2.10. Invasion assay

Cells (1×10^4), in 0.5 ml of medium without growth supplement, were added to each well of a 24-well/8 μm pore invasion membrane chamber coated with Matrigel (BD Discovery Labware, Bedford, MA) [28]. The lower chambers contained medium with a double concentration of growth supplement. Cells were allowed to invade over the course of 48 h, and then fixed with 100% methanol, stained with 0.5% crystal violet, rinsed with water, dried, and examined under a microscope. Values for invasion were obtained by counting the total number of migrated cells in each chamber and presented by the average of three independent experiments.

3. Results

3.1. Establishment of immortalized hSAECs by hTERT

It has been shown that both hTERT expression and inactivation of either RB or p16 are required for the immortalization of primary human keratinocytes and mammary epithelial cells [29,30]. Introduction of hTERT into hSAECs led to their immortalization with a retarded growth until PD = 35. Then, fast growing cells emerged and continued to grow up to PD = 120 (Fig. 1A). Therefore, immortalized cells were cloned and karyotyped. Among 5 clones analyzed, one clone designated as hSAEC-T1 showed minimal chromosomal changes. This clone was almost diploid with trisomies of chromosomes 5 and 9 and an isochromosome of chromosome 20 (Fig. 1B). The other 4 clones carried some additional chromosomal changes, in addition to these changes (Supplementary Table 1). Since inactivation of the p16/RB and p53 pathways is required for and enhances the process of epithelial cell immortalization, respectively, the status of the p16 and p53 genes was examined in hSAEC-T1 cells. p16 was not expressed due to promoter methylation, as treatment with a DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine restored p16 expression, confirmed by RT-PCR and Western blot analyses (Fig. 1C and D). The p53 gene was mutated in this cell line. C to T transition of nucleotide 817 in exon 8, resulting in the amino acid change from arginine to cysteine (R273C), was detected by genomic PCR and sequencing. No wild-type allele was detected, therefore, only a mutant p53 protein was expressed in this cell line. The results indicate that methylational inactivation of the p16 gene, mutational inactivation of the p53 gene and several chromosomal alterations have occurred in hSAEC-T1 cells during cultivation, and the cells carrying these alterations were selected in the process of immortalization.

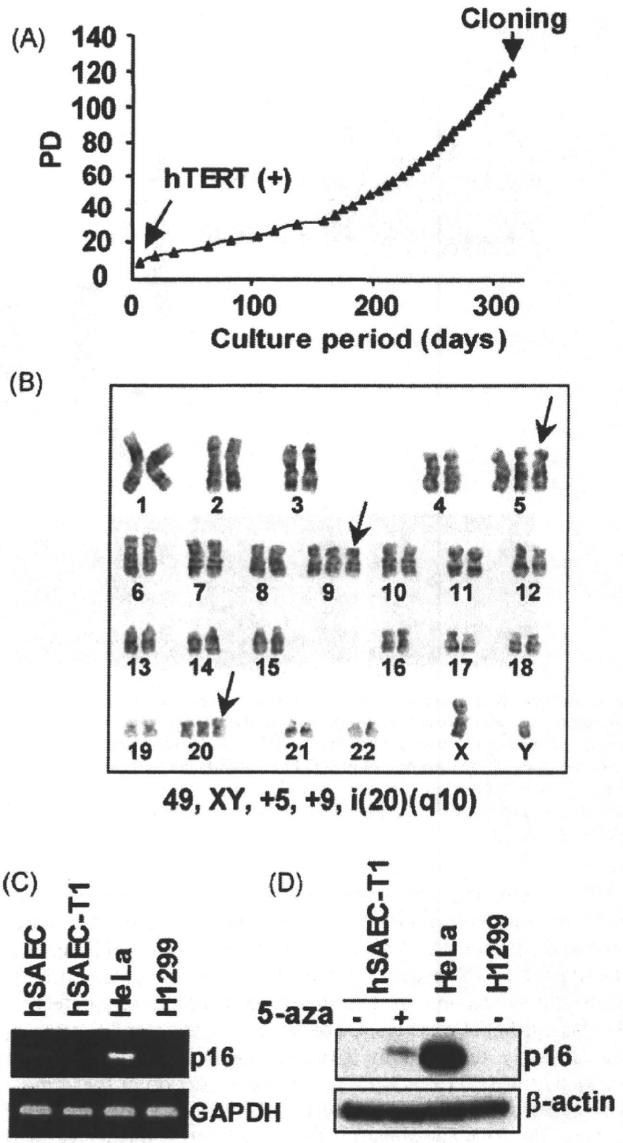

Fig. 1. Immortalization of human small airway epithelial cells (hSAEC) by hTERT. (A) Growth of hSAEC with introduction of hTERT. hSAEC was infected with the retroviral hTERT expression vector at PD = 9, cultured for up to PD = 120, and cloned. (B) Karyotype analysis of hSAEC-T1 cells. A minimal change with 49 chromosomes was observed in this clone. (C) RT-PCR analysis of p16 mRNA in hSAEC-T1 cells and primary cultured hSAEC. (D) Western blot analysis of p16 protein in hSAEC-T1 cells after 5-aza-2′-deoxycytidine (5-aza) (1 μM) treatment. β-actin was used as a loading control. HeLa and H1299 cells were used as a positive and a negative control for p16 expression in C and D, respectively.

3.2. Motility and invasiveness enhanced by LKB1 knockdown in hSAEC-T1 cells

Using the immortalized hSAEC-T1 cells, we established several stable LKB1 knockdown clones that express a small and negligible amount of LKB1 protein by introducing an shRNA mediated silencing vector, pTER-LKB1-sh#4. Suppression of LKB1 expression in individual clones was confirmed by Western blot analysis (Fig. 2A). Clone sh#4-9 expressed a little lower level of LKB1 protein than parental cells, whereas clones sh#4-8 and sh#4-12 expressed almost negligible levels of LKB1 protein. Under standard culture conditions, there were no remarkable changes in the growth of LKB1 knockdown clones (Supplementary Fig. S1A). Morphological observation of these clones revealed that LKB1 knockdown caused an increase in the population of spindle shaped cells (Fig. 2D). This finding led us to examine the motility of these clones. We used a wound healing assay to monitor the effect of LKB1 on cell motility. The velocity of LKB1 knockdown clones sh#4-8 and sh#4-12 was significantly increased (~3-fold) in comparison with that of clone sh#4-9 or parental cells (Fig. 2B; also Supplementary Fig. S1B). We then examined the invasiveness of these cells by a Matrigel invasion assay. Parental cells along with clone sh#4-9 showed a low invasiveness, whereas LKB1 knockdown clones sh#4-8 and sh#4-12 exhibited increased invasiveness (Fig. 2C). Thus, in hSAEC-T1 cells, the population of spindle shaped cells was increased and motility and invasiveness were enhanced by suppression of LKB1 expression.

3.3. Epithelial–mesenchymal transition (EMT) induced by LKB1 knockdown in hSAEC-T1 cells

EMT is often associated with spindle shaped morphology and with enhanced motility and invasiveness of cells. To assess whether

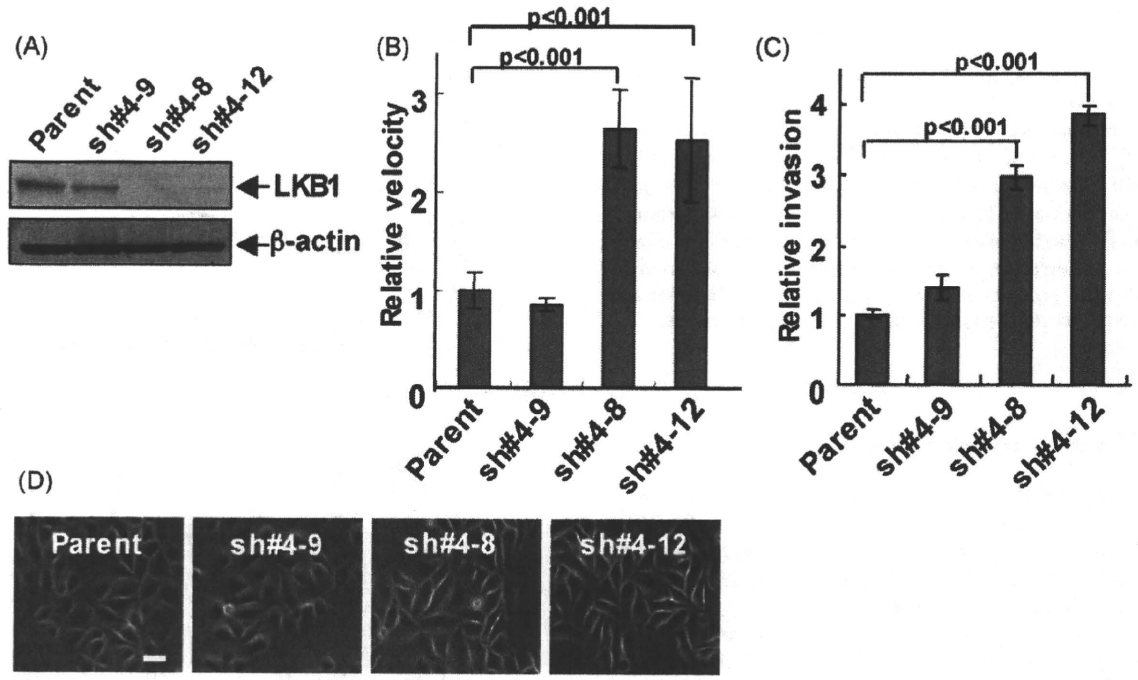

Fig. 2. Motility and invasiveness enhanced by LKB1 knockdown in hSAEC-T1 cells. (A) Western blot analysis for LKB1 expression in hSAEC-T1 cells with and without LKB1 knockdown. β-actin was used as a loading control. (B) Motility of hSAEC-T1 cells with and without LKB1 knockdown. Motility is expressed by the degree of wound closure assessed by measuring the distance between wounded edges at 1 h intervals. Data are presented by means ± SD of three independent measurements and expressed as ratios to parental cells. (C) Invasiveness of hSAEC-T1 cells with and without LKB1 knockdown. Invasiveness is expressed by the number of migrated cells in each chamber. Data are presented by means ± SD of three independent measurements and expressed as ratios to parental cells. Statistical significance for the difference was determined using a Student's two-tailed *t*-test. (D) Morphology of hSAEC-T1 cells with and without LKB1 knockdown. Morphologies of cells under standard culture conditions are shown. Bar, 25 μM.

hSAEC-T1 cells had undergone an EMT by LKB1 knockdown, we examined the expression of several epithelial and mesenchymal marker proteins by Western blot analysis. Parental hSAEC-T1 cells showed an epithelial-like phenotype. Mesenchymal markers, fibronectin, N-cadherin and vimentin, were very faintly detected, whereas epithelial markers, E-cadherin, α-catenin and γ-catenin, were strongly detected in hSAEC-T1 cells. It was noted that knockdown of LKB1 resulted in a strong induction of these mesenchymal markers and a marked reduction of these epithelial markers (Fig. 3A). The Western blot results were further verified by immunostaining with antibodies against fibronectin and E-cadherin (Fig. 3B). Increased fibronectin deposition was observed in these LKB1 knockdown clones. E-cadherin staining was intense and mainly restricted to cell-cell contacts in the parental cells and clone sh#4-9, and a disappearance of E-cadherin at cell-cell contacts was observed in LKB1 knockdown clones, sh#4-8 and sh#4-12.

Several transcription factors, including Snail, Slug, Twist and ZEB1, have been shown to promote cell transitions from an epithelial to a mesenchymal phenotype [2–4]. We then examined the expression of these proteins in these LKB1 knockdown clones by Western blot analysis (Fig. 3C). Only the zinc finger and homeodomain transcriptional repressor ZEB1 was induced in LKB1 knockdown clones. Snail and Slug were not detected in these cells. Twist was detected, but the amount was not changed by LKB1 knockdown. Next, we measured the amount of mRNA for these transcription factors by quantitative RT-PCR analysis (Fig. 3D). ZEB1 was most highly induced, and the level of ZEB1 mRNA expression showed the significant inverse correlation to the level of LKB1 mRNA expression in these clones. The levels of Snail and Slug mRNA expression were also changed but did not show significant correlation to that of LKB1 mRNA expression. The level of Twist mRNA expression was not changed, as the amount of Twist protein. E-cadherin has been shown to be a target of transcriptional repression by ZEB1. Our Western blot and immunostaining results also indicated an inverse correlation of ZEB1 expression with E-cadherin expression in these cells (Fig. 3A–C). Quantitative RT-PCR results further supported this finding. E-cadherin mRNA was decreased and N-cadherin mRNA was increased in the cells with ZEB1 induction (Fig. 3D). Thus, it was concluded that LKB1 knockdown induces ZEB1 expression at both mRNA and protein levels in hSAEC-T1 cells. A similar result was found using several stable clones (sh#1-2, sh#1-5, sh#1-14 and control sh#1-7) generated by a different targeting construct pTER-LKB1-sh#1 (Supplementary Fig. S2), confirming that induction of ZEB1 as well as reduction of E-cadherin were not due to the off-target effect of siRNA but a specific effect of LKB1 knockdown. Thus, we concluded that ZEB1 mediated EMT had occurred in SAEC-T1 cells by LKB1 knockdown.

