The Pennsylvania State University

The Graduate School

College of Medicine

COOPERATION OF P120-CATENIN DOWNREGULATION AND PIK3CA MUTATIONS IN PROGRESSION OF HEAD AND NECK SQUAMOUS CELL CARCINOMA

A Dissertation in

Biomedical Science

by

Michal A. Kidacki

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

August 2017

The dissertation of Michal A. Kidacki was reviewed and approved* by the following:

Douglas B. Stairs
Assistant Professor of Pathology, Pharmacology, and Biochemistry & Molecular Biology
Medical Director, Morphologic and Molecular Core Research Laboratory
Dissertation Advisor
Chair of Committee

Edward J. Gunther
Professor of Medicine

Diane Thiboutot
Professor of Dermatology

Lisa M. Shantz
Associate Professor of Cellular & Molecular Physiology

Todd D. Schell
Professor of Microbiology and Immunology

Ralph L. Keil
Chair, Biomedical Sciences Graduate Program
Associate Professor of Biochemistry and Molecular Biology

*Signatures are on file in the Graduate School

Abstract

Head and neck squamous cell carcinoma (HNSCC) is a highly invasive cancer diagnosed in over half a million people around the world every year. This deadly disease originates from squamous epithelia in the head and neck region and is associated with tobacco smoking, alcohol consumption, and Human Papilloma Virus (HPV) and Epstein Barr Virus (EBV) infections. HNSCC develops due to many different genetic aberrations. Loss of p120-catenin (P120CTN), a tumor suppressor, is one of such events. P120CTN is downregulated or lost in many cancers of epithelial origin. This loss frequently correlates with decreased HNSCC patient survival. In addition to P120CTN dysregulation, *PIK3CA* mutations are often found in HNSCC. For the scope of this dissertation we demonstrate two approaches to studying HNSCC. Our first approach focuses on a P120CTN-null mouse model and the immune milieu, while our second approach focuses on the interactions of P120CTN downregulation with the most common oncogene found in HNSCC, mutant PIK3CA. We demonstrate that activating PIK3CA mutations cooperate with P120CTN in promoting cell migration and invasion.

Deletion of P120CTN in the mouse oral and esophageal epithelium leads to formation of squamous tumors with a concomitant increase in Myeloid-derived Suppressor Cells (MDSCs). We have investigated whether a specific subset of MDSCs is upregulated in the P120CTN-null mice. Although we observed several

trends in our results, we did not identify any significant differences in the subset frequency between P120CTN-null and P120CTN-wild-type mice. We have also studied the effects of epithelial cells on MDSCs *in vitro*. P120CTN-null epithelial cells did not increase MDSC survival, activation or directional migration compared to P120CTN-wild type cells. Finally, we have assayed MDSC counts from P120CTN-wild-type and P120CTN-null tissues. Previous studies determined that P120CTN-null organs experience an increase in MDSCs. We have determined that this increase is systemic and can be seen in all of the tissues of a P120CTN-null mouse.

We have also investigated the cooperation of P120CTN with the most common oncogene found in HNSCC, *PIK3CA*. We have utilized two different activating mutations of PIK3CA, E545K and H1047R, to investigate the cooperation of PIK3CA and P120CTN on anchorage-independent growth, migration, and invasion. We performed our experiments using two cell lines, normal oral keratinocytes (NOK) and normal esophageal keratinocytes (EPC1). We used cell lines that are as close as possible to normal cells to limit interference from other mutations. Additionally, we have confirmed the findings from our normal oral keratinocyte cell line in a normal esophageal keratinocyte cell lines, the most closely related keratinocyte cell line not derived from tumor samples. We have discovered that P120CTN downregulation and *PIK3CA* mutations cooperate to induce migration and invasion, while only minimally

increasing the anchorage-independent growth. P120CTN downregulation and *PIK3CA* mutations increase invasion via increased MMP1 expression. Inhibition of MMP1 abrogates the invasion induced by P120CTN downregulation and *PIK3CA* mutations. The *in vitro* data were confirmed by analyzing The Cancer Genome Atlas (TCGA) database of HNSCC tumors. We have confirmed that MMP1 expression is increased in tumors with low P120CTN expression and enhanced PI3K pathway activity compared to tumors with high P120CTN expression and basal PI3K pathway activity. The role of MMP1 in HNSCC invasion provides a possible target for future drug development that could limit invasion of this cancer.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

7AAD	7-Aminoactinomycin D
ADAM	a Disintegrin and Metalloproteinase
ADAM12	a Disintegrin and Metalloproteinase 12
ADAMT	ADAM with Thrombospondin Motifs
AKT	Protein Kinase B
ANGPTL2	Angiopoietin Like 2
ARP2/3	Actin-Related Proteins 2 and 3
CAN	Copy Number Alterations
CCL15	C-C Motif Chemokine Ligand 15
CCL2	C-C Motif Chemokine Ligand 2
CCL26	C-C Motif Chemokine Ligand 26
CD11b	Integrin αM
CDC42	Cell Division Control Protein 42 homolog
cFOS	Fos Proto-Oncogene, AP-1 Transcription Factor Subunit
cJUN	Jun Proto-Oncogene, AP-1 Transcription Factor Subunit
c-MET	Hepatocyte Growth Factor Receptor
COX2	Prostaglandin-endoperoxide Synthase 2
CXCL2	Chemokine Ligand 2
DAMP	Damage Associated Molecular Patterns
DIAPH1	Protein Diaphanous homolog 1
DMEM	Dulbecco's Modified Eagle's Medium
ECM	Extracellular Matrix
EDA	Extra Domain A
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EIF4E	Eukaryotic Translation Initiation Factor 4e
EMMPRIN	Extracellular Matrix Metalloproteinase Inducer
EMT	Epithelial-Mesenchymal Transition
EPC1	Esophageal Keratinocytes
EPHA3	EPH Receptor A3
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ESCC	Esophageal Squamous Cell Carcinoma
FBS	Fetal Bovine Serum
FGFR	Fibroblast Growth Factor Receptor

FHIT	Fragile Histidine Triad
GAP	RHO-GTPase Activating Proteins
GDI	RHO-GDP Dissociation Inhibitors
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GM-CSF	Granulocyte Macrophage Colony-stimulating Factor
Gr-1	Myeloid-differentiation Antigen
GRP-1	General Receptor of Phosphoinositides 1
GSK3β	Glycogen Synthase Kinase-3β
GTP	Guanosine Triphosphate
GTPase	Small Guanosine Triphosphatase
HER2	Human Epidermal Growth Factor Receptor 2
HNSCC	Head and Neck Squamous Cell Carcinoma
HPV	Human Papilloma Virus
HRAS	Harvey Rat Sarcoma Viral Oncogene homolog
IACUC	Institutional Animal Care and Use Committee
IDO	Indoleamin-2,3-dioxygenase
IL-10	Interleukin-10
IL-3	Interleukin-3
IL-4	Interleukin-4
IL-8	Interleukin 8
JNK	c-JUN N-terminal Kinase
KSFM	Keratinocyte Serum-free Medium
LPS	Lipopolysaccharide
Mac-1	Macrophage-1 Antigen
MDSC	Myeloid-derived Suppressor Cells
MLCK	Myosin Light Chain Kinase
MMP	Matrix Metalloproteases
MRCK	Myotonic Dystrophy Kinase-related CDC42-binding kinase α
MT-MMP	Membrane-type MMPs
mTORC1	Mechanistic Target of Rapamycin Complex 1
mTORC2	Mechanistic Target of Rapamycin Complex 2
NLS	Nuclear Localization Sequence
NFkB	Nuclear Factor Kappa B Subunit 1
NOK	Normal Oral Keratinocytes
N-WASP	Neural Wiskott-Aldrich Syndrome Protein

P120CTN	p120-Catenin
PAK1,2,3	P21-activate Kinases
PAR1	Protease Activated Receptor 1
PDK-1	Phosphoinositide-dependent Kinase-1
PGE2	Prostaglandin E2
PLCγ	Phospholipase C γ
PI3K	Phosphoinositide 3-kinase
PI4P5K	Phosphatidylinositol-4-phosphate 5-kinase
PIK3CA	p110α
PIP2	Phosphatidylinositol 4,5-bisphosphate
PIP3	Phosphatidylinositol Trisphosphate
pro-TNFα	pro-Tumor Necrosis Factor Alpha
PTEN	Phosphatase and Tensin homolog
RAC1	Ras-related C3 Botulinum Toxin Substrate 1
Rb	Retinoblastoma
RHOA	Ras Homolog Family Member A
RHOC	Ras Homolog Family Member C
ROCK1	RHO-associated Protein Kinase 1
ROCK2	RHO-associated Protein Kinase 2
RT	Room Temperature
RTK	Receptor Tyrosine Kinases
SGK-1	Serum/Glucocorticoid Regulated Kinase 1
SOX2	SRY-Box 2
SPHK1	Sphingosine Kinase 1
SRC	Avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog
STAT3	Signal Transducer And Activator Of Transcription 3
TCGA	The Cancer Genome Atlas
TGFα	Transforming Growth Factor α
TGFβ	Tumor Growth Factor β
Th2	CD4+ T helper 2 cells
TNFα	Tumor Necrosis Factor α
TRAIL	TNF-related Apoptosis Inducing Ligand
Treg	CD4+ T regulatory cells
TSC1	Tuberous Sclerosis 1
TSC2	Tuberous Sclerosis 2
uPA	Urokinase

VAV2	Vav Guanine Nucleotide Exchange Factor 2
WASF1	WAS Protein Family Member 1
XIRP1	Xin Actin Binding Repeat Containing 1

Acknowledgements

I would like to take this opportunity to thank everyone that helped me through these trying times of my graduate career. I would first and foremost like to thank my parents for their support through my PhD years even though they had no clue why anyone in their sound mind would ever want to get a PhD. Now I somewhat get it. Well, Mom and Pops, now I have a piece of paper that proves that I'm stubborn, so look at that.

I would like to thank my advisor, Doug, for being the flame in my own potato-egg-coffee fable. I am leaving your lab a better scientist. The experience of being in your lab has allowed me to grow up a lot. I am now more determined, self-critical and I know my limitations. By the way, while I still can't properly pronounce words like "astute", "Schuylkill" or "Worcestershire", I definitely got the "th" sound down like a native now thanks to your coaching!

I would also like to thank Heather for being the best person I have had the fortune to meet at work. Heather, you are hard-working, intelligent, and humble. You are the big sister I always wanted. The sheer amount of work that I see you accomplish while simultaneously juggling responsibilities of motherhood is simply astounding. I wish you the best and I know you will change many lives in the course of your career for the better. I know you have changed mine.

Final thanks come to my support system. Christina, thank you for being there for me through these demanding times, and for motivating me to strive for

more. You are truly one of a kind and I love that you are not afraid to speak your mind. Please don't change. Ron, you are my brother from another mother, I know I can always count on you. Arthur and Mo, even though you guys live far away from me now, I know I can always reach out to you and get some good advice. Bill, thank you for having an unusual sense of humor and for showing me that you can still be cool while being a dad. Pat, thank you for showing me what love of research really looks like. Pat you are extremely humble and that quality, I believe, allowed you to be an amazing scientist.

Finally, I wanted to thank Kat, Kuba and Bartek. All of you grew into amazing adults. Your self-reliance in tough situations taught me that no matter the odds, hard work pays off eventually. I would like to dedicate this dissertation to the three of you, so you know even though I couldn't see you as often as I would like, I always thought of you.

I would like to finish with a quote that guided me through my tough times.

"Sucking at something is the first step to becoming sorta good at something"

Jake the Dog

Chapter 1

Literature review

1.1 Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) is a deadly disease that is diagnosed in half a million people worldwide every year [1]. Lack of specific symptoms leads to HNSCC often being diagnosed at an advanced stage, with metastatic spread already present at the time of diagnosis. Many genetic events can lead to tumorigenesis in the squamous epithelium of the head and neck region. The development of HNSCC is sometimes foreshadowed by the presence of neoplastic lesions, such as leukoplakia and erythroplakia. Moreover, HNSCC is also more likely to develop in the background of tobacco smoking, alcohol consumption and HPV infection [2].

Local invasion is the leading factor responsible for the deadly nature of HNSCC. Active Phosphoinositide 3-kinase (PI3K) pathway and the activation of RHO-family of GTPases frequently contribute to increasing migration and invasion in HNSCC. Aberrations to p120-catenin (P120CTN), a tumor suppressor, have been identified in many epithelial cancers. P120CTN loss or mislocalization contribute to activation of RHO-GTPases and also modification of the PI3K signaling. Furthermore, invasive cells require Matrix Metalloproteases (MMP) to digest their way out of the primary site. Many different MMPs so far have been shown to increase with the development of HNSCC [3]. MMP1 appears to be the most commonly increased MMP in HNSCC [4]. The work outlined in this dissertation demonstrates that MMP1 promotes invasion in

HNSCC and is upregulated cooperatively by both P120CTN loss and active PI3K signaling. HNSCC, like many other cancers, evades immune destruction. Myeloid-derived suppressor cells (MDSCs) limit the activity of cytotoxic T cells. Circulating MDSCs are present in HNSCC patients, and their activity has been linked to tumor progression [5]. Furthermore, MDSCs increase in the mouse oral and esophageal epithelium in the P120CTN-null mouse model [6]. This dissertation demonstrates the results of P120CTN loss in epithelial cells on the MDSCs.

1.2 Head and Neck Squamous Cell Carcinoma

1.2.1 World and US Epidemiology

Head and neck squamous cell carcinoma is a term covering a group of cancers arising from the squamous epithelia in the head and neck region. This cancer occurs in the oral and nasal cavities, pharynx, larynx and sinuses (Figure 1.1). Over half a million people are diagnosed with HNSCC every year. Ten percent of HNSCC cases occur in the United States alone with about 20% of newly diagnosed patients being diagnosed with stage IV tumors with distant metastases. The public health burden of HNSCC is profound, with ~300,000 people living with the disease in the United States. Approximately a third of HNSCC patients will die within 5 years of diagnosis [7]. HNSCC in the United States is the 9th most common cancer [7]. In South and South-East Asia, HNSCC is the most commonly diagnosed cancer. HNSCC accounts for approximately 40-

50% of all cancers in countries such as Vietnam, Sri Lanka and India due to their extensive use of smokeless tobacco and areca nut [8-10]. Treatment of HNSCC frequently entails large, disfiguring surgical excisions; thus, of those who survive the disease, a large fraction has substantial functional and cosmetic morbidity. Precise characterization of the pathways involved in HNSCC invasion may be very useful, as this understanding may allow for earlier diagnosis and better treatment.

1.2.2 Preneoplastic changes

HNSCC does not have overt symptoms in earlier stages and as a result is often discovered once the disease has advanced. The symptoms include dyspnea, loose teeth, dysphagia, and a change to patients general condition [11]. There are a few pre-neoplastic lesions which foreshadow the development of HNSCC. One of them is a white discoloration to the mucous membrane called leukoplakia. Leukoplakia, upon biopsy, commonly contains dysplasia and hyperplasia. Leukoplakia generally carries a 5% chance of turning into a carcinoma. Patients with leukoplakia are more likely to develop HNSCC due to the following risk factors: female gender, increased size of the lesion and the presence of high-grade dysplasia [12]. The diagnosis of dysplasia is based on the appearance of cells visualized by microscopic examination of biopsy specimens. Dysplastic cells have an increased prominence of nuclei and nucleoli and do not maintain the normal distribution of strata in the epithelium leading to

cellular disorganization. Often dysplasia does not extend throughout the entire thickness of the tissue; however, when dysplasia spans the epithelium from the basal layer to the superficial layer of the mucosa, it is defined as carcinoma *in situ*. The diagnosis of dysplasia alone is correlated with progression to carcinoma in up to 30% of cases. Erythroplakia is the other pre-neoplastic lesion that can develop into HNSCC. It reddish patch that commonly contains dysplastic and hyperplastic cells. Hyperplasia indicates an increase in the number of normal looking cells. Carcinoma *in situ* or an invasive tumor can be found in up to 40% of erythroplakia cases [2]. Although the presence of pre-neoplastic lesions has allowed for early detection, a large number of tumors go undetected until they are at an advanced stage.

1.2.3 Patient evaluation

Due to a large percentage of new patients presenting at late stages of disease, it is recommended that every new HNSCC patient receive a workup to evaluate for metastasis. Panendoscopy, CT scan or PET scan are recommended to identify metastases. The most common sites of distant metastasis include the lungs, liver, and bone. Second primary malignancies occur in the epithelia exposed to the same carcinogens, and are primarily the head and neck, lungs and esophagus. Hence, patients with HNSCC might at a later time develop completely new tumors or recurrences of the original tumor. This complicates

patient management, since a large area of the body needs to be surveilled regularly.

1.2.4 Field cancerization

Field cancerization is a concept that is important in cancers in which heavy exposure to a carcinogen is observed, like HNSCC. This notion was first described by Slaughter and it pertains to genetic changes in the peritumoral mucosal epithelium [13]. After tumor excision, the peritumoral epithelium is left behind. Although this tissue generally lacks obvious biomarkers of disease, it does harbor genetic alterations that increase its chances of developing into carcinoma. These unexcised modified epithelia or “cancerized fields” attribute to local recurrences of second primary malignancies, which eventually lead to death in HNSCC [14, 15]. This underscores the severity of this disease. Even if the tumor is completely excised prior to metastasis, the patient still has a high risk of developing a new tumor from the same epithelium.

1.2.5 Genetic alterations

HNSCC can arise in the background of many diverse genetic changes. Copy number alterations (CNAs) are commonly found in almost all of the chromosomes, with predominance in chromosomes 3, 8 and 11 [16-22]. Deletion of chromosome 3p arm leads to the loss of many tumor suppressors such as Fragile Histidine Triad (FHIT), EPH Receptor A3 (EPHA3), and Xin Actin Binding Repeat Containing 1 (XIRP1). Region 3q is commonly found to be

amplified. 3q contains oncogenes such as SRY-Box 2 (SOX2) and p110α (PIK3CA). Also, amplifications and deletions are found in 11q which harbors Erb-B2 Receptor Tyrosine Kinase 2 (ERBB2) oncogene and P120CTN tumor suppressor. Hence, many different genetic aberrations occur in the background of HNSCC.

A more comprehensive look at HNSCC genetics was done thanks to efforts of TCGA, which analyzed genomes of 279 patients using several methods, such as whole-exome sequencing, microarray analysis, and RNA-seq [18]. TCGA study was of paramount importance in the field for multiple reasons. The approach the TCGA undertook displayed a new methodology that differed from the approach we have originally used to identify genetic changes important to cancer biology. In the history of cancer research, V-SRC was the first viral oncogene to be identified as a product of a viral oncogene [23, 24]. This viral oncogene via molecular hybridization studies was found to have a homologue – C-SRC, which is found widely in eukaryotes [25]. Stemming from this methodology many oncogenes were discovered. Some were discovered via homology to viral DNA, but others thanks to the study of insertional mutagenesis. This process is induced by abnormal regulation of a protooncogene due to viral transcriptional regulatory elements [26]. Many other genes that are relevant for tumor biology emerged as a result of virus research. For example, *TP53* was

initially identified to bind the T antigen of SV40 virus and much later was discovered to actually be a tumor suppressor [27, 28].

The development of DNA sequencing and other various forms of genetic, epigenetic or transcriptome analysis has shifted our approach on how we study oncogenes. Based on the present state of literature we can predict which pathway aberrations could potentially play a role in tumor biology. Moreover, if specific genes are modified frequently in cancers, then that generates new targets for our future studies. TCGA is a great example of multiple modalities being combined to identify new genetic aberrations and confirm the presence of already known genetic changes in cancers. According to the study by TCGA, the frequency of genomic aberrations in HNSCC was found to be, on average, 141 CNAs (amplifications or deletions) and 62 structural aberrations (chromosomal fusions) per tumor. They also demonstrated that *TP53* and *CDKN2A* tumor suppressors are almost universally lost in HNSCC. The most commonly mutated and amplified oncogenes were *PIK3CA*, *CCND1*, *EGFR* and *MYC.* Overall, the TCGA studies confirmed already suspected involvement of aberrations of PIK3CA or TP53 in HNSCC, but thanks to large sample size, this study allowed us to more accurately estimate the frequency of these genetic changes.

1.2.6 Risk Factors

Multiple risk factors have been found to contribute to the development of HNSCC. An estimated 73% of all HNSCCs are due to the combined effects of

tobacco smoking and alcohol consumption [29]. In addition to tobacco and alcohol, many other risk factors like viral infections (Epstein Barr Virus and Human Papillomavirus), radiation, chemical exposures due to occupational hazards and diet can contribute to the development of this deadly disease [30-34]. Some of these risk factors even affect signaling pathways that are involved in HNSCC metastasis.

1.2.7 Tobacco smoking

Tobacco smoking is the leading risk factor for HNSCC [35]. A life time value of tobacco exposure is estimated by pack-years. The increased amount of smoke pack-years is correlated with a higher risk of developing HNSCC [36]; therefore, quitting smoking can reduce the chance of developing HNSCC [35]. Moreover, the longer a patient has abstained from smoking until tumor development and treatment, the better the patient's outcome [37]. Tobacco smoke contains many carcinogens, including nitrosamines and polycyclic aromatic hydrocarbons [38]. It has also been shown to work via activating MUC1 protein, which leads to the loss of tight junctions [39]. In addition, MUC1 interaction with P120CTN leads to the alteration of cell motility [40]. The mechanism responsible for MUC1 and P120CTN effect on motility is still unknown, but we know that this effect is independent of E-cadherin [41]. Interestingly, like P120CTN, PI3K pathway is affected by tobacco smoke. However, in the case of PI3K, the tobacco smoke increases the activation of

PI3K pathway, which then mediates its transformative effect via FRA1 and NFkB pathways [42, 43]. Hence, tobacco smoke can modulate HNSCC tumorigenesis not by providing carcinogens, but also by activating important oncogenic signaling pathways.

1.2.8 Alcohol Consumption

Alcohol consumption is the second largest risk factor of HNSCC [35]. Furthermore, alcohol consumption and tobacco smoking synergize to promote HNSCC formation [44]. Alcohol consumption paired with tobacco smoke exposure increases the risk of developing HNSCC approximately thirty five times [44]. Byproducts of alcohol, such as acetaldehyde, can cause N(2)-ethyl-2'-deoxyguanosine DNA adducts, which can lead to carcinogenesis [45, 46]. Alcohol also irritates the tobacco-exposed area and allows for carcinogens to better penetrate into the tissue. Very often the irritation is enough to induce inflammation leading to increased oxidative stress, which can also play a role in the process of carcinogenesis [47].

1.2.9 Human Papilloma Virus

Human Papilloma Virus (HPV) has been commonly associated with HNSCC tumorigenesis [48]. Two thirds of oropharyngeal tumors have been reported to be HPV-positive, compared to six percent of non-oropharyngeal HNSCC tumors[18]. HPV viral proteins E6 and E7 promote tumor progression by inhibiting tumor suppressors, namely retinoblastoma (Rb) and p53 protein (*TP53*)

[49-51]. HPV-positive tumors do not have mutations in either *TP53* or *CDKN2A,* since the function of these tumor suppressors is inactivated by the presence of HPV viral proteins E6 and E7. Moreover, HPV related HNSCC cases are now on the rise due to increased incidence of oral sexual habits [52]. Fortunately, HPV-positive HNSCC has a higher overall survival rate than HPV-negative tumors [53]. We can speculate that the higher overall survival of patients with HPV-positive HNSCC is likely to be due to those tumors being more responsive to the treatment due to lower genomic instability. In HPV-positive tumors, the viral proteins are responsible for inactivating key tumor suppressors, while in HPV-negative tumors random mutations inactivate the same gene, which indicates that there are other mutations in the genome. Due to the likely presence of additional mutations in the genome we can assume that the genome is more unstable and hence more likely to adapt to the cancer treatment. In addition, the amount of HNSCCs due to alcohol consumption and tobacco smoking appears to be on the decline, which bodes well for future patient outcomes as these are the main risk factors leading to carcinogen-induced HNSCC [54].

1.3 Metastasis in HNSCC

1.3.1 Metastatic cascade

Metastatic progression is estimated to be responsible for ninety percent of all cancer deaths [55]. In HNSCC, locoregional metastasis—in which the tumor invades heavily into adjoining tissues and lymphatics—occurs at very high rates

making the tumor very difficult to surgically resect. Metastasis is still a poorly understood process. In particular, it is still unclear why certain tumors are highly metastatic (i.e. melanoma) while others rarely metastasize (i.e. basal cell carcinoma of the skin). Although there is still much unknown about the mechanism driving differential rates of metastasis, what is known about metastasis has been well summarized by the metastatic cascade theory.

Metastatic cascade outlines the steps required for a cancer to escape the primary site and establish new metastatic foci at distant sites. Migration and invasion are included in the first step of metastasis. During this step, cancer cells detach themselves from the basement membrane and other cells by secreting proteases which digest extracellular matrix (ECM) and allow the cancer cells to invade into adjacent tissues. The second step includes intravasation into the blood vessels and lymphatics. Cancer cells enter the circulation by squeezing in between endothelial cells that form blood vessels. The third step involves circulation, in which the tumor cells freely float through the bloodstream and are subject to high levels of oxygen and cytotoxic T cells. The two factors have been shown to promote cancer cell death; thus, it is believed that the majority of cancer cells die in circulation. Next, the cells must extravasate, or leave the bloodstream, in order to colonize a secondary site. During colonization, metastasized cancer cells proliferate and establish new foci [56]. Presence of

metastatic foci in HNSCC patients corresponds with high mortality and poor prognosis [57].

Migration is one of many steps that cancer cells need to take in order to successfully invade into adjacent or distant tissues. Epithelial cells are commonly non-migratory due to the high density of cell-cell connections, such as adherens junctions, tight junctions and desmosomes, as well as the presence of hemidesmosomes which attach cells to the basement membrane. Epithelial cells often form a barrier facing the outside environment and are found lining structures such as the gastrointestinal tract and the bronchi. During embryological development, wound-healing, and cancer, epithelial cells undergo the Epithelial-Mesenchymal Transition (EMT) and acquire characteristics of mesenchymal cells such as increased motility, invasiveness, and production of extracellular matrix proteins [58]. In HNSCC, EMT can be induced by diverse stimuli. To name a few, increased expression of surface receptor NOTCH1 or transcription factor Fos Proto-Oncogene, AP-1 Transcription Factor Subunit (cFOS) induce EMT in HNSCC cell lines with a concurrent expression of cancer stem-like cell markers such as *C-MYC*, *SOX2*, and *NANOG* [59, 60]. Overexpression of various signaling molecules stimulates reprogramming of epithelial cells to a more mesenchymal state; however, this state is not uniform between experimental systems. Due to a wide range of stimuli, the mesenchymal phenotype will differ between cell lines. This variability makes EMT difficult to

study and limits its use as a determinant of cells with high motility. Furthermore, the rate of migration can be altered in the absence of EMT. Abnormal activation of various signaling pathways can impact the expression, activity, and arrangement of cytoskeletal proteins which subsequently alter cell motility.

Despite differences in the types of risk factors and anatomical origins, the majority of HNSCCs appear to have modifications in the same signaling pathways. PI3K and the Rho family of GTPases are the two most commonly modified signaling molecules in HNSCC [18, 61, 62]. Mutations of these molecules and their downstream targets have been found to be responsible for neoplastic transformation, increased tumor cell motility and invasion.

1.3.2 PI3K pathway

The PI3K pathway is the most commonly activated pathway in HNSCC [18]. PI3K signaling occurs downstream of cell surface receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR) or fibroblast growth factor receptor (FGFR) (Fig. 1.2). Small guanosine triphosphatase (GTPases) like Harvey rat sarcoma viral oncogene homolog (HRAS) can also activate the PI3K pathway. PI3K is a protein kinase that phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) into its active form, phosphatidylinositol-3,4,5-trisphosphate (PIP3). PI3K is a protein complex composed of a regulatory and a catalytic subunit. PI3Ks that facilitate conversion of PIP2 to PIP3 belong to class I PI3Ks. Interestingly, class II and III PI3Ks have not been found to be involved in

carcinogenesis. Class I PI3Ks are composed of two groups: IA and IB. Group IA PI3Ks are made of one regulatory subunit and one catalytic subunit. The regulatory subunits of Group IA are p85α, p55α, p50α, p85β, and p55γ. The catalytic subunits of Group IA are p110α (PIK3CA), p110β, p110δ. PI3Ks of group IB are made of one catalytic subunit, p110γ, which simultaneously binds two regulatory subunits, p101 and p84 [63].

The opposite activity to PI3K is performed by phosphatase and tensin homolog (PTEN), which works by dephosphorylating PIP3 to deactivate it. Conversion of PIP2 to PIP3 leads to recruitment of phosphoinositide-dependent kinase-1 (PDK-1) and protein kinase B (AKT) to the plasma membrane. PDK-1 phosphorylates threonine 308 of AKT resulting in AKT activation. Activated AKT phosphorylates tuberous sclerosis 1 (TSC1) and tuberous sclerosis 2 (TSC2) resulting in the activation of the mechanistic target of rapamycin complex 1 (mTORC1) (Figure 1.2). mTORC1 signaling through eukaryotic translation initiation factor 4e (EIF4E) results in increased protein synthesis while mTORC1 signaling through S6K has been associated with increased mRNA synthesis, cap dependent translation and elongation, and translation of ribosomal proteins. Additionally, conversion of PIP2 to PIP3 has been associated with activation of other pathways such as Phospholipase C γ (PLCγ), Vav Guanine Nucleotide Exchange Factor 2 (VAV2), General Receptor of Phosphoinositides 1 (GRP-1), and TEC [64-68].

Interestingly, PI3K pathway members are activated by HNSCC's primary risk factor, tobacco smoking. Nicotine contained in tobacco smoke binds nicotinic acetylcholine receptors leading to activation of AKT and mTORC1 [69]. This emphasizes the importance of PI3K pathway activity for tumorigenesis. This pathway can be activated transiently by tobacco smoking. If the nicotine levels drop, in the cancerized field with modified epithelium, the oncogene addiction can push the cells to select for tumor cell subsets with aberrantly activated PI3K pathway. In a mouse model of breast cancer, withdrawal of Wnt pathway activation leads to tumor regression followed by evolution of tumor subclones that re-establish Wnt pathway activity, which results in a relapse [70]. A similar situation could be seen in the case of HNSCC, where the near constant stimulation of PI3K pathway by nicotine, might lead to oncogene addiction to the PI3K pathway.

PI3K pathway activation plays a prominent role not only in tumor initiation, but also in the migration and invasion of HNSCC. This pathway can be activated by a variety of RTKs, including epithelial growth factor receptor (EGFR). Ninety one percent of HNSCC tumors have increased levels of transforming growth factor α (TGFα), an EGFR ligand [71, 72]. In addition, treatment of HNSCC cell lines with EGFR ligands (e.g., epidermal growth factor (EGF), betacellulin, TGFα, heparin binding EGF and amphiregulin) increased cell migration and invasion [73, 74]. Conversely, PI3K pathway activity can result in RTK overexpression.

RTK overexpression results in spontaneous receptor dimerization events which consequently lead to ligand-independent receptor activation. Overexpression of RTKs like FGFR1, FGFR2, and FGFR3 on the HNSCC cell surface leads to increased migration and invasion and correlates with higher rates of metastasis [75-77]. Similarly, upregulation of human epidermal growth factor receptor 2 (HER2) and hepatocyte growth factor receptor (c-MET) expression has been shown to increase cell migration and invasion *in vitro* [78, 79]. While these reports do not demonstrate whether PI3K pathway drives the observed phenotype, we can speculate that it is likely that PI3K pathway is activated in many of the given examples and in those instances it plays a role in modulation of motility and invasion.

PI3K pathway activation can occur due to activating mutations or overexpression of PI3K. The PI3K heterodimer is composed of an 85 kDa regulatory and a 110 kDa catalytic subunit. *PIK3CA* codes for the 110 kDa subunit. This gene was originally identified by Cantley [80]. Its activity was demonstrated to be increased by viral oncoproteins, and some viruses even carried mutant versions of PIK3CA [81, 82]. This established the possibility that PIK3CA might be important in the field of cancer research. Later it was demonstrated that *PIK3CA* is the most commonly mutated oncogene overall in all cancers [83]. This gene is overexpressed and mutated in 56% and 21% of HNSCC, respectively [18]. Seventy three percent of all PIK3CA mutations are

localized to three hotspots - residues 542, 545, and 1047 and cause constitutive activation of PI3K [18]. Mutations E542K and E545K are in the helical domain, while the H1047R mutation is in the kinase domain of the 110 kDa catalytic subunit. The gain-of-function helical domain mutations lead to PI3K activation by binding Ras and are not affected by the regulatory 85 kDa subunit. The mutations in the kinase domain activate PI3K independently of Ras via interaction with the regulatory 85 kDa subunit [84]. E542K, E545K, and H1047R PIK3CA mutants increase the invasive phenotype of HNSCC cell lines [85]. However, E545K and H1047R induced different rates of metastasis in a breast cancer model indicating that while all these mutations induce constitutive activation of PIK3CA and increased invasion, the location of each mutation within the gene affects the observed *in vivo* phenotype; therefore, additional work is needed to characterize these mutants [86]. PIK3CA overexpression results in increased activation of the PI3K pathway and correlates with increased lymph node metastases in HNSCC [87]. This indicates that any change that results in PI3K pathway activation in HNSCC promotes an invasive phenotype in HNSCC.

The PI3K pathway's involvement in invasive properties of HNSCC also comes from the pathway's effects on proteins involved in basement membrane breakdown and actin mobilization. PI3K pathway activation can increase the expression of MMP1, MMP2, MMP3, MMP9, MMP12 and Disintegrin And Metalloproteinase Domain-Containing Protein 12 (ADAM12) by increasing the

expression of prostaglandin-endoperoxide synthase 2 (COX2) and extracellular matrix metalloproteinase inducer (EMPPRIN) [78, 79, 88, 89]. MMP9 is also induced due to mTORC1 activity through EIF4E [90]. MMP9 degrades collagen IV, the main component of the basement membrane, facilitating cell invasion [91]. Interestingly, ADAM12 cleaves stromal proteins akin to metalloproteases, but can also increase HER2 expression, further activating the PI3K pathway [78]. In addition, the activity of Serum/Glucocorticoid Regulated Kinase 1 (SGK-1) and glycogen synthase kinase-3β (GSK3β), proteins downstream of mTORC1, led to invasion or EMT in other cell types [92, 93]. Further studies are required to evaluate the role of SGK-1 and GSK3β in HNSCC invasion.

PI3K pathway activity has been shown to result in activation or increased expression of the proteins regulating cytoskeleton rearrangement like Ras Homolog Family Member C (RHOC), VAV2, and Fibronectin [88, 94]. Increased activity of RHOC, VAV2 and Fibronectin correlates with increased cell invasion [74, 88, 94]. Activated RHOC increased invasion through E-cadherin downregulation in HNSCC [94]. VAV2 functions are still largely unknown. VAV2 interacts with both PI3K and EGFR to become activated, and acts as a guanine nucleotide exchange factor to activate small Guanosine Triphosphatases (GTPases). Some of the VAV2 regulated GTPases belong to the RHO family [95, 96]. Finally, work in HONE1 cells, an HNSCC cell line, indicates that PI3K signaling enhanced Fibronectin expression which activated the Cell Division

Control protein 42 homolog (CDC42) and Ras-related C3 Botulinum Toxin substrate 1 (RAC1), increasing cell invasion [88]. Hence, PI3K pathway can modulate motility of cells by utilizing various members of the RHO-family of GTPases.

