This thesis for Doctor of Philosophy degree by

Xi Chen

has been approved for the

Cancer Biology Program

by

Rebecca Schwepppe, Chair

Mary Rayland

Lynn Heasley

Heide Ford

Maranke Koster

Shi-long Lu, Advisor

Date: December 16, 2016
ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) patients have a poor prognosis, with invasion, recurrence and metastasis as major causes of mortality. Currently, effective therapeutic options for advanced HNSCCs are still limited. The phosphatidylinositol 3-kinase (PI3K) pathway regulates a wide range of cellular processes crucial for tumorigenesis, and PIK3CA amplification and mutation are among the most common genetic alterations in HNSCC. Compared to the well-documented roles of the PI3K pathway in cell growth and survival, its roles in tumor invasion, recurrence and metastasis have not been well delineated.

Herein, I used a PIK3CA-genetically engineered mouse model (PIK3CA-GEMM) in which PIK3CA is overexpressed in head and neck epithelium to mimic PIK3CA amplification in human HNSCC patients. I found that PIK3CA overexpression increased tumor invasiveness and metastasis by increasing dedifferentiation and epithelial-to-mesenchymal transition (EMT), and marked inflammation in tumor stroma. Molecular analysis suggested that progression of PIK3CA-driven HNSCC is facilitated by PDK1 and enhanced by TGFβ signaling rather than by AKT. Examination of HNSCC clinical samples revealed that both PIK3CA and PDK1 protein levels correlated with tumor progression, highlighting the
significance of this pathway and providing a rationale for targeting PI3K/PDK1 and TGFβ signaling in advanced HNSCC patients with PIK3CA amplification.

EMT confers cancer stemness phenotype, and is believed to be involved in tumor recurrence and metastasis. Thus, I further explored the role of PIK3CA overexpression in regulating cancer stem cells (CSCs). I found that overexpression of PIK3CA enriches the CSC population. However, knocking down PIK3CA, or components in the PI3K pathway, paradoxically enhances CSC population. Protein array analysis revealed that CSC phenotype is associated with activation of multiple receptor tyrosine kinases pathways with Trk and Ephrin pathways the predominant. Pharmaceutical inhibitors targeting these pathways effectively eliminate CSCs both in vitro and in vivo. I also performed microarray analysis and identified histone deacetylase (HDAC) may contribute to EMT and CSC phenotypes in our models. In supporting this finding, I found that HDAC inhibitors effectively eliminate the CSC population in both murine and human HNSCC cells. Last, I did a microRNA array to identify potential candidates which may be essential for CSC maintenance. Preliminary studies showed that miR-18a-5p and miR-199a-5p may be involved in this process. In summary these data suggested that inhibitors targeting Trk, Ephs or HDAC pathways may be effective to treat recurrence and metastasis in HNSCC patients with PIK3CA amplification.

The form and content of this abstract are approved. I recommend its publication.

Approved: Shi-long Lu
CONTENTS

I. INTRODUCTION .......................................................................................... 1

Head and Neck Squamous Cell Carcinoma .............................................. 1

Etiological Factors for HNSCC ................................................................. 2

Molecular Alterations in HNSCC .............................................................. 2

PI3K Signaling Pathway in HNSCC ......................................................... 6

PIK3CA .................................................................................................. 7

PI3K Isoforms .................................................................................... 9

Key Components in PI3K Signaling (PTEN, AKT, mTOR and PDK1) .............................................................................. 11

Crosstalk between PI3K Signaling and TGFβ Signaling in HNSCC .................................................................................. 13

Mouse Models for HNSCC ...................................................................... 15

Chemically Induced HNSCC Mouse Models ....................................... 15

Xenograft Models for HNSCC ................................................................. 16

Genetically Engineered Mouse Models .............................................. 17

Inducible Head-and-neck Specific Transgenic Mouse Model ........ 17

Inducible Head-and-neck Specific Knockout Mouse Models .... 18

EMT, Cancer Stem Cells and Anti-Cancer Stem Cell Therapy ....... 20

Epithelial-to-mesenchymal Transition (EMT) in HNSCC .......... 20

Cancer Stem Cells in HNSCC ................................................................. 21

EMT and Cancer Stem Cells ................................................................. 22

Anti-Cancer Stem Cell Therapy ........................................................... 23

Epigenetics and Targeted Epigenetic Therapy in Cancer ............. 23
miRNA Dysregulation in HNSCC.......................................................... 25
Current Standard of Care for HNSCC .............................................. 27
Summary.......................................................................................... 28

II. MATERIALS AND METHODS.......................................................... 30
Cell Culture....................................................................................... 30
Cell Proliferation, Migration and Invasion Assays.......................... 31
Stable Knocking Down Using Lentiviral-based shRNAs.................... 32
HNSCC Sphere Formation Assay..................................................... 32
FACS Analysis ................................................................................ 34
Quantitative Real-time PCR ............................................................ 35
Protein Analysis............................................................................... 36
Immunostainings ............................................................................ 42
Receptor Tyrosine Kinase (RTK) Antibody Array ......................... 43
Microarray (Gene or MicroRNA) Analysis..................................... 44
MicroRNA Transfection ................................................................. 44
Inhibitor Treatment ....................................................................... 45
Animal Studies .............................................................................. 45
Generation and Characterization of Inducible Head-and-neck-
specific PIK3CA Genetically Engineered Mouse Model (GEMM) .................................................................................. 45
In-vivo Tumorigenicity Study............................................................ 49
Human Correlation......................................................................... 50
Statistical Analysis......................................................................... 50
III. OVEREXPRESSION OF PIK3CA IN MURINE HEAD AND NECK EPITHELIUM DRIVES TUMOR INVASION AND METASTASIS THROUGH PDK1 AND ENHANCED TGFβ SIGNALING ....................... 51

   Introduction ........................................................................................ 51

   Results ............................................................................................... 53

   Overexpression of PIK3CA in Murine Head and Neck Epithelia Resulted in Increased Susceptibility to Head and Neck Carcinogenesis ................................................................. 53

   Overexpression of PIK3CA in Murine Head and Neck Epithelia Promotes Tumor Invasion and Metastasis ........................................................ 55

   PIK3CA Overexpression Induced De-differentiation, EMT and Enriched CSC Properties ............................................................... 58

   PIK3CA-GEMM Tumors Have Increased PDK1 Expression and Activation .............................................................. 62

   PIK3CA Overexpression Led to Increased Inflammation in HNSCC Tumor Stroma .............................................................. 69

   PIK3CA-GEMM Tumors Have Increased TGFβ1 Ligand, and Increased Smad3 Expression and Activation ....................... 69

   PIK3CA and PDK1 Are Associated with Progression in Human HNSCC .................................................................................. 74

   Discussion ............................................................................................. 76

IV. DISTINCT ROLES OF PIK3CA IN ENRICHING AND MAINTAINING OF CANCER STEM CELLS IN HNSCC ....................................................... 82

   Introduction ........................................................................................ 82

   Results ............................................................................................... 84

   Overexpression of PIK3CA Promotes Cell Proliferation, Migration, Epithelial-to-mesenchymal Transition and De-differentiation .............................................................. 84

   Overexpression of PIK3CA Enriches Putative Head and Neck Cancer Stem Cells .............................................................. 87
Sphere Forming Capacity is a Functional Measurement for Cancer Stemness Properties of HNSCC ........................................  89

Knocking Down PI3K Failed to Reverse EMT Phenotypes and Reduce CSC Population .........................................................  91

Knockdown of Key Components in PI3K Pathway Promotes CSC Population ..................................................................... 100

Targeting Multiple Receptor Tyrosine Kinase Pathways Effectively Eliminates CSC Populations with Inhibiting Ephs, Trks and c-kit the Most Prominent ......................................................... 101

Discussion ....................................................................................... 112

V. INHIBITION OF HISTONE DEACETYLASE ELIMINATES CSC POPULATION THROUGH REVERSION OF EMT IN HNSCC WITH PIK3CA OVEREXPRESSION ......................................................... 121

Introduction ...................................................................................... 121

Results ............................................................................................. 122

Transcriptional Profiling Identified Molecular Pathways Related to PIK3CA Overexpression-induced CSC Properties .......... 122

Inhibition of Histone Deacetylase Eliminates CSC Population through Reversing EMT ........................................................ 123

Identification of Key Molecular Events Responsible for Anti-CSC Effect upon Treatment of HDAC Inhibitors ......................... 126

Discussion ....................................................................................... 129

VI. IDENTIFICATION AND VALIDATION OF MICRORNAS REGULATING CSC PHENOTYPE IN THE PIK3CA-OVEREXPRESSION HNSCC ................................................................. 135

Introduction ...................................................................................... 135

Results ............................................................................................. 136

Identification and Validation of MicroRNAs (miRNAs) Differentially Expressed in CSCs Isolated from the PIK3CA-overexpressing HNSCCs ................................................................. 136
Modulation of miR-18a-5p and miR-199a-5p Expression Interfered CSC Population Isolated from the PIK3CA-overexpressing HNSCCs .......................................................... 137

Discussion ................................................................................................. 140

VII. Discussion ............................................................................................ 143

Novel Mechanisms Underlying PIK3CA Overexpression – driven HNSCC Progression ........................................................................................................ 144

PDK1 and Enhanced TGFβ Signaling Drives PIK3CA Overexpression–mediated HNSCC Progression ................................................................. 144

PIK3CA Overexpression Promotes EMT and CSC Properties to Drive HNSCC Progression ........................................................................... 148

PI3K-independent Mechanisms that Maintain PIK3CA Overexpression–induced EMT and CSC Traits ................................................................. 150

Novel Therapeutic Strategies for Treating HNSCC with PIK3CA Alterations ........................................................................................................ 155

REFERENCES ............................................................................................... 158
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔPR</td>
<td>truncated progesterone receptor ligand binding domain</td>
</tr>
<tr>
<td>4NQO</td>
<td>4-nitroquinoline-1 oxide</td>
</tr>
<tr>
<td>AKT</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>ALDH</td>
<td>aldehyde dehydrogenase</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic proteins</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CDKN2A</td>
<td>cyclin-dependent kinase Inhibitor 2A</td>
</tr>
<tr>
<td>CSC</td>
<td>cancer stem cell</td>
</tr>
<tr>
<td>DMBA</td>
<td>7,12-dimethylbenz[a]anthracene</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E2F1</td>
<td>transcription factor E2F1</td>
</tr>
<tr>
<td>EGFR</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>Ephs</td>
<td>ephrin receptors</td>
</tr>
<tr>
<td>ERBB</td>
<td>receptor tyrosine-protein kinase erbB-2</td>
</tr>
<tr>
<td>EMT</td>
<td>epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FAT1</td>
<td>protocadherin FAT1</td>
</tr>
<tr>
<td>FDA</td>
<td>U.S. Food and Drug Administration</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GEMM</td>
<td>genetic engineered mouse model</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HER</td>
<td>epidermal growth factor receptor</td>
</tr>
<tr>
<td>HNSCC</td>
<td>head and neck squamous cell carcinoma</td>
</tr>
<tr>
<td>HPV</td>
<td>human papilloma virus</td>
</tr>
</tbody>
</table>
IC$_{50}$  half maximal inhibitory concentration
KRAS  Kristen rat sarcoma viral oncogene homolog
MAPK  mitogen-activated protein kinase
MDM2  mouse double minute 2 homolog
MET  hepatocyte growth factor receptor
mRNA  messenger RNA
mTORC1/2  rapamycin (mTOR) complex 1/2
miRNA  microRNA
NSD1  transcription coregulator protein
NFE2L2  nuclear factor (erythroid-derived 2)-like 2
p16  cyclin-dependent kinase inhibitor 2A
p53  tumor protein p53
p85  phosphatidylinositol 3-kinase regulatory subunit
p110  phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit
PI3K  phosphatidylinositol-3-kinase
PDGF  platelet-derived growth factor
PDK1  pyruvate dehydrogenase lipoamide kinase isozyme 1
PIK3CA  phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha
PIK3R1  phosphatidylinositol 3-kinase regulatory subunit alpha
PTEN  phosphatase and tensin homolog
RET  ret proto-oncogene
RNA   ribonucleic acid
RTKs  receptor tyrosine kinases
shRNA  short hairpin RNA
Smad2  SMAD family member 2
Smad3  SMAD family member 3
Smad4  SMAD family member 4
Snail1  Zinc finger protein snail1
Snail2  Zinc finger protein snail2
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRC</td>
<td>SRC proto-oncogene, non-receptor tyrosine kinase</td>
</tr>
<tr>
<td>SP</td>
<td>side population</td>
</tr>
<tr>
<td>TGF-α</td>
<td>transforming growth factors α</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factors β</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>transforming growth factor beta II receptor</td>
</tr>
<tr>
<td>TRAF3</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>Twist1</td>
<td>Twist-related protein 1</td>
</tr>
<tr>
<td>Twist2</td>
<td>Twist-related protein 2</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>Zeb1</td>
<td>zinc finger E-box-binding homeobox 1</td>
</tr>
<tr>
<td>Zeb2</td>
<td>zinc finger E-box-binding homeobox 2</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

Head and Neck Squamous Cell Carcinoma

Head and neck cancers are composed mostly of head and neck squamous cell carcinoma (HNSCC) and salivary gland cancers. HNSCC refers to cancers derived from the squamous epithelium along the head and neck region, including nasal cavity, oral cavity and tongue, pharynx (nasal pharynx, oropharynx, hypopharynx) and larynx. Squamous epithelium is a layer of epithelial cells which can be organized either as a single layer in lung, or as a multiple layers (stratified) in head and neck region, skin, and cervix etc. Cancers developed from these organs are referred to general as squamous cell carcinomas. HNSCC affects 650,000 people and claims 350,000 lives annually. Every year in the United States alone, there are 55,000 new cases of HNSCC and 15,000 deaths. The 5-year survival rate for HNSCC is below 50% and has not been improved over the past several decades, which is largely due to locoregional invasion, recurrence and metastasis. As invasion, recurrence and metastasis are the predominant causes of lethality in HNSCC patients, understanding the molecular mechanisms that regulate these processes is pivotal. A better therapeutic strategy that aims at controlling invasion, recurrence and metastasis is required to ultimately improve the poor prognosis of HNSCC patients.
Etiological Factors for HNSCC

HNSCC is a complex disease characterized by clinical and biological heterogeneity and is frequently caused by tobacco use, alcohol and human papilloma virus (HPV) infection. In addition, there is a high incidence of HNSCC in Fanconi Anemia survivors at their early 20 to 30 years of age, suggests a genetic predisposition confers higher risk for developing HNSCC in this genetic disease. Traditionally, HNSCC is often seen in male over 50 years old who have a heavy smoking and/or drinking history, and poor oral hygiene condition. Tobacco use is the single largest risk factor for HNSCC. Almost 85% of HNSCC is associated with tobacco use and the quantity of tobacco use directly links to the poor prognosis. Alcohol consumption is another major risk factor for HNSCC. Recently study also showed multiplicative joint effect between tobacco use and alcohol consumption on HNSCC risk.

HNSCC due to HPV infection represents another subtype of HNSCC, and incidence of HPV-related HNSCC keep increasing every year. It currently occupies over 25% of whole HNSCC cases. The increased incidence of HPV-related HNSCCs, especially oropharyngeal cancers, among younger patients is likely due to engagement in oral sex. Fortunately, HPV-positive HNSCCs appear to respond more favorably to radiation therapy than HPV-negative ones, and vaccination against HPV is proposed with the hope to reduce HPV-associated HNSCC.

Molecular Alterations in HNSCC

As of 2011, the major molecular alterations predicated in each stage of
HNSCC tumorigenesis are depicted in Figure 1.1. Based on HPV and chromosome status, HNSCCs is divided into HPV+ (~25%) and HPV-/chromosome instability (CIN)+ (~65%) and HPV-/CIN- subgroups (~10%)\(^4\). Most of studies before 2011 focused on HPV-/CIN- HNSCC patients. As shown in Figure 1.1, deregulations of cell cycle –related genes, such as p16, p53, RB, CDKs and cyclinD1 are the major players for early stage of HNSCC tumorigenesis. Among those, inactivation of p16 (mutation, genetic lose, or methylation) and p53 mutations are the most frequent genetic alterations. Dysregulation of transforming growth factor beta (TGF\(\beta\)) signaling, such as loss, or reduced expression of TGF\(\beta\) type II receptor, Smad4, or overexpression of TGF\(\beta\)1 ligand, contributes greatly to malignant transformation and tumor progression\(^{13,14}\). Overexpression of epidermal growth receptor (EGFR) has been suggested as an important molecular event in HNSCC tumorigenesis (Figure 1.1). The prevalence of EGFR overexpression in HNSCC (present in 80-90% of samples) is significant\(^{15}\). However, gene amplification of EGFR is only present in 10–30% of samples and the EGFR mutation occurs in 1-7% of the tumor cell population\(^{15}\). These findings suggest that other mechanisms may be involved in the overexpression of EGFR in HNSCC. Thus, further study of EGFR homeostasis in HNSCC is critical for development of effective therapeutics.

Alterations of the PI3K/AKT/PTEN pathway has been shown to be altered in HNSCC progression as shown in Figure 1.1. This is further validated and highlighted after a few seminal publications by whole-exome sequencing on large cohorts of HNSCC clinical samples from multiple institutions\(^{16–20}\). Two major findings from these studies are: 1) Alterations of PI3K/AKT/PTEN/mTOR pathway are the most
Figure 1.1. A summary of molecular alterations in HNSCC tumorigenesis. Major genes and pathways involved in HNSCCs are depicted. This figure has been adapted from C. René Leemans et al. (2011) *Nature Review Cancer*. 
frequent molecular alterations in human HNSCC patients and 2) Identification of somatic mutations of Notch molecules.

The molecular alterations in HPV positive HNSCC are distinct from HPV negative HNSCC cases. HPV-16 is implicated in the pathogenesis of a HNSCC subgroup and the HPV viral oncogenes E6 and E7 are frequently overexpressed in the oropharynx of these patients\textsuperscript{21,22}. A previous study reported a significant association between oropharyngeal cancer and oral HPV-16 infection and demonstrated that HPV exposure increased the risk of developing oropharyngeal cancer, regardless of tobacco and alcohol use\textsuperscript{11}. Inactivation of p53 and RB by HPV E6 and E7 antigens is a well-recognized molecular mechanism for HPV positive HNSCC tumorigenesis\textsuperscript{10,23,24}. Besides, HPV-positive HNSCCs have unique gene-expression profiles and minimal mutations, while HPV-negative HNSCCs are frequently riddled with molecular and cytogenetic changes described above.

Recurrence and metastasis are two major reasons for cancer death in HNSCC patients. Recurrence is highly associated with stage of primary tumors. For advanced stage of HNSCC patients, recurrence rate is as high as 30% within two years\textsuperscript{4,5}. Node metastasis is frequent in HNSCC patients and correlated with primary tumor size\textsuperscript{25}. Distant metastasis of HNSCC occurs in about 20\% of HNSCC patients, with lung metastasis the most common\textsuperscript{5,26}. The metastatic progression of HNSCC tumor cells is a multistep process that involves intimate interactions between tumor cells and their surrounding microenvironment\textsuperscript{27}. Potential metastasis-related proteins include factors involves in epithelial-to-mesenchymal transition (EMT), cancer stem cells (CSCs), inflammation and angiogenesis\textsuperscript{28–30}. Growth factors, chemokines or
cytokines are also involved in these processes. For example, TGFβ, vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), interleukin-8 etc. have been shown to play important roles in HNSCC progression. Currently, both preclinical and clinical studies are evaluating the efficacy of antiangiogenic compounds (with or without chemotherapy) for the treatment of HNSCC. Hopefully, these studies will spur the development of novel antiangiogenic and anti-metastatic agents for the treatment of HNSCC. Drugs targeting EMT and CSCs are hypothetically effective in preventing/treating recurrence and metastasis in HNSCC patients, in which are the major components of my thesis.

PI3K Signaling Pathway in HNSCC

The phosphatidylinositol-3-kinase (PI3K) signaling pathway is a major signaling component downstream of receptor tyrosine kinases (RTKs), and regulates multiple cellular functions such as survival, growth, migration, and metabolism. The PI3K family is comprised of three subclasses of lipid kinases. Among the three classes of PI3Ks, class IA PI3K is heavily linked to human cancers and is often activated by RTK signaling and Ras. Class IA PI3K, a heterodimer consisting of a p85 regulatory and p110 catalytic subunit, phosphorylates phosphatidylinositol-4,5,bisphosphate [PI(4,5)P2] in the cell membrane and generates the second messenger phosphatidylinositol-3,4,5, trisphosphate [PI(3,4,5)P3]. Following RTK signaling, adaptor subunit p85 binds to either phosphotyrosine residues or adaptor molecules, which recruits the catalytic subunit p110 to form the heterodimer to the
cell membrane. The p110 subunit phosphorylates PI(4,5)P2 to generate PI(3,4,5)P3. Further downstream, PI(3,4,5)P3 interacts with 3-phosphoinositide-dependent protein kinase (PDK1) and protein kinase B (AKT) and binds them to the plasma membrane\textsuperscript{36}. In the plasma membrane, rapamycin (mTOR) complex 2 (mTORC2) phosphorylates AKT at Ser473, while PDK1 phosphorylates AKT at Thr308\textsuperscript{37} (Figure 1.2). Activated AKT directly regulates multiple downstream molecules, such as mTORC1 and p70-S6, to transduce the major downstream PI3K signal pathway\textsuperscript{38–40}. However, it has been shown that, in certain contexts, PI3K signaling and AKT activation can be uncoupled\textsuperscript{41–44}.

PI3K pathway is commonly altered in many human malignancies, including HNSCC. Recent whole-exome sequencing analysis of human HNSCC samples identified numerous molecular alterations in the PI3K pathway, such as gain-of-function mutations and amplifications in PIK3CA and loss of heterozygosity and inactivating mutations in PTEN\textsuperscript{16–20} etc, which I will describe in detail below. These results highlight the importance of this pathway in HNSCC tumorigenesis and provide strong rationale of targeting this pathway for HNSCC treatment\textsuperscript{45}.

**PIK3CA**

PIK3CA, which encodes the PI3K catalytic subunit p110α, is one of the most altered oncogenes in human cancers. Mutation of PIK3CA presents in ~12% of total human cancer samples from the data of the Catalogue of Somatic Mutations in Cancer (COSMIC) Database (http://www.sanger.ac.uk/genetics/CGP/cosmic/). Compared to somatic mutation of PIK3CA, which is about 10% in HNSCC
Figure 1.2. Schematic of PI3K/AKT/mTOR signaling. Following RTK signaling, adaptor subunit p85 binds to either phosphotyrosine residues or adaptor molecules, which recruits the catalytic subunit p110 to form the heterodimer. The p110 subunit phosphorylates PI(4,5)P2 to generate PI(3,4,5)P3. Further downstream, PI(3,4,5)P3 interacts with 3-phosphoinositide-dependent protein kinase (PDK1) and protein kinase B (AKT) and binds them to the plasma membrane. In the plasma membrane, rapamycin (mTOR) complex 2 (mTORC2) phosphorylates AKT at Ser473, while PDK1 phosphorylates Thr308. Activated AKT phosphorylates numerous downstream regulators with the mTOR complex 1 (mTORC1) the most prominent.
patients\textsuperscript{16,17,46,47}, amplification of PIK3CA is more common, reaching to 40% in HNSCC patients\textsuperscript{38,40,48}. Interestingly, PIK3CA amplification is also more frequent in squamous cell carcinomas of lung and cervix, suggesting its unique pathological role in squamous cell carcinomas\textsuperscript{38,40,48}. Clinical correlation studies showed that PIK3CA alterations are associated with cancer recurrence, metastasis and poor clinical outcomes in various human cancers\textsuperscript{49–51}. In HNSCC, a few clinical studies also showed that alteration of PIK3CA were correlated with advanced stage\textsuperscript{52,53}, vascular invasion\textsuperscript{54} and lymph node metastasis\textsuperscript{55}.

Functionally, although the activated forms of PIK3CA transforms cells in vitro\textsuperscript{36}, their oncogenic role in vivo was only recently investigated. In vivo, deletion or inactivation of PIK3CA halts oncogenic transformation and is resistant to Ras-oncogene-induced tumorigenesis\textsuperscript{36,56}. Overexpression of PIK3CA results in hyperplasia in ovarian surface epithelium and predisposes mammary glands to neoplastic transformation\textsuperscript{57}. Additionally, a knock-in PIK3CA H1047R mutant is sufficient for lung and breast cancer development\textsuperscript{58,59}. However, in HNSCC, our recent report showed that PIK3CA overexpression alone is not able to initiate tumor formation, but greatly promotes tumor progression and metastasis\textsuperscript{14}.

**PI3K Isoforms**

The PI3K isoforms is summarized in Table 1.1. Among these isoforms, PIK3R1, which encodes the regulatory subunit p85α of class IA PI3K is also frequently mutated in human cancers. Somatic mutations in PIK3R1 occur in 7% of human HNSCC samples\textsuperscript{60}. It is believed that these mutations weaken an inhibitory
Table 1.1 Summary of PI3K isoforms.

<table>
<thead>
<tr>
<th>Class</th>
<th>PI3Ks</th>
<th>Catalytic subunit: p110α, p110β, p110δ and p110γ</th>
<th>Regulatory subunit: p50α, p55α, p55γ, p85α, p85β, p87, p101</th>
<th>Class I PI3Ks use phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) as their substrate. Catalytic subunits interacts with regulatory subunit.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class I</td>
<td>PI3Ks</td>
<td>Class II PI3Ks use PtdIns as a substrate. Under certain condition, they might also use PtdIns-4-phosphate (PtdIns4P) as substrate. Unlike class I PI3Ks, there is no regulatory subunit for class II PI3Ks.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Class II</td>
<td>PI3Ks</td>
<td>Vps15, Vps34</td>
<td>There is only one catalytic member vacuolar protein sorting 34. Vps34 uses PtdIns as a substrate and interacts with Vps15.</td>
<td></td>
</tr>
</tbody>
</table>
interaction but retain a stabilizing interaction between p85α and p110α, resulting in aberrant activation of PI3K signaling\textsuperscript{62}.

The p110β, p110γ and p110δ isoforms are also oncogenic in experimental settings. However, human cancer samples barely contain genetic alterations in these isoforms\textsuperscript{61}. In a PTEN-GEMM prostate cancer study, ablation of p110β, not p110α, slowed prostate tumorigenesis\textsuperscript{63}. Although, when a constitutively activated form of the p110β isoform was overexpressed, it induced prostate intraepithelial neoplasia in mice\textsuperscript{64}. Furthermore, a breast cancer ERBB2-GEMM study showed that a catalytically inactive p110β knock-in halted tumor development\textsuperscript{65}. For HNSCC, no such studies have been reported.

**Key Components in PI3K Signaling: PTEN, AKT, mTOR and PDK1**

PTEN, the second most commonly mutated tumor suppressor in human cancers, acts as a negative regulator of PI3K signaling by dephosphorylating PI(3,4,5)P3 (Figure 1.2). Inactivation of PTEN results in activation of AKT and mTOR, which are oncogenic in human cancers\textsuperscript{66}. Somatic mutations of PTEN are rare in HNSCC (roughly 3% of cases), but loss of PTEN expression is more common and occurs in 30% of the cases\textsuperscript{67}. A report showed that loss of PTEN expression is associated with tumor prognosis\textsuperscript{67}. However, the mechanisms underlying loss of PTEN expression are still unknown\textsuperscript{68–71}.

AKT, a serine/threonine kinase, is considered the central mediator of the canonical PI3K pathway\textsuperscript{72}. In general, activation of PI3K molecules or inactivation of PTEN will result in AKT activation, making it as a readout for the upstream molecular
changes. AKT activation may be an early event in human HNSCC tumorigenesis, as persistent AKT activation occurs as early as the pre-malignancy stage, which includes dysplasia and carcinoma in situ.

