**Hedgehog Signaling Drives Radioresistance and Stroma-Driven Tumor Repopulation in Head and Neck Squamous Cancers**

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**Abstract**
Local control and overall survival in patients with advanced head and neck squamous cell cancer (HNSCC) remains dismal. Signaling through the Hedgehog (Hh) pathway is associated with epithelial-to-mesenchymal transition, and activation of the Hh effector transcription factor Gli1 is a poor prognostic factor in this disease setting. Here, we report that increased GLI1 expression in the leading edge of HNSCC tumors is further increased by irradiation, where it contributes to therapeutic inhibition. Hh pathway blockade with cyclopamine suppressed GLI1 activation and enhanced tumor sensitivity to radiotherapy. Furthermore, radiotherapy-induced GLI1 expression was mediated in part by the mTOR/S6K1 pathway. Stroma exposed to radiotherapy promoted rapid tumor repopulation, and this effect was suppressed by Hh inhibition. Our results demonstrate that Gli1 that is upregulated at the tumor–stroma intersection in HNSCC is elevated by radiotherapy, where it contributes to stromal-mediated resistance, and that Hh inhibitors offer a rational strategy to reverse this process to sensitize HNSCC to radiotherapy. *Cancer Res; 74(23): 7024-36. ©2014 AACR.*

**Introduction**
Head and neck squamous cell carcinoma (HNSCC) remains a devastating malignancy with approximately 50% five-year overall survival (OS) rates (1). During and immediately after treatment, a process called accelerated repopulation occurs in HNSCC whereby a few surviving cells rapidly proliferate leading to tumor recurrence (2) but this process still requires elucidation.

One proposed mechanism for how tumor epithelial cells become resistant to therapy is transformation into a more mesenchymal phenotype known as epithelial-to-mesenchymal transition (EMT; ref. 3). Recent reports have linked hedgehog pathway (HhP) signaling to EMT induction following (chemo)radiotherapy in gastric (4, 5), esophageal (6), colorectal (7), ovarian (8), and endometrial (9) cancers. Transcriptional activation of pro-EMT genes by Gli1 can lead to mesenchymal changes (i.e., spindle cell shape, pseudopodia formation) and increased tumor invasiveness. Targeting the HhP or its downstream EMT targets can lead to reversal of the mesenchymal (stem cell "like") phenotype and restoration of chemoradiotherapy sensitivity. A retrospective analysis of RTOG 90-03 demonstrated that patients with HNSCC who expressed high levels of Gli1 before treatment had poor local control (LC) rates, more distant metastases, and worse OS; when correlated with those patients also expressing high levels of EGFR, these patients had the worst outcomes overall (10). We previously demonstrated in HNSCC xenografts that EGFR inhibition leads to upregulation of the HhP in EGFR-dependent, human papilloma virus (HPV)-negative HNSCC (11). The interrelatedness of these different pathways suggests that inhibition of both EGFR and the HhP may be necessary to maximize the effectiveness of radiotherapy in HNSCC.

The role HhP plays in radiotherapy resistance and tumor repopulation in HNSCC remains unknown. We hypothesize that radiotherapy similarly modulates the HhP in EGFR-dependent tumors such as HNSCC and could be a mechanism for resistance. Our work examines intratumor regional differences in HhP signaling, how radiotherapy modulates the HhP, and the effect of HhP inhibition with cyclopamine on HhP gene expression, cell survival, and EMT phenotype in the context of radiotherapy. We propose an alternative mechanism where radiotherapy-induced Gli1 nuclear (nGli1) translocation and GLI1 gene expression is mediated through the mTOR pathway. Finally, we show that the tumorigenicity of...
serially cotransplanted tumor and stroma cells increases after radiation and can be abrogated by HhP inhibition.

Materials and Methods

Cell lines and drugs
HN11 and TU167 HNSCC cell lines have been previously described (11–17). siRNA (AKT, S6K1) was completed in serum-free media using 1.3 μL/mL Dharmacet 1 (Thermo Scientific) and 100 nmol/L siRNA. GLI1 silencing was completed using doxyxycycline-inducible (0.5 μg/mL) pTRIPZ lentiviral constructs (RH5469-99637632) that introduced shRNA as previously described (11). Cells were treated with 1 μmol/L cycloamine (LC Laboratories), 1 μmol/L Rapamycin (LC Laboratories), or 1 μmol/L PF-4708671 (Sigma-Aldrich) for a minimum of 12 hours before irradiation. Generation of chronically irradiated cell lines were performed by irradiating cells at 50% to 60% confluency daily at 2 Gy/day for 5 days. Cells were allowed to recover for 1 to 2 weeks before initiating the second irradiation cycle. Cells were allowed 1 to 2 weeks for recovery before initiating any studies.

