Cancer Research

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

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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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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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doi: 10.1158/0008-5472.CAN-09-4063

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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

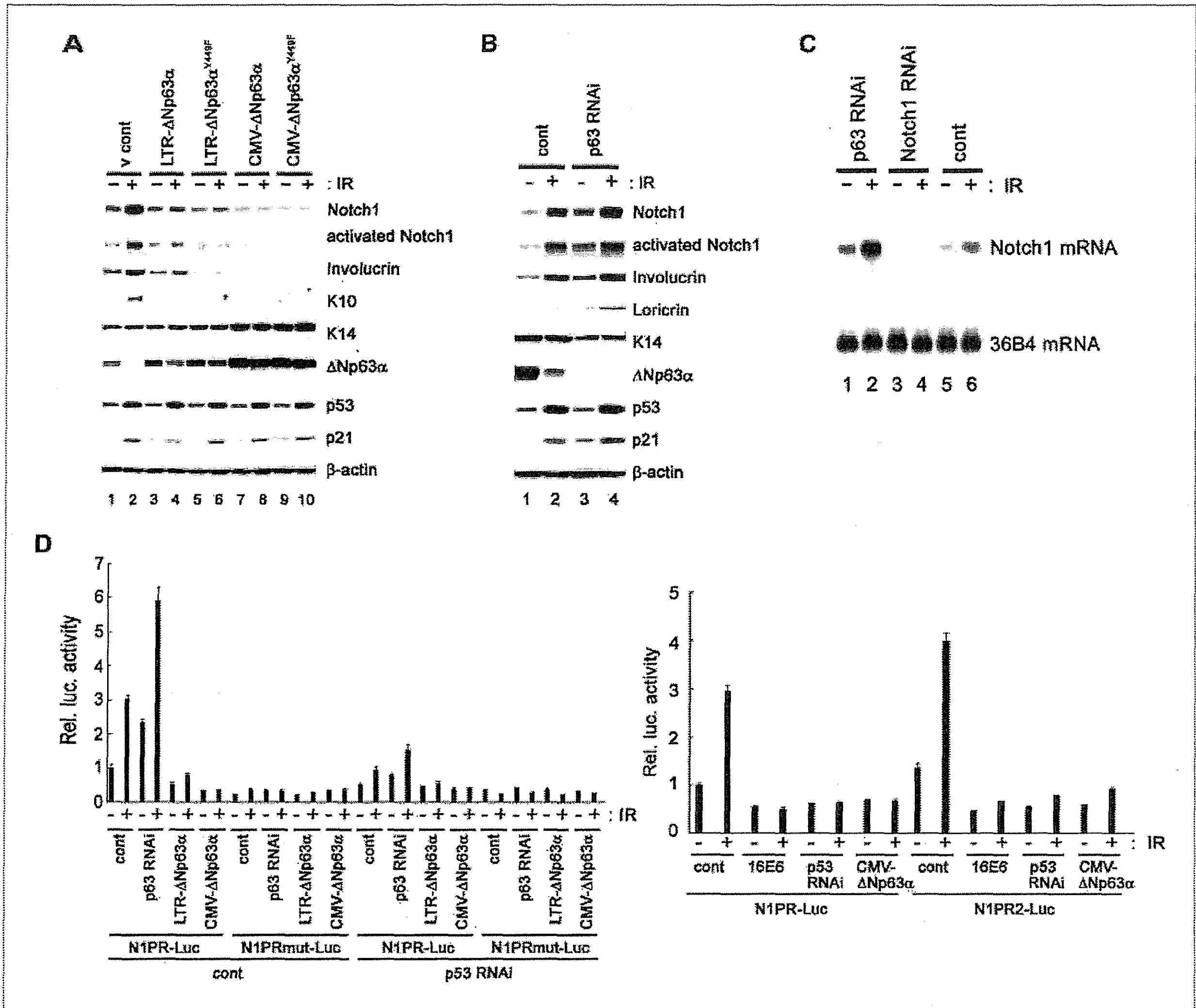

Figure 1. ΔNp63α represses p53-dependent expression of the *Notch1* gene and inhibits differentiation of normal human keratinocytes upon genotoxic stress. A, HCK1T cells were stably transduced with the indicated genes by retroviral gene transfer. v cont, vector control. Cells were exposed to 10 Gy IR (+) 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

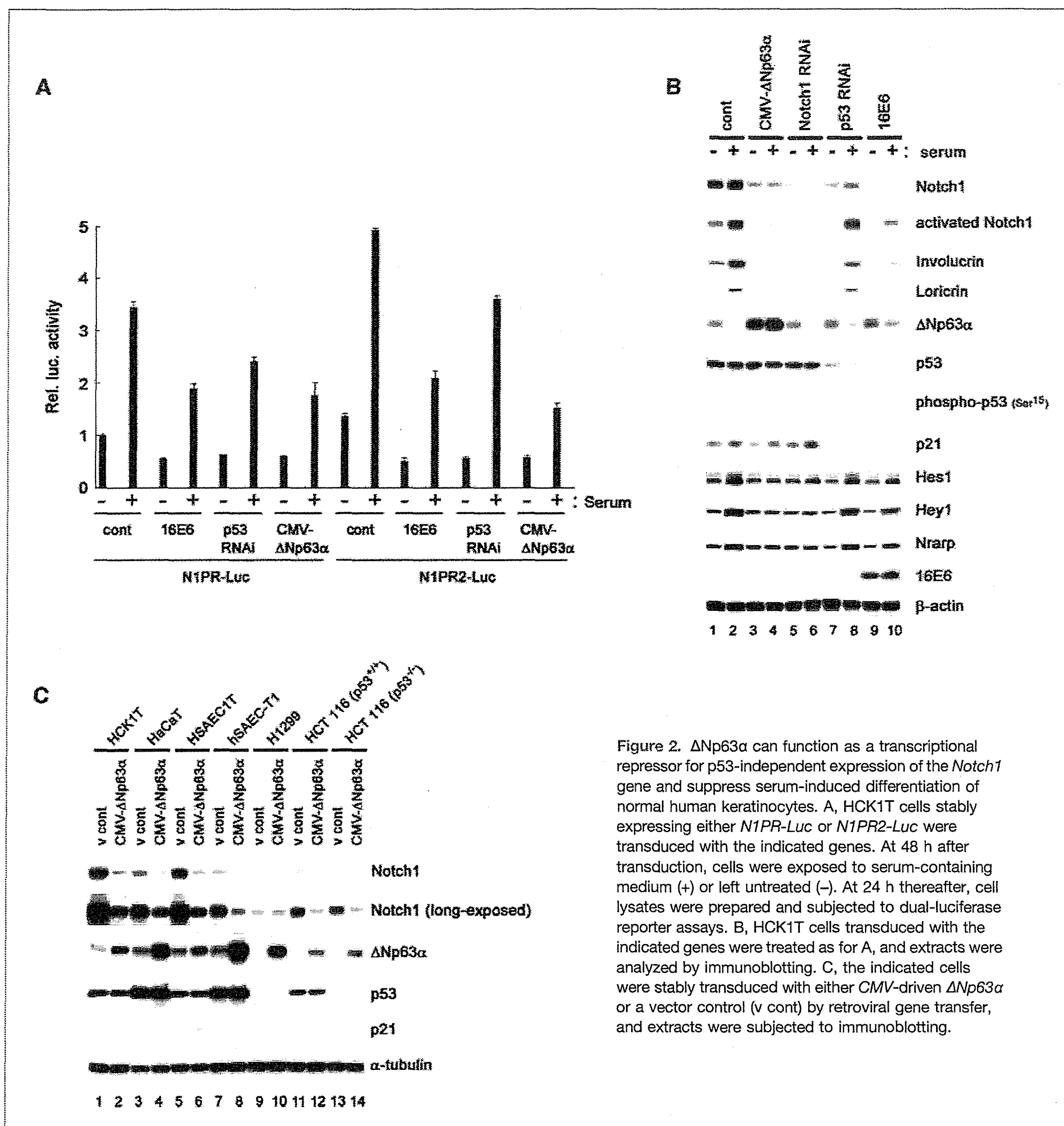

Figure 2. ΔNp63α can function as a transcriptional repressor for p53-independent expression of the *Notch1* gene and suppress serum-induced differentiation of normal human keratinocytes. A, HCK1T cells stably expressing either *N1PR-Luc* or *N1PR2-Luc* were transduced with the indicated genes. At 48 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.