1.3.3 RHO-family of GTPases

The RHO-family of GTPases consists of signaling proteins that have been shown to regulate actin cytoskeleton rearrangement, gene expression and cell proliferation [97]. There are many members belonging to this protein family, e.g., Ras Homolog Family Member A (RHOA), RHOC, RAC1, CDC42 [98-100]. RHO-GTPase activation occurs as a result of a guanosine diphosphate (GDP) being replaced by a guanosine triphosphate (GTP) by a guanine nucleotide exchange factor (GEF). RHO-GTPase activating proteins (GAPs), promote the hydrolysis of GTP to GDP and inactivation of the RHO-GTPase. Further inhibition of RHO-GTPases can occur due to RHO-GDP dissociation inhibitors (GDIs), which sequester RHO-GDP bound proteins away from the cell membrane. Following the activation, RHO-GTPases interact with specific effector proteins that propagate the signal downstream [101]. RHOA and RHOC induce cytoskeletal changes via RHO-associated protein kinase 1 (ROCK1), RHO-associated protein kinase 2 (ROCK2), phosphatidylinositol-4-phosphate 5-kinase (PI4P5K), and protein diaphanous homolog 1 (DIAPH1) (Fig. 1.3). Activated RAC1 primarily signals via P21-activate kinases (PAK1, PAK2, PAK3). PAKs, similarly to ROCK,

phosphorylate LIM kinase, which then phosphorylates cofilin [102].[101]. In addition, PAK inhibits MLCK, acting in an opposite direction to ROCK. CDC42 relies on Neural Wiskott-Aldrich Syndrome Protein (N-WASP), Myotonic Dystrophy Kinase-related CDC42-binding kinase α (MRCK) and PAKs as its main effectors [103] [104] (Figure 1.3).

Multiple factors lying downstream of the RHO family of GTPases have been shown to be activated in HNSCC and their activation have been linked to processes of invasion and migration. RAC1 and CDC42 signaling promote increased migration in HNSCC cell lines SCC25 and FaDu [105, 106]. A guanine exchange factor, TIAM1, which is commonly activated by the PI3K pathway is increased in UM-SCC47 cells. TIAM1 overexpression causes an increase in migration of UM-SCC47 cells, which is speculated to be through the RHO-GTPase family of proteins [107]. In this project we demonstrate insufficient activation of RHOA pathway, but RAC1 and CDC42 signaling still remain to be investigated as drivers of migration in our experimental system.

1.3.4 P120CTN

P120CTN is a major factor that controls the activity of RHO-family GTPases is P120CTN (Figure 1.4) [108-110]. Dysregulation of P120CTN has been identified in a large number of epithelial cancers and the list is still growing. Our current understanding of this protein implicates it as an important tumor suppressor in the pathogenesis of HNSCC. P120CTN is frequently

downregulated or lost in HNSCC, which is correlated with poor prognosis [111]. P120CTN loss in esophagus, skin, salivary gland, mammary, dental enamel, kidney and intestines leads to E-cadherin loss and increase of inflammation [112-117]. Furthermore, P120CTN loss or mislocalization in skin, bladder, breast, gastroesophageal junction, esophagus and oral cavity is correlated with decreased patient survival [117-121]. The effects of P120CTN on motility, invasion and patient survival are even more significant considering that tobacco smoking and alcohol consumption lead to a decrease in P120CTN expression [122-124].

P120CTN is a structural protein that binds the juxtamembrane domain of E-cadherin [125]. P120CTN binding on E-cadherin prevents internalization of E-cadherin and promotes maintenance of adherens junctions [126]. Four main isoforms of P120CTN are commonly found in cells, but due to the presence of alternate splice sites, the actual number of possible isoforms is thirty-two [127]. Each P120CTN isoform consists of an Armadillo domain (consisting of 9 Armadillo repeats – forty-two amino acid long repeats forming triple α helices) flanked by N-terminal and C-terminal regions (Figure 1.5). P120CTN isoform 1 is the longest isoform and it contains the longest N-terminal region. P120CTN isoform 1 is commonly found in fibroblasts. P120CTN isoform 3 has a shortened N-terminal that is deprived of the coiled-coil domain and is commonly found in epithelial cells. P120CTN isoform 4 is missing almost the entire N-terminal, and

this is the reason why this isoform is often used to study the consequences of losing regulatory N-terminal sequences in P120CTN [128].

1.3.5 P120CTN isoforms

Different P120CTN isoforms can have diverse effects in cells, either promoting or inhibiting migration and invasion. During a SNAIL-induced EMT, P120CTN has been demonstrated to switch from isoform 3 (epithelial-like) to isoform 1 (fibroblast-like), which suggests that P120CTN isoforms are integral to EMT induced migration and invasion [129]. In MDA-MB-231 breast cancer cells, P120CTN isoform 1 increases invasion by increasing RHOA activity, P120CTN isoform 3 has no effects, and P120CTN isoform 4 decreased RHOA activity along with the invasion [128]. The effect on RHOA is speculated to be the result of two different P120CTN domains interacting with RHOA and affecting its GDP dissociation. The authors argue that the N-terminal domain is a necessary for RHOA inhibition. They also raise a very interesting point relation to the ratio of P120CTN isoform 1 to N-terminally truncated forms as being responsible for the effects on RHOA. While each P120CTN isoform leads to a different migratory rate in tumor cell lines, P120CTN loss is often correlated with increased metastasis and promotes increased migration and invasion [130]. In addition, P120CTN function can be altered by phosphorylation events. SRC-family kinases phosphorylate P120CTN at Y112 diminishing its effects on RHOA, while phosphorylation at Y217 and Y228 strengthens the effects on RHOA [131].

Additionally, RAC1 activation due to active Wnt signaling is inhibited if P120CTN is phosphorylated by SRC kinases [132]. Hence, P120CTN can be modified in various ways and it is not clear how each modification in different isoforms will impact cell motility.

In addition to regulating RHO-family GTPases, P120CTN can also bind microtubules and motor proteins [133, 134]. P120CTN regulates gene expression by sequestering transcriptional repressors such as Kaiso, Glis2, and REST/coREST at the cell membrane [135-137]. Additionally, P120CTN can be mislocalized from the cell membrane to the cytoplasm or the nucleus. P120CTN mislocalization is speculated to occur if P120CTN is overexpressed, its binding partners are not present, or due to mutation or phosphorylation events. P120CTN mislocalization or P120CTN loss can result in an increase in P120CTN-associated transcriptional repressors in the cytoplasm and the nucleus. Moreover, mislocalized P120CTN aids the transcriptional repressors in their entry into the nucleus because of the Nuclear Localization Sequence (NLS) present in the Armadillo domain of P120CTN [137]. Not much is known about the effects of P120CTN-associated transcriptional repressors on motility and invasion. Kaiso has been demonstrated to bind to the promoter region of MMP7 and inhibit it [138]. However, P120CTN knockdown in prostate cancer demonstrated an increase in MMP7 expression, contrary to the expected outcome, which was a decrease of MMP7 [139]. The authors speculate that due to disinhibiton of Kaiso

the P120CTN knockdown induced an activation of the β-catenin pathway, which resulted in the MMP7 increase. This emphasizes that P120CTN interacts with many different proteins and its effects depend on the background signaling occurring in the cell. It implicates P120CTN as a regulator more than a driver of processes such as migration and invasion.

1.3.6 Matrix Metalloproteases

Matrix Metalloproteases (MMPs) play a significant role in invasion and metastasis. MMP-induced breakdown of the extracellular matrix (ECM) in the stroma allows the tumor cells to escape from the primary site and establish a metastatic focus. MMPs have been a focus of cancer research for over 40 years, but the therapeutics targeting MMPs have not improved patient survival in clinical trials due to issues with side effects, dose titration, and lack of specific inhibitors[140]. MMP research began when researchers tried to understand the mechanism of tadpole tail dissolution [141]. From then on close to thirty MMPs have been identified in the human genome. MMPs are zinc-dependent endopeptidases that are secreted by cells into the extracellular space where they cleave various extracellular matrix (ECM) proteins. Membrane-type MMPs (MT-MMPs) are an exception to that rule. MT-MMPs contain an additional transmembrane domain and a cytoplasmic tail, which allow them to be attached to the cell membrane, while still retaining the ability to cleave ECM proteins. All MMPs contain a pro domain, catalytic domain, and a hemopexin-binding domain.

The active site lies in the catalytic domain and is composed of histidine residues that bind zinc ligands. The hemopexin-binding domain is responsible for substrate specificity. The pro domain must be cleaved in order for the protease to become completely active.

In addition to cleaving ECM proteins, many MMPs cleave latent growth factors, integrins, and other pro-MMPs that reside in the ECM, which then stimulate tumor growth and promote invasion. For example, MMPs can cleave pro-Tumor Necrosis Factor alpha (pro-TNFα) into its active form [142]. In addition, increased MMP7 expression (MMP regulated by P120CTN) has been shown to promote the selection of cells that are more resistant to apoptosis in mammary tumors [143]. MT-MMPs cleave pro-MMP2 into its active form further promoting invasion [144]. When MMPs cleave ECM proteins, the primary goal is thought to clear the path for invasion. However, cleaved ECM proteins often reveal cryptic active domains that can affect the tumor growth, invasion or survival. This is exemplified by MMP1, which digests collagen revealing a cryptic domain that binds αvβ3 integrins, promoting increased melanoma cell survival [145]. Hence, MMPs could possibly aid HNSCCs in more ways than just helping cell invasion.

In HNSCC many MMPs have been demonstrated to promote cell invasion. EGF-induced increase in MMP1, MMP9, MMP11 and MMP14 results in an increased invasive capacity in the UM-SCC-1 HNSCC cell line [3]. Furthermore,

these results have been confirmed independently in HT-1080 and UM-SCC-1 HNSCC cell lines by inhibiting MMP14, which led to abrogation of invasion [146]. Betacellulin, an EGFR ligand, increases the expression of MMP9 via activation of PI3K signaling in HNSCC [147]. Furthermore, blood MMP9 levels increase in HNSCC patients and could be used as a diagnostic test for detection of this deadly cancer [148]. EGF can also induce the expression of MMP1 in HNSCC by signaling via ANGPTL4 [149]. Oleic acid treatment of TU183 oral cancer cells, a model of increased free fatty acids in obese HNSCC patients, also induced ANGPTL4 activity, but in this report instead of MMP1, MMP9 was induced [150]. A literature review by Iizuka et al. presents MMP1 as the most upregulated MMP in HNSCC [4]. Moreover, increased MMP1 mRNA expression in the saliva was demonstrated to detect Oral Squamous Cell Carcinoma [151, 152]. This emphasizes, how prevalent MMP1 increase is in HNSCC and how important it is to study what causes it.

MMP1 (Interstitial collagenase) is a member of the collagenase family, which includes MMP8 and MMP13. Collagenases cleave the collagen triple helix into ¾ and ¼ fragments. Besides digesting collagen, the collagenases can cleave secreted factors such as pro-Interleukin-8, pro-TNFα, and Protease Activated Receptor 1 (PAR1), a unique protease activated G-protein coupled receptor [153-155]. MMP1 cleaves collagen types I, II, III, V, VII, VIII, X and gelatin. It is commonly found in fibroblasts and tumor cells [156]. MMP1 gene is

located at chromosome 11q22 along with MMP3, MMP7, MMP8, MMP10, MMP12, MMP13, MMP20, and MMP27 [157]. MMP1 can be activated by multiple factors such as kallikrein, trypsin, neutrophil elastase, cathepsin G, tryptase, chymase, MMP14 and stromelysins, which are often found at sites of inflammation, a common finding in many cancers [158-160]. Two proenzymes of MMP1 can be concurrently expressed by cells, a 52 kDa unmodified protein and a 57 kDa glycosylated protein [161]. Upon activation, MMP1 can be found in the extracellular space as a 42 kDa or 46 kDa protein [161]. Additionally, MMP1 can also be localized to the cell surface by association with EMMPRIN, which binds MMP1 and activates it [162, 163]. This indicates that MMP1 can be found on the surface of the cell driving its invasive capability.

1.4 Myeloid-derived Suppressor Cells in HNSCC

1.4.1 Immune Evasion in HNSCC

Burnet and Thomas demonstrated that transplanted tumors are destroyed by the immune system, which gave rise to the idea of tumor surveillance [164]. The current thinking is that many aberrant cells that are fated to become cancers are detected and destroyed by the immune system. The significance of the immune system in cancer prevention has been clearly established. With the suppression of the immune system, we observe a large increase in the incidence of many cancers with an infectious etiology [165]. This constant surveillance by the immune system creates an evolutionary pressure for cancer cells to develop

strategies to avoid detection and destruction. There are many ways in which this could be accomplished. HNSCCs often secrete immunosuppressive molecules such as Tumor Growth Factor β (TGFβ), Prostaglandin E2 (PGE2), Interleukin-4 (IL-4), and Interleukin-10 (IL-10) [166]. Furthermore, HNSCCs tend to have defective antigen processing machinery (estimated 80% of tumors), which fails to present tumor-associated antigens efficiently [167]. In addition, HNSCCs can recruit immunosuppressive cells such as $CD4^+$ T helper 2 cells (Th2), $CD4^+$ T regulatory cells (Treg) and Myeloid-derived Suppressor Cells (MDSCs) [5, 168, 169]. MDSCs can promote Treg differentiation, the suppression of cytotoxic T cell proliferation and activity, and even induction of cytotoxic T-cell apoptosis [170, 171]. MDSCs are commonly increased in HNSCC and many other cancers [172-176]. Furthermore, MDSCs are believed to aid in HNSCC tumor progression since increased amounts of MDSCs found at the tumor site correlate with increased tumor stage [5]. Finally, chronic inflammation is often found at the site of many tumors. MDSCs migrate as a response to inflammatory cytokines in order to limit the deleterious effects of inflammation. We can speculate that it is possible that an increase of MDSCs at the tumor sight might be a response to the inflammation found in the tumor microenvironment.

1.4.2 MDSCs

MDSCs were originally discovered by chance in various tumor mouse models [177-179]. MDSCs are believed to be the body's response to chronic

inflammation. MDSCs are believed to act mostly via their effects on $CD8^+$ cytotoxic T cells [171]. These immunosuppressive cells produce Arginase and Reactive Oxygen Species, which reduce T cell metabolism and recognition, and limit the cytotoxic killing of tumor cells [171]. Moreover, MDSCs limit T cell proliferation via production of suppressive cytokines including, Interleukin-10 (IL-10), Transforming Growth β (TGF-β), Indoleamin-2,3-dioxygenase (IDO) [180-182].

MDSCs in mice are a mixture of immature granulocytes and immature monocytes. MDSCs are identified based on the expression of two surface markers, Gr-1 and CD11b. The Gr-1 surface marker is commonly found on the surface of monocytes, macrophages and granulocytes in mice [171]. Further evaluation of this surface marker determined that it actually consists of two distinct antigens, Ly6C and Ly6G. While Gr-1 is still used to characterize MDSC cell subtypes, Ly6C and Ly6G expression were found to distinguish mouse MDSCs into two subtypes, granulocytic ($CD11b^+Ly6G^+Ly6C^{lo}$), and monocytic ($CD11b^+Ly6G^-Ly6C^{hi}$) [171]. CD11b is the second surface marker used for identifying MDSCs. CD11b, also known as macrophage-1 antigen (Mac-1), is used for identifying monocytes, granulocytes, macrophages, and natural killer cells [183]. Unfortunately, MDSCs in humans are identified by a completely different set of surface markers. All human MDSCs are $CD3^-CD19^-CD56^-$ $CD11b^+CD33^+HLA\text{-}DR^-$. Lack of CD3 marker is used to remove lymphocytes,

since CD3 is a part of a T-cell receptor. We also exclude $CD19^+$ cells, which are B cells, and $CD56^+$ cells, which are Natural Killer cells. MDSCs are considered not to have HLA-DR, (Human Leukocyte Antigen - antigen D Related), an MHC class II, which functions to present processed antigens. CD33 surface marker is found on progenitor cells, and undifferentiated myeloid cells, hence MDSCs are expected to express it. Like in mouse MDSCs, human MDSCs are composed of a mixed immature monocyte and granulocyte population and hence we can also divide MDSC into subtypes.

Similarly to mouse MDSCs, human MDSCs can be further subdivided into monocytic ($CD14^+CD15^-CD66^-$) and granulocytic ($CD14^-CD15^+CD66^+$) fractions [184]. CD14 is expressed at ten times higher concentration on monocytes than neutrophils, so it is used as a marker of the monocytic fraction. CD15 and CD66 are surface marker of neutrophils, so they are only present on the granulocytic fraction. Interestingly, in human MDSCs we identify a third subtype, early-stage MDSCs, which are considered to be the least differentiated MDSCs ($CD14^-CD15^-CD66^-$) [184]. Hence, all of the surface markers used in the identification of MDSCs are found only on myeloid cells. To reiterate, MDSCs in mice are a subset of immature myeloid cells with Gr-1 and CD11b surface markers, but not all immature myeloid cells are MDSCs.

As MDSCs are a mixed population of cells, it is unlikely that different cell types contribute equally to their immunosuppressive activity. Many attempts have

been made to further subdivide the MDSCs. Many mouse models have been generated to identify a subpopulation of MDSCs that is responsible for tumor survival. In renal cancer and melanoma the granulocytic MDSCs, and not the monocytic MDSCs, appear to correlate with disease outcomes [185, 186]. However, in other cancers the monocytic MDSCs modulate pro-tumor effects [187]. In HNSCC, one report speculated that the granulocytic MDSCs play a more significant role due to a correlation with advanced clinical stage and poor prognosis [175]. Nevertheless, in order not to overdraw our conclusions we have to take into perspective the small size of the study (n=32) and the fact that all the patients were of Japanese ethnicity. A larger study needs to be performed to draw any substantial conclusions. Hence, it is not clearly established which part of the MDSC population modulates the immunosuppressive effect.

1.5 Overview

1.5.1 Chapter 2 Hypothesis

P120CTN is a tumor suppressor commonly dysregulated in HNSCC and Esophageal Squamous Cell Carcinoma (ESCC) and its loss correlates with poor survival [117, 121]. P120CTN loss in the mouse oral/esophageal epithelium leads to tumor formation with concurrent MDSC infiltration in these tissues [6]. MDSCs are frequently upregulated in many cancers including HNSCC and their immunosuppressive ability is thought to promote immune evasion. The importance of MDSCs in tumor promotion is emphasized by tumor regression

upon MDSC inhibition [188]. We hypothesized that loss of P120CTN in epithelial cells leads to a local MDSC increase in P120CTN-null tissues via process of recruitment or retention. *Chapter 2* describes experiments testing the effects of P120CTN-null epithelial cells on MDSC survival, activation and migration. We also investigate the MDSC increase in the P120CTN-null mouse model at two different stages of disease development. Finally, we analyze MDSC subsets present in the P120CTN-null mouse.

1.5.2 Chapter 3 Hypothesis

In the P120CTN-null mouse model, the oral tumors display minimal invasion, which does not recapitulate the human condition of HNSCC. While P120CTN may create an environment conducive for cancer, it may need an oncogene to induce a more aggressive HNSCC phenotype that resembles human HNSCC. We decided to investigate the role of mutant PIK3CA, the most commonly mutated oncogene in HNSCC, in combination with P120CTN downregulation *in vitro*. We have utilized two PIK3CA activating mutations, E545K and H1047R. *Chapter 3* describes experiments examining the cooperation between P120CTN downregulation and *PIK3CA* mutations in regulating anchorage-independent growth, migration and invasion. We study MMP1, the common target of aberrant P120CTN and PI3K signaling, and its effects on cell invasion. Finally, we verify our *in vitro* results using the TCGA gene expression database of HNSCC tumor samples.

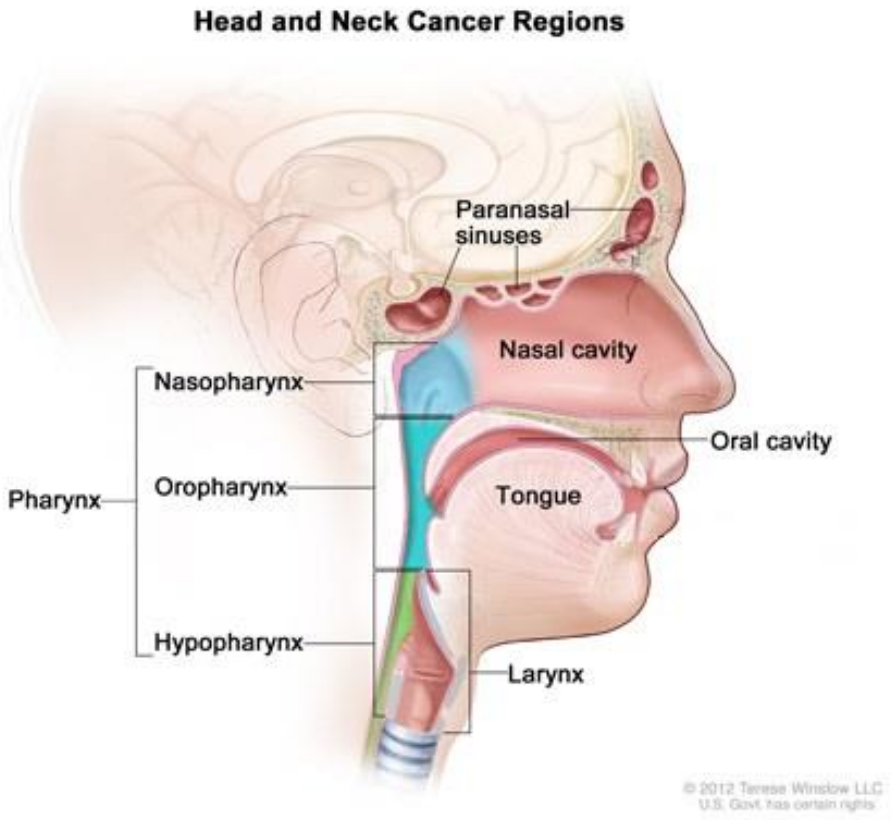

Figure 1.1 Anatomical locations of HNSCC. Adapted from National Cancer Institute. Head and Neck Cancer—Patient Version. Available at: https://www.cancer.gov/types/head-and-neck. Accessed on 3/21/17

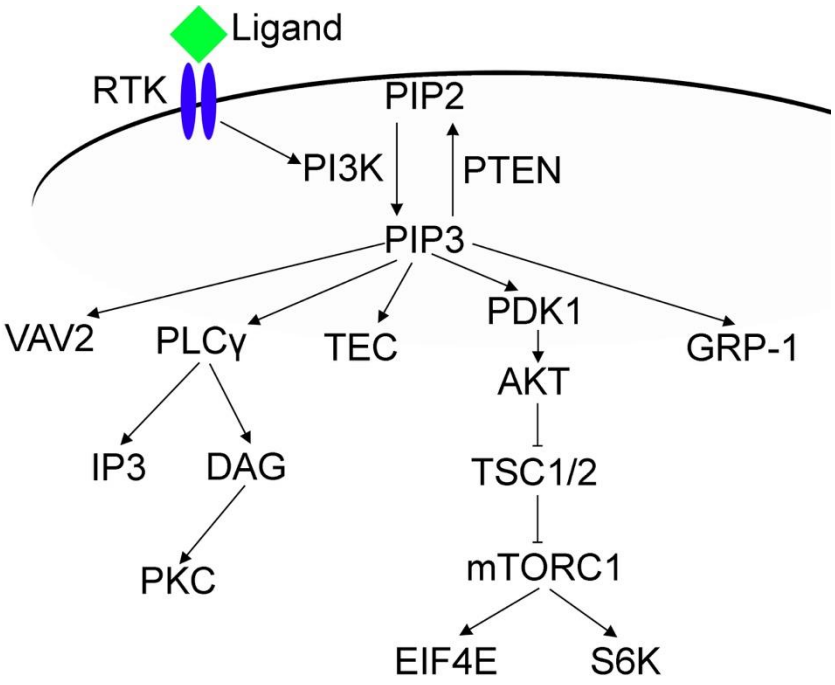

Figure 1.2 The PI3K signaling pathway.

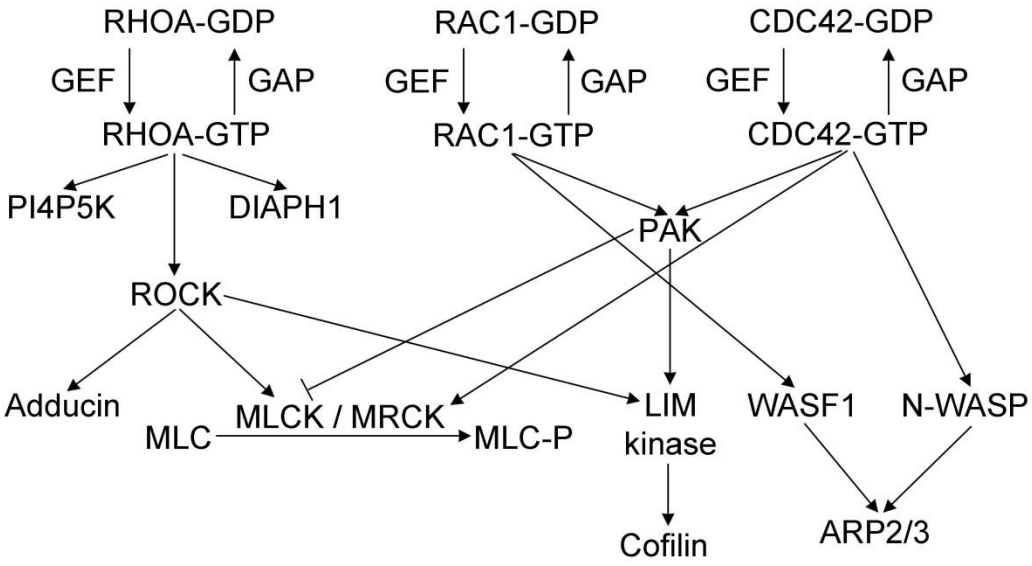

Figure 1.3 Signaling pathway of the RHO-family of GTPases.

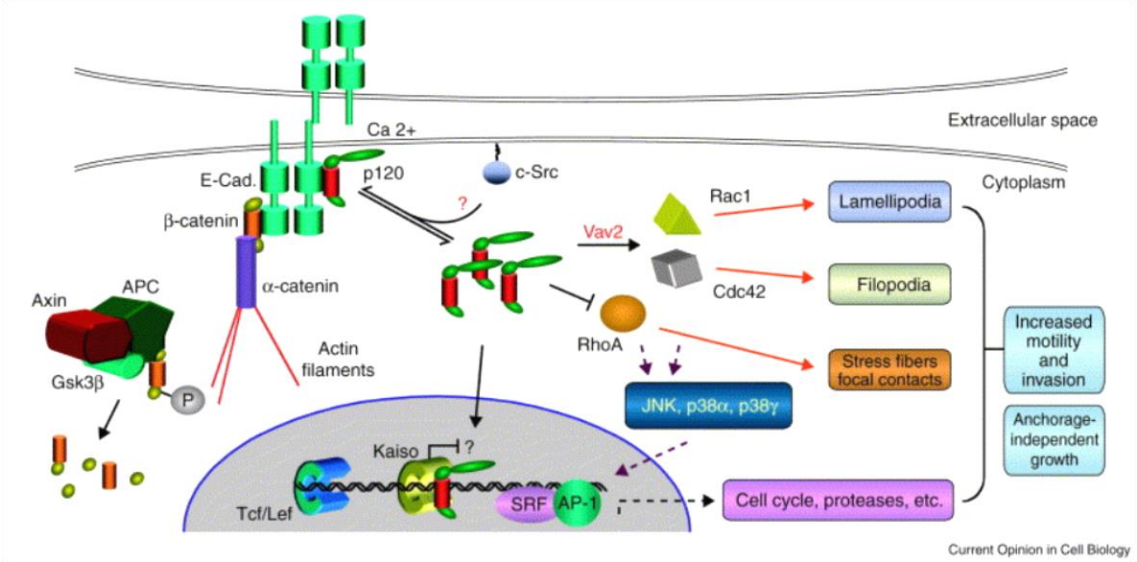

Figure 1.4 P120CTN modulates RAC1, CDC42 and RHOA activity. Adapted with permission from Anastasiadis, Panos Z., and Albert B. Reynolds. "Regulation of Rho GTPases by p120-catenin." *Current opinion in cell biology* 13.5 (2001): 604-610.

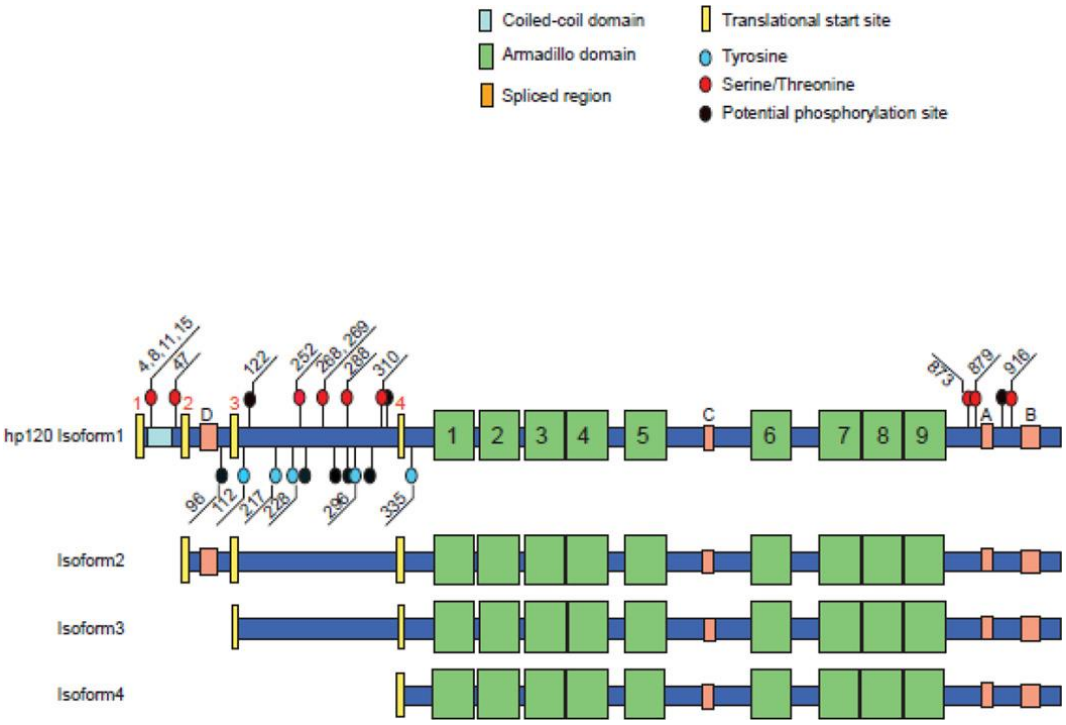

Figure 1.5 P120CTN isoforms and phosphorylation sites. Adapted with permission from Hong, Ji Yeon, Il-Hoan Oh, and Pierre D. McCrea. "Phosphorylation and isoform use in p120-catenin during development and tumorigenesis." *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research* 1863.1 (2016): 102-114.

Chapter 2

p120-Catenin Loss Increases Myeloid-Derived Suppressor Cells in All Organs

2.1 Introduction

2.1.1 Myeloid-derived Suppressor Cells

Myeloid-derived Suppressor Cells (MDSCs) were first discovered in the 1980s in mouse cancer models that demonstrated a rise of immature myeloid cells in the bloodstream. These cells were later determined to have an immunosuppressive capability speculated to aid tumors in immune evasion [177-179]. MDSCs aid tumor cells via production of Arginase and Reactive Oxygen Species, which reduce T cell recognition and limit the cytotoxic killing of tumor cells [171]. Moreover, MDSCs limit T cell proliferation via production of immunosuppressive cytokines including, Interleukin-10, Transforming Growth Factor β, and Indoleamine-2,3-dioxygenase [180-182]. MDSCs have been reported to aid many different cancers in immune system evasion, including head and neck squamous cell carcinoma (HNSCC) [173-176]. Furthermore, studies show that as the tumor grows, more MDSCs accumulate at the tumor site [5]. This suggests that MDSCs may be instrumental in tumor progression or that the accumulation of MDSCs is a result of the increased inflammation present at the tumor site.

2.1.2 MDSC subsets

Since MDSCs are a very heterogeneous group of cells, many attempts have been made to identify a more specific subset of these cells that could be responsible for their tumor-promoting effects. The initial attempts were aimed in

differentiating whether the two main constituents of MDSCs, immature granulocytes and immature monocytes, have different activity in the background of cancer. In renal cancer and melanoma, the increase in granulocytic MDSCs, and not the monocytic MDSCs, correlated with negative outcomes [185, 186]. However, in other cancers, the monocytic MDSCs produced the pro-tumor effects [187]. These findings demonstrated that a crude division based on cell morphology is not enough to identify the active MDSC population. Hence, an approach towards specific surface factors was undertaken by the field. A variety of surface factors such as Platelet Endothelial Cell Adhesion Molecule 1 (CD31), a member of the B7 family – B7-H4, Colony Stimulating Factor Receptor 1 (CD115), and Interleukin 4 Receptor α (CD124) have been independently shown to be the surface markers of the active pro-tumor subset of MDSCs in the setting of different cancers [170, 189-191]. This indicates that tumors preferentially upregulate specific MDSC subsets. It is still unknown which subsets are increased in different cancers and what are the differences between the subsets. Identification of a specific surface marker unique to HNSCC MDSCs would aid in the design of better treatments targeting the active MDSC population.

2.1.3 p120-Catenin

p120-catenin (P120CTN) is classically described as a structural protein that stabilizes cadherin at the cell membrane [125]. In recent years; however, P120CTN has been shown to play the role of a tumor suppressor. Loss or

mislocalization of P120CTN alters negatively patient outcomes in many cancers such as skin, bladder, breast, and esophageal cancer to name a few [118-120, 192]. Loss of P120CTN in a variety of organs leads to the development of chronic inflammation [112-115, 193]. In a mouse model of HNSCC and esophageal squamous cell carcinoma (ESCC), P120CTN loss in the squamous epithelia of the oral cavity, esophagus, and forestomach induces tumor development at these tissues [6]. These mice express L2Cre promoter, which is an EBV promoter that drives the expression of Cre recombinase in the squamous epithelia of the oral cavity, esophagus and the forestomach. Mice develop tumors at 9-12 months of age, with phenotype penetrance of >80% in both males and females. The tumors display a large amount of inflammation with minimal invasion in the underlying tissue. Moreover, P120CTN-null tumor-bearing mice display increased levels of MDSCs in the P120CTN-null organs. It is still unclear how P120CTN loss in the epithelium leads to an increase in tumor MDSCs.

