Overexpression of the Cellular DEK Protein Promotes Epithelial Transformation In vitro and In vivo


Abstract

High levels of expression of the human DEK gene have been correlated with numerous human malignancies. Intracellular DEK functions have been described in vitro and include DNA supercoiling, DNA replication, RNA splicing, and transcription. We have shown that DEK also suppresses cellular senescence, apoptosis, and differentiation, thus promoting cell growth and survival in monolayer and organotypic epithelial raft models. Such functions are likely to contribute to cancer, but direct evidence to implicate DEK as an oncogene has remained elusive. Here, we show that in line with an early role in tumorigenesis, murine papilloma formation in a classical chemical carcinogenesis model was reduced in DEK knockout mice. Additionally, human papillomavirus E6/E7, hRas, and DEK cooperated in the transformation of keratinocytes in soft agar and xenograft establishment, thus also implicating DEK in tumor promotion at later stages. Finally, adenoviral DEK depletion via short hairpin RNA expression resulted in cell death in human tumor cells and in vivo, but did not significantly affect differentiated epithelial cells. Taken together, our data uncover oncogenic DEK activities as postulated from its frequent up-regulation in human malignancies, and suggest that the targeted suppression of DEK may become a strategic approach to the treatment of cancer. [Cancer Res 2009;69(5):1792–9]

Introduction

The human DEK protein was originally identified as a fusion with the CAN/NUP214 nucleoporin in a subset of patients with acute myeloid leukemia (AML; ref. 1). DEK was subsequently reported as a gene that is frequently up-regulated in aggressive human tumors such as glioblastoma, melanoma, and bladder carcinoma (2–4). DEK is an up-regulated target of the human papillomavirus (HPV) E7 oncogene in vitro (5), as well as in HPV E7 transgenic mouse epithelium and in human cervical cancer biopsies in vivo (6, 7). These studies had implicated retinoblastoma family members in the regulation of DEK, and transcriptional regulation of DEK through E2F1/E2F2 was later reported (4). Because retinoblastoma tumor suppressor pathways are inactivated in most human malignancies, E2F-mediated expression is likely one relevant mechanism that drives DEK overexpression. In fact, DEK has been found to be highly up-regulated in retinoblastoma and small cell lung cancers, both of which are strongly associated with retinoblastoma loss (refs. 8–11; Supplementary Fig. S1A).

A number of intracellular DEK activities have been studied extensively in vitro, and DEK was independently discovered as a protein that modulates the topology of SV40 minichromosomes (12). DEK has been proposed to function in replication (12), positive and negative regulation of transcription (13–19), as well as mRNA processing (20–22). DEK likely modulates DNA architecture with a high affinity for chromatin (23), and perhaps as a related property, DEK has recently been implicated in DNA damage responses (24). DEK inhibits senescence and apoptosis, at least in the latter case via the destabilization of p53 (5, 25, 26). The above functions, either individually or in combination, may well contribute to procarcinogenic DEK functions. DEK overexpression correlates strongly with carcinogenesis and although the above intracellular functions of DEK hint at a role in tumorigenesis, the question of whether DEK is indeed an oncogene has not been directly addressed. Based on Oncomine data and tumor cell line analyses, we report that DEK expression is transcriptionally up-regulated in a wide variety of human tumors. This up-regulation is not merely correlated as DEK overexpression in normal immortalized keratinocytes (NIK) cooperated with hRas, HPV E6 and E7 for anchorage-independent growth and tumorigenesis in nude mice. In order to determine whether DEK was important for tumor formation in a murine epithelial cancer model, we subjected DEK knockout and control mice to a two-step 9,10-dimethyl-1,2-benzanthracencene (DMBA)/12-O-tetradecanoyl-phorbol acetate (TPA) protocol. Papilloma formation was significantly decreased in DEK knockout mice compared with wild-type animals and heterozygote controls. Importantly, we show here that differentiated cells are almost completely resistant to DEK depletion compared with their undifferentiated counterparts. Our data is the first to show oncogenic DEK activities according to classical variables, and strengthens the notion that targeting DEK may be a feasible approach for the treatment of cancer.

