Original Manuscript

mTOR inhibition prevents rapid-onset of carcinogen-induced malignancies in a novel inducible HPV-16 E6/E7 mouse model

Juan Luis Callejas-Valera, Ramiro Iglesias-Bartolome, Panomwat Amornphimoltham, Julia Palacios-Garcia, Daniel Martin, Joseph A. Califano, Alfredo A. Molinolo and J. Silvio Gutkind*

Moores Cancer Center, 3855 Health Sciences Dr, La Jolla, CA 92093, USA, Developmental Skin Biology Section (HNB-254), NIH/NIAMS Building 50, Bethesda, MD 20814-4340, USA, Centro de Biología Molecular Severo Ochoa (CSIC-UAM), Madrid, 28049 Spain and Oral and Pharyngeal Cancer Branch, NIH/NIDCR, Building 30, Bethesda, MD 20892-2190, USA

*To whom correspondence should be addressed. Tel: +1 858 534 5980; Fax: +1 8588227519; Email: sgutkind@ucsd.edu

Abstract

The rising incidence of human papillomavirus (HPV)-associated malignancies, especially for oropharyngeal cancers, has highlighted the urgent need to understand how the interplay between high-risk HPV oncogenes and carcinogenic exposure results in squamous cell carcinoma (SCC) development. Here, we describe an inducible mouse model expressing high risk HPV-16 E6/E7 oncoproteins in adults, bypassing the impact of these viral genes during development. HPV-16 E6/E7 genes were targeted to the basal squamous epithelia in transgenic mice using a doxycycline inducible cytokeratin 5 promoter (cK5-rtTA) system. After doxycycline induction, both E6 and E7 were highly expressed, resulting in rapid epidermal hyperplasia with a remarkable expansion of the proliferative cell compartment to the suprabasal layers. Surprisingly, in spite of the massive growth of epithelial cells and their stem cell progenitors, HPV-E6/E7 expression was not sufficient to trigger mTOR activation, a key oncogenic driver in HPV-associated malignancies, and malignant progression to SCC. However, these mice develop SCC rapidly after a single exposure to a skin carcinogen, DMBA, which was increased by the prolonged exposure to a tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA). Thus, only few oncogenic hits may be sufficient to induce cancer in E6/E7 expressing cells. All HPV-E6/E7 expressing SCC lesions exhibited increased mTOR activation. Remarkably, rapamycin, an mTOR inhibitor, abolished tumor development when administered to HPV-E6/E7 mice prior to DMBA exposure. Our findings revealed that mTOR inhibition protects HPV-E6/E7 expressing tissues from SCC development upon carcinogen exposure, thus supporting the potential clinical use of mTOR inhibitors as a molecular targeted approach for prevention of HPV-associated malignancies.

Introduction

Human papillomavirus (HPV) infection affects hundreds of millions of people around the world, and high-risk subtypes, primarily HPV-16 and HPV-18, are associated with multiple human malignancies, including cervical, anal, vulvar and oropharyngeal squamous cell carcinoma (SCC) (1). Around 90% of these HPV-associated cancers involve HPV-16 infection, which hence is considered the most important high-risk subtype (for a review see 1). The incidence of HPV positive (HPV+) head and neck squamous cell carcinomas (HNSCC), particularly in lesions arising in the oropharynx, is rising worldwide, highlighting the increasing importance of HPV infection for HNSCC (2,3). The number of HPV-associated cervical cancer cases has already begun to experience a dramatic decrease due to the implementation of routine cervical cancer screens, such as cytology-based
screening and HPV testing and the recent development of HPV vaccines (4-6). However, no decline in HPV+ HNSCC cases has been observed yet (3). It is expected that the current implementation of HPV vaccination programs in females and males will ultimately reduce the incidence of HPV+ HNSCC, but it may take decades before this impacts the current increasing trend in this HPV-associated malignancy. Thus, there is an urgent need to develop more effective treatment options to prevent and treat the current explosion in HPV+ HNSCC cases.

The HPV genome is a double-stranded DNA of 8 kb containing a non-coding region, named long control region (LCR) that includes key regulatory elements involved in viral DNA replication and transcription (1). The coding region is divided in two areas, the early region encoding E1-E7 proteins and the late region containing the genes that form the viral capsid (L1 and L2). Two proteins of the early region, E6 and E7 are highly conserved among high-risk HPV subtypes, and are the most important virally encoded proteins involved in cancer (7). The main property of E6 is its ability to induce p53 degradation by ubiquitination, while E7 activates E2F through its direct binding to pRb and releasing E2F from pRb complexes (reviewed in 1). However, whether E6/E7 expression is sufficient to initiate carcinogenesis is unclear.

The activation of the PI3K/Akt/mTOR signaling pathway plays a key role in many human malignancies (8,9). Specifically, we have recently shown that p53, a downstream target of mTOR, accumulates in multiple HPV-associated cancers, including HNSCC and cervical SCC, as well as anal SCC (10,11), suggesting that mTOR activation might contribute to these cancers. At the molecular level, the E7 protein can induce Akt activation through pRb binding (12,13), and E6 can stimulate mTOR in its complex 1 (mTORC1) by stimulating Akt activity (14) or by targeting TSC2 for degradation (15). These findings, and our prior reports indicating that mTOR inhibition prevents the growth of HPV+ HNSCC and cervical SCC, suggest that the mTOR signaling pathway represents a potential therapeutic target for HPV-associated cancers.

