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Roles of the PDZ-binding motif of HPV 16 E6 protein in oncogenic transformation of human cervical keratinocytes

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Key words

Cervical cancer, E6, human cervical keratinocytes, human papillomavirus, PDZ domain

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The high-risk human papillomavirus E6 proteins have been shown to interact with and lead to degradation of PDZ-domain-containing proteins through its carboxy-terminal motif. This PDZ-binding motif plays important roles in transformation of cultured cells and carcinogenesis of E6-transgenic mice. However, its biological effects on the natural host cells have not been elucidated. We have examined its roles in an *in vitro* carcinogenesis model for cervical cancer, in which E6 and E7 together with activated HRAS ($HRAS^{G12V}$) can induce tumorigenic transformation of normal human cervical keratinocytes. In this model, E6Δ151 mutant, which is defective in binding to PDZ domains, almost lost tumorigenic ability, whereas E6SAT mutant, which is defective in p53 degradation showed activity close to wild-type E6. Interestingly, we found decreased expression of PAR3 in E6-expressing cells independently of E6AP, which has not been previously recognized. Therefore, we knocked down several PDZ-domain containing proteins including PAR3 in human cervical keratinocytes expressing E7, $HRAS^{G12V}$ and E6Δ151 to examine whether depletion of these proteins can restore the tumorigenic ability. Single knockdown of SCRIB, MAGI1 or PAR3 significantly but partially restored the tumorigenic ability. The combinatorial knockdown of SCRIB and MAGI1 cooperatively restored the tumorigenic ability, and additional depletion of PAR3 further enhanced the tumorigenic ability surpassing that induced by wild-type E6. These data highlight the importance of the carboxy-terminal motif of the E6 protein and downregulation of PAR3 in tumorigenic transformation of human cervical keratinocytes.

Infection with specific human papillomaviruses (HPVs), such as HPV-16 and -18, is a major risk factor for human cancer of uterine cervix. From the fact that E6 and E7 genes are almost exclusively expressed in cervical cancer cells and that they can inactivate tumor suppressors, p53 and pRB, respectively, they are believed to play key roles in cervical carcinogenesis. In addition to inducing p53 degradation, numerous studies have indicated that the E6 protein has many other targets. The C-terminal PDZ-binding motif is specifically conserved among E6 proteins of high risk HPVs, and is essential to bind and enhance degradation of several PDZ domain-containing proteins, including DLG1, DLG4, SCRIB, MAGI1 and PTPN13.[1–6] Accumulating lines of evidence suggest that the PDZ domain-binding motif is particularly important for transformation and tumorigenesis in cultured cells, transformation of primary human keratinocytes, and hyperplasia and carcinogenesis in E6-transgenic mice,[1,7] and some of the PDZ proteins are known to have tumor suppressor functions.[8–12] Interestingly, the E7 protein but not the E6 protein of Rhesus papillomavirus type 1 (RhPV1), which is closely related to HPV-16[13] and causes anogenital malignancy in their host,[14] has the PDZ-binding motif at the C-terminus, and interact with the cell polarity regulator PAR3,[15] belonging to the same pathway of regulation as SCRIB and DLG1. Other viral oncoproteins such as HTLV1 Tax and Adenovirus E4ORF1 also conserve the C-terminal PDZ-binding motif, suggesting a role in their viral life cycle and oncogenic potential of these viruses.[2]

However, little is known about which PDZ domain-containing target(s) are important for HPV-induced cervical carcinogenesis. We previously demonstrated that transduction of oncogenic HRAS ($HRAS^{G12V}$) and MYC together with HPV16 E6E7 is sufficient for highly tumorigenic transformation of primary human cervical keratinocytes (HCKs).[16] More recently, we found $HRAS^{G12V}$ and HPV16 E6E7 is sufficient for tumorigenic transformation of HCKs.[17] Having taken advantage of this system, we found the PDZ domain-binding motif plays an important role in tumorigenic transformation of

primary HCKs, and further investigated which PDZ domain-containing target(s) are critical for HPV-induced cervical carcinogenesis.