3.4. Correlation of miR-200 expression with LKB1 expression in hSAEC-T1 cells

It was recently shown that the miR-200 family and ZEB1 are reciprocally repressed in cancer cells [26,31–33]. To determine whether LKB1 expression is associated with miR-200 family expression in hSAEC-T1 cells, we quantified the expression of miR-200a and miR-200c, as representative members of the miR-200 family, by real-time PCR in parental hSAEC-T1 cells and 7 knockdown clones with various levels of LKB1 expression (Fig. 4). A significant inverse correlation ($P=0.0090$ and $P=0.0210$) was observed between ZEB1 and miR-200a/c expression in these clones (Fig. 4B and C). A similar inverse correlation ($P=0.0012$) was found for the expression of LKB1 and ZEB1 (Fig. 4A). In contrast, miR-200a/c expression was positively correlated ($P=0.0001$ and $P=0.0010$) with LKB1 expression in these clones (Fig. 4D and E). This result further supports that ZEB1 mediated EMT had occurred in SAEC-T1 cells by LKB1 knockdown.

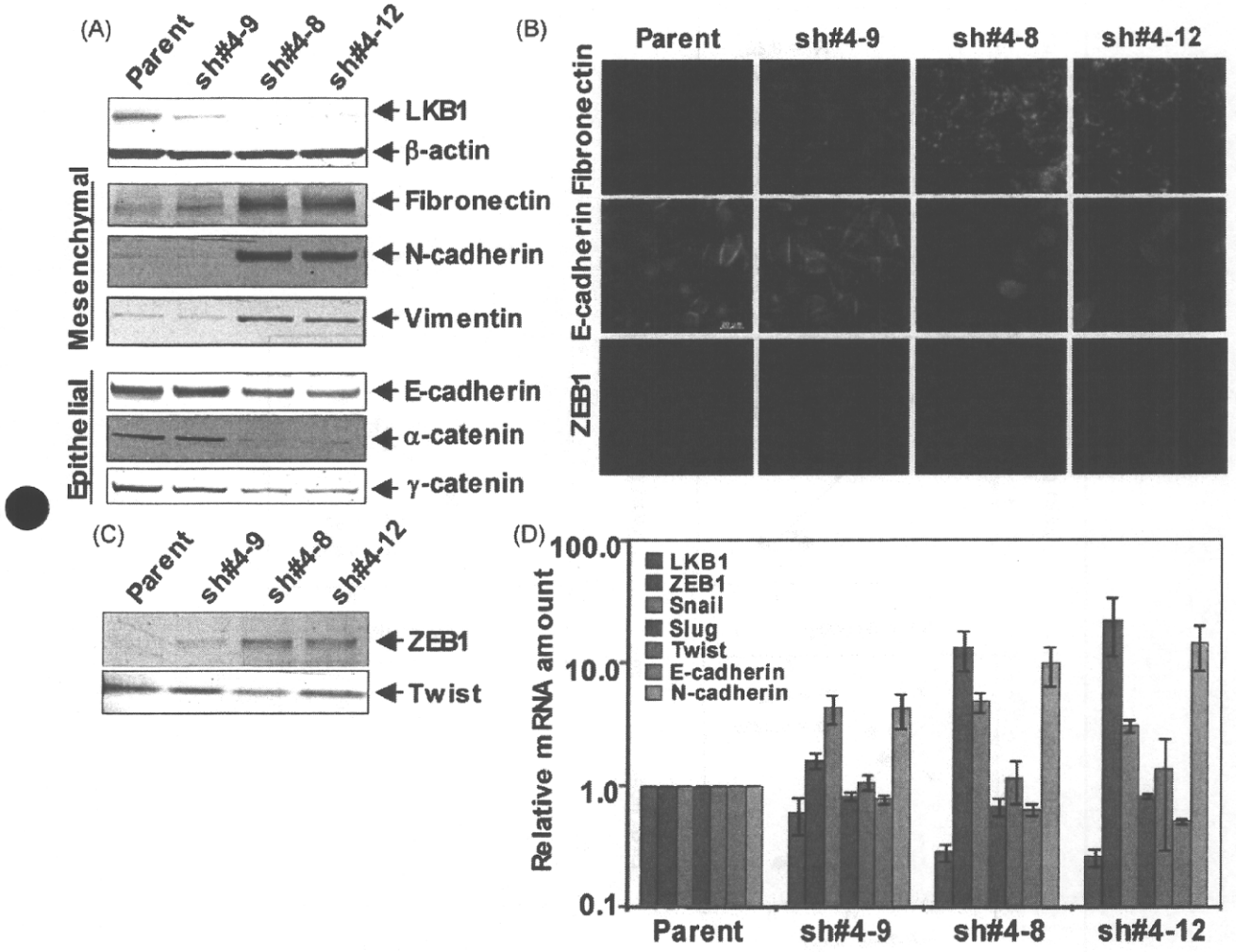

Fig. 3. EMT induced by LKB1 knockdown in hSAEC-T1 cells. (A) Western blot analysis for mesenchymal markers, fibronectin, N-cadherin and vimentin, and epithelial markers, E-cadherin, α-catenin and γ-catenin in hSAEC-T1 cells with and without LKB1 knockdown. β-actin was used as a loading control. (B) Immunohistochemistry for fibronectin, E-cadherin and ZEB1 in hSAEC-T1 cells with and without LKB1 knockdown. (C) Western blot analysis for ZEB1 and Twist in hSAEC-T1 cells with and without LKB1 knockdown. (D) Quantitative RT-PCR analysis for LKB1, ZEB1, Snail, Slug, Twist, E-cadherin and N-cadherin mRNAs in hSAEC-T1 cells with and without LKB1 knockdown. mRNA amounts in LKB1 knockdown clones are shown as a ratio relative to those in parental cells.

3.5. Epithelial–mesenchymal transition induced by LKB1 knockdown in H358 cells

To elucidate whether LKB1 inactivation commonly induces EMT in lung adenocarcinoma cells, we next examined a lung adenocarcinoma cell line H358 carrying the wild-type LKB1 gene [12] and with retention of epithelial phenotypes. As in the case of hSAEC-T1 cells, mesenchymal markers, fibronectin, N-cadherin and vimentin, were faintly or not detected, whereas epithelial markers, E-cadherin, α-catenin and γ-catenin, were intensely detected in H358 cells [6]. Several stable LKB1 knockdown clones derived from H358 cells were generated using two different targeting vectors, pTER-LKB1-sh#1 (sh#1-1 and sh#1-5) and pTER-LKB1-sh#4 (sh#4-7, sh#4-9). Induction of mesenchymal markers such as N-cadherin and vimentin, and reduction of an epithelial marker protein E-cadherin were observed in these clones by Western blot analysis, although induction/reduction was not so evident as in hSAEC-T1 cells (Fig. 5A and data not shown). ZEB1 expression was also induced in these clones. Quantitative RT-PCR analysis for EMT-inducing transcription factors further confirmed ZEB1 to be the most highly induced one among them, and the analysis for E-cadherin and N-cadherin further supported the decrease of E-cadherin mRNA and the increase of N-cadherin mRNA in the cells with LKB1 knockdown (Fig. 5B). Increased cell motility and invasiveness were also observed in these clones (Fig. 5C and D), as observed in hSAEC-T1 cells. Thus, it was concluded that the phenotype of H358 lung AdC cells had changed from an epithelial one to a mesenchymal one accompanied by the induction of ZEB1 expression by knocking down the expression of the LKB1 gene.

3.6. Transient knockdown of LKB1 enhances ZEB1 expression in hSAEC-T1 and H358 cells

Consistent results for the induction of EMT were obtained using several clones with stable LKB1 knockdown derived from both hSAEC-T1 cells and H358 cells. To further elucidate whether such an induction can occur by transient LKB1 knockdown in the cells or not, we transiently suppressed LKB1 expression by introducing siRNAs into both hSAEC-T1 and H358 cells. To exclude the off-target effect of siRNA, siLKB1#1 and siLKB1#2 with different target sequences were used for knocking down the expression of LKB1 in the cells. Then, expression of LKB1, ZEB1, N-cadherin and

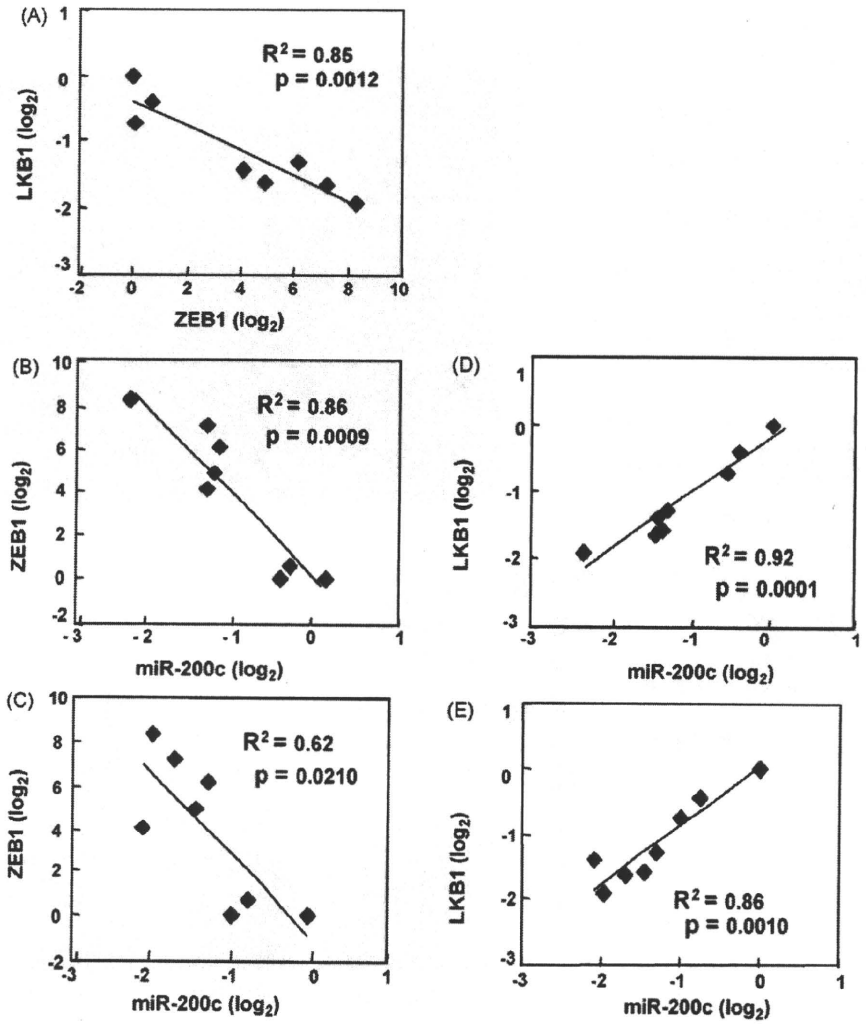

Fig. 4. Association of miRNA-200a/c expression with LKB1 and ZEB1 expression in hSAEC-T1 cells. Levels of mRNA expression were measured by real-time PCR for parental and 7 knockdown clones, and indicated by $\log_2$ ratios. Associations of two variables are shown by Pearson correlation coefficients (R^2) and *P*-values.

E-cadherin were assessed by real-time RT-PCR analysis (Fig. 6). The amount of LKB1 mRNA was markedly reduced by 4 h after siRNA transfection and was persistently suppressed over 24 h in both siRNAs. ZEB1 expression was increased at around 8–16 h of post-transfection, and, at a later period, N-cadherin expression was increased and E-cadherin expression was reduced in hSAEC-T1 cells (Fig. 6A). Similar results were obtained in H358 cells; enhanced ZEB1 expression occurred at around 8–16 h of post-transfection, followed by the increase in N-cadherin expression, although reduction of E-cadherin expression was not so evident as hSAEC-T1 cells (Fig. 6B). The induction of ZEB1 mRNA by transient LKB1 knockdown in these cells was also confirmed in another experiment (Supplementary Fig. S3).

