The p53 Protein Does Not Facilitate Adenovirus Type 5 Replication in Normal Human Cells

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Although several adenovirus type 5 (Ad5) proteins prevent deleterious consequences of activation of p53, it has been reported that viral replication proceeds more efficiently when human tumor cells produce wild-type compared to mutant p53. We have now exploited RNA interference and lentiviral vectors to achieve essentially complete knockdown of p53 in normal human cells: no effects on the kinetics or efficiency of viral gene expression or production of infectious particles were observed.

Infection with human species C adenoviruses, such as adenovirus type 5 (Ad5), leads to the accumulation of viral gene products that modulate the activity of the master regulator of the cellular response to genotoxic stress, p53. The immediate early E1A proteins induce activation and stabilization of p53, resulting in a transient increase in p53 concentration in numerous cell types (1–5). Potential antiviral effects of p53 activity, such as induction of growth arrest or apoptosis, are precluded by several viral early gene products: the E1B 55-kDa and E4 Orf6 proteins and several cellular proteins form an E3 ubiquitin ligase that targets p53 for proteasomal degradation (6–8), the E1B 19-kDa protein blocks apoptosis downstream of p53 (9; reviewed in reference 10), and the E4 Orf3 protein can prevent activation of transcription of p53-dependent genes (11).

Paradoxically, despite these various mechanisms to preclude or circumvent deleterious consequences of p53 activation, there have been reports that functional p53 facilitates adenovirus replication. For example, tumor cell lines that synthesize a mutant p53 (p53mut) were observed to support Ad5 DNA replication and induction of cytopathic effect (CPE) and cell death less efficiently than lines producing wild-type p53 (p53wt) (12–14). The same trend was observed in isogenic p53wt and p53mut HT1080 (13) and K1 (15) cells, and introduction of a dominant-negative, mutant form of murine p53 rendered cells resistant to Ad5-induced death (14). Furthermore, viral late gene expression and yield were reduced in the p53-defective K1 cell line relative to the p53wt line, and ectopic wild-type p53 expression rescued these defects. In contrast to these findings, host cell p53 status was not observed to have an effect on Ad5 replication and appearance of CPE when infections of p53wt (W158 and HNK [human natural killer]) and p53-null (SK-OV-3 and H1299) cell lines were compared (16). Interpretation of many such studies is complicated by the use of nonisogenic, tumor-derived cell lines that carry numerous, non-equivalent genetic alterations affecting stress responses, cell cycle regulation, and metabolism (17). Furthermore, generation of cells devoid of functional p53 and matched to isogenic parental cells by overproduction of dominant-negative mutant p53 would not be expected to simulate accurately the absence of p53 from the virus’s natural host cells.

In order to avoid such complications, and to evaluate the contribution of p53 to Ad5 replication in a physiologically relevant context, we exploited RNA interference and a lentiviral vector to prevent p53 synthesis in primary normal human bronchial epithelial cells (NHBECs) and human foreskin fibroblasts (HFFs).

![FIG 1 Effect of p53 knockdown on Ad5 protein synthesis and replication in NHBECs.](http://jvi.asm.org/...)

A) NHBECs were transduced with lentiviral vectors for production of control (C) or p53-specific (p53) shRNAs and puromycin selected. The steady-state concentrations of p53 and β-actin were examined by immunoblotting. B) Control (lane C) and p53-knockdown (p53) cells were infected with 5 PFU/cell Ad5 for the periods indicated, and the viral E2 DBP and protein V were examined by immunoblotting. Untransduced cells were mock infected (M) as a negative control. C) Viral yields 24 h after infection with 5 PFU/cell Ad5 were determined by plaque assay on 293 cells. The averages of two independent experiments and the combined propagated standard deviations (error bars) are shown.
Lentivirus vectors were generated by ligating a short hairpin RNA (shRNA) construct specific to p53 (targeting the sequence GACTCCAGTGGTAATCTAC) or an shRNA sequence targeting no known human genes (Sigma SCH002; CAACAAGATGAAGACACCAA) into an AgeI/EcoRI-digested pLKO.1 lentiviral expression vector (Sigma) carrying a puromycin resistance gene. Replication-defective lentiviruses were produced by introduction of pLKO.1-shRNA constructs with packaging and envelope vectors (18) into 293FT cells (19, 20). Medium was collected 72 h later and filtered through a 0.45-μm syringe filter (Millipore). Virus particles were purified by centrifugation through a sucrose cushion (21) and resuspended in 20 mM HEPES-NaOH (pH 7.5) containing 0.135 M NaCl, 4 mM KCl, 2 mM CaCl₂, and 1 mM Na₂HPO₄.