Irradiation
Cells and animals were irradiated using a RS-2000 (Rad Source Technologies, Inc). Cells were irradiated with 2 or 10 Gy using a 160 KVp source, at 25 mAmp, and at a dose rate of 1.15 Gy/minute. Mice were irradiated using a customized animal restraint exposing only the head/neck region of the mouse. Radiation dose and dose rate were calculated empirically for the RS-2000.

Colony formation assay
Serial dilutions were performed and single cells were plated on 6-well plates. Cells were allowed to adhere for 16 hours before drug treatment and irradiated with a single dose (0–10 Gy) at least 8 hours after drug treatment. Cells were washed 3 days after initial drug exposure and grown for an additional 7 to 10 days until visible colonies on the control plate could be measured. Cells were fixed in 10% formalin solution, stained with 0.5% crystal violet and washed with cold tap water. Threshold for positive proliferative colonies were ≥50 cells. Experiments were replicated either two or three times to generate the radiotherapy dose response curves or the radiotherapy–cycloamine cell sensitivity assay curves, respectively.

Human xenograft therapeutic studies
HN11 and TU167 parental cell lines were implanted into athymic nude mice using an established floor-of-the-mouth (FOTM) method as previously described (18). Seventy-five thousand cells were resuspended 1:1 in a DMEM/FBS and Matrigel mixture and kept on ice until tumor implantation. A 4-arm study was performed to generate efficacy data. Arms used for the parental tumors were: (i) control; (ii) cycloamine 40 mg/kg by oral gavage everyday for 15 days excluding weekends; (iii) radiotherapy 5 Gy once per week for 3 weeks; or (iv) both. When the average tumors reached 100 mm, mice were distributed into their respective groups. Tumor size was evaluated twice per week using the following formula: volume = [length × width^2]/2. For pharmacodynamic analysis on experimental day 10, tumors were extracted 1 hour after drug administration and/or 48 hours after radiotherapy and divided into one of two groups: formalin fixation or FACS.

We performed a pharmacodynamic study by implanting HNSCC into FOTM and assigning them to one of the four treatment groups (Supplementary Fig. S3A). After receiving the prescribed therapy, tumors were collected from each group, mouse stroma (anti-mouse H2Kd, BioLegend) and human tumor cells (anti-EpCAM; Cell Signaling Technology) were separated by FACS, recombined in a 1:3 ratio (5,000:15,000) with untreated tumor cells, and implanted onto the flanks of athymic nude mice. Tumor regrowth was assessed at 3 and 6 weeks after implantation and all tumors were excised at 6 weeks for analysis.

Statistical analysis
Two-tailed, student paired t test (GraphPad Prism) was used to compare groups for both in vitro and in vivo experiments. A majority of cell-based experiments were replicated three times with a minimum of three replicates per experiment. In vivo animal experiment statistics were performed on a minimum of 5 animals per group for comparison. Statistical comparisons were performed by group. Statistical values were shown only if P < 0.05.

Additional RNA, protein, and immunohistochemical methods are discussed in Supplementary Materials and Methods.

Results

Hhp upregulation in the growing edge of HNSCC tumors
Utilizing our established patient-derived xenografts (PDX) model system (18), mouse stroma, tumor margin, and tumor center were isolated by laser capture microdissection (LCM) from control or irradiated flash-frozen flank tumors (Fig. 1A), and evaluated for species-specific GLI1 expression. In nonirradiated tumors, hGLI1 at the tumor center was 4.7 times higher compared with the tumor center (P = 0.004; Fig. 1B). When comparing irradiated tumor to nonirradiated tumor, normalized hGLI1 was significantly upregulated at the margin (8.8×; P = 0.02) compared with the center (2.7×; P = 0.2). Human sonic hedgehog (SHH), was also significantly upregulated at the tumor margin by 9.2 times in irradiated tumors (P = 0.02). Furthermore, mGLI1 demonstrated a nonsignificant trend toward upregulation (4.1×) in the mouse stroma (Fig. 1C). Results were performed from a single experiment in triplicate. We examined Gl1 expression following radiotherapy by immunohistochemistry (HIC; Fig. 1D) at a single time point that demonstrated upregulation of Gl1 protein but was not differentially increased between the margin and center.