We then measured the expression level of miR-200a/c after transient LKB1 knockdown by real-time PCR analysis. Contrary to the mRNA level of ZEB1, the expression level of miR-200a/c was inconsistent to the results of stable LKB1 knockdown clones in several experiments. A representative result was shown in Fig. 6C. Although miR-200c was not significantly reduced at any time points, miR-200a expression was slightly decreased at 16 h after siRNA transfection. Therefore, it was suggested that ZEB1 rather than miR-200 is a primary target of LKB1 for the induction of EMT in these cells, and that members of the miR-200 family are EMT regulators where ZEB1 is an EMT inducer.

3.7. *Occurrence of EMT by LKB1 knockdown is dependent on ZEB1 expression in H358 cells*

The above results indicate that LKB1 dependent EMT occurs through the induction of ZEB1 expression. Therefore, we next investigated the effect of LKB1 and ZEB1 double-knockdown on the motility of H358 cells. Transient knockdown of LKB1 expression significantly increased the wound healing capacity of H358 cells. The mean velocity of transient LKB1 knockdown cells was significantly higher than that of control cells, as in stable LKB1 knockdown cells (Fig. 5C). Therefore, we next examined whether or not this increase of cell motility was due to ZEB1 induction or not by introducing ZEB1 siRNA into H358 cells. Introduction of ZEB1 siRNA effectively reduced ZEB1 protein expression in H358 cells (Fig. 7A) and resulted in a drastic suppression of cell motility (Fig. 7B and C). Cell morphology also changed significantly; knockdown of LKB1 increased the population of mesenchymal-like spindle shaped cells, whereas knockdown of ZEB1 increased the population of epithelial-like polygonal shaped cells with tight cell–cell adhesions (Fig. 7B). In particular, in LKB1 knockdown cells, further repression of ZEB1 also showed a suppression of cell motility (Fig. 7C). It was confirmed that increased cell motility by LKB1 knockdown was suppressed by ZEB1 repression using another ZEB1 siRNA (Hs_ZEB1_0526 Mission siRNA, Sigma) (data not shown). These results imply that increased

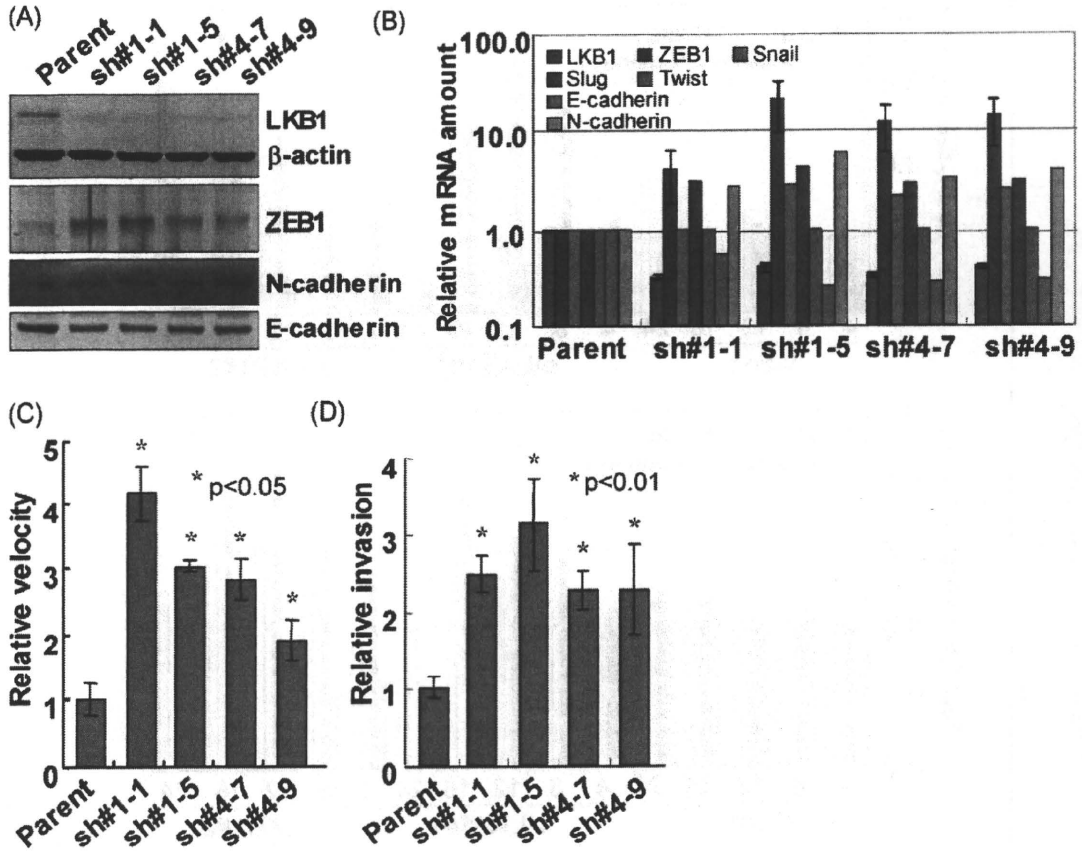

Fig. 5. Epithelial–mesenchymal transition induced by LKB1 knockdown in H358 cells. (A) Western blot analysis for the expression of ZEB1, N-cadherin and E-cadherin, in H358 derived LKB1 knockdown clones. β-actin was used as a loading control. Clones designated sh#1 and sh#4 were generated by targeting vectors pTER-LKB1-sh#1 and pTER-LKB1-sh#4, respectively (see Section 2). (B) Quantitative RT-PCR analysis of mRNAs in H358 cells with and without LKB1 knockdown. Relative mRNA amounts were determined by normalization with the amount of GAPDH mRNA. The mRNA amounts of LKB1 and ZEB1 were obtained from two independent PCR experiments in duplicate and shown as mean ± SD. (C) Motility of H358 cells with and without LKB1 knockdown. Motility is expressed by the degree of wound closure assessed by measuring the distance between wounded edges at 1 h intervals. Data are presented by means ± SD of three independent measurements and expressed as ratios relative to parental cells. (D) Invasiveness of H358 cells with and without LKB1 knockdown. Invasiveness is expressed by the number of migrated cells in each chamber. Data are presented by means ± SD of three independent measurements and expressed as ratios relative to parental cells. Statistical significance for the difference was determined using a Student's two-tailed *t*-test.

cell motility exhibited by LKB1 knockdown was mediated by the induction of ZEB1 expression. Thus, it was concluded that EMT was induced by LKB1 knockdown through the augmentation of ZEB1 expression in H358 cells.

4. Discussion

Inactivation of the p53 and p16 genes is common in lung AdCs, and the majority of AdC cells carry mutations of the p53 gene and either methylation or homozygous deletion of the p16 gene [8]. The LKB1 gene is inactivated in lung cancer generally by either homozygous deletion or hemizygous deletion plus point mutation that results in the absence/truncation of LKB1 protein products, and in most cases, LKB1 inactivation is accompanied by inactivation of both the p53 and p16 genes [10,11]. In this study, to assess the specific contribution of LKB1 inactivation to multistage carcinogenic processes of lung AdC, we first investigated the biological significance of LKB1 inactivation by using a primary hSAEC derived cell line, hSAEC-T1. Introduction of hTERT into primary hSAECs allowed us to obtain the immortalized hSAEC-T1 cell line with inactivation of the p53 and p16 genes and retention of the wild-type LKB1 gene. As a model of LKB1 inactivation in human lung carcinogenesis, LKB1 gene expression was constitutively knocked down in hSAEC-T1 cells. Transition from an epithelial to a mesenchymal phenotype, i.e. EMT, was observed by LKB1 knockdown in hSAEC-T1 cells. Similar phenotypic alterations were also observed by LKB1 knockdown in the H358 lung AdC cell line, in which p53 is homozygously deleted and p16 is methylated [34,35]. The appearance of mesenchymal phenotypes was well correlated with the level of reduced LKB1 expression in two different cell lines, SAEC-T1 and H358, using two different knockdown vectors. Furthermore, such phenotypes were also induced by transient LKB1 knockdown experiments using these two cell lines. Thus, it was strongly indicated that LKB1 inactivation would be one of causative events for the induction of EMT in lung AdC cells.

Several recent studies have implicated the involvement of LKB1 gene inactivation in differentiation, invasion and metastasis of lung cancer cells [10–12,28]. Therefore, the present results are consistent with those findings and further support the involvement of LKB1 inactivation in malignant progression of lung AdC. Previous studies on LKB1 functions using several lung cancer cell lines also showed an increase in invasiveness of the cells in response to LKB1-downregulation. In those studies, invasiveness was indicated to be enhanced through the up-regulation of the PEA3 transcriptional factor or the NEDD9 adaptor protein [12,28]. Therefore, we evaluated the expression levels of PEA3 and NEDD9 in stable and transient LKB1 knockdown hSAEC-T1 and H358 cells. However, significant changes in the amounts of NEDD9 mRNA and those of PEA3 protein were not observed in the LKB1 knockdown cells (data not shown). At present, it is unclear why the mediators of

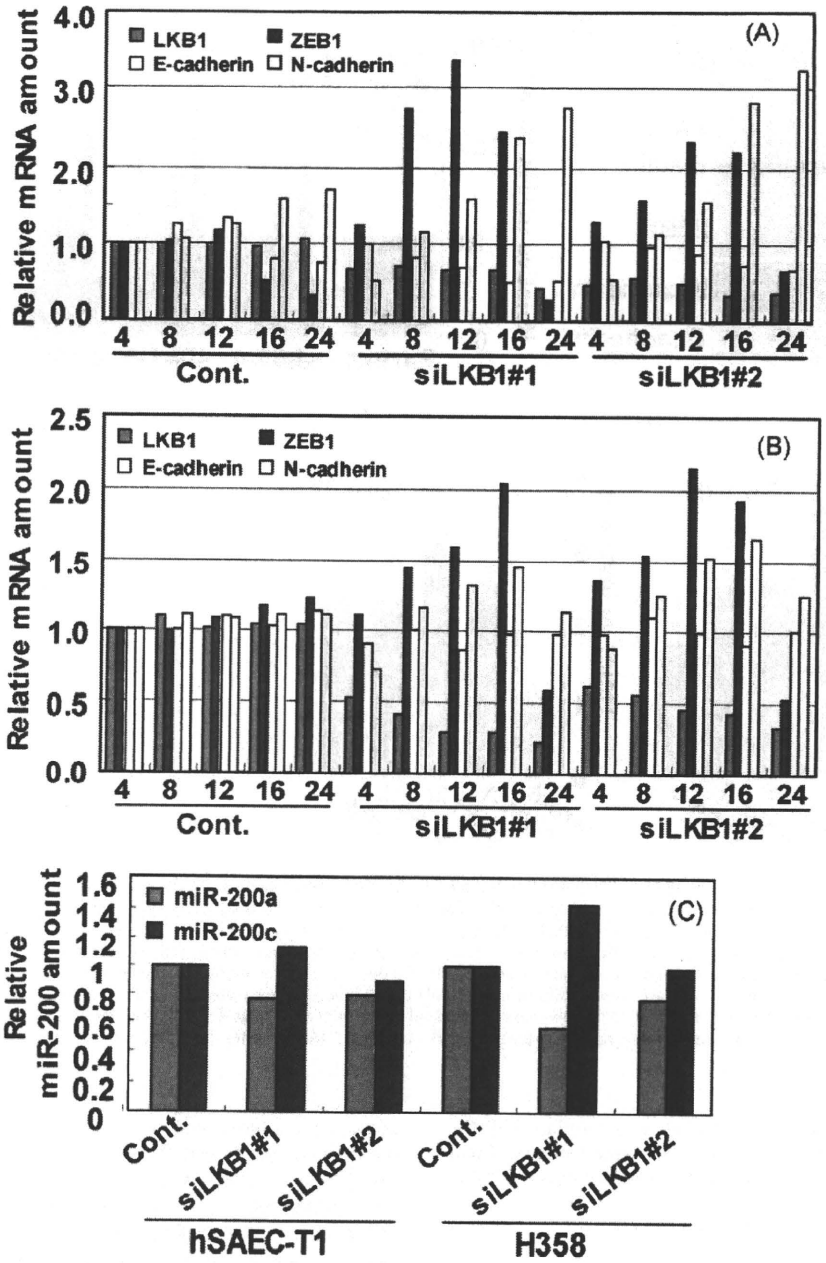

Fig. 6. Enhanced ZEB1 expression by transient LKB1 knockdown in hSAEC-T1 and H358 cells. Quantitative RT-PCR analysis was performed for LKB1, ZEB1, E-cadherin and N-cadherin mRNAs in hSAEC-T1 (A) and H358 cells (B) after LKB1 siRNA transfection. Cells were transfected with a control-siRNA or with two different siRNAs for LKB1, siLKB1#1 and siLKB1#2, respectively. Relative mRNA amounts were determined by normalization with the amount of GAPDH mRNA, and expressed as ratios relative to control samples (4 h after control-siRNA treatment). (C) Relative expression of miR-200a and miR-200c were measured by quantitative RT-PCR at 16 h of post-transfection of siRNAs and expressed as ratios to control samples (16 h after control-siRNA treatment).

