exposure to calcium and serum were largely canceled by MYC^{T58A} expression (Figure 4A). Similar effects of MYC^{T58A} induction were also observed in HCK1T-E cells with AKT or MEK1DD, although they were less marked, probably because E6E7 and AKT or MEK1DD influenced MYC regulation (17,20) (Figure 4B; data not shown). Induction of MYC^{T58A} significantly supported the growth of these cells in differentiating medium containing serum and high calcium, whereas no significant effects were observed in KGM (Figure 4C and D). Thus, MYC confers resistance to calcium- and serum-induced terminal differentiation and activates the mTOR pathway in HCK cells.

Inhibition of tumorigenic potentials of HCK1T-E–HRAS cells by inhibition of MYC or mTOR

Finally, we examined the role of endogenous MYC with HRAS in tumorigenic potential of HCK cells and cervical cancer cell lines (CaSki, SiHa, HeLa and C33A; Supplementary Figure 3B and C is available at *Carcinogenesis* Online; data not shown) by introducing the MYC inhibitor, OmoMYC (6), with Tet-regulated expression system, for which potential tumor-suppressive effects were recently reported in a mouse lung cancer model featuring KRAS mutation (21). OmoMYC induction levels were determined with an anti-MYC monoclonal antibody that recognizes both endogenous MYC and OmoMYC (Figure 5A). In contrast to the observations with MYC^{T58A} induction (Figure 4A and B), OmoMYC induction resulted

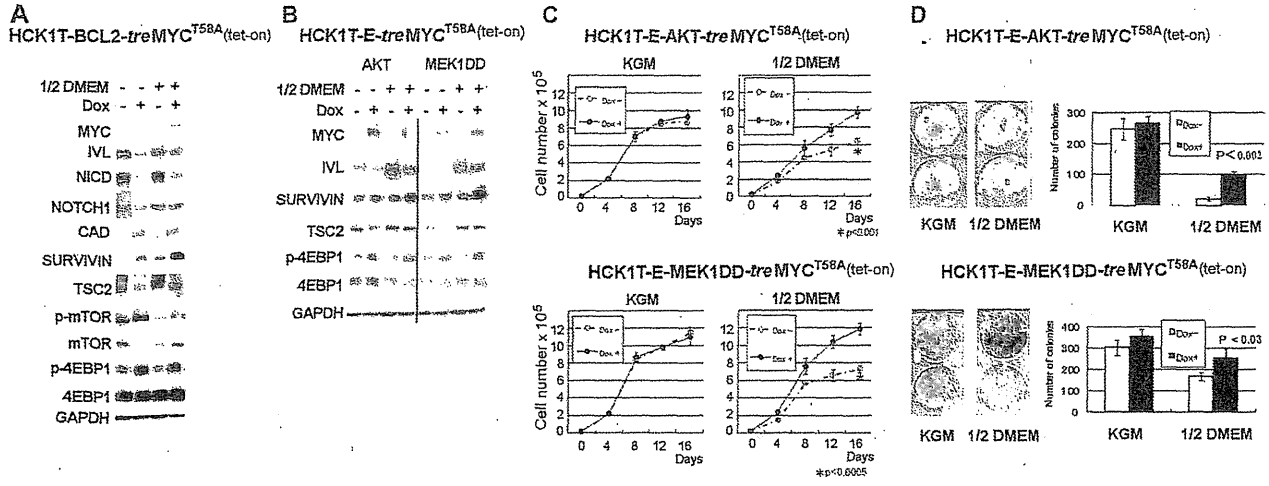

Fig. 4. Resistance to differentiation and mTOR activation by MYC. (**A**) HCK1T with BCL2 cells were treated with 1 μg/ml Dox for 3 days to induce MYC^{T58A} expression. Some cells were exposed to calcium and serum (KGM and DMEM were mixed at one to one ratio; 1/2 DMEM hereafter) for the last 1 day to induce calcium- and serum-induced terminal differentiation. Differentiation markers and MYC regulated genes were determined by western blotting. (**B**) HCK1T-E with AKT or MEK1DD with inducible MYC^{T58A} cells were treated as in (A). (**C**) Growth of cells in KGM (C left) and 1/2 DMEM (right, medium was replaced on day 1) were determined. A total of 2 × 10^4 cells were seeded on 22 mm dishes (BD Bioscience 3043) with the addition of Dox 1 μg/ml after 6 h and then counted on the indicated days. (**D**) The cells were seeded at a cell number of 2000 in wells of 35 mm dishes of six-well plates and 1 μg/ml Dox were added to the medium of three wells (dark bar) after confirmation of cell attachment (5 h). In half of the experiments, medium was replaced with 1/2 DMEM on day 2. After cultivation for 10 days, the cells were stained with Giemsa's dye, and the numbers of colonies were counted. Note that HCK1T-E with MEK1DD cells formed huge dense colonies with induction of MYC^{T58A} in clonogenic assay.

in increased involucrin and TSC2 (Figure 5A), further supporting regulation of these molecules through MYC in HCK cells. With induction of OmoMYC not exceeding endogenous MYC levels, HCK1T-E and HCK8T-E with HRAS cells did not result in significant reduction of growth (Figure 5B). Anchorage-independent growth ability of these cells was dramatically reduced with OmoMYC induction (Figure 5C) and tumorigenic potential was also profoundly reduced (Figure 5D). We obtained essentially the same result by moderate silencing of endogenous MYC in HCK1T-E with HRAS (Supplementary Figure 3A is available at *Carcinogenesis* Online). The induction of OmoMYC in cervical cancer cell lines also resulted in the suppression of their transforming abilities (Supplementary Figure 3B and C is available at *Carcinogenesis* Online). Although overexpression of MYC was not obvious in these cell lines, even in HeLa cells with a low level of MYC amplification (22), MYC might also play a critical role in these cells.

Because we found activation of the mTOR pathway in HRAS-transduced HCK cells, effects of an mTOR inhibitor, Rapamycin, on their transformation were tested. The clonogenicity of HCK1T-E with HRAS cells was reduced with Rapamycin in a dose-dependent manner (Figure 5E). Although either 10 nM Rapamycin or OmoMYC induction alone did not result in complete repression of clonogenic potential, simultaneous use of them blocked clonogenicity completely (Figure 5E), while strongly suppressing tumorigenic potential in nude mice (Figure 5F). These data indicate that the mTOR pathway is a major downstream effector activated by HRAS through MYC.

Discussion

MYC and RAS oncogenes can cooperatively induce full transformation of mouse cells but cause apoptosis and senescence, respectively, when expressed individually. Unlike the mouse cell case, transduction of MYC and RAS oncogenes into human cells does not suffice for full transformation, possibly because of more sophisticated tumor-suppressive failsafe mechanisms. However, we recently demonstrated that MYC and RAS can cooperatively transform human cells (HCKs) with the help of HPV16 E6 and E7 (4). In the development of cervical cancer, deregulated expression of E6 and E7 precedes disease progression, and E6 and E7 can immortalize HCKs and alleviate both MYC-induced apoptosis and RAS-induced senescence, mainly through inactivation of p53 and pRB. Here, we showed that oncogenic RAS on a background of E6E7 expression can induce full transformation of HCKs, and that stabilization of MYC by RAS is critical for tumorigenic transformation. Many mechanisms have been reported to be involved in MYC stabilization. A major ubiquitin ligase of MYC, FBXW7, preferentially recognizes and induces degradation of MYC with phosphorylated Thr58 and unphosphorylated Ser62, and thus the MYC^{T58A} mutant is very stable (23). Phosphorylation of Ser62 by ERK1/2 and inhibition of Thr58 phosphorylation through inactivation of GSK3β by AKT/PI3K are reported to be involved in RAS-induced MYC stabilization (17). Recently, CDK2 and downstream target(s) of PDK1 were also documented to phosphorylate Ser62 (24,25). Activities of these kinases can be regulated by multiple RAS signaling pathways as well.

If we could identify core gene sets, which promote reprogramming of normal human cells into cancer-generating cells, it would be of great advantage to understanding the complicated molecular mechanisms of carcinogenesis. In this study, we clarified that transduction of only three factors, namely oncogenic HRAS, E6 and E7, is sufficient for tumorigenic transformation of HCKs, though early studies have already suggested cooperation between E6E7 and oncogenic RAS (26–29). Thus, E6, E7 and HRAS might constitute one such core gene set. It also proved sufficient to induce full transformation of other normal human cell types, including human tongue keratinocytes, HBECs and HFFs though the HBECs and HFFs had been transduced with hTERT. Our recent study indicates that the role of E6E7 could be largely but not completely replaced by the blockade of the pRB and p53 pathways in human tongue keratinocytes (30). We previously found that ovarian surface epithelial cells could not be fully transformed by transduction of oncogenic KRAS and MYC with blockade of the pRB and p53 pathways by CDK4/CYCLIN D1 and a dominant-negative form of p53 (5). Other than inactivation of p53 and pRB, E6 and E7 proteins have many functions and it is very conceivable that these could be involved in full transformation. Indeed, in HCKs, the PDZ-binding motif of E6 is critical for full transformation of HCKs through degradation of several PDZ-containing proteins (our unpublished results).