Radiotherapy upregulates the Hhp
We used two established human epithelial HPV-negative HNSCC cell lines collected from patients with FOTM SCC (19). HN11 from a primary tumor, and TU167 from a relapsed subject to confirm our in vivo observations. We determined whole transcriptome expression differences quantitatively and found dissimilar gene expression levels in the hedgehog, epithelial cell markers, and mTOR pathways (Supplementary Fig. S1). Consistent with being a relapsed, more transformed cell type, TU167, had decreased epithelial cell markers and...
increased HhP and mTOR pathway expression. After radiotherapy, GLI1 as well as downstream EMT genes ZEB2 and SNAI1 were acutely and significantly upregulated at 24 and 48 hours (Fig. 2A).

EMT is a potential mechanism explaining why LC can be compromised due to accelerated repopulation following prolonged genotoxic stress. We explored whether chronic irradiation modulated HhP and EMT-associated genes. Chronically irradiated HN11 demonstrated upregulation in HhP (GLI1, GLI2) and EMT (ZEB2, VIM) genes compared with the parental line. In TU167, we observed upregulation of the EMT pathway genes GLI2 and TWIST1 (Fig. 2B). FACS of chronically irradiated HN11 demonstrated increased VIM expression (<4% of the total population), indicating that a small fraction of these cells have assumed a mesenchymal phenotype (Fig. 2C). VIM was higher in parental TU167 compared with HN11 and was not significantly upregulated by sustained radiotherapy.

**Effect of cyclopamine on radiotherapy-mediated GLI1 upregulation**

To test whether radiotherapy-induced GLI1 upregulation could be suppressed through HhP inhibition, we used the Smoothened receptor inhibitor cyclopamine. Pretreatment with 1 μmol/L cyclopamine followed by radiotherapy significantly suppressed radiotherapy-induced GLI1 in HN11 but not in TU167 (Fig. 3A). Using shGLI1, we documented partial GLI1 suppression in HN11 but not in TU167 following radiotherapy (Supplementary Fig. S2). Cells pretreated with 1 μmol/L cyclopamine demonstrated no significant change in nGLI1 content at 1, 2, and 4 hours. Despite inhibition with cyclopamine, we still observed a 2-fold increase in nGli1 in HN11 and TU167 (Fig. 3B, first and third panels) after radiotherapy. Similarly, cytoplasmic Gli1 (cGli1) was similarly increased following irradiation and suppressed with cyclopamine pretreatment. Interestingly, cGli1 levels were unable to be fully suppressed following irradiation and suppressive effects were similar to those seen with GLI1 suppression.
orthotopic HNSCC model

In vivo effect of radiotherapy and cyclopamine in an HN11 cell line that induces HhP- and EMT-associated genes were evaluated by qRT-PCR at 0, 24, and 48 hours. B, chronically irradiated cells compared with their original parental cell lines were evaluated for HhP or EMT-gene expression by qRT-PCR. C, expression levels of vimentin were evaluated between parental and chronically irradiated cells by FACS. Statistically significant findings are denoted: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 2. Radiotherapy (RT) induces in vitro GLI1 expression. A, mechanistic studies were performed on two HN11 cell lines, TU167 and HN11. GLI1, ZEB2, and SNAI1 expression was evaluated by qRT-PCR at 0, 24, and 48 hours. B, chronically irradiated cells compared with their original parental cell lines were evaluated for HhP or EMT-gene expression by qRT-PCR. C, expression levels of vimentin were evaluated between parental and chronically irradiated cells by FACScan. Statistically significant findings are denoted: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Despite pretreatment with cyclopamine. This effect was more pronounced in TU167 compared with HN11 (Fig 3B, second and fourth panels). Treating with higher (but not clinically achievable) concentrations of cyclopamine (10 μmol/L) led to significant inhibition of radiotherapy-induced GLI1 expression suggesting a dose–response effect (data not shown).

Using colony formation assay (CFA), the primary cell line HN11 was significantly more radiosensitive than TU167 at lower radiotherapy doses (Fig. 3C). We then evaluated whether cyclopamine inhibition could enhance cytotoxicity at single radiation dose. Combinatorial therapy with cyclopamine and radiotherapy demonstrated a significant effect compared with single modality therapy or no treatment (control vs. 1 μmol/L + radiotherapy; TU167, P = 0.003; HN11, P = 0.0006) with the greatest response seen in HN11 (Fig. 3D) compared with TU167 (Fig. 3E). Chronically irradiated HN11 cells exposed to 1 μmol/L cyclopamine for 12 hours demonstrated a trend towards suppressing GLI1 (1.7 ± 0.3–1.5 ± 0.2 n.s.), GLI2 (1.7 ± 0.03–1.1 ± 0.02, P = 0.0025), and VIM (3.0 ± 0.2–1.1 ± 0.1, P = 0.0043). No trend was observed for TU167 for GLI1, GLI2, or VIM. These findings demonstrated that chronically irradiated HN11 cells that induce HhP- and EMT-associated genes were responsive to cyclopamine inhibition.