LKB1 knockdown induced invasiveness are different among three studies, including the present study. Because LKB1 is not a direct transcriptional factor but a signaling mediator, it is possible that LKB1inactivation could induce mesenchymal phenotypes through multiple pathways in the cells.

EMT is known to be mediated by several transcriptional factors, as described above. Among them, ZEB1 was identified as a major transcription factor induced by stable knockdown of LKB1 expression in both hSAEC-T1 and H358 cells (Figs. 3 and 5). In addition, miR200a and miR200c, which are known to repress ZEB1 expression, were downregulated by LKB1 knockdown in both cells. Thus, acquisition of mesenchymal phenotypes in LKB1 knockdown stable clones might be due to feedforward loop between ZEB1 and miRNAs. Transient LKB1 knockdown studies indicate that the induction of ZEB1 seems to be an earlier event than miR-200a/c repression in the process of EMT induction (Fig. 6), and ZEB1 induction might remain throughout the completion of EMT and ultimately affect the expression of miR-200a/c. This phenomenon seems convincing as transient knockdown of LKB1 in hSAEC-T1 and H358 cells enhanced ZEB1 expression and slightly reduced miR-200a expression, whereas in LKB1 knockdown stable clones, derived from both hSAEC-T1 (Figs. 3 and 4 and Supplementary Fig. S2) and H358 cells (Fig. 5), expression of ZEB1 was increased and that of miR-200a/c was decreased significantly. The same is true for the expression of N-cadherin and E-cadherin; transient knockdown of LKB1 leads to an increase in N-cadherin expression and

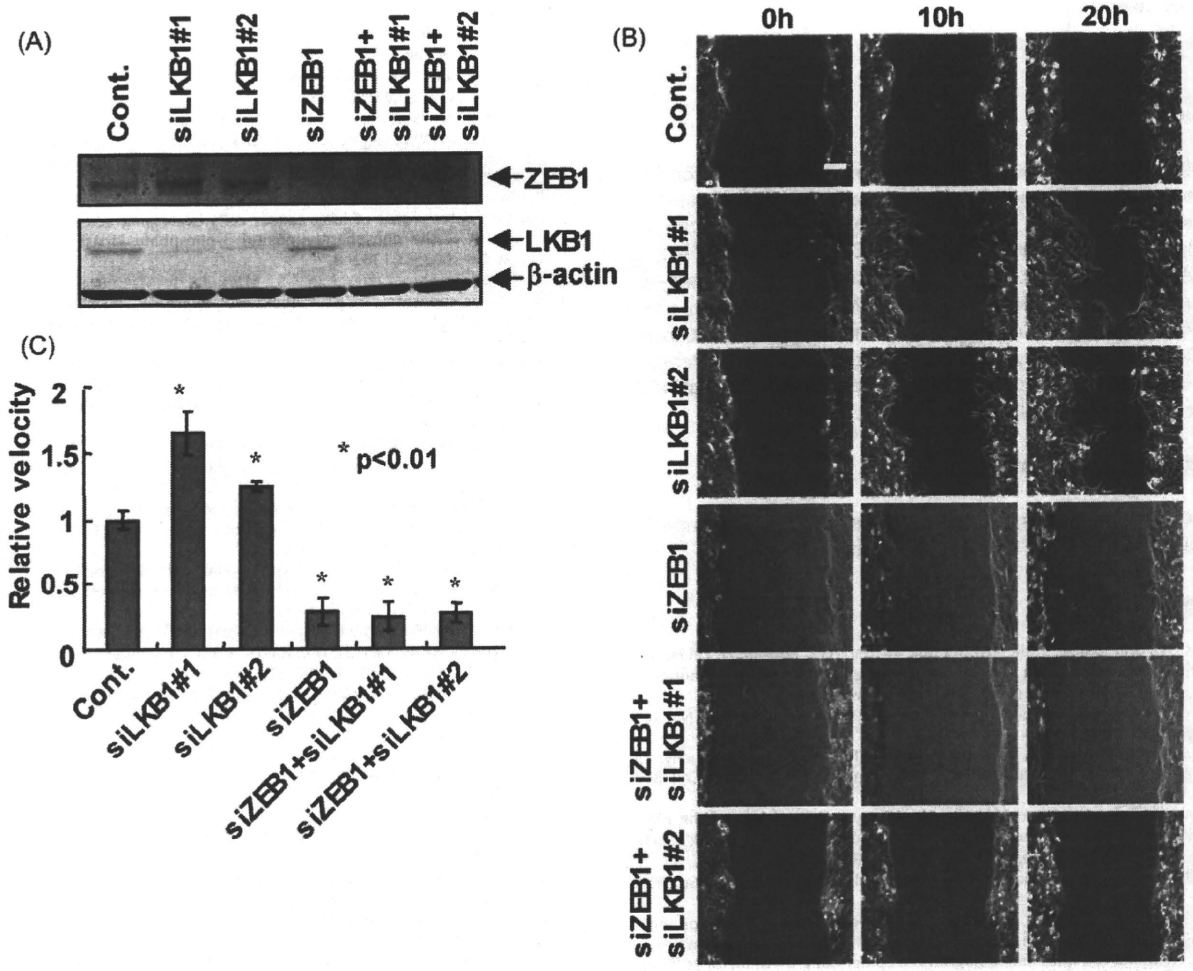

Fig. 7. EMT is dependent on ZEB1 expression in H358 cells.
(A) Western blot analysis for ZEB1 and LKB1 in H358 cells after 72 h of LKB1 and/or ZEB1 siRNA transfection. Two siRNAs for LKB1, siLKB1#1 and siLKB1#2, were used for LKB1 knockdown. β-actin was used as a loading control. (B) Wound healing assay of H358 cells treated with LKB1 and/or ZEB1 siRNA. Representative cells at the wounded edges after 0, 10 and 20 h of wounding are photographed. (C) Motility shown by the degree of wound closure assessed by measuring the distance between wounded edges at 1 h intervals. Data are presented by means ± SD of three independent measurements and expressed as ratios to control (control-siRNA treated). Bar, 100 μM.

a slight decrease in E-cadherin expression, whereas in stable LKB1 knockdown clones, expression of N-cadherin and E-cadherin significantly increased and decreased, respectively (Figs. 3 and 5 and Supplementary Fig. S2). Thus, the induction of ZEB1 could be a primary effect and reduction of miR-200a/c could be a secondary effect of LKB1 inactivation. Recent studies have demonstrated that EMT is associated with breast cancer stem cell function and that downregulation of miRNA-200c is important for tumorigenicity of human breast cancer stem cells [36,37]. It will be interesting to see if LKB1 depletion induces the stem cell-like properties in lung cancer cells.

On the other hand, stable expression of LKB1 did not induce a phenotypic alteration resembling a reversion of EMT, i.e. MET, in lung AdC cell lines with LKB1 inactivation, A549 and H23 (unpublished results). This result is consistent with earlier gene expression studies using exogenous LKB1-expressed cells [12,21]. Moreover, transient knockdown of LKB1 in LKB1 wild-type lung cancer cell lines, such as PC9, H820 and H2009, induced ZEB1 expression, but the magnitude of ZEB1 induction varied among the cell lines. The reason for such a difference is uncertain at present, but could be due to the difference in accumulated genetic alterations in the cells. Indeed, there was no significant correlation between LKB1 status and E-cadherin expression in 33 lung AdC cell lines (Supplementary Fig. S4), although the mean expression level of E-cadherin in LKB1 wild-type cell lines was slightly higher than that in LKB1 mutated cell lines. Thus, various degrees of mesenchymal phenotypes are acquired among lung cancer cell lines, and the acquisition of mesenchymal phenotypes cannot be explained only by LKB1 inactivation. Several other genetic alterations could also induce EMT in lung AdC cells. Therefore, further studies using immortalized lung epithelial cells would be a strong strategy of elucidating molecular pathways of EMT in lung AdC cells. As described above, LKB1 is not a transcriptional factor but a signaling mediator; therefore, loss of LKB1 might induce ZEB1 expression through several other mediators. Therefore, it seems possible that overexpression of LKB1 could not completely reproduce the reverse course of loss-of-function phenotype and that uncertain genetic alterations in the downstream of LKB1 might also affect the EMT induction in lung AdC cells. Recent studies indicated that LKB1 is a regulator of molecules in several signaling pathways, including those of AMPK-related kinases and Cdc42-PAK signals, in cancer cells [38–40]. One hypothesis is that one of the AMPK-related kinases or a Cdc42-PAK pathway protein induces the transcription of ZEB1 by LKB1 depletion. Further studies to elucidate how LKB1 intermediates and regulates ZEB1 expression will provide deep understanding of EMT induction in lung AdC cells.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.lungcan.2010.02.004.

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Molecular and Cellular Pathobiology

Cancer Research

ΔNp63α Repression of the *Notch1* Gene Supports the Proliferative Capacity of Normal Human Keratinocytes and Cervical Cancer Cells

Takashi Yugawa, Mako Narisawa-Saito, Yuki Yoshimatsu, Kei Haga, Shin-ichi Ohno, Nagayasu Egawa, Masatoshi Fujita, and Tohru Kiyono

Abstract

The p53 family member p63 is a master regulator of epithelial development. One of its isoforms, ΔNp63α, is predominantly expressed in the basal cells of stratified epithelia and plays a fundamental role in control of regenerative potential and epithelial integrity. In contrast to *p53*, *p63* is rarely mutated in human cancers, but it is frequently overexpressed in squamous cell carcinomas (SCC). However, its functional relevance to tumorigenesis remains largely unclear. We previously identified the *Notch1* gene as a novel transcriptional target of p53. Here, we show that ΔNp63α functions as a transcriptional repressor of the *Notch1* gene through the p53-responsive element. Knockdown of *p63* caused upregulation of Notch1 expression and marked reduction in proliferation and clonogenicity of both normal human keratinocytes and cervical cancer cell lines overexpressing ΔNp63α. Concomitant silencing of *Notch1* significantly rescued this phenotype, indicating the growth defect induced by *p63* deficiency to be, at least in part, attributable to Notch1 function. Conversely, overexpression of ΔNp63α decreased basal levels of Notch1, increased proliferative potential of normal human keratinocytes, and inhibited both p53-dependent and p53-independent induction of Notch1 and differentiation markers upon genotoxic stress and serum exposure, respectively. These results suggest that ΔNp63α maintains the self-renewing capacity of normal human keratinocytes and cervical cancer cells partly through transcriptional repression of the *Notch1* gene and imply a novel pathogenetical significance of frequently observed overexpression of ΔNp63α together with p53 inactivation in SCCs. *Cancer Res; 70(10); 4034–44. ©2010 AACR.*

Introduction

p63, a member of the p53 family of transcription factors, plays a pivotal role in epithelial development and morphogenesis (1–3). Like other p53 family members, the *p63* gene encodes multiple isoforms and contains two different promoters to drive the expression of two classes of proteins with or without the NH_2-terminal major transactivation domains TAp63 and ΔNp63, respectively. In addition, TAp63 and ΔNp63 each have three variants with different COOH-termini (α, β, and γ) generated through alternative splicing. TAp63 isoforms are expressed in the initiation period of epithelial stratification during embryogenesis (4) and are hardly detectable in adult epidermis (3, 5, 6), although they were very recently reported to be expressed in dermal and epidermal precursors and prevent premature aging of skin by maintaining adult stem cell populations and genomic stability (7). The ΔNp63α, the predominant isoform expressed in developmentally mature keratinocytes, is localized in the proliferative basal layers of stratified epithelia, where it essentially contributes to maintenance of regenerative potential or stemness (5, 8–11). Whereas ΔNp63α was initially shown to have dominant-negative activity toward TAp63 isoforms as well as p53 in a competitive manner (12), studies also suggest the presence of a second transactivation domain in the COOH-terminal region (13). In contrast to *p53*, *p63* is rarely mutated in human cancers, but tumor suppressor functions have been proposed, particularly for TAp63 isoforms (7, 14, 15). p63 expression is diminished during progression to invasion and metastasis of bladder carcinomas, and loss of p63 expression is associated with poor prognosis (5, 16). Sequestering p63 functions with mutant-p53 is also suggested to be linked with metastatic risk in breast cancer patients (17). Furthermore, it has been experimentally shown that loss of p63 in squamous cell lines results in upregulation of genes involved in invasion (18) and an increase in cell motility (18, 19). On the other hand, overexpression of ΔNp63α has been found in >50% of human squamous cell carcinomas (SCC), including examples in the lung, head and neck, and cervix, often as a result of gene amplification (6, 16, 20, 21). However, the pathologic relevance to tumorigenesis remains obscure. In addition, although a number of genes have been

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identified as p63 targets (22–25), the significance of each in epithelial biology and oncology is not fully understood.