Transgenic mice carrying E6 and E7 proteins under the control of constitutive epithelial promoters such as cytokeratin 14 (cK14), cK5, cK10 and cK1 have been developed. Interestingly, the vast majority of these models do not develop SCC lesions spontaneously (16-19). Although these studies suggest that E6/E7 cannot induce cancer alone, a possibility exists that these viral genes may compromise mouse development and/or viability, as cK14/5 and cK10/1 are expressed as early as embryonic (E) days E9 and E15 respectively in developing mouse embryos (20). In this case, transgenic animal lines constitutively expressing high levels of E6/E7 may be selected against by promoting embryonic lethality, as we noticed when attempting to express E6 oncogenes using a constitutive cK5 promoter (21). Furthermore, the onset of HPV infection in humans is limited to adulthood, soon after the initiation of sexual activity with a prevalence between 2-44% in women and 16-32% in men (1,22). Thus, the development of mouse models expressing HPV oncoproteins in adults is expected to be more relevant than its persistent expression during embryo development or early postnatal stages.

To address this limitation, we developed a HPV-16 E6/E7 inducible mouse model bypassing the impact of these viral oncoproteins during development, therefore mimicking the clinical evidence. For this purpose, we expressed E6/E7 in the epithelium of adult animals using a tetracycline-inducible system combined with mice engineered to express the rtTA (Tet-on) transgene under the control of the cK5 promoter (21). Our results indicate that after doxycycline induction, both E6 and E7 are highly expressed in the skin, and that they promote rapid epidermal hyperplasia. Furthermore, E6/E7 expression bypassed the typical basal cell growth restriction, as there was a remarkable increase in cell proliferation in the suprabasal and parabasal epidermal cell layers. Surprisingly, in spite of this massive growth, mTOR activation was only observed in the suprabasal epidermal layers and no tumors were identified after more than one year of observation. However, these mice develop SCC lesions rapidly (9 weeks) after a single exposure to a typical carcinogen, DMBA, which was increased even further by the prolonged exposure to a tumor promoter, TPA (6 weeks). In this case, all HPV-E6/E7 expressing SCC lesions exhibited increased basal mTOR activation. Remarkably, rapamycin abolished tumor development when administered to HPV-E6/E7 mice prior to DMBA exposure. These findings suggest that mTOR inhibition may prevent cancer development caused by carcinogen exposure of pre-existing cells expressing HPV oncoproteins. Overall, our data support the potential clinical use of mTOR inhibitors as a molecular targeted approach for the prevention of HPV-associated malignancies.

Materials and methods

Mice

For the generation of Tet-E6/E7 mice, the open reading frame from the HPV-16 E6/E7 coding region was amplified from the HPV-16 genome and cloned downstream of the seven Tet-responsive element (Tet-O7) in a modified pBSRV vector. The fragment containing the expression cassette was isolated by Pmel digestion from vector DNA and purified for micro-injection into FVB/N mouse fertilized oocytes. Founders were identified for the presence of the transgene by screening genomic DNA from tail biopsies using a PCR reaction. The cK5-rtTA and transgenic FVB/N mice have been previously described (21). A similar number of wild-type animals as well as transgenic mice receiving doxycycline treatment were used as controls. The presence of the E6 and E7 transgenes were determined by PCR with the following primers: forward sequence 5’-TGATCTCTACTGTTATGGACAAATTAAGATG-3’, reverse sequence 5’-TTCTCCCGTGCTGTCGTC-3’, band approximately 125bp. The presence of the rtTA transgene was determined with the following primers: forward sequence 5’-CAGAGTCACGCGGCATGCG-3’, reverse sequence 5’-ATCTGAATGTACTTTTGCTCCATTGCGAT-3’, band approximately 350bp. Both male and female mice were used in the studies. Doxycycline was administered after birth in the food using grain-based pellets (Bio-Serv) at 6 kg. To quantify HF density, we used Lgr5-EGFP-IRES-CreERT2 mice (Jackson Laboratory, Stock 008875 (23). Lgr5 transgene was detected by PCR with the following oligos: Lgr5 wild-type (F) 5’-CTGCTCTGCTCGGCGAC-3’, Lgr5 wild-type (R) 5’-ATACCCGCACTTTTGGAC-3’, and Lgr5 mutant (R) 5’-GAACTTCAGGGTCACGCG-3’. Western blotting

Cells were collected in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with protease (protease inhibitor
cocktail, Sigma) and phosphatase inhibitors (1 mM Na₂VO₃ and 1 mM NaF). Protein quantification was performed by using the BCA Protein Assay Kit (Pierce Cat. 23225). Indicated amounts of protein were loaded onto 10% SDS-PAGE, transferred to PVDF filters and blotted using specific antibodies. Antibody detection was achieved by enhanced chemiluminescence (Amersham, GE Health Care) in a LAS-3000 system (Fujifilm, Japan). Results show a representative experiment of 3.