Materials and Methods

Cell culture and cell lines. Normal human cervical keratinocytes were obtained with written consent from patients who underwent abdominal surgery for a gynecological disease other than cervical cancer. HCK1, HCK4 and HCK12 were used in this study. These cells were established by transduction of human TERT into HCK1, HCK4 and HCK12 cells, and maintained in low-calcium serum-free keratinocyte-growth medium (KGM) (Epilife-KG2 KURABO Industries, Ltd, Osaka, Japan). Cervical cancer cell lines, HeLa, CaSki, SiHa, C33A, OMC4 and Yumoto were maintained in Dulbecco's modified Eagle medium (DMEM; Sigma, St Louis, MO, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS). All cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Vector construction and retroviral infection. Construction of the retroviral expression vectors, pCLXSH-hTERT, pCLXSN-16E6E7, pCLXSN-16E7, pCLXSN-16E6, and its mutant has been described previously[18,19] (Table S1). Similarly, pCLXSN-6E6, -11E6, -18E6, -26E6, 30E6, -31E6, -33E, -35E6, -39E6, -43E6, -45E6, -52bE6, 54E6, -58E6, -59E6 were prepared by using the Gateway system so that each construct contains Kozak consensus sequence and E6open reading frames. Human $HRAS^{G12V}$ cDNA in pBabe-puro-HrasG12V (a gift from Dr. Hahn WC) was cloned and recombined into retroviral expression vectors to generate pCMSCVbsd-$HRAS^{G12V}$. pCMSCVbsd was produced by replacing the puromycin resistant gene in pCMSCVpuro with a segment containing blasticidin-S resistant gene. The construction of the destination vector pDEST-SI-CMSCVpuro, the entry vector pENTR-H1R-stuffer and the short-hairpin RNA (shRNA) retroviral expression vectors has been described previousl.[20] The targeted sequences for MAGI1, SCRIB, DLG1, DLG4, PAR3 and firefly luciferase were listed in Table S2. The production of recombinant viruses and selection of infected HCKs were detailed earlier.[16]

Western analysis. Whole-cell proteins were extracted in lysis buffer (0.5% NP40, 1 mM DTT, 50 mM NaCl, 25 mM Tris-HCl, pH 8.0, 0.02% NaN_3,) supplemented with 5% (v/v) protease inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan). Gels were loaded with 20 μg of total cell lysate per lane as described previously.[19] A monoclonal antibody to HPV16E6 (clone 47A4) was generated by using the N-terminal 16 amino acids peptide of HPV16E6 as an antigen. All other antibodies were purchased as followings: monoclonal antibodies against HPV16E7 (clone 8C9; Invitrogen, Carlsbad, CA, USA), DLG4 (PSD95) (Upstate, K28/43), DLG1 (Santa Cruz, Dallas, TX, USA BD Transduction lab, clone 12) and p53 (Oncogene Science/EMD Millipore, Billerica, MA, USA; Ab6), p21 (Oncogene Science, WAF1 Ab1), GAPDH (Ambion, Austin, TX, USA; AM4300) and polyclonal antibodies against HRAS (Santa Cruz, sc-520), PAR3 (Upstate 07-330 and MBL, D235-3), SCRIB (Becton Dickinson, Franklin Lakes, NJ, USA; C-20), MAGI1 (Sigma, M-5691), α-tubulin (Cell Signaling Technologies, Danvers, MA, USA; 2144S) and β-actin (Santa Cruz, sc-1616). Primary antibodies indicated above were used as probes with horseradish peroxidase-conjugated anti-mouse (Jackson Immunoresearch Laboratories, West Grove, PA, USA; Cat#115), anti-rabbit (Jackson Immunoresearch Laboratories, Cat#111) or anti-goat (Santa Cruz, sc-2033) immunoglobulins as the secondary antibodies. All the antibodies were used at a dilution of 1/1000 for Western blotting. The LAS3000 CCD-imaging system (Fujifilm Co. Ltd., Tokyo, Japan) was employed for detection and quantification of proteins visualized by Lumi-light plus Western blotting substrate (Roche, Basel, Switzerland).

RT-PCR analysis. Total RNA was obtained from cultured each cell lines using an RNeasy kit according to the manufacturer's protocol. Single-stranded cDNAs were synthesized from 500 ng of RNA using a reverse transcription system (Promega, Madison, WI, USA). Fifty nanograms of cDNA template was subjected to PCR amplification with primer sets specific to E6 or acidic ribosomal phosphoprotein P0 (36B4) using a PCR reagents kit (Applied Biosystems, Foster City, CA, USA). Sequences of primer pairs used were as follows: 5′- GCAAC AGTTACTGCGACGTG -3′ and 5′- GGACACAGTGGCTTT TGACA -3′ for E6 and 5′-TCGACAATGGCAGCATCTAC-3′ and 5′-GCCTTGACCTTTTCAGCAAG-3′ for 36B4 as described previously.[4]

Clonogenic assay. Cells were seeded into 6 well plate (Falcon, Corning, NY, USA; cat#353046) containing 2 mL of medium. The number of 500 cells to seed per well in a 5%

