Requirement of Epidermal Growth Factor Receptor for Hyperplasia Induced by E5, a High-Risk Human Papillomavirus Oncogene

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Abstract

Multicellular organisms rely on complex networks of signaling cascades for development, homeostasis, and responses to the environment. These networks involve diffusible signaling molecules, their receptors, and a variety of downstream effectors. Alterations in the expression or function of any one of these factors can contribute to disease, including cancer. Many viruses have been implicated in cancer, and some of these modulate cellular signal transduction cascades to carry out their life cycles. High-risk human papillomaviruses (HPVs), the causative agents of most cervical and anogenital cancers, encode three oncogenes. One of these, E5, has been postulated to transform cells in tissue culture by modulating growth factor receptors. In this study, we generate and characterize transgenic mice in which the E5 gene of the most common high-risk HPV, HPV16, is targeted to the basal layer of the stratified squamous epithelium. In these mice, E5 alters the growth and differentiation of stratified epithelia and induces epithelial tumors at a high frequency. Through the analysis of these mice, we show a requirement of the epidermal growth factor receptor for the hyperplastic properties of E5. (Cancer Res 2005; 65(15): 6534-42)

Introduction

Alterations in cellular signaling pathways that respond to external stimuli and regulate cell growth, differentiation, and death commonly contribute to cancer. For example, in many types of cancers, including breast and head and neck, alterations in the expression and/or activity of epidermal growth factor receptor (EGFR) family members are common, and their activity contributes to tumorigenicity (1). Viruses have evolved mechanisms to modulate cellular signaling pathways to reprogram host cells to support their viral life cycles or modulate host defense responses (2–4). One such case is the human papillomaviruses (HPVs), a subset of which, termed high-risk HPVs (e.g., HPV16), cause cervical cancer, other anogenital cancers, and a subset of head and neck cancers (5–7). HPVs encode three oncogenes, E5, E6, and E7 (8–12). HPV16 E5 is an 83–amino acid membrane-associated protein (13, 14) that is found in detectable levels in the Golgi apparatus, endoplasmic reticulum, and nuclear membrane (15, 16). HPV16 E5 is considered an oncoprotein because in tissue culture it transforms murine fibroblasts and keratinocytes (9, 11, 12), enhances the immortalization potential of E6 and E7 (17), and in cooperation with E7 stimulates the proliferation of human and mouse primary cells (18, 19).

Multiple studies have implicated the EGFR in transformation by the HPV16 E5; however, there is no direct proof to date for a requirement of EGFR in E5-induced transformation. In vitro, E5 induces enhanced EGFR phosphorylation in the presence of EGF (12, 20, 21). Studies monitoring the mitogenic response to EGF in E5-expressing human keratinocytes led investigators to propose a synergistic interplay between E5 and EGF (12). HPV16 E5 also binds to the 16-kDa subunit of the v-ATPase (15) and through this interaction is thought to delay endosomal acidification in human keratinocytes (22). This delay in endosomal acidification has been implicated as a reason for enhanced EGFR phosphorylation in keratinocytes, as failure to acidify endosomes may result in decreased receptor degradation and increased receptor recycling to the cell surface (12, 22).

The in vitro studies described above are consistent with a role of EGFR signaling in E5-mediated effects on epithelial cells. However, no study has yet shown a requirement for EGFR signaling in mediating the phenotypes of E5. To understand the biological properties of E5 in vivo and to test directly for a role of EGFR signaling in mediating the phenotypes of E5, we generated transgenic mice that express the HPV16 E5 gene. These mice (strain K14E5) displayed phenotypes that were strikingly similar to mice overexpressing ligands of the EGFR in the epidermis, such as transforming growth factor-α (TGF-α) and amphiregulin (23–25). These phenotypes included epidermal hyperplasia, hyperkeratosis, enhanced DNA synthesis, aberrant differentiation, and formation of spontaneous skin tumors. Importantly, the epithelial hyperplasia induced by E5 in these mice was attenuated when the K14E5 mice were crossed to mice carrying an allele of Egrf, EgrfIII to release a 2.8-kbp fragment that contains the hK14 promoter and hK14 polyadenylation sequences in plasmid pG1Z-keratin 14 (K14) to generate plasmids pK14E6* and pK14E6*, respectively. The constructs were sequenced to verify the intact state of the E5 ORF. The recombinant plasmids were triple digested in plasmid pG1Z-keratin 14 (K14) to generate plasmids pK14E5* and K14AU1E5* transgenes were constructed similarly to the K14E6* transgene and to test directly for a role of EGFR signaling in mediating the phenotypes of E5, we generated transgenic mice that express the HPV16 E5 gene. These mice (strain K14E5) displayed phenotypes that were strikingly similar to mice overexpressing ligands of the EGFR in the epidermis, such as transforming growth factor-α (TGF-α) and amphiregulin (23–25). These phenotypes included epidermal hyperplasia, hyperkeratosis, enhanced DNA synthesis, aberrant differentiation, and formation of spontaneous skin tumors. Importantly, the epithelial hyperplasia induced by E5 in these mice was attenuated when the K14E5 mice were crossed to mice carrying an allele of Egrf, EgrfIII to release a 2.8-kbp fragment that contains the hK14 promoter and hK14 polyadenylation sequences in plasmid pG1Z-keratin 14 (K14) to generate plasmids pK14E6* and pK14E6*, respectively. The constructs were sequenced to verify the intact state of the E5 ORF. The recombinant plasmids were triple digested