Materials and Methods

Cell culture. U2OS human osteosarcoma cells were maintained in DMEM (Sigma) supplemented with 10% fetal bovine serum (FBS) and antibiotics. NIKs were maintained on irradiated murine J2 3T3 feeder fibroblasts as described previously (27). Primary human foreskin keratinocytes (HFK) were prepared from human foreskins (26) and maintained in Epilife Medium (Cascade Biologics) with antibiotics. For the differentiation of HFKs, cells were overlaid with Epilife containing 10% FBS and 1 μmol/L of CaCl2.

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

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Plasmids and viral constructs. The Dek open reading frame was amplified using the forward primer F-(5’ ATGTCGCCCTCGGCC 3’) and the reverse primer R-(5’ TCAAGAATTCCTTCTTACG 3’). The cDNA was cloned into the pGEM-T easy vector (Promega). The Dek cassette was sequenced, digested with NotI and cloned into a NotI-digested FMEV-type vector pSF91-I-gGFP-PRE (R780) obtained from the Baum laboratory (28). FMEV retroviral vectors combine the long terminal repeat of Friend mink cell focus-forming virus with the 5’ untranslated leader region of the murine embryonic stem cell virus for optimal transgene expression. Vector particles were generated in the Viral Vector Core facility at the Cincinnati Children’s Hospital Medical Center, and were pseudotyped with the feline endogenous virus (RBD14) envelope protein. The pBABE-IRas vector encoding oncogenic H-RasV12 was a generous gift from Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The empty Ad as well as the AdDEKsh vector were described previously (26). Producer cell lines for empty LXSN retrovirus as well as retroviruses expressing HPV16 E6, HPV16 E7, and HPV16 E6/E7, respectively, were a generous gift from Dr. Denise Galloway, University of Washington, Seattle, WA.

Adenoviral and retroviral infections. For adenoviral infections, the cells were washed with PBS and infected with the indicated number of infectious units per cell of adenovirus stock in PBS containing 4% FBS for 1 h as described previously (26). Virus was then aspirated and the cells were washed twice with PBS and overlaid with fresh medium. For retroviral infections, NIHs were transduced with 4 ml of R780 or R780-DEK retroviral supernatant containing 8 μg/ml of polybrene. The cells were washed with PBS after 3 h, and overlaid with fresh medium. Cell pools were sorted for green fluorescent protein (GFP) expression using a fluorescence-activated cell sorter (FACS Vantage SE DiVa; Becton Dickinson). Cells were collected and maintained on plastic or feeder cells (for NIHs) as above. Cells infected with puromycin or neomycin-resistant vectors were selected in 1 μg/ml of puromycin or 650 μg/ml (for NIHs) or 200 μg/ml (for HFKs) of G418, respectively.

Western blot analyses. Western blot analyses were performed as described previously (26). Membranes were probed with either the DEK monoclonal antibody (BD Biosciences), a p53 (Ab-6) monoclonal antibody (Calbiotech), a cyclin A polyclonal antibody (Santa Cruz Biotechnology), proliferating cell nuclear antigen antibody (BD Biosciences), p21 monoclonal antibody (Calbiotech), or an actin-specific monoclonal antibody, a generous gift from James Lessard (Division of Developmental Biology, Cincinnati Children’s Hospital Medical Center, Cincinnati, OH).

Soft agar assays. A total of 5 × 10⁴ NIHs were submersed in 0.4% agarose in F medium and plated on a 0.8% agarose/F medium overlay. Cells were overlaid with 0.4% agarose/F medium twice a week for 4 weeks. Colonies were quantified and pictures were taken at the end of the experiment.

Aptoptosis analysis by flow cytometry. Cells were infected with either empty Ad or AdDEKsh. Cells were harvested after trypsinization on the indicated days, washed in cold PBS, and fixed with BD cytofix/cytoperm (BD PharMingen) for 20 min at room temperature. The cells were then washed with BD perm/wash buffer twice and 20 μL/1 × 10⁶ cells were incubated with biotinylated anti–active caspase 3 antibody (BD PharMingen) for 1 h at room temperature in the dark. Cells were washed and incubated with 5 ng per 1 × 10⁶ cells of streptavidin-APC (BD PharMingen) for 30 min at room temperature. The cells were washed and analyzed using BD CellQuest software on a Flow Cytometer (BD Biosciences).