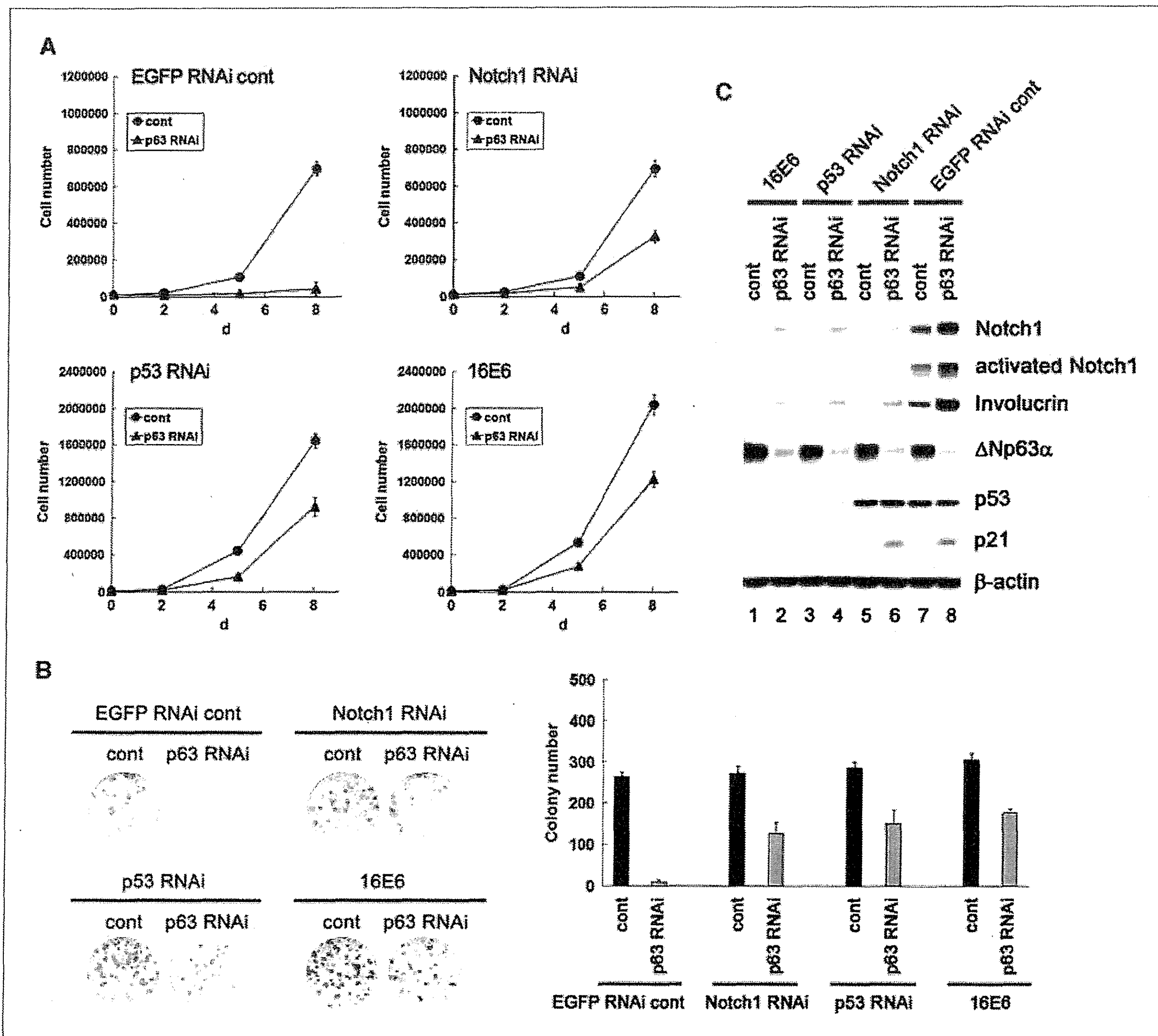

Figure 3. Knockdown of *Notch1* or inactivation of p53 restores the proliferation defect triggered by *p63* silencing. A, HCK1T cells stably expressing *Notch1* shRNA, HPV-16 E6, *p53* shRNA, or *EGFP* shRNA control (cont) were transduced with either *p63* shRNA or control shRNA. At 48 h after transduction, 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,

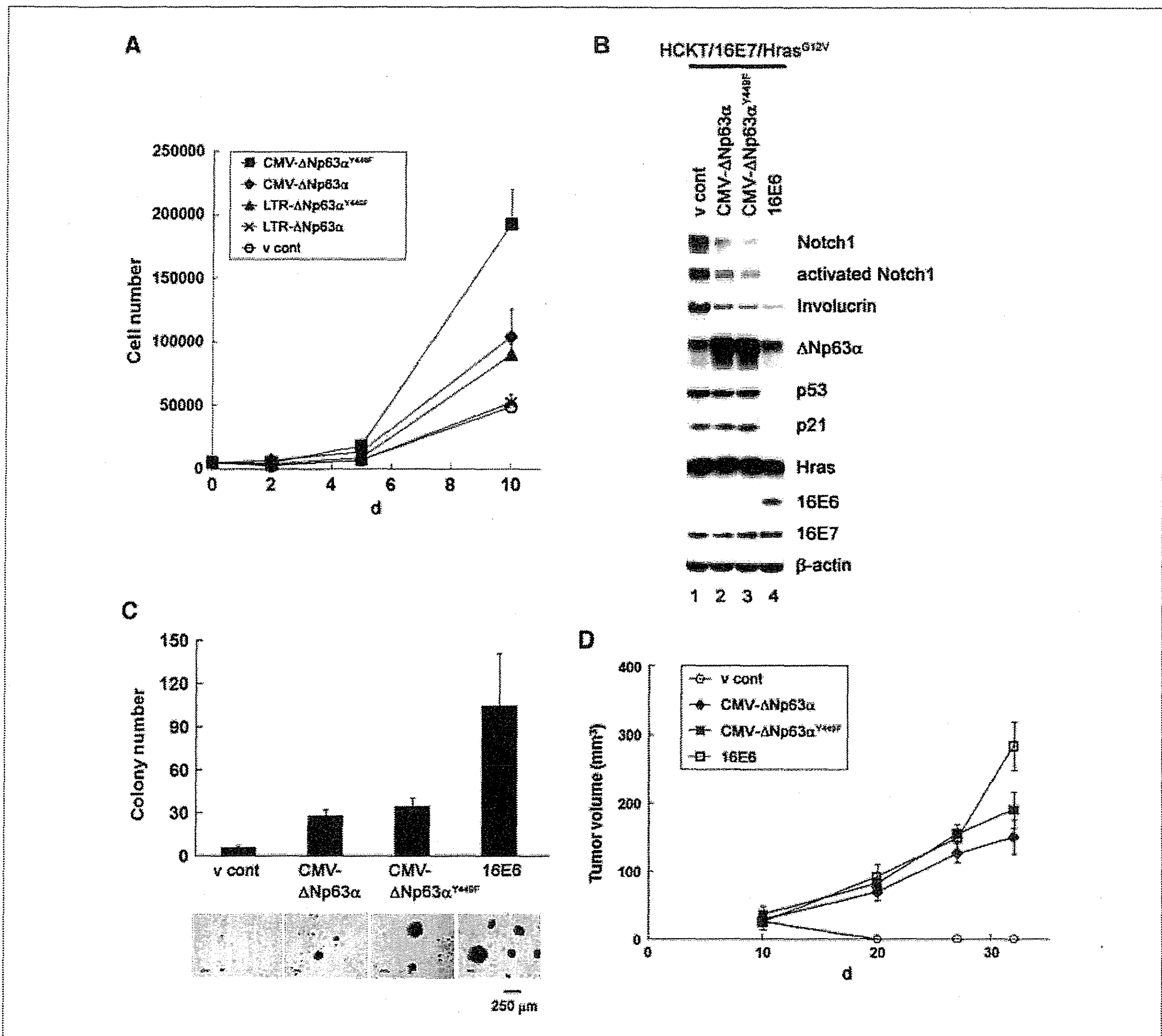

Figure 4. ΔNp63α overexpression endows normal human keratinocytes with increased proliferative capacity and tumorigenic properties. A, HCK1T cells were stably transduced with the indicated genes. Aliquots of 1×10^3 cells were replated on 35-mm dishes, and proliferation was monitored over the next 10 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 mm² 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 (mm³) 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