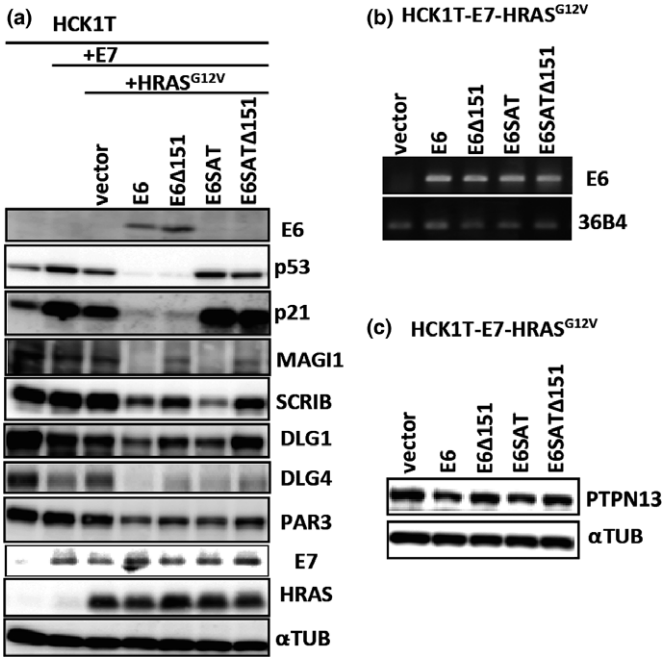

Fig. 1. Establishment of HCKT-E7-$HRAS^{G12V}$ expressing the wild type HPV16E6 or its mutants defective for degradation of p53 and/or PDZ domain containing proteins. Expression of HPV16E7, HRAS mutant, $HRAS^{G12V}$, and HPV16E6 or its mutants was introduced to HCK1T cells by retrovirus mediated transduction as described in Materials and Method. The levels of the wild type 16E6 and its mutant were examined by Western blots (a) and RT-PCR (b). (a) The mouse monoclonal antibody for HPV16E6 was raised against 16 amino acids of its N-terminal region and therefore does not react with HPV16E6SAT which contains R8S/P9A/R10T substitution. While the wild type E6 and E6Δ151 which lacks its C-terminal amino acid induced p53 degradation, E6 mutants with SAT substituions such as E6SAT and E6SATΔ151 did not do so. The levels of PDZ domain containing proteins, such as Scribble (SCRIB), DLG4, MAGI-1 (MAGI) and PAR3 were decreased in wild-type E6, but not E6Δ151 or E6SATΔ151 expressing cells. The level of HPV16E7 and HRAS were not affected by the expression of E6. α-tubulin was detected as a loading control. (b) mRNA levels of the wild type E6 and its mutants were comparable in those cells. 36B4 mRNA was also detected as an internal control. (c) The level of PTPN13 was compared in indicated cells by Western blotting.

CO_2 environment at 37°C. After 2 weeks, colonies were fixed, stained and counted as previously described.[20]

Colony formation in soft agar medium. Cells were seeded at 5×10^4 cells per 6-well plate in an appropriate medium. Colonies were counted after 3 weeks as previously described.[16]

Tumorigenesis in nude mice. All surgical procedures and care administered to the animals were in accordance with institutional guidelines. A 100 μL volume of cells in a 1:1 mixture of Matrigel (BD Biosciences, San Jose, CA, USA) was subcutaneously injected into female BALB/c nude mice (Clea Japan Inc., Tokyo, Japan). The expression of human involucrin in all tumors was determined by Western blots with antibodies against human involucrin (clone SY5; Sigma) that do not react with mouse epidermis to confirm that the tumors were derived from implanted HCKs (data not shown).

Results

The C-terminal PDZ domain-binding motif of HPV16 E6 is critical for inducing enhanced proliferation, anchorage-independent growth and tumorigenic potential of HCK cells expressing E7 and HRAS^{G12V}. Recently, we reported that introduction of HPV16 E6 and E7 (E6E7) and HRAS^{G12V} was sufficient for tumorigenic transformation of HCK1T cells, which are normal cervical keratinocytes transduced with hTERT, and primary normal cervical keratinocytes.[16] In order to examine biological functions of E6 in HPV-induced cervical carcinogenesis, HCK1T cells expressing HPV16 E7 and HRAS^{G12V} were first established and then each of wild type and three mutants of HPV16 E6 were transduced into the cells. 16E6Δ151 is defective in binding to PDZ domains, 16E6SAT is defective in degradation of p53 and 16E6SATΔ151 is defective in both activities (Table S1). As expected, p53 and the downstream effector p21 were downregulated by 16E6 and 16E6Δ151 but not by 16E6SAT containing R8S/P9A/R10T substitutions,[21] and 16E6SATΔ151. On the other hand, DLG1, MAGI1, SCRIB and PTPN13 were downregulated by 16E6 and 16E6SAT but not by 16E6Δ151 and 16E6SATΔ151 (Fig. 1a,c). Interestingly, PAR3 was also decreased in parallel with downregulation of these reported E6 targets. Since the anti-16E6 monoclonal antibody (clone 47A4) raised against the N-terminal 16 amino acids is unable to detect 16E6SAT, which has mutations in the epitope, expression of these mutants were confirmed by RT-PCR (Fig. 1b, upper panel). These cell lines were characterized by proliferation in ordinary culture, clonogenic assay, anchorage-independent growth and tumorigenic potential in nude mice (Fig. 2). Wild type 16E6 induced enhanced proliferation of the cells, and the two mutants defective in p53 degradation showed profoundly reduced activity, whereas 16E6Δ151 showed intermediate activity (Fig. 2a). Similar tendency was observed in clonogenic assay (Fig. 2b,c). However, anchorage-independent growth capacity and tumorigenic potential were strongly reduced in the E6 mutants defective in binding to PDZ domains, whereas E6 SAT mutant clearly enhanced tumorigenic potential of the cells though not to the levels of