Immunofluorescence microscopy. Sections were deparaffinized in xylene and rehydrated. Antigen retrieval was done by heating the sections in 10 mmol/L sodium citrate (pH 6.0), in a rice cooker for 20 min and allowed to cool to room temperature. Terminal nucleotidyl transferase–mediated nick end label staining was performed with the Cell Death Detection Kit (Roche). Sections were washed with PBS, counterstained with DAPI Vector Vectashield mounting medium (Vector Laboratories, Inc.), and coverslipped. Immunofluorescence detection was via a Zeiss fluorescence microscope (Zeiss) and images were captured using 20× and 40× magnification with an Axiovision camera (Lucas Microscope Service) driven by Axiovision software.

DMBA/TPA murine skin carcinogenesis. A schematic of the targeting construct is shown in Fig. 1A. For the generation of a Dek knockout mouse, an 8.6 kb Sall-BamHI fragment from a 129 Sv plasmid vector was excised and cloned into the pZip B vector, and stably transfected into NIH 3T3 cells, containing the 5’ half of the Dek open reading frame, was subcloned into pBlueScript. A 5 kb IRES-LacZ-Neo selectable marker (29) was inserted into the NsiI site in Dek exon 6 (Dek codon 184). The targeting vector was linearized with Sau3A and electroporated into E14 mouse ES cells. G418-resistant colonies were isolated and homologous recombinants were identified by using Southern blot analysis of EcoRI-digested DNA, probed with a 5’ external 320 bp EcoRI-BamHI fragment. The wild-type (9.5 kb) and mutant (8.4 kb) Dek fragments were easily distinguished (data not shown) and seven independent clones with normal karyotypes were identified, four of which were microinjected into C57Black/6 blastocysts. All clones produced chimeric offspring, which gave germ line transmission of the targeted allele. Dek−/− mice were healthy and bred normally. Bone marrow of Dek−/− mice did not express DEK protein as determined by Western blot analysis using a rabbit polyclonal antibody (ref. 12; data not shown). All use and handling of mice were carried out in the American Association for Accreditation of Laboratory Animal Care–approved Cincinnati Children’s Hospital Veterinary Care Facility according to an Institutional Animal Care and Use Committee–approved protocol to S.I. Wells. For genotyping, primer pair A (5’-CGA ACT CGT GGA GAG CAT GTT GA-3’, 5’-ATG TGT CAG GCT GCA TCT CCA ATG-3’) was used for the amplification of the wild-type allele, primer pair B (5’-ATC CAT CAG GTC TGA TGT GCC-3’, 5’-TGG AAG GTA AGT GCC CCT TA-3’) was used for the amplification of the knockout allele. Mice were subjected to a two-step carcinogenesis model adapted from ref. 30. Briefly, 100 μg of DMBA (Sigma) in 100 μL of acetone was applied to the shaved flank of mice once at the age of 2 to 3 months. One week after DMBA application, 30 μg of the tumor promoter TPA in 100 μL of acetone was applied twice a week for 20 weeks. Papilloma counts were performed once a week and recorded. Mice were harvested for analysis after 20 weeks of TPA treatment.

Adenoviral injections into established tumors. For tumorigens studies using an Institutional Animal Care and Use Committee–approved protocol, the flanks of 4- to 8-week-old female athymic nude mice (Harlan Laboratories) were injected with 3 × 10⁵ cells. NIHs containing IRas vector, and either empty R780 backbone or the R780-DEK vector, together with either HPV16 E6, HPV16 E7, and HPV16 E6/E7 were used for the injections. Tumor growth was monitored for 3 months, at which time, tumors were harvested, fixed in 4% paraformaldehyde overnight, washed in PBS, and subsequently dehydrated in a series of alcohols. Tumors were embedded in paraffin, sectioned for analysis using a rabbit polyclonal antibody (ref. 12; data not shown). All use and handling of mice were carried out in the American Association for Accreditation of Laboratory Animal Care–approved Cincinnati Children’s Hospital Veterinary Care Facility according to an Institutional Animal Care and Use Committee–approved protocol to S.I. Wells. For genotyping, primer pair A (5’-CGA ACT CGT GGA GAG CAT GTT GA-3’, 5’-ATG TGT CAG GCT GCA TCT CCA ATG-3’) was used for the amplification of the wild-type allele, primer pair B (5’-ATC CAT CAG GTC TGA TGT GCC-3’, 5’-TGG AAG GTA AGT GCC CCT TA-3’) was used for the amplification of the knockout allele. Mice were subjected to a two-step carcinogenesis model adapted from ref. 30. Briefly, 100 μg of DMBA (Sigma) in 100 μL of acetone was applied to the shaved flank of mice once at the age of 2 to 3 months. One week after DMBA application, 30 μg of the tumor promoter TPA in 100 μL of acetone was applied twice a week for 20 weeks. Papilloma counts were performed once a week and recorded. Tumors were harvested for analysis after 20 weeks of TPA treatment.