RNA reverse transcription and real-time quantitative PCR
Total RNA was obtained and reverse transcription was performed as previously described [24]. Changes in the mRNA expression were examined by Quantitative Real-Time PCR using an ABIPrism 7500 FAST Sequence Detection System (Applied Biosystems). cDNA was amplified using SYBR Green PCR Master Mix (Applied Biosystems) in the presence of specific oligonucleotides. The PCR conditions and quantification was performed as previously described [24]. Oligonucleotides used for amplification were:

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rn18s</td>
<td>5'-GAAACTGGAATG GCTCATTAAC-3'</td>
</tr>
<tr>
<td>Lgr6</td>
<td>5'-GGCTGAGTGA CAACTGACAC-3'</td>
</tr>
<tr>
<td>Lgr5</td>
<td>5'-GGCTGACGCT GTGGCTTCCG-3'</td>
</tr>
<tr>
<td>Sox9</td>
<td>5'-TCGGTGAAGAAC GTGGTGAAGAACG-3'</td>
</tr>
<tr>
<td>Tnc</td>
<td>5'-CAGGATGACT CATTGTCCTAC-3'</td>
</tr>
<tr>
<td>Nfat1</td>
<td>5'-GGCGGAAGAA GATGGTGCTGTC-3'</td>
</tr>
<tr>
<td>Runx1</td>
<td>5'-ACTCATC-3'</td>
</tr>
</tbody>
</table>

Tissue preparation, histology and immunohistochemistry
Tissues were fixed in Z-fix (zinc buffered formaldehyde, Anatech Ltd.) overnight and then transferred to 70% ethanol. Fixed tissues were embedded in paraffin, sectioned to a thickness of 4 μm and stained with hematoxylin and eosin or stained for Ki67, cK10, F4/80 and active phosphorylated forms of S6 (pS6). Histology slides were scanned with Scanscope Digital microscope (Aperio, Vista, CA).

Immunofluorescence
Sections were processed and stained as previously described [10]. Immunofluorescence analysis was performed using P–H2AX and cK15 antibodies. Nuclei were stained with Hoechst 33342 (Invitrogen). Tissue section images were taken with a Zeiss Axio Imager Z1 microscope equipped with an Apotome device (Carl Zeiss) using a Zeiss Plan APOCHROMAT 20×/0.8 na objective and Zen 2012 software (Carl Zeiss). Whole-mount fluorescent images were taken using an inverted Zeiss LSM 700 confocal microscope, coupled to Zen software 2010 (Carl Zeiss); 14–16 Z sections were taken with a Zeiss Plan APOCHROMAT 20×/0.8 na objective and 3D maximum projections were made with Zen software 2010 (Carl Zeiss). Final images were bright contrast adjusted with Zen 2012 (Carl Zeiss). Each immunostaining was repeated at least in five independent mice and three independent experiments and several fields were reviewed.

Tumor induction
Two-stage chemical induced carcinogenesis was performed essentially as previously described [25]. Briefly, mice were shaved in the back and tumors were initiated by the topical treatment with a single dose of DMBA (100 μg/200 μl in acetone) followed by the tumor promotion phase in which mice were treated twice weekly with TPA (12.5 μg/200 μl in acetone) for 21–24 weeks. The number and diameter of each tumor were measured weekly. Animals were euthanized after the last TPA treatment, and treated skin areas were preserved for further analyses.

Rapamycin treatment
Rapamycin treatment was administered after the initial shaving until the end of the study. Animals were randomly divided into two groups and treated with rapamycin (2.5 mg/kg daily) or an equal volume of diluent (an aqueous solution of 5.2% Tween-80 and 5.2% polyethylene glycol) through intraperitoneal injection.

Chemicals and antibodies
Antibodies against active form of S6 and γ-H2AX were from Cell Signaling Technologies (#2111 and #9718). Antibodies against HPV-16 E6, and E7 proteins were purchased from Santa Cruz Technology (sc-460 and sc-51951). Antibody against Ki67 was from Dako (M7249). Antibody for cK10 and cK15 were purchased from Biogenex (#950401 and #831901). Antibody for F4/80 was purchased from Ebioscience (14–4801). DMB and TPA were ordered from Sigma (D3254 and P8139) and rapamycin was from LC Labs (R-5000).

TUNEL assay
To detect apoptotic basal epithelial cells, a TUNEL assay was used in WT and E6/E7 transgenic mice (five mice each) with the ApoTag Plus fluorescein In Situ Apoptosis Detection Kit (S7110; Millipore, Billerica, MA) according to the manufacturer’s instructions. Skin epithelia was flat mounted as described, and co-stained with Hoechst 33342 (Invitrogen).

Hair follicle density quantification
To quantify the hair follicle (HF) density in mice, we crossed cK5-rtTA/Tet-E6/E7 or cK5-rtTA mice with an Lys5-GFP-Cre mice expressing a GFP fluorescence fusion protein only in the bulb of the HF. After birth, mice were treated with doxycycline for 3 months and then sacrificed. The number of HF in 10 different sections was quantified using confocal fluorescent microscope. Raw data were normalized according to the area measure (five mice per group).