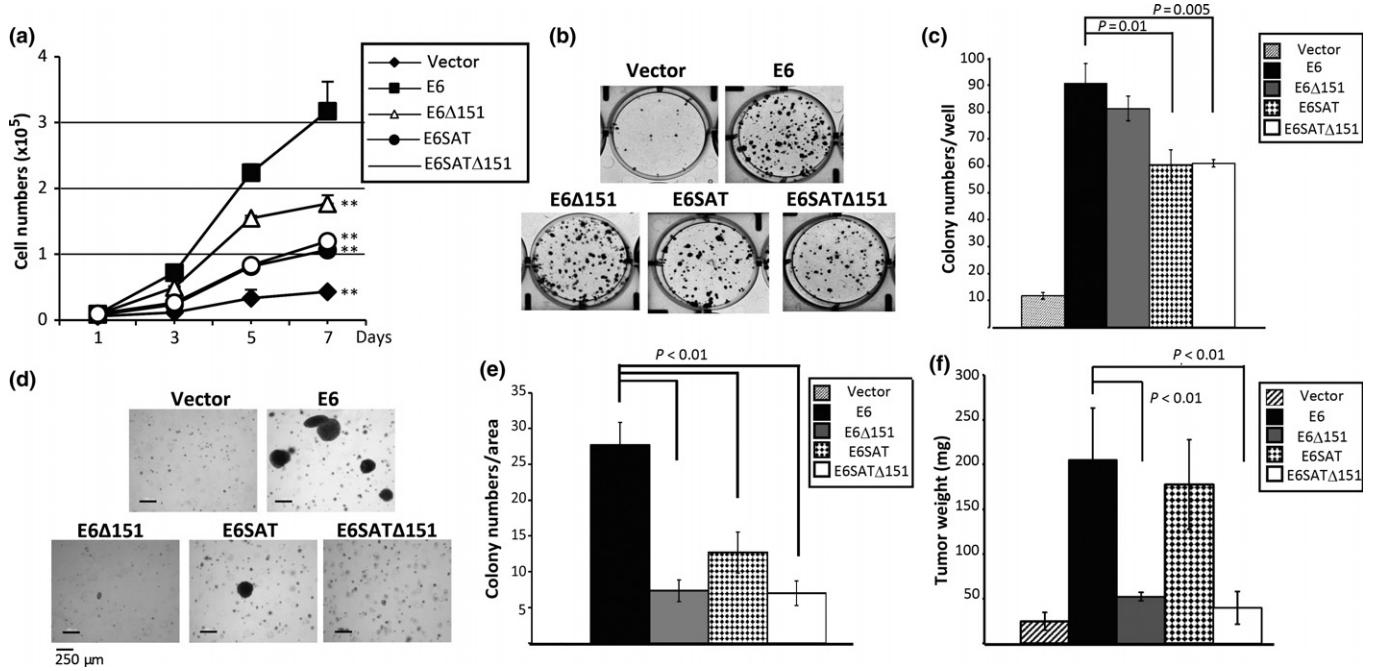

Fig. 2. Tumorigenic potentials of HCK1T-E7-HRAS^{G12V} expressing the wild type or E6 mutants defective for inducing degradation of p53 and/or PDZ domain containing proteins. The ability of E6 mutants to promote cell proliferation was compared to the wild type by examining growth curve (a) and clonogenic potential (b and c). HCK1T-E7-HRAS^{G12V} cells transduced with retrovirus containing an empty vector (vector) or expressing the wild type or mutants of E6 were seeded at 2×10^4 cells/well in 12 well plates at day 0 and cell number in each well was counted at indicated time points. ** indicates *P*-value < 0.05 compared to the wild type E6 expressing cells. (b, c) The cells were seeded on 35-mm dishes under sparse conditions. After cultivating for 2 weeks, the cells were stained with Giemsa's dye, and number of colonies was counted. The photographs are representative dishes (b), and the graph illustrates means + SDs (c). Anchorage independent growth of HCK1T-E7-HRAS^{G12V} expressing wild type or mutants of E6 was examined (d, e). The cells were seeded onto soft agar at 5×10^4 and cultivated for 4 weeks. The representative images are shown (d). Colonies whose size was >50 μm in diameter were counted and the total number of colonies in a 16 mm^2 area was compared (e). Tumor promoting potentials of the E6 mutants were compared to the wild type by mouse xenografts. 1×10^6 cells mixed with Matrigel were subcutaneously injected into nude mice. Mice were sacrificed at 40 days after the injection and tumor weights were compared (f). *P*-value was evaluated by student's *t*-test.

wild type 16E6 (Fig. 2d–f). The results were confirmed by two independently established HCKs, HCK4T and HCK12T (Fig. S1a–c for HCK4T and d–f for HCK12T). Since the expression of cytokeratin 7, which is recently reported as a marker of cells-of-origin of cervical cancer,[22,23] was detected in HCK1T cells but not in HCK4T and HCK12T cells by western blotting (data not shown) and the tumorigenicity was severely impaired in HCK1T-E7-HRAS^{G12V} with 16E6Δ151 mutant, we considered HCK1T to be more relevant to analyze cervical carcinogenesis, and used in the following analyses.