Cervical cancer is thought to arise from cervical keratinocytes, and high-risk human papillomaviruses (HR-HPV), such as HPV-16 and HPV-18, have been well characterized as causative agents (26). Two viral proteins, E6 and E7, are known to inactivate the major tumor suppressors p53 and retinoblastoma protein, respectively, and are considered responsible for both genesis and maintenance of the transformed phenotype. The HR-HPV E6 protein is known to suppress keratinocyte differentiation, although the underlying molecular mechanism has been elucidated to only a limited extent (27).

The *Notch* family genes encode evolutionarily conserved cell surface receptors playing crucial roles in cell fate specification during development as well as in maintenance of self-renewing tissue organization (28). The biological consequence of Notch activation is critically dependent on cell type and cellular context (29–31). In normal keratinocytes, Notch1 has been identified as a key inducer of differentiation (32–34), and accumulating evidence suggests a tumor suppressive role in mammalian postnatal epidermis (29, 31, 35–37).

Previously, through analysis of the E6 proteins of HR-HPVs, we identified the *Notch1* gene as a novel p53 target and showed that genotoxic stress activates the p53-Notch1 pathway to induce differentiation of normal human keratinocytes (38). E6-mediated p53 inactivation can disrupt this pathway, leading to Notch1 downregulation and thus inhibition of differentiation. Others have also reported p53-driven *Notch1* expression contributing to tumor suppression (39). In addition, our previous finding that the p53-responsive element in the *Notch1* promoter is occupied by p63 and replaced by p53 upon genotoxic stress prompted us to investigate the possible involvement of p63 in *Notch1* gene expression. Herein, we show that the *Notch1* gene is a critical negative regulatory target of ΔNp63α and that overexpressed ΔNp63α endows epithelial cells with an increased proliferative potential and tumorigenic properties through constitutive downregulation of Notch1.

Materials and Methods

Cell culture. Normal human cervical keratinocytes (HCK) were obtained with written consent from a patient who underwent abdominal surgery for a gynecologic disease other than cervical cancer and were retrovirally transduced with the catalytic subunit of human telomerase reverse transcriptase for immortalization (HCK1T; ref. 38). HCK1T and primary human dermal keratinocytes (HDK) were cultured in serum-free keratinocyte-SF medium supplemented with 5 ng/mL epidermal growth factor and 50 μg/mL of bovine pituitary extract (Invitrogen). The source, authentication, and methods of maintenance of cell lines used are described in the Supplementary Materials and Methods.

Retroviral vector construction. Retroviral vector plasmids were constructed using the Gateway system according to the manufacturer's instructions (Invitrogen). Segments of *ΔNp63α* and *$\Delta Np63\alpha^{Y449F}$* (Itch E3 ubiquitin ligase-binding site mutant; ref. 40) were cloned and recombined into retroviral expression vectors to generate pCLXSN-ΔNp63α, pCLXSN-$\Delta Np63\alpha^{Y449F}$, PQCXIN-ΔNp63α, and PQCXIN-$\Delta Np63\alpha^{Y449F}$. To generate a *p63*-specific short hairpin RNA (shRNA) expression vector, pCL-SI-MSCVpuro-p63Ri, 5′-GGGTGAGCGTGTTATTGATGCT-3′ was chosen as the targeted sequence.

Immunoblotting. Immunoblotting was conducted as described previously (38). Antibodies used were listed in Supplementary Materials and Methods.

Northern blotting. Total RNA (15 μg) was electrophoresed on 1% agarose-formaldehyde gels, transferred to nylon membranes, and hybridized to ^{32}P-labeled probes. The Notch1 probe was generated by random primer labeling (Amersham Biosciences) of a Notch1 cDNA corresponding to the intracellular domain.

Dual-luciferase reporter assay. Construction of the *Notch1* promoter reporter *N1PR-Luc* and its p53-binding site-mutant *N1PRmut-Luc* was as described previously (38). Another version of reporter harboring a longer region of the *Notch1* promoter *N1PR2-Luc* was constructed by inserting a *Notch1* promoter region spanning −2,153 to −1 relative to the translation initiation site (cloned from BAC clone, RP11-611D20) into a promoterless luciferase reporter plasmid, PGV-B (Toyo Ink). Cells were cotransduced with the *Notch1* promoter reporters and the *Renilla* luciferase construct for normalization, and lysates were harvested and subjected to dual-luciferase reporter assay, following the manufacturer's instructions (Promega).

Clonogenic assay. Aliquots of 500 cells were seeded on 35-mm dishes under sparse conditions. After cultivation for 2 weeks, the cells were stained with Giemsa's dye, and the number of colonies was counted.

Colony formation in soft agar. Aliquots of 5×10^4 cells were suspended in a 0.4% agarose-containing medium and seeded on 35-mm dishes with a 0.7% agarose underlay. The next day, cells were overlaid with 0.6% agarose and then medium was added on top of the agar. Medium was changed weekly to feed cells, and the number of colonies over 50 μm in diameter was counted after 3 weeks.

Tumorigenesis in nude mice. All surgical procedures and care given to the animals were in accordance with institutional guidelines. Cells were resuspended in 50% Matrigel (BD Biosciences) and s.c. injected into female BALB/c nude mice (Clea Japan, Inc.).

Results

ΔNp63α downregulates Notch1 expression and differentiation of normal human keratinocytes upon genotoxic stress. Previously, we detected p63 binding to the p53-responsive element identified in the *Notch1* promoter and its replacement by p53 upon genotoxic stress in normal human keratinocytes (38). The observation prompted us to speculate that the predominant isoform of p63, ΔNp63α, may function as a transcriptional repressor for the *Notch1* gene. To address this, we first examined the effect of ΔNp63α expression on

Notch1 levels in normal human epithelial cells. Because ΔNp63α is the major isoform in keratinocytes and the other isoforms are expressed at low to undetectable levels, hereafter we refer to endogenous ΔNp63α as "p63" except where required. The wild-type ΔNp63α and a degradation-resistant mutant ΔNp63α^{Y449F} (40) were expressed under the control of different promoters, *LTR* or *CMV*, in normal HCKs (HCK1T; ref. 38). Upon *ΔNp63α* transduction, the endogenous level of Notch1 was downregulated to an extent parallel with the ΔNp63α level (Fig. 1A). Notch1 downregulation by overexpressed ΔNp63α was also observed in other different types of normal human epithelial cells, including primary HDKs, bronchial epithelial cells, small airway epithelial cells, and mammary epithelial cells (Supplementary Fig. S1), indicating a common control mechanism for Notch1 expression in human epithelial cells. Consistent with the previous

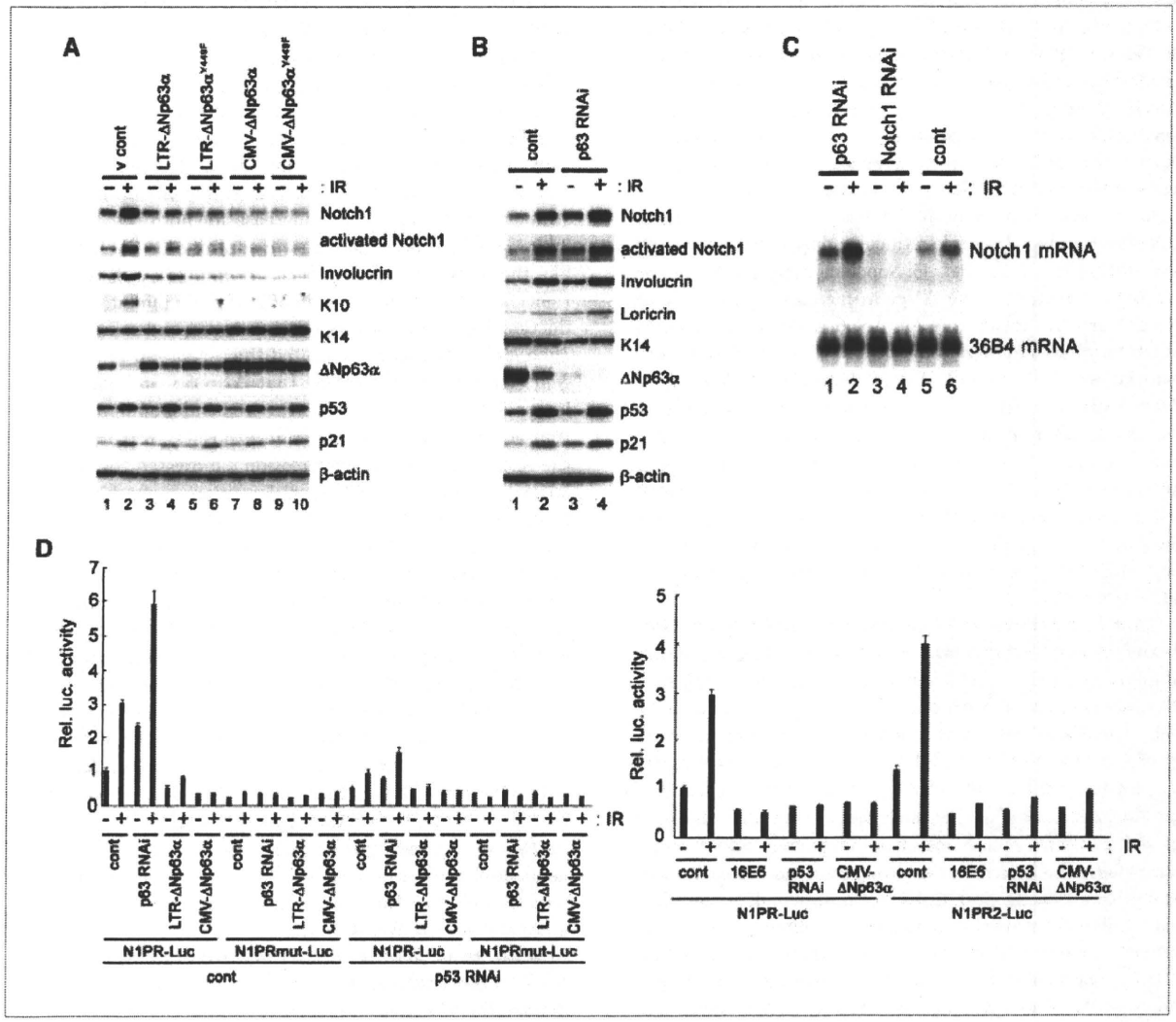

Figure 1. ΔNp63α represses p53-dependent expression of the *Notch1* gene and inhibits differentiation of normal human keratinocytes upon genotoxic stress. A, HCK1T cells were stably transduced with the indicated genes by retroviral gene transfer. v cont, vector control. Cells were exposed to 10 Gy IR (+) or left untreated (–), and cell lysates were prepared at 24 h posttreatment. Extracts were analyzed by immunoblotting with the indicated antibodies. B, HCK1T cells were transduced with either control shRNA (cont)– or *p63* shRNA–encoding retroviral vectors. At 48 h after transduction, cells were exposed to 10 Gy IR (+) or left untreated (–). Cell lysates were prepared after another 24 h of incubation, and immunoblotting was performed. C, *Notch1* mRNA levels in HCK1T cells expressing *p63* shRNA, *Notch1* shRNA, or control shRNA. *36B4* was used as the loading control. D, left, HCK1T cells stably expressing *p53* shRNA or control shRNA were first introduced with the reporters of 1-kb *Notch1* promoter (*N1PR-Luc*) or the 1-kb *Notch1* promoter having the p53-binding site mutated (*N1PRmut-Luc*). Cells were then transduced with retroviral vectors encoding *LTR*-driven or *CMV*-driven *ΔNp63α*, *p63* shRNA, or control shRNA. At 48 h after transduction, cells were exposed to 10 Gy IR (+) or left untreated (–), and 24 h thereafter, cell lysates were prepared. Cell extracts were subjected to dual-luciferase reporter assays. Rel. luc., relative luciferase. D, right, HCK1T cells stably expressing either the 2-kb *Notch1* promoter reporter (*N1PR2-Luc*) or *N1PR-Luc* were transduced with the indicated genes. Cells were processed as for A, and dual-luciferase reporter assays were performed.

reports showing down-modulation of ΔNp63α in response to UV radiation (41, 42), treatment with ionizing radiation (IR) resulted in drastic reduction of ΔNp63α in control cells. However, *CMV*-driven expression of ΔNp63α seemed to be maintained at a higher level and abrogated the p53-mediated Notch1 induction even after IR. We also observed downregulation of a representative differentiation marker, involucrin, in concert with the Notch1 level. Another differentiation marker, K10, was also induced upon IR treatment only in control cells, and this response was diminished in cells expressing exogenous ΔNp63α. In contrast, expression of K14, a marker of the proliferating basal layer and a direct target of ΔNp63α (11), was upregulated by ΔNp63α dose dependently, confirming the functionality of exogenously introduced ΔNp63α. Interestingly, p21 expression seemed to be unaffected by excess ΔNp63α and was induced upon genotoxic stress in ΔNp63α-overexpressing cells, suggesting target specificity of ΔNp63α in this cellular context.