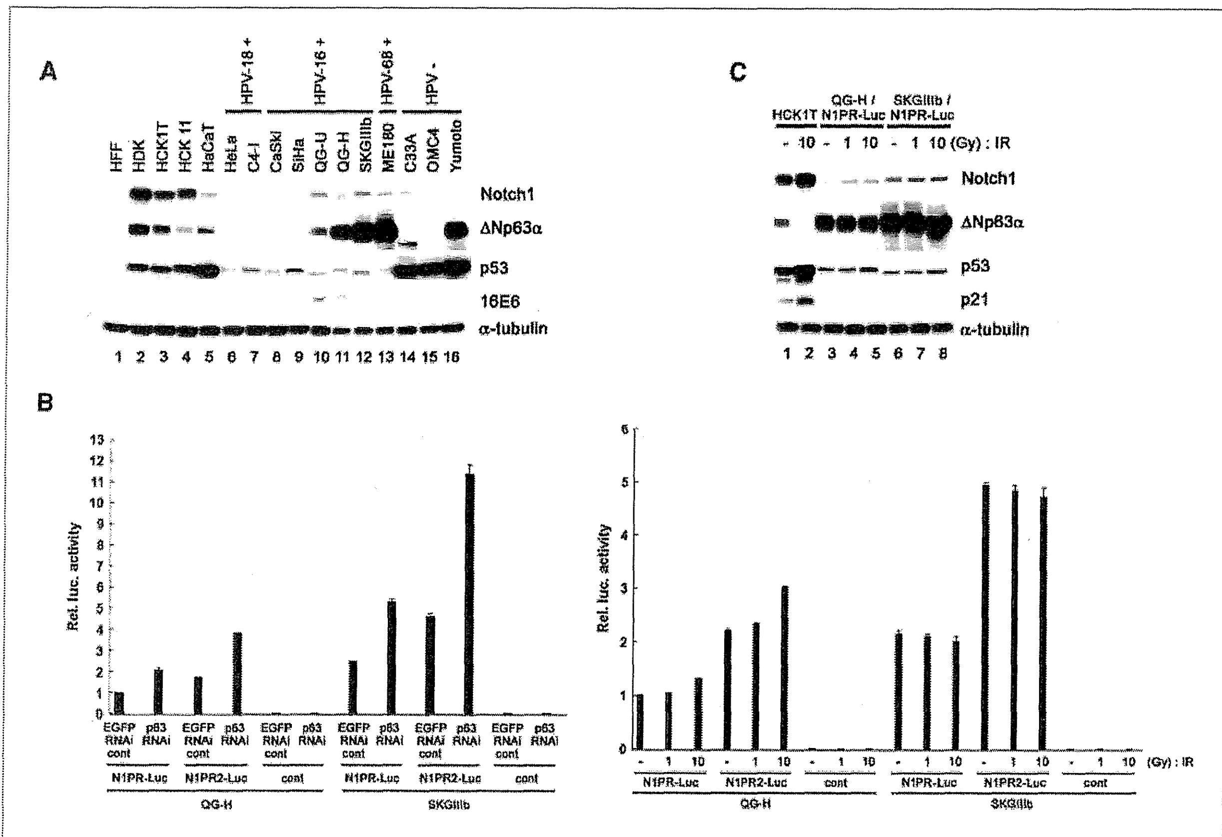

Figure 5. *Notch1* promoter activation is blocked by overexpressed ΔNp63α in cancer cells. A, immunoblotting was performed with the indicated antibodies. B, left, QG-H and SKGIIIb cells were stably introduced with *N1PR-Luc, N1PR2-Luc* reporters, or control construct (cont) and then transduced with retroviral 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.

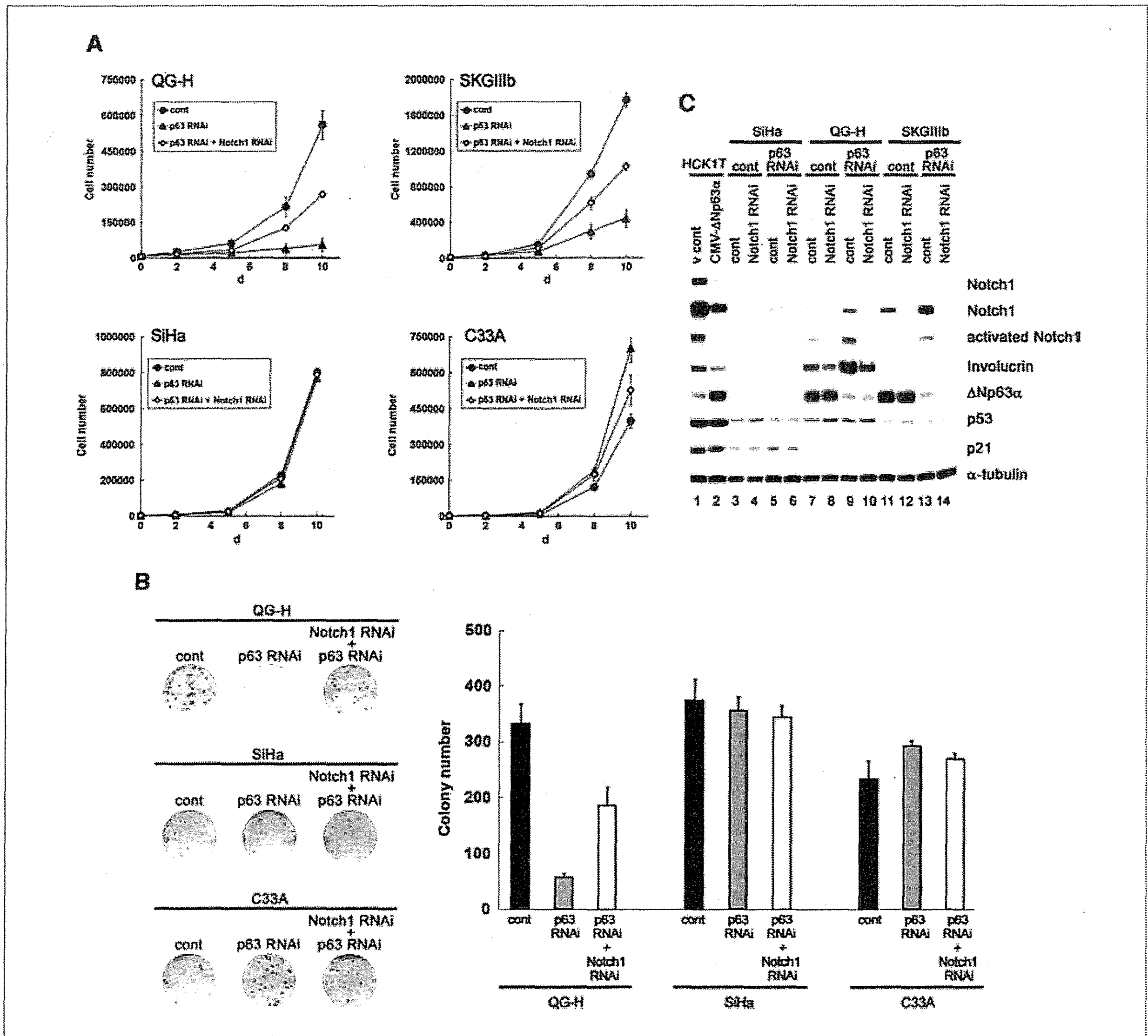

Figure 6. Knockdown of *p63* induces growth suppression in cancer cells, and simultaneous silencing of *Notch1* rescues this proliferation defect. A, QG-H and SKGIIIb cells were introduced with *Notch1* shRNA or control shRNA (cont). Cells were then transduced with either *p63* shRNA or control shRNA. 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.

Acknowledgments

We thank Drs. B. Vogelstein and K.W. Kinzler for HCT116 isogenic cell lines ($p53^{+/+}$ and $p53^{-/-}$); Dr. J. Yokota for hSAEC-T1 cells; Dr. M. Enari for helpful discussion; and T. Ishiyama, A. Noguchi, E. Kabasawa, and T. Shizume for expert technical assistance.