Epithelial cells have been shown to recruit MDSCs and sequester them via various cytokines. Increases in secreted proteins such as Chemokine (C-X-C motif) Ligand 2 (CXCL2), C-C Motif Chemokine Ligand 2 (CCL2), Interleukin 8 (IL-8), C-C Motif Chemokine Ligand 15 (CCL15), C-C Motif Chemokine Ligand 26 (CCL26), Tumor Necrosis Factor α (TNFα), Urokinase (uPA), and Damage Associated Molecular Patterns (DAMPs) result in a higher amount of resident tumor MDSCs [194-201]. Moreover, P120CTN loss can affect gene transcription

through activation of Kaiso, Glis2, and REST/coREST, transcriptional repressors that are normally inhibited by P120CTN [136, 137, 202]. Therefore, it is possible that P120CTN-loss in the mouse epithelium could modulate cytokine expression through one of the associated transcriptional repressors. The increase in expression of cytokines could be the mechanism by which MDSCs are recruited to the P120CTN-null area to aid tumor progression. Additionally, the cytokines expressed in the P120CTN-null tissues could preferentially improve MDSC survival or activity at those sites. Hence, we aimed to investigate which of these three processes occurred at the site of P120CTN loss.

2.2 Methods and Materials

2.2.1 P120CTN-null mouse model

The Institutional Animal Care and Use Committee (IACUC) at the Penn State University Hershey Medical Center approved all animal studies. Mice were housed under a 12hr light/dark cycle and fed *ad libitum*. *L2Cre;P120CTN*$^{Loxp/Loxp}$ mice were a kind gift of Anil Rustgi [6]. Mice were genotyped for the *L2Cre* and the *LoxP* allele as described previously [6]. Control mice for the purpose of our experiments were either *P120CTN*$^{Loxp/Loxp}$ or *L2Cre*;*P120CTN*$^{Loxp/-}$. Tumor-bearing mice were sacrificed at 3-9 months of age when they developed systemic signs of inflammation, as indicated by dermatitis on the face and neck of the animal, which correlates with tumor development. Both sexes of mice were used for all the experiments. Mice were sacrificed using CO_2 gas.

2.2.2 Cell lines

M2 cells were generated by Doug Stairs, Ph.D. by isolating esophageal keratinocytes from male P120CTN$^{Loxp/Loxp}$ mice. M2 cells were then modified in the same manner as F2 cells described previously [6]. M2Neo cells express an empty vector, while M2Cre cells express *nlsCre*, *Cre* recombinase with the attached nuclear localization signal, resulting in P120CTN loss. M2Neo and M2Cre cells were grown in keratinocyte serum-free medium (KSFM; Invitrogen) supplemented with 40 µg/mL bovine pituitary extract (Invitrogen), 1.0 ng/mL EGF

(Invitrogen), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) and maintained at 37°C and 5% CO2.

2.2.3 Western blot analysis

M2Neo and M2Cre cells were plated at $5x10^5$ cells/100 mm dish in KSFM. Cells were harvested after 72 hours and incubated in lysis buffer (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, and protease inhibitors). Protein concentrations were determined and Western blotting was performed as described in [203].

2.2.4 Antibodies

The antibody against P120CTN (#610134) was purchased from BD Transduction Laboratories. Beta-actin (#A5316), used as a loading control for immunoblotting, was purchased from Sigma-Aldrich Corp. Antibodies for CD124-PE (#561695), CD80-BV421 (#562611), CD31-PerCP-Cy5.5 (#562861), and CD11b-FITC (#553310) were purchased from BD. The antibody for CD115- PE-Cy7 (#25-1152-80) was purchased from Ebioscience. The antibody for CD274-APC (#124311) was purchased from Biolegend. The Gr-1-PE antibody (#130-091-932) was purchased from Miltenyi.

2.2.5 MDSC isolation

The MDSC population used for experiments was defined as Gr-$1^+$$CD11b^+$ cells. Splenocytes and bone marrow cells were isolated from control and

P120CTN-null mice. Spleens were crushed through a 40μm sieve using a sterile syringe plunger. Bone marrow was derived from femurs by isolating the femoral diaphysis and flushing the medullary cavity with PBS (Hyclone). Cells were resuspended in MACS BSA buffer (1X PBS (Hyclone; GE Healthcare Life Sciences) pH 7.2, 0.5% Bovine Serum Albumin ((Fisher), 2mM EDTA (Fisher)) and incubated with anti-Gr-1-PE antibody for 15 minutes in the dark at 4ºC followed by an incubation with anti-PE magnetic beads (Miltenyi) for 15 minutes in the dark at 4ºC. Cells were then passaged through a magnetic column twice. After isolation the purity of the isolate was confirmed using flow cytometry staining for Gr-1 and CD11b epitopes. MDSCs were then used immediately in experiments or frozen temporarily.

2.2.6 Flow cytometry

$2.5x10^6$ Splenic and bone marrow MDSCs that were isolated as described in *MDSC isolation* were stained with fluorophore-conjugated antibody mix for 15 minutes at 4°C in the dark. The antibody mix contained Gr-1-PE, CD11b-FITC, CD124-PE, CD80-BV421, CD31-PerCP-Cy5.5, CD115-PE-Cy7, and CD274-APC that were diluted in MACS BSA buffer. After staining, cells were washed with MACS BSA buffer and flow cytometry was performed using a a Becton-Dickinson 17-color LSR II.

2.2.7 MDSC survival and activation analysis

$4x10^5$ MDSCs isolated from spleens of control mice were plated in 24-well plates containing conditioned medium from M2Neo or M2Cre cells or non-conditioned medium. The conditioned medium was derived by plating $5x10^5$ M2Neo or M2Cre cells in 8mL of 10% Fetal Bovine Serum (FBS; HyClone; Thermo Scientific) Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Inc.) medium in a 100mm dish and culturing for 24 hours. The conditioned medium was collected and sterile filtered to remove cell debris and any cells present in the supernatant. MDSCs were cultured in the M2Neo-, M2Cre- or non-conditioned medium for 24, 48, 72, and 96 hours and then cells were aspirated to assay for survival and activation. MDSC survival was assessed by staining with trypan blue and counting the cells using the Countess Cell Counter (Invitrogen). After being counted the cells were stained for a marker of activation, CD124, and flow cytometry was performed using a Becton-Dickinson 17-color LSR II to evaluate the amount of $CD124^+$ cells.

2.2.8 MDSC isolation from tissues

Control and P120CTN-null mice were sacrificed at 3 and 9 months of age. Esophagus, forestomach, spleen, colon, brain, pancreas, lung and kidney were isolated. Spleens were isolated as previously described in the *MDSC isolation* section. All the other tissues were weighed and equal weights were used for further dissociation. Tissues were chopped and dissociated in 1mg/ml of

collagenase type 1 (EMD Millipore) in DMEM for 1hour and 10min at 37°C in a rotator. Larger undigested cell chunks were allowed to sediment to the bottom of the tube during a minute incubation and the supernatant containing the single cells was transferred to a separated tube. Undigested tissue chunks were incubated with 0.25% Trypsin, 2.21 mM EDTA in HBSS (EMD Millipore) for 5 minutes at 37°C. Chunks were vortexed every 60 seconds. Previously collected single cell supernatants and single cells from trypsinized chunks were pooled together and washed twice with MACS BSA buffer. To remove cell debris, cells were passaged through 100µm and 40µm sieves. Cells were stained with antibody cocktails and flow cytometry was performed as described in the *Flow cytometry staining* section. 7-Aminoactinomycin D (7AAD) was added to each sample 10 min before flow cytometry to exclude cells without an intact cell membrane from analysis.

2.2.9 MDSC migration assay

Boyden-chamber migration assays were performed using M2Neo- and M2Cre-conditioned medium as a chemoattractant at the bottom of each well. $7.5x10^5$ MDSCs were placed in the Boyden-chamber inserts in 2.3% FBS DMEM (FBS; HyClone; Thermo Scientific) Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Inc.). MDSCs were allowed to transmigrate through the membrane in the Boyden-chambers for 4 hours and then cells were collected from the bottom

of each well and stained with trypan blue and counted in the Countess Cell Counter (Invitrogen) to assess the amount of migration.

2.2.10 Statistical Analysis

A two-tailed Student's t-test was used for determining significant differences between groups unless otherwise mentioned. ANOVA with Sidak-Holm post-hoc analysis was used for determining significance for multiple comparisons done in MDSC surface marker analysis.

2.3 Results

2.3.1 Isolated MDSCs contain more than 90% of CD11b$^+$ Gr-1$^+$ cells

MDSCs were isolated from tumor-bearing P120CTN-null mice for use in experiments assessing MDSC surface markers and to characterize the effects of P120CTN-null epithelium on MDSCs. MDSCs from the spleens of tumor-bearing P120CTN-null mice were isolated using the Gr-1 marker as previously described [6]. Magnetic isolation using anti-Gr-1 antibody from tumor-bearing P120-CTN null mice resulted in a 90% Gr-1$^+$ final population raising our working concentration of Gr-1$^+$ cells to be significantly higher than unisolated cell ($p<0.05$) (Figure 2.1A). Moreover, flow cytometry analysis confirmed that almost all of the Gr-1$^+$ cells (95%) isolated also express CD11b$^+$, which confirms their status as MDSCs (Figure 2.1B).

2.3.2 MDSC activation surface markers are not changed significantly in P120CTN-null mice

In the P120CTN-null mice we observe the development of oral, esophageal, and gastric tumors with a concurrent increase in MDSCs [6]. In many tumor models, a specific subset of MDSCs can be responsible for the immunosuppressive activity we assign to all MDSCs. Identification of the active MDSC subset would allow for the development of more specific treatments while limiting off-target effects. We decided to test whether the surface markers identified by other groups to be specific to the active subset of MDSCs also apply

in our mouse model. We measured CD31, CD80, CD115, CD124, and CD274 surface marker levels in control and P120CTN-null mice to evaluate whether these surface markers are significantly changed.

We isolated MDSCs from the spleens and bone marrows of control P120CTN-wild-type and tumor-bearing P120CTN-null mice (n=3). Tumor-bearing mice were selected based on signs of systemic inflammation, which in our model was strongly associated with the presence of a tumor. In the P120CTN-null mouse model, the loss of P120CTN induces a very strong inflammatory response in the P120CTN-null tissues and the effects can be seen on adjoining tissues. However, tumor development only occurs in the P120CTN-null tissues, so while other tissues experience increased inflammation, the tumor-promoting effects only occur in the P120CTN-null tissues. This is most likely directly related to the loss of P120CTN, which is a tumor suppressor. P120CTN-null mice loose facial hair and develop scaly pruritic rashes with concurrent development of oral tumors. Consequently, the development of tumors in this mouse model is frequently correlated with outward symptoms, which allow us to select mice with the most advanced disease. Control mice did not experience any inflammation in the head and neck area. Isolated MDSCs were stained for CD124, CD80, CD31, CD115 and CD274, surface factors suspected of being elevated. Flow cytometry demonstrated no statistical difference between the amount of expression of any of the tested surface markers between splenic MDSCs and bone marrow MDSCs

of control and tumor-bearing mice (Figure 2.2A). Splenic MDSCs are measured as a representative of circulating MDSCs, while bone marrow represents mobilization of early MDSCs. Hence, we conclude that P120CTN-loss in the oral and esophageal epithelia does not significantly alter many previously identified activation markers in splenic and bone-marrow MDSCs in the P120CTN-null mouse model.

2.3.3 P120CTN-null epithelium does not promote MDSC survival, activation or recruitment

P120CTN-null mice have increased levels of MDSCs at the P120CTN-null organs [6]. We wanted to investigate whether P120CTN loss in the epithelium can directly affect MDSC numbers in the surrounding stroma. We investigated the effects of P120CTN-null epithelium on MDSCs *in vitro* by using mouse esophageal keratinocytes, M2 cells, as oral mouse keratinocytes were unavailable and esophageal keratinocytes are the most closely related cell type. M2Neo cells have a wild-type expression of P120CTN, while M2Cre cells have a complete loss of P120CTN (Figure 2.3A). We hypothesized that P120CTN-null epithelium increases both the active recruitment and the cell survival of MDSCs. A protein array was done to analyze the difference between M2Neo and M2Cre secreted proteins (Table 2.1). In support of our hypothesis, M2Cre cells secreted many more cytokines, some of which (Interleukin-4 (IL-4), Granulocyte macrophage colony-stimulating factor (GM-CSF), Interleukin-3 (IL-3)) have been

shown to promote MDSC activity and survival [204-206]. Given these results, we tested the effects of M2Neo and M2Cre secreted proteins on MDSC CD124 (marker of MDSC activation) levels and survival. Cultures with either M2Neo or M2Cre conditioned medium significantly increased CD124 levels and cell counts of MDSCs compared to MDSCs cultured in non-conditioned medium over 4 days of culture ($p<0.05$) (Figure 2.3B,C). No difference was observed between MDSCs cultured in M2Cre-conditioned medium and M2Neo-conditioned medium. MDSC counts indicate that MDSCs survive better and become more activated due to proteins secreted by epithelial cells in general, but the P120CTN-null epithelium does not improve these effects further.

Our experiments suggest that retention of MDSCs at the P120CTN-null site is most likely not the mode by which MDSCs increase in P120CTN-null tissues. Therefore, we tested whether MDSCs could be directly recruited by P120CTN-null epithelium. This was accomplished using a Boyden-chamber migration assay to determine MDSC migration towards non-conditioned medium, M2Neo-conditioned medium or M2Cre-conditioned medium. MDSCs in the M2Neo-conditioned medium chambers migrated significantly more than MDSCs in M2Cre-conditioned media ($p<0.05$) and MDSCs in non-conditioned media ($p<0.05$) (Figure 2.3D). Interestingly, unlike in previous experiments where M2Neo- and M2Cre-conditioned media did not differ in effect on MDSCs, our results suggest that P120CTN-null epithelia are not as efficient as P120CTN-

wild-type epithelia in inducing MDSC migration. These results suggest that P120CTN-null epithelium does not recruit MDSCs nor does it increase MDSC survival and activation better than P120CTN-wild-type epithelium.

2.3.4 MDSC increase in P120CTN null animals is not organ-specific and escalates with the progression of the disease

In vitro experiments demonstrated that the P120CTN-null epithelium does not directly recruit MDSCs nor does it increase MDSC survival or activation. Therefore, we investigated whether the observed increase in MDSCs in P120CTN-null mice is systemic or if there is an increased migration of MDSCs localized specifically at the P120CTN-null tissues. Previously, it has been demonstrated that tumor-bearing P120CTN-null mice experience MDSC increases in the esophagi and spleens [6]. Mice used for this experiment were sacrificed at either 3 months (no sign of tumor development yet) or 6 to 9 months of age (tumor with scaly rash present). We isolated esophagus, forestomach, spleen, colon, brain, pancreas, lung, and kidney from control P120CTN-wild-type and P120CTN-null mice. Organs were crushed and dissociated, and flow cytometry was performed to analyze the amount of MDSCs present in each organ. Increases in MDSCs can be seen in some organs at 3 months of age. We expected P120CTN-null tissues, such as the esophagus and forestomach, to display an increase, as it would demonstrate that prior to tumor formation these tissues have increased levels of MDSCs. Conversely, these tissues in non-tumor-

bearing P120CTN-null mice did not display any increase. Interestingly, the kidneys displayed a significant increase in resident MDSCs in non-tumor-bearing P120CTN-null mice as compared to control P120CTN-wild-type mice ($p<0.05$) (Figure 2.4A). One explanation for the specific increase in MDSCs observed in the kidneys is due to the high blood content, as blood was not removed from all of the tissues prior to organ isolation. To confirm this supposition, future studies can acquire blood samples from P120CTN-null and P120CTN-wild type mice to compare MDSC levels. We speculate that most likely the MDSC increase observed in P120CTN-null mice prior to disease development happens in the bloodstream first. Furthermore, other organs that contain large volumes of blood, such as lungs and spleens, are also trending in the same direction as kidneys with respect to MDSC counts.

Next we analyzed MDSC levels in adult 6 to 9 month-old mice. We discovered that MDSC levels increase significantly in all of the organs isolated in tumor-bearing P120CTN-null mice as compared to control P120-CTN-wild-type mice ($p<0.05$) (Figure 2.4B). This finding provides evidence that the MDSC increase observed in P120CTN-null organs of tumor-bearing mice is not specific to these tissues only. With the progression of the disease, all organs experience an increased amount of MDSCs. Hence, we conclude that P120CTN loss increases MDSC levels in all of the tissues of P120CTN-null mice.

2.4 Discussion

2.4.1 MDSC subsets

An increase in immature myeloid cells, now known as MDSCs, was first noticed over 30 years ago in mouse cancer models [177, 179, 207]. Since then, scientists have identified that mouse MDSCs are a set of immature myeloid cells in the monocytic or granulocytic lineage with CD11b and Gr-1 surface markers [171]. Additional surface markers of MDSCs have been further characterized in certain cancer models [170, 190, 204, 208-211]. The presence of these markers identifies the active MDSC population, which is broadly categorized as having an effect on tumor growth, promoting regulatory T cells and inhibiting cytotoxic T cells. We understand that these activities affect different mechanisms involved in tumor immune evasion, but the literature is limited with respect to the amount of MDSC surface factors that have been discovered. Additionally, it is important to note, that these MDSC activation surface factors could be specific for each disease state and cancer type. It is possible that MDSCs may not have a universal surface factor, which could be used to identify the active subset of MDSCs in all cancers. However, new surface factors of MDSCs are of critical importance. Gr-1 and CD11b markers are found on many other cells of myeloid origin. Unfortunately, as far as specific inhibition of MDSCs, anti-Gr-1 antibodies are the mainstay of the experimental approach in this field. We need new targets to limit the off-target effects of our treatments and to be able to study MDSC in a

more rigorous manner to generate results that can translate more readily to humans. Our findings show no differences between the surface markers CD124, CD80, CD31, CD115, and CD273 in control P120CTN-wild-type and tumor-bearing P120CTN-null mice. While these markers have been shown to be increased in many cancers, our *in vivo* model was unable to replicate these findings. In support of our findings, the Gabrilovich lab analyzed many of the same surface markers our lab studied and demonstrated that the MDSC subpopulations, identified by CD124, CD80, CD115, CD273 and CD274, did not define a more active subset [212]. Based on the results from our lab and others, the surface markers identifying active MDSCs in cancer mouse models might not be transferable. For unknown reasons, many of these markers are uniquely specific to their cancer type. Understanding of the mechanism of action of how different MDSC subpopulations occur in the setting of different cancers could aid in delineating the disease progression more closely. We can speculate, that various tumors secrete differing combinations of cytokines that vary in effects on the bone marrow production of MDSCs. Further investigation into why these differences occur could allow us to develop therapeutics that specifically target and inhibit the active MDSC subpopulations. Additionally, we have not performed our subset analysis using tumor-resident MDSCs. B7-H3^{+} MDSCs have been reported to be found exclusively in the tumor environment [213]. It is possible that some of the studied surface markers are not significantly increased when we

study splenic and bone-marrow MDSCs. Isolation of stromal MDSCs could yield a different result since the effects of local environment on the MDSCs are more pronounced.

2.4.2 MDSC increase

Additionally, the experiments described in this chapter have been performed on a mixed C57BL/6:129 mouse background. While it may have been ideal to study the P120CTN-null mouse model on a pure C57BL/6 background, we found that in these mice the tumor penetrance decreased and the tumor latency nearly double when compared to what our lab previously observed [6]. These results suggest that the genetic background of the mice plays a significant role in tumorigenesis resulting from P120CTN-loss. Mice from the mixed C57BL/6:129 background have higher rates of autoimmunity [214]. This is explained by pointing to loci located on chromosome 1, which are related to lupus development. However, we have to also consider that this study was designed specifically to look at antinuclear antibodies and the formation of other inflammation-related effects, such as MDSC modulation, was not analyzed. Therefore, we don't know which part of the genome from the 129 mice is responsible for the increased propensity for inflammation. We, however, speculate that the propensity for an exaggerated immune response could possibly be related to the induction of MDSCs. MDSCs are commonly induced by inflammation; hence, it stands to reason that mice with exaggerated immune

responses would elicit greater MDSC increases. Furthermore, understanding how P120CTN-loss activates the immune response in the context of different genetic backgrounds could aid in identifying genes that could partake in MDSC mobilization.

The results we observed from the tissue dissociation experiments support our previous findings relating to P120CTN-null epithelium. Specifically, the epithelium is unable to directly recruit or induce activation or enhance survival of MDSCs. While other studies demonstrate that MDSCs can be recruited directly to the tumor site by a specific cytokine, our results show that in our model system, the increase in MDSCs that had been originally identified in P120CTN-null tissues is actually a system-wide expansion of the MDSC population. Our results also suggest that P120CTN-null epithelia are less likely to increase MDSC levels specifically because we did not observe any increases in survival or activation of MDSCs in the P120CTN-null epithelium. It is possible that another cell type, which becomes induced in response to P120CTN loss in the epithelium, propagates the general inflammation and is responsible for the systemic MDSC increase. We know that MDSC increase is tied to the inflammation induced by P120CTN loss. When P120CTN-null mice are treated with dexamethasone, a glucocorticoid with anti-inflammatory properties, a decrease in total MDSCs is observed accompanied by tumor regression [6]. We believe, this finding demonstrates how inflammation is a required component of

MDSC induction in the P120CTN-null mouse model. Unfortunately, dexamethasone is not a very specific drug. We do not know whether the effects on the bone marrow or on the P120CTN-null epithelium are more important. Can dexamethasone inhibit MDSC production in the bone marrow or does it mainly function by decreasing production of inflammatory cytokines in the P120CTN-null tissues? We can speculate it is the latter, but further experimentation is necessary to support that claim. Interestingly, depletion of MDSCs in the P120CTN-null mouse model via anti-CD38 antibody (targeting one of the MDSC surface factors) resulted in a tumor regression [188].This finding indicates that MDSCs are more important for tumor growth than the inflammation occurring at the site of P120CTN loss. In summary, MDSCs are instrumental in promoting tumor immune evasion, but not much is still known about different MDSC subsets or what specifically leads to MDSC increase in the setting of P120CTN loss.

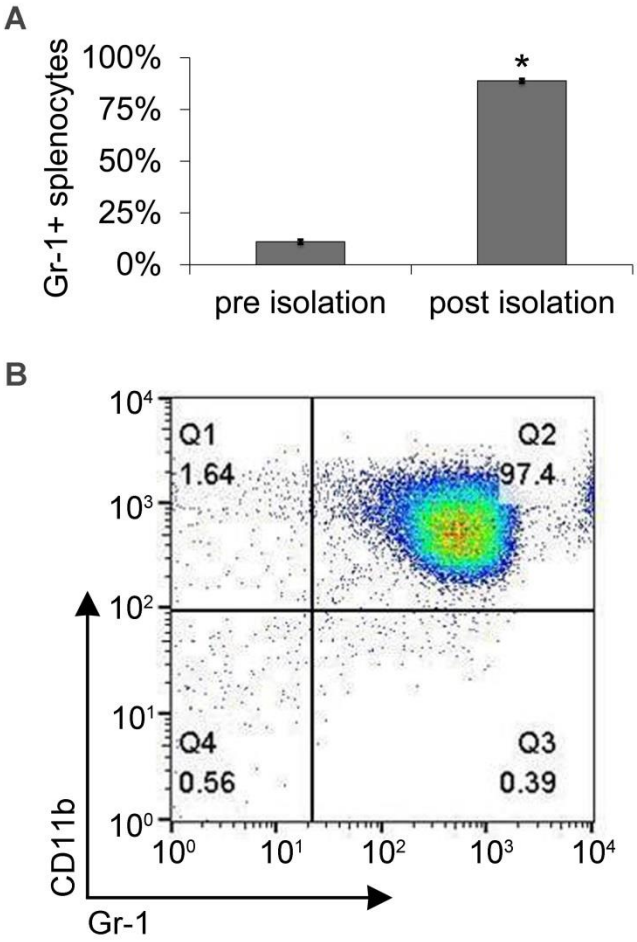

Figure 2.1. Confirmation of MDSC purity after MDSC isolation. A, Quantification of Gr-1$^+$ cells before and after magnetic isolation. **B,** Representative scatter plot depicting that >95% of isolated Gr-1$^+$ cells are also CD11b$^+$. Data presented as means ± SEM. n=6 for all experiments. Student t-test used for statistical analysis. *p<0.05.

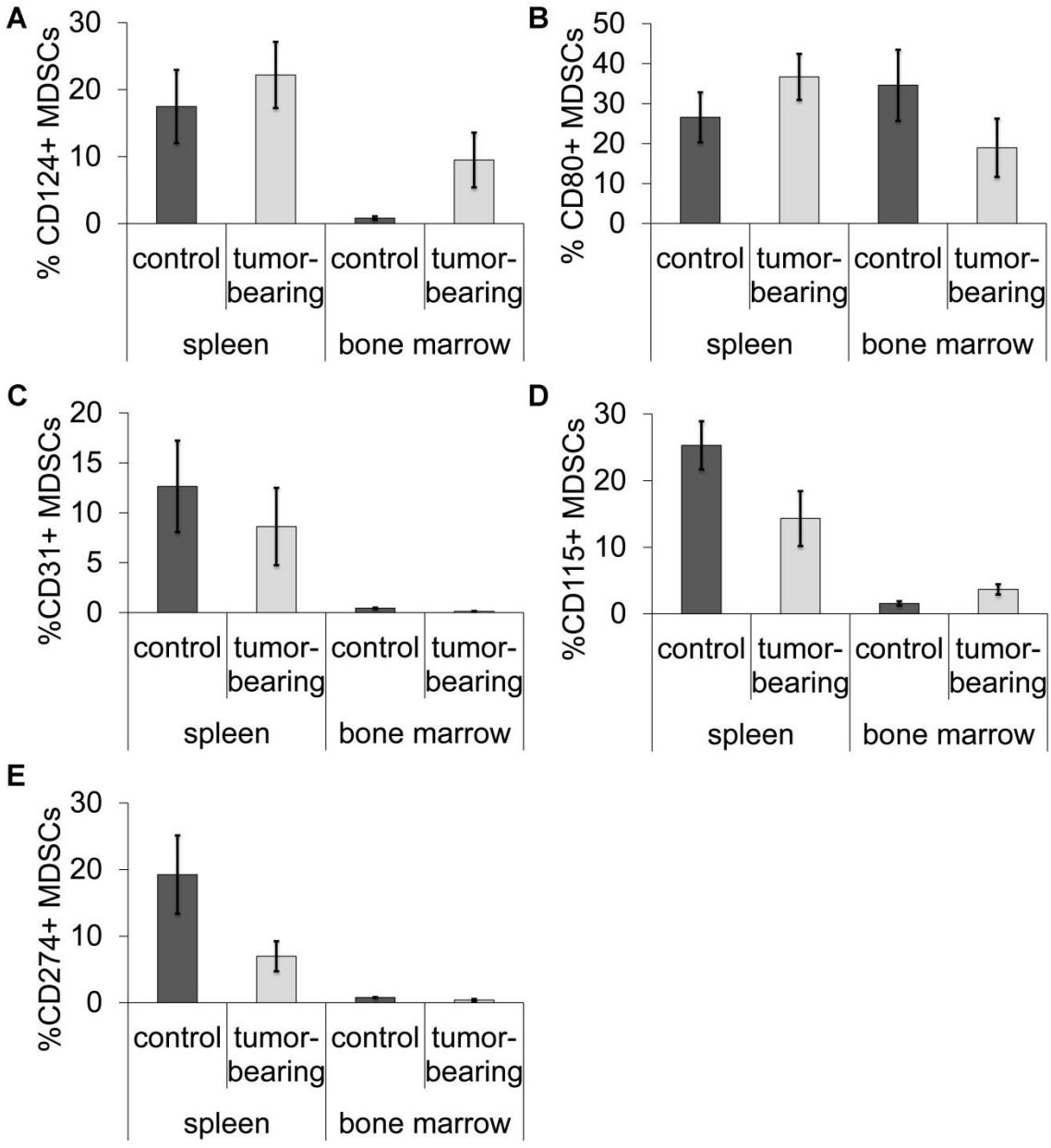
A
% CD124+ MDSCs
30
20
10
0
control
tumor-bearing
control
tumor-bearing
spleen
bone marrow
B
% CD80+ MDSCs
50
40
30
20
10
0
control
tumor-bearing
control
tumor-bearing
spleen
bone marrow
C
%CD31+ MDSCs
20
15
10
5
0
control
tumor-bearing
control
tumor-bearing
spleen
bone marrow
D
%CD115+ MDSCs
30
20
10
0
control
tumor-bearing
control
tumor-bearing
spleen
bone marrow
E
%CD274+ MDSCs
30
20
10
0
control
tumor-bearing
control
tumor-bearing
spleen
bone marrow

Figure 2.2. MDSC surface markers in tumor-bearing mice do not differ significantly from control mice. A, Quantification of CD124, **B,** CD80, **C,** CD31, **D,** CD115, and **E,** CD274 MDSC surface markers in the MDSCs from spleens and bone marrow of control and tumor-bearing mice. Data presented as means ± SEM. n=3. ANOVA used for statistical analysis.

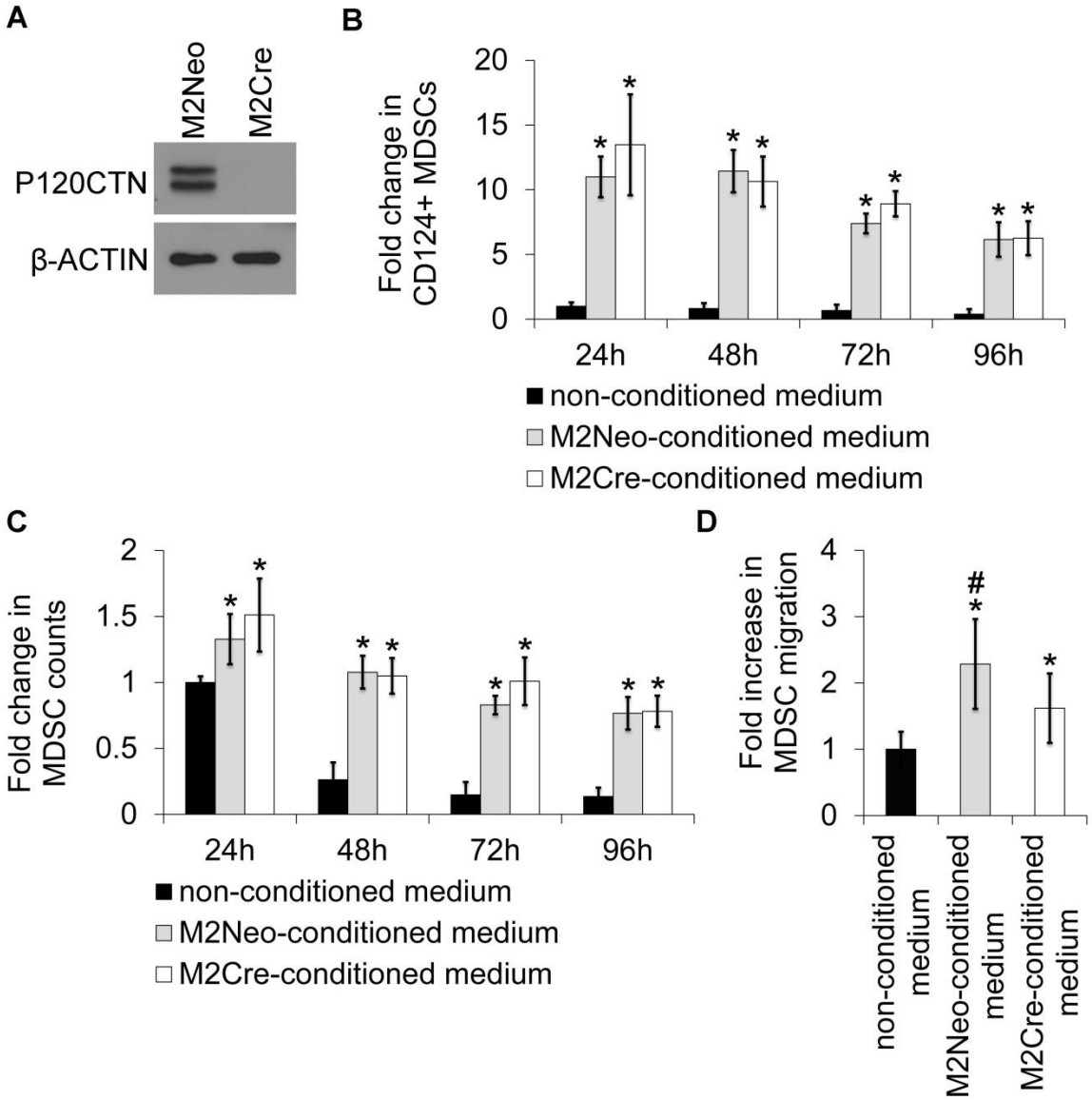
A
M2Neo
M2Cre
P120CTN
β-ACTIN
B
Fold change in CD124+ MDSCs
20
15
10
5
0
24h
48h
72h
96h
non-conditioned medium
M2Neo-conditioned medium
M2Cre-conditioned medium
C
Fold change in MDSC counts
2
1.5
1
0.5
0
24h
48h
72h
96h
non-conditioned medium
M2Neo-conditioned medium
M2Cre-conditioned medium
D
Fold increase in MDSC migration
4
3
2
1
0
non-conditioned medium
M2Neo-conditioned medium
M2Cre-conditioned medium

Figure 2.3. P120CTN loss in keratinocytes does not increase MDSC survival, activation or migration. A, Western blot analysis of P120CTN expression in M2CNeo and M2Cre cells. β-actin used as a loading control. **B,** Quantification of $CD124^+$ MDSCs upon culture in non-conditioned medium or conditioned medium from M2Neo or M2Cre cells. **C,** Quantification of MDSC cell counts upon culture in non-conditioned medium or conditioned medium from M2Neo or M2Cre cells. **D,** Quantification of MDSC migration in Boyden-chamber migration assay in conditioned medium from M2Neo or M2Cre cells. Data presented as means ± SEM. n=3 for all experiments. Student t-test used for statistical analysis. *$p<0.05$ for comparison to non-conditioned medium (B, C, D). #$p<0.05$ for comparison to M2Neo-conditioned medium (D).