It has previously been shown that down-modulation of ΔNp63α is induced upon keratinocyte differentiation (43, 44) and Notch1 activation (ref. 45; Supplementary Fig. S2). Then we further examined the ΔNp63α-mediated repression of Notch1 and the causal role for ΔNp63α downregulation in keratinocyte differentiation by the loss-of-function approach. Knockdown of *p63* by shRNA-mediated RNA interference indeed resulted in upregulation of Notch1 and involucrin, as well as another terminal differentiation marker, loricrin, and this was further enhanced by IR (Fig. 1B). Northern blot analysis validated the Notch1 regulation by p63 at the level of transcription (Fig. 1C). Thus, ΔNp63α negatively regulates the Notch1 level, and its overexpression can suppress induction of Notch1 and differentiation markers in response to genotoxic stress.

ΔNp63α represses Notch1 promoter activity by counteracting p53. To further corroborate transcriptional regulation of the *Notch1* gene by p63, we assessed the effect of p63 expression on *Notch1* promoter reporter activity in HCK1T cells. The *Notch1* promoter was shown to be transactivated by p53 in response to IR (38), whereas ΔNp63α overexpression led to shutdown of *Notch1* promoter activity even after genotoxic stimuli (Fig. 1D, left). Conversely, *p63* silencing caused a marked increment of the promoter activity compared with the control case, this response being further strengthened by IR. Mutations in the previously identified p53-binding sites or *p53* knockdown resulted in lack of response to either *p63* silencing or IR, suggesting dependence of p63 functionality on the p53-responsive element and p53 presence. We also examined the effect of ectopic expression of the TAp63α isoform on *Notch1* promoter activity and found that, in contrast to ΔNp63α, it transactivated *Notch1* promoter in the absence of DNA-damaging stimuli to a level similar to that seen in *p63* knockdown cells (Supplementary Fig. S3). In addition to the p53-responsive element, there are several putative transcription factor binding sites between 1 and 2 kb upstream of the *Notch1* gene, which are conserved among human, mouse, and rat. We therefore aimed to compare the activity of the 2-kb *Notch1* promoter with that of the 1-kb *Notch1* promoter and found similar downregulation by E6 expression, *p53* silencing, or ΔNp63α overexpression in the absence or presence of genotoxic stimuli (Fig. 1D, right). Therefore, we conclude that ΔNp63α functions as a transcriptional repressor for *Notch1* gene expression by counteracting p53 under genotoxic stress conditions.

ΔNp63α can repress p53-independent expression of the Notch1 gene and suppress differentiation of normal human keratinocytes upon serum exposure. Notch1 has been shown to be a key determinant of keratinocyte differentiation (32, 33), and the developmental normality of p53-deficient mice points that p53 is not essential for such differentiation, at least in the developmental stages (46). Accordingly, it is tempting to speculate that ΔNp63α may exert its repressor activity on *Notch1* gene expression independently of competition with p53. To address this possibility, we determined whether ΔNp63α could also inhibit *Notch1* promoter activation in HCK1T cells upon keratinocyte differentiation induced by exposure to serum-containing medium. Serum stimulation caused ~3-fold increase for both the 1-kb and 2-kb *Notch1* promoter activities in control cells (Fig. 2A). Unlike the activation of the *Notch1* promoter by IR (Fig. 1D, right), knockdown of *p53* did not efficiently inhibited the induction. However, ΔNp63α overexpression and expression of 16E6 still substantially inhibited the activation of the 2-kb *Notch1* promoter, indicating that ΔNp63α and 16E6, to a lesser extent, can attenuate the p53-independent *Notch1* transactivation induced by serum exposure. In parallel with the results, ΔNp63α overexpression suppressed the upregulation of Notch1 as well as differentiation markers upon serum-induced differentiation, similarly to the case of *Notch1* silencing (Fig. 2B). We also observed reduced accumulation of Hes1, Hey1, and Nrarp, all of which are known downstream targets of Notch1 signaling, in ΔNp63α-overexpressing cells, confirming the down-modulation of Notch1 signaling activity by ΔNp63α. Importantly, serum stimulation did not activate p53, and *p53* silencing failed to inhibit the induction of Notch1 and differentiation markers. Moreover, ΔNp63α overexpression downregulated the endogenous levels of Notch1 in a *p53*-deficient HCT116 isogenic cell line and those in which mutant forms of p53 are expressed (Fig. 2C). Thus, these data suggest that ΔNp63α can also function as a transcriptional repressor for *Notch1* gene independent of competition with p53 and that its overexpression inhibits both p53-dependent and p53-independent induction of keratinocyte differentiation.

Ablation of p53 or Notch1 significantly rescues the proliferative defect of p63-compromised cells. Next, we addressed the functional importance of the p63-Notch1 axis by rescue experiment. Knockdown of *p63* in HCK1T cells resulted in a virtually complete loss of proliferative ability (Fig. 3A) as well as clonogenicity (Fig. 3B). E6 expression or concomitant silencing of *p53* significantly ameliorated this phenotype, in line with a previous report of p53 dependency for hypoproliferation induced by *p63* knockdown in developmentally mature keratinocytes (9). Importantly, *Notch1* silencing also significantly rescued the ability of *p63*-deficient cells to proliferate, suggesting that the defect in the self-renewing capacity induced by *p63* deficiency is at least

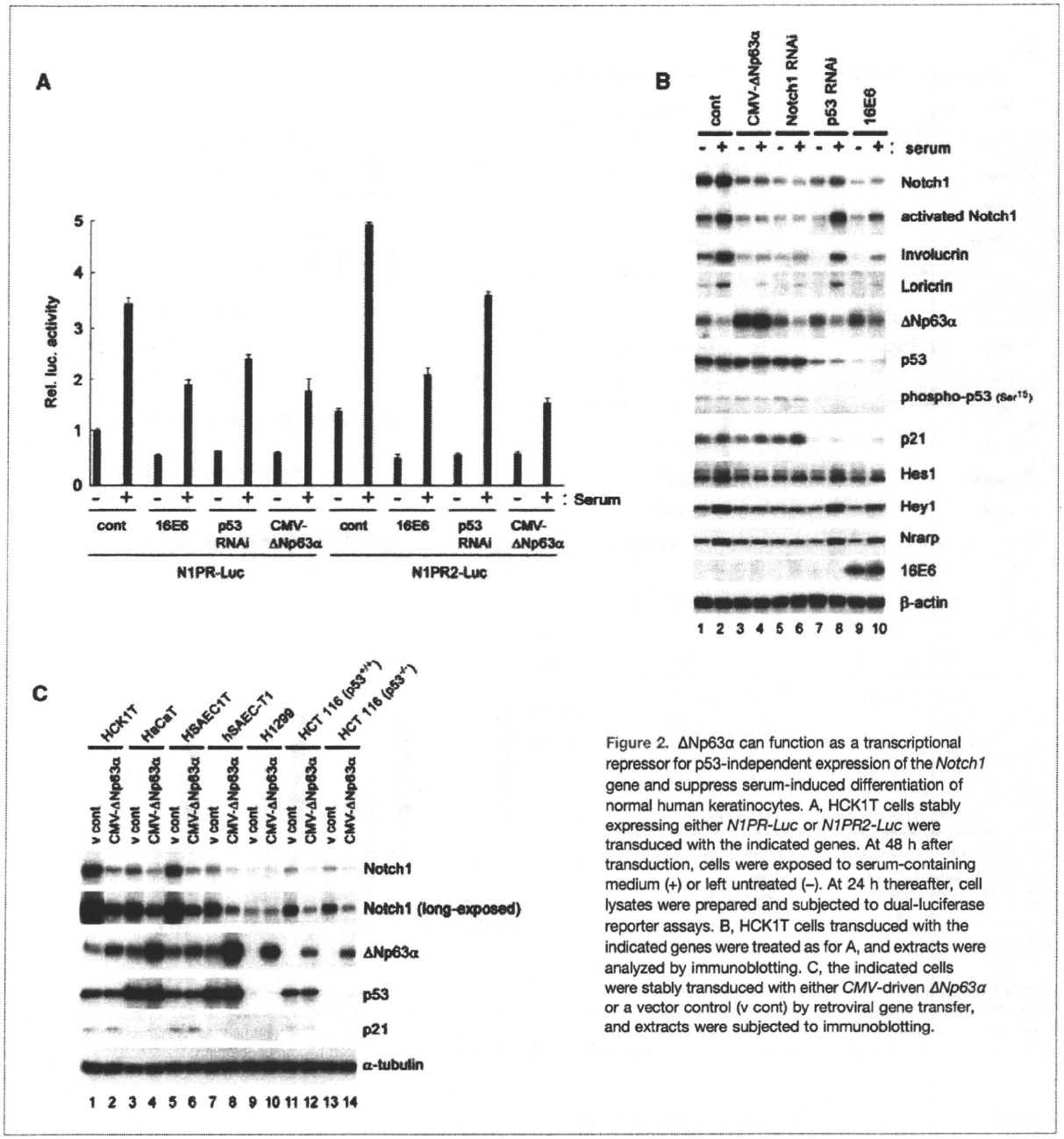

Figure 2. ΔNp63α can function as a transcriptional repressor for p53-independent expression of the *Notch1* gene and suppress serum-induced differentiation of normal human keratinocytes. A, HCK1T cells stably expressing either *N1PR-Luc* or *N1PR2-Luc* were transduced with the indicated genes. At 48 h after transduction, cells were exposed to serum-containing medium (+) or left untreated (–). At 24 h thereafter, cell lysates were prepared and subjected to dual-luciferase reporter assays. B, HCK1T cells transduced with the indicated genes were treated as for A, and extracts were analyzed by immunoblotting. C, the indicated cells were stably transduced with either *CMV*-driven *ΔNp63α* or a vector control (v cont) by retroviral gene transfer, and extracts were subjected to immunoblotting.

in part attributable to Notch1 function. Nevertheless, there was still a large population of cells undergoing p53-independent and Notch1-independent growth suppression after *p63* knockdown.

Previously, we showed the relevance of the p53-Notch1 pathway to both spontaneous differentiation by culture stress and its induction upon DNA damage (38). In marked contrast to control cells in which Notch1 and involucrin were considerably upregulated upon *p63* knockdown, these changes were modest or marginal in cells expressing E6, *p53* shRNA, or *Notch1* shRNA (Fig. 3C). Introduction of a constitutively active form of Notch1 into HCK1T cells did not evoke apoptosis[1] (47) but rather induced differentiation accompanied by massive growth inhibition (ref. 38; Supplementary Fig. S2). Therefore, we infer from these data that endogenous levels of p53 in culture are able to trigger differentiation and thus cause growth suppression through Notch1 induction when p63 is

[1] T. Yugawa, et al., unpublished data.