HF growth cycle analysis
To study the first HF cycle in HPV-16 E6/E7 doxycycline inducible transgenic mice samples were excised from the dorsal skin at day 7 (early anagen), day 14 (late anagen), day 17 (catagen) and day 21 (telogen) after depliation, essentially as described [26]. The skin samples were fixed in Z-fix overnight and transferred to 70% EOH and embedded in paraffin. Then, 4 μm sections were cut and stained with hematoxylin and eosin (H&E), cK5-rtTA mice were used like controls.

Epidermal thickness measurement
The epidermal thickness was measured in sections stained with hematoxylin and eosin, tracing parallel lines between basal layer to the cornified layer using at least 10 measurements per image and at least 7 mice per group.

Statistical analysis
Data are presented as mean ± SE. Statistical significance was evaluated by Student’s t test using the GraphPad Prism 5.00 software. The statistical significance of differences was indicated in figures by asterisks as follows: *P ≤ 0.05, **P ≤ 0.01 and ***P ≤ 0.001.

Results
HPV-E6/E7 expression promotes uncoupling between differentiation and proliferation but it is not sufficient to initiate carcinogenesis
To evaluate the effects of HPV-16 E6/E7 expression in the epidermis, we conditionally expressed the E6/E7 genes in the same background under the control of Tet-O7 (Tet-E6/E7) (Figure 1A). The offspring from this cross followed a normal Mendelian distribution with no lethality. Remarkable, we observed a rapid and clear induction of hair loss together with an increase in skin thickness upon Tet activation using
doxycycline (Figure 1A). The expression of E6 and E7 was evaluated by Western blot analysis. We found that both oncoproteins are highly expressed in the skin of mice fed with doxycycline compared with control mice (Figure 1A). Similar results were obtained by RT-qPCR (data not shown).

Mice expressing E6/E7 developed a marked hyperplasia being the skin thickness almost double when compared with WT mice (Figure 1B). Unlike WT mice where few proliferative (Ki67+) cells are only found in the basal layer of the skin, E6/E7 inducible transgenic mice showed a notable increase in overall cell proliferation, and a decompartmentalization of the proliferative skin layers. Indeed, in these mice many cells actively divide in suprabasal skin layers that are characterized by the co-expression of the epithelial keratinocyte differentiation marker cytokeratin 10 (cK10). However, the hyperproliferation of basal keratinocytes was not accompanied by mTOR activation, which was still restricted to the suprabasal skin layers, following a pattern comparable to that in the WT mice (Figure 1C). This suggests that although E6/E7 expression can stimulate mTOR activity in cellular systems in vitro, it is not sufficient to activate mTOR in vivo. Similarly, mice expressing HPV-16 E6/E7 proteins did not develop carcinoma including the oral cavity, oropharynx and vagina, even after more than one year of observation. This finding suggests that the increase in the proliferative capacity of

Figure 1. Uncoupling between differentiation and proliferation is not sufficient to induce cancer. (A) Schematic representation of the animal model used to express HPV-16 E6/E7 in the basal epidermal compartment. Representative pictures of cK5-rtTA (control littermates) and cK5-rtTA/Tet-E6/E7 animals 3 months after doxycycline induction. Back skin from cK5-rtTA and cK5-rtTA/Tet-E6/E7 mice treated with doxycycline for 3 months was collected and then 50 µg of total cell lysates were analyzed by Western Blotting for E6, E7 and tubulin used as a loading control. (B) Fixed sections of wild-type (WT) and E6/E7 mouse back skin were collected after doxycycline treatment (3 months) and stained with H&E. The dotted line follows the location of the basal membrane. The bar represents 30 µm. Quantification of the thickness of the epidermis (N = 7). (C) IHC from representative skin samples acquired from control littermates and mice expressing E6/E7 doxy-treated for 3 months. The antibodies used are indicated.
the keratinocytes by HPV-16 E6/E7, particularly in the suprabasal layers, is not sufficient to promote tumorigenesis.

HPV-16 E6/E7 expression induces progressive hair loss linked to an increase in the number of apoptotic cells in the HF

To measure HF density we crossed our cK5-rtTA/Tet-E6/E7 animal mouse model with an LGR5-GFP-Cre reporter mouse (Figure 2A). In the skin, these mice express GFP-Cre only in the HF bulb enabling the visualization and measurement of hair density. Compound mice were fed with doxycycline food for 3 months, and HF density determined using littermate mice lacking Tet-E6/E7 as controls. E6/E7 expressing mice showed a clear decrease in the number of HFs (Figure 2A). We used the opportunity to explore the possibility that E6/E7 may affect the normal postnatal HF morphogenesis cycle. The development of