Materials and Methods

Construction of transgene and generation of transgenic mice. The K14E5* and K14AU1E5* transgenes were constructed similarly to the K14E6* (26) and K14E7* (27) transgenes. Briefly, the codon-optimized E5 open reading frame (ORF), E5* (16), and E5* with a 6–amino acid tag (AU1) at the NH2 terminus, AU1-E5*, were individually inserted into the unique HindIII site between the hK14 promoter and hK14 polyadenylation sequences. The fragments were purified by gel electrophoresis and microinjected into fertilized mouse FVB/N eggs as described previously (28). Mice born from these eggs were screened for the presence of transgene in their genome by
Southern analysis of genomic DNA prepared from tail biopsies. The hybridization probe was the 2.8-kbp E5-specific DNA fragment released from plasmid pK14AU1E5* by digestions with ScaI, EcoRI, and HindIII; it was 32P labeled by random primer extension. To estimate the copy number of each of the transgenic lineages, standard DNA of plasmid pK14E5* or pK14AU1E5* equal in amount to 0.1, 1, or 10 copies per mouse genome was included in the Southern analysis. The blot was quantified with a Molecular Dynamics (Piscataway, NJ) PhosphorImager. Multiple lineages of K14E5* and K14AU1E5* mice were bred in the Association for Assessment and Accreditation of Laboratory Animal Care–approved animal facilities in the McArdle Laboratory for Cancer Research (Madison, WI).

Analysis of transgene expression by real-time PCR. Total cellular RNA was extracted from 10-day-old animals using the RNeasy Fibrous Tissue Midi kit (Qiagen, Valencia, CA). Total cellular RNA (3 μg) was reverse transcribed using the Omniscript RT kit (Qiagen). Real-time PCR analysis was done with the E5*-specific primers 5'-TGGATACTGATCCACACACAATCTG-3' and 5'-AGCACCAGAGTATCGAGGA-3' and the Taqman probe 6-FAM-TGCGCTTCTGTTGTCCTTTT-GAMFAM (Applied Biosystems, Foster City, CA). Primers and probe were also used for L32, a housekeeping gene, to account for input variability. Amplification using the Perkin-Elmer sequence detector 7700 was done with the 2x Universal Amplification Mix (Perkin-Elmer, Wellesley, MA). The amplification profile was 45 cycles of 94°C for 15 seconds and 60°C for 1 minute. The threshold (Ct) values for each sample were measured and expression level for each sample was determined by calculating 2ΔΔCt.