There are three isoforms of AKTs (AKT1, AKT2 and AKT3) and all three isoforms are altered, although infrequent, in multiple human cancers. The most common mutation of AKT is an E17K missense mutation. However, there are no reports of the AKT1 E17K mutation in HNSCC, but the mutation was detected in human SCCs of lung. The E17K mutations at AKT2 or AKT3 genes were found in endometrial carcinoma and E17K mutation at AKT3 was also found in melanoma. Mutation of AKT isoforms are barely present in HNSCC. Overexpression of AKT isoforms, especially AKT2, occurs in multiple human cancers. At the mRNA level, AKT2 overexpression occurs in breast and colon cancers and is associated with cancer migration, invasion and metastasis. AKT1 and AKT3 overexpression is only observed in human gastric cancer and melanoma, respectively. In HNSCC, one study reported AKT2 overexpression in tumor samples, but not AKT1 or AKT3.

mTOR is one of the most intriguing signaling mediators of the PI3K/AKT pathway. mTORC1 and mTORC2 are the two distinct mTOR complexes. mTORC2, which contains Rictor, SIN1 and mLST8/GbL, activates AKT by phosphorylating Ser473 of AKT. mTORC1, which contains Raptor, PRAS40 and mLST8/GbL, is activated by AKT to control key eukaryotic translational regulators, including p70-S6 kinase and 4E binding protein 1 (4E-BP1). Additionally, mTORC1 phosphorylates the growth factor receptor-bound protein 10 (Grb10). mTORC1-mediated
phosphorylation stabilizes Grb10, which inhibits the PI3K and ERK-MAPK pathways\textsuperscript{88}. Activation of mTOR/p70-S6/4E-BP1 pathway occurs frequently in human HNSCC specimens and cell lines; additionally, rictor amplification has also been reported\textsuperscript{47,89}. Given its important function in PI3K/AKT pathway, targeting mTOR, such with rapamycin, is a promising approach for cancer therapy\textsuperscript{90}. For example, rapamycin has been reported to be effective in treatment of HNSCC both in vitro and in vivo\textsuperscript{39,89,91}.

PDK1 is another serine/threonine protein kinase that phosphorylates members of the AGC kinase superfamily, including AKT. Interestingly, PDK1 has been shown to regulate cell migration but not proliferation in lymphocytes\textsuperscript{92}, and to promote EMT in cardiac development\textsuperscript{93}. Compared to PTEN, AKT and mTOR, PDK1 is relatively less studied in human cancers. Overexpression of PDK1 has been reported in several human cancers\textsuperscript{67,94,95} and correlates with disease progression in patients with pancreatic cancer or melanoma\textsuperscript{67,96}. Invasion and activation of matrix metalloproteinase occurs during PDK1 overexpression\textsuperscript{97}, while downregulation of PDK1 has an inhibitory effect on human breast cancer cells\textsuperscript{98}. Interestingly, PDK1 is required for Kras-driven pancreatic cancer in murine models but not for the Kras-driven lung cancer, suggesting a context-specific role of PDK1 in human cancers\textsuperscript{95}.

**Crosstalk between PI3K Signaling and TGFβ Signaling in HNSCC**

Transforming growth factor beta (TGFβ) is a multifunctional cytokine that regulates cell proliferation, apoptosis, tissue remodeling, immune function and angiogenesis\textsuperscript{26,99,100}. The TGFβ ligands (TGFβ1, 2 and 3), bone morphogenic
proteins (BMPs) and activins/inhibins are all members of the TGFβ superfamily. The two types of transmembrane serine/threonine kinase receptors for TGFβ signaling are TGFβ receptor I (TGFβRI) and TGFβ receptor II (TGFβRII). Major regulators of TGFβ signaling are Smad family members, including receptor-activated Smads (R-Smads, Smad2 and 3), inhibitory Smads (I-Smads, Smad6 and 7) and the common Smad (Smad4). Phosphorylation of TGFβRI leads to phosphorylation of R-Smads. Phosphorylated R-Smads form a complex with Smad4 and regulate gene expression at Smad binding elements, with interactions between the Smad complexes and other transcription factors. Attenuation of TGFβ signaling is accomplished by I-Smads which bind to TGFβRI and recruit ubiquitin ligases to degrade TGFβRI and R-Smads. Cross talk between PI3K and TGFβ signaling can occur at multiple levels. For example, Ligand-bound TGFβ receptors activate MAP kinases, PI3K/AKT and Rho GTPase. On the other hand, PI3K is able to mediate TGFβ receptor initiated intracellular signaling and antagonize TGFβ-induced cytostasis in some situations.

TGFβ signaling plays a dual role in human cancers with a potent tumor suppressor at the early stage, and tumor promoter at the late stage of human cancers. Epithelial cells with defective TGFβ signaling are hyper-proliferative, resistant to apoptosis and have increased genomic stability. Tumor epithelial cells often exhibit a compensatory increase in TGFβ1 ligand production, which further promotes tumor progression by increasing inflammation, angiogenesis in tumor stromal cells. Besides, TGFβ1 is a potent inducer for EMT and immune suppressor.
Our lab has shown that loss or reduced expression of Smad4 and TGFβRII is common in human HNSCC, and Smad4 or TGFβRII GEMM gave rise to metastatic HNSCC, highlights the importance of this pathway in HNSCC development and progression\textsuperscript{13,107,108}. Conversely, altered TGFβ signaling in tumor epithelial cells elaborate TGFβ1 ligand overexpression in human HNSCC\textsuperscript{104}. The TGFβ/SMAD and PI3K/AKT pathways, interplay each other in human cancer development and progression. In the early stages of cancer, PI3K/AKT pathway activation antagonizes the effects of TGFβ-induced growth-inhibition or cell death\textsuperscript{101}. However, tumor cells are often more aggressive due to crosstalk between the TGF-β/SMAD and PI3K/AKT pathways at late stage\textsuperscript{100,101,104}. Targeting either or both pathways has been proposed including our lab for treating HNSCCs\textsuperscript{14,109}.

**Mouse Models for HNSCC**

In vivo modeling of HNSCC, which recapitulates both the gross pathological and molecular features of human HNSCC patients, provides an excellent platform to investigate molecular mechanisms and testing novel therapeutics for HNSCC. Here, I will detail three types of mouse models for HNSCC.

**Chemically Induced HNSCC Mouse Models**

This refers to induction of murine HNSCC by applying chemical carcinogens to immunocompetent mice. One example is the carcinogen 7,12-dimethylbenz[a]anthracene (DMBA)-induced Syrian hamster cheek pouch model\textsuperscript{110}. For this model, repeated exposure to the carcinogen DMBA for 14 weeks causes
premalignant mucosal lesions and SCCs, which share genetic/epigenetic alterations with human HNSCCs. These changes include H-ras mutation and overexpression of TGFβ1. However, hamster pouch mucosa tumors do not resemble human HNSCC anatomically and histologically.

Another chemically-induced HNSCC mouse model uses 4-nitroquinoline-1-oxide (4NQO), a water-soluble quinolone derivative to induce mouse HNSCC. 4NQO induces DNA adduct forming similar to human tobacco carcinogen, mimicking tobacco exposure in human HNSCC patients. The advantages of the chemical-induced HNSCC mouse models are 1) recapitulating an environment risk factor for HNSCC; 2) recapitulating tumor heterogeneity. The disadvantages are 1) the molecular alterations are not clear and less defined; 2) the doses of carcinogen used for the model may not be physiologically relevant. However, combining chemical exposure with other mouse models such as genetically engineered mouse model may be the best system to illustrate the interaction between environmental risk factors and genetic alterations in HNSCC development or progression. For example, we have recently shown that overexpression of PIK3CA promotes HNSCC progression by PDK1 and enhanced TGFβ signaling by using such combined strategy.

Xenograft Models for HNSCC

This refers to transplanting human HNSCC tumors either cell lines or fresh tumor tissues in immune-compromised mice. The advantages of this model are: 1) the tumor is human origin and is good for human relevance study; 2) the tumors
grow faster and are good for a quick in vivo study and drug screening. The disadvantages are: 1) the model lacks an intact immune system which is a critical part for cancer study; 2) it is not a naturally occurred tumor, and the tumor is a transplant.

**Genetically Engineered Mouse Models (GEMMs)**

GEMMs refer to using genetic engineered techniques to either overexpress a specific gene (transgenic), or delete a certain gene (knockout) in the mouse genome\(^ {118} \). The advantages are: 1) ideal model for investigating causal role of specific molecular alterations identified in human cancer patients. 2) naturally occurred tumors mimicking each stage of human cancer development and progression. The disadvantages are: 1) it relatively lacks of tumor heterogeneity; 2) it is labor intensive and takes longer time.

The earliest GEMM for HNSCC is to overexpress cyclin D1 transgene in oral-esophageal epithelium by using an Epstein-Barr virus lytic promoter\(^ {119} \). However, this model used a conventional transgenic technique, in which the transgene level cannot be controlled, and was over physiologically relevant level in human patients. Therefore, our lab developed inducible head-and-neck-specific mouse models for HNSCC.

**Inducible Head-and-neck-specific Transgenic Mouse Models**

Our model system utilized the progesterone receptor (PR) system to allow for spatial and temporal control of gene expression, as well as the ability to control gene
expression levels\textsuperscript{117,120}. This inducible transgenic system contains both a transactivator line and a target line. As shown in Figure 1.3, the transactivator contains the Gal4 DNA binding domain, a truncated progesterone receptor ligand binding domain (ΔPR) and the NF-κB p65 transactivation domain. Activation of the transactivator only occurs when ΔPR is bound to PR antagonist, RU486. RU486 activates the transactivator in a dose-dependent manner. To target transgene expression in the basal layer of the stratified epithelia, the Keratin 5 (K5) or K14 promoter are used. The target line consists of engineered GAL4 binding sites upstream of a tata minimal promoter and the target gene. When the transactivator and target lines are crossed, it produces double transgenic mice containing both genetic components. Following application of RU486, the transactivator binds the GAL4 binding site upstream of the target gene and drives expression of the target gene\textsuperscript{121} (Figure 1.3). The spatio-temporal control, and the ability to adjust gene expression level make it an ideal model to study the in vivo role of overexpressed genes identified in human HNSCC patients.

**Inducible Head-and-neck-specific Knockout Mouse Models**

As shown in Figure 1.3, this system is based on the modification of the Cre/LoxP system. The Cre enzyme is a DNA recombinase derived from the bacteriophage P1 and works by initiating DNA recombination at loxP sites. The Cre enzyme is fused with ΔPR, which allow it to be controlled by RU486. Gene deletion is accomplished by flanking the target gene with loxP sites and activating Cre enzyme by RU486 to initiate recombination and delete the LoxP flanking part of the
Figure 1.3. Schematic of inducible transgenic/conditional knockout mouse models. (A.) Inducible transgenic mouse models consists of a transactivator line and a target line. In the transactivator line, the keratin 5 or 14 is used to restrict expression of the GLp65 fusion protein to the stratified epithelia. GLp65 consists of a GAL4 DNA binding domain, the NF-kappaB p65 transactivation domain, and a RU486-inducible truncated progesterone receptor ligand binding domain. The target line consists of the gene of interest under the control of minimal tata promoter and four GAL4 DNA binding sites. Crossing of these two lines generates inducible transgenic mouse model. RU486 can be topically applied to induce transgene expression. (B.) For the inducible conditional knockout mouse model, Cre enzyme is fused with ΔPR to allow it to be controlled by RU486. Gene deletion is accomplished with Cre/LoxP system by flanking the target gene with loxP sites and activating Cre enzyme by RU486 to initiate recombination and delete the LoxP flaking region of the target gene.
target gene. Cre/LoxP has also been used in combination with other inducible promoters, such as tetra-cyclin, or estrogen receptor systems\textsuperscript{117,120}. In addition, introduction of a new mutant gene can also be accomplished using the Cre/LoxP system, so called knock-in system. To initiate expression of a mutant gene, a floxed STOP cassette is placed before or after the target gene, which interrupts gene transcription. Once the Cre-PR1 fusion protein is activated, the floxed stop cassette is removed and the mutant gene is expressed.

By using these systems, our lab and others successfully generated inducible head-and-neck-specific TGFβ, PIK3CA and AKT transgenic mouse models, and inducible head-and-neck-specific TGFβRII, Smad4, PTEN, Kras knockout mouse models\textsuperscript{13,14,107,108,122}, which can be used to study molecular mechanism of TGFβ and PI3K pathway in HNSCC, and to test novel therapeutics for HNSCC.

**EMT, Cancer Stem Cells and Anti-Cancer Stem Cell Therapy**

**Epithelial-to-Mesenchymal Transition (EMT) in HNSCC**

Epithelial-to-mesenchymal transition (EMT) is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells. During embryonic development and wound healing, EMT allows for morphological alteration of epithelial cells\textsuperscript{123}. Epithelial morphological changes breakdown cell-to-cell and cell-to-extracellular matrix connections, which allows cell migration during tissue regrowth and repair\textsuperscript{123}. In human cancers, EMT participates in cancer cell migration
and metastasis and is associated with cancer progression. EMT is a feature in a subset of tumors of human HNSCC patients. In HNSCC patients, enhanced tumor cell migration and higher risk of metastasis are associated with E-cadherin loss and gain of vimentin, a molecular feature of EMT, and overexpressed EMT-related transcription factors.

TGFβ1 is a potent EMT inducer in multiple human cancers, including HNSCC. In addition, it has been reported that Bmi1 regulates EMT through activation of AKT, stabilization of Snail, and repression of E-Cadherin in HNSCC. HNSCC cells with strong HIF-1α expression are capable of regulating EMT-associated gene expression. Given the strong association between EMT and HNSCC progression, understanding EMT process in HNSCC tumorigenesis will generate novel therapies to combat HNSCC progression.

**Cancer Stem Cells in HNSCC**

Cancer stem cells (CSCs) refer to cancer cells that possess the pluripotent characteristics of normal stem cells. It is hypothesized that CSCs contribute to cancer progression by forming tumors and sustaining themselves with self-renewal/repair mechanisms. CSCs promote resistance to chemotherapy and radiotherapy, and contribute to recurrence and metastasis. Altogether, these characteristics support a role for CSCs in tumor initiation, recurrence and metastasis.

Surgery and chemo-radiation therapies are still the major standard treatments for HNSCC patients. While these treatments are often effective for patients at their
early stage, over 20% of patients will relapse and die\textsuperscript{138}. Platinum-based therapy provides better control over local disease, but the incidence of recurrence and distant metastases is increasing and the mortality rate for HNSCC patients with distant metastases reaches to $\sim 90\%$\textsuperscript{139,140}. CSCs may contribute to therapeutic resistance, recurrence and the formation of distant metastases\textsuperscript{27,30}. Therefore, we believe that CSCs must be eliminated in order to achieve a cure for HNSCC patients. Currently, the putative markers for identification and isolation/enrichment of CSCs in HNSCC include surface markers CD44\textsuperscript{141,142}, CD166\textsuperscript{143}, CD24\textsuperscript{144}, Side population (SP)\textsuperscript{145}, and aldehyde dehydrogenase 1 (ALDH1)\textsuperscript{146}. CSCs can also be enriched by sphere formation assay\textsuperscript{147}. Besides these methods, c-Met\textsuperscript{148}, CD98\textsuperscript{149}, CD271\textsuperscript{150}, CD200\textsuperscript{151} and CD10\textsuperscript{152} are recently reported as alternative CSC markers for HNSCC, although there markers are still controversial\textsuperscript{142,144,153}.

**EMT and Cancer Stem Cells**

Interestingly, EMT and generation of CSCs are intricately connected in human cancers. The first example was found in breast cancer cells, where generation of stem cell traits in conjunction with EMT induction have been observed in mammary epithelial cells and breast cancer cells\textsuperscript{154}. Further research revealed that EMT induction promoted tumor cell invasion, metastasis, drug resistance and enriched CSC populations in other types of human cancers\textsuperscript{123,155,156}. In HNSCC, CSC-like CD44+ population is capable of switching a proliferative, epithelial-like, non-CSC phenotype into a migratory, mesenchymal and CSC-like phenotype\textsuperscript{157}, suggesting the connection between EMT and CSC traits in HNSCC. In addition,
overexpression of S100A4 in HNSCC cells promoted an EMT phenotype, accompanying with enhanced stem cell-like properties; conversely, S100A4 knockdown reversed EMT and reduced self-renewal and tumorigenic properties in vitro and in vivo\textsuperscript{158}.

**Anti-Cancer Stem Cell Therapy**

Traditional therapies often fail to prevent and/or treat cancer recurrence and metastasis\textsuperscript{30}. CSC theory, although controversial, is believed to contribute to cancer recurrence and metastasis\textsuperscript{159}, providing a promising opportunity for curing cancers.

In general, there are two approaches to eliminate CSCs: 1) Agents that show specific or preferential cytotoxicity to CSCs; 2) agents that restore mesenchymal-to-epithelial (MET) state. The first example is salinomycin, a potassium ionophore that selectively destroys CD44\textsuperscript{high}/CD24\textsuperscript{low} breast CSCs in vitro and inhibits tumorigenesis in vivo\textsuperscript{160,161}. The second example is PKA activation drugs, which are able to reverse EMT, and reduce CSC ability\textsuperscript{162}. Studies on CSCs have also identified several CSC-related signaling pathways, such as Hedgehog\textsuperscript{163}, Notch\textsuperscript{164–166} and Wnt/\(\beta\)-catenin\textsuperscript{167,168} pathways. Drugs designed to target these pathways are developing in the hope of using them as anti-CSC therapies in future.

**Epigenetics and Targeted Epigenetic Therapy in Cancer**

Epigenetics refers to modification of gene expression rather than alteration of the genetic code itself\textsuperscript{169}. Two major epigenetic modifications are histone modification and DNA methylation. Epigenetic changes are implicated in the
pathogenesis of cancer and represent a novel approach to cancer therapeutics\textsuperscript{170}.

Histone deacetylation executed by histone deacetylase (HDAC) is the most important process of histone modification. Histone deacetylation regulates chromatin remodeling and controls transcription in both normal cells and cancer cells\textsuperscript{170,171}. DNA methylation at the CpG-rich regions of promoter also controls transcription of the gene, and has been shown as one of the mechanisms of silencing tumor suppressor genes in human cancers\textsuperscript{169}. Given the important roles of both epigenetic processes in human cancers, targeting either histone deacetylation using HDAC inhibitors, or DNA methylation using demethylating agent have been used clinically for treating certain human malignancies\textsuperscript{172}. For example, HDAC inhibitors have been found to block cancer cell proliferation and differentiation\textsuperscript{173,174} induce apoptosis, and inhibit angiogenesis in human cancers\textsuperscript{175}. Demethylating agents work by covalently bonding with DNA methyltransferase and preventing DNA methylation. Previous research indicates that demethylating agents such as 5-Azacytidine are capable of inhibiting DNA methylation and associated oncogenesis\textsuperscript{176,177}.

Epigenetic modification is also frequently linked to certain key characteristics of CSCs. For example, silencing tumor suppressors with DNA methylation fuels CSC and promote tumor initiation, while hypomethylation of self-renewal and differentiation-associated genes supports CSC proliferation and metastasis\textsuperscript{178}. BMP2/4 promotes differentiation of both normal and cancerous stem cell populations. When BMP receptor 1B (BMPR1B) is silenced, BMP2/4 supports oncogenesis. However, these changes can be countered by demethylating the
miRNA Dysregulation in HNSCC

MicroRNAs (miRNAs) are a class of small non-coding RNAs, ranging from 19-25 nucleotides in length. miRNAs serve as regulators of post-transcriptional modification through targeting mRNAs by binding mRNA to inhibit its translation or target for degradation. A single miRNA can target hundreds of mRNAs and regulate multiple cell signaling pathways. Given the diverse roles of miRNA in cell signaling, it is not surprising that miRNA dysregulation is linked to oncogenesis. In addition, miRNAs represent a new class of molecule for developing targeted therapies in cancer treatment.

Dysregulation of miRNA expression has been linked to tumorigenesis and dysregulated miRNAs may function as either tumor suppressors or oncogenes (Figure 1.4). For example, miR-17-92 cluster promotes tumorigenesis by increasing proliferation of cancer cells and deletion of miR-17-92 cluster slows down Myc-induced oncogenesis. miR-21 overexpression is observed in breast cancer and is associated with advanced stage, lymph node metastasis and poor prognosis; knockdown of miR-21 in breast cancer cells inhibits proliferation, migration, invasion and in vivo tumor growth. miR-200 family miRNAs function as tumor suppressors and loss of these miRNAs promotes EMT and metastasis.

Changes in miRNA expression also commonly occur in HNSCC. The Let-7 miRNA family is down regulated in HNSCC. Let-7a, inhibits expression of genes linked to cancer stem cell-like properties, while Let-7d suppresses EMT in vitro.
Figure 1.4. MicroRNAs targeting multiple hallmarks of cancer. Dysregulated microRNAs may function as either tumor suppressors or oncogenes to mediate cancer progression. This figure has been adapted from Iorio M V. et al. (2012) EMBO Mol Med.
Another miRNA, miR-130b, is frequently overexpressed in HNSCC\textsuperscript{189,191} and is associated with EMT\textsuperscript{192} and downregulating tumor suppressor genes\textsuperscript{193}. Amplification of miRNA locus, including miR-106b, miR-93 and miR-25, also occurs in HNSCC and is related to cancer cell proliferation\textsuperscript{189}.

Large subsets of HNSCC cases are plagued by recurrence, metastasis and subsequent death, which are likely caused by CSCs\textsuperscript{194}. Further research identifying specific miRNAs involved in CSC maintenance will contribute to the development of miRNA-based anti-CSC therapies for HNSCC.

**Current Standard of Care for HNSCC**

Most HNSCC patients present with advanced, stage III or IV disease, which require a combination therapy (usually chemotherapy, radiation and surgery). Stage I or II disease requires less aggressive treatment and have a better prognosis than patients with late stage HNSCC. However, they are still at risk of recurrence or metastasis, which are the two major challenges for treating HNSCC patients\textsuperscript{5}.

Currently, radiation therapy remains the primary treatment for oropharyngeal cancer, advanced hypopharyngeal and laryngeal cancer, while chemotherapy is essential for treating locally advanced HNSCC\textsuperscript{195}. Induction chemotherapy is often administered with or prior to radiotherapy\textsuperscript{195}. The only targeted therapy agents that are approved to treat HNSCC patients by FDA are inhibitors that target EGFR. Despite the usage of the third generation of these inhibitors, the median overall survival for patients with recurrent or metastatic tumors remains less than 1 year\textsuperscript{195}. To advance therapeutic strategies, numerous ongoing clinical trials are evaluating
the safety and efficacy of new treatments for HNSCC. A multicenter, randomized phase 3 trial tested a combination of docetaxel, cisplatin, and fluorouracil as induction chemotherapy for HNSCC patients, but only achieved modest results\textsuperscript{196}. Studies are also analyzing the efficacy of EGFR inhibitors, including cetuximab and other monoclonal antibodies, for HNSCC treatment, but observed resistance after treatment\textsuperscript{197,198}. Another class of potential therapeutic agents to treat HNSCC is tyrosine kinase inhibitors, which are administered orally and include the heavily studied drugs gefitinib\textsuperscript{199} and erlotinib\textsuperscript{200}. Unfortunately, both gefitinib and erlotinib are only modestly effective against HNSCCs and gefitinib doesn’t improve survival rates over those of methotrexate\textsuperscript{201}.

Given the lack of effective treatments for HNSCC by current standard of care and rising incidence of this disease, there is an urgent need for novel therapies. Most recently, a promising avenue to treat HNSCC is the use of immune checkpoint inhibitors to unleash or enhance anti-cancer immune response. Recent clinical trials of PD1 antibody for the first line treatment of HNSCC, especially for the recurrent or metastatic HNSCC achieved some promising results\textsuperscript{202}. There is no doubt that novel and effective therapies are highly desired to combat this devastating disease, particularly for fighting with disease recurrence and metastasis.

**Summary**

Prognosis of HNSCC patients has not been significantly improved over past decades. Treatment plans remain surgery, chemo-radiation and limited options of targeted therapy, which is often less effective to control disease progression.
Investigation of underlying molecular mechanism in HNSCC progression has a hope to develop novel and effective treatment plans to improve patients’ survival. Both PI3K and TGFβ signaling pathways are highly associated with human cancers, including HNSCC. We believe that interplay of these two pathways is at least one of the driven forces to promote HNSCC progression. EMT and CSC are implicated in cancer recurrence and metastasis, which are the major causes for cancer deaths. Understanding molecular mechanisms responsible for these two processes in the context of deregulating PI3K/TGFβ signaling will generate candidates for development as novel therapies to prevent/treat recurrence and metastasis of HNSC patients.
CHAPTER II

MATERIALS AND METHODS

Cell Culture

Cells were cultured from either 4NQO-induced control tongue SCCs (CUCONs) or 4NQO-induced PIK3CA-GEMM tongue SCCs (CU110s). Tumor tissues were minced with blade, and digested in 0.35% collagenase (Gibco, Carlsbad, CA, USA) followed by two rounds of 1% trypsin digestion at 37°C. Single cells were obtained through a cell strainer (70 µm nylon, BD Biosciences, Franklin lakes, NJ, USA), and plated at 1 × 10^5/ml in 10cm dishes. The authentication of the cultured cells was validated by the transgene-specific genotyping PCR. The CU110 and CUCON cells were maintained in DMEM (Corning Cellgro) supplemented with 10% FBS (Sigma-Aldrich), streptomycin (100 mg/mL), and penicillin (100 IU/mL) in a 5% CO2 incubator (37°C).

UMSCC cell lines were obtained from the University of Michigan and VU cell lines from Vrije University at Netherland under MTA agreements Human HNSCC cell lines UMSCCs 1, 2, 47, VU1131, VU1365, Fadu, LNM1, Tu, HN6, CAL27, M4C and M4E were obtained and cultured as recommended by protocols provided by University of Michigan, Vrije University, or ATCC. Basically these cells were cultured in DMEM (Corning Cellgro) supplemented with 10% FBS (Sigma-Aldrich), streptomycin (100 mg/mL), and penicillin (100 IU/mL) in a 5% CO2 incubator (37°C).
**Cell Proliferation, Migration and Invasion Assays**

For the cell proliferation assays, CU110 and CUCON cells were seeded in 12-well plates as a density of $5 \times 10^4$ cells per well in triplicate. For CU110 cell transfected with lentiviral vectors, cells were plated in 12-well plates as $2.5 \times 10^4$ cells per well in triplicate. Cells were then counted by Vi-CELL cell counter (Beckman Coulter, CA, USA) at different time points. Briefly, cells in each well of 24-well plate were harvested by treating with trypsin. 800µL of diluted cell suspension was used for the cell viability analysis by using Vi-CELL counter. We also used Cell Counting Kit-8 (Dojindo, Japan) to quantify cell proliferation and viability according to the manufacturer’s manual. Briefly, $6 \times 10^3$ cells were seeded into each well of 96-well plate. After different time points, 100µL of Cell Counting Kit-8 reagent was added to each well and incubate at 37°C for 1.5 hours. The plate was then read by plate reader (Biotek Synergy II) at 450nM.

For the cell migration assay, cells were plated in 12-well plates and wound scratches were made using a 200µl pipette tip when cells reach confluence. Cells were washed with PBS, photographed and incubated in a complete growth media for 48 hours. Cells were then photographed using Walter Camera Microscope (Walter Technology). The extent of wound closure was quantified by measuring the distance between two sides of the scratched lines before and after 48 hours migration using Photoshop (Adobe Technology). Migrated distance was presented as pixels and multiple measurements were performed.