In vivo effect of radiotherapy and cyclopamine in an orthotopic HNSCC model

To further our in vitro observations, we evaluated tumors in vivo, combining cyclopamine and radiotherapy. Mice were implanted in the FOTM (Fig. 4A) with either HN11 or TU167 cell lines and randomized to one of the four treatment arms (Fig. 4B). Radiotherapy ± cyclopamine led to a reduction in tumor volume, compared with control or cyclopamine alone in HN11 and TU167 parental tumors (Fig. 4C and D). Of note, cyclopamine had no effect on inhibiting tumor growth in HN11 and, similar to the control arm, the tumor continued to grow. It has not been reported in the literature that cyclopamine stimulates tumor proliferation and, given the size of the error bar compared with the control arm, is unlikely to be suggestive of the above hypothesis. In HN11, tumor volumes responded dramatically to radiotherapy (67% reduction) or the combination treatment (77% reduction), which correlated well with their in vitro susceptibility to radiotherapy (Fig. 4C). In TU167, we observed smaller tumor sizes in both the radiotherapy alone and combinatorial therapy arms (radiotherapy: 37% reduction compared with control; cyclopamine + radiotherapy: 49% reduction compared with control; Fig. 4D). TU167-irradiated tumors had a propensity for developing cystic, cavitated lesions (radiotherapy: 3/10 tumors; dual therapy: 8/10; Supplementary Fig. S3A) beginning around day 25. At day 45, representative cystic and noncystic TU167 tumors were harvested from euthanized mice for analysis. The noncystic tumors by hematoxylin and eosin (H&E) were solid throughout compared with the cystic tumors, which were only a thin rim of tissue (Supplementary Fig. S3B). Tumor proliferation evaluated using Ki67 for the noncystic tumors revealed that control (H-score: 80) or cyclopamine-treated (H-score: 80) tumors
Figure 3. Effect of cyclopamine on radiotherapy induced GLI1 gene expression and cytotoxicity. A, cells were pretreated with 1 μmol/L cyclopamine 8 hours before receiving radiotherapy. Cells were harvested at specific time points and GLI1 expression evaluated by qRT-PCR. B, nuclear extracts were generated as previously described from cells/C61 μmol/L cyclopamine/C6radiotherapy. (Continued on the following page.)
Figure 4. In vivo effects of cyclopamine and radiotherapy (RT). A, figure describing location of orthotopic FOTM tumor implanted in athymic nude mouse. B, schema describing cyclopamine and/or radiotherapy dose and duration for animal tumor growth studies. Tumor growth for orthotopically implanted FOTM HN11 (C) and TU167 (D) parental tumors was assessed at 28 days posttreatment initiation.

(Continued.) nGLI1 accumulation was evaluated by immunoblot and temporal fold changes described. C, CFA radiotherapy dose curves were generated from serially diluted cells grown on 6-well plates treated 24 hours after adhesion with 0–10 Gy irradiation. Cells were fixed, stained, and counted approximately 8–10 days after radiotherapy. CFAs were performed to assess the effect of cyclopamine and 2 Gy radiotherapy on cell growth and survival on HN11 (D) and TU167 (E). Eight hours before irradiation, cells were pretreated with 1 μmol/L cyclopamine in low serum media. Low serum media was chosen as it more closely represents the tumor milieu. Furthermore, use of low serum media before drug treatment will prevent exogenous growth factors from competing with the effects of cyclopamine. Statistically significant findings are denoted: *, P < 0.05; **, P < 0.01; ***, P < 0.001. n.s., nonsignificant.

Tumor and Stromal Dependence on Hedgehog Pathway Signaling

demonstrated the highest amount of Ki67 staining compared with radiotherapy (H-score: 65) or dual therapy–treated (H-score: 40) tumors. The dual therapy–treated tumors demonstrated the most reduced Ki67 staining both in the periphery and core (Supplementary Fig. S3C). This suggests that there was both increased necrosis and decreased proliferation in radiotherapy and dual treated TU167 tumors, in spite of small volumetric differences. In comparison, the cystic versus noncystic radiotherapy treatment tumors suggest less proliferating cells in the cystic tumors and growth is restricted to the tumor margin/edge.