Figure 2. HPV-16 E6/E7 expression induces a progressive loss of hair linked with an increase in the number of apoptotic cells in the HF bulb. (A) Schematic representation of the animal model used to measure HPV-16 E6/E7 HF density. Briefly, cK5-rtTA/Tet-E6/E7 were crossed with Lgr5-GFP-Cre and fed with doxycycline food for 3 months. Experiments were performed using a fluorescent microscope. Representative pictures of cK5-rtTA/Lgr5-GFP-Cre (control littermates) and cK5-rtTA/Tet-E6/E7/Lgr5-GFP-Cre animals 3 months after doxycycline induction. Ear skin was used for the experiment. Quantification of HF/mm² of the epidermis (N = 5) is shown. (B) H&E staining of the first synchronous hair cycle from control littermates and Tet-E6/E7 mice at different ages. Postnatal day 7 represents anagen, day 16 late anagen, day 17 shows catagen and 21 telogen. (C) TUNEL assay shows positive nuclear staining for DNA fragmentation in apoptotic cells (green pseudocolored cells) together with nuclear Hoechst 33342 (red pseudocolor cells) in representative back skin sections from cK5-rtTA/Tet-E6/E7 mice versus WT controls (original magnification ×40). (D) cK5-rtTA/Tet-E6/E7 mouse skin quantification by RT-qPCR of transcriptional regulator and markers essential for HF stem cell maintenance and proliferation. N = 5 mice from each genotype (WT and cK5-rtTA/Tet-E6/E7 mice).
HF can be divided into eight distinct stages that are initiated during embryogenesis and culminate soon after animal birth. To analyze the consequences of HPV-16 E6/E7 expression during this first hair cycle, we selected four time points, 7, 14, 17 and 21 days of age. We observed that upon doxycycline treatment, all cK5-rtTA/Tet-E6/E7 transgenic mice showed a similar pattern compared to control littermates suggesting that HPV oncoproteins induction did not affect normal HF development (Figure 2B). Next, we explored whether E6/E7 may induce a premature HF cell death. For this purpose, we measured the number of apoptotic cells in our E6/E7 transgenic mice observing a clear increase in apoptosis in the HF bulb (Figure 2C). Furthermore, we tested HF stem cell progenitor markers and observed significant changes in the expression profiles of Lgr6, Runx1 and Tnc (Figure 2D). This evidence indicates that the progressive loss of HF may result from an increase in apoptosis and alteration in the HF stem cells compartment.

Expression of HPV-16 oncoproteins accelerates tumorigenesis in the context of a two-stage skin carcinogenesis model

As cK5-rtTA/Tet-E6/E7 mice were unable to develop spontaneous SCC, we next challenged our model using a classical two-stage carcinogenesis protocol. The carcinogen DMBA causes multiple mutations in the skin and initiates tumorigenesis, and exposure to multiple TPA treatments acts as a promoter (27). cK5-rtTA/Tet-E6/E7 mice were randomly divided into three groups, DMBA, TPA and DMBA-TPA (Figure 3A). Strikingly, we found that all E6/E7 transgenic mice treated with both DMBA and TPA rapidly developed tumor lesions (less than 6 weeks after DMBA exposure with an average of 15 tumors per mouse), at a time in which most control littermates did not exhibit any tumors. Furthermore, we observed that around 50% of HPV-E6/E7 transgenic mice treated with a single dose of DMBA also developed lesions that progress into SCC (1–3 lesions per mouse around week 9). In contrast, mice treated with TPA only did not develop any tumors (Figure 3B). These studies confirmed that HPV-16 E6/E7 does not initiate carcinogenesis even in the presence of a strong promoter, but revealed that the sole exposure to carcinogen is sufficient to induce tumors, albeit with limited efficiency. Furthermore, the concurrent exposure to carcinogen and tumor promoting stimulation resulted in an early explosive cancer development.

Remarkably, DMBA alone was sufficient to induce squamous carcinogenesis in mice expressing E6/E7, although much fewer than when TPA was used as tumor promoter (Figure 4A). The histopathologic analysis of the tumor lesions demonstrated that the E6/E7 expressing mice exposed to DMBA-TPA developed fully infiltrative SCC lesions compared with E6/E7 mice treated only with DMBA, in which some occasional infiltrative areas were founded. On the other hand, control mice treated with the same protocol did not develop SCC (Figure 4B and C). Detailed analysis of the skin lesions indicated that WT mice treated with DMBA-TPA only developed a small number of papillomas, and retained a compartmentalized proliferation (Ki67+), which is restricted to the basal layer, while mTOR activation (pS6+) was observed in the suprabasal layer (Figure 4B). In contrast, the SCC lesions in our cK5-rtTA/Tet-E6/E7 mice also exhibit Ki67+ expression in suprabasal layers, in the same compartment that shows mTOR activation. These data suggest that a coordinated activation of cellular proliferation (Ki67) and mTOR activation (pS6) might be important for tumor promotion. The latter may facilitate increased macromolecular synthesis and thereby increase tumor mass (8,9) (Figure 4C). We then tested whether H-Ras, which is usually a hallmark of DMBA-TPA induced skin tumors (28), was also mutated in HPV-16 E6/E7 expressing tumors. Skin lesions showed missense mutation in H-Ras exon 2 (codon 61) in 100% of the lesions including papillomas and SCC (N = 8, data not shown). In contrast, we did not find any mutations in N-Ras (exons 1, 2), K-Ras (exons 1, 2), and PIK3CA (exons 9 and 20), supporting that the expression of HPV-16 oncoproteins does not modify the nature of the carcinogen associated cancer drivers. Of interest, no mutations in tp53 (exons 4, 5, 7, 8–9) was found in our HPV-16 E6/E7 lesions, in contrast to frequent tp53 mutations in DMBA-TPA SCC in control mice (29) as we reported in our prior study in SCC lesions (25).