NHBECs, which closely resemble the host cell type infected by adenoviruses during natural infection of the upper respiratory tract (22), were cultured as described previously (23). These cells were transduced with purified lentivirus particles and selected by culture in medium supplemented with 1 μg/ml puromycin. Intracellular p53 concentrations were assessed by immunoblotting, using β-actin as the internal loading control, as described previously (24). As illustrated in Fig. 1A, p53 was present in cells producing the control shRNA, but could not be detected in p53 shRNA-transduced cells, even when blots were overexposed, indicating complete knockdown. Despite the absence of detectable quantities of p53, NHBEC-p53 cells exhibited no reduction in growth rate compared to parental or control shRNA-expressing NHBECs or alterations in morphology. No major differences in the kinetics of accumulation of these viral proteins were observed in the presence and absence of p53 (Fig. 1B). Quantification of such signals by using ImageJ (http://imagej.nih.gov/ij/) indicated that by 24 h postinfection (p.i.), the concentration of the DBP in p53 knockdown cells was 80% of that attained in control cells, but the concentrations of protein V were identical. Furthermore, when cells were harvested 24 h after infection and viral yields were measured by plaque assay on 293 cells (25), viral yield was reduced by less than 1.4-fold in p53 knockdown cells compared to control cells (Fig. 1C). These observations indicate that endogenous p53, which accumulates transiently after infection (Fig. 2A), has no impact on the kinetics or efficiency of Ad5 replication in NHBECs.

The viral life cycle progresses rapidly in NHBECs, but in another primary cell type, HFFs, viral early protein synthesis is delayed significantly (26, 27), and transiently stabilized p53 is present for a greater period prior to decreasing in concentration (Fig. 2B). Such delay in viral gene expression would be expected to permit extensive activation of host responses to viral infection. Supporting this model, the early E1B 55-kDa protein, which inhibits innate immune responses to Ad5 infection (23, 28), is necessary for efficient progression into the late phase in HFFs, specifically when expression of immediate early and early genes is delayed (26). We therefore reasoned that, as a result of the lag in synthesis of viral proteins that inhibit p53, p53 knockdown in HFFs might have a more significant impact on viral replication than in NHBECs. HFFs were therefore transduced with filtered lentiviral particles, and following puromycin selection as described above, the efficiency of p53 knockdown was evaluated by immunoblotting. The concentration of p53 in cells transduced with p53-specific shRNA was less than 3% of that observed in control-transduced cells, (Fig. 3A), as measured by densitometry using ImageJ. These cells, which are poorly infectible (27), were infected with 30 PFU/cell of the phenotypically wild-type Ad5

![FIG 2](https://jvi.asm.org/figure/2)

**FIG 2** Transient stabilization of p53 by Ad5 infection. NHBECs (A) or HFFs (B) were infected for the indicated periods with 5 PFU/cell or 30 PFU/cell Ad5, respectively, or mock infected (M), and p53, E1A, and β-actin proteins were examined by immunoblotting. Note the different time scales in the two experiments.

![FIG 3](https://jvi.asm.org/figure/3)

**FIG 3** Effect of p53 knockdown on Ad5 late protein synthesis in HFFs. (A) HFFs were transduced with lentiviral vectors for production of control (C) or p53-specific (p53) shRNA and puromycin selected, and the steady-state concentrations of p53 and β-actin were examined by immunoblotting. (B) Control and p53-knockdown (p53) HFFs were infected with 30 PFU/cell AdEasyE1 (wild type [WT]) or AdEasyE1Δ2347 (∆E1B) for the periods indicated, and the viral proteins DBP and V were examined by immunoblotting.
derivative, AdEasyE1 (24), and intracellular DBP and protein V concentrations were measured 56 and 72 h after infection as described above. Again, no significant difference in the accumulation of these viral proteins was observed in control compared to p53-knockdown cells (Fig. 3B), indicating that progression into the late phase is unaffected by the absence of p53. Interestingly, the same results were observed in cells infected by the E1B 55-kDa null virus AdEasyE1ΔΔ2347 (24): in agreement with previous observations (27), synthesis of late protein V was impaired in the absence of the E1B protein, but knockdown of p53 had no effect on accumulation of this protein or the DBP (Fig. 3B).

In toto, these data establish that p53 is not required for maximally efficient viral gene expression or replication in normal human epithelial cells or fibroblasts infected with low multiplicities of Ad5. The direct comparisons between cells that differ only in whether p53 is present circumvented both the unknown (and potentially large number of) variables inherent when cell lines that were lacking p53 were compared to the wild type. The direct comparisons between cells that differ only in whether p53 is present also allowed us to avoid the potentially large number of variables inherent when cell lines that were lacking p53 were compared to the wild type. The direct comparisons between cells that differ only in whether p53 is present circumvented both the unknown (and potentially large number of) variables inherent when cell lines that were lacking p53 were compared to the wild type. The direct comparisons between cells that differ only in whether p53 is present also allowed us to avoid the unknown impact on viral replication of overproduction of dominant-negative forms of p53 (12–14). It is likely that these limitations account for the discrepancies between our observations and others (27), synthesis of late protein V was impaired in the absence of the E1B 55kDa protein in the adenovirus type 5 infectious cycle. Virology 203:229–240.