Increased tumorigenic potential by exogenous MYC was observed with variation (Figure 1), indicating certain threshold levels of MYC are required for tumorigenicity depending on the cell type. However,

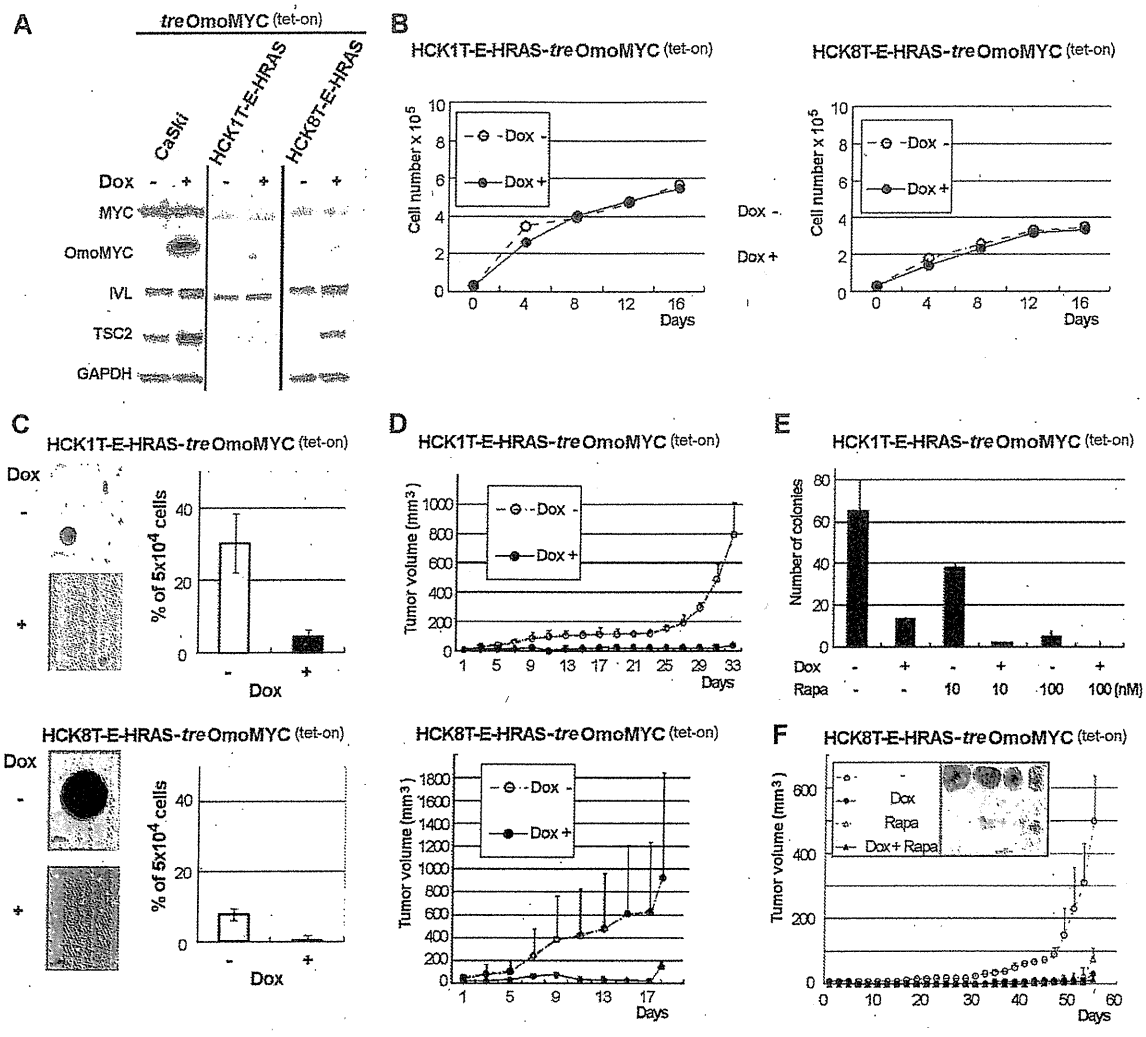

Fig. 5. Inhibition of MYC and/or mTOR pathway repressed tumorigenic potential of HCK cells with E6E7 and HRAS. (**A**) Induction of OmoMYC, an MYC inhibitor (Dox 1 μg/ml 5 days) and alteration of involucrin and TSC2 in HCK and CaSki cells were determined by western blotting. (**B**) Effects of OmoMYC induction on growth of HCK cells with E6E7 and HRAS were determined as for Figure 4C. (**C**) For assessment of anchorage independent growth of HCK cells with OmoMYC induction, aliquots (5×10^4 cells) were seeded in 35 mm dishes. After 3 weeks, the numbers of colonies (≥50 μm in diameter) were counted. (**D**) Effects of OmoMYC on tumor-forming ability of HCK cells were determined as for Figure 3B. (**E**) Clonogenic potential of HCK1T cells with OmoMYC or Rapamycin was determined as for Figure 4D. Indicated concentration of Rapamycin was added to the cells at day 1. (**F**) The effect of OmoMYC with Rapamycin on tumor-forming ability of HCK8T-E with HRAS cells was determined as in D. Mice were treated with 5 mg/kg Rapamycin administered by intraperitoneal injection twice a week.

transduction of four factors, E6, E7, RAS and MYC, proved sufficient for tumorigenic transformation of normal human cells tested here and broader cell types, including colon epithelial cells and pancreatic duct epithelial cells (data not shown), though hTERT might be additionally required for cells, such as HFFs, in which E6 cannot activate telomerase. In our previous study, HCK1T-E–HRAS–MYC cells adapted to DMEM showed much higher MYC expression than those kept in KGM (Supplementary Figure 4 is available at *Carcinogenesis* Online) with higher tumorigenicity, i.e. 10 DMEM-adapted cells formed huge tumors in ~50 days in contrast to the same cell number kept in KGM forming tiny tumors after ~100 days [(4) and data not shown]. DMEM-adapted cells might have gained the capacity to permit high levels of MYC and might give us a clue to understand further malignant conversion. Our preliminary data indicate that the DMEM-adapted cells exhibit epithelial–mesenchymal transition like changes, as determined by immunoblotting and microarray analysis (Supplementary Figure 4 and Supplementary Table 1 are available at *Carcinogenesis* Online).

We tried to dissect the RAS signaling pathways in order to define the critical factors for the promotion of cancer and found that activation of AKT or ERK pathway alone on the background of E6 and E7 expression was insufficient for full transformation. However, with additional induction of MYC^{T58A}, the cells acquired tumorigenicity in nude mice (Figure 3 and Table I, C). These results allow us to hypothesize that one critical player to promote cancer 'stemness' downstream of HRAS signaling is elevated function of MYC. In normal HCK1T, induced expression of MYC^{T58A} inhibited terminal differentiation and increased expression of Survivin, which is implicated as a cancer stem cell marker (31) (Figure 4A). Furthermore, we found that TSC2 expression was repressed with induction of MYC^{T58A}, as reported recently for another cell type (14), accompanied by activation of the mTOR pathway.

It was recently reported that MYC sustains pluripotency of induced pluipotent stem and embryonic stem (ES) cells through repression of the primitive endoderm differentiation regulator, GATA6 (32), and our results indicate that MYC confers resistance to calcium- and

serum-induced terminal differentiation (Figure 4A–D) and the tumorigenic potential on non-tumorigenic HCKs (Figure 3). Although MYC^{T58A} has reduced activity to induce apoptosis compared with wild-type MYC in the mammary gland (23), it does occur in a dose-dependent manner, even in the presence of E6 and E7 (Supplementary Figure 5 is available at *Carcinogenesis* Online) and remaining cells form tumors (Figure 3). We did not observe significant differences in the tumorigenic potential with the induction of MYC^{T58A} in HCK1T-E with HRAS cells (Supplementary Figure 1 is available at *Carcinogenesis* Online). It is also reported that low levels of deregulated MYC are competent to drive ectopic proliferation of somatic cells and oncogenesis, but overexpression of MYC wakes up the apoptotic and ARF/p53 intrinsic tumor surveillance pathways (33). These results clearly indicate that a certain threshold level of MYC is sufficient for tumor development, which is not affected by further overexpression, though such surplus expression of MYC might affect other pathological features such as metastasis.

MYC has been identified as one of four genes, which can reprogram fibroblasts into ES cells (34). Analysis of the ES cell-specific gene expression signature revealed that core pluripotency factors such as OCT4 and SOX2 are active in ES and induced pluipotent stem cells but not in cancer stem cells (35), but MYC regulatory networks are activated in both ES and cancer stem cells. Thus, MYC seems to play a role in normal ES cell biology and also cancer stem cells. MYC expression is deregulated in a wide range of human cancers and the rate of overexpression is generally more than the level of amplification (36). Cancers without amplification of MYC but with alterations in other oncogenes, such as RAS and growth factor receptors, which activate the function of MYC, could also be considered as MYC deregulated. Here, inhibition of endogenous MYC functions with Omo-MYC resulted in significant reduction of tumor formation and when the mTOR pathway activated by MYC was suppressed with Rapamycin, the tumorigenic potential of HCK cells was suppressed profoundly (Figure 5). To our knowledge, this is the simplest *in vitro* carcinogenesis model for human cancer and the first report indicating that endogenous MYC is a critical regulator of HRAS-induced tumor formation by human cells. The contribution of MYC to the cancer stemness might be broader than generally considered, and attempts to inhibit MYC functions with small molecules (37) as cancer therapy might be applicable to a wide range of malignancies.

Supplementary material

Supplementary Table 1 and Figures 1–5 can be found at http://carcin.oxfordjournals.org/.

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Conflict of Interest Statement: None declared.

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Full Paper

Creation of immortalised epithelial cells from ovarian endometrioma

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BACKGROUND: Epithelial cells of endometriotic tissues are difficult to propagate *in vitro* as experimental material is scarce owing to their limited life span. However, there is an increasing concern regarding their malignant transformation in ovaries. The present study sought to generate their stable culture system.

METHODS AND RESULTS: Purified epithelial cells isolated from ovarian endometriomas using microscopic manipulation were successfully immortalised by combinatorial transfection of human *cyclinD1*, *cdk4* and human telomerase reverse transcriptase (*hTERT*) genes, whereas the introduction of *hTERT* alone, or together with *cdk4*, was insufficient for immortalisation, leading to cellular senescence. We confirmed stable cytokeratin expression in the immortalised cells, proving their epithelial origin. These cells expressed progesterone receptor B and showed significant growth inhibition by various progestins. Oestrogen receptor (ER) expression was detected in these cells, albeit at low levels. Additional overexpression of ERα generated stable cells with oestrogen-dependent growth activation. Soft-agar colony formation assay and nude mice xenograft experiments demonstrated that these cells, even those with additional inactivation of *p53*, did not have transformed phenotypes.

CONCLUSION: We for the first time generated immortalised epithelial cells from ovarian endometrioma that retained sex steroid responsiveness. These cells are invaluable tools not only for the consistent *in vitro* work but also for the study of molecular pathogenesis or carcinogenesis of endometriosis.

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Endometriosis is a common gynaecological disorder associated with dysmenorrhoea, pelvic pain and subfertility and is a leading cause of disability and loss of productivity in women of reproductive age (Olive and Schwartz, 1993). Numerous studies have attempted to dissect the biology of endometriosis. These studies mainly use *in vitro* culture of stromal cells rather than culture of epithelial cells from endometriotic tissues, because the former cells are more easily and stably cultured for much longer periods than the latter cells (Noble *et al*, 1997; Gurates *et al*, 2002). In fact, it is difficult to culture endometriotic epithelial cells *in vitro*, because these cells lose their proliferative capacity during ongoing cultivation of primary cultures over several days. The inability of endometriotic epithelial cells to survive *in vitro* is an obstacle to gaining a better understanding of the biology of this disease. In particular, malignant change of endometriosis, especially of ovarian endometrioma, for which epithelial cells are exclusively responsible, has lately attracted considerable clinical attention (Kurman and Craig, 1972; McMeekin *et al*, 1995; Ness, 2003; Oral *et al*, 2003). There is therefore an urgent need to establish a stable system for the culture of endometriotic epithelial cells that can be used for research not only into the biology of endometriosis but also into its carcinogenesis.