Results

The human DEK proto-oncogene is up-regulated in many human cancers. Using the Oncomine research platform, we identified a large number of human malignancies in which DEK was significantly overexpressed (Supplementary Fig. S1A). Interestingly, the degree of DEK up-regulation often correlated with the severity of prognosis as indicated by histopathologic determination of a higher stage and grade, or poor differentiation characteristics (data not shown). For several tumor types including glioblastoma and cervical cancer, DEK up-regulation has already been emphasized in the literature (2, 3). Whereas DEK was highly overexpressed in most malignancies, its expression levels were decreased in prostate cancers, and in a subset of ovarian and adult bone marrow–associated cancers. Decreased DEK expression in AML as

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observed here (Fig. 1A, see GEO web site\(^5\)) contrasts with a previous report by the Knuutila laboratory (31) describing increased DEK expression. Interestingly, a striking difference between the two reports is patient age: DEK down-regulation was observed in pediatric AML, whereas DEK up-regulation was observed in adult AML. Additional experiments are needed to determine whether these differences in DEK expression indeed distinguished pediatric from adult AML, perhaps even contributing to their known biological differences. Regardless, the expression data indicate that high DEK expression is not universally expected for all tumor types. DEK was overexpressed in cervical cancer and head and neck cancers, both of which have been correlated with HPV infection. This association is in agreement with recent data in which DEK was highly expressed in HPV-positive cervical lesions compared with controls (6, 7). As shown in Supplementary Fig. S1B, Western blot analysis of a panel of epithelial primary and cancer cells showed that DEK protein is up-regulated in the majority of human cancer cell lines compared with primary keratinocytes and fibroblasts (compare lanes 4–13 to lanes 1–3). DEK expression seemed to be especially high in cervical cancer cell lines (lanes 4–8) as well as in osteosarcoma cells (lanes 9 and 10). DEK expression was also strongly increased in 293 cells expressing


Figure 1. Dek expression is important for the formation of papillomas in vivo. A, targeting of the mouse Dek gene. Top line, the 5' half of the Dek gene comprising exons 1 to 6. Arrow, the direction of transcription. The NsiI site (N) in exon 6 is indicated as well as the EcoRI sites (E) used for Southern blotting. Middle line, the targeting construct containing 8.6 kb of genomic DNA with the IRES-LacZ-Neo selectable marker inserted into the NsiI site in exon 6. Bottom line, the targeted allele. The 320 bp EcoRI-BamHI fragment used as a probe (P) for Southern blotting. B, DMBA/TPA carcinogenesis model. Dek wild-type vs. heterozygote and knockout littermates (three/group) were subjected to 100 \( \mu \)g of DMBA on the flank, and starting at 1 wk post-tumor initiation, 30 \( \mu \)g of the tumor promoter TPA was applied twice a week for 20 wks. The graph represents the number of papillomas per mouse that were present at the indicated time points. * \( P < 0.05 \) between the knockout and heterozygous mice. Bottom, H&E staining depicts the morphology of a representative papilloma (magnification, \( \times \)50; inset, magnification, \( \times \)200). Different genotypes developed papillomas that were morphologically similar. C, Western blot analysis. Primary keratinocytes from the heterozygous and knockout Dek mice were harvested for the isolation of protein, and Western blot analysis for DEK and actin was carried out. Primary murine keratinocytes were prepared from the skin of newborn Dek heterozygous and knockout mice, floated overnight at 4°C in 0.25% trypsin/EDTA. On the next day, the epidermis was separated from the dermis, minced, filtered, and keratinocytes were plated in defined keratinocyte serum-free medium supplemented with FBS and antibiotics. After 7 d, protein was harvested for Western blot analysis. Bands shown were imaged from the same exposed film. Immunohistochemistry on normal, untreated skin of wild-type, heterozygote, and knockout Dek mice confirms DEK loss in the knockout mice (magnification, \( \times \)1,000). Tissue sections were deparaffinized, rehydrated, subjected to antigen retrieval and processed. Sections were incubated with IgG1 antibody as a control, or with DEK antibody. Sections were washed and incubated in ABC reagent for 30 min, washed again, and incubated with 3,3-diaminobenzidine. Sections were then washed and stained in Nuclear Fast Red prior to mounting and visualization.
the adenoviral E1A protein. E1A, like E7, is known for the potent inhibition of retinoblastoma protein family members. Taken together, these data suggested that DEK is up-regulated in many human malignancies and that it may promote multiple stages of tumor progression.