Grant Support

Grant-in-Aid for Cancer Research from Ministry of Health Labor and Welfare of Japan (T. Kiyono) and Grant-in-Aid for Scientific Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan (T. Yugawa).

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Received 11/07/2009; revised 02/01/2010; accepted 02/23/2010; published OnlineFirst 05/04/2010.

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Open

Cancer Gene Therapy (2010), 1–10
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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.
Cancer Gene Therapy advance online publication, 1 October 2010; doi:10.1038/cgt.2010.53

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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Received 22 November 2009; revised 23 April 2010; accepted 9 June 2010

individuals >30 years (about 80%) have circulating anti-HSV-1 antibodies in Japan.[10] Virus particles injected into the peritoneal cavity are vulnerable to inactivation by complement proteins, uptake by the reticuloendothelial system and neutralization by circulating antibodies.[11] Of these host defenses, antibodies are likely to be the most restrictive barrier to therapy, as they mediate a long-lasting state of immunity to repeated infections.[12] Several widely differing approaches aimed at protecting viral particles within the circulation and ensuring tumor delivery are currently the focus of intense research. For example, one possibility is modification of the viral coat,[13,14] although such technologies are technically challenging. In addition, cellular carriers could be used as Trojan Horse vehicles to shield oncolytic virus (OV) from neutralization following intraperitoneal administration, and act as *in situ* virus factories once arriving at the tumor site.[15,16]

In this study, we demonstrate that the molecular engineering of cellular carriers can increase their ability to support viral replication, promote direct cell-to-cell viral infection of the tumor, and shield oncolytic virus from neutralizing antibodies during delivery *in vitro*. Furthermore, we show the suitability of human peritoneal mesothelial cells (MCs) as a carrier system for delivery of HF10 and Hh101 to maximize the efficacy of oncolytic virus *in vivo*.

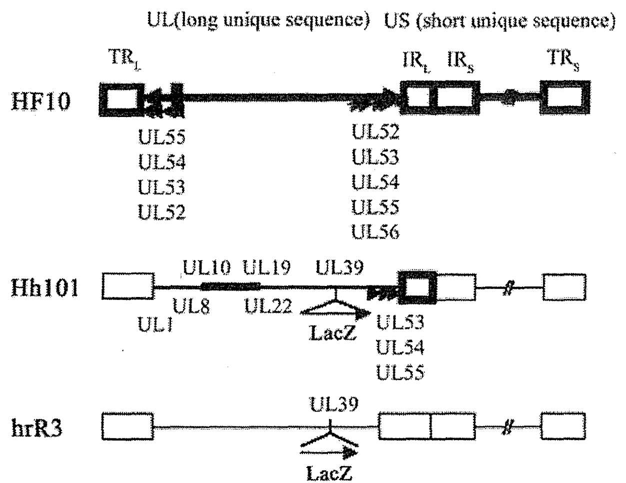

Figure 1 Models of the structures of herpes simplex virus type 1 (HSV-1) mutants. A schematic representation of the structure of the Hh101 and HF10 genomes. The locations of deletions and insertions in the genome of HF10 are shown. Expansions indicate the positions of genes within the deletion and insertion regions. Arrows indicate the position and orientation of genes within the expansions.

Materials and methods

Cell lines and viruses

Human ovarian cancer (SKOV3) cells were generously donated by Memorial Sloan-Kettering Cancer Research Laboratory. African green monkey kidney (Vero) cells were obtained from the Riken Cell Bank (Tsukuba, Ibaragi, Japan). SKOV3 cells were maintained in RPMI 1640 supplemented with 10% fetal calf serum and penicillin–streptomycin. Vero cells were grown in Eagle's minimal essential medium containing 10% calf serum and 1% penicillin–streptomycin. These cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

hrR3, a ribonucleotide reductase (UL39)-deficient HSV-1 mutant, derived from the parental wild-type strain KOS, was kindly provided by Sandra K Weller (University of Connecticut Health Center, Farmington, CT). HF10 is a non-selected clone derived from HSV-1 strain HF and causes extensive cell membrane fusion in infected cells. Hh101 is a recombinant virus clone isolated from Vero cells co-infected with HF10 and hrR3 (Figure 1). The phenotypes of these viruses have been previously described.[17–19]

To visualize viruses *in vitro* and *in vivo*, the green fluorescent protein (*GFP*) gene was inserted into HF10 under control of the cytomegalovirus (CMV) promoter, in which UL43 was deleted. We named this virus HF-GFP.

Establishment and characterization of immortalized MCs

Human peritoneal MCs were isolated from surgical specimens of human omentum after obtaining consent from each patient, as described previously.[20] Briefly, small pieces of omentum were surgically resected under sterile conditions and were trypsinized at 37 °C for 30 min. The suspension was then passed through a 200-μm pore nylon mesh to remove undigested fragments, and centrifuged at 2000 r.p.m. for 5 min. The collected cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum. In the subsequent experiments, cells were used during the second or third passage after primary culture.

Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen, Carlsbad, CA). Briefly, hTERT, human cyclin D1 and human mutant Cdk4 (Cdk4R24C: an inhibitor resistant form of Cdk4) were first recombined into entry vectors by BP reaction (Invitrogen). Then these segments were recombined with a lentiviral vector, CSII-CMV-RfA, by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclin D1 and -hCDK4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously.[21] Following the addition of recombinant viral fluid to MCs in the presence of 4 $\mu g\,ml^{-1}$ polybrene, infected cells were selected in the presence of 250 $\mu g\,ml^{-1}$ of G418, 0.5 $\mu g\,ml^{-1}$ of puromycin, 3 $\mu g\,ml^{-1}$ of blasticidin-S or 50 $\mu g\,ml^{-1}$ of hygromycin-B. These cells are named human omentum mesothelial cells (HOmMCs). The study to establish immortalized MCs was approved by the local ethics committee and institutional review board of our hospital.

Anti-HSV-1 serum

Anti-HSV-1 serum was obtained from mice or guinea pigs by intravenous injection of HSV-1 grown in Vero cells. The neutralizing capacity of antiserum was determined by mixing about 100 plaque-forming units (PFU) of HSV-1 with serial dilutions of antiserum. The serum titer was

expressed as the dilution causing 50% plaque reduction: the neutralization titers of anti-HSV-1 mouse antiserum and anti-HSV-1 guinea pig antiserum were 1000 and 1600, respectively.

Viral replication assay

MCs or HOmMCs were plated on 35-mm dishes at a density of 3.7×10^5 cells per dish in 2 ml of the growth medium under standard conditions overnight. Cells were then infected with HF10 or Hh101 at multiplicities of infection (MOI) ranging between 0.03 and 3. To measure virus replication, cells were scraped from dishes at the indicated times, lysed by freeze–thaw and centrifuged at 3000 r.p.m. for 5 min. Viral titers were determined from the sample supernatants by plaque assay.

In vitro *delivery of HSV using MCs as carriers*

MCs were infected with Hh101 (MOI, 3) for 1 h at 37 °C; free virus was then removed and the cells were washed with phosphate-buffered saline (PBS) three times and resuspended in fresh medium. At 2 h after infection, the infected cells were trypsinized. The suspension was centrifuged at 1300 r.p.m. for 5 min at 4 °C. The collected cells were used as infected carrier cells.

SKOV3 cells were plated on 35-mm dishes at a density of 5.6×10^5 cells per dish in 2 ml of the growth medium. After 24 h, Hh101- (3×10^5 PFU) or Hh101-infected carrier cells (1×10^5 cells, MOI, 3) were added to the media, and we observed any resulting cytopathogenic effects (CPEs). At 24 h after infection, viral titers were determined from the sample supernatants by plaque assay.

In vitro *effects of anti-HSV-1 antiserum on HF-GFP*

HOmMCs were infected with HF-GFP (MOI, 3) for 1 h at 37 °C; free virus was then removed and the cells were washed with PBS three times and resuspended in fresh medium. At 2 h after infection, the infected cells were trypsinized. The suspension was then centrifuged at 1300 r.p.m. for 5 min at 4 °C. The collected cells were used as infected carrier cells.