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There are two major barriers within epithelial cells that inhibit cell division under usual culture conditions: premature senescence and telomere-dependent senescence (Kiyono *et al*, 1998). The former is observed during early passage in primary culture and is caused by the activation of Rb that leads to cell cycle arrest, whereas the latter is found at a later stage of culture and is caused by telomere shortening after a considerable number of cell divisions. Inhibition of both Rb function and telomere shortening is therefore required for long-term culture of epithelial cells. In previous studies, we successfully established a stable system for the culture of primary endometrial epithelial cells, in which the human papillomavirus type 16 *E6/E7* genes and the human telomerase reverse transcriptase (*hTERT*) were introduced to inhibit Rb functions and to activate telomerase, respectively (Kyo *et al*, 2003). These immortal cells were not transformed but retained the original characteristics of endometrial epithelial cells, such as steroid responsiveness. Subsequent studies have demonstrated that overexpression of *cyclin D1* or *cdk4*, instead of HPV *E6/E7*, effectively inhibited Rb activity and might be an alternative method of overcoming premature senescence in primary epithelial cells of other origins (Ramirez *et al*, 2004; Sasaki *et al*, 2009).

In the present study, we sought to generate a stable culture of epithelial cells isolated from the ovarian endometriomas by the introduction of various genetic elements. These cells were successfully immortalised without generation of transformed phenotypes and were responsive to progestin and oestrogen.

These cells are thus potentially useful as an experimental model for analysis of the mechanisms of steroid hormone functions as well as of carcinogenesis arising from ovarian endometrioma.

MATERIALS AND METHODS

Isolation and purification of human endometriotic glands

Human endometriotic tissue samples were obtained from a 27-year-old and a 44-year-old patient undergoing laparoscopic ovarian cystectomy as a treatment for ovarian endometrioma with written informed consent. Briefly, tissues were gently minced into small pieces (1–2 mm^3) and were incubated for 1 h at 37 °C in a shaking water bath in 20 ml Hank's Balanced Salt Solution containing 0.2% collagenase type 3 (Washington Biochemical Corp., Lakewood, NJ, USA) and 1000 U of deoxyribonuclease I (Takara, Otsu, Japan). Epithelial glands were separated from stromal cells, blood cells and debris by serial filtration using narrow gauge sieves with apertures of 40–100 μm. Individual glands on the bottom of the dishes were directly picked up one by one under a microscope, collected into Eppendorf tubes and seeded onto 24-well dishes for subsequent gene transfection by viral vectors. The use of clinical materials obtained with written informed consent was approved by the Institutional Research Ethics Committee.

Vector construction and transfection

Viral construction and transfection of HPV16 *E6/E7* and *hTERT* have been previously reported (Kyo *et al*, 2003). Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen, Carlsbad, CA, USA). Briefly, *hTERT*, *human cyclinD1* and *human mutant Cdk4* (Cdk4R24C: an inhibitor-resistant form of Cdk4 that was generously provided by Dr E Hara (The Cancer Institute of JFCR, Tokyo, Japan)) (Wölfel *et al*, 1995) were first recombined into entry vectors using the BP reaction (Invitrogen). These segments were then recombined with a lentiviral vector, CSII-CMV-RfA (a gift from Dr H Miyoshi (RIKEN BioResource Center, Tsukuba, Japan)) (Miyoshi *et al*, 1998), using the LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclinD1 and -hCDK4R24C. Production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was performed as described previously (Miyoshi *et al*, 1998). A dominant negative form of *p53* (*DN p53*) (Kiyono *et al*, 1994) was cloned into lentiviral vector plasmids by recombination using the Gateway system (Invitrogen). Oestrogen receptor α (ERα) overexpressing cells were established by lentiviral infection of the human ERα expression vector (pCMSCV-EM7bsd-hERα).

Cell culture

Stably established endometriotic epithelial cells were maintained in DMEM supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2 at 37 °C.

Reverse transcriptase–PCR (RT–PCR)

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA), and the first-strand cDNA was synthesised from 1 μg of total RNA by reverse transcription using Superscript II Reverse Transcriptase (Invitrogen) with random primers. Primer sequences and conditions for each gene are listed in Supplementary Table 1.

Western blot analysis

Nuclear extracts from cells were prepared using the method of Schreiber *et al* (1989). Subsequently, 50 μg of nuclear extracts were electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Membranes were blocked in TBST (150 mM NaCl, 20 mM Tris-Cl, pH 7.5 and 0.1% Tween) containing 5% nonfat dried milk and then incubated with specific antibodies against PR (H-190, dilution 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and actin (C-11, dilution 1:1000, Santa Cruz Biotechnology) followed by reaction with anti-rabbit IgG. Immunoreactive bands were visualised using the ECL detection system (GE Healthcare Biosciences, Pittsburgh, PA, USA), as suggested by the manufacturer.

Immunocytochemistry and immunohistochemistry

Cells were cultured on LAB TEK chamber slides (Nalge Nunc International, Naperville, IL, USA) for 24 h, fixed with methanol. For antigen retrieval of CD10, the sections were heated by boiling in 10 mM citrate buffer, pH 6.0 for 20 min followed by cooling at room temperature for 20 min. Slides were incubated for 60 min at room temperature with the following mouse monoclonal antibodies and working dilutions: anti-pan-cytokeratin (4/5/6/8/10/13/18) (C11, dilution 1:500, Santa Cruz Biotechnology) and anti-CD10 (clone 56C6, dilution 1:80, Leica Microsystems Inc., Buffalo Grove, IL, USA). After incubation with an anti-mouse secondary antibody, immune complexes were visualised using the ABC-elite kit (Vector Laboratories Inc., Burlingame, CA, USA).

β-gal assay

The β-gal assay was performed as previously described. Briefly, cells were fixed for 5 min at room temperature in 3% formaldehyde followed by incubation at 37 °C with senescence-associated β-gal stain solution containing 1 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl and 2 mM $MgCl_2$. After 6–12 h incubation, positive staining was confirmed using microscopy.

In vitro growth assay

The proliferative activity of cells treated with progestins or oestrogen was examined by counting the cell number. Briefly, the cells were seeded at a density of 5–10 × 10^4 cells per well in six-well flat-bottomed plates and were grown overnight in normal growth media at 37 °C. Cells that had been pre-incubated in normal growth media or in phenol red-free media containing charcoal-treated fetal bovine serum for 24 h were treated with 17β-estradiol (E2), 6α-methyl-17α-hydroxy-progesterone acetate (MPA), progesterone or dienogest at various concentrations. Ethanol was used as a vehicle control.

Assay of aromatase activity

The aromatase activity of cells was assayed by detecting the formation of tritiated water from [1β-^{3}H]-androstenedione (PerkinElmer Genetics, Bridgeville, PA, USA) as described (Shozu *et al*, 1997). We used a 4-h incubation for the experiment. Aromatase activity was expressed as the rate of incorporation of tritium into water per milligram of protein per 4 h of incubation.

Anchorage-independent growth

A total of 2 × 10^5 Ishikawa cells or immortalised cells were seeded onto 6-cm dishes containing 0.33% Noble agar in DMEM supplemented with 10% fetal calf serum on top of a 0.5% agar base in DMEM supplemented with 10% fetal calf serum. Colonies >0.2 mm were counted after incubation for 2 weeks.

Mice xenograft experiments

Immortalised endometriotic epithelial cells were resuspended in growth media (10^8 cells per ml) and were subcutaneously injected (0.1 ml) into the base of the bilateral flank of female BALB/c nu/nu mice (age range 7–9 weeks, SLC, Hamamatsu, Japan). Tumour growth was monitored weekly until confirmed tumours were visualised or at least for 2 months unless tumour formation was detected. All the experiments have been carried out with the ethical committee approval and meet the standards required by the UKCCCR guidelines (Workman *et al*, 2010).

RESULTS

Generation of immortalised epithelial cells from ovarian endometrioma

Endometriotic tissues were collected from the surface epithelia of ovarian endometrioma of two patients, a 27-year-old (patient 1) and a 44-year-old (patient 2) patient who underwent laparoscopic ovarian cystectomy. These tissues were minced and digested in a collagenase solution. Endometriotic glands were then roughly isolated by serial filtration from the stromal cells, followed by direct pick-up, one by one, using microscopic manipulation (Figure 1A). Approximately 100 glands were individually seeded on the wells of plastic dishes and were infected with various combinations of lentiviral vectors for expression of *cyclinD1*, *cdk4*, *dominant negative p53* and *hTERT*. For comparative purposes, various combinations of retroviral vectors for expression of HPV16 *E6*, *E7* and *hTERT* (Kyo *et al*, 2003) were also introduced (Figure 1). Combinatorial transfection of at least three out of these genes successfully generated a total of five independent cell populations from the two patients that achieved >40 population doubling (PD). Two of these populations were transfectants harbouring the *cyclinD1*, *cdk4* and *hTERT* genes, two harboured the *E6*, *E7* and *hTERT* genes and the other population harboured *cyclinD1*, *cdk4*, dominant negative *p53* (*DN-p53*) and *hTERT* genes. Morphologically, all of these cells exhibited a small round shape that was compatible with an epithelial origin and formed a mesh-like structure on plastic dishes (Figure 1B). Introduction of the *hTERT* gene alone, or together with *cdk4*, generated cells from both patients that passed through 10 PD, but finally led to growth arrest at PD between 15–40, during which they exhibited morphological change to a large and flat shape. This phenomenon was determined to be senescence because these cells stained positive for the senescence-associated β-gal (Figure 1C). The cells derived from patient 1 and 2 that gained an extended life span, following the introduction of *cyclinD1*, *cdk4* and *hTERT* genes (and *DN-p53*), were named as EMosis-CC/TERT1 (and EMosis-CC/TERT/DNp53-1) and EMosis-CC/TERT2, respectively, and the cells into which HPV *E6/E7/TERT* were introduced were named as EMosis-E6/E7/TERT1 and EMosis-E6/E7/TERT2, respectively. These cells continued to grow for over 100 PD (Figure 1E), without any morphological change or senescence-associated β-gal staining (Figure 1D). To date, these cells have grown for over 200 PD and continue to grow. We have therefore concluded that these cells have gained immortal phenotypes. These findings indicate that co-expression of *cyclinD1* and *cdk4* are required in order to overcome the premature senescence of endometriotic epithelial cells and that these genes, combined with the expression of *hTERT*, are sufficient for their immortalisation, whereas the additional inactivation of *p53* is not necessarily required for immortalisation.