Immunohistochemical and immunofluorescence analysis of mouse epidermis. To examine the in vivo properties of HPV16 E5, K14E5* mice were generated expressing a codon-optimized version of the E5 protein referred to previously as E5* (16). This codon-optimized HPV16 E5 gene was used because codons rare in mammalian genes are found commonly in papillomavirus genes (30, 31) and comprise ~40% of the codons in the HPV16 E5 translational ORF. When transfected into human keratinocytes, E5* produces 6- to 9-fold higher protein levels than wild-type E5 without altering mRNA levels (16). We reasoned that the codon-optimized gene would facilitate our ability to express E5 at sufficient levels to study it, because in mice transgenes are often expressed at low levels. We also generated K14AU1E5 transgenic animals that express E5* with a 6-aminocaproic acid tag (AU1) at the NH2 terminus. Other than the epitope tag, the amino acid sequence of this transgene is no different from that of E5*. Importantly, both E5* and AU1-E5* gene products have the same E5 amino acid sequence and possess the same biological and biochemical properties as the natural HPV16 E5 protein (16, 32). E5* and AU1-E5* were cloned into a K14 expression plasmid containing the human K14 promoter and 3' polyadenylation sequences (Fig. 1). The K14 promoter was chosen because it directs expression of the transgene to the basal layer of the epithelium where HPV begins its viral life cycle and from which it is thought that HPV-associated cancers arise. Potential founder mice that were born and survived to the time of weaning were analyzed by Southern analysis to determine their transgene status. From these mice, four transgene-positive founders were identified. All four mice were germ line transgenic; therefore, four independent lineages of K14E5 mice were obtained, with transgene copy numbers ranging from 10 to 50 copies per cell in the hemizygous state (Table 1; Fig. 2). One of these founders, 614, had the transgene integrated at two separate loci. We labeled these two integrations as "a" and "b" and offspring that are hemizygous for these integration events are called sublines 614a or 614b, respectively. Offspring that carry both "a" and "b" integrations are

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EGFR Mediates E5-Induced Phenotypes
and it contains a 6-amino acid tag (AU1) at the NH₂ terminus of E5*. E5, sequences.

Figure 1. K14E5 and K14AU1E5 constructs. A, to create HPV16 E5 transgenic mice, the codon-optimized E5 gene, E5*, was placed into a cassette containing the human K14 promoter, which directs expression of the transgene to the basal layer of the stratified squamous epithelium and K14 polyadenylation sequences. B, an epitope-tagged version of this construct was also developed, and it contains a 6-amino acid tag (AU1) at the NH₂ terminus of E5.*

termed subline 614c mice. When founder 615 was bred to FVB/N males (FVB/N is the inbred genetic background on which the transgenic founders were generated), none of its transgenic pups survived. Some of her transgenic offspring survived when we crossed her to males from the BTBR genetic background. For this reason, line 615 was established on a FVB/N × BTBR mixed genetic background.

K14E5 mice display overt phenotypes. Multiple K14E5 founder mice died before age 13 days and displayed severe phenotypes that included stunted growth, thickened, scaly skin, and inability to move limbs. In the three most extreme cases, the founders died by day 8 after birth. All three of these mice were genotyped by Southern analysis and found to carry the transgene at very high copy numbers (>50 copies per cell; data not shown). Four founders survived to weaning, the scaly tail phenotype often waned but the ears always remained thick, red, and scaly. The 614, mice also had severe phenotypes, which resulted in neonatal death at age 6 to 21 days (23%), wrinkled, scaly skin and tail, thick, scaly ears, closed eyes, alopecia, and curly whiskers (Table 1; Fig. 2A). If the 615 transgenic animals survived past weaning, the scaly tail phenotype often waned but the ears always remained thick, red, and scaly. The 614, mice also had severe phenotypes, which resulted in neonatal death at age 6 to 21 days (23%), wrinkled, scaly skin and tail, thick, scaly ears, closed eyes, alopecia, and curly whiskers (Table 1; Fig. 2A). Line 32 mice had more modest phenotypes. Neonatal death (8%), which occurred between days 6 and 18, was less frequent than in lines 614, and 615, and the scaly skin on the back and top of the tail and curly whiskers were likewise less severe. By age 3 weeks, the scaly skin on the back and tail had waned, but many of the mice in line 32 did not open their eyes until as late as 5 weeks. As early as age 7 months, some of these animals developed thick, red ears. Line 33 animals had no gross phenotypes as pups, except curly whiskers, but as early as age 5 months developed thick, red ears similar to line 32. The severity of the phenotypes among these four lines in general correlated with numbers of transgene copies, with lines 32 and 33 carrying 10 copies (hemizygous state) having less severe phenotypes and lines 614, and 615 carrying 50 copies (hemizygous state) having more severe phenotypes.