Depletion of single PDZ domain-containing proteins partially restored the reduced anchorage-independent growth and tumorigenicity by 16E6Δ151 mutant. In order to examine the effect of knockdown of PDZ domain containing proteins on the tumor promoting ability of 16E6, we designed shRNAs specifically knock down each PDZ domain-containing proteins. These include DLG1, DLG4, MAGI1, SCRIB, PTPN13 and PAR3. Among a few different shRNAs to each target tested, the most potent shRNAs were chosen for transduction into HCK1T cells expressing E7, HRAS^{G12V} and 16E6Δ151 (Fig. 3a). Knockdown of SCRIB, MAGI1 and PAR3 significantly enhanced anchorage-independent growth, while that of DLG1, DLG4 and PTPN13 showed only marginal effects. However, effect of any single shRNA did not completely restored the reduced activity of 16E6Δ151 (Fig. 3b). Consistent with these results, tumorigenicity of the cells was also significantly restored by depletion of SCRIB, MAGI1 or PAR3, while weakly by depletion of DLG1, DLG4 or PTPN13 (Fig. 3c). However, the restoration of tumorigenicity of HCK1T-16E7-HRAS^{G12V}-16E6Δ151 by single knockdown of SCRIB, MAGI1 or PAR3 was partial, indicating that reduction of single PDZ domain containing protein by E6 is not sufficient for its ability to promote tumor growth.

Depletion of MAGI1, SCRIB and PAR3 cooperatively enhanced anchorage-independent growth and tumorigenicity of HCKs expressing E7, HRAS^{G12V} and 16E6Δ151. Since the wild-type E6