SKOV3 cells were plated on 35-mm dishes. After 24 h, HF-GFP (10^5 PFU per dish) or HF-GFP-infected carrier cells (10^4 cells per dish) were added to the media with or without anti-HSV-1 mouse antiserum. At 24 h after infection, SKOV3 cells were photographed using the Leica (Wetglar, Germany) M205FA fluorescence stereomicroscope with a standard GFP filter set. At 30 h, SKOV3 cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet solution. The number of plaques was counted under microscopy. The graphs (Figure 5e) were obtained from two independent experiments.

Animal studies

Animal studies were performed in accordance with guidelines issued by the Animal Center at Nagoya University School of Medicine. Female Balb/c slc nu/nu mice (5 to 6 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). For surgical procedures, mice were anesthetized with an intraperitoneal injection of 7.2% chloral hydrate in sterile PBS (0.005 ml g^{-1} body weight).

Subcutaneous tumor model

To determine the therapeutic efficacy of HF10, we used a subcutaneous (s.c.) tumor model. SKOV3 cells were cultured and passaged twice *in vitro*, and 5×10^6 cells were injected s.c. into the flanks of 5-week-old nude mice. At 8 days after tumor challenge, when s.c. tumors were ~10–15 mm in diameter, mice were treated with intratumoral (i.t.) injection of HF10 (1×10^7 PFU). Animals in the first group were injected on days 8, 10 and 12. Animals in the second group were injected on days 8, 10, 12, 18, 20 and 22. Control mice were treated with i.t. injection of 1 ml PBS. Tumor volume was monitored for the indicated number of days after treatment.

Intraperitoneal tumor model

We confirmed that intraperitoneal (i.p.) injection of SKOV3 cells into 6-week-old female Balb/c nude mice resulted in peritoneally disseminated tumors, ascites, cachexia and death. To assess the efficacy of Hh101, this murine xenograft model was used. Nude mice ($n = 27$) were engrafted i.p. with 2×10^6 SKOV3 cells. HOmMCs were infected for 2 h with MOI = 3 of Hh101 and were used as carriers. After freezing and thawing at 2 h after infection, ~5×10^4 PFU of Hh101 were detected as infectious viruses. To estimate the effect of Hh101 in minimally spread ovarian cancer, HOmMCs (3×10^6 cells) infected with Hh101 were injected i.p. on days 3, 6 and 9. The control groups received 1 ml PBS or Hh101 (5×10^4 PFU ml^{-1}) by i.p. injection on the same days. Mice of each group were followed up to record survival times.

To evaluate the role of Hh101 in more advanced disease, SKOV3 tumors were allowed to grow for 6 days before they were treated. Female Balb/c nude mice (6 weeks old)($n = 10$) were engrafted i.p. with 2×10^6 SKOV3 cells, and this animal group was treated with repeated injection on days 6, 9, 12, 15 and 18. Animals were followed up daily to record survival times.

Localization of virus-associated GFP expression in mice with disseminated ovarian cancer

Female Balb/c nu/nu mice (6 weeks old) were engrafted i.p. with 2×10^6 SKOV3 cells. On day 30, mice were randomized into two cohorts (control, HF-GFP treatment group). The control group received i.p. administration of 1 ml PBS. The HF-GFP treatment group was given 10^7 PFU of HF-GFP i.p. After 24 h, mice were killed and tumors were examined using the Leica M205FA automated fluorescence stereomicroscope with a standard GFP filter set.

To assess the effect of anti-HSV-1 antiserum by using infected carrier cell, we injected control serum or anti-HSV-1 guinea pig antiserum of (500 µl per each mouse, ×1/100 dilution) i.p. into each mouse at 1 h before treatment. HOmMCs were preinfected with HF-GFP (MOI, 3) for 2 h and were washed in PBS. For this experiment, a disseminated ovarian cancer model (1×10^7 SKOV3 cells, i.p.) was established in 6-week-old female Balb/c nu/nu mice. On day 14, mice were randomized qinto control, HF-GFP and HF-GFP-infected HOmMCs groups. The HOmMCs group was treated with 10^7 cells of

HOmMC-infected HF-GFP. Mice were killed after 24 h and the intra-abdominal images were obtained by the fluorescence stereomicroscope. For each experiment, images were captured under identical exposure settings. Overlays were generated using Adobe Photoshop CS software (Adobe Systems, San Jose, CA).

Statistical methods

Data were analyzed using the StatView statistical software package (SAS Institution, Cary, NC). The survival data were analyzed using the Kaplan–Meier method and the log-rank test. Differences in tumor volumes between the treated and control groups were analyzed by the Student's *t*-test. *P*-values <0.05 were considered statistically significant.

Results

Intratumoral Administration of HSV mutants suppresses s.c. tumor growth of human ovarian cancer cells in nude mice

We examined the ability of HF10 to control tumor cell growth in an *in vivo* model. We used an s.c. tumor model, because HF10 is fatal to immunodeficient animals when it is administered intravenously or intraperitoneally. The flanks of Balb/c-nu mice were s.c. injected with 5×10^6 SKOV3 cells. When tumors were palpable (day 8), i.t. injections of PBS or HF10 (1×10^7 PFU) were made on days 8, 10 and 12 for group one, and on days 8, 10, 12, 18, 20 and 22 for group two. Injections (i.t.) with HF10 significantly reduced tumor growth compared with PBS-injected control animals (Figure 2a). Moreover, in group two, complete disappearance of tumors was observed in some animals (Figure 2b). Representative pictures of control and HF10-injected mice are shown (Figure 2c).

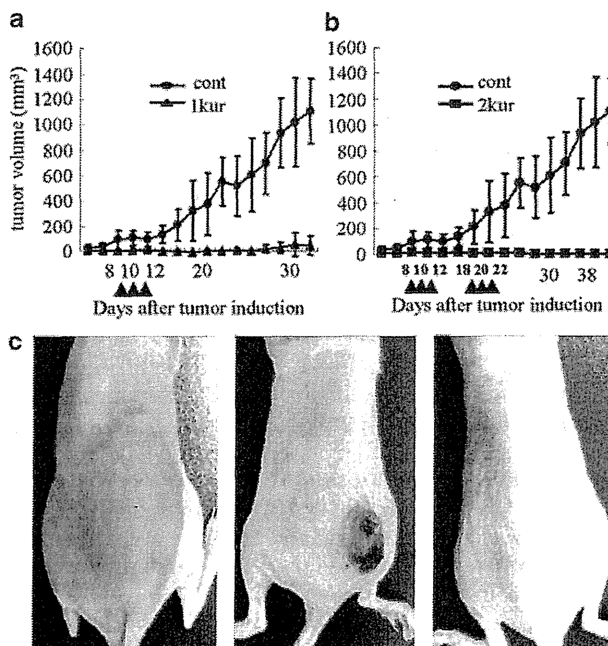

Figure 2 HF10 reduces tumor growth in a subcutaneous (s.c.) ovarian cancer model. (**a**) In all, 5×10^6 SKOV3 cells were s.c. implanted into the flank of 5-week-old nude mice. When s.c. tumors were approximately 10–15 mm in diameter, phosphate-buffered saline (PBS) (control) or 1×10^7 plaque-forming units HF10 were injected intratumorally on days 8, 10 and 12. (**b**) Group two was injected on days 8, 10, 12, 18, 20 and 22. Tumor volume was monitored for the indicated days after treatment ($P<0.01$; control vs HF10 treatment group). Bars represent means + s.e.m. of each group. (**c**) Representative pictures of control (right), group one (middle) and group two (left) at day 30.

In vitro *replication of HSV mutants in MCs, immortalized HOmMCs and SKOV3 cells*

To determine which cell line might be adequate for use as a carrier, we next tested the ability of HF10 and Hh101 to replicate in MCs, immortalized human omentum MCs and in SKOV3 cells. Human MCs may pose considerable advantages as vehicles for oncolytic virotherapy for ovarian cancer. First, MCs can be isolated from patients and grown in culture relatively easily. In addition, if isolated from the same patient that will be treated, autologous transplantation overcomes the difficulties related to immune rejection of the transplanted cells. Cells were infected at MOIs of 3 or 0.03. At MOI of 3, virus titers were ~10-fold higher in HOmMCs than in MCs or SKOV3 cells at 24 h after infection (Figures 3e, f). This correlated with the observation of more extensive and rapid CPEs in HOmMCs than SKOV3 cells (Figure 3c, d). Moreover, viral titers of MCs and SKOV3 cells infected at MOI of 3 and HOmMCs infected at MOI of 0.03 increased equally with time. Virus replication was the most efficient in HomMCs; thus these findings suggested that HOmMCs would be suitable for use as carrier cells.