**DEK is important for papilloma formation in vivo.** Because these and other published data suggested an oncogenic role for DEK, we used a new Dek knockout mouse model to investigate a role for this molecule in the early stages of skin tumorigenesis in vivo. Generation of the Dek knockout mice is described in detail in Materials and Methods, and a schematic of the targeting construct is shown in Fig. IA. A classical two-step epithelial carcinogenesis model was used (30), which consists of a one-time application of DMBA for initiation, followed by TPA promotion twice a week for 20 weeks. We used littermate Dek knockout, heterozygous, and wild-type mice for these experiments. As depicted in Fig. 1B, there was a significant delay in the formation of papillomas in Dek knockout mice compared with wild-type and heterozygous mice. Papillomas ultimately formed in the Dek knockout mice, suggesting a role for DEK in tumor initiation in this model. DEK knockout in primary murine keratinocytes was confirmed by Western blot analysis and by immunohistochemistry on normal, untreated mouse skin (Fig. 1C).

**DEK overexpression promotes transformation in vitro and in vivo.** The finding that DEK supported papilloma formation in vivo suggested but did not prove that it functions as an oncogene. In order to probe such transforming potential directly, we performed soft agar colony assays using normal human keratinocytes (NIKs) that stably expressed oncogenic hRas and HPV16 E6/E7 in the presence or absence of overexpressed DEK (Fig. 2B). NIKs are spontaneously immortalized cells, which are carried on feeder cells and have retained responsiveness to differentiation in organotypic raft culture (32). DEK overexpression dramatically increased colony formation, indicating transforming potential. Similar DEK cooperation was observed in NIKs which were transfected with the full-length, E6/E7-expressing HPV16 genome (data not shown), and subsequently selected to yield a pure population of HPV-positive cells. E7-induced p53 and p21 protein levels were suppressed by the coexpression of HPV E6, suggesting that DEK can promote transformation through p53-independent mechanisms (Fig. 2A).

We next investigated the ability of DEK to promote transformation in vivo. NIKs transduced with hRas, HPV E6 alone, HPV E7 alone, or in combination with E6, and either the R780 empty vector or R780-DEK, were injected into the left and right flank of nude mice, respectively. Tumor formation was monitored for 2 months. No tumors formed in the presence of HPV E6 or HPV E7 alone. However, DEK significantly increased the rate of tumor formation in the HPV E6/E7–expressing tumors from 25% to 75% (Fig. 3A). Tumors that formed from cells transduced with empty R780 vector or R780-DEK–expressing virus were morphologically confirmed to be squamous cell carcinomas; however, DEK-overexpressing tumors were significantly larger than tumors arising from vector-treated control cells (Fig. 3B and C). Moreover, the mitotic index was increased in DEK-expressing tumors compared with controls as assessed by phosphorylated histone H3 expression (Fig. 3D). Collectively, these data show that E6 or E7 is insufficient for inducing NIKs to form tumors, even in the presence of oncogenic ras. In contrast, combined E6 and E7 expression cooperates with ras in inducing tumorigenesis. Furthermore, the incidence and size of E6/E7-induced tumors is increased by DEK overexpression, providing evidence that DEK cooperates with other oncogenes in tumor initiation and progression.