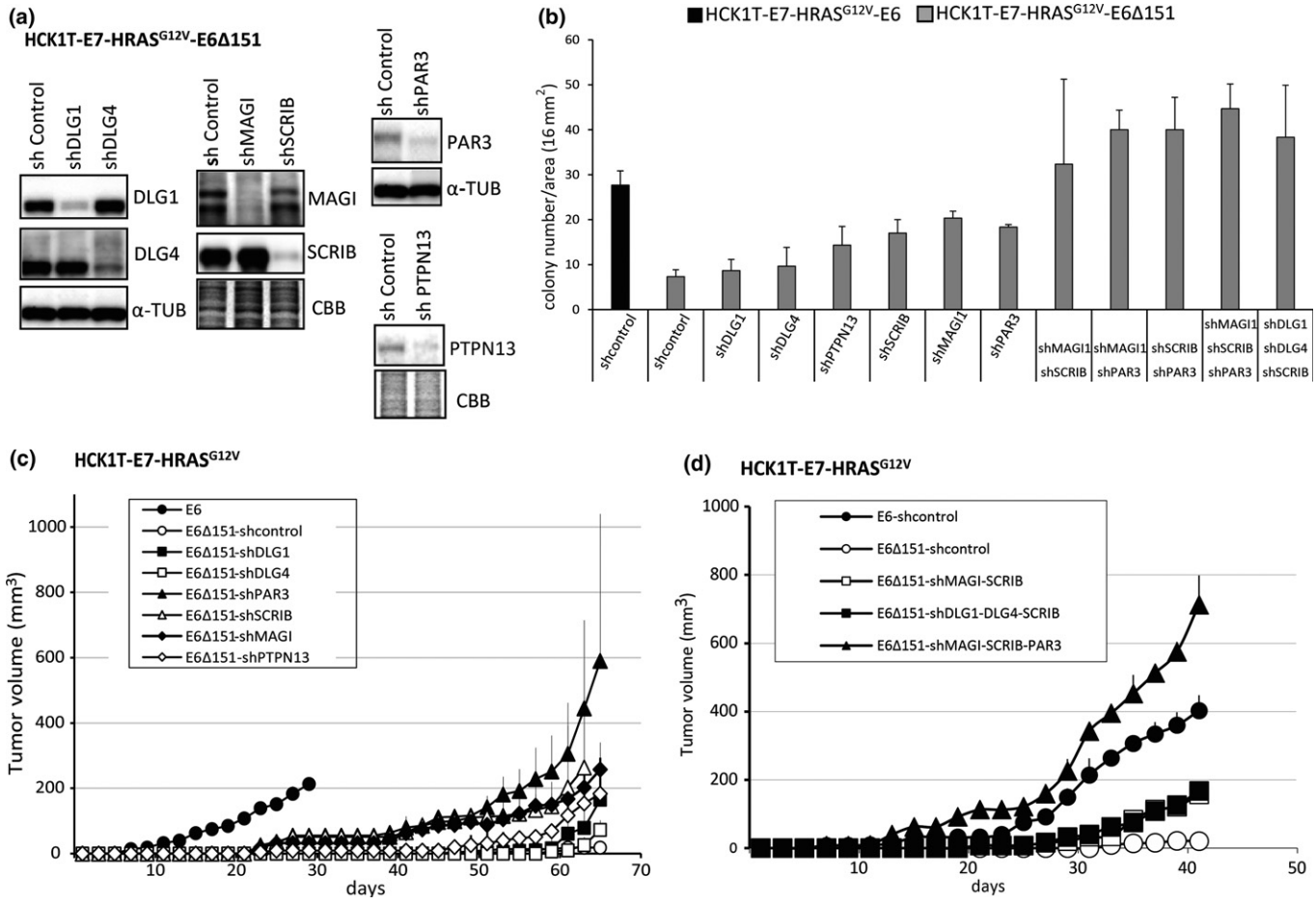

Fig. 3. The effect of knock-down of PDZ domain containing proteins on the tumorigenicity of HCK1T-E7-HRAS^{G12V} expressing E6Δ151. Retrovirus expressing shRNA to the indicated PDZ- domain containing proteins was infected to HCK1T-E7-HRAS^{G12V} expressing E6Δ151 at (a, c). At least two independent shRNA to each PDZ-containing protein were tested for their efficiency to knockdown target proteins and the most efficient shRNA was used for further analysis. A retrovirus expressing shRNA to luciferase was used as a control. For knockdown of multiple target proteins, three retroviruses each expressing shRNA to either PDZ proteins or luciferases were simultaneously infected to the HCK1T-E7-HRAS^{G12V} expressing E6Δ151 at MOI 5 (b, d). (a) Western blot analysis showing knockdown efficiency of shRNA to the indicated target proteins used in the following analysis. α-tubulin was also detected as a loading control. CBB staining was performed to indicate equal loading. (b) Anchorage independent growth of HCK1T-E7-HRAS^{G12V} expressing E6Δ151 with shRNA either to control or PDZ containing proteins was analyzed compared to HCK1T-E7-HRAS^{G12V} expressing wild type E6 with control shRNA. (c) Tumor promoting potentials of HCK1T-E7-HRAS^{G12V} expressing E6Δ151 with knockdown of single PDZ protein was compared to HCK1T-E7-HRAS^{G12V} expressing the wild type E6 by mouse xenograft. The size of tumor was measured at the indicated time points. (d) The effect of knockdown multiple PDZ containing proteins on tumorigenicity of HCK1T-E7-HRAS^{G12V} expressing E6Δ151 was also analyzed. Error bar indicates SD. *P*-value was evaluated by student's *t*-test.

protein inactivates multiple PDZ-domain containing proteins simultaneously, we tried to examine the effect of concurrent depletion of multiple target proteins. To this end, we transduced representative combinations of shRNAs doubly or triply at the multiplicity of infection of 5. Depletion of the target proteins was confirmed by Western blot (data not shown). Every pair of MAGI1-, SCRIB- and PAR3-shRNA examined cooperatively enhanced anchorage-independent growth of the cells, and combination of these three shRNAs further enhanced the anchorage-independent growth to the level that wild type 16E6 could induce (Fig. 3b). In addition, the combination of SCRIB-, DLG1- and DLG4-shRNA resulted in similar level as the wild type E6 did. The tumorigenicity of these cells were then examined in nude mice. The combination of MAGI1- and SCRIB-shRNA cooperatively enhanced the tumor growth more than any single shRNA did, however not to the level as the wild type 16E6 (open square and closed circle, respectively in Fig. 3d). Similarly, triple knockdown of DLG1, DLG4 and SCRIB shRNA enhanced the tumor growth more than any single shRNA did but did not fully restore the wild type level. Notably, triple knock-down of MAGI1, SCRIB and PAR3 surpassed the tumor growth induced by wild type 16E6 (closed triangle in Fig. 3d).