Cell invasion ability was determined using the QCM ECMatrix Cell Invasion Assay Kit (ECM550, EMD Millipore, CA, USA). Briefly, $1 \times 10^5$ cells were seeded in
serum free media to the upper insert and compete media containing 10% FBS was added to the lower chamber. After 48 hours, the invasive cells on the lower surface of the insert were stained according to the manufacturer's manual. The invasive cells were then counted using a microscope at multiple view fields.

**Stable Knocking Down Using Lentiviral-based shRNAs**

The following lentiviral-based vectors containing short hairpin (sh)-RNAs were purchased from Sigma Aldrich (Table 2.1). shRNA lentiviral transduction particles sh-PIK3CA-2 (TL501641V) and its relevant control shRNA (TR30021V, shCntrl-2) were obtained from Origene Technologies. For lentiviral transfection, 2.2 x 10^4 CU110 cells (or 3.5 x 10^4 cells for Fadu/UMSCC47) were plated in each well of 6-well plate and virus-containing media with 8 mg/mL polybrene (Millipore) was added at the second day. 48 hours later, complete media with puromycin (10 mg/mL for CU110 cells and 2 mg/mL for Fadu/UMSCC47 cells) was added. Cells were cultured with puromycin for 1 week to eliminate non-transduced cells. Knockdown efficiency was determined by quantitative real-time PCR and Western blotting analysis.

**HNSCC Sphere Formation Assay**

Either murine or human HNSCC cells or cells pre-treated with inhibitors were suspended at density of 4.0 x 10^5 cells/mL in serum-free DMEM (Corning Cellgro) medium and seeded into 96-well ultra-low attachment plates (Corning Incorporated) as 150µL per well. Cells were cultured in a 5% CO2 incubator (37°C). After 48
### Table 2.1. List of sh-RNAs.

<table>
<thead>
<tr>
<th>sh-RNA</th>
<th>TRC number</th>
</tr>
</thead>
<tbody>
<tr>
<td>sh-PIK3CA-1</td>
<td>TRCN0000361413</td>
</tr>
<tr>
<td>sh-AKT1</td>
<td>TRCN00000039797</td>
</tr>
<tr>
<td>sh-AKT2</td>
<td>TRCN0000265834</td>
</tr>
<tr>
<td>sh-PIK3R1-1</td>
<td>TRCN0000304741</td>
</tr>
<tr>
<td>sh-PIK3R1-2</td>
<td>TRCN00000302323</td>
</tr>
<tr>
<td>sh-PDK1</td>
<td>TRCN0000361331</td>
</tr>
<tr>
<td>sh-FOXC2</td>
<td>TRCN0000084725</td>
</tr>
<tr>
<td>sh-EZH2</td>
<td>TRCN0000039042</td>
</tr>
<tr>
<td>sh-STAT3</td>
<td>TRCN0000071456</td>
</tr>
<tr>
<td>sh-SOX4</td>
<td>TRCN0000012081</td>
</tr>
<tr>
<td>sh-VCAM1</td>
<td>TRCN0000094140</td>
</tr>
<tr>
<td>sh-ITGB4</td>
<td>TRCN0000066877</td>
</tr>
<tr>
<td>sh-LRRK1</td>
<td>TRCN0000362197</td>
</tr>
<tr>
<td>sh-PRL2C2</td>
<td>TRCN0000109931</td>
</tr>
<tr>
<td>sh-OSR1</td>
<td>TRCN0000085912</td>
</tr>
<tr>
<td>sh-DZIP1L</td>
<td>TRCN0000437750</td>
</tr>
<tr>
<td>sh-LPAR6</td>
<td>TRCN0000026397</td>
</tr>
<tr>
<td>sh-NAV3</td>
<td>TRCN0000218037</td>
</tr>
<tr>
<td>sh-BMP4</td>
<td>TRCN0000025957</td>
</tr>
<tr>
<td>sh-MMP14</td>
<td>TRCN0000031264</td>
</tr>
<tr>
<td>sh-ERAP1</td>
<td>TRCN0000324289</td>
</tr>
<tr>
<td>sh-MTHFD1L</td>
<td>TRCN0000075945</td>
</tr>
<tr>
<td>sh-HIF1A</td>
<td>TRCN0000232221</td>
</tr>
<tr>
<td>sh-EPAS1</td>
<td>TRCN0000082304</td>
</tr>
<tr>
<td>pLKO Non-target</td>
<td>SHC216</td>
</tr>
<tr>
<td>shRNA control</td>
<td></td>
</tr>
<tr>
<td>pLKO Non-target</td>
<td>shCntrl-1</td>
</tr>
<tr>
<td>shRNA control</td>
<td></td>
</tr>
</tbody>
</table>
48 hours, spheres (≥ 1.5 microns) were counted as an HNSCC sphere forming unit by using a bright field microscope (Leica). The data was presented by the average of three independent experiments. Spheroids were cultured in the serum-free DMEM medium for 2-12 weeks before mRNA extraction, protein isolation, histology, or in vivo experiments.

**FACS Analysis**

All antibodies used for FACS analysis were purchased from eBioscience unless specified otherwise. In Brief, murine or human HNSCC cells or cells treated with inhibitors were harvested and washed twice in PBS buffer, and suspended in PBS with 1% serum as a density of 1.0 x 10^6 cells/100 µL. Cells were then stained with fluorochrome-conjugated monoclonal antibodies for mouse CD24 (17-0242-82), human CD24 (17-0247-42), mouse/human CD44 (48-0441-82), for 1 hour on ice. After washing twice with ice cold PBS, cells were re-suspended in 400µL ice cold PBS with 1% serum. Propidium iodide (Sigma-Aldrich) was added (1µg/mL) to exclude dead cells for the analysis. A minimum of 50,000 events were recorded for all samples. All FACS analyses were performed on a Gallios (Beckman Coulter, CA, USA) and the data were analyzed using Kaluza (Beckman Coulter, CA, USA).

The assessment of ALDH1 activity was conducted by using ALDEFLUOR assay (StemCell Technologies, Durham, NC, USA). The procedure was followed by the manufacture’s manual. In brief, the single cell suspension was washed twice in PBS buffer and then suspended in ALDEFLUOR assay buffer as a density of 0.8 x 10^6 cells/mL. Activated ALDH substrate BAAA was added as 5µL/mL of cell
suspension and then 500 µL was transferred to a tube containing 5µL of 1.5 mM DEAB, a specific ALDH inhibitor. Cells were incubated at 37°C for 45 minutes. After washing twice with ice cold PBS, cells were re-suspended in 400µL of ice cold ALDEFLUOR assay buffer. Propidium iodide (Sigma-Aldrich) was added (1µg/mL) to exclude dead cells for the analysis. A minimum of 50,000 events were recorded for all samples. All flow cytometric analyses were performed on a Gallios (Beckman Coulter, CA, USA) and the data were analyzed using Kaluza (Beckman Coulter, CA, USA).

For the side population analysis, cells were suspended in the complete DMEM medium as 1.0 x 10^6 cells/mL. Hoechst 33342 (Sigma-Aldrich) was then added at a final concentration of 5 µg/mL for CU110 and CUCON cells and 1.5µg/mL for Fadu and UMSCC47 cells and the samples were incubated for 90 minutes at 37°C. After staining, cells were washed twice with ice cold PBS and re-suspended in 400 µL of ice cold PBS with 1% serum for FACS analysis using MoFlo XDP70 analyzer (Beckman Coulter, CA, USA). Immediate before the analysis, propidium iodide (Sigma-Aldrich) was added (1µg/mL) to exclude the dead cells. For the control reactions, CU110 or CUCON cells were incubated with 1 µmol of verapamil (or 0.7 µmol for Fadu and UMSCC47) for 30 minutes at 37°C prior to the staining by Hoechst 33342.

Quantitative Real-time PCR

Total RNAs were extracted using RNeasy kits (Qiagen). cDNAs for differential gene expression analysis were synthesized using high-capacity cDNA reverse
transcription kit (Applied Biosystems) according to the manufacturer’s instruction. cDNAs for miRNA expression analysis were synthesized using qScript microRNA cDNA synthesis kit (Quanta). Quantitative RT-PCR reactions were carried out in triplicate using iTaq Universal SYBR Green Supermix (Bio-Rad) and were run on CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, INC, Hercules, CA). Gene expression levels were normalized to GAPDH and quantified using comparative CT method. The primer sequences used for gene expression analysis are listed in the Table 2.2. For miRNA expression analysis, miRNA-specific primer paired with universal primer was used to amplify the signal using the SYBR green-based RT-PCR system. miRNA expression levels were normalized to U6 and quantified using comparative CT method. The miRNA specific primer sequences are listed in the Table 2.3.

**Protein Analysis**

Protein was extracted using the methods in our lab\textsuperscript{14}. For western analysis, antibodies against p110α (4249s), total AKT (9272s), AKT1 (2938s), AKT2 (2964s), phospho-AKTSer-473 (3787s), e-cadherin (3195s), vimentin (5741s), SOX2 (3579s), PDK1 (3439), phospho-PDK1 (Ser-241) (3061), phospho-SMAD2 (Ser-465/467) (3101), phospho-SMAD3 (Ser-423/425) (9520) and GAPDH (5174s) were purchased from Cell Signaling. Antibodies against p85α (ab71925) and ki67 (ab16667) were obtained from Abcam. Antibody against CD44 (550536) was obtained from BD Biosciences Pharmingen. Antibody against mouse K1 was purchased from Convance and antibody against K8 was obtained from Fitzgerald.
Table 2.2. Sequence of primers used for qPCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward / Reverse</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDH-1</td>
<td>F</td>
<td>5'-CATGTTCACTGTCAATAGGG</td>
</tr>
<tr>
<td>CDH-2</td>
<td>R</td>
<td>5'-GTGTATGTAGGGTAACCTCTCTC</td>
</tr>
<tr>
<td>CDH-2</td>
<td>F</td>
<td>5'-ACAATCAACAATGAGACTGG</td>
</tr>
<tr>
<td>CDH-2</td>
<td>R</td>
<td>5'-CGTATACTGTGGCACTTTTCTC</td>
</tr>
<tr>
<td>CDH-11</td>
<td>F</td>
<td>5'-CTGTAACATTGGCTGGTTG</td>
</tr>
<tr>
<td>Vimentin</td>
<td>F</td>
<td>5'-GAACCTGAGAGAACTAACC</td>
</tr>
<tr>
<td>Vimentin</td>
<td>R</td>
<td>5'-GATGCTGAGAGTGCTCATTG</td>
</tr>
<tr>
<td>ZEB1</td>
<td>F</td>
<td>5'-ATATGAGCAGACAGGAAGTAGGAG</td>
</tr>
<tr>
<td>ZEB1</td>
<td>R</td>
<td>5'-TTCATGTGTTGAGAGTAGGAG</td>
</tr>
<tr>
<td>ZEB2</td>
<td>F</td>
<td>5'-CAAACAGAAGAATCCAGGAG</td>
</tr>
<tr>
<td>ZEB2</td>
<td>R</td>
<td>5'-TCCAGAGGGTTGCAAGGCTA</td>
</tr>
<tr>
<td>SNAI-1</td>
<td>F</td>
<td>5'-AGTTGACTACCGACCTTG</td>
</tr>
<tr>
<td>SNAI-1</td>
<td>R</td>
<td>5'-AAGGTTGAACCCACACAC</td>
</tr>
<tr>
<td>SNAI-2</td>
<td>F</td>
<td>5'-GACACATTAGAAGACTCAACTG</td>
</tr>
<tr>
<td>SNAI-2</td>
<td>R</td>
<td>5'-GACATTCTGGAAGAGTGGTGT</td>
</tr>
<tr>
<td>TWIST-1</td>
<td>F</td>
<td>5'-GAGACCTAGATGTCATTGTTTC</td>
</tr>
<tr>
<td>TWIST-1</td>
<td>R</td>
<td>5'-GAATTGGTCTCTGCTTCTCT</td>
</tr>
<tr>
<td>TWIST-2</td>
<td>F</td>
<td>5'-CGCATACCTGCTTCTTAC</td>
</tr>
<tr>
<td>TWIST-2</td>
<td>R</td>
<td>5'-CTCTTTATTGTTCTGCTGCTGCC</td>
</tr>
<tr>
<td>ETS-1</td>
<td>F</td>
<td>5'-CGGATTACTTCATCAGCTAC</td>
</tr>
<tr>
<td>ETS-1</td>
<td>R</td>
<td>5'-AGTCGTTCTCATACTTCAGG</td>
</tr>
<tr>
<td>FOXC2</td>
<td>F</td>
<td>5'-ACCAGGCTATTGGTTCTCTG</td>
</tr>
<tr>
<td>FOXC2</td>
<td>R</td>
<td>5'-ATTGATCATGGTGACGTG</td>
</tr>
<tr>
<td>KLF4</td>
<td>F</td>
<td>5'-CCCTTCGGGCATCAGTGTTAG</td>
</tr>
<tr>
<td>KLF4</td>
<td>R</td>
<td>5'-GGACCGCTCTTCTGCTTAAT</td>
</tr>
<tr>
<td>FN-1</td>
<td>F</td>
<td>5'-CCTATAGGATTGGAGACAC</td>
</tr>
<tr>
<td>FN-1</td>
<td>R</td>
<td>5'-GGTGGTAATAGCTGTGTCGG</td>
</tr>
<tr>
<td>BMI1</td>
<td>F</td>
<td>5'-TCAGCAACTTCATCTGGTTAG</td>
</tr>
<tr>
<td>BMI1</td>
<td>R</td>
<td>5'-CTGGACGACAGTCATCGAGTAT</td>
</tr>
<tr>
<td>SOX4</td>
<td>F</td>
<td>5'-CCTCGCTCTCCTCGTCCT</td>
</tr>
<tr>
<td>SOX4</td>
<td>R</td>
<td>5'-TCGTCCTGGAACCTCGTGT</td>
</tr>
<tr>
<td>EZH2</td>
<td>F</td>
<td>5'-GCCCGACCTGGAAATTCCTG</td>
</tr>
<tr>
<td>EZH2</td>
<td>R</td>
<td>5'-CAGACGACCTGGAGCTGCTG</td>
</tr>
<tr>
<td>MK1</td>
<td>F</td>
<td>5'-ACGCAGAGAATGAGTGGCGGTA</td>
</tr>
<tr>
<td>MK1</td>
<td>R</td>
<td>5'-TCAATGCTTGGCGAGGGGCA</td>
</tr>
</tbody>
</table>
Table 2.2. continued.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK5 F</td>
<td>5'-ACCCTCAACAACAAAGTTTGCC</td>
<td></td>
</tr>
<tr>
<td>MK5 R</td>
<td>5'-TCTGCTTTATGGTCTTGTTG</td>
<td></td>
</tr>
<tr>
<td>MK8 F</td>
<td>5'-AGCTGAGGCTGAAACCATGTA</td>
<td></td>
</tr>
<tr>
<td>MK8 R</td>
<td>5'-TCTGGCCTTTGAGGGCTTCAA</td>
<td></td>
</tr>
<tr>
<td>MK13 F</td>
<td>5'-ATTGAGAACCAGTTGATTTG</td>
<td></td>
</tr>
<tr>
<td>MK13 R</td>
<td>5'-TGGTCTGGAACCATTCCTTG</td>
<td></td>
</tr>
<tr>
<td>MK14 F</td>
<td>5'-TCATGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>MK14 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3CA F</td>
<td>5'-TCATGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3CA R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3CB F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3CB R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3CD F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3CD R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3CG F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3CG R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3R1 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3R1 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3R2 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3R2 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3R5 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3R5 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PIK3R6 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PIK3R6 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PI3KC2A F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PI3KC2A R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PI3KC3 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PI3KC3 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PI3KC-2 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PI3KC-2 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PI3R1-3 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PI3R1-3 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>PI3R2-1 F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>PI3R2-1 R</td>
<td>5'-TGTTAGACGCCACCTCCTTG</td>
<td></td>
</tr>
<tr>
<td>β-catenin F</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
<tr>
<td>β-catenin R</td>
<td>5'-GAGCTGAGGCTCTGCATCT</td>
<td></td>
</tr>
</tbody>
</table>
### Table 2.2. continued.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-cadherin</td>
<td>F</td>
<td>ACAATCAACAATGAGACTGG</td>
</tr>
<tr>
<td>N-cadherin</td>
<td>R</td>
<td>CGTATACTGTGTCATTTTCTC</td>
</tr>
<tr>
<td>FoxC2</td>
<td>F</td>
<td>ACCAGGCTATTTTGTCTTG</td>
</tr>
<tr>
<td>FoxC2</td>
<td>R</td>
<td>ATTTTATCATGGTGACGTGG</td>
</tr>
<tr>
<td>CD44</td>
<td>F</td>
<td>TCTGCCCCATCTAGCCTAAAGAGCc</td>
</tr>
<tr>
<td>CD44</td>
<td>R</td>
<td>GTCTGGGTATTTGAAAGGTGAGCC</td>
</tr>
<tr>
<td>CD166</td>
<td>F</td>
<td>CTGTGCTGTGGATGAGGCTTTG</td>
</tr>
<tr>
<td>CD166</td>
<td>R</td>
<td>CCTGAAGAGCAGTTTACACAGA</td>
</tr>
<tr>
<td>CD133</td>
<td>F</td>
<td>CTGCCCAAGCTGGAAGTATA</td>
</tr>
<tr>
<td>CD133</td>
<td>R</td>
<td>CAAACACACACACGAGCAAG</td>
</tr>
<tr>
<td>CD24</td>
<td>F</td>
<td>ACCACGCAGTTTACTGCAAG</td>
</tr>
<tr>
<td>CD24</td>
<td>R</td>
<td>CCCCTCTGGTGATGGCTGTAGA</td>
</tr>
<tr>
<td>EZH2</td>
<td>F</td>
<td>TCAAAACCCTTCTGG</td>
</tr>
<tr>
<td>EZH2</td>
<td>R</td>
<td>TGTCCTCAATGGTGACGA</td>
</tr>
<tr>
<td>BMI1</td>
<td>F</td>
<td>TGGACGAATGCTGGAGAGCTGAGAA</td>
</tr>
<tr>
<td>BMI1</td>
<td>R</td>
<td>TGCCCTAAAAGCTTTGTGGGTGG</td>
</tr>
<tr>
<td>KLF4</td>
<td>F</td>
<td>GCGTGAGGAACCTCTCTCACMTGA</td>
</tr>
<tr>
<td>KLF4</td>
<td>R</td>
<td>AAAGTCTAGGGTCAGGAGTCGTT</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>F</td>
<td>ACTGGCAGGACCTTCTCAAGCAGCA</td>
</tr>
<tr>
<td>Wnt5a</td>
<td>R</td>
<td>GCTATTTTGCGCATACCCCTGGCA</td>
</tr>
<tr>
<td>PDK1</td>
<td>F</td>
<td>AGACCTTTGGGGCCTTGGATGTAT</td>
</tr>
<tr>
<td>PDK1</td>
<td>R</td>
<td>ACAGCCTAAACGCTTTGTGGGATC</td>
</tr>
<tr>
<td>PTEN</td>
<td>F</td>
<td>AATTGGCAATGTCAGGAGCTATGT</td>
</tr>
<tr>
<td>PTEN</td>
<td>R</td>
<td>GATTGGCAATGTCAGGAGCTATGT</td>
</tr>
<tr>
<td>p16</td>
<td>F</td>
<td>ACATCGTGCGATATTTCGCTTCCG</td>
</tr>
<tr>
<td>p16</td>
<td>R</td>
<td>ACATCGTGCGATATTTCGCTTCCG</td>
</tr>
<tr>
<td>EGFR</td>
<td>F</td>
<td>GAAGCCTATGATGGCTAGTG</td>
</tr>
<tr>
<td>EGFR</td>
<td>R</td>
<td>AGGGCATGAGCTGTGAATG</td>
</tr>
<tr>
<td>STAT3</td>
<td>F</td>
<td>TGCTGCAATGGAACCTGTCTTGG</td>
</tr>
<tr>
<td>STAT3</td>
<td>R</td>
<td>AGGAATCGGCTATATTGTGAGCT</td>
</tr>
<tr>
<td>CYCLIND1</td>
<td>F</td>
<td>TGCTGCAAATGGAACCTGTCTTGG</td>
</tr>
<tr>
<td>CYCLIND1</td>
<td>R</td>
<td>TACCATGGAGGGTGTTGGCAAT</td>
</tr>
<tr>
<td>KRAS</td>
<td>F</td>
<td>GGAGGGGCTTTCTTTGTATTTTG</td>
</tr>
<tr>
<td>KRAS</td>
<td>R</td>
<td>GGCAACATCTCTAGACACATTTA</td>
</tr>
<tr>
<td>HRAS</td>
<td>F</td>
<td>CACAGCAGGCTAAAGAGAGATAT</td>
</tr>
<tr>
<td>HRAS</td>
<td>R</td>
<td>CTGCAGGACACTTGTGGTGTG</td>
</tr>
<tr>
<td>NRAS</td>
<td>F</td>
<td>GAAAGCAAGTGGTGATGGATGG</td>
</tr>
<tr>
<td>NRAS</td>
<td>R</td>
<td>TGTCCTCATGACTGCTTCTCT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>TNFα</td>
<td>F</td>
<td>TTGTCTACTCCAGTTCTCT</td>
</tr>
<tr>
<td>TNFα</td>
<td>R</td>
<td>GAGGTTGACTTTCTCTGTTGATG</td>
</tr>
<tr>
<td>NF-κB1</td>
<td>F</td>
<td>GGTGACAGAGCCCTGATTGA</td>
</tr>
<tr>
<td>NF-κB1</td>
<td>R</td>
<td>CTTCTCTCTGCTGCTGAGTT</td>
</tr>
<tr>
<td>IL-1</td>
<td>F</td>
<td>ATGGAGGTGAGAGCTTTCAG</td>
</tr>
<tr>
<td>IL-1</td>
<td>R</td>
<td>TTGAGGTGAGAGCTTTCAG</td>
</tr>
<tr>
<td>IL-6</td>
<td>F</td>
<td>GCTGCAGGAGTCACAGAGG</td>
</tr>
<tr>
<td>IL-6</td>
<td>R</td>
<td>TTGCCGAGTAGATCTCAAAGTG</td>
</tr>
<tr>
<td>IL-6R</td>
<td>F</td>
<td>GCCACCGTACCCTGATT</td>
</tr>
<tr>
<td>IL-6R</td>
<td>R</td>
<td>GTTTCTCTGTGCTGATTCCATCTCT</td>
</tr>
<tr>
<td>CCL2</td>
<td>F</td>
<td>AGTAGGCTGGAGGCTACA</td>
</tr>
<tr>
<td>CCL2</td>
<td>R</td>
<td>GTATGTCTGAGCCATCTCTC</td>
</tr>
<tr>
<td>CCL3</td>
<td>F</td>
<td>CTCTCTGTACCAGACCTGTC</td>
</tr>
<tr>
<td>CCL3</td>
<td>R</td>
<td>GTGGAATCTTCCGGCTGTAG</td>
</tr>
<tr>
<td>CCL9</td>
<td>F</td>
<td>CCACTCTCTTCTCTGACTTC</td>
</tr>
<tr>
<td>CCL9</td>
<td>R</td>
<td>CAGACTGCTTGACTGACTCT</td>
</tr>
<tr>
<td>CCR7</td>
<td>F</td>
<td>GGTGAGCTCTCTTGTCAATT</td>
</tr>
<tr>
<td>CCR7</td>
<td>R</td>
<td>AAGGCCGCCACATCCCTCT</td>
</tr>
<tr>
<td>CXCL1</td>
<td>F</td>
<td>TTCACCTCAAGAACATCCAGAG</td>
</tr>
<tr>
<td>CXCL1</td>
<td>R</td>
<td>AAGCCCTGGAGGCTAc</td>
</tr>
<tr>
<td>CXCL2</td>
<td>F</td>
<td>TCAAGAACATCCAGAGCTTGAG</td>
</tr>
<tr>
<td>CXCL2</td>
<td>R</td>
<td>CTTCAGGGTCAAGGCAACT</td>
</tr>
<tr>
<td>CXCL5</td>
<td>F</td>
<td>AGAAGGAGGTCTGCTGAGGAT</td>
</tr>
<tr>
<td>CXCL5</td>
<td>R</td>
<td>GTGCATTCCGGCTTAGTCTTT</td>
</tr>
<tr>
<td>CXCL12</td>
<td>F</td>
<td>GATCCAAGAGTACCTGGAGAAAG</td>
</tr>
<tr>
<td>CXCL12</td>
<td>R</td>
<td>TTTCTCTCTCTGCTGCGCTCTT</td>
</tr>
<tr>
<td>CXCR2</td>
<td>F</td>
<td>TGGGAGAATCTCAAGGTTGAGAAAG</td>
</tr>
<tr>
<td>CXCR2</td>
<td>R</td>
<td>GACAGCATTCTCGACGAGAATAG</td>
</tr>
<tr>
<td>CXCR4</td>
<td>F</td>
<td>GAATGGGTTCTGGAGACTATG</td>
</tr>
<tr>
<td>CXCR4</td>
<td>R</td>
<td>CACACATGGCATCAAGAGAAG</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>F</td>
<td>TAAAGAGGTCAACCGCGTGCTAAT</td>
</tr>
<tr>
<td>TGFβ1</td>
<td>R</td>
<td>ACTGCTTCGCCGATGTCGTCGTA</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>F</td>
<td>ACGGAGATCTTTGGATGGAATG</td>
</tr>
<tr>
<td>TGFβ2</td>
<td>R</td>
<td>TAGAGACTTTGTGGTGTTGAG</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>F</td>
<td>CCACGAAACTAAGGGTTACTATG</td>
</tr>
<tr>
<td>TGFβ3</td>
<td>R</td>
<td>CTGCGTTCAGGGTGGTGTTGATAG</td>
</tr>
<tr>
<td>TGFβRI</td>
<td>F</td>
<td>GGGCTTTAGTGTCTGGAGGAAA</td>
</tr>
<tr>
<td>TGFβRI</td>
<td>R</td>
<td>CGATGAGTCAAGGAGTACAAG</td>
</tr>
</tbody>
</table>
### Table 2.2. continued.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Type</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGFβRII</td>
<td>F</td>
<td>ACAACATCAACCACAACACGGAGC</td>
</tr>
<tr>
<td>TGFβRII</td>
<td>R</td>
<td>CCGATGGATCAGAAGGTACAAG</td>
</tr>
<tr>
<td>Smad2</td>
<td>F</td>
<td>ATCACAGCTTGGATTTGAGCCAG</td>
</tr>
<tr>
<td>Smad2</td>
<td>R</td>
<td>AGAGCAAGCCTAAGCAGAAGCTCT</td>
</tr>
<tr>
<td>Smad3</td>
<td>F</td>
<td>AGGCGATTCCATTCCCGAGAACACT</td>
</tr>
<tr>
<td>Smad3</td>
<td>R</td>
<td>TGGTTCATCTCGTGGTGCTACTGGTT</td>
</tr>
<tr>
<td>Smad4</td>
<td>F</td>
<td>ACTGCTCAGCCGCTACTTTACCAT</td>
</tr>
<tr>
<td>Smad4</td>
<td>R</td>
<td>GGCTGGAATGCAAGCTCATGTGA</td>
</tr>
</tbody>
</table>

### Table 2.3. Sequence of primers used for qPCR analysis for microRNA.

<table>
<thead>
<tr>
<th>microRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>146b-5p</td>
<td>5’- CCCTGAGAAGCAGAATTCATAGGCT</td>
</tr>
<tr>
<td>214-5p</td>
<td>5’- CCCACTGGAAGTTGGAGTGAGGAC</td>
</tr>
<tr>
<td>378-3p</td>
<td>5’- CCCCCCAGTGTTAGACTACCTGTTC</td>
</tr>
<tr>
<td>199b-5p</td>
<td>5’- CCCGGAAGGCAGAAGCAGAAGCT</td>
</tr>
<tr>
<td>23b-5p</td>
<td>5’- CCCACATGTCTTCACATTTGATT</td>
</tr>
<tr>
<td>199a-3p</td>
<td>5’- CCCAGAATTCATCTCAGGATAG</td>
</tr>
<tr>
<td>194-5p</td>
<td>5’- CCCAAAGTGCTAGATAGGAGAG</td>
</tr>
<tr>
<td>199a-5p</td>
<td>5’- CCCCGGGTTCCTGGCATGCTGATT</td>
</tr>
<tr>
<td>18a-5p</td>
<td>5’- CCCGTAAACGCAACTCCATTGAGG</td>
</tr>
<tr>
<td>20a-5p</td>
<td>5’- CCCGTAAACGCAACTCCCTGTTA</td>
</tr>
<tr>
<td>17-5p</td>
<td>5’- CCCAAAGTGCTAGATAGGAGAG</td>
</tr>
<tr>
<td>708-5p</td>
<td>5’- CCCAAGGAAAGCTCTAGCTGAC</td>
</tr>
<tr>
<td>106a-5p</td>
<td>5’- CCCAAAGTGCTAACTGAGAGGAG</td>
</tr>
<tr>
<td>18b-5p</td>
<td>5’- CCCGTAAACGCAACTCCCTGTTA</td>
</tr>
<tr>
<td>182-5p</td>
<td>5’- CCCCTGGAATGCAAGCTCAGGATAG</td>
</tr>
<tr>
<td>19a-3p</td>
<td>5’- CCCGTGGAATCCATGCAAGGACT</td>
</tr>
<tr>
<td>19b-3p</td>
<td>5’- CCCGTGGAATCCATGCAAGGACT</td>
</tr>
</tbody>
</table>
For the Western blot, equal amount of total protein (30–35 μg) were loaded onto an SDS-PAGE gel. After transferring to PVDF membrane, the blot was incubated with antibodies. Blot images were captured by either a ChemiDoc MP system (Bio-Rad Laboratories Inc.) or X-ray film (Kodak). TGFβ1 ELISA was performed by using the R&D Systems Kit (R&D Systems, Minneapolis, MN, USA) as previously described\textsuperscript{13}.