Adenoviral delivery of DEK short hairpin RNA results in osteosarcoma cell death in vivo. We have shown previously that DEK-specific RNA interference results in cell death in vitro in tumor cell lines, and to a lesser extent, in primary cells (26). Although DEK stimulated tumor growth in xenograft experiments (Fig. 3) and was important for murine papilloma formation in the DMBA/TPA model (Fig. 1), these experiments did not address whether sustained DEK expression was important for tumor maintenance in vivo. We therefore investigated whether DEK depletion might result in tumor cell death. Equal numbers of U2OS osteosarcoma cells were injected into both flanks of athymic nude mice. Tumors were allowed to grow to 200 mm³ and then injected with GFP-expressing replication-deficient empty Ad or AdDEKsh virus using an established fractionated injection
protocol at three different time points (33). The tumors were harvested 1 day after the final viral injection for analysis. After the first injection, GFP expression in the tumors was detectable by bioluminescence whole animal imaging (Fig. 4A), demonstrating that both tumors had received GFP-expressing adenovirus. GFP expression remained detectable even after tumor excision using a dissecting fluorescence microscope (data not shown). After image capture, the tumors were fixed and embedded for analysis. Terminal nucleotidyl transferase–mediated nick end label staining revealed dramatic induction of apoptosis in AdDEKsh-infected compared with Ad control–infected tumors (Fig. 4B). Because these viruses are replication-deficient, thus only allowing for transient DEKsh expression, total tumor growth was not substantially different between the control and DEK-depleted state. Whether uniform DEK depletion will cause tumor regression will therefore await the development of more efficient delivery methods. Nonetheless, our data provide proof of concept that DEK depletion results in cell death in vivo and that the targeting of DEK in human tumors may therefore be a feasible approach to mediate disease regression.

Epithelial differentiation rescues DEK-depleted cells from apoptosis. Previous data showed that DEK overexpression confers protection from apoptosis, and conversely, that acute DEK depletion by RNA interference results in apoptosis in p53-competent cancer cells, and to a lesser extent, in primary cells (26). Together with the above detection of apoptosis following DEK depletion in vivo (Fig. 4), this suggested that DEK targeting strategies may be therapeutically useful, but also that toxicity may be expected from such cancer treatments. To evaluate the degree of toxicity, and considering that differentiated cells comprise the majority of the epithelium, we next asked whether differentiated cells might be protected from cell death. As reported previously, primary keratinocytes were susceptible to a degree of DEK RNAi–associated apoptosis, albeit not to the level of U2OS osteosarcoma cells (Fig. 5A and B). We infected primary keratinocytes with either empty Ad or AdDEKsh and subjected the cells to regular growth medium or to medium containing 1 mmol/L of calcium and 10% FBS in order to induce differentiation (34). Differentiation was verified morphologically and reflected the typical tightening of cell-cell interactions (Fig. 5B). As reported previously, DEK

Figure 3. DEK overexpression stimulates transformation in vivo in cooperation with the HPV oncoproteins and oncogenic ras. A and B, nude mouse injections. NIKs (5 × 10⁶) transduced with hRas, either E7 or E6/E7 and either empty R780 or R780-DEK vector were injected into the flanks of athymic nude mice. The R780 controls were injected into the left flank whereas DEK-overexpressing cells were injected on the right flank of each mouse. Tumor formation (A) as well as tumor volume (B) were monitored over 2 mos. Tumor volume was calculated using the following formula: length × width² × π/6. *, P < 0.05; **, P < 0.01. Experiments containing three to four mice per group were independently performed thrice. C, tumor morphology in the presence and absence of overexpressed DEK. Tumors from A were fixed, embedded in parafin, and sectioned. Sections were then stained with H&E and pictures were taken at 50-fold (top) and 200-fold (bottom) magnification. D, immunohistochemistry for phosphorylated (Ser¹⁰) histone H3 was performed on paraffin sections that were baked, deparaffinized, rehydrated, and subjected to antigen retrieval. Sections were blocked using goat antiserum in PBS. Primary phosphorylated (Ser¹⁰) histone H3 1:1,000 (U.S. Biological) antibodies were diluted in blocking solution, applied to tissue sections, and incubated overnight at 4°C. Antibody staining was detected with Vectastain Elite ABC and DAB substrate kits (Vector Laboratories). Counts represent the evaluation of 300 tumor cells representing two to five tumor sections per mouse and three mice per group. *, P < 0.05, and pictures were taken at 1,000-fold magnification.
DEK Is an Oncogene