PAR3 downregulation by E6 is not dependent on E6AP. Since we found that HPV16 E6 reduced PAR3 level in the PDZ-binding motif-dependent manner (Fig. 1) and depletion of PAR3 had the strongest effect to enhance tumorigenicity of HCK1T cells expressing $HRAS^{G12V}$ and 16E6Δ151 (Fig. 3), we examined whether PAR3 is downregulated in cervical cancer cell lines. Interestingly, in HPV-positive cervical cancer cells, PAR3 were generally reduced in compared to normal keratinocytes such as HCK1T and HaCaT cells and HPV negative cervical cancer cells including C33a, OMC4 and Yumoto cells, similar to DLG4, MAGI1 and p53 (Fig. 4a). Several reports suggested that degradation of not only p53 but also some of PDZ-domain containing proteins by HPV16E6 was mediated by E6AP.[4,24,25] Therefore, we examined whether the reduction of PAR3 in HPV positive cervical cancer cells is dependent on the ubiquitin ligase, E6AP or not. Upon depletion of E6AP, levels of p53 but not PAR3 were increased in HeLa and SiHa cells (Fig. 4b).[4] These results indicate that PAR3 is not a direct target of the E6/E6AP complex. Microarray analysis indicated mRNA levels of PAR3 were comparable among parental HCK1T cells and HCK1T cells expressing HPV16E6 wild type or HPV16E6Δ151 (data not shown). Therefore, we examined whether the treatment of proteasome inhibitors, MG132 and Epoxomicin, can restore protein levels of PAR3 and p53 in HCK1T-E7-$HRAS^{G12V}$ cells expressing HPV16E6 wild type or HPV16E6Δ151 as well as SiHa cells with or without E6AP knockdown. As expected, the levels of p53 were restored partially in HCK1T-E7-$HRAS^{G12V}$ cells expressing HPV16E6 wild type or HPV16E6Δ151 and fully in SiHa cells. The levels of PAR3 were also restored in HCK1T cells expressing HPV16E6 wild type or HPV16E6Δ151 and SiHa cells with and without E6AP knock-down. These results suggest that reduction of PAR3 by E6 is at least in part attributable to enhanced protein degradation by proteasome (Fig. S2). Because PDZ ligand motif in high risk HPV E6 has been associated with tumorigenic potentials, we examined whether reduction of PAR3 by the E6 protein was specific to high-risk HPVs. Indeed, E6 of high risk HPVs except for HPV45 generally induced reduction of PAR3 in HCK1T cells as compared to parental HCK1T. The reduction of PAR3 level was also detected in HCK1T expressing E6 of some low risk HPVs such as HPV43 and 54. Surprisingly, PAR3 was most strongly reduced by E6 protein of HPV54, but not at all by that of HPV6 and 11 (Fig. 4c).

Discussion

We first confirmed downregulation of the PDZ-domain containing proteins, which were reported to be targets of HPV16 E6, in normal cervical keratinocytes, HCK1T cells, by transduction of HPV16 E6 in the C-terminal PDZ-binding motif-dependent manner. Thereby we found that PAR3 was also downregulated by transduction of HPV16 E6. In this study, the C-terminal deletion mutant of E6, E6△151, showed markedly reduced ability to induce anchorage-independent growth and tumorigenicity compared with wild-type E6 in corporation with E7 and $HRAS^{G12V}$. Surprisingly, another mutant of E6, E6SAT, which specifically lacks a function to target p53 for degradation, showed only marginally reduced transforming abilities compared to wild-type E6. The results clearly indicate the importance of the C-terminal PDZ-binding motif of E6 in transformation of cervical keratinocytes.

Then we found that single knockdown of SCRIB, MAGI1 or PAR3 significantly increased the anchorage-independent growth

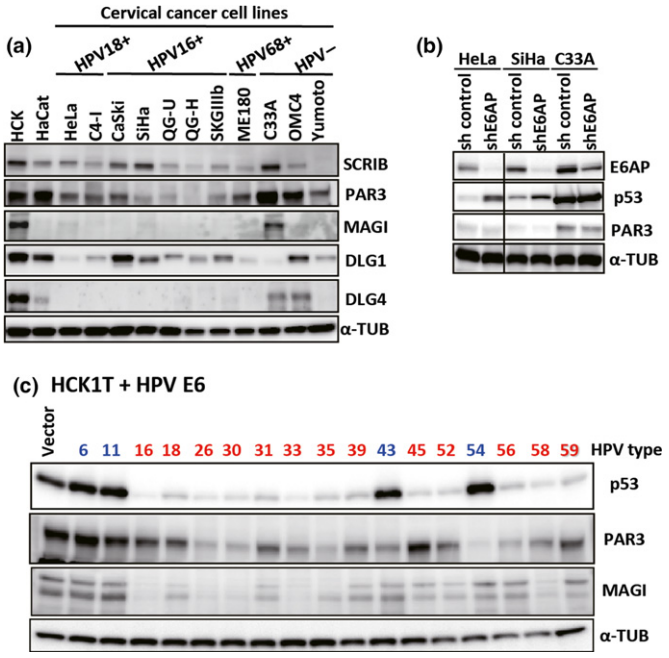

Fig. 4. The protein levels of PDZ containing proteins in cervical cancer cell lines. (a) The levels of PDZ containing proteins in HPV positive-cervical cancer cell lines were compared to that in HPV negative-cervical cancer cell lines or normal human keratinocytes by Western blot analysis. HaCaT cells are spontaneously immortalized human foreskin keratinocytes. (b) The effect of shRNA to E6AP on the level of PAR3 in cervical cancer cells were examined by Western blot analysis. HeLa, SiHa and C33a cells with E6AP shRNA was generated as described previously. p53 was included as a positive control because an E6 and E6AP complex was known to induce degradation of p53. α-tubulin was also detected as a loading control. (c) The ability of E6 to induce reduction of PAR3 level was compared between high and low risk HPVs. The expression E6 of high risk HPVs shown in red (HPV16, 18, 26, 30, 31, 33, 35, 39, 45, 52, 56, 58 and 59 or low-risk HPVs shown in blue (HPV6, 11, 43 and 54) was introduced to HCK1T by retrovirus mediated transduction. A retrovirus carrying empty vector was used as a control (vector). The levels of p53, MAGI-1 and PAR3 were examined by Western blot analysis.