Tumors were harvested for pharmacodynamic analysis 10 days into treatment and immunohistochemical staining performed for EGFR, Gli1, ALDH1, and BMI1. EGFR staining confirmed presence of HNSCC cells over mouse stroma. Faint Gli1 expression was observed in control cells and this was significantly upregulated throughout the tumor following radiotherapy. Radiotherapy-induced Gli1 expression could be partially abrogated by cyclopamine treatment [Fig. 5, HN11 (left) and TU167 (right)]. When evaluating the mouse stroma, we similarly observed an increase in stromal Gli1 following radiotherapy and a pronounced decrease with dual therapy by H-score. In particular, we observed a pronounced effect with cyclopamine alone in suppressing mouse stromal GLI1 in TU167 over HN11 (Supplementary Table S1). Similarly, ALDH1 and BMI1 were upregulated following radiotherapy and partially inhibited by cyclopamine.

Given our in vitro and in vivo findings, we next sought to explore: (i) an alternative mechanism that may drive tumor Gli1 cytoplasmic-to-nuclear translocation independent of the canonical HhP and (ii) whether inhibition of HhP in stromal cells can block tumor repopulation in vivo.

mTOR/S6K1 mediates Gli1 cytoplasmic-to-nuclear translocation

In both cell lines, despite inhibition with significant concentrations of cyclopamine, radiotherapy was still able to induce Gli1 gene expression and increase nGli1 accumulation, suggesting that an accessory pathway (independent of canonical HhP signaling) may be driving radiotherapy-induced nGli1 accumulation. Therefore, we hypothesized that radiotherapy could activate the mTOR/S6K1 pathway and enhance Gli1 cytoplasmic-to-nuclear translocation (Fig. 6A). Following radiotherapy, Gli1 was upregulated by 24 hours in both HN11...
Figure 5. Radiotherapy (RT)-induced GLI1, ALDH1, BMI1 expression. IHC was performed on formalin-fixed tumors and stained with either H&E, EGFR, GLI1, ALDH1, or BMI1. Differences in IHC (×20; bar 100 μm) expression were evaluated by H-score.
and TU167 (Fig. 6B), and pretreatment with 1 μmol/L ZSTK474 (AKT inhibitor) was able to significantly suppress GLI1 expression in HN11 following radiotherapy but not in TU167 (data not shown). This resistance was consistent with the observed overexpression of the mTOR pathway in TU167, and use of a more specific inhibitor was warranted. Following pretreatment with 1 μmol/L rapamycin, we observed suppression of both GLI1 mRNA and Gli1 cytoplasmic-to-nuclear protein accumulation following radiotherapy in TU167 (Fig. 6C). We observe a similar increase in cGli1 following irradiation and the irradiation effect can similarly be suppressed with rapamycin pretreatment. In contrast, HN11 inhibition with rapamycin did not prevent radiotherapy-induced nGli1 accumulation (Supplementary Fig. S4) and is in contrast to our mRNA results. Our transcriptome results may offer an answer for this discrepancy, where TU167 has greater mTOR gene expression compared with HN11 and may be more sensitive to the effects of mTOR inhibition, which may translate into diminished Gli1 accumulation. Cellular Gli1 content is known to be regulated both at the transcriptional and proteasomal level (20). The suppression of GLI1 may suggest that the role of rapamycin may be to regulate gene expression and perhaps is irrespective of nuclear protein accumulation.

Next, we evaluated whether inhibition of the downstream mTOR effector S6K1 could prevent nGli1 accumulation. Similarly, GLI1 expression was significantly reduced in the presence of S6K1 inhibitor (1 μmol/L PF-4708671) following radiotherapy (Fig. 6B). We attempted to determine whether loss of S6K1 could similarly suppress GLI1 expression and prevent nuclear Gli1 protein accumulation. Four siRNA constructs were screened and their ability to suppress S6K1 expression by mRNA and immunoblot were assessed. The two best candidate
constructs were then selected. Both siRNA constructs suppressed expression of S6K1. ppS6K1 was still observed despite knockdown and was completely absent following radiotherapy. Our results suggest that inhibition with one siRNA construct was able to suppress nGli1 accumulation in both HN11 and TU167. However, the other siRNA construct gave contrary results and does not appear to suppress radiotherapy-induced nGli1 nuclear accumulation (Supplementary Fig. S5). Our findings suggest a possible link between mTOR/S6K1 and nGli1 accumulation but must be interpreted with caution.