Expression of epithelial markers and sex steroid receptors

To confirm the origin of the immortalised cells, we next examined the expression of various epithelial and stromal cell markers using RT–PCR analysis. All isolated cells that had an extended life span expressed cytokeratin 8 mRNA, whereas mRNA expression of the stromal marker FSP1 was not observed (Figure 2). The mRNA expression of CD10, a marker that is characteristic of endometrial and endometriotic stromal cells (Sumathi and McCluggage, 2002;

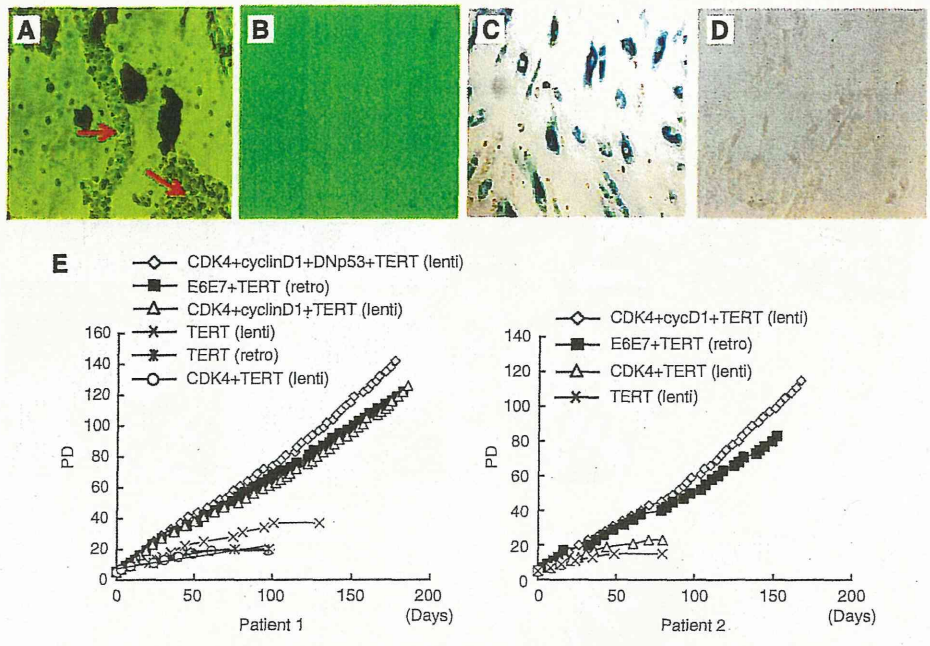


Figure 1 Morphological characteristics and proliferative life span of epithelial cells from ovarian endometrioma transfected with various genetic factors. (**A**) Phase contrast image of glandular clusters isolated from ovarian endometrioma tissues. Individual clusters were directly picked up, one by one, using microscopic manipulation and were transfected with various genetic factors. Glandular clusters are shown in arrows. (**B**) Phase contrast image of representative isolated clones (EMosis-CC /TERT-1 cells) cultured on plastic dishes are shown. (**C**) β-gal staining of cells from patient 1 transfected with *hTERT* alone (population doubling (PD): 20). (**D**) β-gal staining of EMosis-CC /TERT-1 cells (PD: 100). (**E**) The growth characteristics of transfected cells are represented as a growth curve. The genetic factors introduced are shown. Abbreviations: lenti = lentiviral vectors; retro = retroviral vectors.

Toki *et al*, 2002), was not detected in EMOsis-CC/TERT1 or EMOsis-E6/E7/TERT1 cells but was detected in EMOsis-CC/TERT2 and EMOsis-E6/E7/TERT2 cells, whereas CD10 mRNA was not detected in primary endometriotic glands isolated from patients 1 and 2 (Figure 2). We also verified the epithelial origin of these clones using immunocytochemistry. As shown in Figure 3, all of these cells stained positive for pan-cytokeratin. However, although

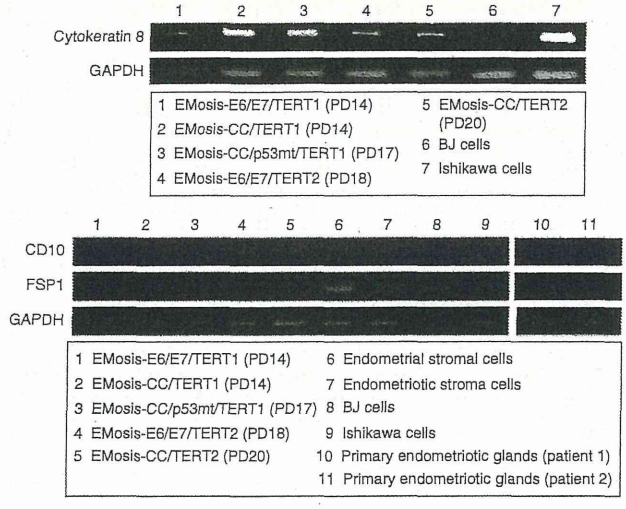


Figure 2 RT–PCR analysis of the expression of epithelial and stromal markers in immortalised epithelial cells from ovarian endometrioma. The expression of cytokeratin 8, CD10 and FSP1 in endometriotic epithelial cells immortalised by various genetic factors was examined using RT–PCR. Ishikawa and BJ cells were used as controls for epithelial and fibroblast cells, respectively. Primary endometriotic glandular cells without transfection, isolated from the ovarian endometrioma of patient 1 or 2, were used as negative controls for CD10 or FSP1 expression. GAPDH expression was assayed as a loading control.

EMOsis-CC/TERT1 cells were negative for CD10, EMOsis-CC/TERT2 cells exhibited apparent CD10 staining.

We further investigated steroid-receptor expression in these cells using RT–PCR. ERα and progesterone receptor B (PRB) were expressed in all cell types that had an extended life span, except for EMOsis-E6/E7/TERT1 that lacked ERα expression (Figure 4A). Because expression of the PR isoform PRA, which has an 164 amino-acid deletion of PRB (Kastner *et al*, 1990), can not be discriminated from that of PRB using RT–PCR because of their identical gene sequences, we performed western blot analysis to distinguish the protein expression of these two PR isoforms. There was no detectable protein expression of PRA or even of PRB in any cell type except for EMOsis-CC/TERT1 that had detectable PRB protein expression (Figure 4B). The expression of ERα was not detected in these immortalised cells by western blot analysis (Figure 4C). These results were summarised in Supplementary Table 2. Aromatase expression is another factor that needs to be considered in relation to steroid-receptor expression. A tritiated water assay revealed that there was no detectable aromatase expression in any of the immortalised cells using assay conditions under which control primary endometriotic stromal cells exhibited significant aromatase activity (Figure 4D).

Lack of transformed phenotypes in immortalised epithelial cells from ovarian endometrioma

We next sought to determine whether these immortal cells had acquired a transformed phenotype. First, their growth properties were examined using a soft-agar colony formation assay. A total of 2×10^5 cells were seeded on soft agar on 6-cm dishes and colonies with diameters >0.2 mm were counted after incubation for 2 weeks. Ishikawa or BJ cells were simultaneously examined as positive or negative controls, respectively. Although Ishikawa cells formed distinct colonies, neither the immortal epithelial cells nor the BJ cells formed colonies (Figure 5A). Tumourigenicity of these cells was also examined using nude mice. Control Ishikawa cells

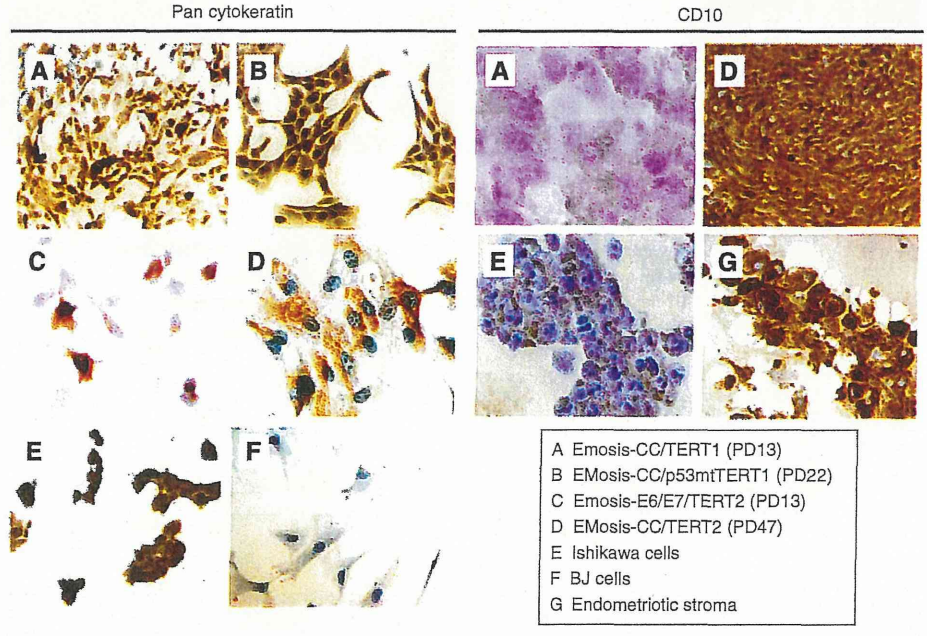


Figure 3 *Immunocytochemical analyses of cytokeratin and CD10 expression in immortalised epithelial cells from ovarian endometrioma. The expression of cytokeratin and CD10 in endometriotic epithelial cells that were immortalised by various genetic factors and cultured on LAB TEK chamber slides was examined using immunocytochemistry. Ishikawa and BJ cells were used as controls for epithelial and fibroblast cells, respectively. Primary stromal cells without transfection, isolated from the ovarian endometrioma of another patient, were used as a positive control for CD10.*