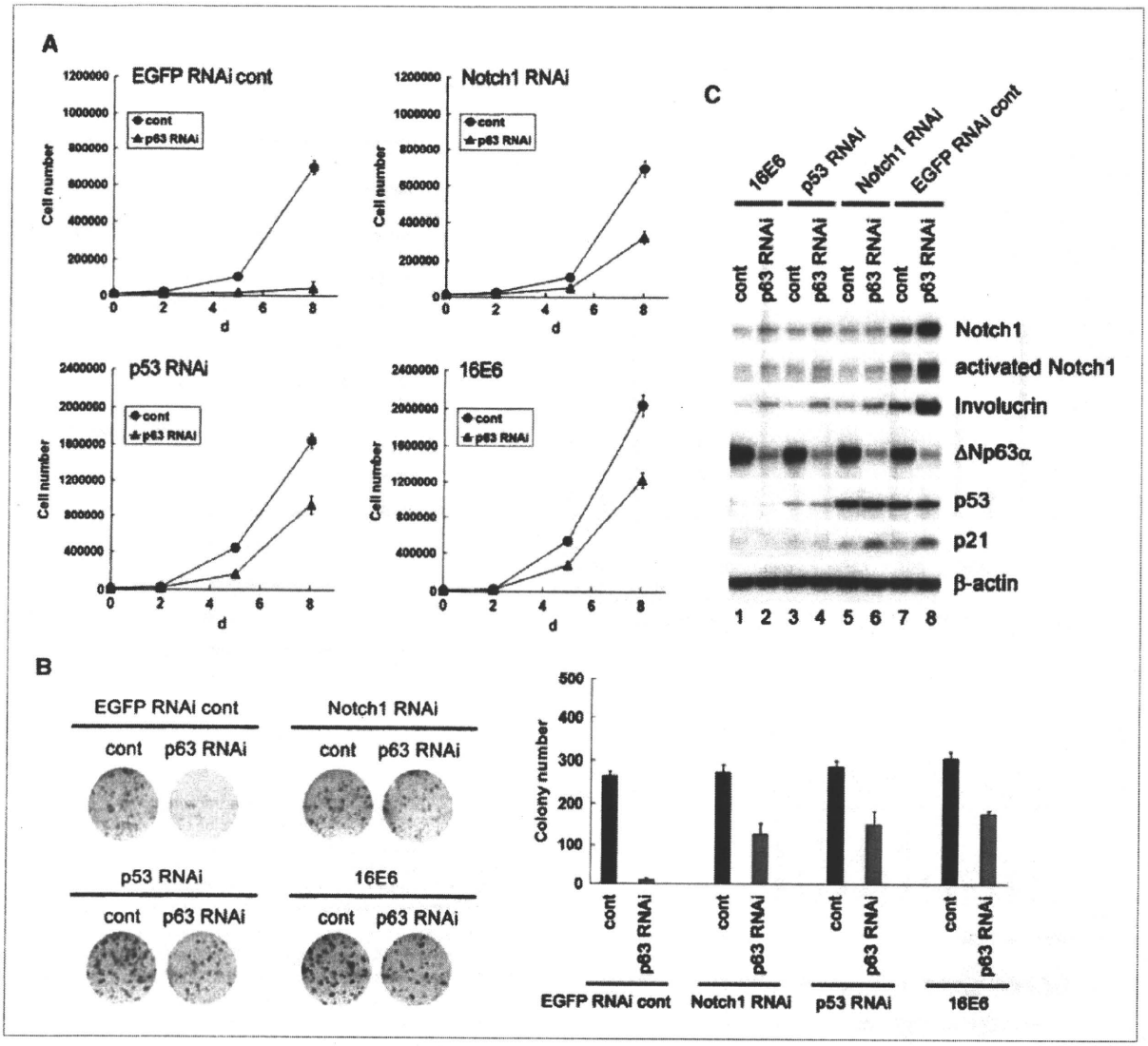

Figure 3. Knockdown of *Notch1* or inactivation of p53 restores the proliferation defect triggered by *p63* silencing. A, HCK1T cells stably expressing *Notch1* shRNA, HPV-16 E6, *p53* shRNA, or *EGFP* shRNA control (cont) were transduced with either *p63* shRNA or control shRNA. At 48 h after transduction, aliquots of 1×10^4 cells were replated on 35-mm dishes, and proliferation was monitored over the next 8 d. B, after treatment as for A, aliquots of 500 cells were seeded on 35-mm dishes to assess clonogenicity. C, after treatment as for A, extracts were analyzed by immunoblotting.

compromised. In this regard, it should be stressed that ΔNp63α can negatively regulate the p53-Notch1 pathway, whereby it supports proliferation by inhibiting differentiation.

ΔNp63α overexpression confers increased proliferative and tumorigenic potential on normal human keratinocytes. We next explored the biological outcomes of ΔNp63α overexpression in HCK1T normal human keratinocytes. Overexpression of ΔNp63α has been previously shown to elicit enhanced clonogenic growth of HCK1T cells after DNA damage (38) or Notch1 activation (45). Here, proliferation assays revealed the proliferation capacity to increase in parallel with ΔNp63α levels (Fig. 4A). We then carried out soft agar colony formation assays to assess the ability of the overexpressed ΔNp63α to induce anchorage-independent growth in combination with the defined oncogenes HPV-16 *E7* and activated *Hras* (48). The expression of the transgenes and the downregulation of Notch1 by ΔNp63α overexpression were confirmed (Fig. 4B). In contrast to vector control cells with which no overt colonies were formed, ΔNp63α and, more clearly, the Y449F mutant elicited colony-forming ability, albeit to a lesser extent than E6 (Fig. 4C). ΔNp63α overexpression also conferred *in vivo* tumorigenicity when cells were s.c. injected into nude mice (Fig. 4D). Thus, ΔNp63α overexpression endows normal keratinocytes with increased proliferative potential in itself and tumorigenic potential together with selected oncogenes.

Overexpressed ΔNp63α in cancer cells has a functional role in proliferation by downregulating Notch1. Next, we aimed to substantiate the functional relevance of overexpressed ΔNp63α in SCCs. Some cervical cancer cell lines, such as QG-H, SKGIIIb, and ME180, were found to exhibit pronouncedly increased levels of ΔNp63α and downregulation of Notch1, relative to those in normal cervical keratinocytes, HCK1T and HCK11, or primary dermal keratinocytes, HDK (Fig. 5A). The activity of the *Notch1* promoter reporter was augmented by *p63* silencing in QG-H and SKGIIIb cells (Fig. 5B, left). Interestingly, genotoxic stress failed to activate the *Notch1* promoter (Fig. 5B, right) wherein the ΔNp63α levels were maintained at high levels (Fig. 5C).

Knockdown of *p63* in a panel of cervical cancer cell lines, including HPV-16–positive lines such as CaSki, SiHa, QG-U, QG-H, and SKGIIIb, and a HPV-negative line, C33A, revealed considerable growth suppression specifically observed in cells overexpressing ΔNp63α (Fig. 6A; Supplementary Fig. S4A, B). Among these are QG-H and SKGIIIb cells. On the other hand, proliferation of CaSki, SiHa, and C33A cells,

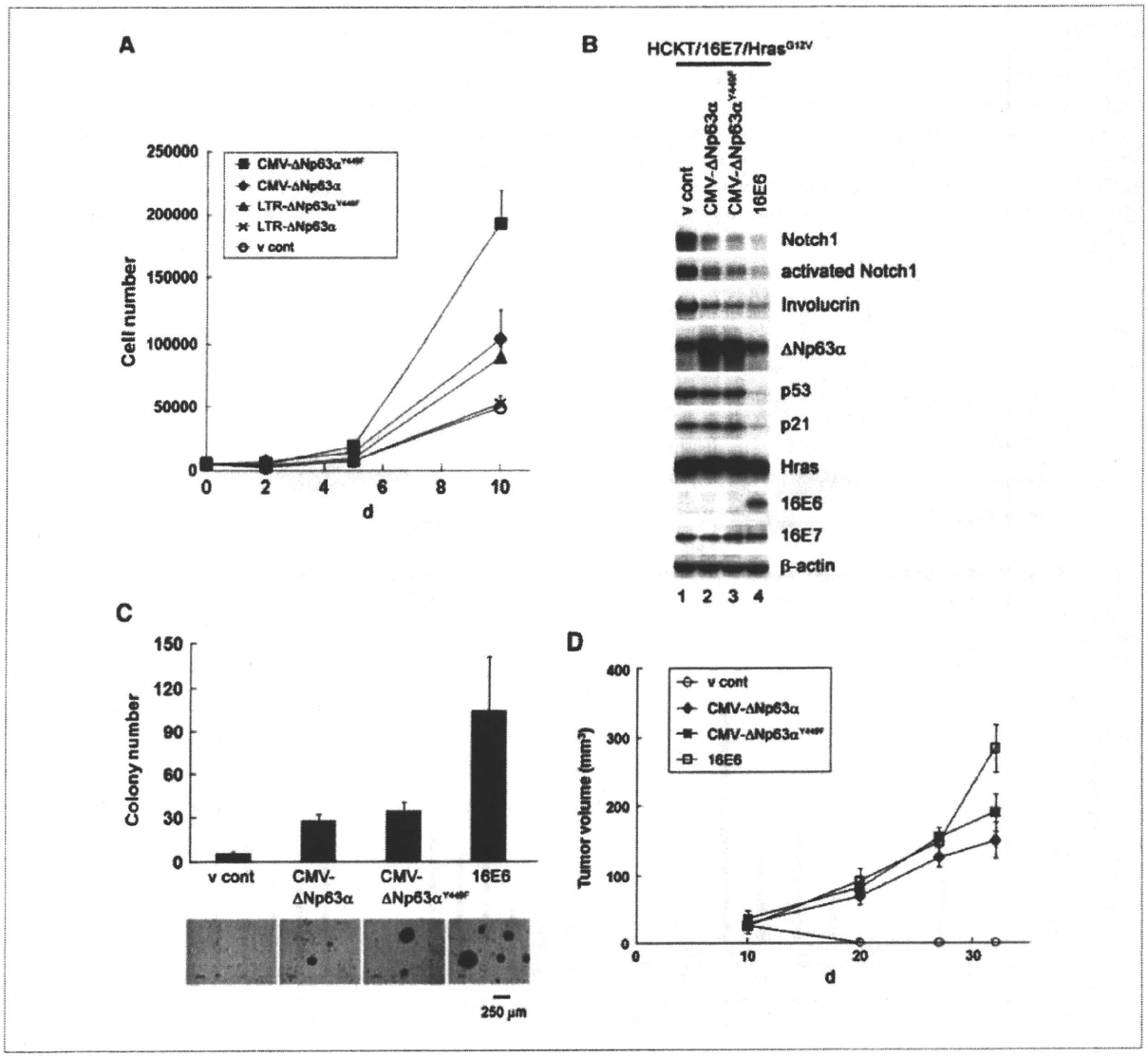

Figure 4. ΔNp63α overexpression endows normal human keratinocytes with increased proliferative capacity and tumorigenic properties. A, HCK1T cells were stably transduced with the indicated genes. Aliquots of 1×10^3 cells were replated on 35-mm dishes, and proliferation was monitored over the next 10 d. B, HCK1T cells expressing HPV-16 *E7* and $Hras^{G12V}$ were transduced with the indicated genes. Cell extracts were analyzed by immunoblotting. C, aliquots of 5×10^4 cells described in B were subjected to soft agar colony formation assay. Typical areas were photographed at 3 wk postplating. The total number of colonies in a 15 mm^2 area was shown. D, *in vivo* tumor-forming abilities of cells described in B. Aliquots of 1×10^6 cells were s.c. injected into nude mice, and tumor size was measured at the indicated time points. The tumor volume (mm^3) was calculated as $L \times W^2 \times 0.52$, wherein L is the longest diameter and W is the shortest diameter.

in which ΔNp63α is undetectable, was not suppressed at all upon *p63* silencing. Much the same was found to be true for their clonogenicity (Fig. 6B; Supplementary Fig. S5). These defects in QG-H and SKGIIIb cells were significantly alleviated by concomitant silencing of *Notch1* (Fig. 6A and B). Taking into account that upregulation of Notch1 was evident upon *p63* silencing in QG-H and SKGIIIb cells (Fig. 6C; Supplementary Fig. S6), we draw the conclusion that the overexpressed ΔNp63α in these cancer cell lines results in robust repression of the *Notch1* gene, which plays an integral role in control of cancer cell growth (Supplementary Fig. S7).

Discussion

In stratified epithelia, negative crosstalk between p63 and Notch1 has been proposed, wherein ΔNp63α expression is suppressed by Notch1 through modulation of IFN-responsive factors and NF-κB signaling in the terminally differentiating layers (45). In addition, p63 has been shown to inhibit Notch1 activity via indirect mechanisms, thereby maintaining the proliferative capacity of keratinocytes (30, 43). In particular, p63 was shown to counteract Notch1 activity by transcriptional repression of the *Hes1* gene, a downstream target of Notch1 signaling (45). However, direct control of Notch1 expression by p63 in developmentally mature keratinocytes has remained elusive. In the present study, using normal human keratinocytes and other different types of normal human epithelial cells, we could show an inverse relationship between ΔNp63α and Notch1 expression and unveil for the first time a repressor function of ΔNp63α for *Notch1* gene expression. Taking this together with our previous finding of p63 binding to the p53-responsive element in the *Notch1* promoter and its dissociation with *Notch1* induction (38), we conclude that *Notch1* gene is a direct negative target of ΔNp63α in human keratinocytes.