**In vivo tumor repopulation following radiotherapy**

Locoregional failure due to tumor repopulation following radiotherapy or chemoradiotherapy remains a significant problem. *In vitro* and *in vivo* studies have implicated that genotoxically stressed tumor or fibroblast cells may play a role in tumor repopulation. In *vivo*, tumor and tumor-associated fibroblasts (stroma) are closely admixed with one another. On the basis of our PDX and TU167 *in vivo* results demonstrating increased mouse stromal Gli1 following radiotherapy, subsequent suppression by pretreatment with cyclopamine and the significant response of TU167 tumors to dual therapy, we hypothesized that cyclopamine may also target the tumor stroma and given its combined affect on TU167 *in vivo*, may inhibit tumor repopulation. We treated orthotopically implanted TU167 tumors with vehicle, cyclopamine, radiotherapy, or the combination, surgically resected them, separated tumor and stroma compartments by flow cytometry, and coimplanted these in different permutations with untreated tumor cells (Supplementary Fig. S6A). This addressed whether the irradiated tumor or stroma could enhance tumor repopulation and whether inhibition of the HhP could abrogate repopulation. We observed a significant 10-fold difference in tumor regrowth at 3 weeks favoring the irradiated stroma combined with fresh tumor cell lines (SRT:T) compared with the nonirradiated control stromatumor (S:C:T) pairing (*P* = 0.01). When stroma cells pretreated with cyclopamine and radiotherapy were combined with fresh tumor (SPT:T), we observed a significant reduction in tumor regrowth rates at 3 weeks when SRT:T was compared against SRT:T (*P* = 0.007) implicating that HhP following radiotherapy may be involved in stroma-mediated tumor repopulation (Fig. 7A). We also evaluated TRT:T pairings and observed a similar though nonsignificant trend in tumor regrowth at 3 weeks. (Fig. 7A; Supplementary Table S2). When we evaluated the same pairings between SRT:T versus TST:T, there was greater than a 4-fold difference in size favoring the SRT:T over the tumor:tumor pairing (*P* = 0.03; Supplementary Table S2). Figure 7B shows representative mice with S:T and T:T pairings at 6 weeks. At 6 weeks, the TST:T pairings continued to demonstrate a significant trend towards smaller tumor sizes (267 ± 27 mm³ vs. 579 ± 102 mm³) compared with the SRT:T pairings (*P* = 0.005). Representative excised tumors from all animals continued to demonstrate that SRT cells conferred a significant growth advantage over S:C or S:C points (nonradiotherapy) stroma. Furthermore, tumors pretreated with cyclopamine were able to inhibit tumor regrowth despite being irradiated (Fig. 7A and C).

**Discussion**

Radiotherapy remains the cornerstone for local management of HNSCC either definitively or in the postoperative setting. Whether radiotherapy can induce Gli1/HhP upregulation through an EMT phenotype as a mechanism for tumor repopulation and the respective relevance of irradiated cancer cells versus intratumor stromal cells for tumor repopulation remain unknown. Accelerated tumor repopulation following radiotherapy is associated with locoregional recurrence in patients and a better understanding of this mechanism is critical to improve LC and OS.

The interface between tumor margin/edge and tumor stroma is an important distinction for cancer biology. First, we demonstrated that Gli1 is significantly upregulated at the tumor edge compared with the core in nonirradiated samples. Second, following radiotherapy, Gli1 gene expression was upregulated at the peripheral tumor edge compared with the tumor core. Of note, this was taken at a solitary time point (48 hours after radiotherapy), and even though overall Gli1 content has increased in both cells, there is no observed preference for staining at the tumor edge. On the basis of this experiment, accumulation of nGli1 preferentially at the tumor edge may occur more rapidly as our immunoblot results suggest and therefore tumor harvested at an earlier time point might illustrate this.

First, the implications of our findings are important because tumors with upregulated Gli1 and/or the EMT pathway have been shown to be more chemo- and radioresistant, more locally invasive, and metastatic (7, 21–23). Second, the peripheral tumor cells model those cell islands left behind after surgical resection (“positive margin”). This is clinically significant as a positive margin indicates a high risk for recurrence and patients with positive margins often experience greater local and distant failure rates when they fail to receive adequate doses of radiotherapy/chemoradiotherapy (24, 25). The change in tumor milieu, increased inflammation, and local hypoxia following surgery may provide additional factors that allow a high Gli1-expressing tumor more opportunity to proliferate/flourish compared with a low Gli1-expressing tumor. Our results demonstrated that Gli1 expression is inherently higher at the tumor margin compared with the core and that radiotherapy can further induce Gli1 overexpression at the tumor margin. However, these findings need to be cautiously interpreted and we caveat that tumor heterogeneity may cause variations in gene expression between tumor margin and center and this may explain the more uniform Gli1 protein expression following irradiation observed by IHC as compared with the quantitative real-time PCR (qRT)-PCR results, which demonstrated greater Gli1 expression at the tumor margin. Another explanation is that radiotherapy leads to Gli1 upregulation, which in turn upregulates certain EMT-associated genes. A limited number of these cells that are more EMT-like can transform to a more mesenchymal phenotype and settle in to the existing environment or translocate away from the source of insult (i.e., local radiotherapy). As they are away from the area of insult, these cells can downregulate various mesenchymal genes and reestablish their epithelial phenotype (mesenchymal-to-epithelial phenotype) allowing them to grow.
in a new microenvironment. This has been illustrated in a squamous cell epithelial cancer model using a conditional doxycycline regulatory system controlling \( TWIST1 \) expression (26). The authors demonstrated that \( TWIST1 \) expression was necessary for EMT but shutting down \( TWIST1 \) was necessary for establishing distant metastasis. This explanation takes into account both the tumor margin and heterogeneity issues providing a possible mechanism for Gli1-mediated treatment failure and distant metastasis.