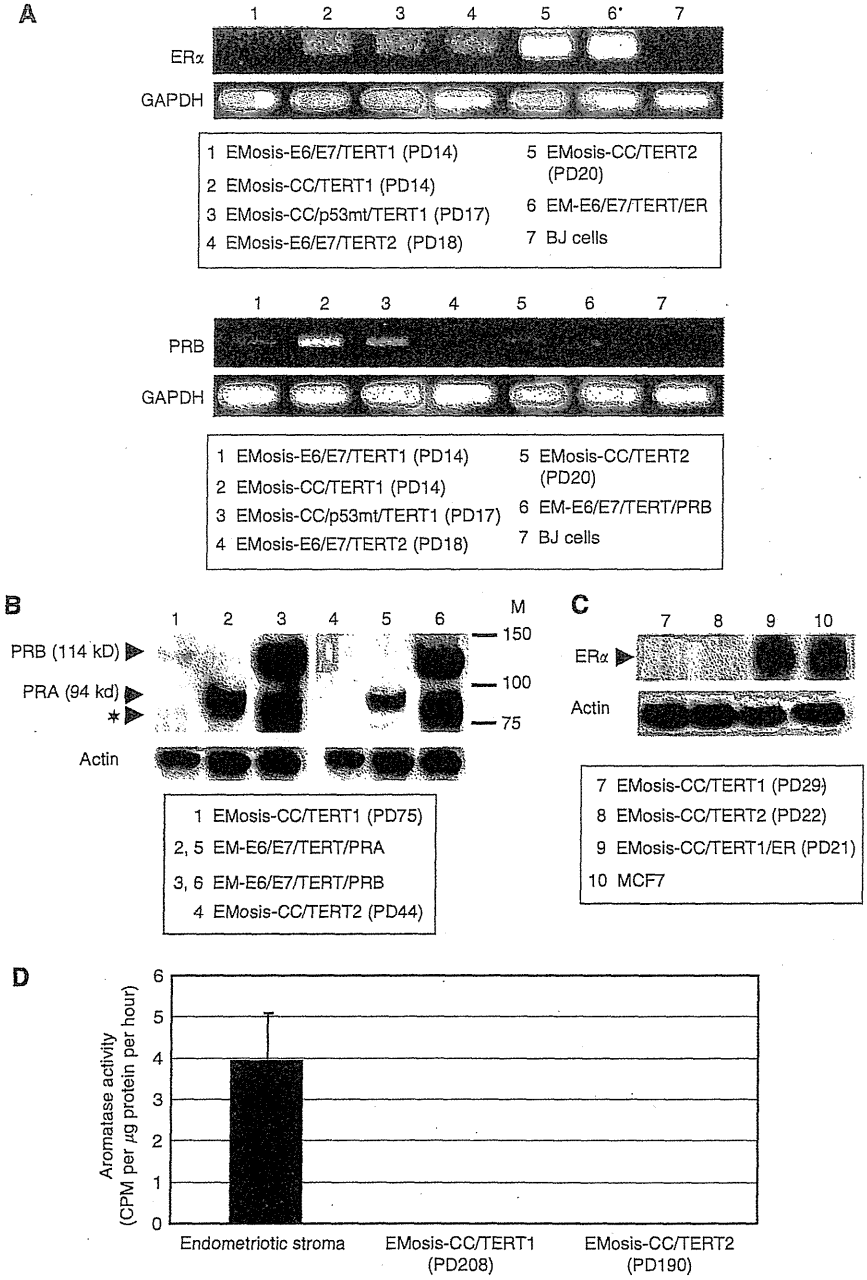
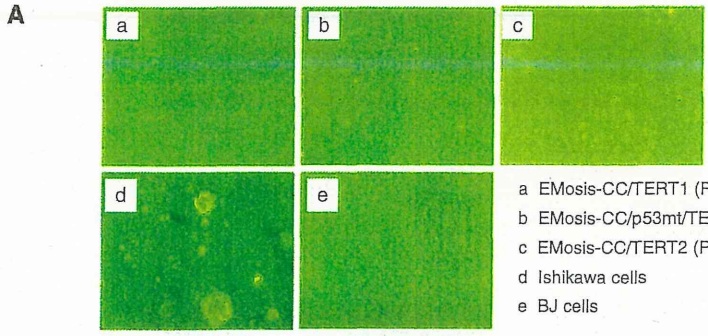
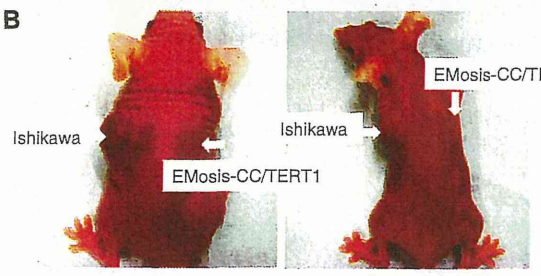


Figure 4 Sex steroid-receptor expression in, and aromatase activity of, immortalised epithelial cells from ovarian endometrioma. (**A**) RT–PCR analysis of expression of the oestrogen receptor α (ERα) or the progesterone receptor B (PRB). EM-E6/E7/TERT/ER cells are immortalised endometrial epithelial cells in which ERα cDNAs were stably transfected and were used as a positive control for ERα. EM-E6/E7/TERT/PRB cells are immortalised endometrial epithelial cells in which PRB cDNAs were stably transfected. Because our primer sets for PRB were designed to amplify the sequences containing *PRB* gene promoter in order to distinguish from PRA transcript, they can detect only intrinsic PRB mRNA but not extrinsic, overexpressed PRB mRNA that lacks promoter sequences. The weak PRB band in EM-E6/E7/TERT/PRB cells is therefore derived from intrinsic PRB. BJ cells were used as a negative control for ERα and PRB expression. GAPDH was used as a loading control. (**B**) Western blot analysis of expression of the progesterone receptor. EM-E6/E7/TERT/PRA or EM-E6/E7/TERT/PRB cells are immortalised endometrial epithelial cells in which PRA or PRB cDNAs were stably transfected and were used as a positive control for PRA or PRB expressions, respectively. Although EM-E6/E7/TERT/PRA cells showed a clear PRA band by western blotting (94 kDa), EM-E6/E7/TERT/PRB cells displayed two bands; one band was of the expected size of intact PRB (114 kDa); the other band was located just below the PRA band (identified by the symbol: ★) and was not a PRA band but a degraded PRB band, which was confirmed by another western blot analysis using a PRB-specific antibody (data not shown). EMosis-CC/TERT1 cells exhibited a weak, but distinct, PRB band but not a PRA band. M: protein weight marker. (**C**) Western blot analysis of expression of the ER. There was no detectable protein expression of ERα in EMosis-CC/TERT1 or EMosis-CC/TERT2 cells. EMosis-CC/TERT1/ER cells, generated by the introduction of ERα cDNA into EMosis-CC/TERT1 cells, were confirmed to have significant ERα expression. MCF7 cells were used as a positive control of ERα expression. (**D**) Analysis of aromatase activity using a tritiated water assay. Primary endometriotic stromal cells isolated from the ovarian endometrioma of another patient were used as a positive control of aromatase activity. Both EMosis-CC/TERT1 and EMosis-CC/TERT2 cells lacked aromatase activity.





Cells	Tumour formation/ inoculation
Ishikawa cell	3/3
HMOsis-CC/TERT1 (PD208)	0/4
HMOsis-CC/TERT2 (PD 190)	0/3

Figure 5 Analysis of the transformed phenotypes of immortalised epithelial cells from ovarian endometrioma. (**A**) Anchorage-independent growth was examined using a soft-agar colony formation assay. A total of 2×10^5 Ishikawa cells or immortalised cells were seeded onto soft agar and colonies >0.2 mm were counted after incubation for 2 weeks. Ishikawa and BJ cells were used as a positive and negative control for colony formation, respectively. (**B**) *In vivo* growth was examined using a tumour formation assay in nude mouse. Immortalised epithelial cells from ovarian endometrioma were resuspended in growth media (10^8 cells per ml) and were subcutaneously injected (0.1 ml) into the base of the bilateral flank of female BALB/c nu/nu mice (age range 7–9 weeks, SLC). Tumour growth was monitored weekly until confirmed tumours were visualised or at least for 2 months unless tumour formation was detected.

formed a subcutaneous tumour in mice 6 weeks after inoculation, but immortal epithelial cells were not able to form any tumour even 2 months after inoculation (Figure 5B).

Responsiveness of immortalised epithelial cells from ovarian endometrioma to progestin and oestrogen

We next examined the responsiveness of the immortalised epithelial cells to progestin. EMOsis-CC/TERT1 and EMOsis-CC/TERT2 cells were treated with MPA, dienogest or progesterone at a concentration of 1 or 100 nM for different time periods. Cell growth was then examined by counting cell numbers. Treatment with MPA or dienogest at a concentration of 10 or 100 nM significantly inhibited the growth of both cell types at 72 h (Figures 6A and B). Treatment with progesterone at 10 or 100 nM significantly inhibited the growth of EMOsis-CC/TERT1 at 72 h but only had a marginal effect on EMOsis-CC/TERT2 cells (Figure 6C). We performed these inhibitory experiments in growth media containing serum, considering clinical situations in which progestin is administrated *in vivo*. However, we also confirmed that progestin inhibited the growth of these cells in phenol red-free media containing charcoal-treated serum, although the extent of inhibition was less than that in normal growth media (data not shown), probably because of the cytostatic conditions of such media. These findings suggest that the immortalised epithelial cells preserved cell responsiveness to progestin.

We further examined the responsiveness of the immortalised epithelial cells to oestrogen. EMOsis-CC/TERT1 and EMOsis-CC/TERT2 cells were treated with 10 or 100 nM of 17β estradiol (E2) for different time periods. We failed to find any effect of E2 on the growth of either cell type (data not shown). This result was likely to be due to the low levels of ERα expression, which could only be faintly detected using RT-PCR. We therefore sought to overexpress ERα in EMOsis-CC/TERT1 cells via lentiviral introduction of ERα cDNA, and obtained ERα-overexpressing EMOsis-CC/TERT1 cells (EMOsis-CC/TERT1/ER). Sufficient expression of ERα in these cells was confirmed by western blot analysis (Figure 4C). The growth of EMOsis-CC/TERT1/ER cells was significantly activated by treatment with 17β-estradiol (E2) at a concentration of 100 nM (Figure 6D). Thus, we had successfully generated immortalised epithelial cells from ovarian endometrioma that still had the property of oestrogen or progestin responsiveness.