**Immunostainings**

For the immunofluorescence (IF) staining, cells grown on the Lab-Tek II chamber slide system (Thermo Fisher Scientific Inc.) or frozen tissue sections of spheres were fixed in ice cold acetone for 10 minutes at 4°C. Slides were washed in PBS and blocked with 5% goat serum for 1 hour at room temperature, then incubated with primary antibodies for 30 minutes. After washing with PBS for 3 times, slides were incubated with either Alexa 488–conjugated (green; Invitrogen) or Alexa 594–conjugated (red; Invitrogen) secondary antibodies for 1 hour. After a final wash with PBS, coverslips were mounted with EverBrite Mounting Medium with DAPI (Biotium Inc.) and examined using a confocal microscopy. Double IF staining was performed as previously described\textsuperscript{13}. Incubation with primary antibodies was as follows: Keratin 1 (K1; Convance Princeton, NJ, USA, PRB-165 P), K8 (Fitzgerald, Acton, MA, USA, 20 R-CP004), E-cadherin (Cell Signaling, Danvers, MA, USA, #3195), Vimentin (BD Pharmingen, San Jose, CA, USA, RV202) and Smad3 (Novus, Littleton, CO, USA, 378611).

For immunohistochemistry (IHC) staining, tissues were fixed in 10% neutral
buffered formalin, embedded in paraffin and sectioned to 5-μm thickness. Sections were stained with hematoxylin and eosin and examined for the presence of hyperplasia, dysplasia, papillomas, carcinomas and metastases. Tumor pathology and metastasis were examined by at least two investigators and confirmed by the head and neck cancer pathologist. IHC was performed as described previously. Incubation with primary antibodies was as follows: PDK1 (Abcam, Cambridge, MA, USA, ab52893), CD45 (eBioscience, San Diego, CA, USA, 14-0451), F4/80 (Serotec, Raleigh, NC, USA, MCA497GA), Gr1 (BD Pharmingen, 550291), CD11b (BD Pharmingen, 550282), p110α (Cell Signaling, #4249), Ki67 (Abcam, ab16667) and pAKTSer473 (Cell Signaling, #3787). Slides were examined with a Leica microscope, and images were taken using the Q Capture Pro software (Q imaging, Surrey, BC, USA). Quantitation of IHC staining of human p110α, pAKT and PDK1 was performed by two independent investigators using methods described previously.

Receptor Tyrosine Kinase (RTK) Antibody Array

The PathScan RTK Signaling Antibody Array Kit was purchased from Cell Signaling Technologies and the procedure was performed according to the instruction of the manufacturer. Briefly, protein lysates were prepared by using RTK Array Lysis Buffer; protein lysate were then quantified and diluted to the same concentration before loading to the array chamber. Detection antibody cocktail was then added to the chamber and incubate overnight at 4°C. After overnight, chamber was washed by using RTK Array Wash Buffer and processed with HRP-linked
Streptavidin solution. Chamber slide was documented by using BIO-RAD ChemiDoc XRD imaging system and dot density was quantified by using Photoshop (Adobe).

**Microarray (Gene or microRNA) Analysis**

Total RNAs (including microRNAs) were extracted using TRIzol (Invitrogen) and RNeasy kits (Qiagen). RNA (including microRNAs) quality was examined using an Agilent 2100 Bioanalyzer. Basically, 200ng total RNA was used to amplify and label for generating biotinylated cRNAs. cRNAs were hybridized onto Affymetrix GeneChip (Mouse Gene 2.0 Chip for genes or miRNA 4.0 GeneChip for microRNAs) at the Genomics and Microarray Core of University of Colorado Anschutz Medical Campus. Scanned microarray data was processed by using GenePattern (Broad Institute) and ExpressionFileCreator to generate raw signal values. Pre-processed data was analyzed by Affymetrix Transcriptome Analysis Console 3.0 to generate hierarchical clustering. Differential gene expression analysis was performed by using Affymetrix Transcriptome Analysis Console 3.0. Differentially expressed gene sets were filtered based on a fold change >2, and the defaulted false discovery rate. Functional clustering analysis was done using both Affymetrix Transcriptome Analysis Console 3.0 and NIH David.

**MicroRNA Transfection**

Transfections of microRNAs or relevant mimics and inhibitors were performed using DpharmFECT Transfection Reagents (GE Healthcare) according to the manufacture’s instruction. In general, 80 nM of each microRNAs or relevant mimics
and inhibitors along with DpharmFECT Transfection Agent (2µL/mL of medium) was used for transfecting $5 \times 10^4$ CU110 cells in a well of 24-well plate. Transfected cells were cultured for 48 hours for further experiments.

**Inhibitor Treatment**

Information of all inhibitors used is listed in Table 2.4. All inhibitors were prepared in DMSO as 10 mM stock. The effect of the various treatments on cell viability was evaluated by using two different methods. First method is based on using the Vi-CELL cell counter (Beckman Coulter, CA, USA). Briefly, cells were plated in 24-well plates at a density of $9.4 \times 10^4$ cells per well in triplicate. Cells were then treated with either vehicle (DMSO) or inhibitors. After 48 hours, cells were trypsinized and cell viability was measured by Vi-Cell cell counter (Beckman Coulter, CA, USA). The percentage of viable cells was defined as the ratio of cell number in inhibitor-treated group to that of the DMSO-treated group. IC50s were calculated using GraphPad Prism software. We also used Cell Counting Kit-8 (Dojindo, Japan) to quantify the cell viability according to the instruction manual.

**Animal Studies**

**Generation and Characterization of Inducible Head-and-neck-specific PIK3CA Genetically Engineered Mouse Model (GEMM)**

All animal experiments were performed in accordance with protocols approved by the Institutional Animal Cancer and Use Committees of University of
### Table 2.4. Information of inhibitors.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Vendor</th>
<th>Cat. No.</th>
<th>Target/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>PX866</td>
<td>LC laboratories</td>
<td>P-7501</td>
<td>pan-PI3K</td>
</tr>
<tr>
<td>gefitinib</td>
<td>Selleck Chemicals</td>
<td>S1025</td>
<td>EGFR</td>
</tr>
<tr>
<td>imatinib</td>
<td>Selleck Chemicals</td>
<td>S2475</td>
<td>c-kit</td>
</tr>
<tr>
<td>axitinib</td>
<td>Selleck Chemicals</td>
<td>S1005</td>
<td>VEGFR</td>
</tr>
<tr>
<td>ponatinib</td>
<td>Selleck Chemicals</td>
<td>S1490</td>
<td>Multi-RTKs</td>
</tr>
<tr>
<td>AZD4547</td>
<td>Selleck Chemicals</td>
<td>S1561</td>
<td>Axl, Ron, Tyro3</td>
</tr>
<tr>
<td>GNF-5837</td>
<td>Selleck Chemicals</td>
<td>S7519</td>
<td>TrkA/B/C</td>
</tr>
<tr>
<td>OSI906</td>
<td>Selleck Chemicals</td>
<td>S1091</td>
<td>IGF-1R, InsR</td>
</tr>
<tr>
<td>PD325901</td>
<td>Selleck Chemicals</td>
<td>S1036</td>
<td>MEK</td>
</tr>
<tr>
<td>Stattic</td>
<td>Selleck Chemicals</td>
<td>S7924</td>
<td>STAT3</td>
</tr>
<tr>
<td>GSK2334470</td>
<td>Selleck Chemicals</td>
<td>S7087</td>
<td>PDK1</td>
</tr>
<tr>
<td>LY2157299</td>
<td>Selleck Chemicals</td>
<td>S2230</td>
<td>TβR1</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Selleck Chemicals</td>
<td>S1150</td>
<td>Microtubule</td>
</tr>
<tr>
<td>Everolimus</td>
<td>Selleck Chemicals</td>
<td>S1120</td>
<td>mTOR</td>
</tr>
<tr>
<td>LDN211904</td>
<td>Merck Millipore</td>
<td>428201</td>
<td>Ephrin receptors</td>
</tr>
<tr>
<td>Apicidin</td>
<td>Cayman Chemical</td>
<td>10575</td>
<td>HDAC</td>
</tr>
<tr>
<td>SAHA</td>
<td>Cayman Chemical</td>
<td>10009929</td>
<td>HDAC</td>
</tr>
<tr>
<td>TSA</td>
<td>Cayman Chemical</td>
<td>89730</td>
<td>HDAC</td>
</tr>
<tr>
<td>VPA</td>
<td>Cayman Chemical</td>
<td>13033</td>
<td>HDAC</td>
</tr>
<tr>
<td>5-aza-2-deoxycytidine</td>
<td>Sigma-Aldrich</td>
<td>A3656</td>
<td>DNA methyltransferase</td>
</tr>
</tbody>
</table>
Colorado Anschutz Medical Campus (UCAMC) and the Oregon Health and Science University (OHSU). The plasmid containing full-length cDNA of PIK3CA (pcDNA3.1-PIK3CA) was obtained from Addgene (Cambridge, MA, USA) under the material transfer agreement. The PIK3CA fragment was subcloned into the target vector (ptata.A) using the XhoI site (Figure 2.1A). A 3.5-kb HindIII/KpnI fragment including Gal4-binding sites, minimal tata promoter and the PIK3CA cDNA was microinjected into the pronucleus of single-cell embryo by the transgenic mouse core facility at OHSU. The embryos were implanted into pseudo-pregnant females and allowed to develop to term. PCR genotyping was performed at the 3 weeks of age using genomic DNA isolated from tail biopsies. Three lines (#5840, #5446, #5703) transmitted the transgene through their germline and was called tata.PIK3CA mouse line thereafter (Figure 2.1B). The transgene-specific primers covering hemagglutinin tag were designed, along the upstream sequence. The primers used to detect the ptata.PIK3CA transgene were: forward: 5′-TGAGCAAGAGGCTTTGGAGT-3′ and reverse: 5′-TGCATTCTAGTTGTTGTTT-3′.

The target mouse line tata.PIK3CA was then bred to the transactivator mouse line - K5.GLp65, which was developed previously. The resulting bigenic mouse line K5.GLp65/tata.PIK3CA is hereafter referred to as PIK3CA-GEMM. The PIK3CA-GEMMs were selected by using genotyping primers specific for GLp65 (forward: 5′-TGTCAGGTTTCTCGAGGA-3′; reverse: 5′-GGCCATACAGTTTGAGTGACA-3′), and the same primers that were used for detection of the PIK3CA transgene.

All mouse experiments were performed in a C57BL/6 background. Adult bigenic K5.GLp65/tata.PIK3CA and monogenic K5.GLp65, tata.PIK3CA and non-
Figure 2.1. Creation and identification of the target mouse line tata.PIK3CA. (A.) Subcloning strategy to make the target vector ptata.PIK3CA. (B.) Genotyping PCR using transgene specific primers to identify candidate mouse lines tata.PIK3CA, in which the transgene is transmitted through the germline. The transactivator line K5.GLp65, and wild type mice were used as negative controls.
transgenic mice (around 1-month-old) were treated with RU486 for PIK3CA gene induction and with the rodent oral carcinogen, 4NQO (Sigma, St Louis, MO, USA). For the transgene induction, 100μl of RU486, dissolved in sesame oil (0.2 μg/ml), was applied in the oral cavity of mouse to induce either acute or sustained PIK3CA transgene expression as described previously\textsuperscript{13}. For the oral carcinogenesis experiment, 4NQO (Sigma) was prepared weekly in propylene glycol at a concentration of 5 mg/ml, and was placed into drinking water in a final concentration of 50 μg/ml. The water was changed once weekly, and was given to mice for 16 weeks. The general health condition of mice was monitored twice weekly, and their body weight was measured once weekly. Mice were given softened food when there were signs of food-intake difficulty, or decreased body weight. At the end of animal experiment, mice were euthanized. Buccal mucosa, tongue, lymph node and lung tissues were harvested for histological analysis and immunostaining.

**In-vivo Tumorigenicity Study**

For in vivo tumorigenicity study of HNSCC spheres, mono-layer growing or sphere-growing CU110 cells were injected subcutaneously to the left and right flanks of C57BL/6 mice at 1 x 10⁴ or 1 x 10³ cells per injection. Tumor growth was monitored for 1 month. After 1 month, mice were euthanized and tumors were harvested for the subsequent volume measurement. Tumor volume was calculated by the formula: tumor volume = (length width width)/2. For the tail vein injection experiment, 1x10⁵ inhibitor-treated or DMSO-treated CU110 cells were intravenously injected into lateral tail veins of C57BL/6 mice. After 3 weeks, the mice were
euthanized and their lung tissues were collected for gross assessment and hematoxylin/eosin (H&E) staining.

**Human HNSCC Clinical Sample Study**

The clinical study protocol was approved by the Institutional Review Board-approved of UCAMC and OHSU, and written informed consent was obtained from all participants before sample collection. HNSCC and case-matched adjacent tissue samples were surgically resected from consenting patients at the Department of Otolaryngology of UCAMC and OHSU. Tissues examined in this study include a total of 74 HNSCCs (26 SCCs of tongue, 19 SCCs of oral cavity, 3 SCCs of nasal pharynx, 7 SCCs of oropharynx, 7 SCCs of hypopharynx, 12 SCCs of larynx) and case-matched tissues adjacent to tumors, and 24 cases of lymph node metastases. A total of 12 normal oropharyngeal samples from sleep apnea patients were used as normal controls.

**Statistical Analysis**

Statistical calculations were performed using Prism 5 software (GraphPad Software Inc.). Data were presented as the mean ± SDs as indicated in the figure legends. The statistical significance of quantitative data was determined using two-tailed Student t test and results were presented as p values (P < 0.05; P < 0.01; and P < 0.001 indicate statistically significant changes).
CHAPTER III
OVEREXPRESSION OF PIK3CA IN MURINE HEAD AND NECK EPITHELIUM DRIVES TUMOR INVASION AND METASTASIS THROUGH PDK1 AND ENHANCED TGFβ SIGNALING¹

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide, with 350,000 deaths every year²⁰³. There are 55,000 new cases and over 15,000 deaths from HNSCC annually in the United States²⁰⁴. The most common etiological factors for HNSCC are tobacco exposure, alcohol and human papilloma virus infection⁴. Although tremendous efforts have been made to improve the treatment for HNSCC patients, HNSCC remains a lethal disease, with 5-year survival rates below 50%, a number that has not significantly changed in several decades²⁰⁴. Locoregional invasion, recurrence and metastasis are largely responsible for the poor prognosis of HNSCC patients. However, the underlying molecular mechanisms for HNSCC progression are still poorly understood.

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is a key mediator of cell survival, growth and metabolism, and is commonly altered in many human malignancies, including HNSCC³⁸,⁴⁰,⁴⁸. The PI3K family is comprised of three subclasses of lipid kinases. Class IA PI3K, composed of a heterodimer between

p110 catalytic subunit and a p85 regulatory subunit, has been intensively studied and demonstrated to be commonly altered in human cancers\textsuperscript{38,40}. PI3K phosphorylates phosphotidylinositol-4,5,bisphosphate to phosphotidylinositol-3,4,5,trisphosphate, a reaction which is antagonized by the tumor-suppressor phosphatase and tensin homolog (PTEN). In turn, phosphotidylinositol-3,4,5,trisphosphate binds a subset of pleckstrin homology domain-containing proteins, including AKT and 3-phosphoinositide-dependent protein kinase (PDK1). AKT is activated by phosphorylation on serine 473 by the mTORC2 complex, and on threonine 308 by PDK1. Activated AKT directly regulates multiple downstream molecules, such as mTORC1 and p70-S6, to transduce the major downstream PI3K signal pathway\textsuperscript{38,40}. However, it has been shown that, in certain contexts, PI3K signaling and AKT activation can be uncoupled\textsuperscript{41,42,205}.

Recent exome sequencing in human HNSCC samples revealed that molecular alterations in the PI3K pathway are the most common genetic changes in HNSCC patients’ cells\textsuperscript{16,17,46}. Among those changes, PIK3CA, the gene coding for the p110\textsubscript{α} catalytic subunit of PI3K, is most frequently altered through either gene amplification or gain-of-function mutations\textsuperscript{16,17,46}. Over 40\% of HNSCC cases exhibit PIK3CA amplification\textsuperscript{38,40,48}, and ~10\% harbor PIK3CA gain-of-function mutations\textsuperscript{16,17,46}. Although several studies suggest PIK3CA alterations correlate with advanced HNSCC stage\textsuperscript{16,52,53}, vascular invasion\textsuperscript{206} and lymph node metastasis\textsuperscript{55}, there are no in vivo studies that have elucidated the mechanisms by which PIK3CA alterations lead to HNSCC development and progression.

To address this, we generated an inducible head-and-neck-specific
genetically engineered mouse model (GEMM), in which PIK3CA is overexpressed in head and neck epithelium. Our results showed that in this GEMM overexpression of PIK3CA alone resulted in head and neck epithelial hyperplasia but was not sufficient to initiate tumorigenesis. However, overexpression of PIK3CA significantly increased susceptibility to oral carcinogen-induced head and neck tumorigenesis. Strikingly, PIK3CA overexpression promoted HNSCC invasion and metastasis through epithelial-to-mesenchymal transition (EMT) and enrichment of putative head and neck cancer stem cells (CSCs). Further molecular studies showed that activation of PDK1 and enhanced transforming growth factor β (TGFβ) signaling play an important part in HNSCC progression. Our findings highlight the key role of PDK1 and TGFβ signaling in HNSCC invasion and metastasis, and suggest that targeting PDK1 and TGFβ signaling may be effective approaches to controlling HNSCC progression, particularly in patients with PIK3CA gene amplification.

Results

Overexpression of PIK3CA in Murine Head and Neck Epithelia Resulted in Increased Susceptibility to Head and Neck Carcinogenesis

Although PIK3CA amplification/mutation has been described in human HNSCC, little is known about its in vivo role during head and neck tumorigenesis. To this end, we developed an inducible, head-and-neck-specific PIK3CA genetically engineered mouse model (PIK3CA-GEMM) using previously published methods\textsuperscript{13}. Briefly, the PIK3CA-GEMM consists a transactivator mouse line (K5.GLp65) and a
target mouse line (tata.PIK3CA; Figure 3.1A). In the transactivator line, the keratin 5 (K5) promoter is used to restrict expression of the GLp65 fusion protein to the basal layer of stratified epithelia (for example, buccal mucosa, tongue). GLp65 consists of a GAL4 DNA-binding domain, the NF-κB p65 transactivation domain and an RU486-inducible truncated progesterone receptor ligand-binding domain (ΔPR-LBD). The K5.GLp65 mice were bred to the tata.PIK3CA mice to generate the bigenic K5.GLp65/tataPIK3CA (hereafter referred to as PIK3CA-GEMM). The monogenic K5.GLp65 and tata.PIK3CA mouse lines were used as experimental controls. Starting at 4 week of age, mice were orally treated with RU486 dissolved in sesame oil. As shown in Figure 3.1B, upon RU486 application, the expression level of PIK3CA mRNA increased about 8- to 10-fold in the buccal mucosa and tongue tissues from the PIK3CA-GEMM compared with those from the control mice or the PIK3CA-GEMM without RU486 application. Correspondingly, the protein level of p110α (encoded by the PIK3CA gene) was also increased in PIK3CA-GEMM head and neck tissues upon RU486 application (Figure 3.1C).

After verifying induced expression of PIK3CA in the PIK3CA-GEMM head and neck epithelia following RU486 administration, we treated PIK3CA-GEMMs with oral RU486 (20 μg/mouse) twice weekly. Epithelial hyperplasia and carcinoma in situ with increased cell proliferation were observed in both buccal mucosa and tongue tissue as early as 6 months after initiation of treatment (Figure 3.1D). However, these premalignant lesions failed to progress into squamous cell carcinomas (SCCs), suggesting that PIK3CA overexpression alone is not sufficient to induce HNSCC formation.
One of the major etiological factors of human HNSCC development is tobacco exposure. To mimic this human situation and investigate the interaction between environmental factors and genetic susceptibility, we applied the 4-nitroquinoline 1 oxide (4NQO)-induced HNSCC carcinogenesis protocol to the PIK3CA-GEMM. 4NQO is a DNA adduct-forming agent widely used as a tobacco surrogate to induce HNSCC in mouse models. As shown in Figure 3.2A, PIK3CA-GEMM and control mice were treated with RU486 applied orally twice weekly and with 4NQO added to drinking water (50 μg/ml) starting when the mice were 1-month old. Mice were exposed to 4NQO for 16 weeks, and received RU486 through the end of the 12-month experiment. PIK3CA overexpression significantly enhanced susceptibility to 4NQO-induced head and neck carcinogenesis (Figure 3.2B). By 5 to 6 months of age, 39% of PIK3CA-GEMMs developed HNSCC, compared with only 8% of control mice (P<0.01). By 10–12 months, all 4NQO-treated control and PIK3CA-GEMM mice developed HNSCC. The authentication of the PIK3CA tumors was validated by transgene-specific genotyping PCR (Figure 3.2G). By the time of tumor harvest, there were no differences in the general health and nutritional status (for example, activity or body weight) between the control mice and the PIK3CA GEMMs.

**Overexpression of PIK3CA in Murine Head and Neck Epithelia Promotes Tumor Invasion and Metastasis**

It was immediately clear that tumors from PIK3CA-GEMMs and those from control mice differed in gross appearance: although most control tumors were
Figure 3.1. Generation and characterization of the inducible, head-and-neck-specific PIK3CA genetically engineered mouse model (PIK3CA-GEMM).
Figure 3.1. Generation and characterization of the inducible, head-and-neck-specific PIK3CA genetically engineered mouse model (PIK3CA-GEMM). (A.) Schematic of generating the PIK3CA-GEMM by breeding the transactivator mouse line K5.GLp65 to the target mouse line tata.PIK3CA. (B.) Fold inductions of the PIK3CA mRNA quantified by qRT–PCR using transgene-specific primers. RU=RU486, n=6, *P<0.05. (C.) Western blot analysis on protein lysates from the buccal mucosa and tongue of the PIK3CA-GEMMs. (D.) Overexpression of PIK3CA resulted in epithelial hyperplasia in buccal mucosa and tongue from the PIK3CA-GEMMs. H&E staining of buccal mucosa and tongue from 7-month-old control mice and PIK3CA-GEMMs. Increased expression of p110α in the PIK3CA-GEMMs detected by IHC. Increased Ki-67 positive cells are evident in the buccal mucosa and tongue of the PIK3CA-GEMMs assessed by IHC.
papillomatous, tumors from the PIK3CA-GEMMs were flat (Figure 3.2C). Histologically, the majority of the control tumors had an out-growth pattern with intact basement membranes (Figure 3.2D and Figure 3.2H), whereas tumors from the PIK3CA-GEMMs predominantly had a down-growth pattern with broken basement membranes and invasion into muscle (right panels of Figure 3.2D and H). Although over 60% of the control tumors were well-differentiated or carcinoma in situ, over 50% of the PIK3CA-overexpressing tumors were poorly differentiated as confirmed by a head and neck pathologist (Figure 3.2D, E and H). Notably, metastases were observed in ~40% of the PIK3CA-GEMMs (regional lymph node or lung metastases) compared with no metastases in control mice (Figure 3.2 and F). These data showed that overexpression of PIK3CA promoted 4NQO-induced HNSCC progression, particularly tumor invasion and metastasis.

PIK3CA Overexpression Induced De-differentiation, EMT and Enriched CSC Properties

The poorly differentiated tumor histology observed in the PIK3CA-GEMMs was further confirmed by immunostaining of several differentiation markers. As shown in Figure 3.3A, while Keratin 1 (K1, green) was still retained in the control tumors, marking well-differentiated SCC, it was lost in the PIK3CA-GEMM tumors. In contrast, Keratin 8 (K8, red), seen in poorly differentiated SCC, was rarely expressed in control tumors, whereas it was highly expressed in tumors from PIK3CA-GEMMs. This differential keratin expression was further confirmed by K and K8 mRNA expression quantified by quantitative PCR (qPCR; Figure 3B). In addition
Figure 3.2. Overexpression of PIK3CA together with 4NQO treatment increases tumor susceptibility and promotes tumor invasion and metastasis of the 4NQO-induced HNSCC.
Figure 3.2. Overexpression of PIK3CA together with 4NQO treatment increases tumor susceptibility and promotes tumor invasion and metastasis of the 4NQO-induced HNSCC.
Figure 3.2. Overexpression of PIK3CA together with 4NQO treatment increases tumor susceptibility and promotes tumor invasion and metastasis of the 4NQO-induced HNSCC. (A.) Schematics of the animal experiment. Starting at 1 month of age, PIK3CA-GEMMs and control mice were given 4NQO (50 μg/ml) in the drinking water for 16 weeks, and RU486 (20 μg/mouse) applied orally twice weekly until harvesting mice at 12 months. (B.) Summary of tumor incidences in the PIK3CA-GEMMs and control mice. All mice underwent a biweekly full oral cavity examination, and any pathologic changes were documented. Tumor onset was significantly earlier for the PIK3CA-GEMMs (5–6 months) than for the control mice. *P<0.01. (C.) Gross morphology of tongue SCCs from control mice (upper) and PIK3CA-GEMMs (lower). Dotted lines delineate the tumor boundary. Note that tumors from the control mice are papilloma-like, whereas those from the PIK3CA-GEMMs are flat. (D.) Hematoxylin and eosin (H&E) staining of representative tongue tumors shows that tumors from control mice have an out-growth pattern with intact basement membrane (upper left panel), and are well-differentiated (lower left panel), whereas tumors from the PIK3CA-GEMMs have a down-growth pattern, with invasion to the tongue muscle (upper right panel), and are poorly differentiated (lower right panel). Scale bar: 100 μm for upper panel, and 25 μm for lower panel. (E.) Histopathological summary of oral tumors from control mice and PIK3CA-GEMMs. CIS, carcinoma in situ; Mod, moderately differentiated tumors; Poorly, poorly differentiated tumors; Well, well-differentiated tumors. (F.) H&E staining of representative lymph node and lung metastases. Dotted line delineates the upper boundary of the tumor. Scale bar: 100 μm. (G.) Validation of PIK3CA transgene in the tumors from the PIK3CA-GEMMs by PIK3CA-trangene specific PCR genotyping. Both K5.GLp65 and PIK3CA transgenes were present in the tumors from the PIK3CA-GEMMs (lane 1-γ). Tumors from control mice (tata.PIK3CA, lane4; K5.GLp65, lane 6; wildtype, lane 5) contained either one or none of the transgenes. (H.) Histopathology of HNSCC tumors from control mice and PIK3CA-GEMMs. While the tongue tumors in the control mice are mostly carcinoma-in situ (top left panel) and well-differentiated tumors (middle and bottom left panels), those from the PIK3CA-GEMMs are mostly poorly differentiated and invasive (all three right panels).
to these findings, EMT was observed in the PIK3CA-GEMM tumors. As shown in Figure 3.3C, in contrast to the universal expression of the epithelial marker E-cadherin (green) in control tumors, E-cadherin expression was lost in the PIK3CA-GEMM tumors. Conversely, the mesenchymal marker Vimentin (red) was expressed only in the stroma of the control tumors, but was expressed in the epithelium of PIK3CA-GEMM tumors, suggesting that EMT had taken place. This was further confirmed by qPCR showing decreased E-cadherin and increased Vimentin mRNA expression in the PIK3CA-GEMM tumors (Figure 3.3D). Last, relative to controls, the PIK3CA-GEMM tumors showed significantly increased expression of several transcription factors regulating EMT, including Twist1, Zeb1, Zeb2 and Snail1 (Figure 3.3E). Thus, the PIK3CA-GEMM tumors exhibited both histopathological and molecular signature of EMT.