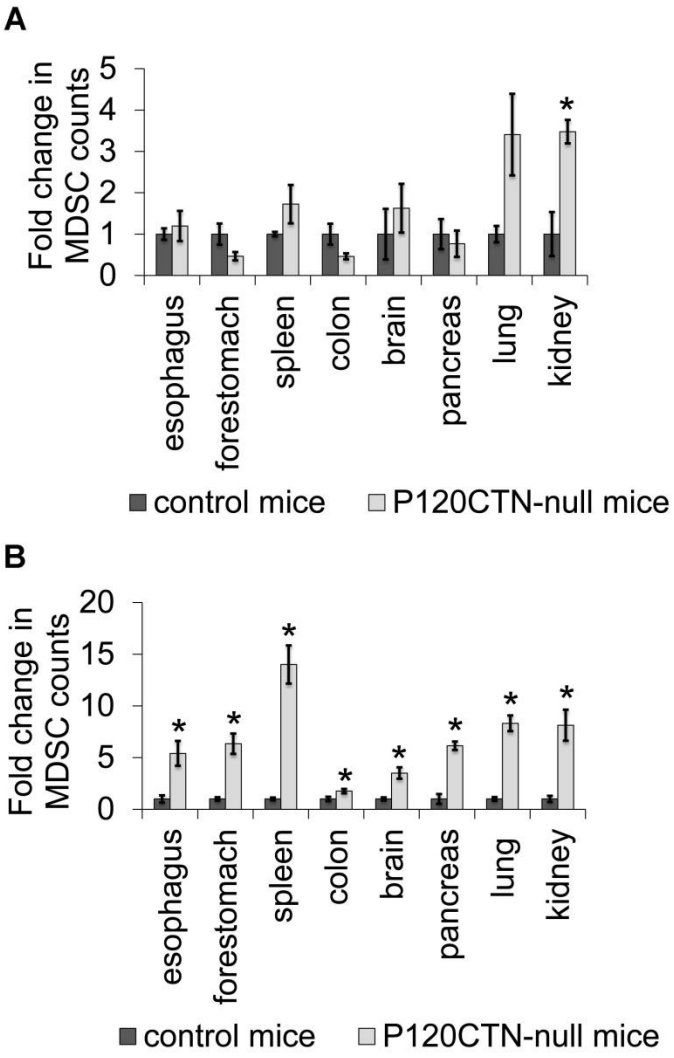

Figure 2.4. P120CTN loss does not specifically increase MDSC counts in P120CTN-null organs. A, Quantification of MDSC levels in 3-month old P120CTN null mice. **B,** Quantification of MDSC levels in 6 to 9-month old P120CTN null mice. Data presented as means ± SEM. n=3 for all experiments. Student t-test used for statistical analysis. *p<0.05 for comparison within the tissue to control mice.

	M2Neo	M2Cre
MIP-1 gamma	1.2	3,321.8
MIP-1 alpha	1.2	1,080.3
RANTES	1.2	978.4
MIP-3 beta	1.2	944.2
RELM beta	1.2	901.0
Insulin	1.2	883.7
MIP-3 alpha	1.2	853.2
Activin C	1.2	813.7
KC	1.2	484.7
IL-1 beta	1.2	475.2
Resistin	1.2	413.3
I-TAC	1.2	406.5
GM-CSF	1.2	404.2
Adiponectin / Acrp30	1.2	382.8
IL-3	1.2	311.9
IL-9	1.2	309.6
TL1A / TNFSF15	1.2	279.1
IL-4	1.2	254.5
IGFBP-6	1.2	207.2
IGF-II	1.2	144.9
IL-17	1.2	77.1
IGF-I	1.2	68.0
S100A10	1.2	41.2
VEGF-D	126.5	800.0
MIG	246.9	1,209.0
IGFBP-rp1 / IGFBP-7	123.0	535.7
Activin A	248.6	841.4

Table 2.1. M2Cre cells demonstrate an increased expression of many cytokines compared to M2Neo cells. Data presented as fold change compared to negative control. n=1.

Chapter 3

p120-Catenin Downregulation and *PIK3CA* Mutations Cooperate to Induce Invasion through MMP1 in Head and Neck Squamous Cell Carcinoma

3.1 Introduction

Head and Neck Squamous Cell Carcinoma (HNSCC) occurs in the squamous epithelia of the head and neck region encompassing the oral and nasal cavities, larynx, and pharynx. While this deadly disease has known premalignant entities, such as leukoplakia, erythroplakia, and dysplasia, molecular mechanisms driving HNSCC disease progression and metastasis are not well known [215]. Every year, half a million people are diagnosed with HNSCC around the world [1, 216]. A tenth of all of those cases occur in the United States, and a fifth of the new patients will present with distant metastasis at the time of diagnosis [217]. The main risk factors contributing to HNSCC are tobacco smoking and alcohol consumption [215]. Unfortunately, the molecular factors predicting decreased patient survival and the mechanisms responsible for that decrease are still largely unknown.

p120-catenin (P120CTN) is a well-established tumor suppressor, which expression is lost or downregulated in 50-60% of the tumors [6, 121]. Additionally, some reports demonstrate that P120CTN is mislocalized from the membrane to the cytoplasm in 75% of Oral Squamous Cell Carcinomas [111]. It is incompletely understood how mislocalization of P120CTN limits its tumor suppressive ability. We know that mislocalized P120CTN no longer sequesters its transcriptional repressors, such as Kaiso, Glis2, and REST/coREST, and it also leads to loss of E-cadherin on the cell surface, but how or whether these

events are tied to its tummor suppressor activity is still unknown. P120CTN expression can be downregulated by tobacco smoke in a MUC1 dependent manner [39]. Moreover, P120CTN loss is correlated with decreased HNSCC patient survival [111]. Unfortunately, the mechanism responsible for the decreased patient survival associated with P120CTN loss is still unknown. To address this gap in knowledge, we previously explored the *in vivo* effects of P120CTN loss in the oral cavity of the *L2Cre;p120*$^{loxP/loxP}$ mouse [6]. We found that P120CTN loss in the murine oral cavity results in the formation of oral tumors. P120CTN loss leads to tumorigenesis in the organ with P120CTN deletion, but the exact mechanism of action is not known. We speculate that since P120CTN is a tumor suppressor, loss of this protein allows tumorigenesis to progress. Furthermore, the tumors that develop due to P120CTN loss exhibited local invasion only. This observation is likely due to single gene alterations often not being sufficient to mimic human carcinogenesis and progression in some mouse cancer models [218]. Human carcinogenesis occurs in the background of multiple gene alterations; hence, the study of multi-gene cooperation should be intensified in the field of cancer research. We previously reported that Epidermal Growth Factor Receptor overexpression and P120CTN downregulation cooperate to promote increased invasion of esophageal squamous cell carcinoma [203]. Moreover, in HNSCC, overexpression of Cyclin D1 alone is not sufficient to induce invasion; however, additional downregulation

of p53 shifts the tumor towards a more invasive phenotype [219]. These data led us to explore the possibility that a similar mechanism occurs with P120CTN in HNSCC. Mutations in the PI3K pathway are the most common aberration involving an oncogenic pathway in HNSCC [220]. Since, both P120CTN loss and *PIK3CA* mutations are frequently found in HNSCC, which increases the likelihood these two events may cooperate to drive disease progression.

We explore the role of P120CTN downregulation in combination with *PIK3CA* mutations as drivers of migration and invasion in oral keratinocytes. To date, no report has investigated the combined effects of P120CTN and PIK3CA. Here, we report that P120CTN and PIK3CA cooperation in oral keratinocytes increases cell migration and invasion. Both of these genetic modifications also increase MMP1. When MMP1 is knocked down, cells with both P120CTN downregulation and *PIK3CA* mutations invade less. Hence, increased MMP1 expression is responsible for the increase in cell invasion due to P120CTN downregulation and *PIK3CA* mutations. Furthermore, analysis of data from HNSCC patient samples, which demonstrates that P120CTN loss and PI3K pathway activation lead to an increase in MMP1 expression, confirmed our *in vitro* findings.

3.2 Materials and Methods

3.2.1 Cell Lines

All cell lines were cultivated as described previously [203]. TRIPZ (Open Biosystems) and pLVX (Clontech) were used to modify h-TERT immortalized cell lines, Normal oral keratinocytes (NOK), a gift of Dr. Karl Munger, and esophageal keratinocytes (EPC1), a gift of Dr. Anil Rustgi [221, 222]. NOK-C cells were generated by infection of parental NOK cells with a pLVX-IRES-Neo vector and a lentiviral inducible scramble shRNA TRIPZ vector (RHS4696-99637087). NOK-P cells were generated by infection of parental NOK cells with a pLVX-IRES-Neo vector and a lentiviral inducible *P120CTN* shRNA TRIPZ vector (V2THS_113295). NOK-545 and NOK-1047 cells were generated by infection of parental NOK cells with a pLVX-IRES-Neo-*PIK3CA*(E545K) or pLVX-IRES-Neo-*PIK3CA*(H1047R) vector and a lentiviral inducible scramble shRNA TRIPZ vector. NOK-545P and NOK-1047P were generated by infection of parental NOK cells with a pLVX-IRES-Neo-*PIK3CA*(E545K) or pLVX-IRES-Neo-*PIK3CA*(H1047R) vector and a lentiviral inducible *P120CTN* shRNA TRIPZ vector. E545K and H1047R PIK3CA mutants were subcloned from pCMV plasmids containing respective mutant PIK3CAs (Addgene), prior to being cloned into the pLVX-IRES-Neo vector. All EPC1 cells were generated by infection with a lentiviral inducible *P120CTN* shRNA TRIPZ vector. EPC1-545 and EPC1-545P cells were generated by infection with pLVX-IRES-Neo-*PIK3CA*(E545K). EPC1-

1047 and EPC1-1047P cells were generated by infection with pLVX-IRES-Neo-*PIK3CA*(H1047R). Down-regulation of P120CTN was induced through 1 µg/ml doxycycline treatment of cells for a minimum of 72 hours. Cell counts and viability assays were performed after three days of cultivation, by harvesting the cells, and staining with trypan blue and counting them in the Countess Cell Counter (Invitrogen). NOK and EPC1 cell counts and viability were then compared to their respective controls, NOK-C and EPC1-C cells.

3.2.2 Western Blot Analysis

NOK and EPC1 cells were plated at $5x10^5$ cells/100 mm dish in 10% FBS DMEM (Dulbecco's Modified Eagle's Medium (DMEM; Mediatech, Inc.) with 10% Fetal Bovine Serum (FBS; HyClone; Thermo Scientific)). Cells were harvested after 72 hours and incubated in lysis buffer (10 mM Tris-HCl at pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 2 mM sodium orthovanadate, and protease inhibitors). Protein concentrations were determined and Western blotting was performed as described in [203].

3.2.3 Anchorage-independent growth assay

A mixture of 1% agar (Fisher) in 10% FBS DMEM at a temperature of 40°C was plated into 60mm dishes and allowed to solidify. $2.5x10^4$ NOK or EPC1 cells were mixed with a mixture of 0.67% agar in 10% FBS DMEM at 40°C and immediately plated over the solidified mixture of 1% agar in 10% FBS DMEM. After the agar containing cells solidified, 10% FBS DMEM was added to the top

and replaced every other day for 30 days. Plates were then stained with a 0.005% crystal violet (VWR), 10% methanol (Fisher) solution and 10 representative images were taken per plate per replicate. Colony number was determined by manual count and cell size was calculated based on measurements made in Photoshop (Adobe).

3.2.4 RhoA-GTP pulldown assay

NOK cells were plated at $1.2x10^6$ cells/150 mm dish in KSFM. Cells were harvested after 72 hours as described in the Western Blot Analysis section. Protein concentrations were determined and 800 µg of NOK lysates were incubated with 50µg Rhotekin-RBD beads (Cytoskeleton) at 4°C on a rotator for 1hr according to manufacturer's instructions (Cytoskeleton). The beads were pelleted, washed and Western blotting was performed as described in [203]. In addition to Rhotekin-RBD beads pulldown blots, whole cell lysate blots were probed for total RhoA as a loading control.

3.2.5 ROCK activity assay

$2.5x10^5$ NOK cells were plated and collected as described in Western Blot Analysis section. Protein concentrations were determined and 50 µg of NOK lysates were mixed with ROCK assay dilution buffer, 75mM $MgCl_2$, and 0.5mM ATP in the provided 96-well plate and incubated for 30min at room temperature (RT) according to manufacturer's instructions (EMD Millipore). Wells were then washed and incubated with α-phospho MYPT1 antibody followed by anti-rabbit

IgG HRP antibody. TMB/E substrate was added to each well and allowed to develop in the dark at 37°C for 9 min. Absorbance of the plate was read at 450 nm using a BioTek Synergy H1 Hybrid Multi-mode microplate reader.

3.2.6 Migration and Invasion Assays

Wound healing assays (scratch assays) were performed on confluent monolayers of NOK cells cultured in 10% FBS DMEM. The monolayers were scratched and images were taken every 4 hours for 12 hours of two specific wounded areas. Migration assays were performed using Boyden chambers with 8μm pore filters (BD Biosciences). Invasion assays were performed using BD Biocoat Matrigel Invasion Chambers with 8-micron pore filters (BD Biosciences). For both assays, the bottom wells of the companion plate were filled with serum-containing medium (10% FBS DMEM) to act as a chemoattractant for the cells. NOK and EPC1 cells were harvested and resuspended in serum-free DMEM and added to each insert at a concentration of $2.5x10^5$ for migration or $5x10^4$ for invasion assays.

Migration assays were incubated for 6 hours and invasion assays were incubated for 16 hours (NOK) or 20 hours (EPC1) at 37°C and 5% CO_2. Nonmigratory and noninvasive cells on the upper surface of the filter were removed with a cotton swab. Migratory and invasive cells were stained with a Diff-Quick stain set (Siemens). Migrated or invaded cells were counted in five

representative high-power fields and all experiments were performed a minimum of three times.

3.2.7 3D Organotypic Culture

NOK cells were grown in organotypic culture on a 3D matrix as described previously with following minor modifications [203, 223]. The collagen/Matrigel fibroblast layer was created by using $1.5x10^5$ human fetal dermal fibroblasts. On day 13, cultures were raised to an air-liquid interface and cultured for four days in Epidermalization III medium. This medium is identical to Epidermalization I medium except that it does not contain progesterone and 2% unchelated newborn calf serum is added. On day 15, medium was replaced to a serum-free Epidermalization III medium. This medium is identical to Epidermalization III medium except it does not contain unchelated newborn calf serum. Cultures were grown for a total of 17 days and harvested on day 17 by fixing in 10% neutral buffered formalin (Fisher Scientific) and paraffin-embedding followed by hematoxylin-eosin staining.

3.2.8 Antibodies

The antibodies against P120CTN (#610134) and PIK3CA (#611398) were purchased from BD Transduction Laboratories. Antibodies for AKT (#4691), pAKT (#4060), MMP2 (#13132), STAT3 (#9132), pSTAT3 (#9145), pNFkB (#3033), p-cJUN(#3270) and E-cadherin (#3195) were from Cell Signaling. MMP1 and MMP11 antibody are respectively from Abcam (#ab134184) and

Epitomics (#1881-1). β-actin (#A5316) used as a loading control for immunoblotting, was purchased from Sigma-Aldrich Corp.

3.2.9 Gelatin Zymography

Gelatin zymography was performed as described previously [224] with minor modifications. Cell lysates were collected in the same manner as described for Western blot analysis and mixed with 4X non-reducing sample buffer (Tris 0.1M, pH 6.8; 0.8% SDS; 40% glycerol; 0.1% bromophenol blue (w/v)) . A total protein amount of 30 µg per lane was loaded onto a 10% SDS gel containing 0.1% gelatin (Fisher Scientific). Gels were washed once in zymogram renaturing buffer (2.5% Triton) for 30 minutes at RT with agitation. Enzymatic digestion of gelatin occurred through incubation in zymography buffer (50mM Tris-HCl, pH7.8, 200mM NaCl; 5mM CaCl2; 0.02% Tween-20) at RT for 30min and then in 37°C for 16 hours. The gels were incubated in staining solution (0.5% Coomassie Brilliant Blue R-250 (Amresco), 5% Methanol, 10% glacial acetic acid) for 30 min at RT. The gels were then destained with 5% Methanol, 10% glacial acetic acid for 2hr at RT with agitation and imaged using an 8 Megapixel camera.

3.2.10 Drug treatment

Cells were cultivated as described [203]. Two days prior to harvest, media was replaced to 10% FBS DMEM containing either 1µM batimastat (ApexBio),

2μM MK2206 (AdooQ), 50μM S3i-201 (AdooQ) or DMSO. Cells were collected two days after the addition of the drugs to the media.

3.2.11 Nucleofection

NOK and EPC1 cell transfections were done using a Nucleofector II device and the Nucleofector V kit (Amaxa Biosystems). NOK cells were transfected using program X-001. EPC1 cells were transfected using program T-030. To reduce off-target effects and maximize the possible knockdown, we used MMP1 siRNAs (ON-Target PLUS) (Dharmacon), which are a pool of four MMP1 siRNAs. 10^6 cells were transfected per reaction in 100μL of transfection buffer with the addition of 400ng of ON-Target PLUS scramble siRNA (D-001810-10-05) or ON-Target PLUS *MMP1* siRNA (L-005951-00-0005). Cells were collected 60 hours post-nucleofection for Western blot, zymography, and invasion assay analysis.

3.2.12 Human MMP Antibody Array

Lysates (25μg) from NOK-C, P, 545, 545P, 1047, 1047P were incubated overnight at 4°C in duplicate on the MMP array slide (ab197453) (Abcam) according to manufacturer's instructions. The detection antibody was applied for 1hr RT and the array slide was read on the Axon GenePix 4000B microarray scanner.

3.2.13 TCGA data analysis

472 HNSCC cases from the Cancer Genome Atlas (TCGA) database have been retrieved and separated into normal PI3K/high *P120CTN* and active PI3K/low *P120CTN* groups. Normal PI3K were defined as having both wild-type *PIK3CA* and *PTEN*, while active PI3K had either an activating mutation in *PIK3CA* or an inactivating mutation in *PTEN*. *P120CTN* was divided into quartiles, and “low” denotes the bottom and “high” denotes the top quartile. Log2 *MMP1* expression was normalized using a z-score.

3.2.14 Statistical Analysis

A one-tailed Student’s t-test was used for determining significant differences between groups unless otherwise mentioned. ANOVA analysis was performed to find significant differences in cell counts and viability assays. TCGA data statistical analysis was done using Wilcoxon rank sum test. Pearson correlation analysis was performed to determine the significance of fold invasion versus MMP1 expression. Grubbs test was used to find outliers (1 outlier in a set of 48 values). A p-value of $p<0.05$ was considered statistically significant.

3.3 Results

3.3.1 Generation of NOK and EPC1 cell lines

Immortalized oral keratinocytes were used to investigate the effects of P120CTN downregulation and *PIK3CA* mutations due to a lower level of background mutations compared to cancer cell lines. NOK-hTERT cells were infected with lentiviral constructs to downregulate P120CTN and/or overexpress PIK3CA. E545K and H1047R mutations in *PIK3CA* were confirmed via sequencing by Eurofins Scientific (Figure 3.1B,C). The control NOKs (NOK-C) contain empty vectors. NOK-P, NOK-545P, and NOK-1047P cells contain inducible TetOn *P120CTN* shRNA. Additionally, NOK-545 and NOK-545P cells contain E545K mutant PIK3CA overexpression, while NOK-1047 and NOK-1047P cells contain H1047R mutant PIK3CA overexpression (Table 3.1).

Immortalized EPC1-hTERT cells were used as a second cell line to confirm the findings observed in NOK cells. These cells were infected with lentiviral constructs containing TetOn *P120CTN* shRNA and PIK3CA mutants or empty vectors. Since all of the EPC1 cells contain the inducible TetOn *P120CTN* shRNA, we achieved P120CTN knockdown by adding doxycycline only to EPC1-P, EPC1-545P, and EPC1-1047P cells. EPC1-545 and EPC1-545P cells contain E545K mutant PIK3CA overexpression, while EPC1-1047 and EPC1-1047P cells contain the H1047R mutant PIK3CA overexpression (Table 3.1).

Once generated, the cell lines were assessed for P120CTN inactivation and PIK3CA overexpression by Western blot analysis. Loss of P120CTN destabilizes E-cadherin which results in its internalization and degradation [225]; therefore, we anticipated a downregulation of E-cadherin expression in cells where P120CTN was downregulated. Upon induction of *P120CTN* shRNA, we observed a decrease in P120CTN and E-cadherin protein levels in both NOK and EPC1 cells (Figure 3.2A,B), demonstrating that the P120CTN knockdown functions in the predicted way.

PIK3CA codes for the p110α subunit of PI3K. There are two common mutations found in *PIK3CA*, the E545K and H1047R mutations. E545K is in the helical domain, while H1047R is in the catalytic domain of the protein. Both of these mutations result in the activation of PI3K and its downstream targets, one of which is AKT [226]. We found that NOK and EPC1 cells infected with PIK3CA mutants expressed higher levels of PIK3CA, as well as phosphorylated AKT(Ser473) (Figure 3.2A,B). Since the PI3K-AKT pathway regulates several cellular processes, including survival and proliferation, we tested the cells for changes in the cell number and viability using cell counts and trypan blue staining. Differences in P120CTN and PIK3CA expression did not result in changes in cell counts or viability in all cells with P120CTN knockdown and/or PIK3CA overexpression compared to their controls in NOK (Figure 3.2C) or

EPC1 (Figure 3.2D) cells. Therefore, all functional assays presented here are not impacted by potential differences in cell number.

3.3.2 P120CTN downregulation and PIK3CA mutations increase anchorage-independent growth colony number and size

The potential for anchorage-independent growth is higher in cells that are more likely to metastasize [227]. Given that P120CTN loss and PIK3CA overexpression are tied to increased levels of metastasis in HNSCC, we hypothesized that P120CTN downregulation and *PIK3CA* mutations increase anchorage-independent growth [121, 228, 229]. Therefore, we assayed the effects of P120CTN downregulation and *PIK3CA* mutations on anchorage-independent growth of NOK and EPC1 cells. Thus far, the effects of P120CTN loss on anchorage-independent growth in keratinocytes have not been sufficiently investigated. Data from breast cancer cell lines show that P120CTN knockdown can cause a decrease, an increase or no effect on anchorage-independent growth [130, 230, 231]. On the other hand, *PIK3CA* mutations have been shown to increase anchorage-independent growth in chicken embryo fibroblasts [232]. Moreover, PIK3CA overexpression can increase anchorage-independent growth in cervical keratinocytes [233].

NOK cells demonstrated a significant increase ($p<0.05$) in the amount of colonies present in cells with either P120CTN downregulation or *PIK3CA* mutations (NOK-P, NOK-545P, NOK-1047, NOK-1047P) compared to the

controls (NOK-C) (Figure 3.3A). In EPC1 cells, only EPC1-545 and EPC1-1047P cells had significantly ($p<0.05$) more colonies than EPC1 cells (Figure 3.3B). NOK cells with *PIK3CA* mutations (NOK-545, NOK-545P, NOK-1047, NOK-1047P) had significantly ($p<0.05$) larger colonies than NOK-C (Figure 3.3C). In EPC1 cells, only EPC1-545P cells had significantly ($p<0.05$) larger colonies than EPC1-C cells (Figure 3.3D). However, in both cell lines combination of P120CTN downregulation and *PIK3CA* mutations did not produce effects larger in magnitude than 1.5 fold, which makes it difficult to draw conclusions from these results. These data suggest that P120CTN downregulation and PIK3CA mutations induce statistically significant changes in anchorage-indpendent growth, which most likely do not play a biologically significant role due to the observed magnitude of change.

3.3.3 P120CTN downregulation and PIK3CA mutations enhance cell migration

Although P120CTN loss or downregulation and *PIK3CA* mutations have been shown to affect cell migration individually, no work has explored the impact of combining P120CTN downregulation with *PIK3CA* mutations in HNSCC [85, 234]. We utilized a wound healing assay to test the combination of P120CTN downregulation and *PIK3CA* mutations on HNSCC cell migration. After 12 hours post-wounding, NOK-C cells did not migrate. NOK cells with single genetic manipulations, P120CTN downregulation or *PIK3CA* mutations (NOK-P, NOK-545, NOK-1047), displayed a significant increase in migration post-wounding

compared to the controls (NOK-C) ($p<0.05$). Interestingly, cells with both P120CTN downregulation and *PIK3CA* mutations (NOK-545P and NOK-1047P) displayed the highest amount of migration, which was statistically significant ($p<0.05$) compared to cells with P120CTN knockdown only or PIK3CA overexpression only (NOK-P, NOK-545, NOK-1047) (Figure 3.4 A,B). These data indicate that P120CTN knockdown and *PIK3CA* mutations have a cooperative effect on the migratory capabilities of NOK cells. To further validate our findings, we assessed NOK and EPC1 cell migration using Boyden-chamber migration assays. Results from Boyden-chamber migration assays in the NOK (Figure 3.4C) and EPC1 (Figure 3.4D) cells were similar to the results from the wound-healing assay. Thus, both migration assays demonstrate that P120CTN downregulation and *PIK3CA* mutations have a greater effect on migration together than they do alone. However, the effects are not additive, which would imply that the same pathway is utilized by both P120CTN and PI3K pathways. The effect observed is limited when both genetic modifications are present due to the ceiling effect.

3.3.4 Increased cell migration due to P120CTN downregulation and PIK3CA mutations is not modulated via the STAT3 pathway

We performed a literature search to identify potential signaling pathways downstream of P120CTN and PIK3CA that could be responsible for the observed increase in migration. P120CTN loss and mutant PIK3CA have been shown to

increase Signal Transducer And Activator Of Transcription 3 (STAT3) phosphorylation at the Y705 residue, which is a marker of STAT3 activity [6, 235, 236]. Furthermore, increased STAT3 activity has been previously demonstrated to increase cell migration in HNSCC cell lines and STAT3 knockdown abrogated that effect [237, 238]. This poised STAT3 as a promising candidate to explain the increase in migration due to P120CTN downregulation and *PIK3CA* mutations.

We assayed phosphorylated STAT3 (Y705) and total STAT3 expression by Western blotting. Results of these experiments demonstrated an increase in phosphorylated STAT3 (Y705) in NOK cells with single genetic manipulations, *P120CTN* downregulation or *PIK3CA* mutations (NOK-P, NOK-545, NOK-1047), compared to control keratinocytes (NOK-C) ($p<0.05$) (Figure 3.5A). Moreover, NOK cells with both P120CTN knockdown and *PIK3CA* mutations (NOK-545P, NOK-1047P) exhibited higher levels of phosphorylated STAT3 compared to cells with P120CTN downregulation (NOK-P) and cells with *PIK3CA* mutations (NOK-545, NOK-1047) (Figure 3.5A). We significantly inhibited STAT3 activity in NOK cells by using a STAT3-specific inhibitor, S3i-201 ($p<0.05$) (Figure 3.5B). We tested the effects of STAT3 inhibition in a Boyden-chamber migration assay. The results demonstrated that cells treated with S3i-201 did not migrate less than their DMSO-treated controls. On the contrary, a significant increase in the migration was observed in the S3i-201-treated NOK cells with *PIK3CA* mutations only (NOK-545, NOK-1047) compared to their DMSO-treated controls ($p<0.05$)

(Figure 3.5C). The other modified cell lines treated with S3i-201 (NOK-P, NOK-545P, NOK-1047P) demonstrated an upward trend in migration compared to their DMSO-treated controls, which did not achieve a statistical significance (Figure 3.5C). Hence, we conclude that the observed increase in phosphorylated STAT3 (Y705) due to P120CTN downregulation and PIK3CA mutations is not responsible for the increased migration.

3.3.5 Increased cell migration due to P120CTN downregulation and PIK3CA mutations is not modulated via the RHOA pathway

Our experiments demonstrated that cells with P120CTN downregulation and *PIK3CA* mutations do not rely on STAT3 signaling to increase migration. Therefore, the role of RHOA signaling in the induction of migration was investigated in our experimental system. The RHOA pathway has been extensively studied in relation to cytoskeletal rearrangement and cell migration [239]. RHOA is a small GTPase, and remains active when bound to GTP [240]. P120CTN inhibits RHOA activity in a variety of cell types [109, 241]. Due to P120CTN downregulation or loss, RHOA activity increases resulting in an increase in cell motility. Mutant PIK3CAs, unlike P120CTN, have been shown to increase RHOA activity [242, 243]. Moreover, PI3K, a heterodimer formed by the catalytic subunit (PIK3CA) and a regulatory subunit, is involved in the spatiotemporal activation of RhoA [244].

We measured levels of activated RHOA by performing a RHOA-GTP pulldown followed by Western blotting. RHOA-GTP levels were increased in NOK cells with both *P120CTN* downregulation and *PIK3CA* mutations (NOK-545P, NOK-1047P) as compared to the control (NOK-C) (Figure 3.6A). Our results indicated that active RHOA is only activated in cells with both P120CTN downregulation and *PIK3CA* mutations and might be the reason why NOK cells with both genetic manipulations (NOK-545P, NOK-1047P) migrate quicker than NOK cells with single genetic manipulations only (NOK-P, NOK-545, NOK-1047). We decided to test RHO-kinase (ROCK), the immediate downstream target of RHOA. ROCK is the main effector of RHOA and is responsible for regulating RHOA's effects on cell motility [239]. A ROCK activity assay demonstrated that NOK cells with both genetic manipulations (NOK-545P, NOK-1047P) do not differ from control cells (NOK-C) in levels of ROCK activity (Figure 3.6B). Hence, we conclude that ROCK signaling is not activated due to P120CTN downregulation and *PIK3CA* mutations. This indicates that RHOA pathway is not sufficiently activated to support the observed increase in migration. We have not tested other effects of RHOA, such as PI4P5K or DIAPH1, since the majority of RHOA control of migration is mediated with ROCK. It is also unlikely that RHOA would only activate ancillary pathways without activating ROCK. Even if that is the case we do not think activation of these pathways would be sufficient to produce the effects of the magnitude we have observed in our migration assays. Additionally,

the subdued level of RHOA activity in the presence of large increase in migration points towards another GTPase, such as RAC1 or CDC42, playing a role in modulating migration in this experimental system.

3.3.6 Increased cell migration due to PIK3CA mutations is not modulated via the AKT pathway

The AKT pathway is the main signaling pathway downstream of PIK3CA [245]. It has been implicated in regulating a multitude of cell mechanisms, including cell motility [246]. We hypothesized that the increased migration observed in NOK cells with *PIK3CA* mutations could be explained in part by increased AKT activity.

We have confirmed that *PIK3CA* mutations increase the levels of phosphorylated AKT (S473) in NOK cells (Figure 3.2A). To test the role of AKT in migration we used a specific inhibitor of AKT, MK2206. Treatment of NOK cells with MK2206 reduced phosphorylated AKT (S473) levels in cells with *PIK3CA* mutations (NOK-545, NOK-1047) down to baseline levels (NOK-C) (Figure 3.7A). Boyden-chamber migration assay results demonstrated that MK2206-treated NOK cells had similar migratory rates as the DMSO-treated controls (Figure 3.7B). This experiment was performed n=2 times and MK2206 treatment did not reduce cell migration in any samples. These data, therefore, suggest that the AKT activation due to mutant PIK3CAs is not responsible for increased migration.

3.3.7 P120CTN downregulation and PIK3CA mutations increase p-cJUN in NOK cells

While pursuing potential pathways in charge of increasing migration in our experimental model, we have measured the activity of Jun Proto-Oncogene, AP-1 Transcription Factor Subunit (cJUN) and Nuclear Factor Kappa B Subunit 1 (NFkB) pathways. cJUN pathway activation occurs as a result of phosphorylation of cJUN on serine residue 73 (p-cJUN). Moreover, p-cJUN has been shown to increase the migratory capacity of murine osteosarcoma cells [247]. Similarly, activation of NFkB pathway happens upon phosphorylation of NFKB on serine residue 536 (pNFkB). Active NFkB is also correlated with increased migratory capacity in oral carcinoma [248]. Increases in p-cJUN are similar to the increases in pSTAT3 observed between NOK cell lines. Cells with either P120CTN downregulation or PIK3CA mutation have increased p-cJUN levels above that of the control NOK cells. However, cells with both P120CTN downregulation and PIK3CA mutations display the highest level of p-cJUN (Figure 3.8). pNFkB is only increased in cells with either P120CTN downregulation (NOK-P), E545K PIK3CA mutation (NOK-545), or cells with both P120CTN downregulation and E545K PIK3CA mutation (NOK-545P) (Figure 3.8). This difference in activation of NFkB between the two PIK3CA mutants makes NFkB less likely to be involved in promoting either migration or invasion. The role of these proteins in migration downstream of P120CTN and PIK3CA remains to be tested. Treatment of cellsin

migration assay by small molecule inhibitors targeting cJUN could elucidate the role of this protein in this experimental system.

3.3.8 Invasion is increased in cells with P120CTN downregulation and PIK3CA mutations

Another characteristic of an aggressive, more metastatic cancer is its ability to invade through the extracellular matrix. To assess whether P120CTN downregulation and *PIK3CA* mutations also regulate HNSCC cell invasion, we utilized Boyden-chamber Matrigel invasion assays. Results of these experiments demonstrated an increase in the invasion of NOK and EPC1 cells with single genetic manipulations, *P120CTN* downregulation or *PIK3CA* mutations (NOK-P, NOK-545, NOK-1047 or EPC1-P, EPC1-545, EPC1-1047), compared to control keratinocytes (NOK-C or EPC1-C) ($p<0.05$) (Figure 3.9A,B). Moreover, NOK cells with both P120CTN knockdown and H1047R *PIK3CA* mutation (NOK-1047P) exhibited significantly higher levels of invasion compared to cells with P120CTN downregulation (NOK-P) or H1047R *PIK3CA* mutation (NOK-1047) ($p<0.05$) (Figure 3.9A). Similar results were observed in EPC1 cells (Figure 3.9B). While both cell lines demonstrate similar trends we wanted to emphasize that the NOK cell line had a greater magnitude of effect compared to EPC1 cell line. Additionally, EPC-545P is not significantly larger in magnitude compared to EPC-P as is seen between NOK-545P and NOK-P. This is most likely the result

of the tissue origin and the fact that PIK3CA mutations are more common in oral epithelium compared to esophageal epithelium.

Boyden-chamber Matrigel invasion assays demonstrated that P120CTN downregulation and *PIK3CA* mutations cooperate to induce invasion in NOK and EPC1 cells. 3D organotypic culture assay was used to further explore the role of P120CTN downregulation and *PIK3CA* mutations on cell invasion. In this assay, keratinocytes are cultured on the surface of a collagen and fibroblast matrix. This allows for the interaction of our modified keratinocytes with fibroblasts present in the matrix, which is important for invasion [249, 250]. The keratinocyte-fibroblast interaction allows the 3D organotypic culture assay to mimic the *in vivo* tumor environment, and provides a better estimate of the invasive capacity of cells with P120CTN downregulation and *PIK3CA* mutations. Hematoxylin and eosin staining of 3D cultures demonstrated that all of the cell lines formed a stratified squamous epithelium reminiscent of the nonkeratinized stratified squamous epithelium of the oral cavity (Figure 3.10,B). NOK-C cells exhibited minimal to no invasion into the underlying matrix. In contrast, cells with single genetic manipulations, *P120CTN* downregulation or *PIK3CA* mutations (NOK-P, NOK-545, and NOK-1047), displayed significant invasion. Moreover, cells with both P120CTN downregulation and *PIK3CA* mutations (NOK-545P and NOK-1047P), exhibited a greater amount of invasion into the underlying matrix compared to cells with P120CTN knockdown only or *PIK3CA* mutations only (NOK-P, NOK-

545, NOK-1047) (Figure 3.10A). Similar results were observed in EPC1 cells (Figure 3.10B). Overall, our data indicate that combined P120CTN downregulation and *PIK3CA* mutations have a greater effect on invasion together than they do alone.