DISCUSSION

Although stromal cells in endometriotic tissues are easily isolated and grown under usual culture conditions, epithelial cells are hard to purify and propagate *in vitro*. This difficulty is mainly because of the rarity of epithelial cells in endometriotic tissues as well as to their shorter life span due to two barriers against their *in vitro* growth. To overcome these barriers, we first purified glandular fragments from endometriotic tissues that were treated with collagenase-based reagents via microscopic manipulation. We succeeded in immortalising endometriotic glandular cells through combinatorial introduction of the two genetic factors (*cyclin D1/cdk4*) that inhibit Rb functions together with *hTERT*. Special attention must be paid to contamination of the purified glandular fragments by stromal cells. This is because small amounts of stromal cells might possibly attach to the epithelial clusters providing a limitation to the purification of epithelial cells in glandular clusters. We therefore carefully judged whether the immortalised cells that we obtained were of epithelial origin. RT-PCR and immunocytochemical analyses confirmed the expression of epithelial markers in these cells. The introduction of *hTERT* alone, or the combination of *CDK4* and *hTERT*, failed to immortalise these cells. This result might provide further evidence of the epithelial origin of these cells, because stromal cells are usually immortalised by the introduction of *hTERT* alone (Kiyono *et al*, 1998; Morales *et al*, 1999). Indeed, Krikun *et al* (2004)

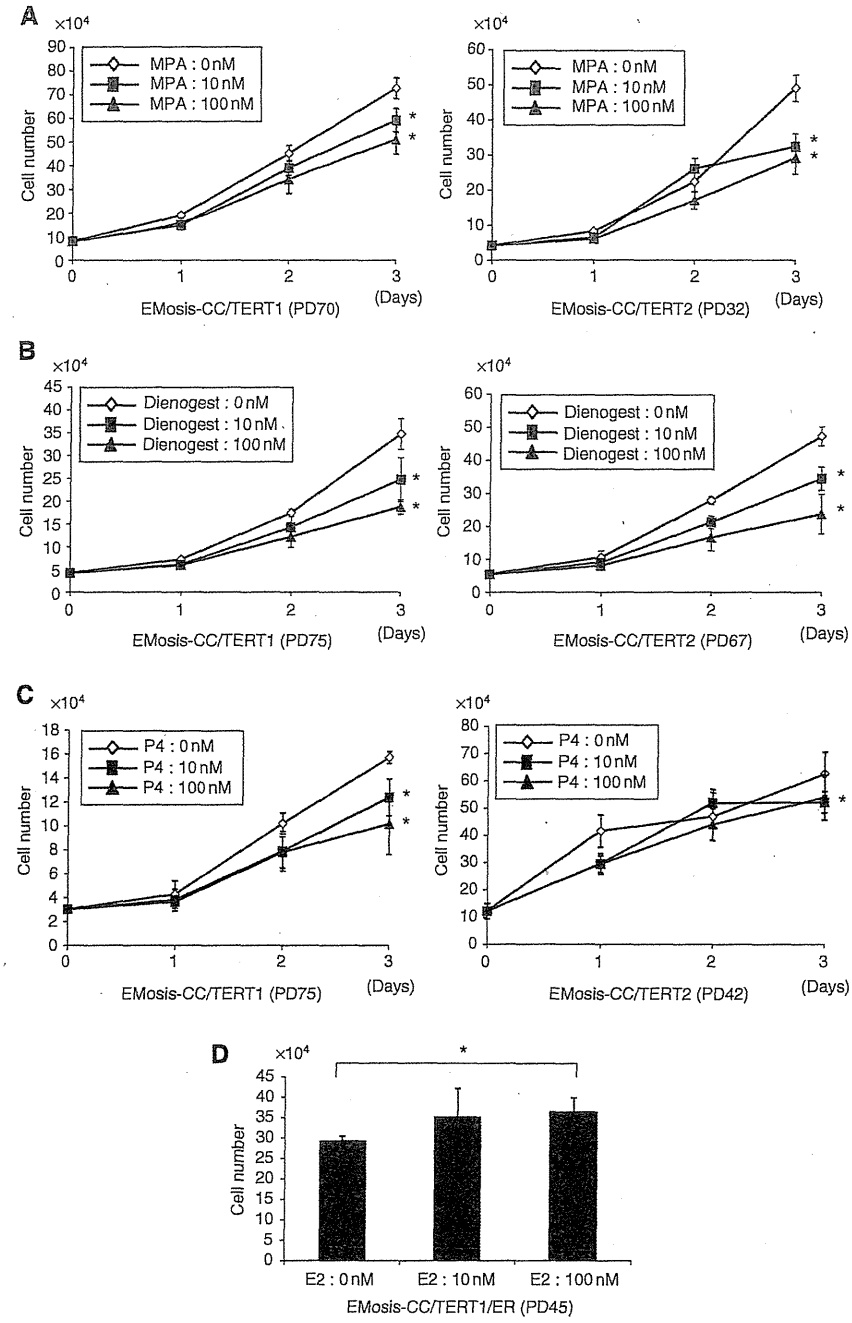


Figure 6 Effect of progestin and oestrogen on the growth of immortalised epithelial cells from ovarian endometrioma. EMosis-CC/TERT1 or EMosis-CC/TERT2 cells were seeded on six-well dishes and were treated with or without MPA (**A**), dienogest (**B**) or progesterone (P4) (**C**) at a concentration of 10 or 100 nM for the indicated number of days. Cell growth was monitored by counting cell numbers. Data are presented as means ± s.d. of three independent experiments. *$P < 0.05$. (**D**) EMosis-CC/TERT1/ER cells were generated by the introduction of ERα cDNA into EMosis-CC/TERT1 cells and confirmed to have significant ERα expression (Figure 4C). EMosis-CC/TERT1/ER cells were seeded on six-well dishes and were treated with or without MPA estradiol (E2) at a concentration of 10 or 100 nM for the indicated number of days. Cell growth was monitored by counting cell numbers on day 5 after treatment. Data are presented as means ± s.d. of three independent experiments. *$P < 0.05$.

confirmed immortalisation of endometriotic stromal cells by the introduction of *hTERT* alone. The requirement of two genetic factors that inhibit Rb function in addition to *hTERT* for cell immortalisation is consistent with observations in other epithelial cell types (Kiyono *et al*, 1998). Even the additional introduction of *DN-p53* failed to immortalise these cells, suggesting that they do not have high malignant potential, despite their invasive behaviour *in vivo*, which is reminiscent of cancer.

Although some studies showed that endometriotic tissues expressed PRB (Shen *et al*, 2008), others demonstrated that PRA was predominantly expressed and that PRB expression was low or absent (Attia *et al*, 2000; Wu *et al*, 2006). In the present study, one strain of the immortalised cells (EMosis-CC/TERT1) expressed PRB that was detectable using western blot analysis, whereas the other strain (EMOsis-CC/TERT2) did not. The reason why PRA was not detected in our western analysis remains unclear, but the

expression might weaken or diminish during *in vitro* culture and/or the subsequent immortalisation step. Both EMosis-CC/TERT1 and EMosis-CC/TERT2 cells responded well to progestin, exhibiting significant growth retardation. It is of particular interest that, even though EMosis-CC/TERT2 cells only weakly expressed PRB, which was only detectable using RT–PCR, they were responsive to progestin, suggesting that such a low level of PRB expression was sufficient for a progestin effect. To our knowledge, this is the first demonstration of cultured epithelial cells from ovarian endometrioma that have stable progestin responsiveness. These cells are therefore a valuable tool for the study of progestin action in endometriosis. Progestin resistance is one of the characteristics of this disease (Vercellini *et al*, 2003; Bulun *et al*, 2006). However, some patients (approximately 50–70%) respond well to progestin-related agents, whereas others do not (Vercellini *et al*, 2003; Momoeda *et al*, 2009). Although the molecular mechanisms of this diversity among patients are not fully understood, some studies have indicated that the absence of, or decrease in, PR expression, possibly via promoter hypermethylation, has a key role in progestin resistance (Wu *et al*, 2006; Burney *et al*, 2007). We recently reported that fork head protein O1 (FOXO1) is a direct target of progestin for inhibiting endometrial epithelial growth (Kyo *et al*, 2011). Phosphorylated Akt has a critical role in this pathway by inhibiting FOXO1 activity, and the status of Akt is a predictor of progestin responsiveness in this cell type. It is therefore of interest to know whether a similar scenario of FOXO1 regulation by progestin exists in endometriotic epithelial cells, and this possibility is under investigation.

CD10 is a characteristic marker of both endometrial and endometriotic stromal cells (Toki *et al*, 2002, Sumathi and McCluggage, 2002). Although the endometriotic epithelial cells were isolated from patient 2 lacked CD10 expression before transfection, the EMosis-CC/TERT2 cells did express CD10 and, in addition, retained CK8 expression as demonstrated using RT–PCR (Figure 3). One possible explanation of these inconsistent results is that the contaminated stromal cells might have had a growth advantage during the immortalisation steps and therefore became the predominant population in the immortal cell culture. However, this possibility is not likely because these immortalised cells continued to express cytokeratin, which was confirmed using both RT–PCR and immunocytochemistry. Alternatively, the process of epithelial mesenchymal transition might be involved in this inconsistent expression of CD10, and this possibility is also under investigation. Recently, several novel mechanisms have been proposed to explain endometrial and endometriotic regeneration. One study showed that epithelial cells in the endometrium might have originated from stromal cells via cellular transdifferentiation (Garry *et al*, 2010). Another study suggested that both epithelial and stromal cells in eutopic/ectopic endometrium might arise from a common cell type with stem-like properties (endometrial stem/progenitor cells) (Maruyama *et al*, 2010). Both studies thus proposed a common origin of epithelial and stromal cells in eutopic/ectopic endometrium. The expression of the stromal marker (CD10) in endometriotic epithelial cells that was observed in the present study may be consistent with a hypothesis. We consider that this phenomenon is interesting and that it will be worthwhile to carry out further extensive analysis to uncover the origin of endometriotic cells.