In the chronically irradiated cells, we observed that HhP genes, \( GLI1 \) and \( GLI2 \), and EMT genes, \( ZEB2, TWIST1 \), and \( VIM \), were upregulated though not as robustly as when cells were acutely irradiated. These genes remained elevated 2 to 8 weeks after radiotherapy, suggesting that a surviving population of irradiated cancer cells adapt gene expression, allowing for survival and repopulation. Other groups, including our own, have demonstrated that inhibition of HhP or EMT pathway genes, \( TWIST, SNAI1, \) or \( SLUG \), can reverse (chemo)radiotherapy resistance and decrease tumor invasiveness, progression, and metastasis (8, 9, 11, 22, 27). Therefore, we decided to evaluate whether HhP inhibition could enhance tumor control by either increasing radiosensitivity or preventing tumor repopulation in vivo. A recent article indicated that inadequate radiotherapy doses in other tumor cell lines can lead to
increased tumor regrowth mediated by the HhP (21). Therefore, direct inhibition of GLI1 activation in vivo may lead to enhanced tumoricidal effects when combined with radiotherapy. When we treated our chronically irradiated HN11 cells with cyclopamine, downregulation of GLI2 and VIM occurred, indicating that HhP inhibition in HNC may be an appropriate anticancer target when combined with radiotherapy.

Our IHC results demonstrated that GLI1 is upregulated in both tumors and in the tumor stroma following radiotherapy and this upregulation was mitigated with cyclopamine. Interestingly, the downstream stem cell marker and DNA damage response protein ALDH1 and BMI1, respectively, were both downregulated following cyclopamine therapy, suggesting that these genes are regulated by GLI1 and that HhP inhibition may prevent or slow the transition to a more "stem cell–like" state. In one study evaluating patients with high versus low ALDH1 expression pre- and postsurgery, patients with colorectal cancer undergoing neoadjuvant chemoradiotherapy who expressed high levels of ALDH1 had greater rates of local failure and recurrence (28). Expression of ALDH1 in HNSCC and colorectal cancer is associated with increased nodal and distant metastasis, worse OS, and is mediated through TWIST1 and SNAI1 expression (29, 30). These findings highlight the concern that chemoradiotherapy can induce ALDH1 (and BMI1) expression which is a poor prognostic factor. Our results are the first to demonstrate that adding cyclopamine to radiotherapy can suppress GLI1 expression in HPV-negative HNC and thereby downregulate ALDH1 and BMI1 expression in vivo.

Accelerated tumor repopulation following radiotherapy is a well-known phenomenon but its mechanism remains unknown. Our in vitro results suggest that both HN11 and TU167 upregulate the HhP acutely and chronically following radiotherapy. Furthermore, cyclopamine can suppress radiotherapy-induced GLI1 in HN11 but not in TU167 despite our in vivo results, and this is likely due to lower baseline levels of HhP expression in HN11 compared with TU167. Given the difference in our in vitro versus our in vivo data, this led us to question whether an alternative target may be the stroma. Our own LCM qRT-PCR and IHC results (Figs. 1C and 4E) show the mouse stroma also upregulated GLI1 following radiotherapy but that GLI2 is suppressed following cyclopamine treatment. One possible explanation is that irradiated stroma may provide a nurturing environment to allow circulating mesenchymal tumor cells to nidus to grow. Researchers have demonstrated that irradiated fibroblasts undergo changes, including matrix remodeling (i.e., release of MMPs) and cytokine release (i.e., TGFβ, basic FGF). This may allow for an altered albeit rich growth environment allowing tumor cells to flourish. A growing body of literature in prostate, breast, pancreas, and other cancers suggests that HhP upregulation in the tumor microenvironment through paracrine signaling plays a substantial role in tumor growth, survival, angiogenesis, and metastasis (27, 31–34). More specifically, cancer-associated fibroblasts (CAF) have been shown to upregulate both the Smoothened receptor and in turn GLI1 (35) and SNAI1 (36), whereas another group demonstrated that inhibition of Smoothened on CAFs in turn translated to decreased GLI1 expression and the downstream EMT gene SNAI1 in pancreatic cancer cells (37), supporting a paracrine signaling model for EMT. These findings indicate that both stromal and tumor GLI1 may play an important role in tumor treatment resistance and repopulation and targeting them may not only enhance CAF sensitivity to genotoxic agents but also inhibit the EMT process in tumors.