The human DEK gene is widely referred to as a proto-oncogene in the literature because of its involvement in a chromosomal translocation in AML as well as its up-regulated expression in multiple human malignancies. Together with reported intracellular DEK activities that inhibit senescence (5, 25), apoptosis (26), and cellular differentiation (6), oncogenic DEK activities have been suspected but never formally shown. Because Dek knockout mice were relatively resistant to the formation of benign papillomas (Fig. 1), we suggest that DEK is an active contributor at early tumor stages. To directly assess such putative activities, we used NIKs to show that DEK (a) contributes to human tumor formation, (b) cooperates with known oncogenes in transformation, and (c) is required for the growth of primary proliferating, but not differentiated human keratinocytes. We chose NIKs for these studies because they are a widely used model for keratinocyte differentiation in two-dimensional and three-dimensional skin models. This spontaneously immortalized human keratinocyte cell line exhibits a near-diploid, stable karyotype, wild-type p53, normal responses to squamous differentiation in three-dimensional organotypic rafts models and is nontumorigenic in athymic mice (36).

The cooperating high-risk HPV E6 and HPV E7 oncogenes exhibit well-documented cellular immortalization and transformation properties (37). HPV E7 alone can support the immortalization of primary keratinocytes under certain circumstances, and coexpression of E6 greatly facilitates the process. E7 proteins bind to and degrade cellular retinoblastoma family members, thus stimulating cellular proliferation (38, 39). Inappropriate proliferation in response to E7 expression is met by cellular defense mechanisms that at least in part involve up-regulated p53 levels, thus predisposing cells to cell death and differentiation (40, 41). Coexpression of the high-risk E6 proteins counteracts this reported trophic sentinel response through ubiquitin-mediated degradation of p53 (42, 43).

Given the presence of E6 in our transformation experiments (Figs. 2 and 3), an additional, and perhaps p53-unrelated role emerged for DEK. The HPV oncogenes alone were not sufficient for depletion caused a 3% to 5% increase in apoptosis in the undifferentiated HFK population, compared with 15% apoptosis in osteosarcoma cells on day 3 post-infection (Fig. 5A). However, there was only a 1% gain in apoptosis following DEK depletion in the differentiated HFKs, and the observed increase between Ad and AdDEKsh-infected differentiated cells did not increase to the level of statistical significance (Fig. 5B). The expected decrease in the levels of DEK protein was confirmed by Western blot analysis in both populations (Fig. 5B). Differentiation itself suppressed DEK protein expression slightly (compare lanes 1 and 3), even though the precise timing of DEK suppression was often donor-dependent (data not shown). This repression occurs presumably through the up-regulation of retinoblastoma protein function during this process. It is possible that DEK overexpression in cancer renders cells more dependent on DEK, thus supporting new cancer treatments via the targeting of DEK. Detection of the cyclin/cdk kinase inhibitor p21 in DEK-positive and DEK-depleted keratinocytes revealed up-regulation of p21 by DEK depletion in undifferentiated, but not in differentiated cells. This was presumably a consequence of p53 activation as previously published (26), and indeed, p21 induction was preceded by increased p53 protein levels as predicted (data not shown). The extracts were also probed for the expression of the p53-related p63 isoform ΔNp63, a stem and progenitor cell marker in mouse and human skin, and critical regulator of basal cell proliferation and differentiation (35). As expected for differentiating keratinocytes, ΔNp63 expression was distinctly down-regulated, and interestingly, was reduced in response to DEK depletion in both differentiated and undifferentiated cells. These data indicate a role for DEK in the repression of squamous cell differentiation. Our recent findings describe the up-regulation of ΔNp63 and decreased differentiation as a consequence of DEK overexpression (6). Whether ΔNp63 is a critical DEK mediator or simply marks differentiation inhibition in response to DEK overexpression remains to be determined. Our findings show that differentiating epithelial cells are less sensitive to DEKsh-dependent apoptosis compared with cancer cells, suggesting that targeting DEK may offer a therapeutic window for cancer treatment.