3.3.9 Protease activity is increased due to P120CTN downregulation or PIK3CA mutations

Matrix metalloproteinases (MMPs) are known to play a role in cell invasion. P120CTN downregulation and *PIK3CA* mutations with regards to MMP expression have not been studied extensively in HNSCC. Hence, as a first step, we compared the proteolytic activity in our NOK cells by gelatin zymography. This technique allows for testing of gelatinolytic activity, which is correlated with the presence of MMPs. Additionally, based on the kDa size of the bands present on the gel, we can estimate what MMP could be responsible for the formation of that band. A double band was visible at approximately 50-55 kDa and demonstrated a stronger intensity in all cells with P120CTN downregulation and/or *PIK3CA* mutations (NOK-P, NOK-545, NOK-545P, NOK-1047, NOK-1047P) compared to control cells (NOK-C) (Figure 3.11A). The presence of a double band indicated either elevation of multiple MMPs or elevation of single MMP that undergoes posttranslational modification. To confirm that these bands on the zymogram were a result of increased MMP activity, we treated the cells with 1μM Batimastat, a pan-MMP inhibitor. Upon treatment with Batimastat, we

observed a loss of the double band in all cells indicating an attenuation of protease activity, likely from an MMP (Figure 3.11B,C). This reduction of protease activity corresponded with a decrease in cell invasion. Cells treated with Batimastat exhibited a significant decrease in invasion in Boyden-chamber Matrigel invasion assays ($p<0.05$) (Figure 3.11D). Overall, these data indicate that the invasion caused by P120CTN downregulation and/or *PIK3CA* mutations may be driven by MMP(s). Similarly as with migration, we do not see an additive relationship, indicating that both P120CTN downregulation and *PIK3CA* mutations share a pathway downstream that regulates the invasion. This is the most likely explanation for the observed ceiling effect in the magnitude of invasion in our cells with both P120CTN downregulation and *PIK3CA* mutations.

3.3.10 MMP1 is the shared downstream target of both P120CTN downregulation and PIK3CA mutations

Given that Batimastat caused a decrease in NOK cell invasion, we assayed which MMPs were elevated in cells with P120CTN downregulation or *PIK3CA* mutations and then further tested which of those MMPs affect invasion. Analysis of a Human MMP Array revealed three MMPs that were increased in our cells, MMP1, MMP2, and MMP10. Of these three proteases, MMP1 and MMP2 exhibited the most consistent increase in expression in cells with either P120CTN downregulation and/or *PIK3CA* mutations (Table 3.2). In addition, both of these proteases are known to have the broadest range of activity; thus, we

chose to investigate the role of MMP1 and MMP2 in NOK and EPC1 cell invasion. We also chose to examine MMP11, as its size is consistent with the sizes of the bands present on the gelatin zymography and it wasn't included on the MMP array.

We assessed the protein expression levels of MMP1, MMP2, and MMP11 by Western blot analysis. MMP1 levels are increased in all genetically modified NOK cells compared to the control cells (NOK-C) ($p<0.05$), with the greatest increase observed in NOK cells with both P120CTN downregulation and *PIK3CA* mutations (NOK-545P and NOK-1047P) ($p<0.05$) (Figure 3.12A,C). Similar observations were made in EPC1 cells (Figure 3.12B,D). In contrast, MMP2 and MMP11 expression did not change significantly in NOK and EPC1 cells with P120CTN knockdown or *PIK3CA* mutations compared to control cells (Figure 3.12A-D). Overall, these data indicate that P120CTN downregulation and *PIK3CA* mutations increase the levels of MMP1.

3.3.11 MMP1 inhibition and invasion

Given that Batimastat inhibited invasion and P120CTN downregulation and *PIK3CA* mutations increase MMP1 expression, we decided to examine the role of MMP1 in invasion. We used pooled *MMP1* siRNAs (ON-Target PLUS siRNA) to knock down MMP1 in NOK and EPC1 cells. Transient nucleofections achieved a statistically significant decrease in MMP1 levels in NOK (Figure 3.13A,C) and EPC1 cells (Figure 3.13B,D). Data from gelatin zymography using

nucleofected NOK-1047 cells (used due to their moderate levels of MMP1 expression) demonstrate that MMP1 knockdown reduces the intensity of the double band at 50-55 kDa (Figure 3.14A,B). This suggests that the observed double band was due to MMP1 activity. Furthermore, the decrease in MMP1 levels was associated with a decrease in cell invasion in all NOK (Figure 3.15A) and EPC1 (Figure 3.15B) cells with P120CTN knockdown and/or *PIK3CA* mutation.

Interestingly, *MMP1* siRNA treatment of 545P cells (for both NOK and EPC1 cell lines) did not achieve as great a knockdown of MMP1 expression as was observed in our other cell lines due to its very high expression in these cells. We hypothesized that this may be why a significant decrease in invasion was not achieved. If MMP1 is a regulator of the invasive potential in 545P cells as it is in all the other cell lines analyzed, inhibition of invasion in NOK-545P should be proportional to MMP1 inhibition in a similar manner as the other NOK cells. We, therefore, performed a Pearson correlation analysis of invasion and MMP1 expression in nucleofected NOK cells. Every replicate of our assay was found to positively correlate with the amount of MMP1 expression and the amount of invasion present ($p<0.05$). If our hypothesis were true, we would expect to see NOK-545P cells nucleofected with *MMP1* siRNA lay within the 95% confidence interval signifying that the invasion in these cells is proportionate to their residual MMP1 expression. Interestingly, NOK-545P cells nucleofected with *MMP1* siRNA

resided within the 95% confidence interval of our trendline in three of the four replicates (Figure 3.16A-D). This indicates that the amount of invasion seen with these cells is proportionate to the residual high level of MMP1 after the knockdown. Altogether, our data demonstrate that MMP1 inhibition leads to a decrease in cell invasion in both NOK and EPC1 cells. Thus, P120CTN downregulation and *PIK3CA* mutations cooperate to increase cell invasion by increasing MMP1 expression.

3.3.12 TCGA data analysis

Our cell line experiments determined that P120CTN downregulation and *PIK3CA* mutations increase invasion by increased MMP1 expression in HNSCC. To validate these results in patient samples, we analyzed data from the TCGA databank of HNSCC [18]. *P120CTN* expression was divided into 4 quartiles and we analyzed the tumors in the top (high *P120CTN*) and bottom (low *P120CTN*) quartiles. Tumors with wild-type *PTEN* and *PIK3CA* were treated as having normal PI3K activity, while tumors with either inactivating *PTEN* mutations or activating *PIK3CA* mutations were considered as active PI3K pathway. *MMP1* expression was compared between tumors with active PI3K pathway and low *P120CTN* (n=23) and tumors with normal PI3K pathway and high *P120CTN* (n=94). We observed that tumors with active PI3K pathway and low *P120CTN* expressed increased levels of *MMP1* mRNA compared to tumors with normal PI3K and high *P120CTN* ($p<0.05$) (Figure 3.17). These data significantly

strengthen our cell line data and demonstrate that the relationship of *P120CTN* and *PIK3CA* to *MMP1* exists in HNSCC patient samples. Therefore, MMP1 is most likely increased in HNSCC due to P120CTN downregulation and overactivity of the PI3K pathway. Furthermore, these data suggest that low *P120CTN* expression and PI3K pathway activation may be used to predict *MMP1* expression and the propensity to invade. Also, these data may begin to explain why P120CTN downregulation or PI3K pathway activation are associated with poor prognosis or the advanced stage of HNSCC.

3.4 Discussion

3.4.1 PI3K and P120CTN in HNSCC

HNSCC develops in the context of multiple genetic alterations that culminate in metastasis. Both P120CTN downregulation and *PIK3CA* mutations are commonly found in HNSCC [18, 111]; however, most studies take a monogenetic approach to investigating genetic changes found in HNSCC. To date, the only other HNSCC study that adopts a similar approach to ours utilizes the combination of p53 loss with Cyclin D1 overexpression [219]. To our knowledge, this is the first report to investigate the effects of both P120CTN downregulation and *PIK3CA* mutations on migration and invasion.

Aside from HNSCC, P120CTN loss or *PIK3CA* mutations correlate with decreased patient survival in many types of epithelial cancers [117, 251-255]. At first glance, some cancers, such as colon cancer, show no statistical significance

of *PIK3CA* mutations on patient outcomes [256]. However, *PIK3CA* mutations correlate with decreased patient survival in colon cancer patients who have undergone monoclonal EGFR therapy [257]. Hence, it is possible the negative effects of *PIK3CA* mutations on patient outcomes might be unmasked if we focus on tumor subtypes in which *PIK3CA* mutations might be cooperating with other genetic changes. Thus, this finding supports the rationale for our study design in which we explored a combination of P120CTN downregulation and *PIK3CA* mutations.

3.4.2 Invasion and MMPs in HNSCC

Migration and invasion are fundamental processes of metastasis. We found that P120CTN downregulation and *PIK3CA* mutation not only increase cell migration and invasion, but their effects work in a cooperative manner. In support of our findings, downregulation of P120CTN has been correlated with increased stage and a higher rate of lymph node metastasis in HNSCC [121, 258-260]. Additionally, overexpression of PIK3CA has been linked to decreased patient survival; however, there is little known about the mechanistic role of mutated PIK3CA in HNSCC disease progression [261].

Effects of P120CTN downregulation and *PIK3CA* mutations on MMPs are poorly studied in HNSCC. Only one report demonstrates that P120CTN loss leads to an increase of MMP1 mRNA in endothelial cells, but no functional consequences have been demonstrated for this relationship [262]. Our studies

are the first to link P120CTN downregulation and PIK3CA mutations to MMP1 expression in HNSCC in invasion. In addition, MMP1 is a marker of late stage HNSCC, which further emphasizes the importance of our findings [263]. Increased MMP1 expression in late stage HNSCC suggests that MMP1 may take part in processes that occur during late stages of carcinogenesis, i.e. metastasis. Our cell line data further strengthens that assertion since MMP1 inhibition leads to a decrease in invasion. Additionally, TCGA data from HNSCC patient tumor samples support the findings from our engineered cells with increased MMP1 expression in tumors with activated PI3K signaling and low *P120CTN*. Altogether, these findings implicate MMP1 as a possible mechanism by which HNSCC tumors with low *P120CTN* and activated PI3K pathways invade, and therefore may be an attractive therapeutic target in the future for HNSCC.

3.4.3 Migration in the background of P120CTN downregulation and PIK3CA mutations

While we provide significant evidence for the mechanism responsible for increased invasion due to P120CTN downregulation and *PIK3CA* mutations, our findings of migration being independent of STAT3 activation are also important. Phosphorylated STAT3 in HNSCC has been shown to increase cell migration [237, 238]. Inhibition of STAT3 using siRNAs and small molecule inhibitors in the above mentioned reports resulted in a significant decrease of migration. Our results demonstrate the opposite. STAT3 inhibition increases the amount of

migration in our experimental system. This is not the first time that STAT3 inhibition lead to an increase in migration. Findings similar to ours have been observed in fibroblasts [264]. Moreover, the increase in STAT3 phosphorylation can be a result of the activation of one of the Ras GTPases such as RHOA, CDC42 or RAC1 [264, 265]. Furthermore, when one of these GTPases is active and STAT3 is knocked down an increase in migration can be observed. We have confirmed that RHOA is not significantly active in our modified keratinocytes. Therefore, our findings point to the possibility of P120CTN downregulation and *PIK3CA* mutations activating RAC1 or CDC42 to increase cell migration. P120CTN overexpression in gastric cancer suppresses RAC1 and CDC42 [266]. Therefore, loss of P120CTN could potentially increase RAC1 activation as has been demonstrated in lung cancer [267]. PIK3CA loss has been shown to limit RAC1 activity in pancreatic cancer [268]. Moreover, PDK1, an effector of the PI3K pathway, can activate RAC1 [269]. Therefore, either the overexpression or presence of constitutively active mutant PIK3CA might result in increased RAC1 activity.

In addition, we have demonstrated that AKT inhibition does not have an effect on mutant PIK3CA driven migration. Studies in other cancers also suggest that AKT is not the pathway used by PIK3CA to propagate migration [242, 270, 271]. To the contrary, AKT inhibition in the background of mutant PIK3CA can increase the amount of migration [270]. The fact that AKT does not play a role in

PIK3CA-driven migration in HNSCC further limits the amount of possible proteins that could be responsible for the increased migration in our system. Since AKT and anything downstream of AKT is most likely not driving migration in our experimental system, we have to look upstream of AKT and downstream of PIK3CA. This limits the most likely candidates to immediate binding partners of PIK3CA or PDK1. Further investigation is required to confirm our prediction.

3.4.4 Anchorage-independent growth in the background of P120CTN downregulation and PIK3CA mutations

Similarly to migration and invasion, increased anchorage-independent growth has been correlated with cancer cells being more metastatic [227]. This result is not surprising. Keratinocytes cannot leave their native environment without risking anoikis, a type of apoptosis induced by lack of cell adhesion to extracellular matrix or other cells. Therefore, the anchorage-independent growth assay indirectly measures the ability of the cells to avoid anoikis. Our results reveal an increase in the amount and the size of colonies formed due to P120CTN downregulation or *PIK3CA* mutations when compared to the control cells. However, the magnitude of the change is minimal and does not extend beyond that of 1.5 fold. While statistically significant, the results bring into question the biological significance of the finding. It is possible that the effects of cooperating P120CTN downregulation and *PIK3CA* mutations are not sufficient to fully transform keratinocytes and these changes do not affect anoikis

significantly *in vivo*. Given that P120CTN downregulation and *PIK3CA* mutations significantly affect migration and invasion, the lack of a profound effect on anchorage-independent growth might not have clinical significance. This also raises a possibility of a third genetic change regulating anchorage-independent growth in low-P120CTN -mutant-PIK3CA HNSCC tumors, which could further increase the metastatic potential.

3.4.5 P120CTN and PI3K in other cancers

Furthermore, loss of P120CTN or constitutive activation of the PI3K pathway is common in many cancers besides HNSCC [272]. Many cancers with P120CTN loss or PI3K pathway activation have been independently reported to have increased MMP1 expression which is correlated with negative patient outcomes [273-279]. Thus far, the mechanism responsible for increased MMP1 expression in many of these cancers is still unknown. We speculate that in a portion of these cancers, P120CTN loss and PI3K activation could be responsible for increased MMP1 expression. While further investigation is required to confirm that hypothesis, the discovery of additional cancers that exploit P120CTN loss and PI3K pathway activation would emphasize the importance of studying P120CTN and PIK3CA simultaneously.

In summary, P120CTN downregulation and PIK3CA mutations increase cooperatively migration and invasion in keratinocytes. We have demonstrated that these genetic events increase MMP1, which upon knockdown reduces the

amount of invasion. Additionally, we confirmed our findings demonstrating that tumors with low P120CTN and active PI3K signaling have an increased level of *MMP1* mRNA. Therefore, in the scope of this chapter we have presented genetic aberrations common to HNSCC, the effects that they have on cell migration and invasion and the causal agent for the increased invasion.

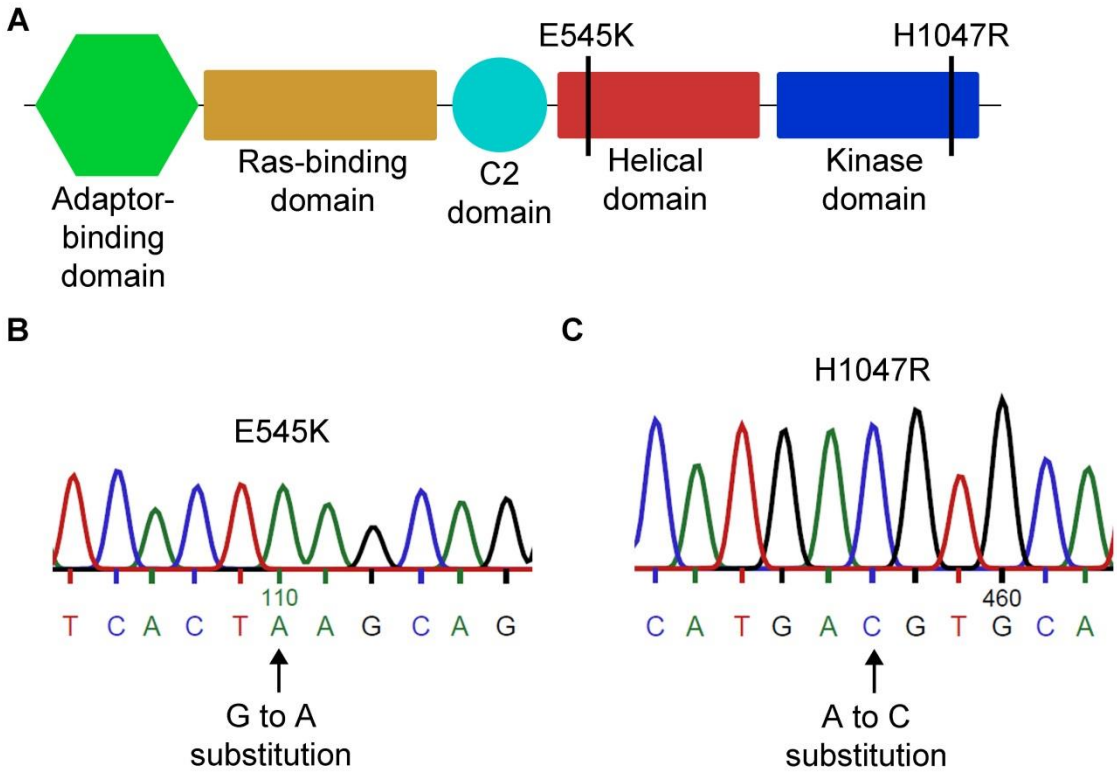

Figure 3.1. Confirmation of *PIK3CA* E545K and H1047R mutations. A, Depiction of PIK3CA domains with the location of E545K and H1047R mutations marked. **B,** Sequencing of PIK3CA constructs used to infect cells confirms the presence of point mutations in the E545K and **C,** H1047R *PIK3CA* mutants.

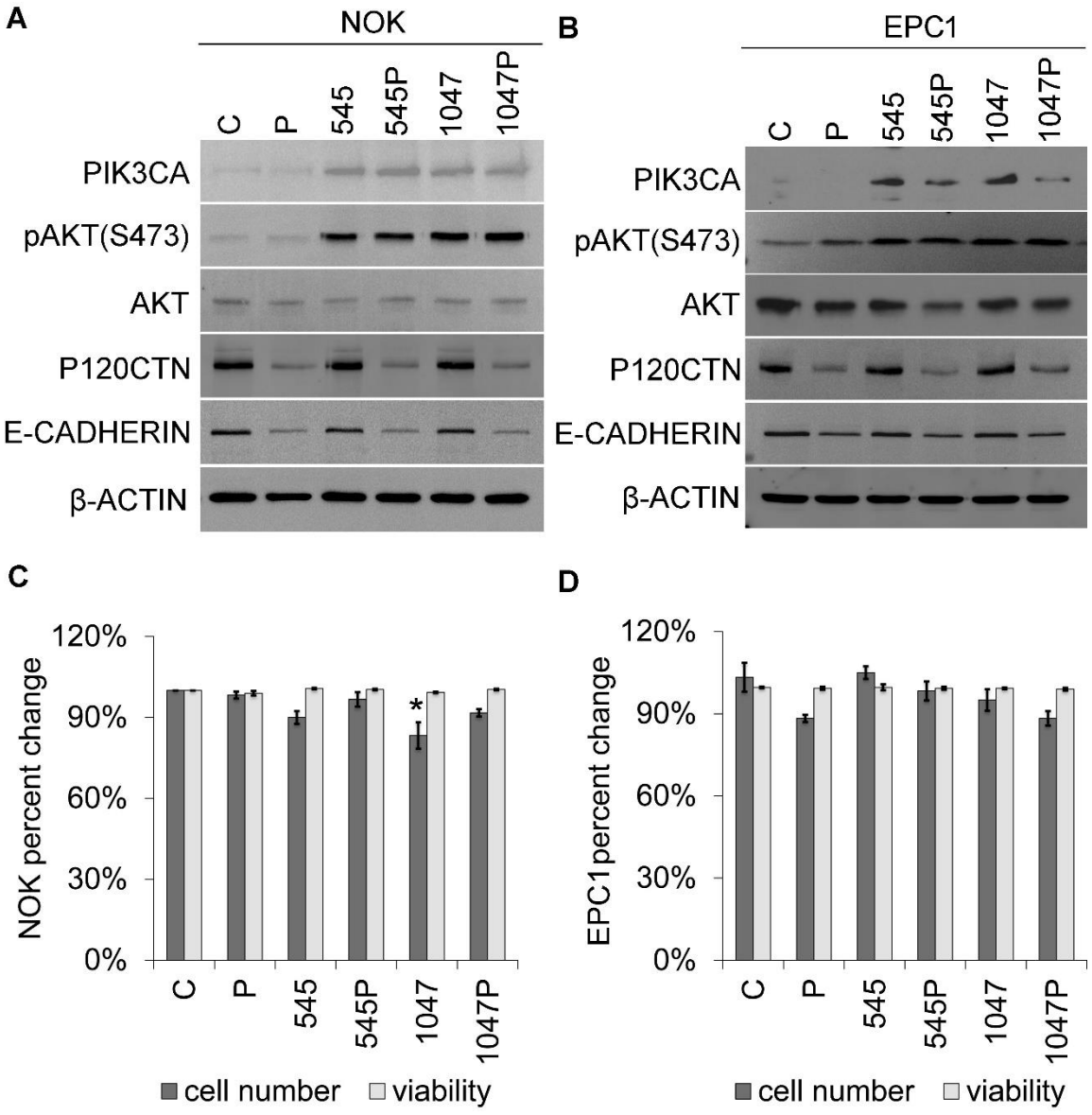
A
NOK
C
P
545
545P
1047
1047P
PIK3CA
pAKT(S473)
AKT
P120CTN
E-CADHERIN
β-ACTIN
B
EPC1
C
P
545
545P
1047
1047P
PIK3CA
pAKT(S473)
AKT
P120CTN
E-CADHERIN
β-ACTIN
C
NOK percent change
120%
90%
60%
30%
0%
*
C
P
545
545P
1047
1047P
cell number
viability
D
EPC1 percent change
120%
90%
60%
30%
0%
C
P
545
545P
1047
1047P
cell number
viability

Figure 3.2. Characterization of modified NOK and EPC1 cells. A, Confirmation of P120CTN downregulation and PIK3CA overexpression by Western blot analysis in NOK cells and **B,** in EPC1 cells. β-actin is used as a loading control. **C,** Cell count and viability assays in NOK cells and **D,** in EPC1 cells. Data presented as means ± SEM. ANOVA used for statistical analysis. n=3 for all experiments.* $p<0.05$

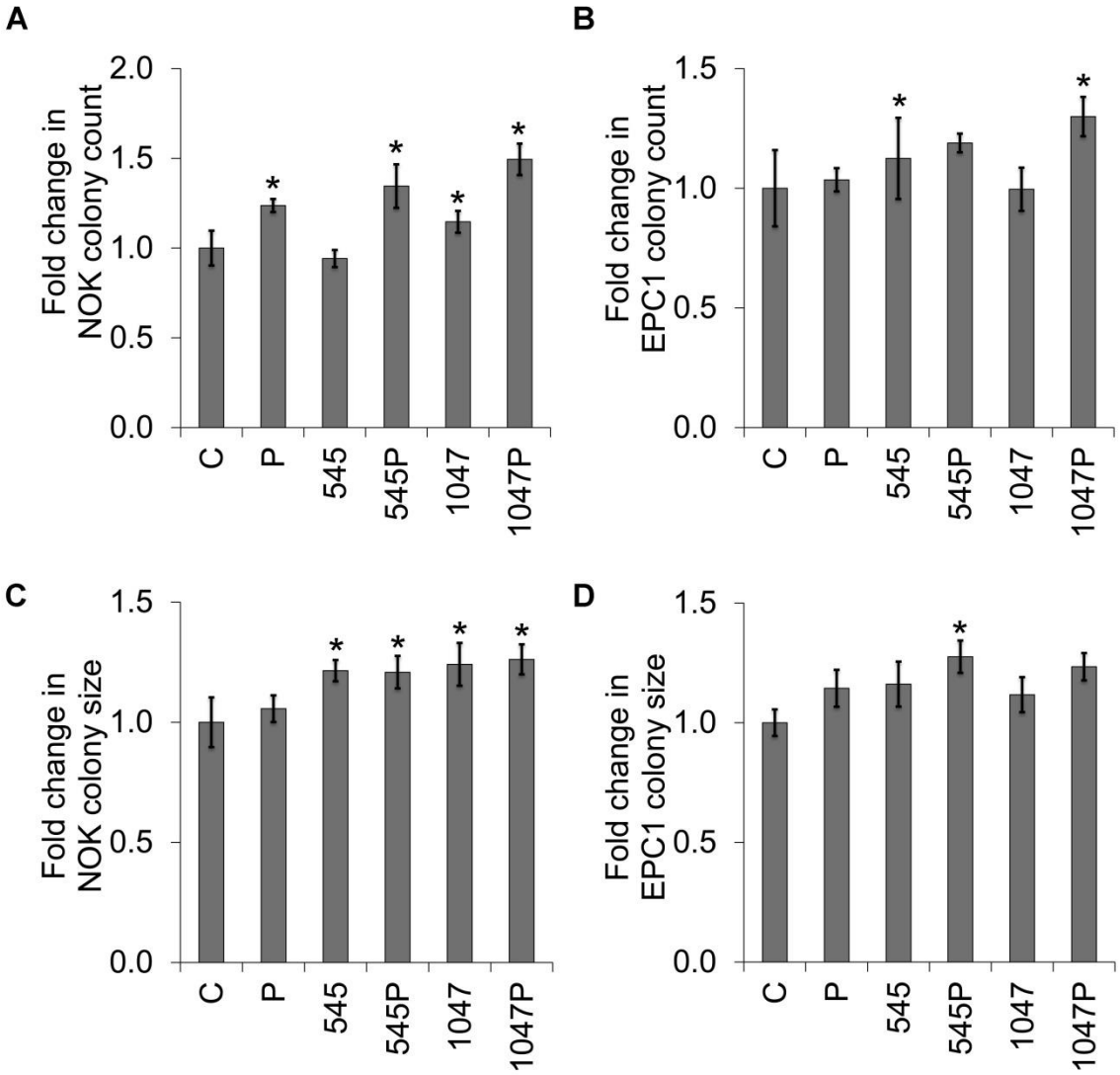
A
Fold change in NOK colony count
2.0
1.5
1.0
0.5
0.0
C
P
545
545P
1047
1047P
B
Fold change in EPC1 colony count
1.5
1.0
0.5
0.0
C
P
545
545P
1047
1047P
C
Fold change in NOK colony size
1.5
1.0
0.5
0.0
C
P
545
545P
1047
1047P
D
Fold change in EPC1 colony size
1.5
1.0
0.5
0.0
C
P
545
545P
1047
1047P

Figure 3.3. Anchorage-independent growth of modified NOK and EPC1 cells. A, Quantification of the number of colonies in the anchorage-independent growth assay in NOK cells and **B,** in EPC1 cells. **C,** Quantification of the size of colonies in the anchorage-independent growth assay in NOK cells and **D,** in EPC1 cells. Data presented as means ± SEM. Student t-test used for statistical analysis. (n=4 for A,C; n=4 for B,D) * $p<0.05$ compared to C cells.

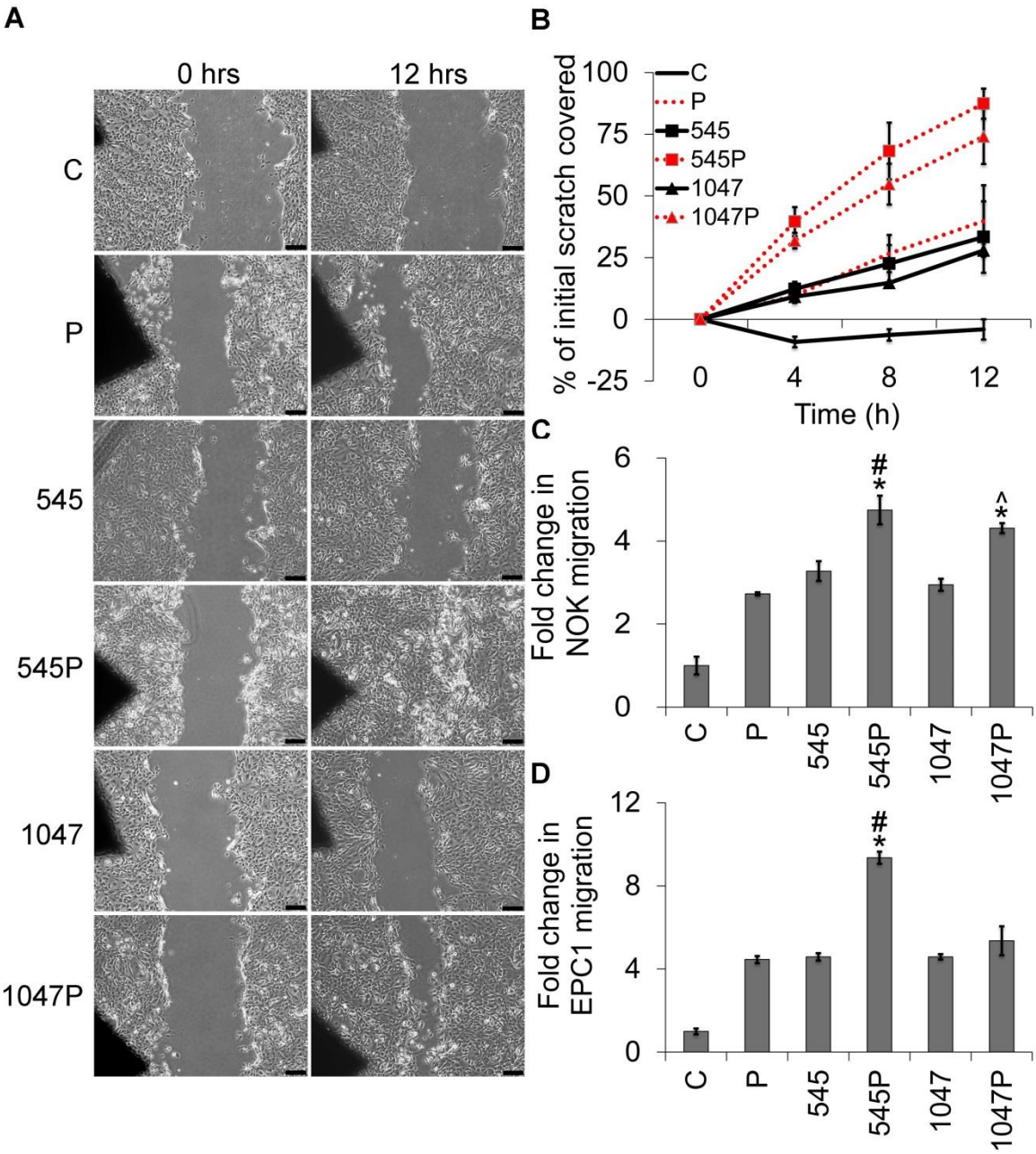
A
0 hrs
12 hrs
C
P
545
545P
1047
1047P
B
% of initial scratch covered
100
75
50
25
0
-25
0
4
8
12
Time (h)
C
P
545
545P
1047
1047P
C
Fold change in NOK migration
6
4
2
0
#
*
*>
C
P
545
545P
1047
1047P
D
Fold change in EPC1 migration
12
8
4
0
#
*
C
P
545
545P
1047
1047P

Figure 3.4. P120CTN downregulation and *PIK3CA* mutations increase cell migration. **A,** Representative images from the wound healing assay. **B,** Quantification of migration in the wound healing assay. Scale bars = 200μm. **C,** Quantification of Boyden-chamber migration assay in NOK cells and **D,** in EPC1 cells. Data presented as means ± SEM. Student t-test used for statistical analysis. n=3 for all experiments. All cell lines are significantly different when compared to C cells ($p<0.05$). * $p<0.05$ compared to P cells. # $p<0.05$ compared to 545 cells. ^ $p<0.05$ compared to 1047 cells.

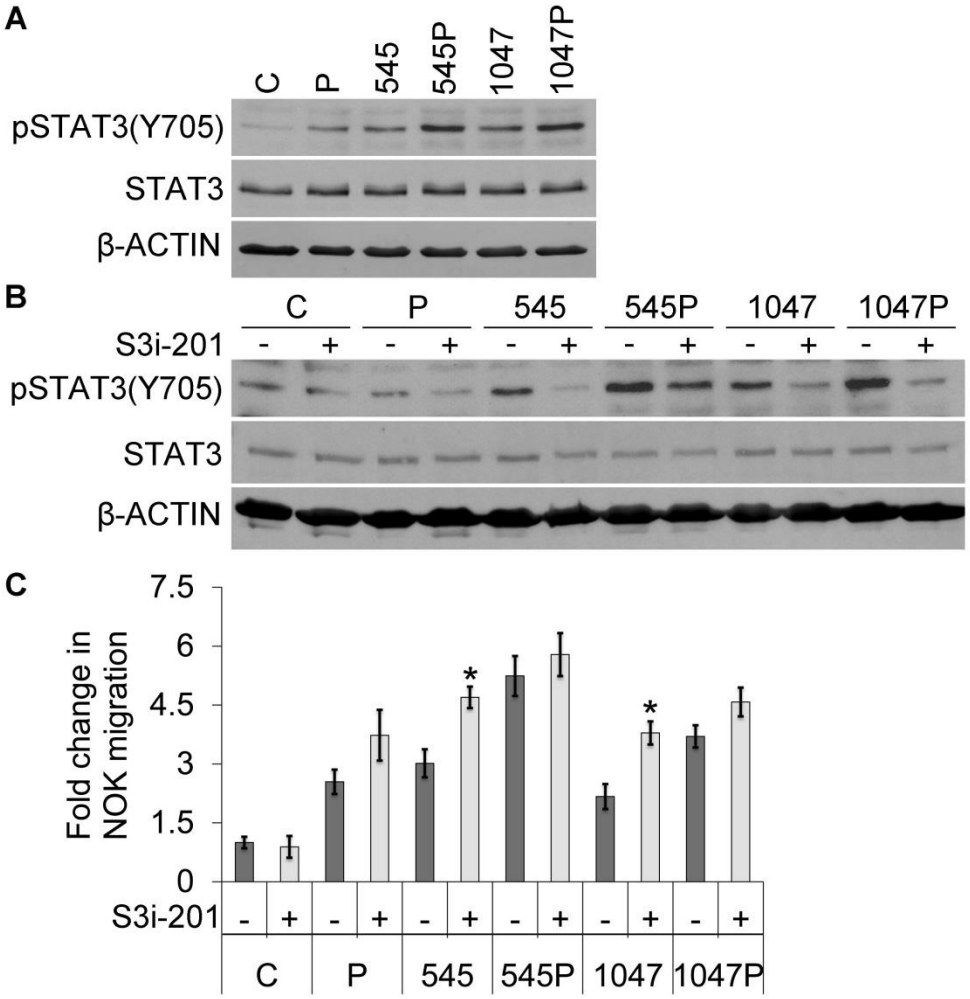
A
C
P
545
545P
1047
1047P
pSTAT3(Y705)
STAT3
β-ACTIN
B
C
P
545
545P
1047
1047P
S3i-201
- + - + - + - + - + - +
pSTAT3(Y705)
STAT3
β-ACTIN
C
Fold change in NOK migration
7.5
6
4.5
3
1.5
0
*
*
S3i-201
- + - + - + - + - + - +
C
P
545
545P
1047
1047P

Figure 3.5 STAT3 phosphorylation does not mediate the increase in cell migration due to P120CTN downregulation and *PIK3CA* mutations. A, Examination of pSTAT3(Y705) expression by Western blot analysis in NOK cells. **B,** Representative image of pSTAT3(Y705) expression in NOK cells treated with S3i-201. β-actin is used as a loading control. **C,** Quantification of Boyden-chamber migration assay upon S3i-201 treatment of NOK cells. Data presented as means ± SEM. Student t-test used for statistical analysis. n=3 for all experiments.* $p<0.05$ for S3i-201 treated cells compared to DMSO control.