Level of E5 expression correlates with severity of phenotypes. Transgenic expression in each line was quantified by using real-time PCR with probe and primers specific for the E5 transgene (Fig. 2B). The E5-specific real-time PCR results for each line is reported relative to that observed in line 32, which had the lowest amount of E5 expression (Table 1, column 4; Fig. 2B). Lines 32 and 33 had relatively low levels of transgene expression compared with lines 614 and 615, and this correlated with the mild to moderate phenotypes that we observed in these lines (Table 1, column 6). Lines 614 and 615, displaying severe phenotypes, had the highest expression levels of E5. The correlation between severity of phenotypes and expression levels of the E5 transgene was made

<table>
<thead>
<tr>
<th>Line</th>
<th>Transgene</th>
<th>Copy number</th>
<th>Transgene expression level*</th>
<th>% Suprabasal BrdUrd incorporation</th>
<th>Gross phenotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>K14AU1E5</td>
<td>10</td>
<td>1.0</td>
<td>14 ± 4.0¹</td>
<td>Infrequent neonatal death (8%) between days 6 and 18, stunted growth, mildly scaly skin and top of tail, closed eyes, curly whiskers</td>
</tr>
<tr>
<td>33</td>
<td>K14AU1E5</td>
<td>10</td>
<td>2.0</td>
<td>17 ± 5.0¹</td>
<td>Curly whiskers</td>
</tr>
<tr>
<td>614²</td>
<td>K14E5</td>
<td>50</td>
<td>3.4</td>
<td>27 ± 1.8³</td>
<td>Neonatal death (23%) between days 6 and 21, stunted growth, wrinkled, scaly skin and tail, thick, scaly ears, closed eyes, alopecia, curly whiskers</td>
</tr>
<tr>
<td>615</td>
<td>K14E5</td>
<td>50</td>
<td>8.0</td>
<td>38 ± 5.2³¹</td>
<td>Frequent neonatal death (73%) between days 6 and 21, stunted growth, wrinkled, scaly skin and tail, thick, scaly ears, closed eyes, alopecia, curly whiskers</td>
</tr>
</tbody>
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*Relative level of transgene-specific RNA quantified by real-time PCR (see Materials and Methods for details). The level of expression quantified for line 32 was set to the value of 1.0 and those of the other lines are indicated relative to that for line 32.

¹Not significantly different from nontransgenic animals (14 ± 4.0).

²Attributes of subline 614c, mice.

³Significantly different from nontransgenic animals with Ps of 0.01 (line 614) and 0.02 (line 615).
more apparent by our analysis of the offspring of the 614 founder, which carried two different integration events. The 614a and 614b offspring, which were hemizygous for one or the other integration event, had low levels of expression of the transgene and displayed mild phenotypes. The 614c offspring, which carried both integration events, had high levels of expression of the transgene and displayed moderate to severe phenotypes (Fig. 2). As is the case in human keratinocytes expressing E5 from the intact HPV16 genome (29), the levels of E5 proteins in the skin of these transgenic mice were below the level of detection using Western blot analysis, immunohistochemistry, or immunofluorescence with available antibodies (data not shown). This finding is consistent with there being very low levels of E5 proteins expressed in these transgenic mice as in the natural infection. However, because the levels of expression of E5 protein in our mice and in HPV-infected human cells are below the level of detection, we are unable to determine whether the levels of expression in the E5 transgenic mouse lines are below, equal, or above that observed in HPV-infected human cells.

**E5 induces suprabasal DNA synthesis in the epidermis of mice.** One of the first phenotypes observed in our transgenic mice, wrinkled skin, was similar to that observed in our K14E7 mice, expressing the HPV16 E7 oncogene (27). The epithelial hyperplasia observed in the E7 transgenic mice correlated with an increase in the frequency of cells supporting DNA synthesis within the differentiating compartment of the epithelium (33). This phenotype correlated with a hallmark of the productive stage of the HPV16 life cycle where viral proteins reprogram cells in the suprabasal (terminally differentiating) compartment to support DNA synthesis, a process termed suprabasal DNA synthesis that is thought to contribute to viral DNA amplification. We had shown previously that both HPV16 E7 and HPV16 E5 contribute to this process in the context of the viral life cycle (29, 34). To determine whether the expression of E5 in the epidermis of transgenic mice results in increased suprabasal DNA synthesis, we monitored DNA synthesis in 10-day-old transgenic mice. Mice were injected with BrdUrd, a nucleotide analogue, 1 hour before harvest. The percentage of BrdUrd-positive cells in the suprabasal compartment of the epithelium was calculated by counting the number of BrdUrd-positive cells in the suprabasal compartment of the epithelium in 10 frames at ×40 magnification and dividing that number by the total number of BrdUrd-positive cells in 10 frames at ×40 magnification. Percent suprabasal BrdUrd-positive cells in lines 615 and 614 high and moderate expressing mice was significantly different from nontransgenic with two-side Ps of 0.02 and 0.01, respectively.