and tumorigenic ability of HCK1T cells expressing $HRAS^{G12V}$, HPV16 E7 and HPV16 E6Δ151, and the combinatorial knockdown of SCRIB and MAGI1 markedly restored the tumorigenic ability. Additional knockdown of PAR3 further enhanced both anchorage-independent growth and tumorigenicity which is rather higher than that induced by wild type HPV16 E6. Though RhPV1 E7 but not E6 conserves PDZ-binding motif at the C-terminus and targets PAR3 for degradation[15] and HPV18 E6 can bind and induce mislocalization of PAR3 protein in a PDZ-dependent manner,[26] PAR3 has not been reported as a degradation target of E6 proteins of high-risk HPVs. We found levels of PAR3 protein were decreased in HCK1T cells by HPV16 E6 in the C-terminal PDZ-binding motif dependent manner. Furthermore, the E6 proteins of many other HPVs with the exception of those of HPV6, 11, 45 also induced reduction of PAR3 in HCK1T cells.

E6 proteins of HPV16 and 18 target similar but different sets of cellular proteins with different specificities.[27] Not only a group of HPVs including high-risk mucosal HPVs, but also some other HPVs such as HPV40 can target a similar set of PDZ proteins and can disturb epithelial polarity.[28] Interestingly, CRPV E6 can also associate with DLG1 through the C-terminal PDZ-binding motif which is required for transformation of NIH3T3 cells.[29] Thus, papillomaviruses in different cradles have evolved to target similar set of proteins with different strategies, indicating evolutional importance of this function in the viral life cycle. These target proteins have an important role in epithelial polarity which is maintained by interdependent control of three complexes, PATJ complex, SCRIB complex and PAR3 complex.[30]

In transformation of mouse primary tongue epithelial cells, inactivation of PTPN13 has almost equivalent function to HPV16 E6 in cooperation with Ras^{G12V}.[6] PTPN13 (FAP1) is suggested as a potential E6 target when DLG1 was identified as the first example of the E6 target.[1] In colorectal cancers, mutations of many protein tyrosine phosphatases including PTPN13 were identified.[31] However, knockdown of PTPN13 had only marginal effects on anchorage-independent growth as well as tumor growth (Fig. 3b,c). Thus, relevant target(s) of HPV16 E6 might depend on cell types. HPV16 is a causal agent for development of not only cervical cancer but also oral squamous cell carcinomas including tongue cancer. Thus it will be intriguing to examine whether PTPN13 functions as a tumor suppressor in human tongue keratinocytes.

We found downregulation of PAR3 by E6 proteins of HPV16. HPV18 E6 was reported to induce mislocalization of PAR3 and to inhibit tight junction formation without reducing the protein levels in 293T and HaCaT cells.[26] In our study, PAR3 levels were clearly decreased by HPV16 E6 at least partly in the PDZ-binding motif-dependent manner. Since depletion of E6AP did not recover the expression of PAR3 and proteasome inhibitors restored levels of PAR3 in HCK1T-E7-$HRAS^{G12V}$ cells expressing wild type E6 or E6Δ151 and SiHa cells, E6 may facilitate proteasomal degradation of PAR3 in an E6AP independent manner (Figs. 4a,b and S2). Some of the E6 target proteins including PATJ are suggested to be E6AP-independent.[32–34] We initially considered that degradation of other PDZ-domain-containing proteins might indirectly decrease the stability of the PAR3 complex. However, surprisingly, the E6 protein of low-risk HPV54, which does not conserve the C-terminal PDZ-domain binding motif, had the strongest ability to downregulate PAR3 protein in HCK1T cells among 17 types of HPVs examined. These results imply that E6 is able to enhance proteasomal degradation of PAR3 with multiple mechanisms. Further study is required to elucidate the precise mechanisms how E6 proteins of these HPVs can downregulate PAR3.

Clearly, our study indicates that the C-terminal PDZ-binding motif of E6 protein plays important roles in the development of cervical carcinogenesis. Thus it is conceivable to consider that difference in oncogenic potential among high-risk HPVs at least partly depends on the difference in the function of the C-terminal motif of the E6 proteins. Targeting this function of E6 as well as the well-known function of E6 to inactivate p53 could be a therapeutic option in HPV-positive cancers.

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Disclosure Statement

Y. I. is an employee of Promega corporation and S-I. O. is an employee of BML, Inc.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Tumorigenic potentials of expression of E7-HRASG12V with the wild type or E6 Δ151 were examined in two independently established HCKs, HCK4T (a, b, c) and HCK12T (d, e, f).

Fig. S2. The effects of proteosomal inhibitors, MG132 or Epoximicin on PAR3 level was examined in indicated cells.

Table S1 Biological activities of HPV16E6 and its mutants used in this study.

Table S2. Sequences of the targeted sites.