Aromatase p450 is expressed in a number of tissues such as ovarian granulose cells, adipose tissue, skin fibroblasts and brain (Simpson *et al*, 1994). Aromatase catalyses the conversion of androstenedione to estrone, which is further converted to the potent oestrogen E2 by the enzyme 17β-hydroxysteroid dehydrogenase type 1. It is known that aromatase is absent in normal endometrium, whereas it is expressed in eutopic/ectopic endometrium in patients with endometriosis (Bulun *et al*, 1993; Noble *et al*, 1997; Zeitoun *et al*, 1999), where it increases local oestrogen production and thereby contributes to the development of this disease. Although it is well established that aromatase activity exists in stromal cells in endometriotic tissues, and this activity is well characterised, the presence and the role of aromatase in epithelial cells are largely unknown. Although a few immunohistochemical studies have shown aromatase expression in endometriotic epithelial cells (Kitawaki *et al*, 1997; Bulun *et al*, 2001), no study has confirmed this finding in *in vitro* cultures, probably because of the difficulty of *in vitro* culture of these cells. Our cell system gave us the opportunity to explore this point and demonstrated that the immortalised epithelial cells from ovarian endometrioma completely lacked aromatase activity, as determined using a tritiated water assay. Therefore, our result strongly supports the absence of aromatase activity in endometriotic epithelial cells.

Although ovarian endometrioma is a benign tumour, some endometriomas have been known to develop into malignant tumours with clear cell or endometrioid-type histology (Wölfler *et al*, 2005; Mandai *et al*, 2009). Although some genetic factors, including *PTEN* mutation, are known to be associated with ovarian cancers arising from endometrioma (Anglesio *et al*, 2010; Jones *et al*, 2010; Wiegand *et al*, 2010), the molecular mechanisms of carcinogenesis are largely unknown. The immortalised cells we established were found to lack transformed phenotypes. These cells might therefore be an ideal model for the study of carcinogenesis, in which candidate genetic factors can be introduced or knocked down, enabling the identification of genetic factors required for transformation. In particular, the ERα-expressing EMosis-CC/TERT1/ER cells appear to be suitable for such purposes. We are currently testing *in vivo* propagation of these cells in NOG mice together with endometriotic stromal cells to reconstitute endometriotic tissues with glandular structures, or hopefully, cancerous tissues after various genetic manipulations. Thus, these cells will be essential for the complete understanding of the multistep carcinogenesis of ovarian endometrioma and hopefully may be useful for identification of novel molecular therapeutic targets.

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Conflict of interest

The authors declare no conflict of interest.

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Characterization of Gut-Derived Intraepithelial Lymphocyte (IEL) Residing in Human Papillomavirus (HPV)-Infected Intraepithelial Neoplastic Lesions

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C-C chemokine receptor type 9, cervical intraepithelial neoplasia, genital tract, integrin αEβ7, intraepithelial lymphocyte, mucosal immunity

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Problem
Mucosal T cells are the most likely direct effectors in host anti-human papillomavirus adaptive immunity and regression of cervical intraepithelial neoplasia (CIN) lesions. There are no studies addressing intraepithelial lymphocytes (IELs) in CIN lesions.

Method of study
Cervical lymphocytes were collected using cytobrushes from patients with CIN and analyzed by FACS analysis. Comparisons were made between populations of cervical T cells in CIN regressors and non-regressors.

Results
A median of 74% of cervical lymphocytes were $CD3^+$ T cells. Populations of integrin $\alpha E\beta 7^+$ IEL in CIN lesions varied markedly among patients (6–57%). Approximately half of integrin $\beta 7^+$ T cells were CD45RA-negative memory T cells. The number of integrin $\alpha E\beta 7^+$ cells among cervical T cells was significantly higher in CIN regressors when compared to non-regressors.

Conclusion
Higher cervical IEL numbers are associated with spontaneous regression of CIN. Accumulation of cervical integrin $\alpha E\beta 7^+$ IEL may be necessary for local adaptive effector functions.

Introduction

Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues (MALT) and in a variety of effector sites such as the mucosa of the intestine, respiratory tract, and genital tract.[1] The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin α4β7 and C-C chemokine receptor type 9 (CCR9). Lymphocyte-expressed integrin α4β7 and CCR9 bind to their natural ligands, mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCL25 (TECK), respectively, which are expressed on the cell surface of

endothelial cells in submucosal post-capillary venules.[1] In the intestine, mucosal dendritic cells (DCs) in gut-associated lymphoid tissues (GALT) regulate the expression of integrin α4β7 on activated effector and regulatory lymphocytes in a retinoic acid–dependent manner.[1–3] Mucosal T cells expressing integrin $\alpha4\beta7^+$ are known to circulate in peripheral blood from inductive sites and to home to the lamina propria (LP) at effector sites via α4β7-MAdCAM-1 and CCR9-CCL25 interactions.[4] Integrin $\alpha4\beta7^+$ T cells can differentiate into $\alpha E\beta7^+$ T cells upon exposure to TGF-β,[5] and the expression of integrin αEβ7 facilitates the retention of lymphocytes in the epithelium via interactions with E-cadherin.[4] Integrin αEβ7 is a specific marker of intraepithelial lymphocytes (IELs) residing in mucosal epithelia, and those cells expressing this antigen on their surface were initially educated in the gut.

The cervical mucosa is a very common site for pathogen invasion and is the primary transmission site for human papillomavirus (HPV), *Chlamydia trachomatis*, and human immune deficiency virus type 1 (HIV-1). A well-organized mucosal defense system in the cervical mucosa is critical to human health. Mucosal epithelial cells in the human cervix are active participants in such immunological protection.[6] However, the lymphocytes populating the cervical mucosal tissues, especially cervical IELs, have been poorly studied. Mucosal T cells in the murine genital tract express a large amount of integrin α4β7 on their cell surface,[7] and MAdCAM-1 is expressed on endothelial cells in the submucosa of murine fallopian tubes infected with *C. trachomatis*.[8] Several studies have demonstrated that human genital mucosa expresses MAdCAM-1 endogenously[9] and that GALT-derived integrin $\alpha4/E\beta7^+$ T cells home to the genital mucosa.[10,11] This T-cell homing and the expression of integrin αE increase in the presence of cervicitis and vaginitis.[10,11] Although integrin $\beta7^+$ mucosal T cells have been found in the cervical mucosa, a local inductive site (i.e., MALT) has never been demonstrated histologically.[11] We hypothesized that GALT may act as the inductive site for cervical IELs.

Human papillomavirus infection is a major cause of cervical cancer, and its precursor lesion, cervical intraepithelial neoplasia (CIN), develops in the epithelium. Natural history studies of CIN[12,13] show that most infections and CIN lesions resolve spontaneously but some persist and progress to cervical cancer. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions[14,15] suggest that cell-mediated immune response against HPV antigens is important in the control of HPV infection and progression to CIN. More controversial are the relative roles of systemic and local mucosal immune responses in the HPV pathogenesis. Trimble et al.[16] reported that naturally occurring systemic immune responses to HPV antigens do not predict regression of CIN 2/3 lesions, but Nakagawa et al.[17] demonstrated a positive association between systemic cell-mediated immune responses to HPV E6 and HPV/CIN regression.

We studied the local mucosal cell–mediated immune response to HPV antigens by characterizing cervical mucosal immune cells collected non-invasively, using only a cytobrush. We confirmed that the collected $CD3^+$ cervical T cells were intraepithelial in origin (integrin $\alpha E\beta7^+$ IELs). Approximately half of the integrin $\beta7^+$ T cells were memory T cells. Finally, integrin $\beta7^+$ intraepithelial T cells increased significantly in the patients whose CIN lesions regressed spontaneously regardless of HPV genotype.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 86 patients under observation after being diagnosed with CIN by colposcopically directed biopsy. All women gave written informed consent, and the Research Ethics Committee of the University of Tokyo approved all aspects of the study. Patients with known, symptomatic, or macroscopically visible vaginal inflammation or sexually transmitted infections were excluded from our study. Samples for HPV genotyping were collected at the first follow-up examination after diagnosis. Cervical lymphocytes were collected from non-menstruating patients at their latest follow-up visit. To study the potential association between cervical IEL characteristics and CIN progression, CIN patients with the regression of cervical cytology (cases) were matched with control patients who did not exhibit cytologic regression over the same time period (measured from initial detection of abnormal cytology). In this study, cytological regression was defined as normal cytology at two or more consecutive evaluations conducted at 3 to 4-month intervals. Thirteen patients were enrolled in the regression group, and the median

follow-up duration was 27 (12–38) months. Thirteen pairs of follow-up time-matched patients with persistent cytological abnormalities were enrolled in the non-regression group, and the median follow-up time was 24 (12–40.5) months.

HPV Genotyping

DNA was extracted from cervical smear samples using the DNeasy Blood Mini Kit (Qiagen, Crawley, UK). HPV genotyping was performed using the PGMY-CHUV assay method.[18] Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and human leukocyte antigen-DQ (HLA-DQ) primer sets. Reverse blotting hybridization was performed. Heat-denatured PCR amplicons were hybridized to specific probes for 32 HPV genotypes and HLA-DQ reference samples. The virological background (HPV genotyping) of 86 patients in our study was shown in Table I. Here, HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs according to International Agency for Research on Cancer multicenter study.[19]

Table I Human Papillomavirus (HPV) Genotype Distribution

HPV type	Total numbers (%)
16	19 (18.4)
18	7 (6.8)
31	2 (1.9)
33	1 (1.0)
35	1 (1.0)
39	1 (1.0)
45	1 (1.0)
51	7 (6.8)
52	20 (19.4)
53	4 (3.9)
56	3 (2.9)
58	12 (11.7)
59	3 (2.9)
68	3 (2.9)
82	1 (1.0)
6	2 (1.9)
54	1 (1.0)
55	1 (1.0)
66	4 (3.9)
69	1 (1.0)
70	3 (2.9)
83	3 (2.9)
84	2 (1.9)
Total	103 (100)

Patients infected with multiple HPV types were included. Of 86 patients, 32 (37%) were infected with multiple types. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs.[19]

Collection and Processing of Cervical Specimens

Cervical cells were collected using a Digene cytobrush as described previously.[20] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was placed in a 15-mL tube containing R10 media [RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B] and an anticoagulant (0.1 IU/mL of heparin and 8 nM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37°C for 15 min with shaking, the cytobrush was removed. The tube was centrifuged at $330 \times g$ for 4 min. The pellet was resuspended in 10 mL of 40% Percoll, layered onto 70% Percoll, and centrifuged at $480 \times g$ for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was >95%, as confirmed by trypan blue exclusion test, and fresh samples were immediately used for further analysis.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled, incubated on ice for 30 min, washed twice with FACS buffer (10% FCS, 1 mM EDTA, and 10 mM NaN_3) and fixed by adding paraformaldehyde in PBS to a final concentration of 1%.