In vitro *delivery of HSV using MCs as carrier cells*

We estimated the efficacy of tumor killing caused by virus-loaded carrier cells *in vitro*. To this end, the oncolytic effects of tumor cells cocultured with virus-loaded MCs was compared with direct infection by virus. We administered Hh101- (3×10^5 PFU) or Hh101-infected carrier cells (1×10^5 cells; MOI of 3) to the media of SKOV3 cells. At 24 h after infection, virus titers in the media were 6.3×10^4 PFU ml^{-1} when Hh101-infected carrier cells were administered, and only 2.1×10^2 PFU ml^{-1} when Hh101 virus was administered. Moreover, CPE was observed ~12 h after infection when Hh101-infected carrier cells were administered (Figure 4a), whereas it was observed 24 h after infection with Hh101 virus alone (Figure 4b). CPE was spread more rapidly and extensively in the case of Hh101-infected carrier cells (Figure 4a). Carrier cells therefore supported sufficient viral replication and could contact target cancer cells efficiently.

In vitro *immune evasion by cell-based delivery of HSV*

As the majority of human adults has been exposed to HSV and has anti-HSV antibodies, it is theoretically possible that oncolytic virus would be attenuated by circulating antibodies. To examine the effect of antibodies

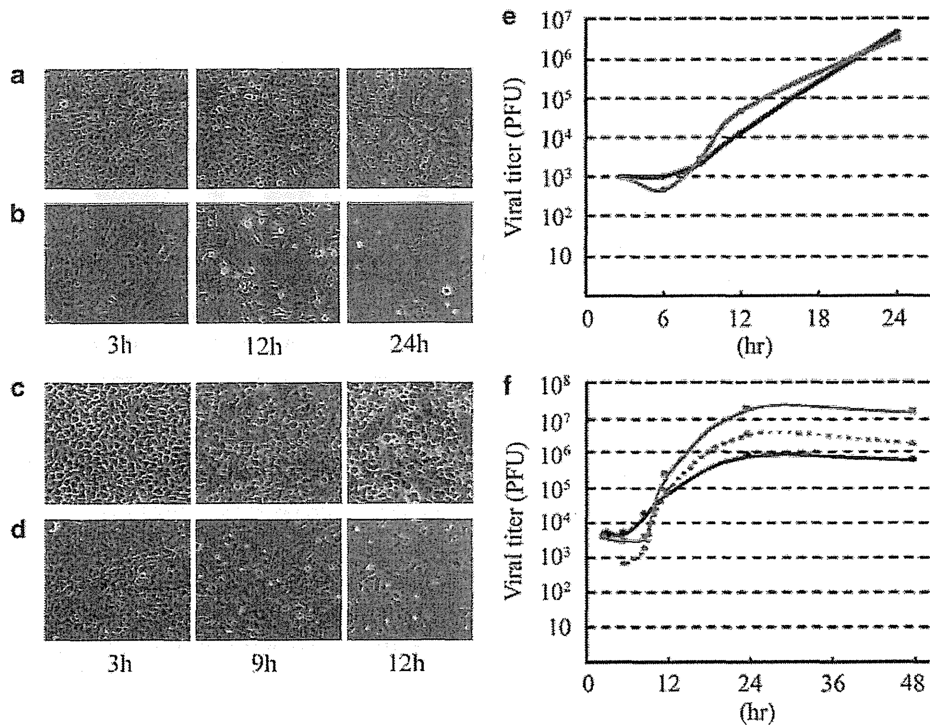

Figure 3 HF10 and Hh101 replication in mesothelial cells (MCs), human omentum mesothelial cells (HOmMCs) and SKOV3 cells. SKOV3 cells (**a**, black line) and MCs (**b**, red line) were infected with HF10 at multiplicities of infection (MOI) 3. SKOV3 cells (**c**, black line) and HOmMCs (**d**, red line) were infected with Hh101 at MOI 3. Representative cytopathogenic effects (CPE) are shown as time series. Cells were harvested and virus titer was determined by plaque assay. The red dotted line shows virus titers in MCs infected with Hh101 at MOI 0.03 (**e**, **f**). The values represent the mean of samples tested in triplicate. PFU, plaque-forming unit.

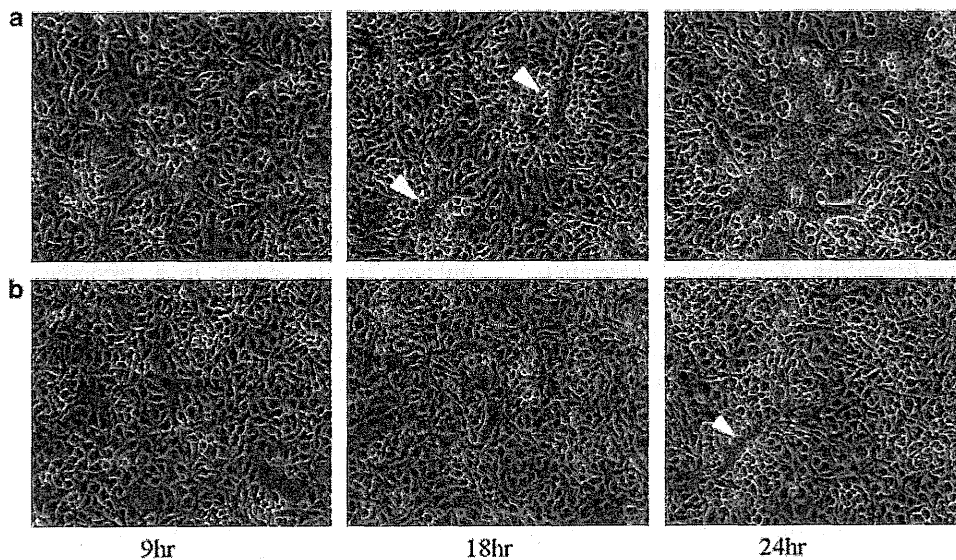

Figure 4 *In vitro* delivery of herpes simplex virus (HSV) using mesothelial cells as carriers. Hh101-infected carrier cells (**a**) or cell-free Hh101 (**b**) were added to the media of SKOV3 cells. Representative cytopathic effects (CPEs) are shown as time series. The white arrow heads show small CPEs.

against virus delivery by carrier cells, we performed *in vitro* experiments. We plated SKOV3 cells on 35-mm dishes, and after 24 h, HF-GFP (10^5 PFU per dish) or HF-GFP-infected carrier cells (10^4 cells per dish) were added to the culture media containing with anti-HSV-1 antiserum or control serum. As evidenced by virus-associated fluorescence, extensive replication was seen in SKOV3 cells 24 h following administration of HF-GFP-

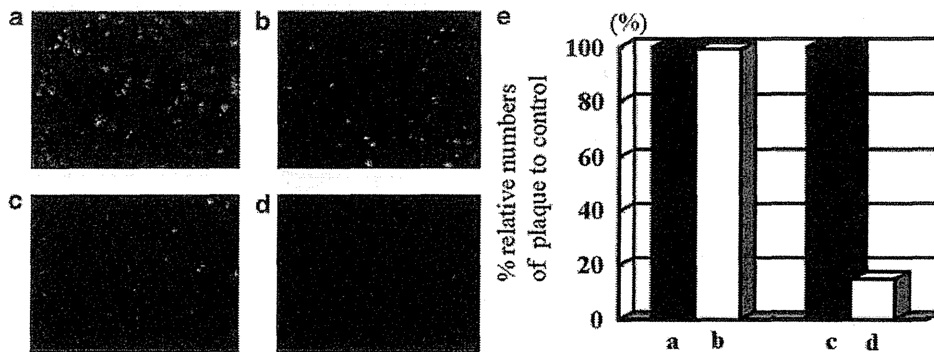

Figure 5 *In vitro* effects of anti-herpes simplex virus type 1 (HSV-1) antibody on HF-green fluorescent protein (GFP). SKOV3 cells were plated for 24 h, and HF-GFP-infected carrier cells (**a**, **b**) or HF-GFP (**c**, **d**) were added to the media containing control serum (**a**, **c**) or anti-HSV-1 antiserum (**b**, **d**). Anti-HSV-1 antiserum was added to the media to give a final dilution of 1:50. Representative cytopathogenic effect (CPE) images are taken at 24 h using the Leica M205FA fluorescence stereomicroscope with a standard GFP filter set. SKOV3 cells were then fixed, stained with 0.2% crystal violet solution and observed at 30 h. The number of plaques was counted and expressed as a percentage of number obtained in control cultures (**e**).