Building on our findings, we hypothesized that the HhP may play a role in the HPV negative tumor microenvironment and tumor repopulation following radiotherapy. When we irradiated stroma and recombined them with fresh tumor cells, it resulted in significant rapid tumor regrowth after reimplantation compared with control or cyclopamine alone. Compared with the irradiated tumor recombined with fresh tumor cells, we observed a nonsignificant trend at 3 weeks and a more pronounced regrowth effect at 6 weeks. Interestingly, we did not see substantial tumor regrowth in the irradiated stroma (or tumors) treated with cyclopamine when combined with fresh tumor cells at either time point. Our findings are novel as they implicate GLI1 as a potential target in the stromal microenvironment important for radiotherapy-induced HNSCC tumor (re)growth. However, additional work is needed to understand what may be driving this regrowth but our findings suggest that HhP may be involved. As the tumor microenvironment decisively contributes to therapy resistance (38), modulating its signaling through targeted therapy may enhance LC rates. Our findings suggest that irradiated HPV-negative HNSCC stroma (and to a lesser degree, irradiated HNSCC tumor) utilize the HhP to mediate tumor repopulation. Identifying the paracrine signaling molecule(s) interacting with the tumor stroma will provide valuable information regarding tumor biology that could be applied towards novel drug discovery. There is a distinct lack of information regarding HPV-positive HNSCC and the HhP in the literature. Given their excellent biology that could be applied towards novel drug discovery. Their excellent sensitivity to genotoxic agents but also inhibit the EMT process in tumors.

Our observation that GLI1- and EMT-associated genes and proteins can be similarly upregulated following acute or chronic irradiation implicates their importance as radiosensitive genes. Our transcriptome analysis of TU167 showed that it was less epithelial compared with HN11 and had greater gene enrichment for the Hedgehog and mTOR pathways. Higher basal expression of HhP genes in TU167 may explain its relative insensitivity to cyclopamine in vitro but this was overcome with higher doses of drug. In esophageal cancer, TGFα has been shown to activate/stabilize GLI1 via mTOR/S6K1 and not through the canonical Smoothened pathway (6). Furthermore, radiotherapy, either through EGFR phosphorylation (39) or directly through DNA damage response proteins such as DNA-PKcs (40), has been shown to activate the PI3K/AKT/mTOR pathway. Our pharmacologic findings suggest that radiotherapy-induced nGLI1 translocation may signal through the mTOR/S6K1 pathway in more mesenchymal appearing cell lines such as TU167 and may be the primary driver for radiotherapy-induced nGLI1 accumulation. However, in more epithelial cell lines such as HN11, rapamycin was unable to suppress nGLI1 accumulation yet GLI1 mRNA remained suppressed following pharmacologic inhibition. One possible explanation is that rapamycin may affect GLI1 expression...
irrespective of nuclear accumulation. One hypothesis is that mesenchymal versus epithelial cells likely signal differently upstream but converge through mTOR/S6K1. Others have similarly demonstrated that the mTOR pathway is upregulated in head and neck cancers and may be an attractive clinical target. One group has shown that rapamycin combined with an AKT inhibitor, enzastaurin, can decrease survival, angiogenesis, and proliferation in preclinical HNC models (41). Furthermore, two phase I clinical studies have been completed: the first in patients with resected, locally advanced head and neck cancer combining everolimus, weekly cisplatin, and radiation and the second, combining everolimus with docetaxel and cisplatin as part of an induction chemotherapy regimen. Both clinical studies have demonstrated tolerability and safety and are presumed to be progressing to phase II (42, 43). It is enticing to consider that the combination of an mTOR inhibitor, cyclopamine, and radiotherapy in patients with recurrent or locally advanced head and neck cancer may be more efficacious as it targets both the tumor and stroma-mediated components involved with tumor repopulation.

Our results show that the HhP and Gli1 play an important role in HNSCC tumor biology. We have demonstrated that the mTOR/S6K1 pathway may contribute to radiotherapy-induced Gli1 signaling in vitro. Radiotherapy combined with targeting HhP led to increased HNSCC cytotoxicity in vitro and in vivo and limited tumor repopulation in vivo. In locally recurrent or metastatic head and neck tumors that may be mTOR driven, combining mTOR and HhP inhibitors with radiotherapy may allow an opportunity to target primary and alternative mechanisms, including stroma signaling, which may lead to improved therapeutic efficacy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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