The following fluorochrome-conjugated mouse monoclonal antibodies specific for human leukocyte surface antigens were used: a fluorescein isothiocyanate (FITC)-conjugated pan leukocyte marker (FITC-anti CD45), a B lymphocyte marker (FITC-anti CD19), a cytotoxic T-cell marker (FITC-anti CD8), a helper T-cell marker (FITC-anti CD4), an integrin β7 marker (FITC-anti integrin β7), a phycoerythrin (PE)-conjugated integrin α4 marker (PE-anti integrin α4), an integrin αE marker (PE-anti integrin αE), a C-C chemokine receptor type 9 marker (PE-anti CCR9), a marker for naïve cells (PE-anti CD45RA), a phycoerythrin cyanine 5 (PC5)-conjugated pan T lymphocyte marker (PC5-anti CD3), a natural killer cell marker (PC5-anti CD56), and an allophycocyanin (APC)-conjugated pan T lymphocyte marker (APC-anti CD3). Cell preparations were labeled in parallel with appropriate isotype control

antibodies. Antibodies were purchased from eBioscience (San Diego, CA, USA) and Beckman Coulter (Brea, CA, USA). Data were acquired using three-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). The positions of lymphocytes and monocytes were determined on the forward scatter versus side scatter (SSC) profile. The positions of pan-lymphocytes and T lymphocytes were determined by CD45 and CD3 gating, respectively. As the percentage of B cells among cervical lymphocytes is known to be low (less than a few percentage) when compared to the 20% level seen in peripheral blood,[20] the presence of elevated $CD19^+$ B cells in cervical specimens would indicate contamination with peripheral blood. For our investigations, cervical samples with more than 3% B cells were excluded from analysis.

Statistical Analysis

Statistical analyses, including calculation of medians and interquartile ranges (IQRs), were performed using the commercial statistical software package JMP® (SAS, Cary, NC, USA). Wilcoxon rank sum test or Fisher's exact test was applied for matched paired comparisons. *P*-values ≤0.05 were considered significant.

Results

Purification of Cervical Leukocytes Collected from CIN Lesions

To characterize mucosal cellular immune responses in HPV-infected lesions, cervical samples, including exfoliated epithelial cells and cervical lymphocytes, were collected from CIN lesions positive for any HPV genotype using a cytobrush. Cervical samples were fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells, and the layer between Percoll and culture medium was collected. Cervical lymphocytes were identified among isolated cells using standard SSC and CD45 gating (Fig. 1). Approximately 10^4–10^5 $CD45^+$ cells were isolated from patients' cervices. $CD45^+$ cells primarily consisted of lymphocytes (Fig. 1, circle) and granulocytes (Fig. 1, square). A minority of the cells included in the square in Fig. 1 were monocytes (data not shown). Two representative cases are provided in Fig. 1: the upper panel represents a patient with numerous granulocytes and a relatively small population of $CD45^+$ lymphocytes (10%), whereas the lower panel represents a patient with few granulocytes and a high number of lymphocytes (30%).

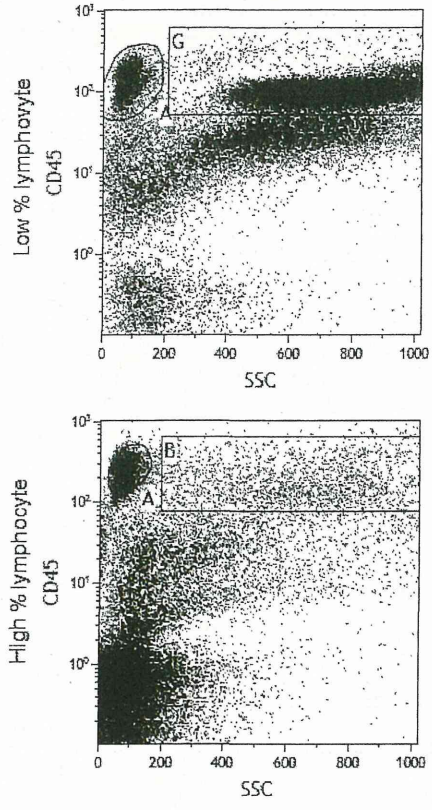

Fig. 1 Flow cytometric analysis of cervical mucosal cells using CD45/SSC gating. Processed cervical specimens were analyzed by flow cytometry and CD45/SSC gating. $CD45^+$ cervical leukocytes are comprised of lymphocytes (circle) and granulocytes/monocytes (square). Upper and lower panels were representative of patients with low (about 10%) and high (about 30%) numbers of lymphocytes among their $CD45^+$ cervical leukocytes, respectively. The absolute number of isolated cervical lymphocytes remained relatively constant among study subjects.

Characterization of Cervical T Cells in CIN Lesions

The majority of cervical lymphocytes isolated from CIN lesions were $CD3^+$ T cells [median 74% (IQR: 59–82)]. $CD19^+$ B cells were rarely found [median 0.45% (IQR: 0.04–1.40)]. In Fig. 2, CD3-gated cervical T cells were characterized by flow cytometry, and each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively. A median of 54%

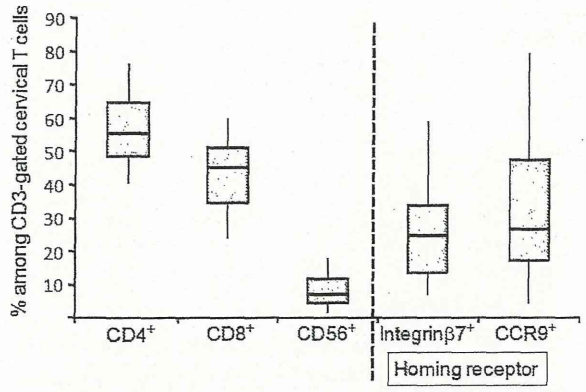

Fig. 2 Characterization of the cervical $CD3^+$ lymphocytes. CD3-gated cervical T cells consisted of $CD3^+$ $CD4^+$ T cells [median 54% (IQR: 49–65), $n = 28$], $CD3^+$ $CD8^+$ T cells [median 46% (IQR: 35–51), $n = 28$], and $CD3^+$ $CD56^+$ natural killer T cells [median 5.6% (IQR: 4.5–12), $n = 17$]. Twenty-four percentage (IQR: 13–34, $n = 43$) and 27% (IQR: 17–47, $n = 27$) of cervical T cells were integrin $\beta7^+$ and $CCR9^+$, respectively. Each median, IQR, and maximum/minimum range is indicated using horizontal lines, boxes, and vertical length lines, respectively.

(IQR: 49–65) of cervical T cells were $CD3^+$ and $CD4^+$, while a median of 46% (IQR: 35–51) expressed CD3 and CD8, demonstrating that $CD8^+$ T cells are relatively abundant among cervical T cells. The CD4/CD8 ratio of 1.15 in the cervix was clearly lower than the value of 2.0 found in peripheral blood. Among $CD3^+$ cells, a median of 5.6% (IQR: 4.5–12) were $CD56^+$ natural killer T (NKT) cells.

Those cervical $CD3^+$ T cells that were originally derived in the gut were defined by expression of the gut mucosa–specific cell-surface antigens integrin $\beta7^+$ and $CCR9^+$. A median of 24% (IQR: 13–34) of cervical T cells expressed integrin β7 and 27% (IQR: 17–47) expressed CCR9 (Fig. 2).

Notably, more than 90% (median: 99.1, IQR: 95.3–100) of the integrin $\beta7^+$ cells co-expressed the αE subunit (integrin $\alpha E\beta7^+$ cells; Fig. 3a). Integrin $\alpha4^+$ cells were rarely present among the integrin $\beta7^+$ cells (Fig. 3b). Approximately 40% (median: 40.1, IQR: 33.2–44.2) of cervical integrin $\beta7^+$ cells were integrin $\alpha E\beta7^+$ $CCR9^+$ double positive (Fig. 3c).

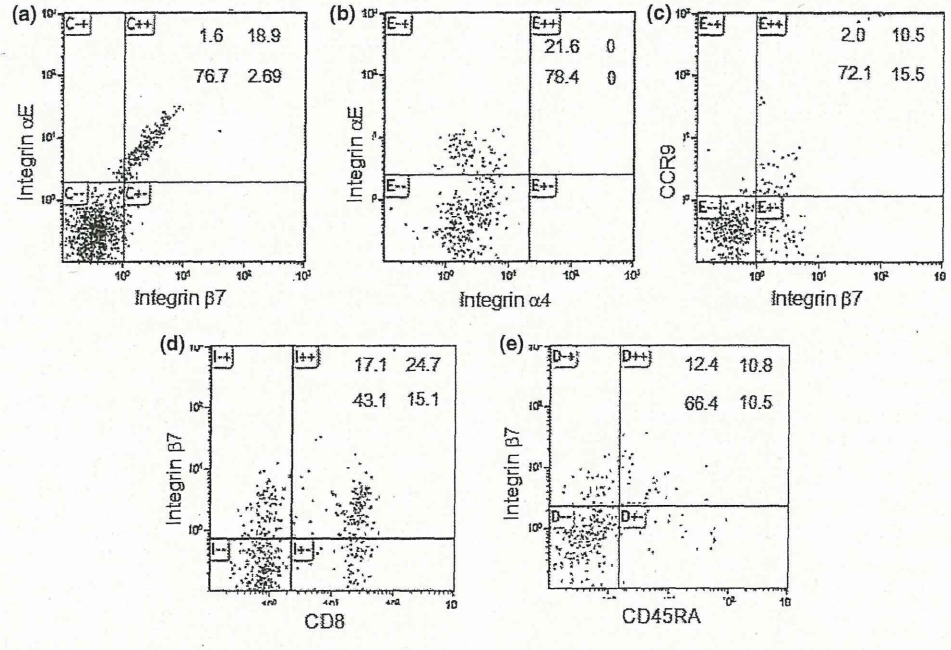

Fig. 3 Characterization of CD8, CD45RA, and homing receptors specific for gut-derived mucosal T cells among $CD3^+$ cervical T cells. Representative flow cytometry analyses of CD3-gated cervical T cells. (a) More than 90% of integrin $\beta7^+$ cervical T cells were integrin αE^+ intraepithelial lymphocyte. (b) Integrin $\alpha4^+$ LPL were negligible in our cervical samples. (c) Among integrin $\beta7^+$ cells, approximately 40% were $CCR9^+$. (d) Forty-two percentage of total cervical T cells and 53% of integrin $\beta7^+$ T cells were $CD8^+$. (e) About half of the integrin $\beta7^+$ T cells were CD45RA-negative memory cells.