Rapamycin treatment abolishes SCC induced by DMBA-TPA

In prior studies, we have shown that mTOR inhibition with rapamycin can induce the regression of established skin SCC lesions promoted by the DMBA-TPA carcinogenesis model (25). These findings have been confirmed and extended by multiple laboratories (30–32). Based on this information, we next asked if rapamycin can be used for the prevention of carcinogen-induced SCC development in HPV-16 E6/E7 expressing tissues. For these studies, we first promoted the expression of E6/E7 using doxycycline, and then treated the mice with rapamycin (2.5 mg/kg daily) after shaving prior to DMBA treatment until the end of the protocol (Figure 5A). Noteworthly, we found that none of the mice treated with rapamycin developed SCC lesions after 21 weeks compared with E6/E7 expressing mice not treated with rapamycin (Figure 5B and C). We also analyzed the skin thickness and found that mTOR inhibition was able to block epidermal hyperproliferation in response to DMBA-TPA exposure (Figure 5D). Surprisingly, rapamycin had very limited impact on the fraction of basal and suprabasal Ki67+ proliferative cells in E6/E7 expressing mice, suggesting that rapamycin does not act primarily by reducing overall cell proliferation (Figure 5E). E6/E7 expressing mice treated with rapamycin showed mTOR activation only in the suprabasal layers of the skin comparable to E6/E7 control mice and in contrast to mTOR pathway activation in DMBA-TPA induced premalignant (papillomas) and SCC lesions.

Rapamycin blocks DNA damage avoiding the DMBA carcinogenic effect

The remarkable absence of SCC lesions in HPV-16 E6/E7 expressing mice after DMBA-TPA treatment prompted us to explore whether mTOR inhibition interfere with the initial carcinogenic process in E6/E7 expressing cells. E6/E7 oncoproteins themselves are able to induce DNA damage and chromosome instability in vivo (33). To test whether mTORC1 inhibition may interfere with DMBA-induced carcinogenesis, we pre-treated mice expressing HPV-16 E6/E7 with rapamycin and then applied DMBA (Figure 6A). After 2 h, we collected back skin and analyzed for DNA damage using γ-H2AX, which detects double-strand DNA breaks (34). Interestingly, we found that rapamycin was able to reduce the number of cells staining positive for the active form of γ-H2AX after DMBA treatment in HPV-E6/E7 expressing tissues, supporting that mTOR blockade could protect against DNA damage in HPV+ cells caused by carcinogen exposure (Figure 6B). Langerhans cells (LC), a subtype of macrophages, may increase epithelia DNA damage after DMBA treatment by producing active metabolites (35). However, no differences were observed in LC numbers in tissues treated with DMBA with or without rapamycin (Figure 6C). These findings suggest that rapamycin may prevent the carcinogenic effect of DMBA by blocking DNA damage in E6/E7 expressing cells directly.
The rising incidence of HPV-associated malignancies has highlighted the urgent need to understand how the interplay between high risk HPV oncogenes and carcinogenic exposure results in SCC development. Here, we describe a novel HPV-16 E6/E7 inducible mouse model under the control of cK5 promoter.

Our mouse model rapidly develops skin hyperplasia, aligned with previous findings using HPV constitutive mice models that display similar phenotype at more advanced age or lower percentage (19,36,37). This rapid development of skin hyperplasia involves the increase in the number of proliferative cells in the basal epithelial layer, which occurred concomitant with a

Discussion

The rising incidence of HPV-associated malignancies has highlighted the urgent need to understand how the interplay between high risk HPV oncogenes and carcinogenic exposure results in SCC development. Here, we describe a novel HPV-16 E6/E7 inducible mouse model under the control of cK5 promoter.

Our mouse model rapidly develops skin hyperplasia, aligned with previous findings using HPV constitutive mice models that display similar phenotype at more advanced age or lower percentage (19,36,37). This rapid development of skin hyperplasia involves the increase in the number of proliferative cells in the basal epithelial layer, which occurred concomitant with a
remarkable expansion of the proliferative cell compartment in the suprabasal layers. Surprisingly, this decompartimentalization of the proliferative layers and clearly increased cell growth was not sufficient to induce cancer. Furthermore, unlike previous studies suggesting that the E6 oncoprotein may induce mTORC1 activity directly (14) or through TCS2 degradation (15), we found that expression of HPV-16 E6/E7 did not induce mTOR activation, as judged by no pS6 accumulation in the basal compartment of the epithelial cells in vivo. Thus, the inducible expression of HPV-16 E6/E7 and thus the activation of their intervening molecular mechanisms, including p53 and pRb inhibition, may promote the upregulated growth of epithelial cells and their stem cell progenitors, but is not alone sufficient to trigger mTOR activation and their malignant conversion to SCC.