**Figure 2.** Severity of phenotypes correlate with level of expression of E5 transgenes. A, phenotypes observed in nontransgenic (NTG), line 615, and line 614 animals with varying degrees of severity. In the most severely affected animals, we observe wrinkled, scaly skin and tails, and alopecia on the back (arrows). Note that line 615 mouse is of a FVB × BTBR mixed background and therefore has darkly pigmented hair. Other mice shown are FVB inbred mice, with white hair. B, quantification of transgene expression by real-time PCR. Total RNA from the skin of 10-day-old pups from each transgenic line was reverse transcribed into cDNA. The abundance of E5 cDNA in each line was then quantified by real-time PCR analysis with a FAM-labeled probe specific for the transgene. Relative transgene expression levels for nontransgenic (no expression), line 615, and line 614 animals with no phenotype, mild phenotype, moderate phenotype, and severe phenotype compared with line 32 (see Table 1, column 3). C, percent suprabasal DNA synthesis correlates with severity of phenotypes (A) and expression level of E5 (B). Ten-day-old nontransgenic and transgenic mice were injected with BrdUrd 1 hour before harvest. The percentage of BrdUrd-positive cells in the suprabasal compartment of the epithelium was calculated by counting the number of BrdUrd-positive cells in the suprabasal compartment of the epithelium in 10 frames at ×40 magnification and dividing that number by the total number of BrdUrd-positive cells in 10 frames at ×40 magnification. Percent suprabasal BrdUrd-positive cells in lines 615 and 614 high and moderate expressing mice was significantly different from nontransgenic with two-side Ps of 0.02 and 0.01, respectively.
38 ± 5.2, respectively, compared with nontransgenic animals, which had a percentage suprabasal BrdUrd-positive cells of 14 ± 4.0. These differences were statistically significant with two-sided P values of 0.01 and 0.02, respectively. There were no statistically significant differences in the percentage of BrdUrd-positive suprabasal cells in animals from lines 32 and 33 compared with nontransgenic animals. Indeed, the percentage of suprabasal BrdUrd-positive cells correlated well with severity of phenotypes and levels of expression of the E5 transgene as illustrated in Fig. 2C. The animals with the most severe phenotypes and highest expression levels of E5 displayed the highest percentage of suprabasal DNA synthesis.

E5 induces an aberrant differentiation pattern in the epidermis of mice. To determine if E5 was disrupting normal differentiation of the epithelium, we stained ear sections from 10-day-old nontransgenic and E5 transgenic animals with an antibody to K14. K14 is a protein that normally is detected only in the undifferentiated cells in the basal layer of the normal epithelium as seen in nontransgenic mice (Fig. 3C). However, in E5 transgenic epithelium, K14 expression was expanded throughout the entire epidermis (Fig. 3D). In contrast, the K14E7 mice displayed a normal pattern of expression of K10, a marker for early stages in epithelial differentiation (first being expressed in the stratum spinosum). This finding is in contrast to what has been observed by us with the K14E7 transgenic mice, which displayed a delayed onset of expression of K10 within the hyperplastic epithelium (27).

Another distinct feature of the thickened epithelium in the K14E5 mice was the presence of multiple layers of cells containing many pyknotic nuclei, located directly above the proliferating compartment (Fig. 3B, red arrows). This abundance of pyknotic nuclei was absent in nontransgenic mice (Fig. 3A). To determine if these cells were undergoing nuclear DNA fragmentation, TUNEL assays were done. As is evident in Fig. 3J, there was a dramatic increase in TUNEL-positive cells in the E5 transgenic animals compared with the nontransgenic mice (Fig. 3I). TUNEL-positive cells are normally found at a very low frequency within the granular layer of the epithelium (e.g., see Fig. 3I), and this DNA fragmentation reflects the normal process of differentiation-dependent nuclear breakdown in the stratified squamous epithelium.