infected carrier cells in spite of anti-HSV-1 antiserum. In the absence of anti-HSV-1 antiserum (Figure 5a), however, virus-associated fluorescence was larger and brighter than in the presence of anti-HSV-1 antiserum (Figure 5b). In contrast, little virus-associated GFP was observed in SKOV3 cells 24 h following administration of HF-GFP in the presence of anti-HSV-1 antiserum (Figure 5d), although a number of GFP-expressing cells were detectable in the absence of anti-HSV-1 antiserum (Figure 5c). These results indicated that cellular carriers can efficiently shield oncolytic virus from neutralizing antibodies.

Localization of virally infected cell delivery in the presence of anti-HSV-1 antiserum

In order to visualize the distribution of the cellular vehicles in mice, we used HF-GFP, which allowed us to follow the biodistribution of virus-associated fluorescence using an *in vivo* imaging system. To assess the localization of intraperitoneally injected virus, we established a mouse model using ovarian cancer cells, in which 2×10^6 SKOV3 cells were inoculated into the peritoneal cavity of nude mice, leading to the formation of peritoneal disseminations. HF-GFP was injected into the peritoneal cavity 30 days after the initial inoculation of cancer cells. At 24 h after HF-GFP injection, nearly all visible tumor nodules in the peritoneal cavity were GFP positive. We detected GFP expression even in small tumors and the brightness of GFP varied in each tumor (Figure 6b). In contrast, no GFP expression was seen in the tumors in animals of the control group (Figure 6a). The GFP expression persisted for 7 days after HF-GFP injection; however, the brightness of GFP weakened as time passed. GFP expression persisted longer in mice injected with HF-GFP-infected carrier cells than in mice injected with HF-GFP alone. These findings suggested that viruses injected into the peritoneal cavity exhibited preferential and specific distribution in disseminated cancer foci in HSV-1 naïve animals.

Next, to examine the impact of a pre-existing immune response, we used the passive immunization method in which anti-HSV-1 antiserum was injected i.p. into mice. Intraperitoneal tumor-bearing mice were given cell-free HF-GFP or HF-GFP-infected HOmMCs i.p at 1 h after treatment with anit-HSV-1 antiserum or control serum. No clear GFP signal was observed in disseminated tumors when HF-GFP was given to mice pretreated with anti-HSV-1 antiserum (Figure 6c). In contrast, a significant GFP signal was detected in peritoneal tumors in spite of pretreatment with anti-HSV-1 antiserum, suggesting that infected carrier cells could bypass circulating antibodies and transfer virus to intraperitoneally disseminated ovarian tumors (Figure 6d).

Intraperitoneal administration of HSV-1 mutant-infected carrier cells improved survival of mice with ovarian cancer

To assess the suitability of carrier cells for the delivery of HSV-1 mutants *in vivo*, we conducted survival experiments to compare the effects of Hh101 administered directly with the effects of Hh101 delivered via HOmMCs. Because HF10 is lethal to immunodeficient animals, we utilized Hh101, which is a recombinant virus clone isolated from HF10 and hrR3 and is less virulent than HF10. PBS, Hh101 or Hh101-infected carrier cells were injected three times 3 days after the i.p. injection of 2×10^6 SKOV3 cells, a time at which tumors would be invisible to the naked eye. Three repeated therapeutic injections of Hh101-infected HOmMCs significantly improved the mean survival time of ovarian cancer-engrafted nude mice (55 days, $n = 10$) compared with the administration of Hh101 alone (46 days, $n = 9$; $P < 0.05$) (Figure 7a). Two-tenth of the animals in the carrier cell-treated group were completely protected from relapse of peritoneal tumor and ascites.

Next, PBS or Hh101-infected carrier cells were injected five times 6 days after the injection of tumor cells, a time at which numerous macroscopic white, 2 mm diameter tumors were seen at the diaphragm, at the mesentery and occasionally at the omentum. As shown in Figure 7b, all mice, irrespective of treatment, developed macroscopic

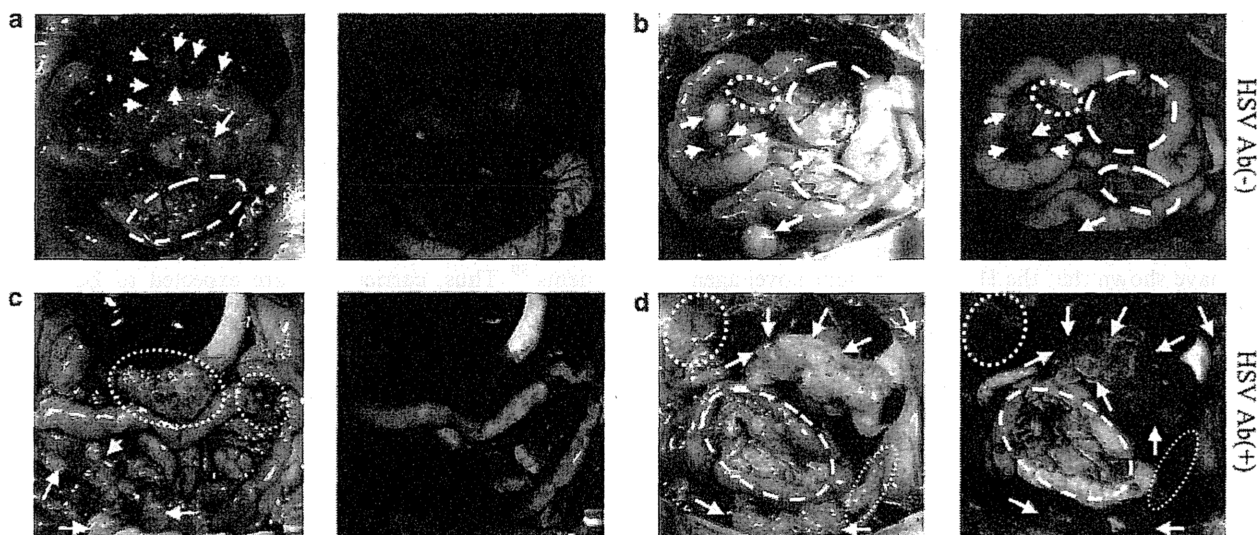

Figure 6 *In vivo* visualization of virally infected cell delivery in the presence of anti-herpes simplex virus type 1 (HSV-1) antiserum. Mice were bearing disseminated SKOV3 ovarian tumors. Mice were randomized into non-treatment group (**a**, **b**) and treatment group with anti-HSV-1 antiserum (**c**, **d**). Intraperitoneal tumor-bearing mice were given phosphate-buffered saline (PBS) (**a**), cell-free HF-green fluorescent protein (GFP) (**b**, **c**) or HF-GFP-infected human omentum mesothelial cells (HomMCs) (**d**) intraperitoneally 1 h after treatment. Representative photographs showing between tumor location and GFP signal. Each picture taken 24 h after viral injections is shown. The yellow arrows and dotted-line circles indicate disseminated ovarian tumor.