It is noteworthy that our system has a progressive hair loss linked to an increase in the apoptotic cells in the HF. In addition, we also found changes in HF stem cell populations as indicated by the analysis of specific markers. For example, we observed an increase in Runx1 and Tnc together with a decrease of Lgr6. Interestingly, Runx1 and Tnc play an important role in the correct homeostasis of the HF suggesting that an aberrant increase may induce HF stem cell exhaustion (38,39). Furthermore, Lgr6 positive cells have an important role in long-term wound repair, including new HF formation (40). Taken together, our data suggest that specific expression of HPV-16 oncogenes in the HF and the surrounding epithelia may affect interfollicular and follicular stem cell subsets inducing progressive apoptosis, and loss of their ability to maintain and regenerate all required HF structures.

While expression of HPV-16 E6/E7 proteins was not able to promote the development of spontaneous SCC, we then asked if these proteins would sensitize to cancer development after
carcinogen exposure. Using the well-established DMBA-TPA carcinogen-tumor promoter model, we found that HPV-16 E6/E7 transgenic mice develop tumors quite rapidly, almost in an explosive fashion after DMBA-TPA treatment, especially when compared with control mice that did not develop tumors at the time that the experiment had to be terminated due to the excessive tumor burden in E6/E7 mice. Interestingly, in our model all mice expressing E6/E7 oncoproteins were able to develop SCC supporting the additive effect that HPV-16 expression plays in tumor carcinogenesis. In contrast, in previous constitutive E6/E7 mouse models used in skin carcinogenesis studies no increased conversion of papillomas to carcinomas was observed (36), or the number of SCC and percentage of HPV-E6/E7 mice developing SCC was low (41). We also found that a single dose of DMBA was sufficient to induce SCC in 50% of E6/E7-expressing mice. Thus, these findings suggest that only few oncogenic hits may be sufficient to induce cancer in E6/E7 expressing cells.

Figure 5. Long-term rapamycin treatment protects against malignant transformation mediated by the chemical induced carcinogenesis protocol in HPV-16 E6/E7 transgenic mice. (A) Schematic representation of long-term rapamycin treatment combined with DMBA-TPA (D-T) chemical induced carcinogenesis in the HPV-16 E6/E7 inducible animal mouse model. Briefly, after initial shave mice were treated with 2.5 mg/kg of rapamycin (Rapa) daily and in parallel DMBA-TPA protocol was performed as described in materials and methods. (B) Representative picture of cK5-rtTA/Tet-E6/E7 mouse treated with rapamycin compared with cK5-rtTA/Tet-E6/E7 mice treated with vehicle control. (C) Graphics depict the impact in disease free survival and average of lesions per mouse of prolonged rapamycin treatment in DMBA-TPA HPV-16 E6/E7 transgenic mice (red line) versus DMBA-TPA HPV-16 E6/E7 mice treated with control vehicle (blue line). (D) Quantification of epidermal thickness was performed as in Figure 1 comparing E6/E7 transgenic mice (control cohort) with DMBA-TPA chemical-induced carcinogenesis in HPV-16 E6/E7 animal mice with or without long-term rapamycin treatment. (E) Cross-sections, evaluating histologically by H&E, pS6 and Ki67 staining in DMBA-TPA-induced skin lesions with or without rapamycin treatment.
This observation is aligned with recent cancer genome sequencing efforts that revealed mutations in genes encoding PI3K, K-Ras and PTEN, which converge in mTOR pathway induction (42), are a frequent event in human HPV+ HNSCC (43–45). In agreement with this, in our animal model we observed that mTOR activation was restricted to the suprabasal non-proliferating epithelial cell layers even when E6/E7 provoked a clear hyperplasia. Instead, mTOR was activated in the basal and invading epithelial layers in all SCC tumors examined. This supports further our prior results documenting an important role for mTOR in HPV-associated human cervical and oral cancers (10).

These findings prompted us to test whether rapamycin, which blocks mTOR as part of its complex mTORC1 (8,9), can be used to prevent HPV-16 associated SCC. Remarkably, we found that long-term treatment with rapamycin initiated prior to carcinogen exposure was able to abolish tumor formation by DMBA-TPA in HPV-E6/E7 expressing mice. Certainly, further studies may be required to clarify whether rapamycin treatment can also prevent the development of cancer lesions when administered after carcinogen exposure. Interestingly, our histological analysis reflected a clear restoration of the normal mTOR activation pattern compared with control untreated E6/E7 transgenic mice exposed to DMBA-TPA. However, rapamycin...
 did not diminish the E6/E7 induced hyperploproliferation, thus pro-
viding the first evidence that mTOR inhibition can protect from
carcinogen-induced malignant conversion in the absence of a
cytostatic effect.