It has been shown that de-differentiation and EMT correlate with enhanced CSC/progenitor activity\textsuperscript{154}. We thus examined putative CSC markers of HNSCC by qPCR. As shown in Figure 3.3F, overexpression of PIK3CA significantly increased the mRNA expression of CD44 and CD166, and reduced the expression of CD24, all of which indicate an enhanced CSC phenotype. This result is further supported by increased mRNA expression of embryonic stem cell markers Nanog, Oct4 and EZH2. Interestingly, increased expression of Wnt5a, which is implicated in migration/invasion, was also found in the PIK3CA-GEMM tumors (Figure 3.3F).

**PIK3CA-GEMM Tumors Have Increased PDK1 Expression and Activation**

To investigate the molecular mechanisms of PIK3CA-driven HNSCC
Figure 3.3. PIK3CA overexpression in tumor tissue promotes cellular dedifferentiation, epithelial-mesenchymal transition (EMT) and enrichment of cancer stem cell properties. (A.) Double immunofluorescence (IF) staining of keratin 1 (K1, green) and keratin 8 (K8, red) markers on tongue tumor samples from control mice and PIK3CA-GEMMs (n=7). K1 indicates well-differentiated cells, whereas K8 indicates poorly differentiated cells. Scale bar: 25 μm. (B.) Relative mRNA levels of K1 and K8 measured by qRT–PCR, normalized to β-actin. Results are averages of triplicate experiments. *P<0.05 (n=6 for each group). (C.) Double IF staining of epithelial marker E-cadherin (green) and mesenchymal marker Vimentin (red) on tongue tumor samples from control mice and PIK3CA-GEMMs (n=12). Scale bar: 25 μm. (D.) Relative mRNA levels of E-cadherin and Vimentin measured by qRT–PCR, normalized to β-actin. Results are averages of triplicate experiments. *P<0.05 (n=6 for each group). (E.) Relative mRNA levels of EMT-related molecules measured by qRT–PCR, normalized to β-actin. Results are averages of triplicate experiments. *P<0.05 (n=6 for each group). (F.) Relative mRNA levels of putative markers for cancer stem cell of HNSCC (left panel) and molecules regulating CSC phenotype (right panel) measured by qRT–PCR. Results are averages of triplicate experiments. *P<0.05 (n=6 for each group).
progression, we examined several key molecules in the PI3K pathway. It has been shown that the change of mRNA expression ratio between AKT1 and AKT2 regulates EMT in breast cancer cells\textsuperscript{208}. However, there were no significant changes of mRNA expression of AKT1, AKT2, PDK1 or PTEN in the PIK3CA-GEMM tumors compared with control tumors (Figure 3.4A). AKT1 and AKT2 protein levels were similar between control and PIK3CA-GEMM tumors. However, PDK1 protein levels were increased in PIK3CA-GEMM tumors (Figure 3.4I). We then examined AKT and PDK1 protein activation by western blotting. Surprisingly, we did not detect significant differences in AKT phosphorylation at either Ser473 or Thr308 between the control and the PIK3CA-GEMM tumors. However, PDK1 activation by phosphorylation at Ser241 was significantly enhanced in PIK3CA-GEMM tumors (Figure 3.4B). Similarly, immunohistochemistry (IHC) staining showed an increase of total PDK1 protein in the PIK3CA-GEMM tumors (Figure 3.4C). These findings were unexpected and suggested that PDK1 may mediate oncogenic signaling in PIK3CA-driven HNSCC progression. Last, we examined several molecules that are commonly altered in human HNSCCs. 4NQO exposure causes the formation of DNA adducts, resulting in mutations\textsuperscript{207}. We first examined two oncogenes that are commonly mutated in HNSCC, that is, Ras and PIK3CA. We sequenced eight PIK3CA-GEMM tumors and eight control tumors for mutations at codons 12, 13 and 61 of the K-ras and H-ras genes, and exons 9 and 20 of the PIK3CA gene. The only mutation we found was a G to A point mutation in codon 12 of mouse K-ras gene, which changes glutamic acid to aspartic acid (Figure 3.4J). This mutation was found only in one control tumor. We then examined the mRNA levels of p16, EGFR, Stat3,
CyclinD1, K-ras, H-ras and N-ras in PIK3CA-GEMM and control tumors. We found increased Stat3 and decreased p16 mRNA in the PIK3CA-GEMM tumors compared with control tumors (Figure 3.4D).

To further examine whether PDK1 is involved in the tumor progression of the PIK3CA-GEMM mice, we cultured primary tumor cells from both PIK3CA-GEMM tumors (CU110 cells) and control tumors (CUCON cells). As shown in the Figure 3.4K-a, consistent with the EMT changes in the tumor tissues, CU110 cells were more spindle-like, whereas CUCON cells were more round and epithelial-like. Overexpression of p110α was observed in the CU110 cells compared with that in CUCON cells (Figure 3.6E, left panel). We then stably knocked down PDK1 in the CU110 cells (CU110-shPDK1), along with CU110 cells with a scramble insert (CU110-SCR) as a control (Figure 3.4K-b). Knocking down PDK1 in the CU110 cells increased E-cadherin expression and decreased Vimentin expression (Figure 3.4E), but had no effects on phosphorylation of Smad2 or Smad3 (Figure 3.4K-b). In addition, knocking down PDK1 (CU110-shPDK1) significantly reduced cell proliferation compared to the control cells (CU110-SCR) (Figure 3.4F). Moreover, knocking down PDK1 in the CU110 cells moderately attenuated cell migration (Figure 3.4G) and invasion (Figure 3.4H) abilities. These data suggested that PDK1 contributed to the tumor progression phenotypes observed in the PIK3CA-overexpression HNSCCs.
Figure 3.4. Overexpression and activation of PDK1 but not AKT in PIK3CA-GEMM tumors.
Figure 3.4. Overexpression and activation of PDK1 but not AKT in PIK3CA-GEMM tumors.
Figure 3.4. Overexpression and activation of PDK1 but not AKT in PIK3CA-GEMM tumors. (A.) Relative mRNA levels of PIK3CA, AKT1, AKT2, PDK1 and PTEN measured by qRT–PCR. Results are averages of triplicate experiment. *P<0.05 (n=6 for each group). (B.) Western blotting analysis of tumor samples from control mice and PIK3CA-GEMMs using the antibodies as indicated. (C.) Immunohistochemistry (IHC) staining of p110α, pAKTser473 and PDK1 in tumors from PIK3CA-GEMMs and control mice. Scale bar: 50 μm. (D.) qRT–PCR examination of mRNA levels of molecules commonly altered in human HNSCCs. Results are averages of triplicate experiments. *P<0.05 (n=6 for each group). (E.) Relative mRNA levels of PDK1, E-cadherin and Vimentin in CU110 cells with PDK1 knockdown (CU110-shPDK1) or with a scramble control (CU110-SCR) measured by qRT–PCR. *P<0.05. (F.) Cell proliferation assay of CU110-shPDK1 and CU110-SCR measured by the Cell Counting Kit-8. *P<0.05. (G.) Migration assay in CU110-shPDK1 and CU110-SCR cells. Dotted line defines the edges of cells. All experiments were performed in triplicate. *P<0.05. (H.) Invasion assay in CU110-shPDK1 and CU110-SCR cells. All experiments were performed in triplicate. *P<0.05. (I.) Western blot analysis of AKT1, AKT2, PDK1 and total AKT in tumors from PIK3CA-GEMMs and control mice. (J.) DNA sequencing results from a control tumor. The results showed a G to A mutation in codon 12 of the mouse K-ras gene, which changes glutamic acid to aspartic acid. (K.) a. Bright field images of cells cultured from either a control tumor (CUCON) or a PIK3CA-GEMM tumor (CU110). b. Western blot analysis of PDK1, pSmad2, pSmad3 in the CU110-shPDK1 and the CU110-SCR cells. c. Cell proliferation assay in CU110 cells treated with TGFβ type I receptor inhibitor, LY2157299 relative to a DMSO control.
PIK3CA Overexpression Led to Increased Inflammation in HNSCC Tumor Stroma

In addition to the de-differentiation and EMT changes in the PIK3CA-GEMM tumor cells, we observed numerous infiltrated leukocytes in the stroma of the PIK3CA-GEMM tumors on histological sections examined by hematoxylin and eosin staining (Figure 3.5C) and IHC with CD45 (Figure 3.5A). We then examined subtypes of infiltrated leukocytes using IHC. Tumor-associated macrophages are stained by F4/80 antibody, and myeloid-derived suppressor cells in a tumor microenvironment are stained by granulocyte markers Gr1 and CD11b\textsuperscript{209}. Both cell types were increased in PIK3CA-GEMM tumors compared with control tumors (Figure 3.5A). Last, we evaluated which cancer-associated inflammatory cytokines were upregulated in PIK3CA-GEMM tumors. Among the 15 inflammatory chemokines/receptors we evaluated, mRNA levels of CCL3, CCR7, CXCL12 and CXCR4 were all significantly increased in the PIK3CA-GEMM tumors compared with the control tumors (Figure 3.5B).

PIK3CA-GEMM Tumors Have Increased TGFβ1 Ligand, and Increased Smad3 Expression and Activation

The EMT change in tumor cells and the enhanced leukocyte infiltration in PIK3CA-GEMM tumors are similar to what we observed previously in HNSCC mouse models with aberrant TGFβ signaling\textsuperscript{13,107,108}. This prompted us to further investigate whether TGFβ signaling contributes to PIK3CA-driven HNSCC progression. We first used quantitative reverse transcription–PCR (qRT–PCR) to
Figure 3.5. Increased leukocyte infiltration and inflammation in tumors from PIK3CA-GEMMs. (A.) Detection of leukocyte subtypes in tumors from PIK3CA-GEMMs and control mice using IHC with CD45, F4/80, Gr1 and CD11b. Scale bar: 50 μm. (B.) qRT–PCR examination of inflammatory-related cytokines/chemokines and receptors. Results are averages of triplicate experiments. *P<0.05 (n=6 for each group). (C.) HE staining of PIK3CA-GEMM tumors showing increased leukocyte infiltration (marked as *) compared to the control tumors.
examine several TGFβ signaling pathway molecules known to be altered in human cancers. We found that mRNA levels of TGFβ1 and Smad3 were significantly higher in PIK3CA-GEMM tumor tissues than in control tumor tissues (Figure 3.6A). TGFβ1 protein level was also increased in the PIK3CA-GEMM tumor tissues, as determined by ELISA (Figure 3.6B). We then performed double IF of Smad3 and the epithelial marker E-cadherin. As shown in Figure 3.6C, the Smad3-positive area was inversely correlated with E-cadherin-positive staining, suggesting that Smad3 may contribute to the EMT changes in the PIK3CA-GEMM tumors. Increased phosphorylation of Smad3 in the PIK3CA-GEMM tumors compared with the control tumor tissues was also observed through western blot analysis (Figure 3.6D).

To further assess the role of TGFβ signaling in mediating the tumor-promoting effects of PIK3CA overexpression, we created CU110 cells in which the PIK3CA gene was knocked down (CU110-shPIK3CA), along with CU110 cells with a scramble insert (CU110-SCR) as a control (Figure 3.6E, right panel). To examine the effect of PIK3CA overexpression or knockdown on TGFβ pathway in the cultured HNSCC cells to check whether PIK3CA-mediated regulation of TGFβ pathway is cell autonomous in cancer cells, we compared TGFβ1 mRNA levels in CUCON, CU110, CU110-shPIK3CA and CU110-SCR cells. As shown in Figure 3.6F, TGFβ mRNA levels in the CU110 cells were about four times than that in the CUCON cells. Conversely, knocking down PIK3CA significantly reduced TGFβ1 mRNA levels in CU110-shPIK3CA cells compared with the CU110-SCR cells, suggesting that the PIK3CA-mediated regulation of TGFβ pathway is cell autonomous in cancer cells. Last, we treated the PIK3CA-overexpressing CU110 cells with a TGFβ type I
Figure 3.6. Increased TGFβ ligand and smad3 expression and activation in tumors from PIK3CA-GEMMs.
Figure 3.6. Increased TGFβ ligand and smad3 expression and activation in tumors from PIK3CA-GEMMs. (A.) qRT–PCR examination of molecules in the TGFβ signaling pathway. *P<0.05 (n=6 for each group). (B.) ELISA quantification of TGFβ1 ligand (n=5). (C.) Double IF staining of epithelial marker E-cadherin (green) and Smad3 (red). Note the inverse correlation between Smad3 and E-cadherin in control and PIK3CA-GEMM tumors. (n=4), scale bar: 25 μm. (D.) Western blot analysis of phosphorylated Smad3. (E.) Western blot analysis of p110α in cells cultured from a 4NQO-induced control tumor (CUCON) and a 4NQO-induced PIK3CA-GEMM tumor (CU110), and CU110 cells with either a PIK3CA knockdown (shPIK3CA) or a scramble control (SCR). (F.) qRT–PCR examination of TGFβ1 mRNA levels in CUCON and CU110 cells (left panel), and CU110 cells with PIK3CA knockdown (shPIK3CA) or scramble control (SCR; right panel). *P<0.05. (G.) Western blot analysis of phosphorylation of Smad2 and Smad3 in CU110 cells treated with a TGFβ type I receptor inhibitor, LY2157299, or dimethyl sulfoxide (DMSO; control). (H.) Migration assay in CU110 cells treated with a TGFβ type I receptor inhibitor, LY2157299, or DMSO (control). Dotted line defines the edges of cells. All experiments were performed in triplicate. *P<0.05. (I.) Invasion assay in CU110 cells treated with a TGFβ type I receptor inhibitor, LY2157299, or DMSO (control). All experiments were performed in triplicate. *P<0.05.
receptor kinase inhibitor, LY2157299, to address the contribution of TGFβ pathway to the PIK3CA-driven HNSCC progression. Treatment with LY2157288 effectively reduced Smad2 and Smad3 phosphorylation, indicating the effectiveness of the inhibition of the TGFβ signaling pathway (Figure 3.6G). Following the treatment, both cell migration and invasion were significantly hampered (Figure 3.6H and I), with a minor effect on cell proliferation (Figure 3.4K-c), indicating that the TGFβ pathway contributes to migration and invasion of PIK3CA-driven HNSCC tumorigenesis.

**PIK3CA and PDK1 Are Associated with Progression in Human HNSCC**

To address the clinical relevance of our results from the PIK3CA-GEMM, we examined PIK3CA, PDK1 and pAKT expression levels in primary human HNSCC samples, case-matched adjacent mucosa and metastatic lesions, compared with normal oropharyngeal samples from patients with sleep apnea as controls. We first performed qRT–PCR to measure PIK3CA mRNA levels. Compared with normal controls, PIK3CA mRNA was increased in both adjacent mucosa and HNSCC samples (Figure 3.7A). Interestingly, increased PIK3CA expression positively correlated with reduced tumor differentiation and increased lymph node metastasis (Figure 3.7A). We then carried out IHC staining of p110α, PDK1 and pAKT. Figure 3.7B summarizes the percentage of cases with immunostaining 2+ in HNSCC tumors and metastases. Consistent with PIK3CA qRT–PCR results, we found increased p110α immunostaining both in poorly differentiated HNSCCs and in metastases (Figure 3.7B and C). Interestingly, PDK1 expression positively correlated with PIK3CA expression and advanced disease (Figure 3.7B and C).
Figure 3.7. PIK3CA, PDK1 and pAKT alterations in human HNSCCs. (A.) qRT–PCR quantitation of PIK3CA mRNA expression in human HNSCCs. Error bars indicate mean±s.d. *P<0.05. Adj, adjacent mucosa; LN mets, lymph node metastases; Mod, moderately differentiated tumors; NN, normal controls; Poorly, poorly differentiated tumors; Well, well-differentiated tumors. (B.) Percentage of human HNSCC cases which exhibited greater than or equal to2+ IHC staining of p110α, PDK1 and pAKT. *P<0.05. (C.) IHC staining of serial sections of a poorly differentiated HNSCC. Scale bar: 50 μm.
However, AKT activation showed the opposite pattern, and demonstrated reduced expression in poorly differentiated tumors and metastases compared with early-stage lesions. These data from human tissue sample support our mouse model data that PIK3CA-driven HNSCC progression is predominantly mediated through PDK1, but not through AKT.

Discussion

Previous mouse models were generated by knocking in PIK3CA mutant H1047 into different tissue contexts\textsuperscript{210–214}. However, somatic mutations of PIK3CA are infrequent (~10\%) compared with PIK3CA gene amplification (~40\%) in HNSCC patients\textsuperscript{38,40}. Thus, our PIK3CA-GEMM, in which the wild-type PIK3CA is overexpressed specifically in murine head and neck epithelium, more closely mimics the majority of human HNSCC patients. As this transgene can be induced in a spatio-temporal manner, our model is an ideal platform to assess the role of PIK3CA overexpression at each stage of HNSCC tumorigenesis. This also represents the first GEMM in head and neck tissue for studying the in vivo role of PIK3CA. In the PIK3CA-GEMM, we found that PIK3CA overexpression alone is not sufficient to initiate tumor formation in head and neck tissue. This is in contrast to the results of tumor formation in breast and lung cancer mouse models\textsuperscript{210–212}. One explanation is that previous mouse models were based on gene mutations compared with our model, which is based on gene overexpression; these two types of gene alterations may play distinct roles in tumorigenesis. The other possibility is that PIK3CA may
have a context-specific role in tumorigenesis. For example, the same mutant PIK3CA-H1047 that causes spontaneous breast and lung tumor formation is not sufficient to initiate tumor formation in ovarian or colon tissues unless coupled with either a PTEN or APC deletion\textsuperscript{213,214}. Our data suggest that PIK3CA overexpression may not initiate tumorigenesis, but instead it promotes tumor progression in HNSCC.

Compared with the well-documented mitogenic signaling of PI3K in cell growth and survival, the role of PI3K in tumor invasion and metastasis has not been well delineated. In a subset of aggressive breast cancer, which exhibits EMT and CSC characteristics, the authors found the mutation rate of PIK3CA reached nearly 50%, significantly higher than in any other types of breast cancer\textsuperscript{215}. Knocking in the PIK3CA mutant into a breast cancer cell line also resulted in an EMT phenotype\textsuperscript{216}. In line with these data, our PIK3CA-GEMM provides the first in vivo evidence that PIK3CA alteration is able to drive tumor progression at least partially through enriched EMT and CSC characteristics, which may be responsible for the invasive and metastatic phenotype observed in the PIK3CA-GEMM.

AKT is largely regarded as the dominant mediator of oncogenic PI3K signaling\textsuperscript{38}. However, studies suggest that the link between PI3K and AKT can be uncoupled\textsuperscript{41–44}. Recent reports showed that AKT can directly phosphorylate Twist1 to promote EMT\textsuperscript{217}, and that the ratio of AKT1 and AKT2 and its regulated microRNAs are responsible for EMT and CSCs\textsuperscript{208}. However, we found no differences in AKT activation or expression of AKT isoforms between control and PIK3CA-GEMM tumors. Lack of AKT activation has also been observed in subset of PIK3CA-mutated breast cancer\textsuperscript{42}, PTEN-null lung cancers\textsuperscript{218} and BRAF-
initiated melanoma. AKT activation can be detected in mouse models as early as pre-neoplastic lesions upon either 4NQO or NNK treatment and AKT activation in the oral cavity can initiate benign tumor formation. However, it fails to promote tumor progression unless combined with p53 loss suggesting that AKT activation may be involved in early events in HNSCC carcinogenesis, but not in tumor progression. In some breast cancers with PIK3CA mutations, PDK1, but not AKT, is activated. Similarly, our results suggest that PDK1, rather than AKT, facilitates progression of PI3K-driven HNSCC, which raises the possibility of PDK1 as a therapeutic target in HNSCC patients with PIK3CA alterations.

PDK1 is a serine/threonine protein kinase that phosphorylates members of the AGC kinase superfamily, including AKT, and is implicated in cell proliferation, survival and metabolism. Interestingly, PDK1 has been shown to regulate cell migration but not proliferation in lymphocytes and to promote EMT in cardiac development. Compared with the extensive research into AKT, less is known about the role of PDK1 in human cancers. Overexpression of PDK1 has been reported in several human cancers and correlates with disease progression in patients with pancreatic cancer or melanoma. Interestingly, PDK1 is required for Kras-driven pancreatic cancer in murine models but not for the Kras-driven lung cancer, suggesting a context-specific role of PDK1. In HNSCC, PDK1 has been shown to mediate G-protein-coupled receptor and epidermal growth factor receptor (EGFR) cross-talk as well as cell growth, both in vitro and in vivo. It has become evident recently that the role of PDK1 in physiological and pathological conditions is not limited to AKT activation; it may also evoke other signaling for tumorigenesis. For
example, PDK1 has been shown to activate SGK3 in a PI3K-dependent, AKT-independent manner in breast cancer\textsuperscript{42}. PDK1 can also directly activate PLCγ1 to mediate cancer cell invasion\textsuperscript{223}. Moreover, PDK1 directly phosphorylates polo-like kinase 1 to induce an embryonic stem cell-like gene signature associated with aggressive tumor behaviors and CSC self-renewal\textsuperscript{224}. Further investigation on the downstream molecular mechanisms underlying PI3K/PDK1 signaling in the PIK3CA-GEMM will provide important insights into PIK3CA-driven HNSCC progression and identify novel therapeutic targets to control HNSCC progression.

The downstream mechanisms of PI3K/PDK1 in HNSCC invasion and metastasis are largely unknown. In this study, we found that the TGFβ1 ligand and its downstream mediator Smad3 are overexpressed in PIK3CA-GEMM tumor tissues. The positive correlation between PIK3CA and TGFβ1 levels was further detected in both PIK3CA-overexpressing tumor cells and PIK3CA-knockdown tumor cells, suggesting that the PIK3CA-mediated regulation of TGFβ pathway is cell autonomous. The contribution of TGFβ pathway to mediate migration and invasion of PIK3CA-overexpressing tumor cells was validated by treating these cells with a TGFβ pathway inhibitor. Cross talk between PI3K and TGFβ signaling can occur at multiple levels\textsuperscript{102}. For example, PI3K is able to mediate TGFβ-receptor-initiated intracellular signaling\textsuperscript{225}. In addition, PI3K can antagonize TGFβ-induced cytostasis and cause the shift in TGFβ signaling to tumor progression\textsuperscript{102}. However, prior reports on PI3K and TGFβ interplay were almost exclusively related to AKT signaling\textsuperscript{102}. Thus, it remains unclear how PI3K/PDK1 interacts with TGFβ/Smad3 signaling during HNSCC progression.
Currently, the only Food and Drug Administration-approved targeted therapy for HNSCC is EGFR inhibitors\textsuperscript{33}. Targeting the PI3K pathway represents a promising new strategy for treating HNSCC. The role of PDK1 in mediating invasion and metastasis of PIK3CA-driven HNSCC suggests it may be useful as a therapeutic target. Targeting PDK1 using either genetic approaches or pharmaceutical inhibitors has been shown to inhibit CSC self-renewal and metastasis\textsuperscript{96,224,226}. In one pre-clinical study on HNSCC, PDK1 inhibition showed anti-tumor effects either by itself or in combination with EGFR inhibition\textsuperscript{222}. Thus, targeting PIK3CA or PDK1 presents an important new avenue for HNSCC treatment. Targeting TGF\(\beta\) as anti-cancer therapy has been evaluated for a decade in multiple cancers\textsuperscript{227}. However, it remains unclear how it may best be used and such therapies are highly stage- and patient-specific. The possible synergistic role of over-activated PI3K and TGF\(\beta\) signaling in HNSCC progression demonstrated in this study has prompted testing of combination therapy. If proven effective in pre-clinical trials with PIK3CA-GEMM, this combination therapy will be immediately translated into clinical trials for treating advanced HNSCC patients who have PIK3CA alterations.

In summary, we report a GEMM in which the PIK3CA is overexpressed in head and neck epithelia. Although overexpression of PIK3CA alone is not sufficient to initiate HNSCC formation, it significantly increased the susceptibility to the 4NQO-induced HNSCC carcinogenesis. More importantly, PIK3CA overexpression promotes EMT and CSC properties and drives tumor invasion and metastasis, which is likely mediated by increased PDK1 expression and activation, and subsequently increased TGF\(\beta\)1 ligand and Smad3 expression and activation. Our study lays the
foundation for future investigations into the mechanism of PDK1 and its interplay with TGFβ signaling in PIK3CA-driven HNSCC tumorigenesis. Moreover, it suggests that therapeutic targeting of PDK1 and/or TGFβ signaling may effectively control HNSCC progression, particularly in patients with PIK3CA amplification.
CHAPTER IV
DISTINCT ROLES OF PIK3CA IN ENRICHING AND MAINTAINING OF CANCER STEM CELLS IN HNSCC

Introduction

Head and neck squamous cell carcinoma (HNSCC) accounts for approximately 6% of all cancer cases and is the sixth most common cancer worldwide. The overall 5-year survival rate of HNSCC is 40-50%, and has not been significantly improved over the past several decades. The main reasons for the poor prognosis of HNSCC patients are loco-regional invasions, recurrence, and distant metastasis. On initial presentation, ~10% of HNSCC cases show metastases and the survival rate for these patients is less than 1 year. Additionally, ~30-40% of post-treated HNSCC patients develop recurrence or metastasis. Thus, therapies controlling HNSCC recurrence and/or metastasis are pivotal to improve poor survival of HNSCC patients.