Figure 3. Analysis of histologic phenotypes in 10-day-old ear epithelium in K14E5 transgenic mice. High-power magnifications of cross-sections of skin from the ear of nontransgenic (A, C, E, G, and I) and line 615 K14E5 transgenic (E5; B, D, F, H, and J) mice. The black dotted line or white dotted line indicates the location of the basement membrane separating the epidermis from the underlying dermis. A (nontransgenic) and B (K14E5 transgenic) are sections stained immunohistochemically for BrdUrd (BrdU). BrdUrd-positive nuclei are brown (black arrows; red arrows, pyknotic nuclei). BrdUrd was injected 1 hour before harvesting tissue from mice. Sections were counterstained with hematoxylin, which stains nuclei blue. C (nontransgenic) and D (K14E5 transgenic) are sections stained immunofluorescently for K14 (red) and counterstained with DAPI (blue). E (nontransgenic) and F (K14E5 transgenic) are sections stained immunofluorescently for K10 (red) and counterstained with DAPI (blue). G (nontransgenic) and H (K14E5 transgenic) are sections stained immunofluorescently for filaggrin (red) and counterstained with DAPI (blue). I (nontransgenic) and J (K14E5 transgenic) are sections of skin immunofluorescently stained for DNA fragmentation by TUNEL (green) and counterstained with propidium iodide (red).
epithelium. The heightened frequency of nuclear breakdown in the K14E5 epithelium likewise arose within the granular layer as evidenced by staining with the granular specific marker, filaggrin (Fig. 3E and F), and confirmed by double immunofluorescence for TUNEL and filaggrin (data not shown).

**E5 transgenic mice develop spontaneous skin tumors.** To determine whether HPV16 E5 contributed to tumorigenesis in the mouse epithelium, K14E5 mice from each of the four lines successfully established were allowed to age and were monitored for spontaneous tumor development. Most line 615 animals did not live past age 20 weeks due to severe hyperplasia and hyperkeratosis. These animals either died or had to be euthanized for humane reasons. Of those line 615 mice that survived to age 20 weeks, three of the five mice developed apparent skin tumors, and by age 22 weeks, all five of these mice had apparent skin tumors. Sections from two line 615 animals with apparent skin tumors were scored histopathologically, and both were confirmed to have at least one skin papilloma. An example of a papilloma from line 615 is shown in Fig. 4A. In line 614c, 9 of 23 (39%) animals at age 20 weeks had developed apparent tumors. Sections from two of these animals, one just 7 weeks old, were scored histopathologically and confirmed to have at least one papilloma per animal. An example of such a papilloma is shown in Fig. 4B. By age 40 weeks, an additional 9 animals (18 of 23 or 78%) had at least one apparent tumor. To date, we have not observed any tumors arising in lines 32 and 33 mice, the lines that displayed mild to moderate phenotypes.

E5-associated phenotypes are similar to phenotypes in mice overexpressing epidermal growth factor receptor ligands in the epidermis. The E5 transgenic mice had strikingly similar phenotypes to mice overexpressing ligands of the EGFR in the epidermis, such as TGF-α and amphiregulin (23–25). A comparison of the phenotypes in our K14E5 mice to those seen in mice overexpressing EGF receptor ligands is provided in Table 2. In one model, TGF-α was directed in its expression by the human K14 promoter to the basal layer of the epithelium, K14-TGF-α, whereas in the other model TGF-α was directed in expression to the spinous layer of the epithelium using the human K1 promoter, HK1.TGF-α. Both models exhibited wrinkled, scaly skin with diffuse alopecia, an aberrant differentiation pattern, and benign papilloma formation (23, 25). In the amphiregulin transgenic model, amphiregulin was directed in expression to the basal layer of the epithelium with the human K14 promoter, K14-ARGE (24). In this model, mice had shorten life spans, scaly skin and alopecia, an aberrant differentiation pattern, and benign papilloma formation (24).

**E5-associated phenotypes are dependent on the epidermal growth factor receptor.** Due to the similarity of phenotypes in our E5 transgenic animals and the mice transgenic for the EGFR ligands (Table 2), we took a genetic approach to determine