Figure 4. DEK depletion in xenograft tumors results in cell death in vivo. A, in vivo imaging system (IVIS) analysis. Nude mice were injected with 5 × 10⁶ U2OS tumor cells into each flank and injected with either empty Ad (left) or AdDEKsh (right) when tumors reached 200 mm³. GFP expression in the tumors was determined and analyzed by IVIS software. Color bars represent minimum to maximum levels of GFP expression. B, immunofluorescence. Tumors from A were harvested, fixed, and embedded in paraffin and sectioned for analysis. Sections were analyzed by immunofluorescence microscopy for the detection of cell death. The experiment was performed twice, using four mice for each experiment. Similar results were seen in both experiments.
transformation (data not shown), and the V12 oncogenic form of the cellular Ras GTPase was therefore coexpressed. RasV12 represents a prominent cooperating oncogene in the multistep process of human carcinogenesis (44). Expression of E6/E7 and Ras was not sufficient for colony formation in soft agar over the course of 3 weeks, but was sufficient for causing tumors in 25% of immunodeficient mice over the course of 3 months. Importantly, the additional overexpression of DEK resulted in a dramatic stimulation of colony formation. This stimulation occurred despite maximal repression of p53 in the presence of E6 (Fig. 2A). DEK overexpression also increased tumor frequencies and volume (Fig. 3). Collectively, these data indicate that DEK overexpression induces tumor growth through a mechanism that is independent of p53 repression. Moreover, the results further support a positive correlation between high levels of DEK expression and more aggressive later stage tumors in some human malignancies such as breast cancer (45).

High levels of DEK expression have been associated with numerous cancers including glioblastoma, hepatocellular carcinoma, AML, and breast cancer. Until recently, it was unclear whether this association was merely a correlative event, or whether DEK expression was functionally important in the genesis of such tumors (1, 3, 31, 45–47). We show here that DEK exhibits oncogenic activities at multiple stages of tumorigenesis. A need for sustained and high level DEK expression in many cancer cell lines is reflected by our previous data whereby DEK depletion resulted in cancer cell death in vitro (26). The finding that DEK depletion in preformed U2OS cell tumors results in apoptosis (Fig. 4) provides proof of concept for the treatment of cancer via DEK suppression. Future approaches may involve more efficacious ways to deliver DEK-specific short hairpin RNA, alone and in combination with other treatments, and may explore the development of small molecules for the specific inhibition of oncogenic DEK activities.

DEK depletion resulted in the death of primary human keratinocytes in vitro (26), suggesting that a degree of toxicity would be expected after DEK targeting. Interestingly, differentiated keratinocytes were almost completely resistant to DEK depletion in comparison with their proliferating counterparts (Fig. 5). Thus, targeted DEK therapy may provide a therapeutic window between cancerous and normal cells. Taken together, our data provide the first evidence to implicate DEK as a bona fide oncogene, and suggest that DEK inhibition in human cancer may be a viable approach for the treatment of benign and malignant lesions.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Figure 5.** Epithelial differentiation rescues cells from DEKsh-induced apoptosis. A, HFKs and U2OS cells were infected with either empty Ad or AdDEKsh and subjected to anti–active caspase 3 antibody and flow cytometry on day 3. The cells were photographed after 4 d. B, HFK differentiation. HFKs were infected as in A and either subjected to normal medium, or differentiated upon the addition of 1 mmol/L of calcium and 10% FBS. Cells were harvested for apoptosis assays as in A, subjected to DEK, p21, ΔNp63, and actin-specific Western blot analyses on day 3, and analyzed for cellular morphology. *, P < 0.05; **, P < 0.01. The Ad-infected populations were not statistically different from each other nor were the Ad- and DEKsh-infected differentiated cell populations.

References


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