The phosphatidylinositol 3-kinase (PI3K) signaling pathway is the most frequently altered oncogenic pathway in HNSCCs. Recent whole-exome sequencing of HNSCC samples has identified mutations (10%) and/or amplification (40%) of PIK3CA, the gene encoding for p110α subunit of PI3K, making it the most common altered oncogene in human HNSCC patients. To better understand the role of PIK3CA in the development and progression of HNSCC in vivo, in last chapter, we overexpressed PIK3CA in the head-and-neck epithelium using an inducible head-and-neck-specific genetically engineered mouse model.
We showed that while overexpression of PIK3CA was not sufficient to initiate tumorigenesis, it markedly promoted HNSCC progression manifested as poorly-differentiated, metastatic tumors with a phenotype of epithelial-to-mesenchymal transition (EMT) and increased gene expression related to head and neck cancer stem cells (CSCs). This data suggest that EMT and CSC traits may drive tumor invasion and metastasis in HNSCC patients with PIK3CA amplification.

EMT is a process by which epithelial cells lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal cells. CSCs refer to a subset of cancer cells that possess the pluripotent characteristics, such as self-renewal or regeneration of normal stem cells. Cancer cells can hijack EMT program to become more migratory, invasive, resistant to anoikis/chemotherapies, or gain CSC properties, in which all these EMT characters can contribute to cancer progression. In general, CSCs are intend to be quiescent, Since CSCs possess self-renewal and tumorigenic properties, and are in general quiescent and required less nutrients, it is believed that they are more suitable to survive in a harsh environment, resistant to chemo-radiation therapies, and can be “seeds” for tumor formation either primarily (tumor initiation), secondarily (recurrence), or distantly (metastasis).

To further understand the role of EMT and CSCs traits in HNSCC progression, in this chapter, we further investigate these two processes using both murine HNSCC cells isolated from the PIK3CA-GEMM I presented in last chapter, and human HNSCC cell lines with PIK3CA genetic alterations. Our data revealed that PIK3CA overexpression promotes EMT and enriches CSC population in both
murine and human HNSCC cell lines. Surprisingly, inhibition of PIK3CA or key components in the PI3K pathway failed to reduce, but rather promote CSC population. To reveal the molecular mechanism of this PI3K-independent CSC phenotype, we performed a receptor tyrosine kinase (RTK) array, and found that multiple RTKs were activated in the PIK3CA-overexpressing HNSCCs. Pharmaceutical inhibitor screening showed the effectiveness of targeting Ephrin receptors (Ephs) or tropomyosin receptor kinases (Trks) in reducing CSC populations in HNSCC.

Results

Overexpression of PIK3CA Promotes Cell Proliferation, Migration, Epithelial-to-mesenchymal Transition and De-differentiation

We have recently reported a genetically engineered mouse model (GEMM) in which the PIK3CA transgene is overexpressed in murine head and neck epithelia. Although overexpression of PIK3CA alone is not sufficient for HNSCC development; it significantly promotes HNSCC invasion and metastasis. Epithelial-to-mesenchymal transition (EMT) and enriched cancer stem cell phenotype were observed in the PIK3CA-overexpressing HNSCC tumors compared to the 4-NQO induced control HNSCC tumors\(^\text{14}\). To further investigate the molecular mechanisms underlying the PIK3CA-driven HNSCC progression, we established primary murine HNSCC cell lines from two individual PIK3CA-overexpressing HNSCC tumors (Hereafter referred to as CU110-1 and CU110-2) and two individual 4-NQO induced control HNSCC
tumors (Hereafter referred to as CUCON-1 and CUCON-2). (Figure 4.1A). As shown in the Figure 4.1A, consistent with the EMT changes in the tumor tissues, CU110 cells were more spindle-like, while CUCON cells were more round and epithelial-like. The authentication of these cell lines was validated by the transgene-specific genotyping PCR. As anticipated, the resulting CU110 cells showed higher expression of p110α and enhanced phospho-AKT level compared with CUCON cells (Figure 4.1B), indicating that PI3K/AKT signaling is highly active in the cell lines established from tumors of PIK3CA-GEMM. Accompanying with the elevated PI3K/AKT signaling, CU110 cells also displayed higher proliferative and migratory capacities compared with CUCON cells (Figures 4.1C and D).

We further characterized EMT phenotype as we observed in the tumor tissues and the cell morphologies of control and PIK3CA-overexpressing HNSCCs. Western blotting results showed that overexpression of PIK3CA in CU110 cells promotes EMT shown as loss of the epithelial marker, E-cadherin and increase expression of the mesenchymal marker, Vimentin (Figure 4.2A). Immunofluorescence staining for E-cadherin and Vimentin further confirmed EMT phenotype in CU110 cells compared to CUCON cells (Figure 4.2B). Next, we analyzed expression of various transcription factors known to regulate EMT program by using quantitative RT-PCR. We found that Zeb1, Twist 1, Snail1 and Ets1 expression but not that of Twist2 and Snail 2, was significantly higher in CU110 cells compared to CUCON cells (Figure 4.2C). As the EMT process generates poorly differentiated tumor cells that are defined by losing cytokeratins230, we further assessed expression of a panel of cytokeratins in both CU110 and CUCON cells.
Figure 4.1. Overexpression of PIK3CA promotes cell proliferation and migration (A.) Culture of murine HNSCC cells from either 4NQO-induced tumors or PIK3CA-overexpressing tumors. Left: H&E staining of tumor samples; Right: phase contrast images of CUCON and CU110 cells. (B.) Western blotting of p110α in murine CUCON and CU110 cells. GAPDH was used as a loading control. (C.) Cell proliferation assay quantified by Vi-cell viability analyzer (Beckman Coulter). Cells were initially seeding as equal numbers and were quantifying live cell number at day 1, day 3, day 6 and day 8 (D.) Wound healing assays for CU110 and CUCON cells. For each assay, 3 individual experiments were performed; distance migrated was measured on the picture taken on day 1 and day 3 and reported as pixels.
Here we found that expression of several cytokeratins was significantly decreased in CU110 cells compared to the CUCON cells (Figure 4.2D), suggesting that CU110 cells are poorly differentiated. Together, these data demonstrate that overexpression of PIK3CA promotes cellular proliferation, migration, EMT, and de-differentiation.

**Overexpression of PIK3CA Enriches Putative Head and Neck Cancer Stem Cells**

It has been shown that EMT and de-differentiation generate cells possessing properties of stem cells and confer cancer cells with stem cell-like characteristics\textsuperscript{154}. To examine if the EMT and de-differentiation phenotypes of CU110 cells generate more cancer stem cells (CSCs), we utilized several putative surface markers reported for CSC isolation in HNSCC. CD44 and CD24 have been widely used individually or in combination to isolate CSCs from several solid tumors\textsuperscript{231,232}, including HNSCC\textsuperscript{142,144}. As shown in Figure 4.3A, FACS results showed significantly higher numbers of CD44+ and lower number of CD24+ cells in CU110 cells compared to CUCON cells. Side population (SP) as measurement of Hoechst dye effluxing activities and aldehyde dehydrogenase (ALDH) activity were also used for isolating CSCs in several solid tumors, including HNSCC\textsuperscript{145,146}. As shown in Figure 4.3B, about 1.4% of SP cells were identified in the CU110 cells, whereas no SP cells were identified in the CUCON cells. The specificity of SP cells was further validated by verapamil elimination experiment. Similarly, a moderate increase of ALDH activity was observed in CU110 cell compared to CUCON cells (Figure 4.3C).
Figure 4.2. Overexpression of PIK3CA promotes epithelial to mesenchymal transition and de-differentiation. (A.) Western blotting of E-cadherin and Vimentin in CUCON and CU110 cells. GAPDH was used as a loading control. (B.) Immunofluorescence staining of E-cadherin (Green) and Vimentin (Red) in CUCON and CU110 cell lines. DAPI stain (Blue) was used to visualize the nucleus. (C.) qRT-PCR analysis of transcriptional factors regulating EMT. Fold changes are relative to CUCON-1 cells. (D.) qRT-PCR analysis of cytokerkatins in CUCON and CU110 cell lines. Fold changes are relative to CUCON-1 cells.
Together, these data showed that overexpression of PIK3CA enriches putative CSCs in HNSCC.

**Sphere Forming Capacity is a Functional Measurement for Cancer Stemness Properties of HNSCC**

To determine if the enriched CSC-like population of CU110 cells exhibits efficient self-renewing capacity, we evaluated the sphere-forming capacities of CU110 and CUCON to assess the self-renewal ability of CSCs\(^{147}\). Consistent with the enrichment of CSCs in CU110 cells, the CU110 cells, but not CUCON cells were able to form spheres in ultralow-attachment, serum-free condition (Figure 4.4.1A). To further examine if the spheres possess CSC properties, we measured mRNA expression of embryonic stem cell markers Nanog, Oct4, and Sox2. As shown in Figure 4.4.1B, expression of these markers were significantly increased in spheres compare to monolayer cultured CU110 cells. We further performed hematoxylin and eosin (H&E) staining on sections of spheroids and observed distinguishable layers with a hollow center. Moreover, the middle and inner layers of these spheroids featured intensive extracellular matrix (Figure 4.4.2A). In consistent with higher mRNA expression of Sox2 in spheroids, the outer layer contained many SOX2+ cells observed by immunofluorescence (Figure 4.4.2B). However, not all CD44+ cells were SOX2+ (Figure 4.4.2B, lower panel). Moreover, some SOX2+ cells were Ki67+ (Figure 4.4.2B, top panel), suggesting that not all CSCs are quiescent and some maybe highly proliferative, questioning if quiescence or slow-cycling is a putative characteristic of CSCs. We also found that some SOX2+ cells
Figure 4.3. Overexpression of PIK3CA enriches putative head and neck cancer stem cell population. (A.) FACS analysis of cell surface marker, CD44 and CD24 in CUCON and CU110 cells. Quantification data are represented as mean ± SD. (B.) Side population (SP) fraction detected by Hoechst dye-effluxing assay of CUCON and CU110 cells. Left panel: representative FACS plots of CUCON and CU110 cells treated with Hoechst33342 in the presence and absence of verapamil. Right panel: quantification of gated SPs of CUCON and CU110 cells. (C.) ALDH activity assay using Aldefluor staining and quantification of ALDH+ cells of CUCON cells and CU110 cells; error bars indicates SD; *, p < 0.05.
were also CD34+ or CD133+ (Figure 4.4.2C). In addition, almost all outer-layer cells were Vimentin+ and K1+, and cells near the hollow center showed caspase 3 positive staining, suggesting that these cells were undergoing apoptosis (Figure 4.4.2C).

To evaluate tumorigenicity of spherical cells in vivo, we injected $10^5$ and $10^3$ cells from either sphere-, or monolayer- cultured CU110 cells into the flanks of syngeneic BL6 mice. While both sphere- and monolayer- cultured CU110 cells generated tumors at similar kinetics, and sizes of all mice injected, cells from spheres developed larger tumors in all mice injected compared to only one mouse developed smaller tumor after injection of monolayer-cultured CU110 cells. The average volumes of tumors from sphere was about 7-fold higher than that from the tumor of monolayer cells (Figure 4.4.3A). Last, we investigated whether sphere forming ability is correlated with p110α expression in human HNSCC cell lines. Among the 17 human HNSCC cell lines we screened, the sphere-forming ability was found to be well correlated with p110α expression levels, suggesting that overexpression of PIK3CA enriches CSC population in human HNSCC (Figure 4.4.3B).

**Knockingdown PI3K Failed to Reverse EMT Phenotype and Reduce CSC Population**

Next, we examined whether PIK3CA is required for maintaining EMT phenotype and CSC population in the PIK3CA-overexpressing CU110 cells. We stably knocked down PIK3CA expression in CU110 cells by using shRNA against
Figure 4.4.1. Sphere forming capacity is a functional measurement for cancer stemness properties of HNSCC. (A.) HNSCC sphere-forming ability of two CUCON and two CU110 cells. Left panel: phase contrast images of CUCON and CU110 cells in serum-free and ultralow attachment culture condition. Right panel: quantification of sphere numbers for two CUCON and two CU110 cells. Spheres with diameter ≥ 30 microns were counted. (B.) qRT-PCR for expression of embryonic stem cell genes in the monolayer- or sphere-cultured CU110 cells. GAPDH was used as an internal control. n=3; error bars indicate SD.
Figure 4.4.2. Sphere forming capacity is a functional measurement for CSC-like properties of HNSCC. (A.) H&E staining for HNSCC sphere section. (B.) Immunofluorescence staining on sphere section using the antibodies as indicated in figures. (C.) Immunofluorescence staining on sphere sections using the antibodies as indicated in figures. K8 or Vimentin positive layer was delineated by white dotted lines in middle panel (right) and lower panel (left); hollow center (lower portion) of spheroid was delineated by white dotted lines in lower panel (right).
Figure 4.4.3. Sphere forming capacity is a functional measurement for cancer stemness properties of HNSCC. (A.) In vivo tumorigenicity by subcutaneously injection of either sphere-or monolayer cultured CU110 cells to the flanks of BL6 mice. Upper: tumor incidence of monolayer- or sphere- cultured CU110 cells. Lower: left: Images of tumors developed from monolayer or sphere- cultured CU110 cells. Right: quantification of tumor volume. **, p < 0.01. (B.) Correlation between p110a expression and sphere-forming ability in human HNSCC cell lines. Left panel: Western blotting and HNSCC sphere forming assay were performed in 17 human head and neck cancer cell lines (UMSCC1/2/10A/10B/22A/22B/47, VU1131/1365, SCC9, Fadu, HN6, Tu, Cal27, M4C/4E and LNM1). Relative quantification of p110a was done by quantifying band intensity of p110a and GAPDH, and is shown as percentage of GAPDH band intensity. **, p < 0.01. Right panel: representative images of sphere-forming capacity of HNSCC cell lines in serum-free and ultralow attachment culture condition. Spheres with diameter ≥ 30 microns were counted.
PIK3CA. Surprisingly, knocking down of PIK3CA failed to induce E-cadherin or reduce Vimentin expression as shown by Western and IF staining (Figure 4.5.1A and B), indicating PIK3CA is not required for maintaining the EMT phenotype in PIK3CA-overexpressing cells. We further examined the effects of knocking down PIK3CA on CSC phenotype in CU110 cells. As shown in Figure 4.5.1C to E, instead of reducing CSC population, knocking down of PIK3CA unexpectedly promoted CSC population in CU110 cells. In the HNC sphere assay, knocking down of PIK3CA moderately increased HNC sphere numbers (Figure 4.5.1C). In the CSC cell surface marker assays, while knocking down of PIK3CA had no effects on CD44+ population, it significantly increased and CD24- populations (Figure 4.5.1D). Furthermore, PIK3CA knockingdown caused about 5-fold increase in side population (Figure 4.5.1E).

We further examined effects of knocking down PIK3CA in two human HNSCC cell lines, Fadu and UMSCC47 (Figure 4.5.2B). The Fadu cell line contains a gain-of function mutation of PIK3CA233, and was originated from a HPV negative HNSCC patient. The UMSCC47 cell line overexpresses PIK3CA the most among 8 HNSCC cell lines we screened (Figure 4.5.2A), and it was originated from a HPV positive HNSCC patient. Knocking down PIK3CA in either Fadu or UMSCC47 cell lines reduced cell proliferation and migration (Figure 4.5.2C and D). However, similar to the effect we observed in the murine CU110 cells, knocking down PIK3CA failed to reduce HNSCC sphere forming abilities in both Fadu and UMSCC47 cell lines (Figure 4.5.2E).
Figure 4.5.1. Knocking down PIK3CA failed to reverse EMT phenotype and reduce CSC population.
Figure 4.5.1. Knocking down PIK3CA failed to reverse EMT phenotype and reduce CSC population. (A.) Western Blot of p110α, E-cadherin, and vimentin in CU110-2 cells stably transfected lentiviral-mediated shRNA (shPIK3CA) or a scrambled control (SCR). GAPDH was used as a loading control. (B.) Immunofluorescence staining of E-cadherin or Vimentin in CU110-2 cells stably transfected with either sh-PIK3CA or a scrambled control (SCR). DAPI staining (blue) was used to visualize the nucleus. (C.) HNSCC sphere-forming assay of CU110 cells stably transfected either shPIK3CA or SCR lentivirus. Spheres with diameter ≥ 30 microns were counted; Quantification of sphere is shown in right, n=3; error bars indicate SD. (D.) FACS analysis of CD44 and CD24 in CU110 cells stably transfected either shPIK3CA or SCR lentivirus. Quantification of CD44 ad CD24 population is shown in right. n=3; error bars indicates SD. (E.) Side population fraction using Hoechst dye-effluxing analysis in CU110 cells stably transfected either shPIK3CA or SCR lentivirus. Quantification of SP fraction is shown in right.
Figure 4.5.2. Knocking down PIK3CA reduced cell proliferation and migration, but not CSC population in human HNSCC cell lines.
Figure 4.5.2. Knocking down PIK3CA reduced cell proliferation and migration, but not CSC population in human HNSCC cell lines. (A.) Screening of PIK3CA expression in 8 human head and neck cancer cell lines (as indicated) by using qRT-PCR. (B.) Western blotting in Fadu (left) and UMSCC47 cell lines (right) stably transfected with either lentiviral - mediated short hairpin sh-PIK3CA or scramble control (SCR). (C.) Cell proliferation assay of Fadu (left) or UMSCC47 (right) cell lines stably transfected with either sh-PIK3CA or scramble control. (D.) Cell migration assay of Fadu or UMSCC47 cell lines stably transfected with either sh-PIK3CA or scramble control. (E.) HNSCC sphere forming assay of Fadu or UMSCC47 cell lines stably transfected with either sh-PIK3CA or scramble control (SCR). The quantification is shown in right. n=3; error bars indicate SD.
Knocking Down Key Components in PI3K Pathway Promotes CSC Population

The effect on CSC population upon PIK3CA knocking down is unexpected, and promoted us to examine this effect upon knock down other key components in PI3K pathway. We first examined the expression levels of several PI3K isoforms in PIK3CA-overexpressing cells. As shown in Figure 4.5.3 A and B, PIK3R1, the gene encoding for the p85α adaptor protein of PI3K, is concurrently highly expressed in CU110 cells as measured by qRT-PCR and Western blotting. Interestingly, the level of p85α is also correlated with the abilities of sphere forming in human HNSCC cell lines (Figure 4.5.3C). We then stably knocked down PIK3R1 in CU110 cells. Similar to the effect of knocking down PIK3CA, PIK3R1 knock down had no effect on reverse EMT shown as qRT-PCR analysis on E-cadherin, Vimentin and several transcriptional factors regulating EMT (Figure 4.5.4A and B). Knocking down PIK3R1 also failed to reduce CSC population as shown by HNSCC sphere forming assay (Figure 4.5.4C), FACS analysis of surface markers CD44, and CD24 (Figure 4.5.4D), and SP fraction (Figure 4.5.4E). Instead, similar to PIK3CA knocking down, knocking down PIK3R1 promoted CSC population (Figure 4.5.4C to E).

It has been reported that the ratio of AKT1 and AKT2 expression regulates EMT and CSC properties in a breast cancer model. Thus, we further knocked down AKT1 or AKT2 in the CU110 cells (Figure 4.5.5A). Similar to the effects of PIK3CA or PIK3R1 knock down, AKT1 or AKT2 knocking down had no effects on EMT as shown by qRT-PCR of E-cadherin, Vimentin and several transcriptional factors regulating EMT (Figure 4.5.5B). Knock down either AKT1 or AKT2 also modestly increased the CSC population as shown by HNSCC sphere forming assay.
(Figure 4.5.5C), FACS analysis of surface markers CD44 and CD24 (Figure 4.5.5D), and SP fraction (Figure 4.5.5 E).

Last, we have recently showed that PDK1 contributes to tumor progression in the PIK3CA-GEMM study as evidenced by reduced proliferation, migration and reverse EMT upon PDK1 knocking down\textsuperscript{14}. However, knocking down PDK1 had no effect on HNSCC sphere forming abilities, on putative CSC surface markers CD44 and CD24 (Figure 4.5.6A and B), and increases SP fraction in the PIK3CA-overexpressing cells (Figures 4.5.6C).

**Targeting Multiple Receptor Tyrosine Kinase Pathways Effectively Eliminates CSC Populations with Inhibiting Ephs, Trks and c-kit the Most Prominent**

Since the maintenance of PIK3CA overexpression-induced CSC properties is no longer dependent on PI3K signaling, we sought to identify other mechanisms important for maintaining the CSC phenotype. We thus performed a receptor tyrosine kinase protein array to compare the differences between the PIK3CA-overexpressing cells (CU110) and the control cells (CUCON). As shown in Figure 4.6A, compared to CUCON cells, there were multiple activated RTKs in CU110 cells, i.e. EGFR, FGFRs, InsR, TrkA and B, c-Kit, Ephrin receptors, Tyro3, Axl, VEGFRs, S6 ribosomal protein, and AKT. We further examined the RTK profiles of inhibition of PI3K pathway either by genetic knocking down of PIK3CA or using PI3K inhibitor PX866. Our results showed that there were in general no changes of RTK profiles upon these two conditions, suggesting these activated multiple RTKs are not regulated by PI3K signaling pathway (Figure 4.6 B and C).
Figure 4.5.3. Examination of PI3K isoforms in murine HNSCC cells. (A.) qRT-PCR results of PI3K isoforms in two CUCON and two CU110 cells. Fold changes are relative to CUCON-1 cells; GAPDH was used as an internal control and results indicate means of triplicate experiments. Results of PIK3CG, PIK3C2A and PIK3C3 are not shown due to no amplification in both CUCON ad CU110 cells. (B.) Western blotting of p110α and p85α in two CUCON and two CU110 cells. GAPDH was used as a loading control. (C.) Correlation between p85α protein and sphere-forming ability in human HNSCC cell lines. Western blotting and HNSCC sphere forming assay were performed in 12 human head and neck cancer cell lines (VU1131, VU1365, UMSCC1, UMSCC2, UMSCC47, Fadu, Tu, HN6, LNM1, CAL27, M4C, and M4E). Relative quantification of p85α was done by quantifying band intensity of p85α and GAPDH, and is shown as percentage of GAPDH band intensity.
Figure 4.5.4. Knocking down PIK3R1 failed to reverse EMT but promoted CSC population.
Figure 4.5.4. Knocking down PIK3R1 failed to reverse EMT but promoted CSC population. (A.) Western blotting in CU110 cells stably transfected with either lentiviral - mediated short hairpin sh-PIK3R1 or scramble control (SCR). GAPDH was used as a loading control. (B.) qRT-PCR results of E-cadherin, vimentin and EMT-related transcription factors in CU110 cells stably transfected with either sh-PIK3R1 or SCR lentivirus. (C.) HNSCC sphere forming assay of CU110 cells stably transfected with either sh-PIK3R1 or SCR lentivirus. The quantification is shown in right. Spheres with diameter ≥ 30 microns were counted. n=3; error bars indicate SD. (D.) FACS analysis of CD44 and CD24 population in CU110 cells stably transfected with either sh-PIK3R1 or SCR lentivirus. (E.) Side population (SP) fraction using Hoechst dye-effluxing analysis in CU110 cells stably transfected either shPIK3R1 or SCR lentivirus. Quantification of SP fraction is shown in right.
Figure 4.5.5. Knocking down AKT1 or AKT2 failed to reverse EMT but promoted CSC population.
Figure 4.5.5. Knocking down AKT1 or AKT2 failed to reverse EMT but promoted CSC population. (A.) Western blotting of CU110 cells stably transfected with either lentiviral -mediated short hairpin sh-AKT1, AKT2 or scramble control (SCR). GAPDH was used as a loading control. (B.) qRT-PCR results of E-cadherin, vimentin and EMT-related transcription factors in CU110 cells stably transfected with either sh-AKT1, sh-AKT2 or SCR lentivirus. (C.) HNSCC sphere forming assay of CU110 cells stably transfected with sh-AKT1, sh-AKT2 or SCR lentivirus. The quantification is shown in right. Spheres with diameter ≥ 30 microns were counted. n=3; error bars indicate SD. (D.) FACS analysis of CD44 and CD24 population in CU110 cells stably transfected with either sh-AKT1, sh-AKT2 or SCR lentivirus. (E.) Side population (SP) fraction using Hoechst dye-effluxing analysis in CU110 cells stably transfected sh-AKT1, sh-AKT2 or SCR lentivirus. Quantification of SP fraction is shown in right.
Figure 4.5.6. Knocking down PDK1 promoted CSC population. (A.) HNSCC sphere forming assay of CU110 cells stably transfected with either lentiviral-mediated short hairpin sh-PDK1 or scramble control (SCR). The quantification is shown in right. Spheres with diameter ≥ 30 microns were counted. n=3; error bars indicate SD. (B.) FACS analysis of CD44 and CD24 population in CU110 cells stably transfected with either sh-PDK1 or SCR lentivirus. (C.) Side population (SP) fraction using Hoechst dye-effluxing analysis in CU110 cells stably transfected sh-PDK1 or SCR lentivirus. Quantification of SP fraction is shown in right.
To identify key pathway(s) regulating CSC phenotype, we utilized a pharmaceutical inhibitor screen approach to assess their effects on inhibiting HNSCC sphere forming. We selected inhibitors targeting the activated RTK pathways from the RTK protein array experiment described above. In addition, to evaluate the effects of targeting PDK1 and TGFβ signaling, two targets we have recently reported which contribute to PIK3CA-overexpressing driven HNSCC progression, we included inhibitors targeting PDK1, i.e., GSK2334470 and TGFβ type I receptor i.e., LY2157299. Furthermore, chemotherapy drug Paclitaxel was also included to assess its effects on HNSCC sphere forming ability (Table 4.1). As shown in Figure 4.7.1A, while PX866 increased the numbers of HNSCC spheres, inhibitors targeting Ephrin receptors (LDN211904), Trk/B (GNF5837), and c-kit (Imatinib) effectively reduced or eliminated HNSCC sphere formation. In addition, treating CU110 cell by LDN211904 also reduced CD44+, and ALDH+ populations (Figure 4.7.1B), treating CU110 cell by GNF5837 significantly reduced ALDH+ population (Figure 4.7.1C), and treating CU110 by Imatinib reduced CD44+ population and SP+ fraction (Figure 4.7.1D).