We previously tested the effects of exogenous *LTR*-driven expression of ΔNp63α on Notch1 expression in HCK1T cells and found them to be marginal in the steady-state. Here, we applied a *CMV* promoter to overexpress ΔNp63α to a level comparable with those observed in some cervical cancer cell lines (Figs. 1A, 2B, 4B, 5A, and 6C). We thereby found that

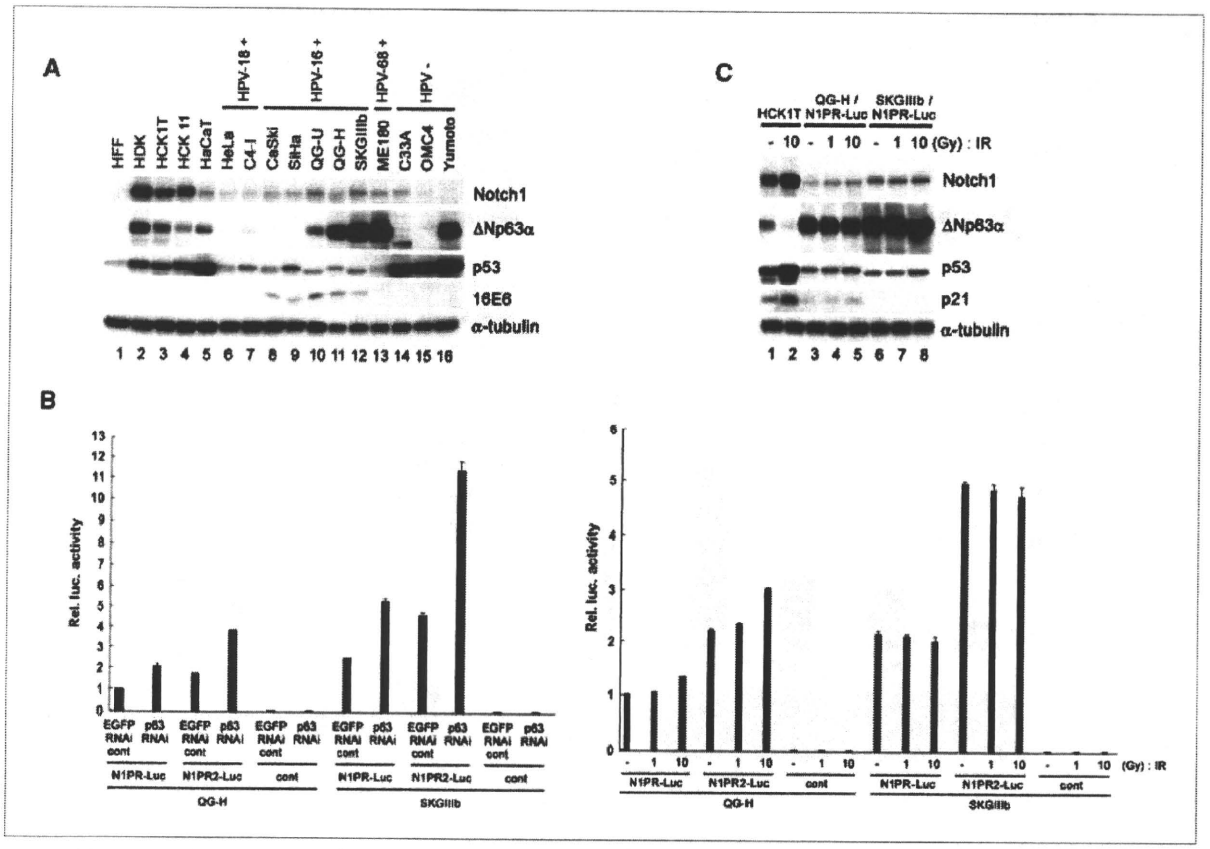

Figure 5. *Notch1* promoter activation is blocked by overexpressed ΔNp63α in cancer cells. A, immunoblotting was performed with the indicated antibodies. B, left, QG-H and SKGIIIb cells were stably introduced with *N1PR-Luc, N1PR2-Luc* reporters, or control construct (cont) and then transduced with retroviral vectors encoding either *p63* shRNA or *EGFP* shRNA control. At 48 h after transduction, cell lysates were prepared and subjected to dual-luciferase reporter assays. B, right, QG-H and SKGIIIb cells stably expressing either *N1PR-Luc, N1PR2-Luc* reporters, or control construct were exposed to 1 or 10 Gy IR or left untreated (–). At 24 h thereafter, cell lysates were prepared and subjected to dual-luciferase reporter assays. C, cells were treated as in B, and extracts were analyzed by immunoblotting.

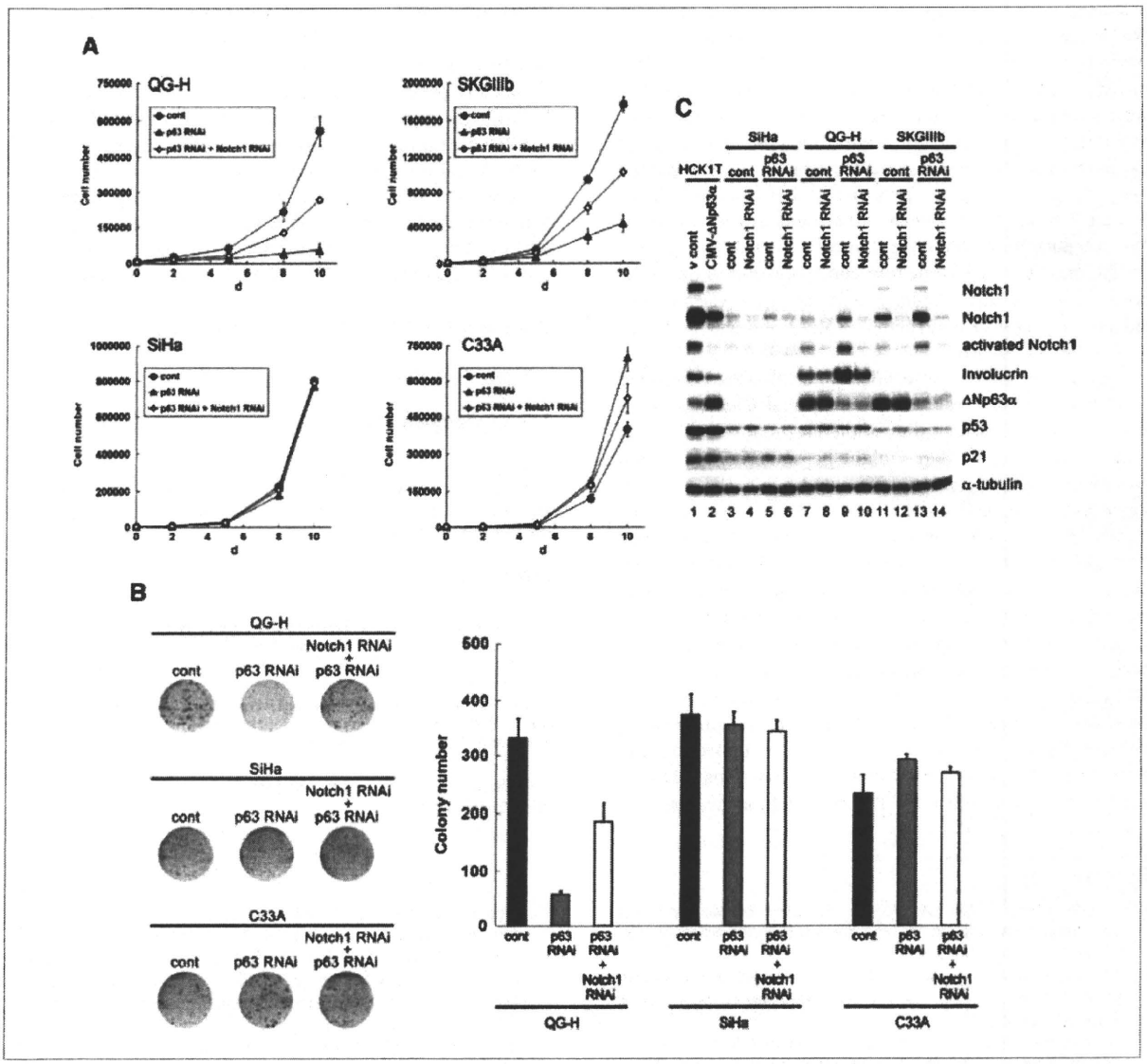

Figure 6. Knockdown of *p63* induces growth suppression in cancer cells, and simultaneous silencing of *Notch1* rescues this proliferation defect. A, QG-H and SKGIIIb cells were introduced with *Notch1* shRNA or control shRNA (cont). Cells were then transduced with either *p63* shRNA or control shRNA. At 48 h after transduction, aliquots of 8×10^3 cells for QG-H and SKGIIIb, 2×10^3 cells for SiHa, and 1×10^3 cells for C33A were replated on 35-mm dishes, and proliferation was monitored over the next 8 d. B, cells were treated as for A, and aliquots of 500 cells were seeded on 35-mm dishes to assess clonogenicity. C, cells were treated as in A, and extracts were analyzed by immunoblotting.

such overexpressed ΔNp63α has a pronounced effect on *Notch1* gene expression (Fig. 1A, D and Fig. 2A–C), where overexpression of ΔNp63α reduced basal levels of Notch1 and inhibited both p53-dependent and p53-independent *Notch1* induction upon genotoxic stress and serum exposure, respectively. The notion for p53-independent function of ΔNp63α can also be supported by the fact that ΔNp63α overexpression frequently coexists with *p53* mutations in primary lung SCCs (21).

The observation of *Notch1* promoter activation by ectopic expression of TAp63α (Supplementary Fig. S3) is consistent with defective *Notch1* expression in the *p63*-null embryonic epidermis (3), assuming that the TAp63 isoform specifically transactivates *Notch1* gene expression in the developmental stage. Given that transcriptional control of the *Notch1* gene remains largely unknown, the possible involvement of TAp63 or other unidentified transcription factors during development as well as under normal physiologic conditions in the postdevelopmental stage is of particular interest.

It was recently reported that the catastrophic epithelial phenotype of the *p63*-null mouse was partially ameliorated by inactivation of either *Ink4a* or *Arf*, with p63 directly repressing *Ink4a* and *Arf* gene expression (49). However, we failed to detect upregulation of these proteins upon *p63*

silencing (Supplementary Fig. S8). Furthermore, in apparent discordance with our observations (Fig. 3A–C) and the observations of others (9), loss of *p53* failed to restore defective proliferation of keratinocytes from *p63*-null mouse (49). We speculate that acute knockdown of *p63 in vitro* recapitulates physiologic differentiation of keratinocytes in suprabasal layers through induction of *Notch1*, which is also regulated by p53, whereas phenotypes in *p63*-deficient or *TAp63*-deficient mice rather reflect impairment in the long-term maintenance of keratinocyte stem cells by p63 through epithelial-mesenchymal interaction and repression of *Ink4a/Arf* locus. However, further studies certainly seem warranted to define the importance of the p63-Notch1 pathway in keratinocyte biology *in vivo*.

Intriguingly, we have also noted that ΔNp63α expression is very low or even undetectable in several lines of cervical cancer cells. In consideration of the essential role of p63 in control of stemness, there might be some compensation mechanism for its loss of function, and this is clearly an area of future research. Given the observed association between p63 loss and metastasis appearance (5, 16), it can be hypothesized that overexpressed ΔNp63α promotes the early stages of carcinogenesis as an oncogene by increasing self-renewing capacity. Once cells become permissive to p63 loss due to some additional genetic or epigenetic alterations, they may acquire invasive features with the epithelial-to-mesenchymal transition (18). In such situations, concomitant loss of TAp63 could be beneficial for cancer progression, because TAp63 isoforms could be induced by wound and stress (7) and possess tumor suppressor functions (15) similar to p53 and TAp73 (50). The presence of multiple isoforms with opposing and/or overlapping functions and their balance might provide explanations for the unique signature of p63 in tumorigenesis.

Taken together, our data establish a direct role of p63 in *Notch1* gene expression in human epithelial cells and provide a molecular rationale for maintenance of proliferative potential by ΔNp63α through *Notch1* repression in normal human keratinocytes as well as cancer cells. Our findings suggest a biological effect of increased ΔNp63α expression together with inactivation of p53 on carcinogenesis by means of persistent downregulation of the *Notch1* tumor suppressor.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ORIGINAL ARTICLE

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

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

Introduction

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

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

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

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