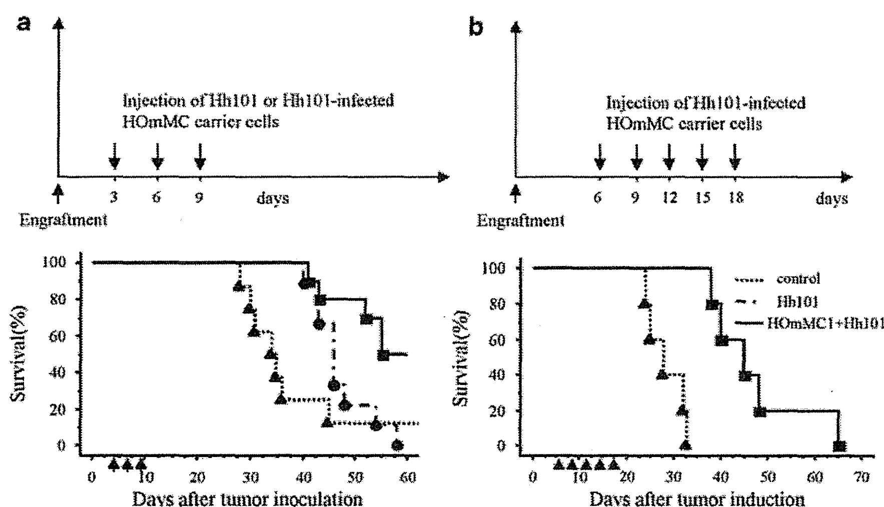

Figure 7 Therapeutic effects of Hh101-infected carrier cells in an ovarian cancer model. Nude mice were engrafted intraperitoneally with SKOV3 cells and (**a**) treatment was started 3 days later using three repeated i.p. injections. Three repeated therapeutic injections of Hh101-infected human omentum mesothelial cells (HOmMCs) improved the survival of ovarian cancer-engrafted nude mice. Injections of Hh101-infected carrier cells were more effective than Hh101 injections alone. Some of the mice treated with Hh101-infected carrier cells survived without symptoms or site injection tumors for >80 days. (**b**) Therapy was started on day 6 with five repeated i.p. injections. Median survival was significantly ($P=0.0018$) prolonged for the group of carrier-cell-treated animals compared with the control group (median survival, 45 days vs 28 days).

tumors in the peritoneal cavity and subsequently died. However, the survival time was extended by treatment with Hh101-infected carrier cells. This resulted in an approximate doubling of the median survival time (45 days; $n=5$) compared with that for control animals receiving PBS alone (28 days; $n=5$; $P<0.01$). Thus, in both experiments the prognosis was significantly improved by treatment with Hh101-infected carrier cells.

Discussion

Genetically engineered, conditionally replicating HSV-1 is a promising therapeutic agent for cancer therapy. The main antitumor mechanism of oncolytic viruses results from viral replication within infected tumor cells, resulting in cell destruction, and liberation of progeny virus particles that can directly infect adjacent tumor cells.[7]

Most clinical trials using oncolytic viral therapy have been performed using direct i.t. injection. However, almost all HSV-1 mutants were not so effective as expected when used clinically as antitumor cytolytic agents.[22–24] In an effort to develop more effective, well-tolerated, novel viral therapeutic agents, we have focused a highly attenuated oncolytic HSV-1 mutant, which lacks four accessory genes (*UL56*, *UL43*, *UL49.5* and *UL55*) and LAT (latency-associated transcript).[17–19] Our previous observation have shown that the HF10 is a potent novel agent for oncolytic therapy that is safe and effective for colon cancer, sarcoma and melanoma treatment in mouse models.[25–27] We have also performed a clinical trial of the treatment of recurrent breast cancer and head and neck cancer using i.t. injection of HF10.[28–30] These results revealed a potent oncolytic effect of HF10 without any side effects in human. Currently HF10 is being tested in the United States for the patients with advanced head and neck cancer in a Phase I clinical trial. Here, we have examined the ability of HF10 to control ovarian cancer in order to apply the HF10 therapy for peritoneally disseminated ovarian cancer. Firstly, we performed i.t. therapy of s.c. xenograft tumors using HF10, and tumor growth was significantly reduced. Moreover, in animals treated with six injections, a complete disappearance of the tumor was observed in some animals.

As a strategy to potentially enhance the delivery of HSV to disseminated tumors and to protect the virus from inhibitory factors (complement, anti-HSV antibodies) in the peritoneal cavity, we and others are exploring the use of carrier cells as Trojan Horses to deliver virus to tumors. The optimal carrier cell should be highly susceptible to HSV infection, not be rapidly killed by the virus, traffic to tumors and transfer the viral infection to the tumor cells via cell-to-cell heterofusion and/or by production of virus progeny.[11,31,32] An assortment of cells have been explored in this regard, including tumor cells,[24,33–35] outgrowth endothelial cells[36] and T cells.[31,37] In this work, we observed an ~10-fold higher amplification of the virus in HOmMCs than SKOV3 cells *in vitro*. Virus replication was the most efficient in HOmMCs, so we decided to use these cells as HSV carrier cells. Next, we estimated the efficacy of tumor killing caused by virus-loaded carrier cells *in vitro*. Our *in vitro* studies clearly demonstrated the efficacy of spread of infection between tumor cells and carrier cells. The transfer and spread of infectivity by MCs derived from the omentum was much higher than infectivity transfer by cell-free viruses. Taken together, these findings suggested that HOmMCs would be suitable for use as carrier cells to treat peritoneally disseminated ovarian cancer. However, the ultimate fear of carrier cells after intraperitoneal inoculation may pose some safety concerns. In this study, we immortalized normal human peritoneal MCs with non-viral human genes (mutant Cdk4, cyclin D1 and hTERT) and utilized as carrier cells. Such as the case, a carcinogenic potential of HOmMCs would not emerge thus far (data not shown). In the previous study,[21] we have developed immortalized ovarian surface epithelium with the same gene sets, and we did not observe any tumorigenesis up to doublings 60. The possibility of clinical application of carrier cells warrants that safety would be ensured.

Ascites frequently accumulate in patients who have tumor spread in the peritoneal cavity, and this fluid is expected to be rich in anti-HSV antibodies because the immunoglobulin G content of ascites fluid is known to reflect that of blood.[38] Also, it has been shown that pre-existing neutralizing antibodies in ascites may prevent initial adenovirus vector delivery in ovarian cancer patients.[39] Thus, carrier cells are expected to be useful not only for systemic virus delivery but also for intraperitoneal administration in patients with peritoneal metastases and pre-existing humoral immune response. We also examined the effect of antibodies against virus delivery by carrier cells. Our *in vitro* data demonstrated that direct cell-to-cell transfer of infectivity by HOmMCs was five to six times more resistant to neutralizing antibodies than infectivity transfer by naked virus. Thus, once infection is successfully transferred to the tumor, it is expected that antibodies will not stop i.t. virus spread. We also showed that HOmMCs infected with HF-GFP could target pre-established ovarian tumor nodules in mice (Figure 6), and this result is consistent with that of measles virus-infected cell carriers.[40] These data support the potential use of HSV oncolytic therapy using carrier cells in humans with pre-existing immunity to HSV.

This study supports the concept that the utilization of carrier cells may have a role in HSV-based oncolytic therapies. Inoculation of HOmMCs infected at MOI 3 or the equal titer of HSV particles should represent comparable viral loads initially administered to the animals. In the SKOV3 model, the carrier cell strategy led to a significant prolongation of animal survival compared with virus alone. Earlier treatment (3 days after engraftment) with infected carrier cells was even more effective. Hh101-infected carrier cells rescued few of the animals, because Hh101 is more attenuated than HF10, which is lethal for immunodeficient mice. If we use HF10 for carrier cell-based therapy in an immunocompetent model, we anticipate that the therapeutic effect would be better and be enhanced. Moreover, the immunogenicity of carrier cells may enhance therapy, as the activation of antitumor immunity during virotherapy appears to contribute to some degree to eliminating tumors and may help to protect from disease. To estimate the role of the immune response in oncolytic viral therapy, we would need to investigate this theory in a syngeneic immunocompetent mouse model of disseminated peritoneal ovarian carcinoma.

In conclusion, we establish that human peritoneal MCs are useful for carrier cells of oncolytic HSV in treating peritoneally disseminated ovarian cancer. Infected MCs and HOmMCs have the unique ability to produce a burst of virus upon delivery to the tumor site. In addition, this strategy allowed oncolytic HSV to escape neutralization by antibodies and complement, and subsequently to transfer the virus to tumor cells by *in situ* cell fusion. These findings may have significant implications for oncolytic virotherapy for ovarian cancer.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This study was supported, in part, by a research grant (number 21592128) from the Ministry of Education, Science and Culture of Japan.

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