The attenuation of CSC phenotype upon inhibition of Ephs, Trks and c-kit molecules led us to hypothesize that inhibitor(s) co-targeting all three molecules may have the maximum effects on the CSC phenotype. Ponatinib was chosen given its inhibition on multiple RTKs, with potency of inhibition on Ephs, Trk and c-kit below 50 nM level. The result is astonishing; with nearly complete elimination of HNSCC sphere formation, ALDH+ and SP fraction (Figure 4.7.2A-C). To assess the in vivo effect of ponatinib treatment on HNSCC metastasis, we treated CU110 cells with
Table 4.1. Summary of IC$_{50}$ for various inhibitors used in the screening and their major targets.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC$_{50}$ (µM)</th>
<th>Target/s</th>
<th>Inhibitor</th>
<th>IC$_{50}$ (µM)</th>
<th>Target/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gefitinib</td>
<td>9.56</td>
<td>EGFR</td>
<td>Axitinib</td>
<td>1.80</td>
<td>VEGFR</td>
</tr>
<tr>
<td>AZD4547</td>
<td>4.40</td>
<td>FGFR</td>
<td>PX866</td>
<td>5.89</td>
<td>pan-PI3 kinase</td>
</tr>
<tr>
<td>OSI906</td>
<td>24.0</td>
<td>IGF-1R, InsR</td>
<td>Everolimus</td>
<td>12.92</td>
<td>mTOR</td>
</tr>
<tr>
<td>GNF5837</td>
<td>6.15</td>
<td>TrkA/B/C</td>
<td>GSK2334470</td>
<td>6.20</td>
<td>PDK1</td>
</tr>
<tr>
<td>BMS777607</td>
<td>7.12</td>
<td>Axl, RON, Tyro3</td>
<td>LY2157299</td>
<td>&gt;50</td>
<td>TGFβ receptor</td>
</tr>
<tr>
<td>Imatinib</td>
<td>33.0</td>
<td>c-Kit, PDGFR</td>
<td>Paclitaxel</td>
<td>0.08</td>
<td>Microtubule</td>
</tr>
<tr>
<td>LDN211904</td>
<td>8.73</td>
<td>Ephrin receptors</td>
<td>Ponatinib</td>
<td>0.54</td>
<td>Ephs, Trks, etc.</td>
</tr>
</tbody>
</table>
Figure 4.6. Activation of multiple receptor tyrosine kinases (RTK) in PIK3CA-overexpressing cells.
Figure 4.6. Activation of multiple receptor tyrosine kinases (RTK) in PIK3CA-overexpressing cells. (A.) Chemiluminescent image of RTK protein array in the PIK3CA-overexpressing cells (CU110) and control cells (CUCON) using the Pathscan RTK Signaling Antibody Array Assay Kit. The quantification is shown under the image. (B.) Quantification of RTK protein array in CU110 cells stably transfected with either short hairpin sh-PIK3CA or scramble control (SCR). (C.) Quantification of RTK protein array in CU110 cells treated with either DMSO or a pan-PI3K inhibitor-PX866 for 48 hours to achieve 40% cell growth inhibition.
either DMSO, or ponatinib for 48 hours. Cell were then harvested and viability was determined by Vi-cell cell analyzer, 1 x 10^5 viable CU110 cells after DMSO or ponatinib treatment were injected into the syngeneic C57Bl6 mice. 21 days later lung tissues were harvested from these two groups of mice. As shown in Figure 4.7.2D, CU110 cells generated multiple lung nodules in all four mice injected. In contrast, none of the lungs have visible lung modules in the CU110 cell treated with ponatinib.

To further validate this result in human HNSCC cell lines, we treated UMSCC47 and Fadu cells with PX866, LDN211904, GNF5837 and Ponatinib. In consistent with the results from CU110 cells, the only effective inhibitors on HNSCC sphere formation on either UMSCC47 or FaDu cell lines were LDN211904 (inhibitor on Ephrin receptors), GNF5837 (inhibitor on TrKs), and ponatinib (pan-inhibitor on Ephrin receptors, TrKs and c-Kit) (Figure 4.7.3).

**Discussion**

The major causes of mortality in HNSCC patients are recurrence and metastasis, which are highly associated with EMT and CSC traits as shown in our study^3–5. Studies suggest that HNSCC tumor cells undergo EMT to infiltrate the blood supply and gain CSC properties to survive and generate new tumors either locally or distantly^123. PIK3CA gene amplification is perhaps the most common genetic change in human HNSCC patients^38,40,48. To understand the role of PIK3CA amplification in HNSCC formation and progression, we overexpressed PIK3CA in murine head and neck epithelium, and found that it significantly promoted HNSCC progression, manifested as more migratory and invasive, poorly differentiated,
Figure 4.7.1. Targeting Ephrin receptors, Trks or c-kit effectively reduce CSC population in HNSCC.
Figure 4.7.1. Targeting Ephrin receptors, Trks or c-kit effectively reduce CSC population in HNSCC. (A.) Screening for pharmaceutical inhibitors effectively reducing CSC population in CU110 cells using HNSCC sphere forming assay. The quantification is shown in right. (B.) FACS analysis of CD44 (Left) or ALDH (Right) in CU110 cells treated with LDN211904. (C.) FACS analysis of ALDH in CU110 cells treated with GNF5837. (D.) FACS analysis of CD44 (Left) or SP fraction (Right) in CU110 cells treated with imatinib. All experiments were done by using CU110 cells treated with specified inhibitor for 48 hours to achieve 40% cell growth inhibition.
Figure 4.7.2. Ponatinib, a multi-kinase inhibitor targeting Ephs, Trk and c-kit, effectively eliminates CSC population in HNSCC. (A.) HNSCC sphere forming assay of CU110 cells treated with ponatinib. The quantification is shown in right. Spheres with diameter ≥ 30 microns were counted. (B.) FACS analysis of ALDH in CU110 cells treated with ponatinib. The quantification is shown in right. (C.) FACS analysis of SP fraction in CU110 treated with ponatinib (left). The quantification is shown in right. (D.) Gross pictures of lung tissues harvested from mice received tail vein injection of CU110 cells treated with DMSO or ponatinib. All experiments were done by using CU110 cells treated with specified inhibitor for 48 hours to achieve 40% cell growth inhibition.
Figure 4.7.3. Targeting Ephrin receptors and Trks effectively eliminates CSC population in human HNSCC cell lines. Screening for pharmaceutical inhibitors reducing CSC population in human HNSCC cell lines: Fadu and UMSCC47, using sphere forming assay. The quantification is shown in right. n=3; error bars indicate SD. All experiments were done by using either Fadu or UMSCC47 cells treated with specified inhibitor for 48 hours to achieve 40% cell growth inhibition.
EMTed, and metastatic HNSCC. In this chapter, we focus our studies on understanding the role of PI3K pathway in regulating EMT and CSC traits using both murine and human HNSCC cell lines.

Several studies have shown that PI3K pathway promotes EMT and CSC properties in human cancers. For example, PIK3CA mutation is most frequently seen (nearly 50%) in aggressive breast cancers that display EMT and CSC properties. Breast cancer cell lines engineered with a knocking-in PIK3CA mutation display an EMT phenotype. PI3K signaling is critical for prostate CSC maintenance and targeting PI3K signaling eliminate these CSCs. Additionally, PI3K signaling cooperates with MEK signaling to maintain self-renewal and tumorigenic capacity of glioblastoma CSCs. PI3K signaling also plays a critical role in maintaining colorectal CSCs. In line with these reports, our study presented here shows that overexpression of PIK3CA promotes EMT and CSC phenotype in HNSCC.

However, we surprisingly found that although overexpression of PIK3CA promotes EMT and CSC in HNSCC, knocking down PIK3CA failed to reverse either EMT or CSC phenotypes. Instead, it appeared to promote the CSC population of HNSCC. The PI3K-independent mechanism has also been observed in a PIK3CA-mutatant breast cancer mouse model. Liu et al observed a subset of recurrent breast cancers that no longer responded to PI3K treatment. These findings together with us warrant a precision treatment of using PI3K inhibitors in clinical setting, particularly when using PI3K inhibitors for recurrent or metastatic cancer patients. The ratio of AKT1 and AKT2 has been reported to regulate EMT and CSC traits.
through regulating miR-200s in breast cancer\textsuperscript{208}. In addition, we found that the level of PIK3R1 is increased in the PIK3CA-overexpressing HNSCCs. However, knocking down AKT1, AKT2 or PIK3R1 didn’t reverse EMT and reduce CSC population. Instead, it promoted CSC population in the PIK3CA-overexpressing HNSCCs. Although genetic suppression of PDK1 reversed EMT\textsuperscript{14}, it failed to affect CSC phenotype. Similar to our finding of knocking down PIK3CA, this data raised a concern when targeting AKT or other components in PI3K pathway to treat recurrent or metastatic HNSCC patients.

Although it still remains unknown, we suspect that multiple feedbacks and pathway crosstalk may be one of the explanations for the PI3K-independent mechanism observed in this chapter. One such example is that activation of mTOR initiates a feedback loop that attenuates PI3K/AKT activities in breast cancer cells. The consequence is enhanced PI3K/AKT activity upon mTOR inhibitor treatment\textsuperscript{239}. Another example is in cancer cells bearing mutant RTKs, blocking PI3K signaling resulted in upregulation of Raf-MAPK pathway to confer tumor growth\textsuperscript{40,240,241}. Recently, Pandolfi and colleagues also showed alternative activation of MAPK signaling as a compensation mechanism for the mTOR inhibition\textsuperscript{242}. In these cases, inhibiting one target/pathway resulted in activating another target/pathway to compensate the initial inhibition. In our case, blocking of PI3K signaling may potentially activate another molecule/pathway to maintain the CSC population in the PIK3CA-overexpressing HNSCCs.

In light of these results, we speculate that PI3K or AKT inhibitors may not be effective to treat recurrent or metastatic HNSCC patients. Indeed, a phase II trial of
the anti-mitotic agent, docetaxel with or without the pan PI3K inhibitor PX-866 in patients with relapsed or metastatic HNSCC showed no difference in response rate or overall survival due to the combination treatment with PX-866\textsuperscript{243}. Although PI3K or AKT inhibitors are actively undergone clinical trials in human cancers, understanding the role of this pathway at different stages of tumor development or progression is essential to develop precision treatment on human cancer patients.

To identify these compensatory mechanisms of PI3K-independency, we screened RTKs in this chapter since it is closest signaling potentially interact with PI3K pathway. We found that multiple RTKs were activated in the PIK3CA-overexpressing HNSCCs, with Ephs and Trks signaling the most prominent. Ephs are highly expressed in stem cells and in CSC in breast, ovarian, lung, glioma and melanoma, and are associated with tumor growth and metastasis\textsuperscript{244,245}. Mutations in Trks have also been reported in multiple cancers, including ovarian, colorectal, melanoma, and lung\textsuperscript{246,247}. Recent studies have also shown that TrkA and C overexpression promotes growth and invasion in a breast cancer model\textsuperscript{248,249}. A report also showed that TrkB and C promotes brain tumor-initiating cells\textsuperscript{250}. The role of either Ephs or Trks in maintenance of CSC population has been demonstrated in this chapter. However, how PIK3CA-overexpression activates these two particular pathways in compensating the maintenance of CSC trait in the PIK3CA-overexpressing HNSCC remains investigation. In addition, the potent elimination of CSC population in the PIK3CA-overexpressing HNSCC by ponatinib provide a possibility that co-targeting both pathways may achieve the maximum effects of eliminating CSCs, although it is possible that other pathways in addition to Ephs and
Trk may also be affected by ponatinib treatment. Nonetheless, it seems that ponatinib can be one of candidates for clinical trials to control HNSCC recurrence and metastasis, given that this drug has been approved and used in clinic to treat leukemia patients with well tolerance\textsuperscript{251}.

Taken together, our results show that while PIK3CA overexpression promotes EMT and enriches CSC population in both murine and human HNSCCs, the maintenance of CSC population in HNSCC becomes PI3K pathway-independent. Multiple RTK activation, particularly the Ephs and Trk signaling may be alternatives to maintain CSC population in the PIK3CA-overexpressing HNSCCs. Thus, targeting Ephs and/or Trk pathway may be effective in eliminating the PI3K-independent CSC population, and may be developed into novel therapies to treat recurrent and/or metastatic HNSCC patients, particularly in patients with PIK3CA amplification.
CHAPTER V

INHIBITION OF HISTONE DEACETYLASE ELIMINATES CSC POPULATION THROUGH REVERSION OF EMT IN HNSCC WITH PIK3CA OVEREXPRESSION

Introduction

HNSCCs remain challenging to treat, largely due to cancer recurrence and metastasis. These complications significantly contribute to the poor survival rates for HNSCC patients. Development of effective therapies that treat HNSCC recurrence and metastasis is urgently needed.

As discussed in the previous chapters, we showed that both EMT and CSC traits in the PIK3CA-overexpressing HNSCCs significantly impact cancer progression, which can be regulated by both genetic and epigenetic events. Epigenetic alterations refer to gene expression changes rather than changes of nucleotide sequences and are mainly regulated through both histone modification and DNA methylation. Histone acetylation and deacetylation are the major processes of histone modification to regulate gene expression. Histone deacetylases (HDAC) deacetylates lysine residues to condensate chromatin to suppress transcription, while histone acetyltransferase acetylates and de-condensates chromatin to release transcription. DNA methyltransferase transfers a methyl group to DNA to repress transcription. Epigenetic modulation is associated with the regulation and maintenance of EMT and CSCs. As tumor cells underwent EMT or CSCs frequently possess unique and reversible epigenetic profiles, the enzymes involved in these epigenetic modifications, such as HDAC and DNA
methyltransferases, present possible targets for new anticancer therapies.

In previous chapters, we have shown that PIK3CA overexpression promotes EMT and enriches CSC population in HNSCCs. However, the maintenance of CSC population in these tumors becomes PI3K pathway-independent. We further identified Ephs and/or Trk pathway may be important in maintaining CSC traits in the PIK3CA-overexpressing HNSCCs. To further identify other molecules/pathways responsible for maintaining CSC traits, in this chapter, we performed an mRNA microarray to compare global expression profiles between CSC and non-CSC population isolated from PIK3CA-overexpressing HNSCC cells. Histone deacetylation (HDAC) was one of the pathways differentiated regulated in the CSC population. Targeting this pathway using HDAC inhibitors effectively eliminated CSC population in the PIK3CA-overexpressing HNSCCs, suggesting that HDAC inhibitor may be valuable for treating recurrence and metastasis of HNSCC patients.

Results

Transcriptional Profiling Identified Molecular Pathways Related to PIK3CA Overexpression-induced CSC Properties

As discussed in Chapter IV, compare to monolayer culture, sphere forming in CU110 cells enriches CSC properties by increased expression of embryonic stem cell markers Sox2, Nanog, and Oct4a, and enhanced in-vivo tumorigenicity, and can be used as a platform for CSC study. We thus performed a gene expression array to compare the transcriptional profiles between HNSCC sphere- and monolayer-
cultured cells. As shown in Figure 5.1A, compared with monolayer-cultured CU110 cells, there are 1448 genes that are differentially expressed in sphere-cultured cells, including 712 upregulated and 736 downregulated genes (genes that have a twofold or greater difference in gene expression). Functional cluster analysis using NIH DAVID and Affymetrix Transcriptome Analysis Console identified several pathways potentially related to CSC phenotype, including cell cycle, DNA replication, ESC pluripotency, epigenetic regulation, focal adhesion-mediated RTK signaling, Adar1 editing deficiency immune response, and cytokine regulation (Figure 5.1B). The slowing cell cycle, DNA replication and enhanced pluripotency reflect general characters of quiescent, slow cycling, and pluripotent CSCs. In addition, the changes in epigenetic regulation, particularly the upregulation of HDAC9 draw us a great attention.

Inhibition of Histone Deacetylase Eliminates CSC Population through Reversing EMT

Aberrant regulation of histone deacetylases (HDACs) have been observed in several cancers, and emerging evidence suggests that histone modification may regulate programs related to EMT and CSC phenotype. The general changes of histone modification, particularly the upregulation of HDAC in HNSCC sphere promoted us to test whether interfering histone modification affects CSC phenotype. We thus applied an HDAC inhibitor apicidin, which targets both class I and class II histone deacetylase to the PIK3CA-overexpressing CU110 cells and assessed the HNSCC spheres forming ability upon the apicidin treatment. As shown in Figure
Figure 5.1. Transcriptional profiles of HNSCC sphere- or monolayer-cultured CU110 cells. (A.) Hierarchical cluster analysis of Affymetrix Mouse Gene 2.0 array showing differentially expressed genes in sphere- or monolayer-cultured CU110 cells. (B.) Pathways enriched in sphere-cultured CU110 cells identified by NIH David and Affymetrix Transcriptome Analysis Console. Genes up-regulated (red), or down-regulated (blue) by more than two-fold in each pathway are listed.
5.2A, in contrast with no effect upon PX866 treatment, apicidin treatment completely eliminated HNSCC sphere forming ability of CU110 cells. Apicidin treatment also significantly increased CD24+ non-CSC population, with no significant effect on CD44+ CSC population in CU110 cells. In contrast, PX866 treatment had no effects on CD24 or CD44, populations (Figure 5.2B). To assess whether apicidin treatment is able to reverse EMT, we examined E-cadherin expression upon apicidine treatment. As shown in Figure 5.2C, apicidin treatment on CU110 cells restored E-cadherin expression at both mRNA and protein levels. In contrast, apicidn treatment had no effect on AKT activation (Figure 5.2D), suggesting the ability of eliminating CSC population is likely through reversion of EMT, but not through PI3K/AKT pathway. We further tested several other HDAC inhibitors, i.e Trichostatin A (TSA), Valproic acid (VPA), and Vorinostat (also called SAHA) on HNSCC sphere forming abilities. Similar to the effect of apicidin treatment, treatment of CU110 cells using these HDAC inhibitors also effectively eliminate HNSCC sphere forming abilities (Figure 5.2E). In contrast, treatment with a demethylation agent, 5-azacytidine, didn’t affect HNSCC sphere formation in CU110 cells (Figure 5.2E). These data suggest that instead of DNA methylation, histone modification may play an important role for maintaining CSC phenotype.

To further expand our observation in murine to human HNSCC cells, we treated Fadu and UMSCC47 cells with Apicidin and PX866 and assessed the sphere forming capacities upon treatment. Similar to the results observed in the murine HNSCC cells, apicidin treatment potently eliminated HNSCC spheres while PX866 showed no effect on HNSCC spheres (Figure 5.2F).
Identification of Key Molecular Events Responsible for Anti-CSC Effect upon Treatment of HDAC Inhibitors

The opposite effects on CSC upon treatment by either HDAC inhibitors or PI3K inhibitor PX866 provide an opportunity to identify key molecular events responsible for the potent anti-CSC effect by HDAC inhibitors. We thus performed another microarray analysis on CU110 cells treated by either an HDAC inhibitor, apicidin or a PI3K inhibitor, PX866. We hypothesized that genes exclusively affecting by apicidin, but not by PX866 treatment may be responsible for anti-CSC effect upon treatment of HDAC inhibitor, and we focused on genes exclusively downregulated by apicidin treatment. A total of 1413 and 2857 genes were downregulated by PX866 and apicidin treatment, respectively. Among these genes, 748 were found to be downregulated by either treatment, while 2109 genes were downregulated exclusively by apicidin (Figure 5.3A). Gene ontology analysis revealed these exclusively downregulated genes to be involved in chromatin modification, transcriptional regulation, developmental regulation, regulation of kinase activities, cell mobility, regulation of phosphate metabolic processes, apoptosis, and regulation of cell junctions and focal adhesions (Figure 5.3A). These findings validated effect of apicidin treatment, since the HDAC inhibitor targets multiple cellular functions by altering the chromatin status. From these exclusively downregulated genes by apicidin, we selected 12 top candidate genes which were downregulated the most upon apicidin, but were either upregulated or no significant change (<2 folds) upon PX866 treatment. (Figure 5.3B). To assess the role of individual gene in maintaining CSC phenotype, we stably knocked down each gene
Figure 5.2. HDAC inhibitors reverse EMT and eliminate CSC population in the PIK3CA-overexpressing cells.
Figure 5.2. HDAC inhibitors reverse EMT and eliminate CSC population in the PIK3CA-overexpressing cells. (A.) HNSCC sphere-forming assay in CU110 cells treated with apicidin or PX866 for 48 hours with 40% cell growth inhibition. The quantification is shown in right. Spheres with diameter ≥ 30 microns were counted. (B.) FACS analysis of CD24 (left), or CD44 (right) in CU110 cells treated with apicidin or PX866 with the same method. (C.) qRT-PCR (left) or western blotting (right) of E-cadherin in CU110 cells treated with apicidin with the same method. (D.) Western blotting of AKT and phosphorylated AKT in CU110 cells treated with Apicidin or PX866 with the same method. (E.) HNSCC sphere-forming assay in CU110 cells treated HDAC inhibitors Trichostatin A (TSA), Valproic acid (VPA), and Vorinostat (SAHA) and demethylation agent 5-azacytidine with the same method. The quantification is shown in right. Spheres with diameter ≥ 30 microns were counted. (F.) HNSCC sphere-forming assay in human Fadu and UMSCC47 cell lines treated with apicidin or PX866.
using lentiviral-based shRNA technique. Among the 12 shRNAs targeting for each individual gene, we were able to knock down 9 genes with over 60% efficacy. We then cultured these cells in an ultralow, and serum free condition. As shown in Figure 5.3C, only cells with VCAM1 knocking down were not able to form HNSCC sphere.

Initial experiment showing the effectiveness of eliminating CSC population by knocking down VCAM1 (sh-VCAM1-139) was exciting. However, when we tried to validate this result using another shRNA (sh-VACM1-142) targeting VCAM1, we didn’t observe similar effect of inhibiting sphere forming as sh-VCAM1-139, although the knocking down efficacy in sh-VCAM1-142 was even better than sh-VCAM1-139 (Figure 5.4B). Whether the sh-VCAM1-139 has off-target effect on other unknown gene(s), or the level of VCAM1 is bi-directional in maintaining CSC phenotype needs further investigation.

Discussion

It has been shown that epigenetic regulation controls EMT and stem cell traits in both normal stem cells and CSCs\textsuperscript{171,178,253}. The potent effects of HDAC inhibitors, but not the demethylation agent in abrogating CSC population indicated targeting HDAC pathway is one of the approaches which can be developed into anti-CSC therapy in human cancers. Moreover, the reversion of EMT upon HDAC inhibitor treatment highlighted the close link between EMT and CSC phenotypes in this scenario. In supporting our notion, several publications have showed the effectiveness of HDAC inhibitor in reversing EMT and eliminating CSC population in
Figure 5.3. Screening of key molecular events responsible for anti-CSC effect upon treatment of HDAC inhibitors.
Figure 5.3. Screening of key molecular events responsible for anti-CSC effect upon treatment of HDAC inhibitors. (A.) Upper: Venn diagram revealing changes of global transcriptional profiles upon treatment of a HDAC inhibitor, apicidin, or a PI3K inhibitor PX866 by microarray analysis. Lower: functional clustering analysis using NIH David software. (B.) Top 12 candidate genes downregulated the most upon apicidin, but upregulated or no significant changes (<2 folds) upon PX866 treatment. (C.) HNSCC sphere forming assay in CU110 cells transfected with individual shRNAs as indicated in image. The quantification of HNSCC sphere is shown in right. Spheres with diameter ≥ 30 microns were counted.
Figure 5.4. Validation of VCAM in maintaining CSC phenotype. (A.) HNSCC sphere forming assay in CU110 cells transfected with either sh-VCAM1-139, or sh-VCAM1-142. The quantification of spheres is shown in right bottom. Spheres with diameter ≥ 30 microns were counted. (B.) Western blotting of VCAM1 in CU100 cells transfected with either sh-VCAM1-139, or sh-VCAM1-142. SCR: scramble control; NSC: non-specific sequence control. GAPDH was used as a loading control.
human cancers\textsuperscript{254,255}. pan-HDAC inhibitors have already been approved by the FDA for the treatment of certain cancers, such as cutaneous T-cell lymphoma\textsuperscript{256,257}. However, HDAC inhibitors cause global, nonselective gene changes, which brings the concerns of its low specificity\textsuperscript{258}. To this point, HDAC9 we have identified from microarray analysis becomes an interesting molecule for further investigation. HDAC9 is highly expressed in CSC population and that each of the HDAC inhibitors we tested is able to target HDAC9. This suggests that it is possible that the anti-CSC and EMT-reversing effect of these inhibitors might be at least, partially through targeting HDAC9. Interestingly, it has been recently shown that HDAC9 expression is associated with poor prognosis in breast cancer and in the response to HDAC inhibitors\textsuperscript{259}. In addition to targeting particular HDAC, we also hypothesize that certain molecules/pathways downstream of HADC inhibition may be more selective and effective to eliminate CSC population of HNSCC. We thus screened genes exclusively downregulated upon HDAC inhibitor treatment. However, of the top eight candidate genes we screened, genetic knockdown did not reduce CSC population of HNSCC. Although there is still possibility that other key molecule(s) we haven’t screened might be the essential “linchpin” gene, it is also possible that the anti-CSC effect of HDAC inhibitor may be mediated through multiple molecules/pathways.

Taken together, the data in this chapter demonstrated the connection between EMT and CSC traits in HNSCC, and highlighted effectiveness of HDAC inhibition in eliminating CSC population. Further investigation on particular isoforms of HDAC, such as HDAC9 identified in this chapter, or downstream molecule(s)/pathway(s) mediating the function of HDAC inhibition may led to
development of more specific and low toxic therapies for treatment of HNSCC recurrence and metastasis.
CHAPTER VI
IDENTIFICATION AND VALIDATION OF MICRORNAS REGULATING CSC PHENOTYPE IN THE PIK3CA-OVEREXPRESSING HNSCC

Introduction

Previous chapters showed that PIK3CA overexpression promotes EMT and enriches CSC population. However, the maintenance of CSC traits is no longer dependent on PI3K pathway. By using RTK protein array and mRNA microarray, we found that targeting Ephs and/Trk pathway, or histone deacetylation effectively eliminated CSC population in the PIK3CA-overexpressing HNSCCs. In this chapter, we hypothesize that certain microRNAs are essential to such maintenance of CSC population and modulating these microRNA expression may be effective to eliminate CSC population.

MicroRNAs (miRNAs) are short non-coding RNAs, roughly 20-24 nucleotides in length, which regulate mRNA expression. miRNAs participate in multiple signaling cascades, such as differentiation, proliferation, apoptosis, and metabolism. Dysregulation of miRNA expression has been linked to tumorigenesis and dysregulated miRNAs may function as either tumor suppressors or oncogenes. For example, miR-17-92 cluster promotes tumorigenesis by promoting proliferation of cancer cells and deletion of the complete miR-17-92 cluster slows down Myc-induced oncogenesis\textsuperscript{183}. miR-21 overexpression is observed in breast cancer and is associated with advanced stage, lymph node metastasis and poor prognosis\textsuperscript{184}; knockdown of miR-21 in breast cancer cells inhibits proliferation, migration, invasion
and in vivo tumor growth\textsuperscript{185}. miR-200 family miRNAs function as tumor suppressors and loss of these miRNAs promotes EMT and metastasis\textsuperscript{186}. CSCs, which are linked to cancer recurrence and metastasis, are also regulated by miRNAs in oncogenic conditions. For example, overexpression of miR-34 reduced CSCs in pancreatic cancer\textsuperscript{260} and miR200 family microRNAs were significantly lost in breast CSCs\textsuperscript{261}.

To identify microRNAs that are essential for the maintenance of CSC population in the PIK3CA-overexpressing HNSCCs, we performed a microRNA array to compare the differential expression between CSC population and non-CSC population isolated from PIK3CA-overexpressing HNSCCs. Among the candidates we screened, we validated the differential expression of miR18a-5p, miR182-5p, miR199a-5p and miR199a-3p in CSC population by qRT-PCR. Our preliminary study further found that introducing miR18a-5p mimics or miR199a-5p inhibitor reduced CSC population, suggesting these two microRNAs may be important to maintain the CSC phenotype in the PIK3CA-overexpressing HNSCCs. Further investigation of downstream target genes regulated by these two microRNAs is undergoing.

Results

Identification and Validation of MicroRNAs Differentially Expressed in CSC Population Isolated from the PIK3CA-overexpressing HNSCCs

microRNA (miRNAs) have been reported as important regulators of CSCs\textsuperscript{182,262}. In light of this, we postulated that PIK3CA overexpression-induced CSC population may distinguish its non-CSC progeny with a unique miRNA expression. In
Chapter V, we discussed that enhanced expression of Sox2, Nanog, and Oct4a was evident in the spheres formed by CU110 cells. In addition, enriched SOX2+ cells were identified in the spheres, strongly suggesting that spheres enrich CSC populations. Based on this, we performed Affymetrix miRNA Array analysis to compare the differential expression of miRNAs between HNSCC sphere and monolayer-growing CU110 cells. For this analysis, there were 143 miRNAs found to be differentially expressed (greater than two folds) in HNSCC sphere compared with monolayer-growing cells, including 81 upregulated and 62 downregulated miRNAs (Figure 6.1). We selected 18 highly differentially expressed miRNAs as candidates for further validation. (Figure 6.2A). qRT-PCR confirmed the differential expression results of these candidate miRNAs from miRNA array (Figure 6.2B). We then selected 4 microRNAs (miR-18a-5p, miR-182-5p, miR-199a-3p and miR-199a-5p) for further functional analysis.