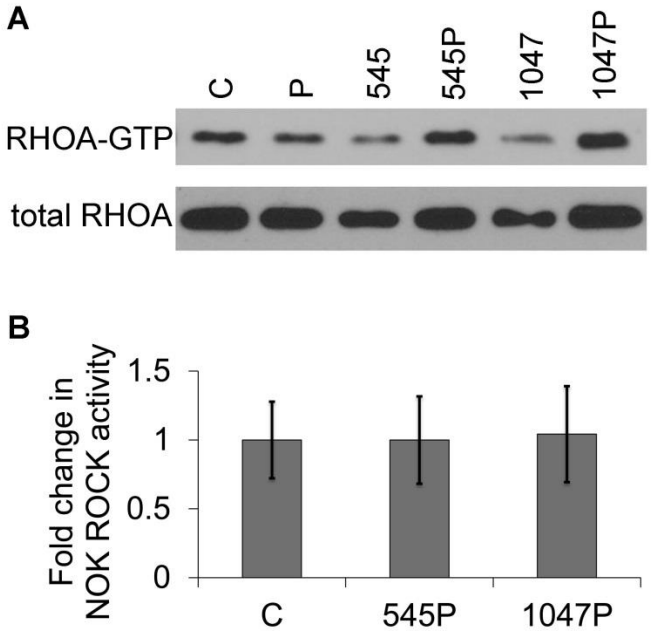

Figure 3.6. P120CTN downregulation and *PIK3CA* mutations in NOK cells do not activate RhoA sufficiently to result in the activation of its effector, ROCK. A, Examination of RHOA-GTP levels by Western blot analysis in NOK cells. Total RHOA is used as a loading control. **B,** Quantification of the ROCK activity assay in NOK cells. Data presented as means ± SEM. Student t-test used for statistical analysis. n=3 for all experiments.

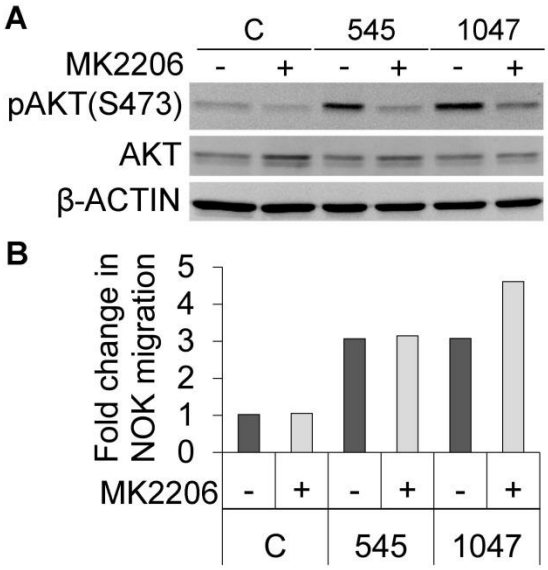

Figure 3.7. AKT phosphorylation does not mediate the increase in NOK cell migration due to *PIK3CA* mutations. A, Examination of pAKT(S473) levels by Western blot analysis in NOK cells treated with MK2206. **B,** Quantification of the Boyden-chamber migration assay upon MK2206 treatment of NOK cells. Data presented as means. No statistical analysis performed. n=2 for all experiments.

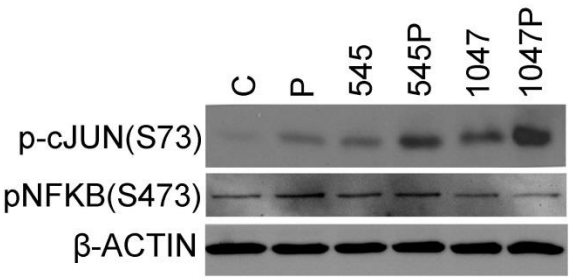

Figure 3.8. cJUN and NFkB pathways are activated by P120CTN downregulation and PIK3CA mutations in NOK cells. Examination of pCJUN (S73) and pNFkB (S536) levels by Western blot analysis in NOK cells. n=3.

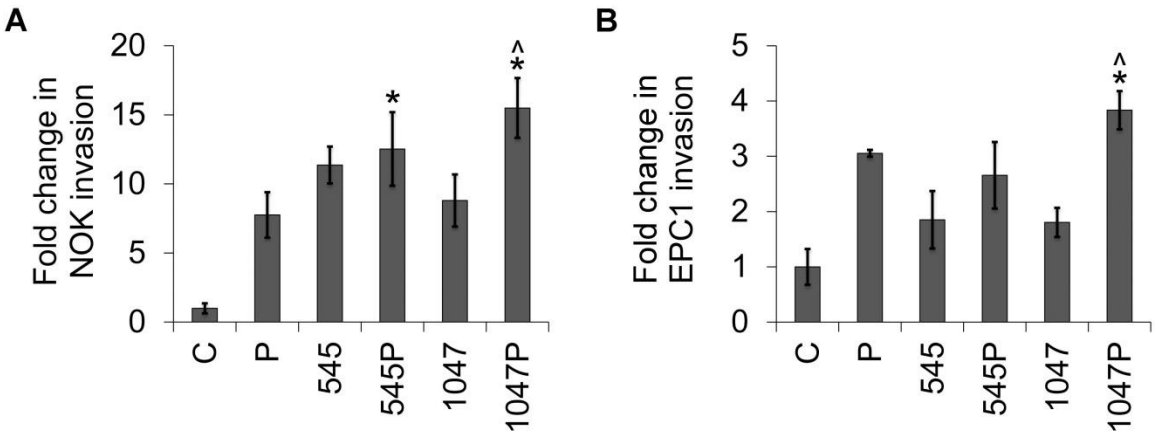

Figure 3.9. P120CTN downregulation and *PIK3CA* mutations increase cell invasion. A, Quantification of Boyden-chamber Matrigel invasion assay in NOK (n=6) and **B,** in EPC1 (n=3) cells. All cell lines are significantly different when compared to C cells ($p<0.05$). Data presented as means ± SEM. Student t-test used for statistical analysis. * $p<0.05$ compared to P cells. ^ $p<0.05$ compared to 1047 cells.

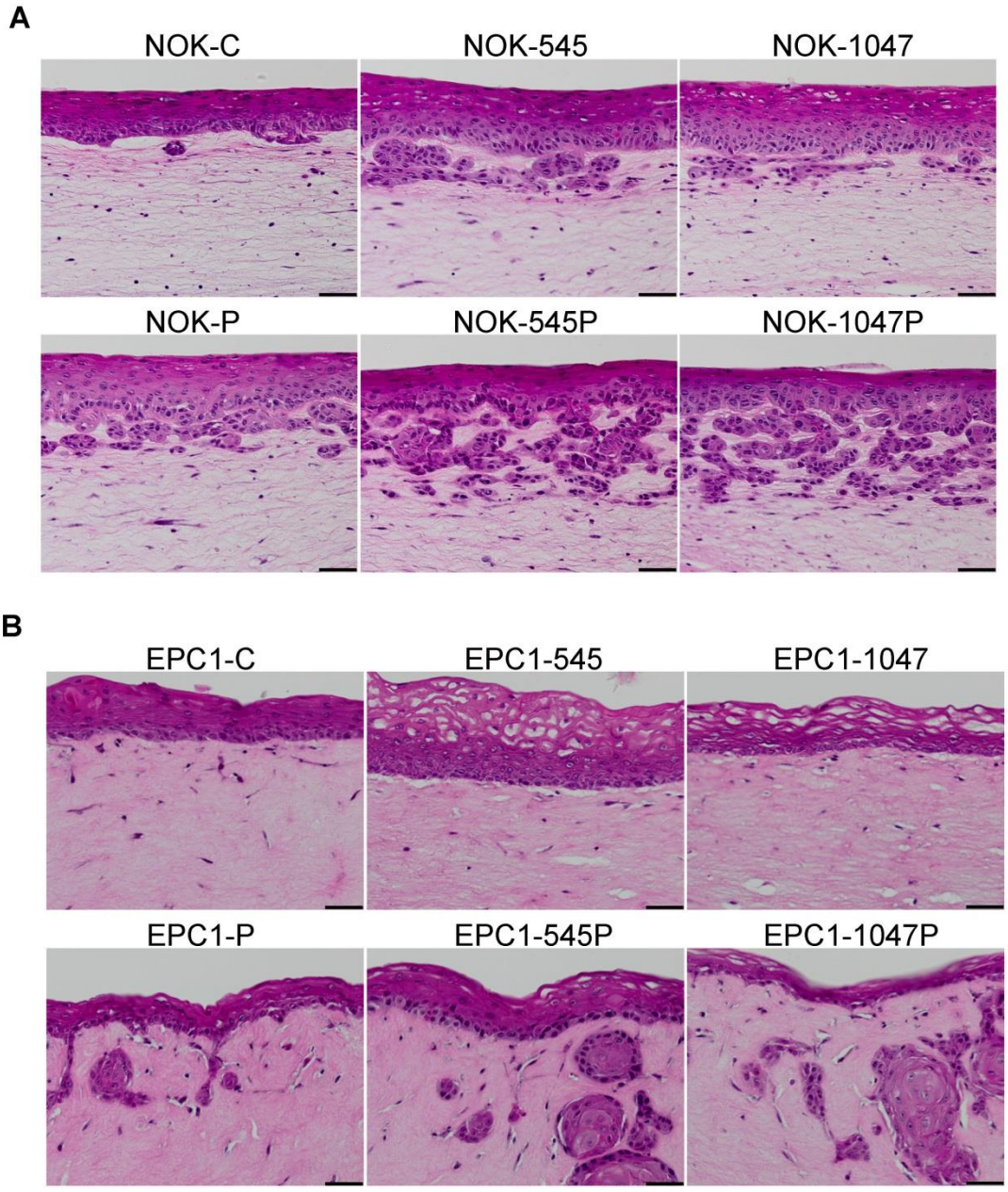
A
NOK-C
NOK-545
NOK-1047
NOK-P
NOK-545P
NOK-1047P
B
EPC1-C
EPC1-545
EPC1-1047
EPC1-P
EPC1-545P
EPC1-1047P

Figure 3.10. P120CTN downregulation and *PIK3CA* mutations increase cell invasion in 3D organotypic cultures. A, Representative images of the H&E staining of 3D organotypic cultures in NOK cells (n=3) and **B,** in EPC1 cells (n=1). Scale bars = 50μm.

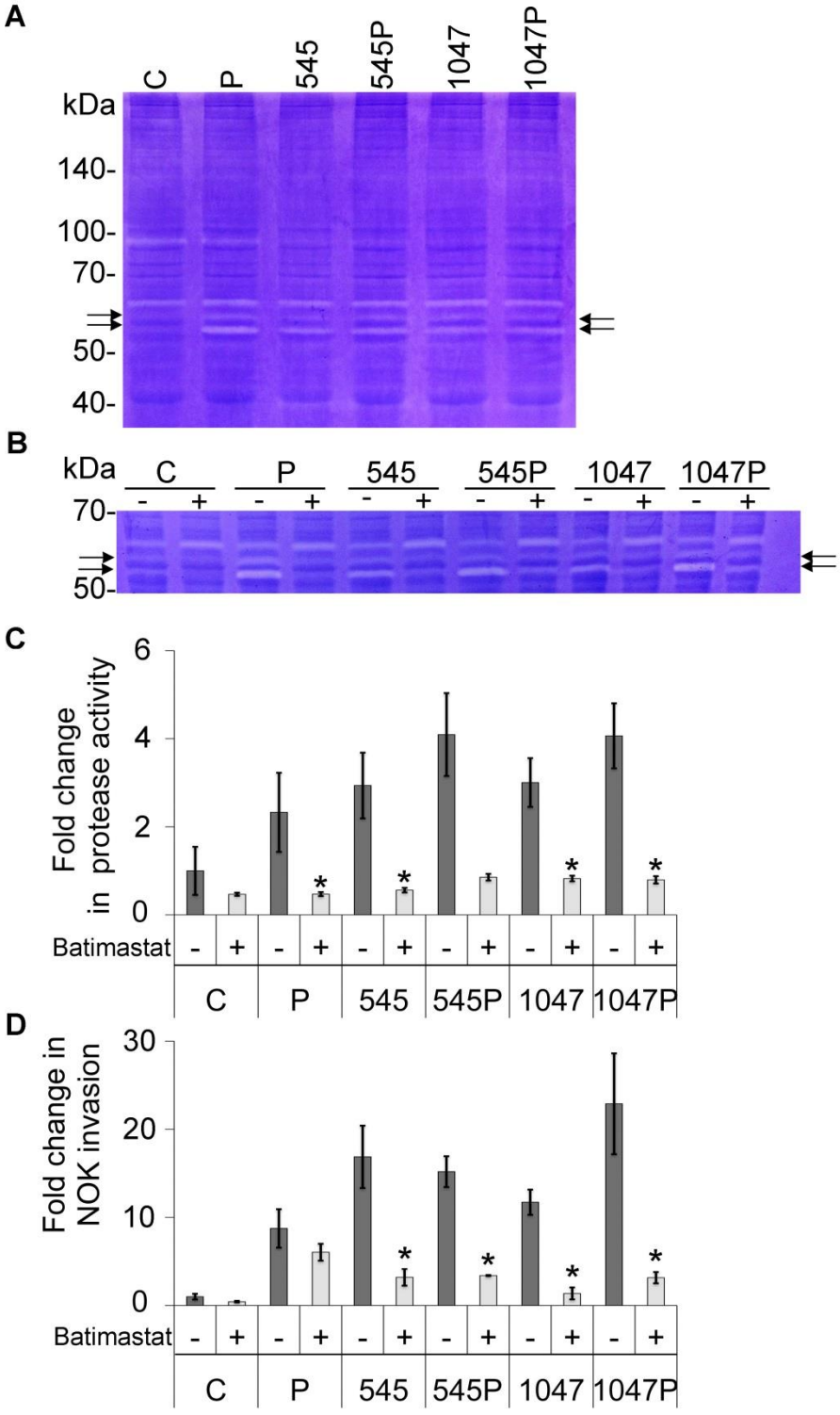
A
C
P
545
545P
1047
1047P
kDa
140-
100-
70-
50-
40-
B
kDa
C
P
545
545P
1047
1047P
- + - + - + - + - + - +
70-
50-
C
Fold change in protease activity
6
4
2
0
Batimastat
- + - + - + - + - + - +
C
P
545
545P
1047
1047P
D
Fold change in NOK invasion
30
20
10
0
Batimastat
- + - + - + - + - + - +
C
P
545
545P
1047
1047P

Figure 3.11. P120CTN downregulation and *PIK3CA* mutation-driven increase in protease activity is responsible for increased invasion. A, Representative image of gelatin zymography. Increase in protease activity indicated by arrows. **B,** Representative image of gelatin zymography of NOK cells treated with Batimastat. **C,** Quantification of gelatin zymography of NOK cells. DMSO treatment represented by (-). Batimastat treatment represented by (+). **D,** Quantification of the Boyden-chamber matrigel invasion assay upon Batimastat treatment of NOK cells. Data presented as means ± SEM. Student t-test used for statistical analysis. n=3 for all experiments. For DMSO-treated cells, all cell lines are significantly different when compared to C cells ($p<0.05$). *$p<0.05$ for Batimastat treated cells compared to DMSO control.

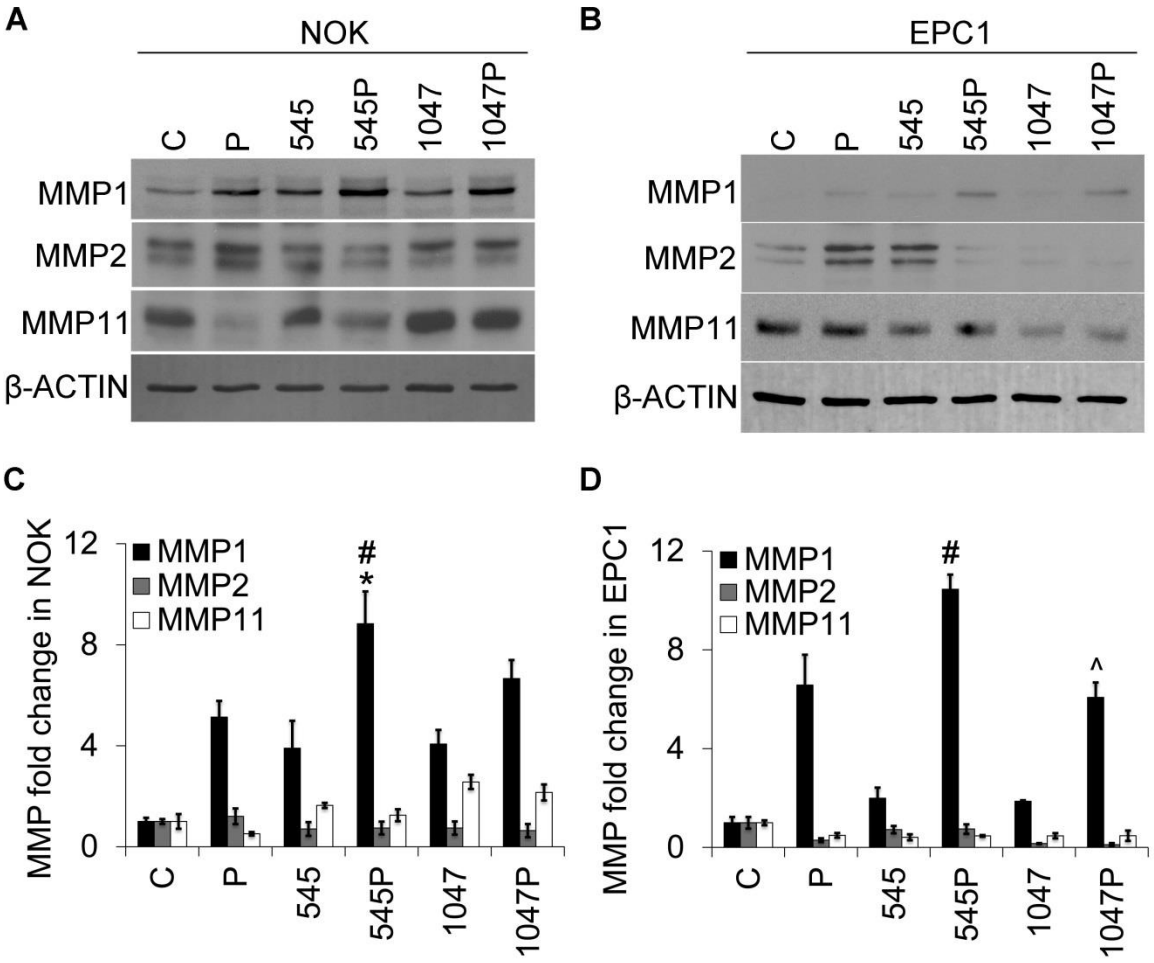
A
NOK
C
P
545
545P
1047
1047P
MMP1
MMP2
MMP11
β-ACTIN
B
EPC1
C
P
545
545P
1047
1047P
MMP1
MMP2
MMP11
β-ACTIN
C
MMP fold change in NOK
12
8
4
0
MMP1
MMP2
MMP11
#
*
C
P
545
545P
1047
1047P
D
MMP fold change in EPC1
12
8
4
0
MMP1
MMP2
MMP11
#
>
C
P
545
545P
1047
1047P

Figure 3.12. P120CTN downregulation and *PIK3CA* mutations increase MMP1 expression. A, Examination of MMP1, MMP2, MMP11 expression by Western blot analysis in NOK cells and **B,** in EPC1 cells. β-actin is used as a loading control. **C,** Quantification of Western blots from NOK cells and **D,** in EPC1 cells. Data presented as means ± SEM. Student t-test used for statistical analysis. n=3 for all experiments. MMP1 expression in all cell lines is significantly different when compared to C cells ($p<0.05$). * $p<0.05$ compared to P cells. # $p<0.05$ compared to 545 cells. ^ $p<0.05$ compared to 1047 cells.

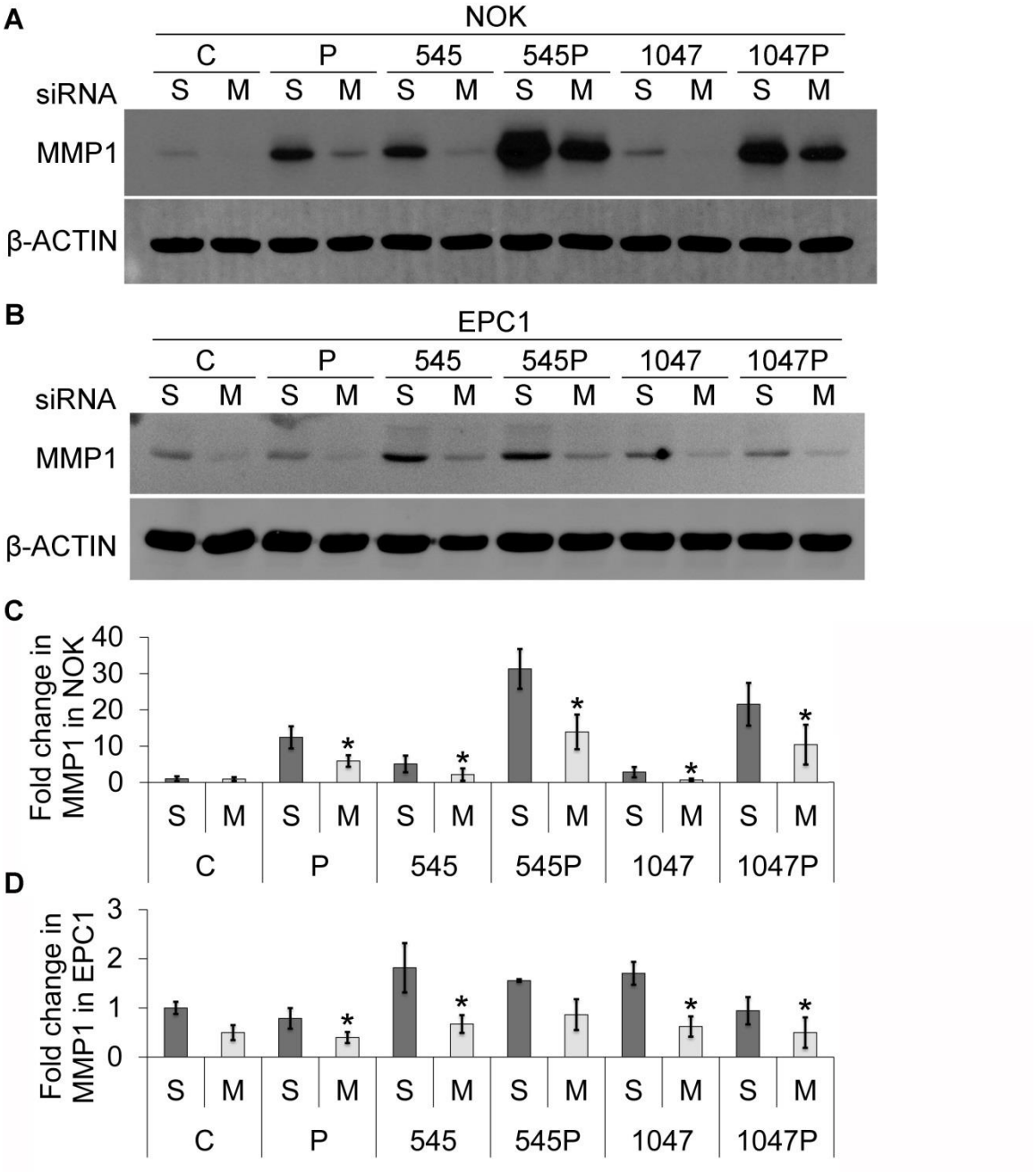
A
NOK
C
P
545
545P
1047
1047P
siRNA
S M S M S M S M S M S M
MMP1
β-ACTIN
B
EPC1
C
P
545
545P
1047
1047P
siRNA
S M S M S M S M S M S M
MMP1
β-ACTIN
C
Fold change in MMP1 in NOK
40
30
20
10
0
S M S M S M S M S M S M
C P 545 545P 1047 1047P
D
Fold change in MMP1 in EPC1
3
2
1
0
S M S M S M S M S M S M
C P 545 545P 1047 1047P

Figure 3.13. MMP1 siRNA decreases MMP1 expression in NOK and EPC1 cells. A, Western blot analysis of nucleofected NOK cells and **B,** in EPC1 cells. β-actin is used as a loading control. **C,** Quantification of Western blots from NOK cells and **D,** in EPC1 cells. “S” denotes scrambled siRNA. “M” denotes *MMP1* siRNA. Data presented as means ± SEM. *p<0.05. Student t-test used for statistical analysis. n=4 for all experiments.

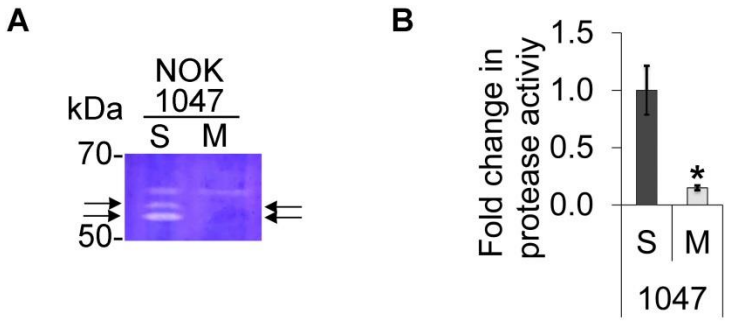

Figure 3.14. MMP1 siRNA decreases protease activity at 50-55kDa in NOK-1047 cells. A, Representative image of gelatin zymography of *MMP1* siRNA nucleofected NOK-1047 cells. MMP protease activity indicated by arrows. **B,** Quantification of gelatin zymography of *MMP1* siRNA nucleofected NOK-1047 cells. Data presented as means ± SEM. *p<0.05. Student t-test used for statistical analysis. n=3.

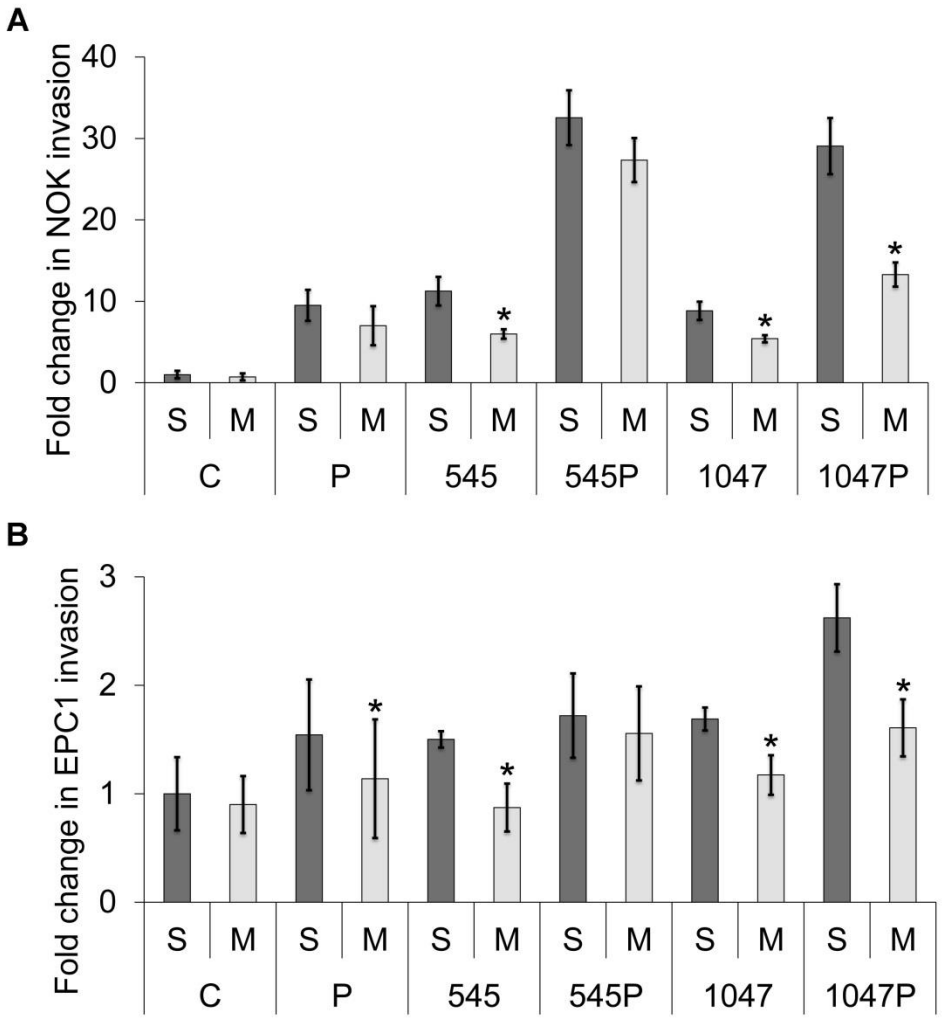

Figure 3.15. MMP1 inhibition decreases invasion in NOK and EPC1 cells. A, Quantification of the Boyden-chamber Matrigel invasion assay in NOK cells and **B,** in EPC1 cells. *p<0.05. Student t-test used for statistical analysis. n=3.

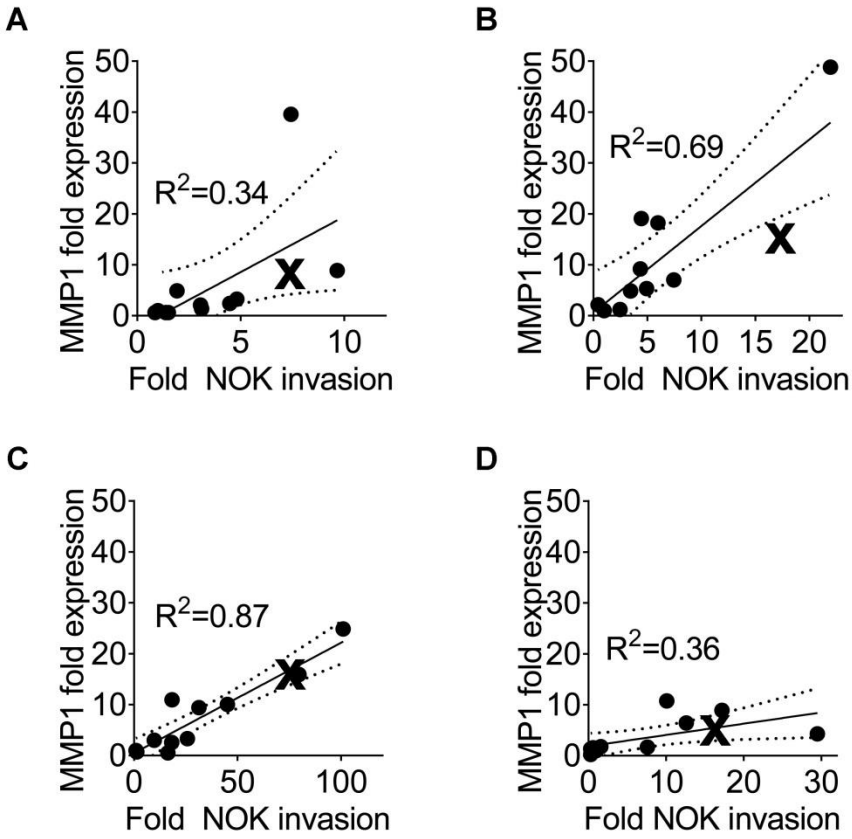

Figure 3.16. MMP1 inhibition is proportional to the amount of invasion in NOK cells. A-D, Pearson correlation analysis of individual replicates of Boyden-chamber migration analysis in NOK cells. "X" denotes *MMP1* siRNA transfected NOK-545P cells. The dotted line represents the 95% confidence interval. R^2 value depicted on the graph represents the fit of the trend line. Pearson correlation used for statistical analysis.

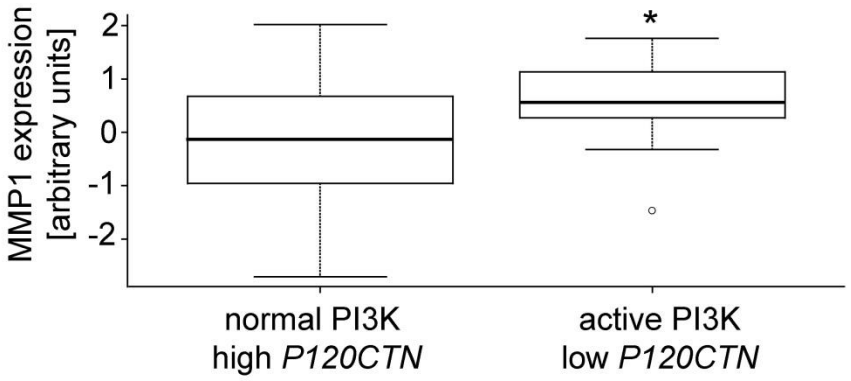

Figure 3.17. MMP1 expression is increased in HNSCC tumor samples with active PI3K signaling and low *P120CTN* expression. Boxplot of the TCGA data of HNSCC. *p<0.05. Wilcoxon rank sum test used for statistical analysis. normal PI3K / high *P120CTN* (n=94). active PI3K / low *P120CTN* (n=23).

cell lines (EPC1/NOK)	P120CTN	PIK3CA
C	wild-type	wild-type
P	knockdown	wild-type
545	wild-type	E545K
545P	knockdown	E545K
1047	wild-type	H1047R
1047P	knockdown	H1047R

Table 3.1. P120CTN and PIK3CA status in NOK and EPC1 cells.

	MMP1	MMP2	MMP3	MMP8	MMP9	MMP10	MMP13
C	1.0	1.0	1.0	1.0	1.0	1.0	1.0
P	1.0	0.9	0.9	0.7	0.6	0.8	0.8
545	1.5	1.4	0.9	0.9	0.5	1.0	0.7
545P	2.1	1.0	0.9	0.8	0.5	0.9	0.7
1047	1.3	1.3	0.9	0.8	0.5	1.1	0.8
1047P	2.0	1.3	1.0	1.0	0.6	1.4	0.6

Table 3.2. Fold expression of MMPs in NOK cells from MMP array. n=2.

Chapter 4

Overall Discussion

4.1 Introduction

4.1.1 Chapter 2

Head and Neck Squamous Cell Carcinoma (HNSCC) originates in the squamous epithelium of the head and neck region and results from multiple genetic events which promote cell transformation. Aberrations of P120CTN and PIK3CA appear to play a significant role in tumorigenesis. Data from the oral/esophageal tumor P120CTN-null mouse model indicate that the loss of P120CTN results in an alteration/aberration in the immune milieu of tumors. Myeloid-derived Suppressor Cells (MDSCs) have been shown to be recruited to the tumor site and have been implicated in supporting carcinogenesis. Whether MDSCs potentiate disease development or are simply a marker of disease is unclear. To better understand the role of MDSCs in HNSCC development, we utilized the P120CTN-null mice and assessed the localization and function of MDSCs at various phases of disease progression. As shown in *Chapter 2*, infiltrating MDSCs were observed in all organs in adult mice with fully developed HNSCC. This contrasted with published reports indicating that larger numbers of MDSCs were recruited to tumors, and instead demonstrates that MDSCs are increased in all organs due to P120CTN loss in the oral epithelium. Additionally, in disease-free three-month old mice, MDSCs were found in organs exposed to higher volumes of blood, such as the kidney. This suggests that MDSCs circulate

in the bloodstream prior to infiltrating various organs and that the presence of MDSCs in the blood precedes tumor formation.