![Figure 4](image_url)
whether EGFR contributed to E5-induced phenotypes we observed in vivo. Specifically, we made use of a mouse strain with an antimorphic mutation in the Egfr, Egfr\textsuperscript{Wa5}, characterized by a missense mutation (D857G, GAT to GGT) within a highly conserved region of the tyrosine kinase domain and whose product functions as a dominant negative (35). The product of the Egfr\textsuperscript{Wa5} allele selectively inactivates the EGFR but not other receptors that are part of the EGFR family.\textsuperscript{4} We crossed our K14E5 transgenic mice to the Egfr\textsuperscript{Wa5}\textsuperscript{+/+} mice, which were on the B6 background. We first validated, by monitoring the levels of P-EGFR in response to stimulation with EGF ligand, that the in vivo activity of the EGFR was attenuated by Egfr\textsuperscript{Wa5} (Fig. 5A). This attenuation was observed on both nontransgenic and K14E5 transgenic backgrounds. Correspondingly, we saw a similar reduction in the levels of phosphorylated mitogen-activated protein kinase in nontransgenic and E5 transgenic mice on the Egfr\textsuperscript{Wa5} background (data not shown). The decrease in P-EGFR was not due to a decrease in overall EGFR (Fig. 5B). We next monitored the overt phenotypes of the K14E5 mice on the Egfr \textsuperscript{+/+} versus Egfr\textsuperscript{Wa5}\textsuperscript{+/+} genetic background. We observed a reduction in E5-induced phenotypes (thick, wrinkled, scaly skin, legs and tail) in the E5:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} mice compared with the E5:Egfr\textsuperscript{+/+} mice (Fig. 6, ii and iii). This reduction in overt phenotypes was also observed at a histologic level, as E5:Egfr\textsuperscript{+/+} mice displayed a much thicker epidermis than E5:Egfr\textsuperscript{+/+} mice (Fig. 6B, i and iii). We quantified the degree of hyperplasia in the K14E5 mice on Egfr \textsuperscript{+/+} versus Egfr\textsuperscript{Wa5}\textsuperscript{+/+} genetic backgrounds by counting the number of cells present in the epidermis (Fig. 6C). There was a statistically significant difference (two-sided \(P = 0.0044\)) in the number of cells in the epithelium of the E5:Egfr\textsuperscript{+/+} versus E5:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} mice (Fig. 6C). Furthermore, the number of cells in the epithelium of the E5:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} mice was not significantly different than that seen in the nontransgenic:Egfr\textsuperscript{+/+} mice (two-sided \(P = 0.13\)) and the nontransgenic:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} mice (two-sided \(P = 0.18\); Fig. 6C).

Discussion

We have shown that HPV16 E5 induced multiple phenotypes in the epidermis of transgenic mice that correlated with the level of expression of the E5 transgene. Interestingly, these phenotypes were very similar to phenotypes observed in mice overexpressing ligands of the EGFR in the epidermis, such as amphiregulin (23–25). This similarity in phenotypes led us to test the hypothesis that the activities of E5 are dependent on EGFR signaling. When we crossed the K14E5 transgenic mice to mice that were heterozygous for a dominant-negative mutation in the Egfr, Egfr\textsuperscript{Wa5}/+, we observed an attenuation of E5-induced phenotypes (Fig. 6A, i), including a complete reduction in the thickness of the epidermis induced by E5 (Fig. 6B). This seems not to be a consequence of some nonspecific effect of Wa5 on epithelial hyperplasia, as the Egfr\textsuperscript{Wa5}\textsuperscript{+/+}, nontransgenic mice were not affected in the thickness of the epidermis compared with nontransgenic mice that were wild-type for Egfr (Fig. 6B). These E5:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} mice provided the first demonstration that the activities of E5 in vivo require functional EGFR.

There is good reason to believe that EGFR mediates, at least in part, the tumorigenesis observed in K14E5 mice given that EGFR is required for E5-induced epithelial hyperplasia. Another papillomaviral oncoprotein, E6, also induces epithelial hyperplasia and tumorigenesis in vivo, and induction of hyperplasia by E6 correlates with its role in tumor promotion (36).\textsuperscript{5} Interestingly, the ability of HPV16 E6 and E7 to induce tumor formation in mouse epithelial cells has been found to be dependent on EGFR (37). Furthermore, in human epithelial cells transformed by HPV16 E6 and E7, EGFR mRNA levels are induced (38). Thus, multiple HPV oncogenes may rely on EGFR signaling for their tumorigenic properties. As with E6 and E7, the requirement of the EGFR for the induced phenotypes of E5 does not necessarily mean that there is a direct effect of E5 on this growth factor receptor, although with E5 there is certainly an abundance of evidence supporting this hypothesis. At this time, only 2 of 14 E5:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} animals have survived past weaning. These E5:Egfr\textsuperscript{Wa5}\textsuperscript{+/+} animals were euthanized at 5 and 15 weeks for other reasons. Neither had developed papillomas. Further studies are necessary to determine whether there is a correlation between E5 EGFR-dependent induction of hyperplasia and tumorigenesis.