**Modulation of miR-18a-5p and miR-199a-5p Expression Interfered CSC Population Isolated from the PIK3CA-overexpressing HNSCCs**

Among the four miRNAs we selected, miR18a-5p and miR182-5p were downregulated while miR199a-5p and miR199-3p were upregulated in HNSCC sphere. We then transfected miRNA mimics of miR185-5p and miR182-5p, and miRNA inhibitors of miR199a-5p and miR199a-3p into CU110 cells and performed HNSCC sphere formation assay subsequently. As shown in Figure 6.3A, while introducing miR182-5p or miR199a-3p inhibitor barely affected HNC sphere forming abilities, introducing miR18a-5p or miR199a-5p inhibitor significantly reduced
Figure 6.1. Identification and validation of microRNAs (miRNAs) differentially expressed in HNSCC sphere of CU110 cells. (A.) miRNA profile of HNC sphere- or monolayer-growing Cu110 cells using affymetrix microRNA microarray 4.0 chips. Left panel: hierarchical clustering of miRNA profile. Right panel: list of miRNA candidates for further validation; linear fold change was calculated by using bi-weight average signal (Log2) from HNSCC sphere- or monolayer-growing Cu110 cells. (B.) Validation of miRNA candidate expression in HNSCC sphere- or monolayer-growing Cu110 cells by qRT-PCR. The experiments were performed in triplicate and data are presented as mean ± SD.
Figure 6.2. Modulation of miR-18a-5p and miR-199a-5p expression interfered HNSCC sphere formation in Cu110 cells. (A.) HNSCC sphere forming assay in Cu110 cells transfected with miR-18a-5p mimic, miR-182-5p mimic, miR-199a-3p inhibitor or miR-199a-5p inhibitor respectively. The quantification of sphere is shown in right. Spheres with diameter ≥ 30 microns were counted. (B.) Target genes of miR18a-5p or miR-199a-5p and relevant implication in cancers; target genes was identified by using TargetScan.
HNSCC sphere forming abilities, suggesting the important role of miR18a-5p and miR199a-5p in maintaining the CSC phenotype in the PIK3CA-overexpressing HNSCC.

We are currently utilizing other CSC markers, and performing similar experiments in human HNSCC cell lines. In addition, we used TargetScan database to predict the potential target genes regulated by these two miRNAs (Figure 6.3B). For the miR-18a-5p, there are 248 predicated transcripts with conserved sites. Some of the target genes have been implicated in promoting cancer progression\textsuperscript{263–266}. For example, NEDD9 promotes lung cancer metastasis through promoting EMT phenotypes\textsuperscript{263}. For the miR-199a-5p, there are 516 predicated transcripts with conserved sites. In contrast to the miR-18a-5p, miR199a-5p potentially targets multiple tumor suppressor such as Sun1\textsuperscript{267–269}.

**Discussion**

Emerging evidence suggest miRNAs as key regulators for CSCs which has been implicated in cancer recurrence and metastasis. In HNSCC, reduced expression of Let-7 miRNA family members contributes to oncogenesis. Let-7a inhibits CSC-like properties in developing tumor populations\textsuperscript{188} and Let-7d suppresses EMT in oral squamous cell carcinoma cell lines\textsuperscript{187}. These data support our hypothesis that deregulation of miRNA in HNSCC contributes to the acquisition of CSC-like properties. Similar to these findings, results from our study suggest that miR-18a-5p and miR-199a-5p may be essential for maintaining CSC population in the PIK3CA overexpression HNSCCs. Although studies involving miR-18a-5p in
cancer are very limited, it has been shown that upregulated miR-199a-5p functions as an oncomir in different cancers\textsuperscript{270,271}. TargetScan analysis revealed that miR-18a-5p targets multiple oncogenes such as CTGF, ORAI3, NEDD9 and IRF2. CTGF has been shown to activate pluripotency genes and EMT program in head and neck cancer cells\textsuperscript{264} and ORAI3, NEDD9 and IRF2 have been implicated to promote proliferation, migration and invasion in different cancers\textsuperscript{263,265,266}. Thus, miR-18a-5p may regulate CSC state through targeting multiple downstream oncogenes and could be considered as a tumor suppressor for CSC. In contrast to the miR-18a-5p, miR-199a-5p may function as an oncomirs as it targets multiple tumor suppressors such as Klotho and SULF1. Klotho has been shown as a tumor suppressor by inhibiting insulin/IGF1, p53/p21, and Wnt signaling\textsuperscript{267} and SULF1 negatively modulate many growth factor induced oncogenic signaling\textsuperscript{272}.

Our current data on miRNAs is still preliminary, in addition to further validate the role of miR18a-5p and miR199-5p in maintenance of CSC population in human HNSCCs, we will study the downstream molecules directly regulated by these two miRNAs both in murine and human HNSCCs. Besides, we will determine how these candidate miRNA expression levels correlate with patient prognosis using a large cohort of HNSCC samples or through available bioinformatics databases. These experiments may lay the foundation for novel therapies for CSC in HNSCC.

Directly utilizing miRNAs as therapy has been tested both in academy and industry. Currently, modified miRNA inhibitors or mimics such as locked nucleic acids (LNAs) -based therapeutic agents\textsuperscript{273}, or packaged with nanoparticles\textsuperscript{274–276} have been tested successfully in multiple experiments and are currently being
assessed for clinical purposes\textsuperscript{273}. For example, miR-34 mimics as a therapeutic against primary and metastatic liver cancer has been conducted in phase I clinical trial and no harmful events were observed\textsuperscript{276}. In addition, phase II trial of microRNA-based anti-HCV therapy-miravirsen has been successfully completed and patients who received miravirsen showed a dose-dependent reduction in HCV levels without major adverse events\textsuperscript{277}. These promising results highlight miRNA therapy as an efficient, specific and safe approaches against various pathological conditions. In the future, we hope that miRNA-based therapies may also be able to directly eliminate CSC population, and to develop as one of the novel treatments for HNSCC recurrence and metastasis.
CHAPTER VII

DISCUSSION

The central theme of this thesis is to understand role of PIK3CA amplification, the most common genetic changes in human HNSCC patients in progression of this devastating disease using in vivo, in vitro and clinical samples approaches.

In chapter III of this thesis, I presented our study on in vivo role of PIK3CA overexpression in HNSCC progression by generating and characterizing a head-and-neck specific genetically engineered mouse model (GEMM) in which PIK3CA is overexpressed. Although overexpression of PIK3CA in murine head and neck epithelium didn’t cause tumor formation spontaneously, overexpression of PIK3CA significantly promoted tumor progression through increased PDK1 and enhanced TGFβ signaling. Examination of human HNSCC clinical samples validated the finding from the PIK3CA-GEMM. Further characterization of the PIK3CA-overexpesing mouse tumor tissues revealed that these tumors underwent changes of epithelial-to-mesenchymal transition (EMT) and de-differentiation, and gained cancer stem cells (CSC) property. In Chapter IV, I further studied the role of PIK3CA overexpression in EMT and CSCs phenotypes by using both murine HNSCC cells derived from the PIK3CA-GEMM tumor tissues and human HNSCC cell lines. Similar to the findings from the PIK3CA-GEMM tumor tissues in vivo, we found that overexpression of PIK3CA promoted EMT and CSC phenotypes in vitro. However, knocking down PIK3CA or key components in PI3K pathway failed to reduce, but rather enriched the CSC population in the PIK3CA-overexpressing tumors. We thus applied three different approaches to identify and validate molecule(s) or pathway(s)
responsible for maintaining the CSC population in the PIK3CA-overexpressing HNSCCs. In chapter IV, we utilized a receptor tyrosine kinase a protein (RTK) array to identify and validate that targeting multiple RTK, with Ephrin receptor and Trk the most prominent, effectively eliminate CSC population in the PIK3CA-overexpressing HNSCCs. In Chapter V, we utilized a mRNA microarray to identify and validate that targeting histone deacetylation (HDAC) potently eliminate CSC population in the PIK3CA-overexpressing HNSCCs. In Chapter VI, we utilized a microRNA (miRNAs) microarray to identify a few candidate miRNAs, e.g. miR18a-5p or miR199a-5p potentially involved in the maintenance of CSC phenotype in the PIK3CA-overexpressing HNSCCs. The major discoveries of my thesis are summarized in Figure 7.1.

**Novel Mechanisms Underlying PIK3CA Overexpression-driven HNSCC Progression**

**Increased PDK1 and Enhanced TGFβ Signaling Drive PIK3CA Overexpression – mediated HNSCC Progression**

One of the major findings in chapter III is that instead of AKT, increased PDK1 expression level and activation contribute significantly to HNSCC invasion and metastasis. Although AKT is taken as the dominant mediator of oncogenic PI3K signaling, we didn’t observe differences in AKT activation or expression of AKT isoforms between control and PIK3CA-overpressing tumors. This data suggest that the link between PI3K and AKT is uncoupled in this scenario. In support this, it has
Figure 7.1. Summary of major findings from chapter 3 to 6 of this thesis.
been shown that knock-in of hyper active AKT mutant in breast epithelium cells could not recapitulate oncogenic PIK3CA mutation\textsuperscript{44}. In addition, AKT activation has also been moderately detected in subset of PIK3CA-mutated breast cancer\textsuperscript{42}. Interestingly, PDK1, but not AKT is highly active in these breast cancers bearing PIK3CA mutations\textsuperscript{42}. Consistent with this finding, in our model system, PDK1, rather than AKT, has been identified to facilitate PIK3CA overexpression-driven HNSCC progression.

PDK1 is a serine/threonine protein kinase that phosphorylates members of the AGC kinase superfamily, including AKT. Although PDK1 has been implicated in cell proliferation, survival and metabolism\textsuperscript{220}, less is known about its role in cancers. Recent studies indicate that PDK1 regulates cell migration in lymphocytes\textsuperscript{92} and induces EMT in cardiac development\textsuperscript{93}, suggesting that dysregulation of PDK1 could be a potential oncogenic driver for cancers. Indeed, overexpression of PDK1 has been observed in human cancers\textsuperscript{67,94,96}; PDK1 expression also correlated with pancreatic cancer and melanoma progression\textsuperscript{67,96}. Emerging evidence suggest that the role of PDK1 in regulating multiple cancerous phenotypes is not limited to activating the AKT. Novel downstream targets of PDK1 include SGK3\textsuperscript{42}, PLC\textgreek{1}\textsuperscript{223}, and polo-like kinase 1\textsuperscript{224}. For example, PDK1 directly activates PLC\textgreek{1} to mediate cancer cell invasion and metastasis\textsuperscript{223}.

In Chapter III, we also found that overexpression of PIK3CA enhanced TGF\beta signaling, including elevated production of TGF\beta1 ligand and increased Smad3 expression and activation. In the study, knocking down of PIK3CA attenuated TGF\beta1 ligand production and reduced activation of its downstream mediator Smad3,
suggesting that the PIK3CA-mediated regulation of TGFβ signaling is in a cell autonomous manner. In addition, pharmacological inhibition of TGFβ signaling attenuated tumor invasion and metastasis, highlighting the critical role of TGFβ signaling in mediating PIK3CA overexpression-driven HNSCC progression. TGFβ signaling and PI3K/AKT pathway are tightly linked and frequently co-activated during tumor progression\textsuperscript{103,105}. Recent studies have revealed multiple insights into cross talk between PI3K and TGFβ signaling. For instance, PI3K/AKT-regulated signaling is able to mediate TGFβ receptor-activated signaling at multiple levels\textsuperscript{225}. Interfering with PI3K/AKT pathway attenuated TGFβ-induced invasive phenotype and reversed EMT\textsuperscript{278}. Although crosstalk between PI3K/AKT and TGFβ/Smad3 signaling is evident, less is known for the interplay between PI3K/PDK1 axis and TGFβ/Smad3 signaling. It will be interesting to see whether PI3K/PDK1 signaling interplays with TGFβ/sm ad3 signaling and how PI3K/PDK1 drives oncogenic TGFβ/Smad3 signaling during the HNSCC progression.

In our model system, overexpression of PIK3CA activates PDK1 and enhances TGFβ signaling to confer HNSCC invasion and metastasis, suggesting therapeutic targeting of PDK1 and/or TGFβ signaling may effectively control HNSCC progression. As the downstream molecular mechanisms underlying PI3K/PDK1 signaling is unknown. It is critical to investigate these mechanisms as outcomes from these studies would provide insights into PIK3CA overexpression-driven HNSCC progression and identify novel targets for better therapeutics.
PIK3CA Overexpression Promotes EMT and CSC Properties to Drive HNSCC Progression

In Chapter III and IV, we found that overexpression of PIK3CA promoted EMT phenotype and enriched CSC population of HNSCC both in vivo and in vitro. EMT is an orderly and polygenic process that contributes to tumor invasion and metastasis. It has been shown that PI3K signaling interacts with Twist or Snail transcriptional factors to regulate EMT in many different cancer types. PI3K also directly/indirectly cooperates with other signaling such as TGFβ, NF-κB, Ras and Wnt/β-catenin to induce and maintain the EMT. These studies disclose the complexity of PI3K-mediated EMT mechanisms. CSCs are subsets of cancer cells that possess traits of normal stem cells such as self-renewal activity and mediate tumor initiation and progression. Several reports recently suggested a link between PI3K signaling and CSCs. For example, PI3K signaling is critical for prostate CSCs maintenance and targeting PI3K signaling eliminate these CSCs. PI3K signaling cooperates with MEK signaling to maintain self-renewal and tumorigenic capacity of glioblastoma CSCs. PI3K signaling also play critical role in maintaining ALDH+ colorectal CSCs. However, It has also been shown that inhibition of PI3K/mTOR pathway induces the generation of gastrointestinal and liver CSCs. These studies suggest that PI3K may have a context/tissue-specific role in regulation of CSCs. Indeed, we found that overexpression of PIK3CA is necessary to confer EMT and CSC characteristics in HNSCC, but is required as a maintainer of these phenotypes. In addition, knocking down of PIK3CA or major components of PI3K signaling such as PIK3R1 or AKT1/2, or treatment with PI3K inhibitor appeared to
promote the CSC-like phenotypes in our model system. This finding further support the notion that PI3K pathway is not required for maintaining CSC phenotype in the PIK3CA-overexpressing HNSCCs.

Failure of affecting EMT and CSC traits by interfering of PI3K signaling could be explained by two reasons. One explanation is that inhibition of PI3K signaling may activate compensatory pathways that lead to the maintenance of the observed EMT and CSC characteristics. Compensatory feedback loops is evident in PI3K signaling. One example is that activation of mTOR in some breast cancer cells initiates a feedback loop that attenuates PI3K/AKT activities. So by treating these tumors with mTOR inhibitors, the cumulative effect is an increase in the activity of PI3K/AKT that enhances tumor growth\textsuperscript{239}. Another example is Ras that activates both Raf-MAPK and PI3K pathways. In this case, inhibiting the PI3K pathway is compensated by an increase in Raf-MAPK signaling that actually drives tumor growth\textsuperscript{240,241}. In both these cases, inhibiting the target to stop tumor growth resulted in the opposite of desired effects highlighting the complexity and inter-connectively of feedback loops and crosstalk between PI3K and other signaling. In our study, blocking of PI3K signaling may potentially activate other alternative pathways to confer the PIK3CA overexpression-induced EMT and CSC traits. The other possibility is that some PI3K-independent mechanisms may solely regulate PIK3CA overexpression-induced EMT and CSC properties. In this case, hyperactive PI3K signaling may only function as an early trigger for activating other pathways to maintain EMT and CSC traits.
PI3k-independent Mechanisms that Maintain PIK3CA Overexpression – induced EMT and CSC Traits

In Chapter IV, V and VI, we reported identification and validation of alternative molecule(s)/pathway(s) responsible for the CSC phenotype which becomes PI3K-independent. Such examples were recently reported in other human malignancies. For example, using a PIK3CA-mutant GEMM for breast cancer, Liu et al found that the recurrence of the PIK3CA-driven breast cancer became PIK3CA-independent. In this study, subsets of PIK3CA-driven mammary tumors show resistance to PI3K inhibition and c-MYC take over the oncogenic role\(^{210}\). In another example of a BCR-ABL-driven chronic myeloid leukemia study, CSC subset never acquires addiction to the BCR-ABL fusion that drives disease development\(^{287}\). These studies, along with our findings strongly suggest that oncogene-driven tumors may not always rely on the same oncogene or related pathways for survival advantages.

In this thesis, three approaches have been used to identify the potential target(s)/pathway(s) responsible for the PI3K-independent CSC phenotype. We first utilized a RTK protein array to compare PIK3CA-overexpressing HNSCCs and control HNSCCs. Our results showed that multiple oncogenic RTKs were activated by overexpressing PIK3CA. Since PI3K is an important signaling node that interacts with other cellular pathways, it is not surprising to observe that multiple RTKs were activated upon overexpression of PIK3CA. In line of this observation, knocking-in of PIK3CA mutant in mouse models of human cancers or cancer cell lines also result in additional mutations in several other genes and activate alternative pathways along the cancerous progression\(^{288–291}\).
Utilization of pharmaceutical inhibitors targeting RTKs identified that ephrin receptors (Ephs) and Trk activation are the prominent effectors for maintaining the CSC phenotype in the PIK3CA-overexpressing HNSCCs. Indeed, Ephs are highly expressed in stem cells and in CSC in breast, ovarian, lung, glioma and melanoma, and are associated with tumor growth and metastasis\textsuperscript{244,245}. Not much is known about the Trk receptors in stem cell and CSC biology. Although mutations in Trk receptors have also been reported in multiple cancers, including ovarian, colorectal, melanoma, and lung\textsuperscript{246,247,292}. Recent studies have also shown that TrkA and C overexpression promotes growth and invasion in breast cancer model\textsuperscript{248,249}. A report also showed that TrkB and C are implicated in regulating brain tumor initiating cells\textsuperscript{250}. For HNSCC it was recently shown that over-expression of TrkB resulted in altered expression of molecular mediators of EMT, including down regulation of E-cadherin and up-regulation of Twist\textsuperscript{293}. This report was also confirmed in a murine model that reduced expression of TrkB suppressed tumor growth\textsuperscript{293}. It still remains unknown how PIK3CA overexpression activates Ephs or Trk pathways. Further investigation on the molecular mechanisms underlying PI3K and Ephs or Trk activations will provide important insights into PI3K-driven HNSCC progression and identify novel therapeutic targets to control HNSCC progression.

The second approach we used to identify potential target(s)/pathway(s) responsible for the PI3K-independent CSC phenotype was to compare the global expression profiles between CSC population and non-CSC population isolated from the PIK3CA-overexpressing HNSCC cells by mRNA microarray analysis. Several pathways were identified in the CSC population including cell cycle, DNA replication,
ESC pluripotency, epigenetic regulation, focal adhesion-mediated RTK signaling, Adar1 editing deficiency immune response, and cytokine regulation. The slowing cell cycle, DNA replication and enhanced pluripotency reflect general characters of quiescent, slow cycling, and pluripotent CSCs. Aberrant regulation of histone deacetylases (HDACs) have been observed in several cancers, and emerging evidence suggests that histone modification may regulate programs related to EMT and CSC phenotype. In the study, we found that targeting epigenetic regulation by HDAC inhibitors reversed PIK3CA overexpression-induced EMT and eliminated CSC population. In contrast, demethylating agent 5-azacytidine didn’t affect the EMT and CSC phenotypes in the PIK3CA-overexpressing HNSCCs, suggesting that the epigenetic factors involved in maintaining PIK3CA overexpression-induced EMT and CSCs are those in histone modification rather than DNA methylation. Indeed, it has been recently shown that HDAC inhibition impedes EMT, decreases the CSC population and halts clonogenic sphere formation in human cancers.

While broad-spectrum HDAC inhibitors have already been approved by the FDA for the treatment of T-cell lymphoma, the mechanisms of action of these drugs are not yet fully understood. HDAC inhibitors cause global, nonselective gene changes, which brings the concerns of its low specificity. Determining which specific pathways are involved in the HDAC inhibition’s effect on affecting PIK3CA overexpression-induced EMT and CSCs could lead to identify more specific and effective therapeutic targets. To this end, we determined genes affected by HDAC inhibition by using mRNA microarray analysis. This analysis found that 2109 genes
were exclusively downregulated by Apicidin treatment and that can be grouped into multiple functional clusters such as chromatin modification, transcriptional regulation, developmental regulation, regulation of kinase activities, cell mobility, regulation of phosphate metabolic processes, apoptosis, and regulation of cell junctions and focal adhesions. This finding, once again highlighted the multiple effects of the HDAC inhibitor. Unfortunately, targeting each candidate genes by a shRNA approach failed to abrogate CSC population in the PIK3CA-overexpressing HNSCCs, although initial experiment showed some promising result of targeting VCAM molecule. These data suggest that there is a possibility that a combinatorial effect targeting multiple molecules might be effective although we cannot rule out the possibility of the mechanism involving a single essential “linchpin” gene. Future studies into the roles of these identified genes and their relevant pathways may yield novel specific therapies for targeting CSC population in the PIK3CA overexpressing HNSCCs.

Emerging evidence suggest certain microRNAs may regulate CSCs in human cancers. For example, overexpression of miR-34 reduced CSCs in pancreatic cancer\textsuperscript{260} and miR200 family microRNAs were significantly lost in breast CSCs\textsuperscript{186,295}. Thus, the third approach we used to identify potential target(s)/pathway(s) responsible for the PI3K-independent CSC phenotype was to compare the global expression profiles between CSC population and non-CSC population isolated from the PIK3CA-overexpressing HNSCC cells. Using microRNA microarray analysis, we identified over 100 miRNAs were differentially expressed in CSC-enriched spheroid cells compared with monolayer-growing non-CSC cells. Of these differentially expressed miRNAs, introducing miR-18a-5p mimic or miR-199a-5p inhibitor into
CU110 cells significantly reduced sphere-forming capacity. These data suggest a suppressive role for miR-18a-5p and a promoting role for miR-199a-5p in maintaining PIK3CA overexpression-induced CSC state. Although there are few studies involving miR-18a-5p in cancer, it has been shown that upregulated miR-199a-5p functions as an oncomir in gastric cancer\textsuperscript{270}. In addition, miR-18a-5p targets multiple oncogenes such as CTGF, ORAI3, NEDD9 and IRF2\textsuperscript{263–266} while miR-199a-5p targets multiple tumor suppressors such as Klotho and SULF1\textsuperscript{267,272}. Further investigation of the potential target genes regulated by these miRNAs is ongoing. These experiments may lay the foundation for identifying miRNAs and targets to improve current therapy for HNSCCs.

Interestingly, relevant works present in Chapter V revealed that reversing EMT program by HDAC inhibitor reduces CSC properties, which indicates that EMT and CSC properties are tightly linked process. However, relevant data from Chapter IV also indicate that attenuation of CSC properties is not necessarily associated with reversing of EMT program, which suggest that EMT and acquisition of CSC properties can be also uncoupled processes. Although different groups have recently revealed that acquisition of CSC properties by activating stemness-related signaling pathways such Wnt, has also been involved in some aspects of the EMT program\textsuperscript{296–298}. Other groups also have indicated that EMT can suppress major attributes of stem cell properties\textsuperscript{299}. Indeed, overexpression of EMT-related transcription factors in CSC-enriched populations induced EMT phenotypes, but suppresses their CSC properties such as self-renewal and metastatic phenotypes\textsuperscript{300}. Conversely, knockdown of EMT-related transcription factors in a mesenchymal-like
cancer cell population induces epithelial phenotype and properties of CSC\textsuperscript{300}. These data suggest the sophistication and complexity of the cross-talk between EMT/MET and stemness and its relevance in cancer progression and metastasis. PIK3CA overexpression-induced EMT process clearly contributes to the acquisition of CSC properties. However, whether the acquired CSC properties still depends on the EMT left a question that necessitates further studies.

**Novel Therapeutic Strategies for Treating HNSCC with PIK3CA Alteration**

Currently, the only Food and Drug Administration-approved targeted therapy for HNSCC is EGFR inhibitors\textsuperscript{33}. However, resistance, recurrence and metastasis still remain the major factors for treatment failure of HNSCC patients\textsuperscript{196,197}. For example, RTK inhibitors such as gefitinib and erlotinib are extensively studied for treating recurrent and/or metastatic HNSCC. Unfortunately, both drugs were barely effective for controlling HNSCC progression and gefitinib doesn't improve survival rates over those of methotrexate\textsuperscript{201}. As PIK3CA appears to be the most frequently altered oncogene in HNSSC, PI3K-targeted therapy are currently evaluated in clinical trials as either monotherapy or in combination with radiation and/or chemotherapy regimens in patients with HNSCC\textsuperscript{301}. Although there is a biologic basis for the use of PI3K inhibitors to treat HNSCC, the efficacy of these agents is less satisfactory. For example, in a randomized phase II trial in recurrent and/or metastatic HNSCC patients, the pan -PI3K inhibitor PX866 was administered with cetuximab, but it did not show any clinically benefit\textsuperscript{302}. In addition, a phase II trial of the anti-mitotic agent, docetxel with or without the PX866 in patients with relapsed or
metastatic HNSCC showed no difference in response rate or overall survival upon the combination treatment\(^{243}\). Given the lack of effective treatments for HNSCC and the rising incidence of disease, there is an urgent need for novel therapies particularly effective in controlling HNSCC recurrence and metastasis.

In our study, one of the promising targets to control HNSCC progression is PDK1. In supporting this, genetic or pharmacological suppression of PDK1 showed to reduce CSCs and metastasis in several human cancers\(^{96,224,226}\). In addition, targeting PDK1 by pharmaceutical inhibitors has been shown to be effective to reduce tumor size of HNSCC\(^{222}\). The other potential target is TGF\(\beta\) signaling. Although TGF\(\beta\)-targeted therapy has been evaluated for a decade in multiple cancers, it remains unclear how it may be efficiently used\(^{227}\). TGF\(\beta\)-targeted therapies are highly stage- and patient-specific. Thus, identifying appropriate patients by using stage-wise biomarkers are critical for the success of such therapies. As both activated PDK1 and enhanced TGF\(\beta\) signaling are concurrent in our model system, combinatorial targeting of PDK1 and TGF\(\beta\) may effectively control PIK3CA-driven HNSCC progression.

In our study, we found that inhibition of PI3K pathway either genetically or pharmaceutically failed to reduce but rather promote the CSC population in the PIK3CA-overexpressing HNSCC. This finding raised a question about how to utilize PI3K inhibitors for clinical management of HNSCC patients. Although PI3K inhibitors are still remain a promising targeted therapy, our findings questioned the effectiveness of this treatment for controlling recurrence and metastatic HNSCC patients. Instead, multiple RTK activation, particularly the Ephs and Trk pathways we
identified suggest that targeting Ephs and/or TrK may be effective to control recurrence and metastasis of HNSCC patients. Although using pharmaceutical inhibitors targeting either Ephs or TrK were effective in reducing CSC population, this is further exemplified by the potent abrogation of CSC population using ponatinib. Ponatinib has been approved by FDA for the treatment of adults with chronic myeloid leukemia and acute lymphoid leukemia with well tolerance. Another potential therapeutic drug is HDAC inhibitors, which are currently actively developed for multiple clinical trials with vorinostat and romidepsin are approved by FDA for treating refractory cutaneous T-cell lymphoma. Thus, our data provide experimental evidence of applying ponatinib or HDAC inhibitors for clinical trials to treat HNSCC progression, particularly to treat recurrent or metastatic HNSCC patients with PIK3CA amplification.
REFERENCES


166


http://dx.doi.org/101056/NEJMo071028. 2009.


199. Takeuchi K, Ito F. Receptor tyrosine kinases and targeted cancer therapeutics. 