4.1.2 Chapter 3

We also investigated the impact of combining P120CTN downregulation with *PIK3CA* mutations. Together, the loss of P120CTN and mutation of PIK3CA resulted in increased cell migration, which appeared to be independent of STAT3 or RHOA-mediated signaling. Both STAT3 and RHOA signaling were previously shown to increase migration in HNSCC cell lines [237, 238]. However, under conditions in which P120CTN is downregulated and *PIK3CA* mutated, inhibition of STAT3 was not sufficient to reduce cell migration. Moreover, inhibition of STAT3 in NOK cells resulted in enhanced cell migration. These results suggest that STAT3 may have opposing effects on migration depending on the genetic background of the cell. Reports that demonstrate a decrease in migration in HNSCC cell lines due to STAT3 inhibition propose targeting the STAT3 pathway as a possible therapeutic target [237]. Our findings emphasize that STAT3 activation might play a different role depending on the genetic context and STAT3 inhibition in some patients might actually worsen the disease outcomes.

In addition, we have identified that P120CTN downregulation and *PIK3CA* mutations increase cell invasion via an increase in MMP1 production. We are the first to demonstrate increased MMP1 expression due to aberrations in P120CTN and PIK3CA in HNSCC. In Esophageal Squamous Cell Carcinoma (ESCC), a

cancer closely related to HNSCC, MMP1 expression predicts decreased patient survival [280]. This suggests that MMP1 expression could also predict negative outcomes in HNSCC. Moreover, in our experimental system P120CTN downregulation and *PIK3CA* mutations cooperatively increase MMP1 expression. This in turn indicates that the combination of P120CTN loss and active PI3K signaling might also be correlated with poor patient survival in HNSCC. In addition, signaling pathways downstream of both of these genetic aberrations most likely intersect at some point to increase the expression of their common target, MMP1.

Interestingly, MMP1 is one of the most commonly upregulated MMPs in HNSCC [4]. Until now, its expression in HNSCC has not been correlated with PI3K pathway activity nor P120CTN loss/downregulation. It is surprising, considering that PI3K pathway alterations and P120CTN downregulation occurring in 30% and 50% of HNSCC tumors, respectively [18, 121]. This suggests that in many cases in which MMP1 has been shown to be increased, the causal agents might be aberrations to PI3K and P120CTN signaling. Analysis of the TCGA database of HNSCC tumors provides further validation for that assertion. Tumors with low *P120CTN* and active PI3K signaling had higher expression of *MMP1* than tumors with high *P120CTN* and normal PI3K signaling. Finally, our results indicate that development of an anti-MMP1 treatment could aid in reducing metastasis, the leading cause of death in the majority of cancers.

4.2 MDSC Discussion

4.2.1 MDSC data in the context of literature

We have determined that P120CTN loss in epithelial cells does not improve MDSC survival, activation or directional migration; however, MDSCs did survive better, became more activated and exhibited increased migration when exposed to conditioned medium from P120CTN-null or P120CTN-wild-type epithelial cells. This indicates that other cells present in the tissue stroma can have significant effects on MDSCs. The fact that normal keratinocytes upregulated CD124 so drastically compared to non-conditioned medium indicates that studies with MDSCs have to be put in perspective of neighboring cells. The Gabrilovich group demonstrated that MDSC subpopulations identified by CD124, CD80, CD115, CD273 and CD274 in ten different mouse tumor models did not define a more active subset [212]. We came to similar conclusions, which differ from the conclusions established by earlier studies that proposed these surface factors as markers of active MDSCs [170, 189-191]. It is possible that each mouse model and disease have their own specific surface markers that identify the active MDSC subset. After all, each of these pathologic entities will differ with regards to cytokine expression. Different cytokines will promote MDSC differentiation and recruitment using alternate signaling pathways. We do not know much about the specific signaling pathways that are responsible for MDSC induction, but we can speculate that dissimilar signaling

pathways will induce MDSCs that will differ in their surface marker expression. However, if that is the actual case, data identified in mouse MDSCs will be even more difficult to translate to humans. In order for mouse MDSC studies to be more translatable they need to identify signaling pathways involved in MDSC differentiation or the mechanism of action of the surface factor that defines the active subset.

Additionally, our results indicate that an increase in MDSCs is systemic and not specific to the organs studied. This emphasizes the importance of testing additional organs when studying tumor-resident MDSCs. If our findings apply to other tumor models, blood draws would be sufficient for MDSC isolation and subtyping. If indeed MDSCs are increased in all organs in other cancers, then the field should focus on studying blood resident MDSCs due to their easy accessibility. We have to also consider another possibility. We have assumed MDSCs play primarily an immunosuppressive role, but it is possible they function in additional ways to modify the tumor microenvironment, for example by facilitating angiogenesis.

4.2.2 Implications of MDSC data in future therapeutic design

MDSCs are a poorly understood group of immunosuppressive cells that have been extensively studied in cancer with the hopes that MDSC inhibition will lead to tumor immune destruction. However, MDSCs are not the only immunosuppressive immune cell present in mammals. Regulatory T cells, and

tumor-associated macrophages and neutrophils, promote tumor growth by inhibiting cytotoxic killing of tumor cells [281, 282].

Our mouse model implicates that a systemic increase in MDSCs is sufficient in supporting tumor growth. This notion could be exploited in organ transplantation. If tumor survival can depend completely on the presence of systemic MDSCs, it would be possible to support organ transplants with a similar approach. MDSC increases can be systemic and not specific to the organ being transplanted and still have an effect. Maybe even cytokines that are responsible for the systemic increase of MDSCs could be used to support organ transplants. Similar approaches have been tried in the organ transplant field. Transfer of regulatory macrophages, which are closely related to MDSCs, produced tolerance in renal transplant patients with decreased use of immunosuppressive drugs [283]. Moreover, mobilization of MDSCs in a heart transplant model is indispensable to tolerance [284]. Therefore, MDSC research groups should focus their efforts on the identification of cytokines that lead to systemic increases of MDSC that still support tumor growth. MDSC-induction therapy could be a safer alternative to the present immunosuppressive regimen in prevention of transplant rejection. The benefit of using MDSCs over immunosuppressive drugs could be in limiting the common side effects patients experience when immunosuppressed. For example, Tacrolimus and Cyclosporine carry a risk of nephro- and neurotoxicity in transplant patients [285]. Replacing these drugs with

an MDSC therapy would not remove all of the negative effects of the drug, but would instead limit them.

Moreover, MDSC-induction therapy could be used in other diseases with an overactive immune system. Asthma is an inflammatory disorder suffering from chronic airway inflammation leading to a reduction in lung function. MDSCs can be used in asthma as a treatment. Studies in mice demonstrated that adoptive transfer of MDSCs or Lipopolysaccharide (LPS)-induction of MDSCs is sufficient to reduce asthma symptoms [286, 287]. Interestingly, LPS treatment of mice mimics a phenomenon observed in young children from rural areas. These children are exposed to less sterile environments and often encounter high doses of LPS during their development. This environment immunizes them against developing asthma [288]. This further strengthens the role that MDSCs play in healthy immune responses. However, we have to take under consideration that increased MDSC levels might have unwanted side effects associated with immunosuppression such as decreased immune surveillance potentially resulting in increased cancer incidence.

Thus far, targeting MDSCs in clinical trials has not shown great efficacy. Sunitinib, a tyrosine kinase inhibitor, decreases MDSC counts in tumor-bearing mice [289]. However, single agent sunitinib clinical trials in HNSCC patients did not provide any benefit to patient outcomes and necessitated early closure of the trial [290]. On the other hand, a combination therapy of sunitinib and radiotherapy

has demonstrated a complete response in 44% of cancer patients with historically incurable metastatic disease [291]. The patients recruited to this study had different cancers and had between one and five distant metastasis. After nine months of treatment no sign of distant disease could be found in 44% of the patients. Unfortunately, the study authors did not specify which cancers performed better due to the treatment. What complicates further the analysis of these results is that none of these studies targeted MDSCs specifically. Hence, currently it is hard to determine if MDSC inhibition holds promise in future HNSCC treatment. However, we can speculate that some of the HNSCC immunotherapies could be potentially targeting MDSCs. Pembralizumab is one of the two immunotherapies offered for HNSCC patients. It inhibits binding of Programmed Death receptors (PD-1) found on cytotoxic T cells to their ligands, which are expressed by tumors and can also be found on the surface of MDSCs. Inhibition of MDSCs appears to potentiate PD ligand blockade, implying that combination therapy targeting both MDSCs and PD-1 could have a synergistic effect [292]. This effect could potentially be found in HNSCC patients allowing for better treatment options.

4.3 Discussion of cooperation of P120CTN downregulation and PIK3CA mutations

We have identified that P120CTN downregulation and *PIK3CA* mutations increase invasion via MMP1 expression. Moreover, both of these genetic

manipulations increase cell migration. The mechanism of action responsible for the migration increase due to P120CTN downregulation and *PIK3CA* mutations in oral keratinocytes is yet to be determined. However, the increase in migration and invasion very likely has an effect *in vivo,* which could be tested by a tail vein assay or *in vivo* microneedle invasion assay [293, 294]. After all, migration and invasion are the fundamental processes of metastasis. Cancer cells that migrate faster and invade more, are likely to have a greater rate of metastasis and therefore a poorer prognosis. We found that P120CTN downregulation and *PIK3CA* mutations not only increase cell migration and invasion, but their effects work in a cooperative manner leading to a ceiling effect, which implicates convergence on the same signaling pathway. The implications of P120CTN on metastasis are emphasized in reports that correlate P120CTN downregulation with a higher rate of metastasis in HNSCC [121, 258-260]. Moreover, PIK3CA overexpression correlates with lymph node metastasis in HNSCC [87]. This emphasizes the importance of PI3K signaling in HNSCC metastasis.

4.3.1 P120CTN aberrations in migration and invasion

Our findings implicating P120CTN downregulation and activating *PIK3CA* mutations in increasing migration and invasion are in accordance with the literature. Aberrations to either P120CTN or PIK3CA in other cell types have also been linked to changes in metastatic potential. P120CTN loss in noninvasive mouse mammary tumors via anoikis-resistance transformed the cells into a

metastatic cancer that metastasized into the lungs and lymph nodes [130]. In our system, P120CTN downregulation led to increased cell invasion. Additionally, we did not observe large changes in anchorage-independent growth assay, which tests resistance to anoikis, indicating that the changes induced by P120CTN loss are cell type specific.

Interestingly, besides P120CTN loss or downregulation, P120CTN isoform-switch and mislocalization can also increase cell invasion. In prostate carcinoma, P120CTN isoform-switch from isoform 3 (epithelial-like) to isoform 1 (fibroblast-like) correlates with an E-cadherin to N-cadherin switch. This P120CTN isoform-switch characterizes a more invasive tumor cell line, which the authors speculate has undergone EMT [295]. Similar findings were postulated by Anastasiadis et al., who demonstrated that P120CTN isoform 1 via RHOA induces an invasive phenotype [128]. P120CTN isoform 1 has been regarded as the isoform that promotes migration, so these findings are not surprising. However, P120CTN isoforms need to be studied in the context of a specific cell type. While Anastasiadis et al. demonstrated an increase in invasion due to P120CTN isoform 1 in breast cancer cells, Liu et al. presented evidence demonstrating the opposite effect in lung cancer cells [296]. P120CTN isoform 1 expression induced small amount of RHOA activation and inhibited invasion. Expression of P120CTN isoform 3 induced a large increase in the activity of RHOA but it had no effect on invasion. These findings emphasize that the same

P120CTN isoforms might play opposite roles in regulation of invasion depending on cell type. Moreover, activation of one of the RHO GTPases does not always lead to increased migration and invasion due to the complex interplay between many RHO GTPases acting concurrently in the same cell. In addition, P120CTN mislocalization can also modulate invasion and migration. Mislocalization of P120CTN has been correlated with lymph node metastasis in ESCC [296]. In addition, basal-like breast cancer cell lines that are more invasive often have mislocalized P120CTN [297]. Therefore, almost any aberration to P120CTN can result in modification of migration and invasion in the cell. This emphasizes the importance of P120CTN as a master regulator of cell motility and invasion. Its role as a regulator implicates the need for other ongoing processes that drive the motility and invasion.

4.3.2 PIK3CA aberrations in migration and invasion

PI3K pathway activity regulates a multitude of mechanisms controlling cell behavior from survival to motility. PI3K pathway activation induced by PIK3CA overexpression or activating mutations modulates tumor cell invasive capability in many cancers. ESCC tumors express increased levels of PIK3CA staining compared to adjacent normal tissue and upon PIK3CA knockdown in these tumors cell motility is decreased [298]. An HNSCC mouse model, induced by 4-nitroquinolone exposure, demonstrated that the addition of PIK3CA overexpression increases the amount of metastases from 0% to 40% [228].

Besides overexpression of PIK3CA, activating mutations of PIK3CA are commonly found in HNSCC and other cancers. PIK3CA mutations increase migration and invasion in breast cancer cells on a mutant Ras, mutant p53 background [299]. Ovarian cancer cells with E545K PIK3CA mutation migrated more than cells with wild-type PIK3CA [300]. This same mutation in cholangiocarcinoma was shown to promote migration and invasion in an AKT-dependent manner [301]. Therefore, PI3K pathway activation due to PIK3CA mutations commonly leads to increased cell migration and invasion.

It is important to mention that PIK3CA mutants appear to work either via canonical-AKT signaling pathway or via an unknown non-AKT pathway. While some of the reports provide evidence that AKT inhibition is sufficient to limit the migration, others do not replicate those findings [301]. Interestingly, reports from breast and lung cancers suggest that AKT is not the pathway used by PIK3CA mutants to propagate migration [242, 270, 271]. Similar findings were demonstrated by our experiments, where inhibition of pAKT with MK2206 did not decrease NOK migration. Moreover, it is likely that the actual rate of PIK3CA-driven migration and invasion via the non-AKT pathway is much higher than reported due to the present publishing bias that discourages publishing of negative or difficult to explain data.

4.3.3 Effects on patient survival

Aside from HNSCC, P120CTN loss or *PIK3CA* mutations correlate with decreased patient survival in many cancers, such as those of lung, breast, pancreas, bladder, and anus [117, 251-255]. At first glance, some cancers, such as colon cancer, show no statistical significance of *PIK3CA* mutations on patient outcomes [256]. However, *PIK3CA* mutations correlate with decreased patient survival in colon cancer patients who have undergone monoclonal EGFR therapy [257]. It is possible that PIK3CA mutations provide resistance to anti-EGFR therapy by relieving the tumors oncogene addiction. EGFR signaling partially signals via PIK3CA and presence of tumors cells with PIK3CA mutations will allow these cells to survive in the absence of EGFR activation. Additionally, many cancers with P120CTN loss or PI3K pathway activation have been independently reported to have increased MMP1 expression which is correlated with negative patient outcomes [273-279]. Thus far, the mechanism responsible for increased MMP1 expression in many of these cancers is still unknown. We speculate that in a portion of these cancers, P120CTN loss and PI3K activation could be responsible for increased MMP1 expression. While further investigation is required to confirm that hypothesis, the discovery of additional cancers that exploit P120CTN loss and PI3K pathway activation would emphasize the importance of studying P120CTN and PIK3CA simultaneously.

The effects of P120CTN downregulation and PIK3CA mutations on MMPs are poorly studied in HNSCC. Only one report demonstrates that P120CTN loss leads to an increase of MMP1 mRNA in endothelial cells, but no functional consequences have been demonstrated for this relationship [262]. Our studies are the first to link P120CTN downregulation and PIK3CA mutations to MMP1 expression in HNSCC. However, MMP1 alone has been previously reported on in HNSCC. In fact, a literature review by Iizuka et al. claims that MMP1 is the most upregulated MMP in HNSCC [4]. This is very interesting considering how prevalent P120CTN aberrations and activation of PI3K signaling pathway are in HNSCC. It is very likely that both aberrant P120CTN and PI3K signaling pathways are responsible for the majority of observed MMP1 expression. Additionally, it is important to identify what other events also contribute to increased MMP1 expression. So far only P120CTN loss and downregulation have been demonstrated to increase MMP1 expression. P120CTN signaling can be affected by phosphorylation, isoform switching and mislocalization, but no data exists on the effect of these changes on MMP1 expression.

We have demonstrated the functional role of increased MMP1 expression in driving cell invasion. However, MMP1 can be used also as a biomarker. MMP1 mRNA levels in saliva can be used to detect oral squamous cell carcinomas [302]. In addition, MMP1 is a marker of late stage HNSCC, which further emphasizes the importance of our findings [263]. Increased MMP1

expression in late stage HNSCC suggests that MMP1 may take part in processes that occur during late stages of carcinogenesis, i.e. metastasis. Therefore, inhibition of MMP1 could potentially limit the amount of metastasis.

Many clinical trials have been undertaken to evaluate effects of MMP inhibition in human cancers, but none of them produced positive results [303]. It was speculated that pre-clinical trials in mice were administered at an early stage of the disease, which lead to a greater efficacy of the treatment. This was confirmed by Batimastat treatment of tumor-bearing mice at various stages of disease. Batimastat retained its efficacy in mice with early disease, but it had no effects if the mice were treated at an advanced stage of cancer [304]. Additionally, many of the clinical trials faced issues of bioavailability and side effects that limited the dose of the available treatment.

A successful anti-MMP treatment in HNSCC needs to meet certain criteria. First, all of the MMP inhibitors used in clinical trials are pan-MMP inhibitors, meaning they target all of the MMPs. Pan-MMP inhibitors do not inhibit all the MMPs equally and therefore it is hard to achieve complete inhibition in all the targeted MMPs without causing toxic effects. Furthermore, it is difficult to identify *in vivo*, which MMPs specifically are inhibited due to the treatment. In order to avoid these issues, anti-MMP1 therapy in HNSCC has to consist either of a selective MMP1 inhibitor or an inhibitor to the transcription factor / signaling

pathway inducing MMP1 in the cancer. The use of selective inhibitors will allow for much higher dosing and possibly efficacy of the treatment.

Second, MMP inhibition works better at an early stage than at the late stage. If we assume MMP inhibition blocks the formation of new metastases, the micrometastases that have formed before the start of the MMP treatment will still be there and will continue to develop and grow and give rise to new metastatic foci. Even in this worst case scenario, if we inhibit seeding of new micrometastases, treatment of the primary tumor and a limited amount of metastases with a combination of surgery and radiotherapy could prolong patient survival. If we can eliminate the rise of new metastases with the anti-MMP treatment, resection of the tumor and all the metastatic foci could be curative. The drawback of this approach is exposed if we consider the fact that HNSCC might initiate a large number of micrometastases. Then even if we stop future metastatic seeding, the patient would still be at risk for tumor recurrence for the rest of their lives.

Finally, MMP1 is not the only MMP expressed by HNSCC tumors. Identification of the MMP profile of each tumor and treatment with specific inhibitors to the tumor-secreted MMPs would increase the treatment efficacy. Alternatively, treatment of many MMPs with inhibitors of transcription factors / signaling pathways might be actually more pragmatic. MMPs are located in clusters throughout the genome. MMP1 is located on chromosome 11q22 along

with MMP3, MMP7, MMP8, MMP10, MMP12, MMP13, MMP20, and MMP27 [157]. Very often the same transcription factors induce the activation of multiple MMPs in the same cluster at the same time. Additionally, the effect of transcription factors that induce MMPs can extend into other aspects of cell biology as apoptosis or motility. Hence, by using inhibitors of transcription factors, we might be able to limit the expression of more than one MMP at a time without affecting MMPs not associated with the tumor and possibly affect additional processes that could also be helping the tumor cells to survive or invade.

The search for new therapeutics in HNSCC is especially important. Thus far only two immunotherapy drugs are offered for patients with HNSCC – Pembrolizumab and Cetuximab. Pembrolizumab works by inhibiting the interacton of PD-1 surface receptor (found on T cells) with PD-L1 (excreted by tumor cells) to limit the immunosuppressive effect of PD-1 activation on the T cells. In 16% of treated HNSCC patients with metastatic disease, Pembrolizumab induced a tumor response lasting from 6 to 24 months, with some patients achieving a complete regression. Cetuximab targets EGFR and in the EXTREME trial has been demonstrated to increase median overall survival from 7.4 months to 10.1 months in HNSCC patients with metastatic disease [305]. In summary, the immunotherapy treatments we currently possess provide limited effect to a small subset of patients. Hence, developing additional treatments is of utmost

importance, so we can start curing HNSCC patients instead of delaying the time of their death due to cancer.

4.3.4 Alternative proteolytic activity present in our experimental system

P120CTN downregulation and *PIK3CA* mutations increase invasion via MMP1 expression. We identified MMP1 as the downstream target in our experimental model based on the MMP array we have performed. P120CTN signaling is poorly studied with respect to MMPs. The only published connection exists to MMP7, which is inhibited by Kaiso, a P120CTN binding partner [138]. P120CTN is theorized to sequester Kaiso and allow for expression of its targets. However, P120CTN knockdown in prostate cancer results in MMP7 increase speculated to result due to modification of Wnt pathway activity [139].

Activating PIK3CA mutations have not been yet studied with regards to the effect on MMP expression. However, active PI3K signaling pathway is correlated with increased expression of multiple MMPs. Epidermal Growth Factor and Fibronectin independently induce MMP9 expression via activation of PI3K signaling in ovarian cancer [306, 307]. Similar findings have been demonstrated in HNSCC, where betacellullin, an EGFR agonist, induced MMP9 via the PI3K/Akt pathway [147]. In the endothelium, PI3K signaling promoted MMP2 and MMP14 expression [308, 309]. Interestingly, MMP1 expression in prostate cancer appears to be regulated by FAK and PI3K activity [310]. Furthermore, data from inflammatory joint disease demonstrate that PI3K signaling induced not

only MMP1 expression but also MMP13 expression [311]. Interestingly, small RHO GTPases such as RHOA and CDC42 appear to play opposing roles in regulating MMP2 and MMP14 [312]. While in our experimental system we have not established the status of RAC1/CDC42 signaling, RHOA activation appears to be minimal, which favors the possibility of its antagonists, RAC1 and CDC42 being active and contributing to MMP expression. MMP14 is a membrane bound MMP. This MMP digests ECM directly in front of the cell and is considered to play an important role in clearing the path for the invading HNSCC cells [313]. Furthermore, evidence exists of MMP14 being regulated by PI3K signaling [314]. Interestingly, PI3K signaling also increases the expression of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) [89]. MMP1 can be localized to the cell surface by association with EMMPRIN, which binds MMP1 and activates it [162, 163]. Therefore, MMP1 in the presence of EMMPRIN becomes a cell surface MMP just like MMP14. Finally, a look at other MMPs located at chromosome 11q22 along with MMP1 could be a good way to identify other MMPs that are increased in our experimental system [157].

4.3.5 Implications on other diseases

P120CTN and PIK3CA are proteins that are widely expressed in many different cell types. While it is of great interest that P120CTN and PIK3CA dysregulation promote increased migration and invasion in HNSCC, diseases other than cancer could also be found to be affected by these genetic changes.

Similarly, it is possible that in those diseases P120CTN downregulation and activating *PIK3CA* mutations can lead to a cooperative increase of the disease severity. Since P120CTN is a structural protein forming adherens junctions, its mislocalization or loss affects epithelial barriers. P120CTN loss leads to intestinal barrier dysfunction and improper glomerulogenesis [115, 116]. The effects on vascular permeability of P120CTN are mediated through effects on VE-cadherin [315]. Similarly to P120CTN loss, PI3K activation also promotes leaky vasculature. In the brain PI3K activity leads to opening up of tight junctions in the blood-brain barrier [316]. PIK3CA activity is closely related to proper functioning of endothelial adherens junctions [317]. The presence of E545K and H1047R mutations in the *PIK3CA* gene results in lymphatic and arteriovenous malformations [318]. It is possible that *PIK3CA* mutations in cancer cells could lead to similar effects in the surrounding vasculature by secreting exosomes, but this has not been yet evaluated. Hence, both P120CTN and PIK3CA play a role in establishing properly functioning vasculature. Interestingly, in some instances PI3K activity is viewed as beneficial. After a traumatic brain injury, the increased PI3K activity and increased P120CTN levels correlate with improved blood-brain barrier function [319].

P120CTN and PIK3CA can also modulate processes ongoing in neurons. P120CTN and PI3K signaling are involved in synapse formation. P120CTN uncoupling from the membrane allows synapses to form, which is very similar to

synapse formation induced by PI3K activation via RAC1 [320, 321]. Hence, a decrease in P120CTN and increased PI3K activity can lead to increased synaptic density. Autism is a disease that has been hypothesized to suffer from too many synapses. PI3K signaling is very likely to take part in processes leading to autism [322]. The autism mouse model is induced by inhibition of Tuberous Sclerosis 2 (TSC2), a protein in the PI3K signaling pathway that is inhibited upon PI3K activation [323]. Conversely, disruption of functioning synaptic connections has been implicated in Alzheimers and Parkinsons disease [324, 325]. Hence, the proteins we have studied might have an effect in various neurological disorders. More importantly, we have demonstrated that *PIK3CA* mutations and P120CTN downregulation can induce MMP1, if not many more MMPs. It is not beyond the realm of possibility that the combination of these two genes affects more than one protease at one time. For example, MMP9 expression in the brain has been implicated in receptor availability and memory function [326]. Therefore, aberrations to P120CTN and PIK3CA in the brain could be a potential target for investigation. Furthermore, effects of MMP1 or other MMPs downstream of P120CTN and PIK3CA could be studied with respect to neurological disorders.

Finally, PIK3CA-induced MMP1 potentially plays a role in autoimmune disorders like asthma and arthritis [327-329]. Increased pro-MMP1 levels are found in asthmatics, and activation of MMP1 correlates with exacerbations [330]. Furthermore inhibition of MMP1 activity reduced smooth muscle contraction in

asthma [329]. It is possible that MMP1 in asthma is induced via a similar mechanism to the one we studied. PI3K pathway inhibitors have been shown to improve lung function in COPD patients [331]. Moreover, airway smooth muscle proliferation is controlled by PIK3CA activation in asthma [332]. Therefore, it would be worthwhile to see if P120CTN undergoes any aberrations in asthma since PIK3CA activity and P120CTN downregulation cooperate in inducing MMP1.

In addition, the PI3K signaling pathway is involved in destructive joint diseases such as osteoarthritis and rheumatoid arthritis. Active PI3K signaling leads to an increase in MMP1 levels [311]. Juvenile arthritis and adult arthritis both report an increase in MMP1 levels in patients [333-335]. Similarly as in asthma, it would be interesting to see if P120CTN dysregulation plays any role in promoting a more severe phenotype of the disease.

4.4 Future directions

4.4.1 P120CTN-null mouse model

We have determined that mouse P120CTN-null esophageal keratinocytes do not increase MDSC migration, survival or activation as compared to P120CTN-wild-type keratinocytes (Figure 2.3). Moreover, P120CTN loss does not lead to an MDSC increase in P120CTN-null tissues only (Figure 2.4). Hence, the increased MDSC counts in the P120CTN-null tissues are most likely a result of a global MDSC increase due to inflammation induced by P120CTN loss [336].

MDSCs are vital to tumor development in the P120CTN-null mouse, since depletion of MDSCs results in reduced tumor growth [188]. However, it is unclear what triggers MDSC upregulation/recruitment. Therefore, it is important to identify cells responsible for the increased production of MDSCs.

We have demonstrated that P120CTN-null epithelial cells do not improve MDSC survival, activation or migration compared to P120CTN-wild-type epithelial cells. However, we have not studied the interaction of P120CTN-null epithelial cells with immune progenitor cells residing in the bone marrow. Cytokines secreted by P120CTN-null epithelial cells could shift immune cell differentiation resulting in increased MDSC levels. We have performed a protein array of conditioned media from P120CTN-null and P120CTN-wild-type epithelial cells to identify the differences in cytokine expression. Many cytokines were upregulated in conditioned media from P120CTN-null epithelial cells. To name a few, IL-3, IL-4 and GM-CSF were increased and reports from other mouse models indicate that these cytokines might stimulate MDSC production [204-206]. Results of this array need to be reproduced and validated before we can proceed to the next step of investigation. MDSC-inducing capability of cytokines can be tested by using recombinant cytokines and neutralizing antibodies towards those cytokines. A recombinant version of the identified cytokine(s) can be injected into P120CTN-wild-type mice and bloodstream MDSC levels can be measured.

Additionally, P120CTN-null mice can be treated with a neutralizing antibody to the specific cytokine followed by bloodstream MDSC count.

Alternatively, immune cells could be a potential mediator of MDSC upregulation. P120CTN-null tissues contain various immune cells, such as macrophages, dendritic cells, natural killer cells, T cells and B cells [6]. Individual immune cell lineages can be knocked out in P120CTN-null animals to test the effect on MDSC increase and tumor growth. Immune depletion and knockout mouse models of macrophages [337, 338], dendritic cells [339], natural killer cells [340], T cells [341], and B cells [342] can be used to cross with the P120CTN-null mouse model. Counts of circulating MDSCs can be made at multiple time points until the nine-month time point at which the mice would be sacrificed and the oral and esophageal tumors isolated for size and stage comparison. If the findings in this experiment are positive, we can confirm the results by isolating immune cells responsible for the increase in MDSCs and injecting them into P120CTN-wild-type and P120CTN-null mice and testing whether our treatments increased MDSC counts in the bloodstream. Follow-up work can consist of identifying cytokines secreted by these immune cells, which lead to an increase in MDSCs.

Fibroblasts can also be another potential mediator of MDSC increase in the P120CTN-null mouse model. Fibroblasts isolated from P120CTN-wildtype or P120CTN-null mice can be injected under the kidney capsule of a P120CTN-

wild-type mouse to ensure the highest chance of graft survival. The blood levels of MDSCs can be monitored over the span of nine months. If fibroblasts from P120CTN-null mice are responsible for MDSC upregulation, we should observe increased MDSC levels in the bloodstream. Follow-up experiments are identical to the ones proposed for epithelial and immune cells.

If the epithelial cells, fibroblasts and immune cells are not responsible for the elevation of MDSC levels, we may investigate the role of bioactive fragments that are released from the matrix during MMP-mediated cleavage. We have demonstrated that P120CTN-knockdown leads to an increase in MMP1 in epithelia (Figure 3.11). MMP1 has been shown to proteolytically process collagen to reveal matricryptic sites that bind αvβ3 integrins promoting increased melanoma cell survival [145]. In addition, MMP1 can weakly cleave Fibronectin [343]. Fibronectin fragments can promote tumor growth via different mechanisms. TNF-related apoptosis inducing ligand (TRAIL) has recently emerged as a targeted therapy that only induces cell death in tumors, while sparing normal tissues [344]. Many cancers, which *in vitro* were determined to be TRAIL-sensitive, appeared to be TRAIL-resistant in the clinical trials. Fibronectin fragment, III-1c, has been demonstrated to promote TRAIL-resistance in lung cancer [345]. Fibronectin fragments can increase $CD4^+$ T cell proliferation and polymorphonuclear leukocyte chemotaxis [346]. Extra Domain A (EDA) Fibronectin fragment promotes MDSC differentiation from bone marrow cells

[347]. This indicates that matricryptic sites revealed by MMP digestion can modify the immune milieu. Moreover, EDA Fibronectin fragment directly affects MDSC production in the bone marrow. If EDA Fibronectin fragment is produced in the P120CTN-null mice that could explain the observed increase in MDSCs. Additionally, P120CTN-null mice experience a large amount of inflammation in the P120CTN-null tissues as indicated by increased pNFkB staining and infiltrating immune cells [6]. This suggests that additional MMPs are activated due to signaling pathway activation in the P120CTN-null cells and cytokines secreted by immune cells. Additional MMPs present at the site increase the range of possible cryptic sites that can be revealed.

To determine what ECM fragments are responsible for MDSC increase we need to isolate the ECM from P120CTN-null tissues (buccal and esophageal epithelium) and their P120CTN-wild-type controls. Tissues will be decellularized, digested with pepsin and proteins will be precipitated out with ammonium sulfate. Proteins will be separated using ion exchange and reverse phase chromatography and fractions will be added to CD11b-depleted mouse bone marrow cells *in vitro*. Bone marrow cells will be evaluated for the increase in $CD11b^{+}$ $Gr\text{-}1^{+}$ cells (MDSCs) and the peptide that reveals the maximal capacity to induce MDSCs will be further characterized using mass spectrometry.

4.4.2 Migration regulated by P120CTN downregulation and PIK3CA mutations

The downregulation of P120CTN and mutant PIK3CAs induce increased migration independent of STAT3, RHOA, and AKT activity. The inhibition of STAT3 activity induced an opposite effect, by increasing the amount of migration in NOK cells. Therefore, there is a possibility that STAT3 has an inhibitory role in our system. We can speculate that STAT3 binding at the promoter region limits the binding of the transcription factor that induces migration to a greater amount. Conversely, STAT3 signaling could be also downregulating our unknown transcription factor or limiting the cell motility by competing for signaling pathway involved in migration. In order to confirm the role of STAT3, we first need to confirm that STAT3 knockout in cells with P120CTN knockdown and *PIK3CA* mutations increases migration. Moreover, overexpression of a constitutively active STAT3 mutant should lead to an opposite effect, limiting migration.

In addition to STAT3 activation we have also tested levels of p-cJUN and pNFkB in cells with P120CTN downregulation and *PIK3CA* mutations. We saw increases in p-cJUN similar to the increases in pSTAT3 observed between NOK cell lines. Cells with either P120CTN downregulation or *PIK3CA* mutations have increased p-cJUN levels above that of the control NOK cells. However, cells with both P120CTN downregulation and *PIK3CA* mutations display the highest level of p-cJUN (Figure 3.8). pNFkB is only increased in cells with either P120CTN downregulation (NOK-P), E545K *PIK3CA* mutation (NOK-545), or cells with both

P120CTN downregulation and E545K *PIK3CA* mutation (NOK-545P) (Figure 3.8). This difference in activation of NFkB between the two PIK3CA mutants makes NFkB less likely to be involved in promoting either migration or invasion. However, activation of either NFkB or cJUN pathway has been demonstrated to result in increased cell migration [247, 248].