Cervical cancer is a progressive disease that takes on average one to two decades to develop. During that period, the HPV genome is maintained as a nuclear plasmid in the infected cervical epithelium, and early genes, including E5, are expressed. Thus, E5 has the potential to contribute to the HPV-associated carcinogenic process. In cervical cancer itself, however, HPV genomes commonly are found integrated into the host cellular genome, and this occurs such that the E6 and E7 ORFs remain intact, but E5 is lost or, if present, underexpressed compared with E6 and E7 (39–41). This suggests that E5 may play a critical role in the genesis of cervical cancer but less of a role in its persistence or permanence.

\textsuperscript{4}D. Lee and D.W. Threadgill, unpublished data.

\textsuperscript{5}S. Simonson and P.F. Lambert, manuscript submitted.
progression. Our K14E5 mice provide an opportunity to assess directly the role of E5 in cervical carcinogenesis. In the original HPV transgenic mouse model for cervical cancer, estrogen was found to synergize with the early region of HPV16 present in K14HPV16 mice, including the E5, E6, and E7 oncogenes, to induce cervical cancer that bore histopathologic features very similar to that seen in human cervical cancer (42). More recently, the individual roles of E6 and E7 oncogenes have been elucidated using our previously generated K14E6 and K14E7 mice that express the HPV16 E6 and E7 oncogenes individually (43). It is interesting to note that the estrogen-treated K14E6/K14E7 doubly transgenic mice had lower mean numbers of cancers per mouse than seen with the K14HPV16 mice expressing all three oncogenes (43). This difference in tumor number could be due to a difference in the expression level of the two transgenes or due to the participation of another early viral protein, such as E5, in the genesis of cervical cancer. What is striking about our initial studies reported here is that the early age of onset and high penetrance of spontaneous skin tumors arising in our K4E5 mice indicates that HPV16 E5 is far more potent an oncogene than we have observed for either HPV16 E6 or E7 in our existing K14E6 and K14E7 mouse models.

Our data suggest that there may exist EGFR-independent activities of E5, as the high mortality rate observed in E5 transgenic mice is retained on a background that is mutant for the EGFR. Seventy-three percent of K14E5 mice on an \( \text{Egfr}^{+/+} \) background die before age 21 days compared with 86% of \( \text{E5};\text{Egfr}^{wa5}/+ \) mice. This retention of high morbidity and mortality may arise from the fact that E5 can alter cells through an EGFR-independent manner or because the \( \text{Egfr}^{wa5} \) allele only has a semidominant effect over that of the \( \text{Egfr} \) allele. Consistent with this latter possibility is the fact that \( \text{Egfr} \)-null mice are embryonic.

Figure 6. E5-induced phenotypes are dependent on the EGFR. A, phenotypes that we observe on the back and tail of E5;\( \text{Egfr}^{+/+} \) (i), nontransgenic; E5;\( \text{Egfr}^{wa5}/+ \) (ii), and nontransgenic; E5;\( \text{Egfr}^{wa5}/+ \) (iv) mice at age 10 days. Note that the phenotype that we observe in the E5;\( \text{Egfr}^{+/+} \) mouse (i) is markedly reduced in the E5;\( \text{Egfr}^{wa5}/+ \) (iii) mouse. B, H&E-stained sections of torso epithelium from E5;\( \text{Egfr}^{+/+} \) (i), nontransgenic; E5;\( \text{Egfr}^{wa5}/+ \) (ii), E5;\( \text{Egfr}^{wa5}/+ \) (iii), and nontransgenic; E5;\( \text{Egfr}^{wa5}/+ \) mice at age 10 days. Note that the epidermis of E5;\( \text{Egfr}^{+/+} \) mice (i) is markedly hyperplastic in comparison with the other genotypes (ii, iii, and iv). C, quantification of the average number of cells that we observe in E5;\( \text{Egfr}^{+/+} \), nontransgenic; E5;\( \text{Egfr}^{wa5}/+ \), and nontransgenic; E5;\( \text{Egfr}^{wa5}/+ \) torso skin at age 10 days. There is a statistically significant difference between the average number of cells in the torso skin of E5;\( \text{Egfr}^{+/+} \) mice compared with E5;\( \text{Egfr}^{wa5}/+ \) mice (\( P = 0.0044 \)).
lethal, whereas Egr\textsuperscript{Prel}/+ mice are viable. The underlying cause of the morbidity and mortality associated with the expression of E5, and the mechanism by which E5 causes this, has yet to be elucidated.

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References


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Requirement of Epidermal Growth Factor Receptor for Hyperplasia Induced by E5, a High-Risk Human Papillomavirus Oncogene

Sybil M. Genther Williams, Gary L. Disbrow, Richard Schlegel, et al.


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