In this regard, we observed that rapamycin protects against
dNA damage in response to DMBA, as judged by the finding that
mTOR inhibition reduced the number of P–γ-H2AX foci in epithe-

cial cells of E6/E7 expressing mice after DMBA expose. While the
precise mechanism by which mTOR inhibition diminishes DNA
 damage caused by DMBA in E6/E7 expressing cells is unclear, es-
pecially considering that rapamycin does not decrease the
elevated cell proliferation in HPV-16 oncogene expressing mice,
our findings may have important therapeutic implications.
Specifically, taking into account that around 90% of cervical can-
cers, 50% of penile/vulvar carcinomas and 20% of oropharyngeal
cancers are caused by HPV infection (1–3), and that there is an
increasing awareness that persistency of HPV infection is a risk
factor for cancer development when expose to other cofactors,
such as tobacco carcinoma (46,47). This rises the exciting possi-
bility of developing new therapeutic approaches for patient sub-
populations with high HPV infection prevalence (1–3). Indeed, the
observation that rapamycin protects from DNA damage and can-
cer initiation in the highly proliferative HPV-16 E6/E7 cells suggest
that mTOR inhibition may represent a suitable chemopreventive
strategy to protect infected tissues from cancer development.

In addition, our findings may provide a possible thera-

tic option for HPV+ HNSCC patients who are often treated with

surgery combined with radiotherapy, but it is possible that resid-
ual non-neoplastic HPV+ infected cells may already be present
beyond the surgical margin and irradiated field. These cells may
be more prone to cancer development after carcinogen expo-
sure due to their high proliferation rate and disabled tumor sup-
pressive functions. In this scenario, mTOR inhibition by the use
of rapamycin or its analogs (rapalogs) or more recently devel-
oped mTOR kinase inhibitors may prevent the appearance of
second primary lesions, or delay cancer recurrence at the pri-
mary disease site.

In summary, we show here the oncogenic effect of E6/E7 pro-
teins in an inducible animal mouse model exposed to DMBA-
TPA, and how these viral proteins induced aberrant proliferation
and hair loss but are not sufficient to promote mTOR activation
and spontaneous SCC development. However, HPV-16 E6/E7
expressing tissues are highly susceptible to cancer progression
upon carcinogenic exposure, which can be prevented by mTOR
inhibition with rapamycin. These finding support that mTOR
inhibition may represent a promising therapeutic option to pre-
vent HPV-associated human malignancies in high risk patient
populations, as well as cancer recurrence and appearance of
second primaries in prior treated HPV+ SCC patients.

Conflict of Interest Statement: None declared.

References
3. Chaturvedi, A.K. et al. (2011) Human papillomavirus and rising or-
4294–4301.
5. Pahud, B.A. et al. (2015) The expanded impact of human papillomavi-
6. Richardson, L.A. et al. (2015) HPV DNA testing with cytology triage in
cervical cancer screening: Influence of revealing HPV infection status.
Cancer Cytopathol., 123, 745–54.
7. Ghiotti, R. et al. (2010) The biological properties of E6 and E7 onco-
8. Sabatini, D.M. (2006) mTOR and cancer: insights into a complex rela-
10. Molinolo, A.A. et al. (2012) mTOR as a molecular target in HPV-asso-

2558–2568.
and HPV oncoproteins by targeting mTOR signaling with metformin in
8, 197–207.
12. Pim, D. et al. (2005) Activation of the protein kinase B pathway by the
HPV-16 E7 oncoprotein occurs through a mechanism involving interac-
tion with PP2A. Oncogene, 24, 7830–7838.
13. Menges, C.W. et al. (2006) Human papillomavirus type 16 E7 up-regu-
lates AKT activity through the retinoblastoma protein. Cancer Res., 66,
5535–5539.
protein activates mTORC1 signaling and increases protein synthesis. J.
Virol., 84, 9398–9407.

turnover in the presence and absence of HPV16 E6. Genes Cells, 13,
16. Auewarakul, P. et al. (1994) Targeted expression of the E6 and E7 onco-
genese of human papillomavirus type 16 in the epidermis of transgenic
mice elicits generalized epidemideral hyperplasia involving autocrine
17. Carraresi, L. et al. (2001) Thymic hyperplasia and lung carcinomas in
a line of mice transgenic for keratin 5-driven HPV16 E6/E7 oncogenes.
Oncogene, 20, 8148–8153.
18. Schreiber, K. et al. (2004) Strong synergy between mutant ras and
HPV16 E6/E7 in the development of primary tumors. Oncogene, 23,
3972–3979.
cytotoxic T-lymphocyte epitope in transgenic mice expressing the
E7 and E6 oncogenes of human papillomavirus type 16. J. Virol., 71,
3998–4004.
20. Lu, H. et al. (2005) Type II keratins precede type I keratins during early
thelial compartment that includes the stem cells is sufficient to pro-
suppressor pathway in skin stem cells initiates basal-cell carcinogen-
25. Amorphphilotham, P. et al. (2008) Inhibition of Mammalian target of
rapamycin by rapamycin causes the regression of carcinogen-induced
27. Rundhaug, J.E. et al. (2010) Molecular mechanisms of mouse skin

28. Stowers, S.J. et al. (1987) The role of oncogenes in chemical carcinogen-
esis. Environ. Health Perspect., 75, 81–86.

and HPV oncogenes by targeting mTOR signaling with metformin in
8, 197–207.