In addition to p-cJUN, CDC42 and RAC1 activity should be considered with respect to the increased migration observed in our experimental system. The increase in STAT3 activity in our system could be the result of the activity of one of the RHO GTPases such as RHOA, CDC42 or RAC1 [264, 265] Interestingly, STAT3 knock down in the background of constitutively active RHOA increases cell migration [264]. The increase of migration upon STAT3 knock down is a similar result to ours. We also see an increase in migration upon STAT3 inhibition, but in our system we did not observe the RHOA signaling to be significantly activated. RAC1 and CDC42 are similar to RHOA with respect to their signaling pathways and effects on migration. It is therefore possible that STAT3 inhibition could have similar effects in the presence of active RHOA as in the presence of active RAC1 or CDC42. Additionally, our findings point to the possibility of P120CTN downregulation and *PIK3CA* mutations activating RAC1 or CDC42. P120CTN overexpression in gastric cancer suppresses RAC1 and CDC42 [266]. Therefore, loss of P120CTN could potentially increase RAC1 activation as it has been demonstrated in lung cancer [267]. PIK3CA loss has

been shown to limit RAC1 activity in pancreatic cancer [268]. Moreover, PDK1, an effector of the PI3K pathway, can activate RAC1 [269]. Therefore, either the overexpression or presence of constitutively-active mutant PIK3CA might result in increased RAC1 activity. All these data point to a strong possibility that RAC1 or CDC42 are activated in cells with P120CTN downregulation and *PIK3CA* mutations. If RAC1 and CDC42 are active in our experimental system, then they could be responsible for the observed increases in migration and invasion.

We need to confirm the role of p-cJUN, RAC1 and CDC42 in our experimental system. p-cJUN has been shown to increase the migratory capacity of murine osteosarcoma cells [247]. Therefore, p-cJUN could play a similar role in HNSCC. Another consideration has to be made for the protein that phosphorylates cJUN, c-JUN N-terminal Kinase (JNK). If we observe increased phosphorylation of cJUN, it is very likely that JNK is also phosphorylated at threonine residue 183 and tyrosine 185, and therefore activated [348]. Confirmation of JNK activity is crucial for our study of migration, since JNK signals independently of cJUN by phosphorylating Paxillin at serine 178 to increase migration [349]. Therefore, we need to test the levels of pJNK and phosphorylated Paxillin by Western blotting in cells with P120CTN downregulation and *PIK3CA* mutations. Treatment with small molecule inhibitors and siRNA knockdowns targeting cJUN, JNK and Paxillin can be performed to

confirm that these proteins are responsible for the increased migration in cells with low P120CTN and mutant PIK3CA.

Additionally, RAC1 and CDC42 status need to be evaluated in our experimental system. RAC1 and CDC42 increase migration in HNSCC cell lines [105, 106]. Moreover, RAC1 and CDC42 are activated by P120CTN and PI3K signaling [108-110, 350]. Hence, it is possible that RAC1 or CDC42 are activated in our system. To determine whether RAC1 and CDC42 play a role in HNSCC, we need to measure the levels of RAC1-GTP and CDC42-GTP in cells with P120CTN downregulation and *PIK3CA* mutations. If they are increased, RAC1- and CDC42-specific inhibitors will be used to determine whether RAC1 or CDC42 inhibition can reduce the amount of migration and invasion in cells with P120CTN downregulation and *PIK3CA* mutations.

4.4.3 Invasion regulated by P120CTN downregulation and PIK3CA mutations

P120CTN downregulation and *PIK3CA* mutations increase MMP1 expression in a cooperative manner. We have performed a pilot experiment testing eight MMPs and we have also confirmed that both MMP2 and MMP11 are not significantly increased due to P120CTN downregulation and PIK3CA mutation. However, our pilot study was not exhaustive and was only done on n=2 samples. There is a strong possibility that additional MMPs are increased in our experimental system. Upon MMP1 knock down we do not observe a decrease of invasion down to the level of control cells (Figure 3.12, 3.14). Moreover, besides

MMPs, other proteases such as membrane bound MMPs, ADAMs (a disintegrin and metalloproteinase) and ADAMTs (a disintegrin and metalloproteinase with thrombospondin motifs) could also play a role in our system. To identify proteases responsible for the increased invasion, we can use RNA-seq data acquired from the NOK cell lines. Following identification of increased proteases in cells with both P120CTN downregulation and *PIK3CA* mutations, we need to confirm their expression levels via Western blotting. Once the expression level is confirmed, siRNA knockdown followed by Boyden-chamber invasion assays will be used to evaluate their effect on invasion.

Since we have already demonstrated that invasion is driven by increased MMP1 expression regulated by P120CTN downregulation and *PIK3CA* mutations, it would be of great benefit to identify the signaling pathway(s) connecting both P120CTN, PIK3CA and MMP1. Identification of the transcription factors responsible for MMP1 increase would not only help in identifying potential drug targets to inhibit metastasis, but also in identifying what other genes are differentially regulated in our experimental system. AP-1, cJUN, FRA-1, NFkB, STAT3, p53, ATF, cFOS, and SRF transcription factors can all bind to the promoter region of the *MMP1* gene [351]. Most of these transcription factors are activated upon phosphorylation. We already know that STAT3 and cJUN are phosphorylated in cells with P120CTN downregulation and *PIK3CA* mutations. We also have demonstrated that NFkB is phosphorylated in some of our cell

lines, namely in cells with P120CTN downregulation or E545K *PIK3CA* mutation or in cells containing both of these genetic aberrations. However, the lack of phosphorylation in cells with H1047R *PIK3CA* mutations makes NFkB a poor candidate. Both E545K and H1047R *PIK3CA* mutations demonstrate an increased MMP1 level. Therefore, it is unlikely that NFkB signaling would induce MMP1 expression in only of the mutants.

To determine what transcription factor is responsible for MMP1 increase we need to measure the level of phosphorylation of FRA-1, ATF, cFOS and SRF transcription factors and their cellular localization using Western blotting in cells with P120CTN downregulation and *PIK3CA* mutations. Assessment of phosphorylation and localization of transcription factors will help in identifying which transcription factors are active in our experimental system. Furthermore, we have already demonstrated an increase in phosphorylation of STAT3 and cJUN transcription factors, so we need to inhibit them using small molecule inhibitors. MMP1 expression analysis on the cells treated with inhibitors can be performed via Western blotting. If STAT3 or cJUN control MMP1 expression in our experimental system, we should see a decrease of MMP1 upon treatment with specific inhibitors. Even though STAT3 inhibition did not decrease the amount of migration in our previous tests, it is possible that STAT3 inhibition could decrease the amount of invasion by regulating MMP1. Moreover, RNAseq of NOK cells could point to additional differentially expressed transcription targets

that control migration and invasion in our experimental system. Confirmation of changes found in the RNAseq followed by inhibition or overexpression of these targets would aid in identifying additional pathways that are changed due to P120CTN downregulation and *PIK3CA* mutations.

Furthermore, the effects of P120CTN downregulation and *PIK3CA* mutations need to be investigated *in vivo*. Xenografts of NOK cell lines in nude mice can be used to establish the tumor forming potential. Additionally, tail vain injections of labeled NOK cells can be used to measure the metastatic capacity to form liver and lung metastases. Successful development of xenografts would also allow us to study the ability of cells to metastasize out of the primary tumor. That metastatic capacity of cells due to P120CTN downregulation and *PIK3CA* mutations can be tested by treating the mice with inhibitors of MMP1 or inhibitors of transcription factors inducing MMP1.

Finally, the TCGA databank contains patient outcomes which have not been explored in terms of the cooperative effect of P120CTN loss and active PI3K pathway. We can mine this database and see if patients with P120CTN downregulation and *PIK3CA* mutations differ in overall survival compared to the rest of HNSCC patients. This would demonstrate to us, whether the tumor subset demarked by aberrations of P120CTN and *PIK3CA* mutations is more deadly.

4.5 Conclusion

Our MDSC findings indicate that in the P120CTN-null mouse model the tumor development induces a systemic and not a local increase in MDSC as described in *Chapter 2*. P120CTN-null mice still develop tumors, even though P120CTN-loss does not directly recruit MDSCs to the P120CTN-null site. This finding indicates that a global MDSC increase can be sufficient to halt many of the local inflammatory processes. Therefore, if we can identify cytokines responsible for the global increase in MDSCs, we could potentially use it in the treatment of inflammatory disorders such as asthma. Furthermore, our MDSC subset analysis failed to identify MDSC activation markers discovered in other tumor mouse models. This indicates that MDSC activation markers could be specific to each disease state. This puts the study of MDSC activation markers in mice in question due to the effects of translatability. Human MDSCs differ in almost every single marker from mouse MDSCs, so it is unlikely that specific MDSC activation surface markers could translate to human MDSCs.

Our findings implicate the downstream signaling of P120CTN and PIK3CA in the modulation of migration and invasion in HNSCC as described in *Chapter 3*. The findings demonstrated should be studied in other cancers containing similar genetic aberrations and MMP changes. Moreover, our findings providence evidence for the cooperation between two completely unrelated genes. It is possible that such a phenomenon occurs with respect to P120CTN in other

diseases too, such as asthma, arthritis or maybe even autism. The identification of MMP1 is important due to the fact that this protein increases invasion. Future research should identify how P120CTN and PIK3CA signaling increase MMP1 levels. After identification of those pathways, we should focus on methods to inhibit them to limit metastasis. If MMP1 expression is downstream of P120CTN and PIK3CA in some of the above mentioned diseases, the future treatments targeting MMP1 and its upstream signaling could be applicable in many more cancer types and diseases.

As mentioned previously, our work identifies that a subset of HNSCC tumor samples with low P120CTN and active PI3K signaling has increased MMP1 expression. MMP1 has been identified as a marker of late stage of HNSCC and its mRNA levels are increased in saliva allowing for detection of HNSCC [263, 302]. It is unlikely that MMP1 expression is caused only by alteration to P120CTN and PI3K. Therefore, while it appears MMP1 is a significant player in the pathogenesis of HNSCC, work needs to be done to identify other potential pathways that also increase MMP1.

Finally, the origins of this project stem from observations made in the P120CTN-null mouse model. Oral tumors from the P120CTN-null mouse did not display a large amount of invasion and did not recapitulate the invasiveness observed in human tumors. We hypothesized that addition of an oncogene is required to mimic the human condition. Therefore, we looked for potential

candidates that were most likely to co-occur in tumors with aberrant P120CTN. PIK3CA is the most commonly mutated oncogene in HNSCC and therefore the most likely candidate. P120CTN downregulation and mutant PIK3CA combination confirmed our hypothesis and revealed a link to MMP1. This emphasizes the need to study gene combinations and the need to follow the data via analytic step-wise investigation.

References

1. Michmerhuizen, N.L., et al., *Genetic determinants in head and neck squamous cell carcinoma and their influence on global personalized medicine.* Genes Cancer, 2016. **7**(5-6): p. 182-200.
2. Colin S Poon, K.M.S. *Overview of the diagnosis and staging of head and neck cancer*. 2016 9-12-16 [cited 2017 3-29-17]; Available from: https://www.uptodate.com/contents/overview-of-the-diagnosis-and-staging-of-head-and-neck-cancer?source=search_result&search=hnscc&selectedTitle=1~150.
3. Rosenthal, E.L., et al., *Role of the plasminogen activator and matrix metalloproteinase systems in epidermal growth factor- and scatter factor-stimulated invasion of carcinoma cells.* Cancer Res, 1998. **58**(22): p. 5221-30.
4. Iizuka, S., N. Ishimaru, and Y. Kudo, *Matrix metalloproteinases: the gene expression signatures of head and neck cancer progression.* Cancers (Basel), 2014. **6**(1): p. 396-415.
5. Chen, W.C., et al., *Inflammation-induced myeloid-derived suppressor cells associated with squamous cell carcinoma of the head and neck.* Head Neck, 2017. **39**(2): p. 347-355.
6. Stairs, D.B., et al., *Deletion of p120-catenin results in a tumor microenvironment with inflammation and cancer that establishes it as a tumor suppressor gene.* Cancer Cell, 2011. **19**(4): p. 470-83.
7. *http://seer.cancer.gov/statfacts/html/oralcav.html*. Cancer Stat Facts: Oral Cavity and Pharynx Cancer 3-29-17]; Available from: http://seer.cancer.gov/statfacts/html/oralcav.html.
8. Reichart, P.A. and X.H. Nguyen, *Betel quid chewing, oral cancer and other oral mucosal diseases in Vietnam: a review.* J Oral Pathol Med, 2008. **37**(9): p. 511-4.
9. Warnakulasuriya, K.A., et al., *Utilization of primary health care workers for early detection of oral cancer and precancer cases in Sri Lanka.* Bull World Health Organ, 1984. **62**(2): p. 243-50.
10. Saranath, D., et al., *High frequency mutation in codons 12 and 61 of H-ras oncogene in chewing tobacco-related human oral carcinoma in India.* Br J Cancer, 1991. **63**(4): p. 573-8.
11. Adrien, J., et al., *Why are head and neck squamous cell carcinoma diagnosed so late? Influence of health care disparities and socio-economic factors.* Oral Oncol, 2014. **50**(2): p. 90-7.
12. Napier, S.S. and P.M. Speight, *Natural history of potentially malignant oral lesions and conditions: an overview of the literature.* J Oral Pathol Med, 2008. **37**(1): p. 1-10.

13. Slaughter, D.P., H.W. Southwick, and W. Smejkal, *Field cancerization in oral stratified squamous epithelium; clinical implications of multicentric origin.* Cancer, 1953. **6**(5): p. 963-8.
14. Partridge, M., et al., *A case-control study confirms that microsatellite assay can identify patients at risk of developing oral squamous cell carcinoma within a field of cancerization.* Cancer Res, 2000. **60**(14): p. 3893-8.
15. Tabor, M.P., et al., *Persistence of genetically altered fields in head and neck cancer patients: biological and clinical implications.* Clin Cancer Res, 2001. **7**(6): p. 1523-32.
16. Califano, J., et al., *Genetic progression model for head and neck cancer: implications for field cancerization.* Cancer Res, 1996. **56**(11): p. 2488-92.
17. Lee, D.J., et al., *Multiple tumor-suppressor genes on chromosome 3p contribute to head and neck squamous cell carcinoma tumorigenesis.* Cancer Biol Ther, 2010. **10**(7): p. 689-93.
18. *Comprehensive genomic characterization of head and neck squamous cell carcinomas.* Nature, 2015. **517**(7536): p. 576-82.
19. Baltaci, E., et al., *Analysis of gene copy number changes in head and neck cancer.* Clin Otolaryngol, 2016.
20. Park, S.W., et al., *Chromosomal aberrations and prognosis in patients with concomitant chemoradiotherapy for resected head and neck cancer.* Oncol Rep, 2016. **35**(4): p. 2207-15.
21. Jin, C., et al., *Cytogenetic abnormalities in 106 oral squamous cell carcinomas.* Cancer Genet Cytogenet, 2006. **164**(1): p. 44-53.
22. Hermsen, M., et al., *New chromosomal regions with high-level amplifications in squamous cell carcinomas of the larynx and pharynx, identified by comparative genomic hybridization.* J Pathol, 2001. **194**(2): p. 177-82.
23. Rous, P., *A Sarcoma of the Fowl Transmissible by an Agent Separable from the Tumor Cells.* J Exp Med, 1911. **13**(4): p. 397-411.
24. Czernilofsky, A.P., et al., *Nucleotide sequence of an avian sarcoma virus oncogene (src) and proposed amino acid sequence for gene product.* Nature, 1980. **287**(5779): p. 198-203.
25. Stehelin, D., et al., *DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA.* Nature, 1976. **260**(5547): p. 170-3.
26. Hayward, W.S., B.G. Neel, and S.M. Astrin, *Activation of a cellular onc gene by promoter insertion in ALV-induced lymphoid leukosis.* Nature, 1981. **290**(5806): p. 475-80.

27. Kress, M., et al., *Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum.* J Virol, 1979. **31**(2): p. 472-83.
28. Lane, D.P. and S. Benchimol, *p53: oncogene or anti-oncogene?* Genes Dev, 1990. **4**(1): p. 1-8.
29. Day, G.L., et al., *Racial differences in risk of oral and pharyngeal cancer: alcohol, tobacco, and other determinants.* J Natl Cancer Inst, 1993. **85**(6): p. 465-73.
30. Saku, T., et al., *Salivary gland tumors among atomic bomb survivors, 1950-1987.* Cancer, 1997. **79**(8): p. 1465-75.
31. Luce, D., et al., *Sinonasal cancer and occupational exposures: a pooled analysis of 12 case-control studies.* Cancer Causes Control, 2002. **13**(2): p. 147-57.
32. Chien, Y.C., et al., *Serologic markers of Epstein-Barr virus infection and nasopharyngeal carcinoma in Taiwanese men.* N Engl J Med, 2001. **345**(26): p. 1877-82.
33. Maden, C., et al., *Human papillomaviruses, herpes simplex viruses, and the risk of oral cancer in men.* Am J Epidemiol, 1992. **135**(10): p. 1093-102.
34. Bravi, F., et al., *Foods, nutrients and the risk of oral and pharyngeal cancer.* Br J Cancer, 2013. **109**(11): p. 2904-10.
35. Weber, A., et al., *Carcinogen-induced site-specific mutagenesis and genetic susceptibility in squamous cell carcinoma of the head and neck.* Onkologie, 2002. **25**(1): p. 8-13.
36. Gillison, M.L., et al., *Distinct risk factor profiles for human papillomavirus type 16-positive and human papillomavirus type 16-negative head and neck cancers.* J Natl Cancer Inst, 2008. **100**(6): p. 407-20.
37. Sterba, K.R., et al., *Smoking status and symptom burden in surgical head and neck cancer patients.* Laryngoscope, 2017. **127**(1): p. 127-133.
38. Caroff J, V.J., Badre R, Guillerm R, *Direct chromatography of the gas phase of cigarette smoke: hydrocarbons and compounds containing oxygen and nitrogen* 1965.
39. Zhang, L., et al., *Cigarette smoke disrupts the integrity of airway adherens junctions through the aberrant interaction of p120-catenin with the cytoplasmic tail of MUC1.* J Pathol, 2013. **229**(1): p. 74-86.
40. Liu, X., et al., *Interactions between MUC1 and p120 catenin regulate dynamic features of cell adhesion, motility, and metastasis.* Cancer Res, 2014. **74**(5): p. 1609-20.
41. Liu, X., et al., *Loss of E-cadherin and epithelial to mesenchymal transition is not required for cell motility in tissues or for metastasis.* Tissue Barriers, 2014. **2**(4): p. e969112.

42. Zhang, Q., et al., *A Phosphatidylinositol 3-kinase-regulated Akt-independent signaling promotes cigarette smoke-induced FRA-1 expression.* J Biol Chem, 2006. **281**(15): p. 10174-81.
43. Yang, C.M., et al., *Cigarette smoke extract induces COX-2 expression via a PKCalpha/c-Src/EGFR, PDGFR/PI3K/Akt/NF-kappaB pathway and p300 in tracheal smooth muscle cells.* Am J Physiol Lung Cell Mol Physiol, 2009. **297**(5): p. L892-902.
44. Dal Maso, L., et al., *Combined effect of tobacco smoking and alcohol drinking in the risk of head and neck cancers: a re-analysis of case-control studies using bi-dimensional spline models.* Eur J Epidemiol, 2016. **31**(4): p. 385-93.
45. Brennan, P., et al., *Pooled analysis of alcohol dehydrogenase genotypes and head and neck cancer: a HuGE review.* Am J Epidemiol, 2004. **159**(1): p. 1-16.
46. Brooks, P.J. and J.A. Theruvathu, *DNA adducts from acetaldehyde: implications for alcohol-related carcinogenesis.* Alcohol, 2005. **35**(3): p. 187-93.
47. Seitz, H.K. and F. Stickel, *Molecular mechanisms of alcohol-mediated carcinogenesis.* Nat Rev Cancer, 2007. **7**(8): p. 599-612.
48. Kreimer, A.R., et al., *Human papillomavirus types in head and neck squamous cell carcinomas worldwide: a systematic review.* Cancer Epidemiol Biomarkers Prev, 2005. **14**(2): p. 467-75.
49. Munger, K., et al., *Interactions of HPV E6 and E7 oncoproteins with tumour suppressor gene products.* Cancer Surv, 1992. **12**: p. 197-217.
50. Crook, T., J.A. Tidy, and K.H. Vousden, *Degradation of p53 can be targeted by HPV E6 sequences distinct from those required for p53 binding and trans-activation.* Cell, 1991. **67**(3): p. 547-56.
51. Dyson, N., et al., *The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product.* Science, 1989. **243**(4893): p. 934-7.
52. Schwartz, S.M., et al., *Oral cancer risk in relation to sexual history and evidence of human papillomavirus infection.* J Natl Cancer Inst, 1998. **90**(21): p. 1626-36.
53. Dayyani, F., et al., *Meta-analysis of the impact of human papillomavirus (HPV) on cancer risk and overall survival in head and neck squamous cell carcinomas (HNSCC).* Head Neck Oncol, 2010. **2**: p. 15.
54. Vidal, L. and M.L. Gillison, *Human papillomavirus in HNSCC: recognition of a distinct disease type.* Hematol Oncol Clin North Am, 2008. **22**(6): p. 1125-42, vii.
55. Mehlen, P. and A. Puisieux, *Metastasis: a question of life or death.* Nat Rev Cancer, 2006. **6**(6): p. 449-58.

56. Leber, M.F. and T. Efferth, *Molecular principles of cancer invasion and metastasis (review).* Int J Oncol, 2009. **34**(4): p. 881-95.
57. Neville BW, D.D., Chi AC, Allen CM, *Oral and maxillofacial pathology*. 2015: Elsevier Health Sciences.
58. Kalluri, R. and R.A. Weinberg, *The basics of epithelial-mesenchymal transition.* J Clin Invest, 2009. **119**(6): p. 1420-8.
59. Muhammad, N., et al., *Involvement of c-Fos in the Promotion of Cancer Stem-like Cell Properties in Head and Neck Squamous Cell Carcinoma.* Clin Cancer Res, 2016.
60. Inamura, N., et al., *Notch1 regulates invasion and metastasis of head and neck squamous cell carcinoma by inducing EMT through c-Myc.* Auris Nasus Larynx, 2016.
61. Abraham, M.T., et al., *Motility-related proteins as markers for head and neck squamous cell cancer.* Laryngoscope, 2001. **111**(7): p. 1285-9.
62. Bian, Y., et al., *Loss of TGF-beta signaling and PTEN promotes head and neck squamous cell carcinoma through cellular senescence evasion and cancer-related inflammation.* Oncogene, 2012. **31**(28): p. 3322-32.
63. Carpenter, C.L. and L.C. Cantley, *Phosphoinositide kinases.* Biochemistry, 1990. **29**(51): p. 11147-56.
64. Laplante, M. and D.M. Sabatini, *mTOR signaling at a glance.* J Cell Sci, 2009. **122**(Pt 20): p. 3589-94.
65. Rameh, L.E., et al., *Phosphoinositide 3-kinase regulates phospholipase Cgamma-mediated calcium signaling.* J Biol Chem, 1998. **273**(37): p. 23750-7.
66. Paruchuri, S., et al., *The pro-inflammatory mediator leukotriene D4 induces phosphatidylinositol 3-kinase and Rac-dependent migration of intestinal epithelial cells.* J Biol Chem, 2005. **280**(14): p. 13538-44.
67. Guillou, H., et al., *Use of the GRP1 PH domain as a tool to measure the relative levels of PtdIns(3,4,5)P3 through a protein-lipid overlay approach.* J Lipid Res, 2007. **48**(3): p. 726-32.
68. Lu, Y., et al., *Phosphatidylinositol 3-kinase is required for CD28 but not CD3 regulation of the TEC family tyrosine kinase EMT/ITK/TSK: functional and physical interaction of EMT with phosphatidylinositol 3-kinase.* J Immunol, 1998. **161**(10): p. 5404-12.
69. Clark, C.A., et al., *Curcumin inhibits carcinogen and nicotine-induced Mammalian target of rapamycin pathway activation in head and neck squamous cell carcinoma.* Cancer Prev Res (Phila), 2010. **3**(12): p. 1586-95.
70. Cleary, A.S., et al., *Tumour cell heterogeneity maintained by cooperating subclones in Wnt-driven mammary cancers.* Nature, 2014. **508**(7494): p. 113-7.

71. Rubin Grandis, J., et al., *Levels of TGF-alpha and EGFR protein in head and neck squamous cell carcinoma and patient survival.* J Natl Cancer Inst, 1998. **90**(11): p. 824-32.
72. Grandis, J.R. and D.J. Tweardy, *Elevated levels of transforming growth factor alpha and epidermal growth factor receptor messenger RNA are early markers of carcinogenesis in head and neck cancer.* Cancer Res, 1993. **53**(15): p. 3579-84.
73. P, O.c., et al., *Epidermal growth factor-like ligands differentially up-regulate matrix metalloproteinase 9 in head and neck squamous carcinoma cells.* Cancer Res, 2000. **60**(4): p. 1121-8.
74. Patel, V., et al., *Persistent activation of Rac1 in squamous carcinomas of the head and neck: evidence for an EGFR/Vav2 signaling axis involved in cell invasion.* Carcinogenesis, 2007. **28**(6): p. 1145-52.
75. Nguyen, P.T., et al., *The FGFR1 inhibitor PD173074 induces mesenchymal-epithelial transition through the transcription factor AP-1.* Br J Cancer, 2013. **109**(8): p. 2248-58.
76. Hase, T., et al., *Correlation of basic fibroblast growth factor expression with the invasion and the prognosis of oral squamous cell carcinoma.* J Oral Pathol Med, 2006. **35**(3): p. 136-9.
77. Vairaktaris, E., et al., *FGFR-2 and -3 play an important role in initial stages of oral oncogenesis.* Anticancer Res, 2006. **26**(6B): p. 4217-21.
78. Rao, V.H., et al., *A positive feedback loop between HER2 and ADAM12 in human head and neck cancer cells increases migration and invasion.* Oncogene, 2012. **31**(23): p. 2888-98.
79. Lim, Y.C., et al., *Overexpression of c-Met promotes invasion and metastasis of small oral tongue carcinoma.* Oral Oncol, 2012. **48**(11): p. 1114-9.
80. Whitman, M., et al., *Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate.* Nature, 1988. **332**(6165): p. 644-6.
81. Cantley, L.C., et al., *Oncogenes and signal transduction.* Cell, 1991. **64**(2): p. 281-302.
82. Chang, H.W., et al., *Transformation of chicken cells by the gene encoding the catalytic subunit of PI 3-kinase.* Science, 1997. **276**(5320): p. 1848-50.
83. Samuels, Y. and T. Waldman, *Oncogenic mutations of PIK3CA in human cancers.* Curr Top Microbiol Immunol, 2010. **347**: p. 21-41.
84. Zhao, L. and P.K. Vogt, *Helical domain and kinase domain mutations in p110alpha of phosphatidylinositol 3-kinase induce gain of function by different mechanisms.* Proc Natl Acad Sci U S A, 2008. **105**(7): p. 2652-7.
85. Murugan, A.K., et al., *Oncogenic mutations of the PIK3CA gene in head and neck squamous cell carcinomas.* Int J Oncol, 2008. **32**(1): p. 101-11.

86. Pang, H., et al., *Differential enhancement of breast cancer cell motility and metastasis by helical and kinase domain mutations of class IA phosphoinositide 3-kinase.* Cancer Res, 2009. **69**(23): p. 8868-76.
87. Fenic, I., et al., *Analysis of PIK3CA and Akt/protein kinase B in head and neck squamous cell carcinoma.* Oncol Rep, 2007. **18**(1): p. 253-9.
88. Hsu, J.Y., et al., *Epidermal growth factor-induced cyclooxygenase-2 enhances head and neck squamous cell carcinoma metastasis through fibronectin up-regulation.* Oncotarget, 2015. **6**(3): p. 1723-39.
89. Suzuki, S. and K. Ishikawa, *Combined inhibition of EMMPRIN and epidermal growth factor receptor prevents the growth and migration of head and neck squamous cell carcinoma cells.* Int J Oncol, 2014. **44**(3): p. 912-7.
90. Das, G., et al., *Rictor regulates MMP-9 activity and invasion through Raf-1-MEK-ERK signaling pathway in glioma cells.* Mol Carcinog, 2011. **50**(6): p. 412-23.
91. Birkedal-Hansen, H., et al., *Matrix metalloproteinases: a review.* Crit Rev Oral Biol Med, 1993. **4**(2): p. 197-250.
92. Schmidt, E.M., et al., *Serum- and glucocorticoid-dependent kinase-1-induced cell migration is dependent on vinculin and regulated by the membrane androgen receptor.* FEBS J, 2012. **279**(7): p. 1231-42.
93. Zhou, B.P., et al., *Dual regulation of Snail by GSK-3beta-mediated phosphorylation in control of epithelial-mesenchymal transition.* Nat Cell Biol, 2004. **6**(10): p. 931-40.
94. Hornstein, I., A. Alcover, and S. Katzav, *Vav proteins, masters of the world of cytoskeleton organization.* Cell Signal, 2004. **16**(1): p. 1-11.
95. Tamas, P., et al., *Mechanism of epidermal growth factor regulation of Vav2, a guanine nucleotide exchange factor for Rac.* J Biol Chem, 2003. **278**(7): p. 5163-71.
96. Yang, H.W., et al., *Cooperative activation of PI3K by Ras and Rho family small GTPases.* Mol Cell, 2012. **47**(2): p. 281-90.
97. Sadok, A. and C.J. Marshall, *Rho GTPases: masters of cell migration.* Small GTPases, 2014. **5**: p. e29710.
98. Tumur, Z., et al., *RhoC mediates epidermal growth factor-stimulated migration and invasion in head and neck squamous cell carcinoma.* Neoplasia, 2015. **17**(1): p. 141-51.
99. Besson, A., et al., *p27Kip1 modulates cell migration through the regulation of RhoA activation.* Genes Dev, 2004. **18**(8): p. 862-76.
100. Worthylake, R.A. and K. Burridge, *RhoA and ROCK promote migration by limiting membrane protrusions.* J Biol Chem, 2003. **278**(15): p. 13578-84.
101. Ridley, A.J., *Rho GTPases and actin dynamics in membrane protrusions and vesicle trafficking.* Trends Cell Biol, 2006. **16**(10): p. 522-9.

102. O'Connor, K. and M. Chen, *Dynamic functions of RhoA in tumor cell migration and invasion.* Small GTPases, 2013. **4**(3): p. 141-7.
103. Rohatgi, R., et al., *The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly.* Cell, 1999. **97**(2): p. 221-31.
104. Leung, T., et al., *Myotonic dystrophy kinase-related Cdc42-binding kinase acts as a Cdc42 effector in promoting cytoskeletal reorganization.* Mol Cell Biol, 1998. **18**(1): p. 130-40.
105. Skvortsov, S., et al., *Radioresistant head and neck squamous cell carcinoma cells: intracellular signaling, putative biomarkers for tumor recurrences and possible therapeutic targets.* Radiother Oncol, 2011. **101**(1): p. 177-82.
106. Arnold, C.R., et al., *Rac1 as a multifunctional therapeutic target to prevent and combat cancer metastasis.* Oncoscience, 2014. **1**(8): p. 513-21.
107. Xiao, P., et al., *[Effect of Tiam 1 on cell proliferation and migration in head and neck squamous cell carcinoma cells].* Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi, 2015. **29**(7): p. 633-7.
108. Anastasiadis, P.Z., et al., *Inhibition of RhoA by p120 catenin.* Nat Cell Biol, 2000. **2**(9): p. 637-44.
109. Wildenberg, G.A., et al., *p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho.* Cell, 2006. **127**(5): p. 1027-39.
110. Zhang, L., et al., *Rac1 and Cdc42 differentially modulate cigarette smoke-induced airway cell migration through p120-catenin-dependent and -independent pathways.* Am J Pathol, 2013. **182**(6): p. 1986-95.
111. Lo Muzio, L., et al., *Is expression of p120ctn in oral squamous cell carcinomas a prognostic factor?* Oral Surg Oral Med Oral Pathol Oral Radiol, 2013. **115**(6): p. 789-98.
112. Bartlett, J.D., et al., *Targeted p120-catenin ablation disrupts dental enamel development.* PLoS One, 2010. **5**(9).
113. Davis, M.A. and A.B. Reynolds, *Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland.* Dev Cell, 2006. **10**(1): p. 21-31.
114. Kurley, S.J., et al., *p120-catenin is essential for terminal end bud function and mammary morphogenesis.* Development, 2012. **139**(10): p. 1754-64.
115. Marciano, D.K., et al., *p120 catenin is required for normal renal tubulogenesis and glomerulogenesis.* Development, 2011. **138**(10): p. 2099-109.
116. Smalley-Freed, W.G., et al., *p120-catenin is essential for maintenance of barrier function and intestinal homeostasis in mice.* J Clin Invest, 2010. **120**(6): p. 1824-35.

117. Chen, T., et al., *Altered localization of p120 catenin in the cytoplasm rather than the membrane correlates with poor prognosis in esophageal squamous cell carcinoma.* PLoS One, 2015. **10**(3): p. e0118645.
118. Ishizaki, Y., et al., *Reduced expression and aberrant localization of p120catenin in human squamous cell carcinoma of the skin.* J Dermatol Sci, 2004. **34**(2): p. 99-108.
119. Silva Neto, B., et al., *Prognostic significance of altered p120 ctn expression in bladder cancer.* BJU Int, 2008. **101**(6): p. 746-52.
120. Wijnhoven, B.P., et al., *Reduced p120ctn expression correlates with poor survival in patients with adenocarcinoma of the gastroesophageal junction.* J Surg Oncol, 2005. **92**(2): p. 116-23.
121. Jiang, Y., et al., *Reduced expression of E-cadherin and p120-catenin and elevated expression of PLC-gamma1 and PIKE are associated with aggressiveness of oral squamous cell carcinoma.* Int J Clin Exp Pathol, 2015. **8**(8): p. 9042-51.
122. Iwata, T., F. Nozu, and M. Imawari, *Ethanol impairs the assembly and disassembly of actin cytoskeleton and cell adhesion via the RhoA signaling pathway, catenin p120 and E-cadherin in CCK-stimulated pancreatic acini.* Biochem Biophys Res Commun, 2011. **405**(4): p. 558-63.
123. Zhang, L., et al., *Pivotal role of MUC1 glycosylation by cigarette smoke in modulating disruption of airway adherens junctions in vitro.* J Pathol, 2014. **234**(1): p. 60-73.
124. Krais, A.M., et al., *CHRNA5 as negative regulator of nicotine signaling in normal and cancer bronchial cells: effects on motility, migration and p63 expression.* Carcinogenesis, 2011. **32**(9): p. 1388-95.
125. Reynolds, A.B., et al., *Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes.* Mol Cell Biol, 1994. **14**(12): p. 8333-42.
126. Gu, C., et al., *Protective role of p120-catenin in maintaining the integrity of adherens and tight junctions in ventilator-induced lung injury.* Respir Res, 2015. **16**: p. 58.
127. Paredes, J., et al., *Expression of p120-catenin isoforms correlates with genomic and transcriptional phenotype of breast cancer cell lines.* Cell Oncol, 2007. **29**(6): p. 467-76.
128. Yanagisawa, M., et al., *A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease.* J Biol Chem, 2008. **283**(26): p. 18344-54.
129. Ohkubo, T. and M. Ozawa, *The transcription factor Snail downregulates the tight junction components independently of E-cadherin downregulation.* J Cell Sci, 2004. **117**(Pt 9): p. 1675-85.

130. Schackmann, R.C., et al., *Loss of p120-catenin induces metastatic progression of breast cancer by inducing anoikis resistance and augmenting growth factor receptor signaling.* Cancer Res, 2013. **73**(15): p. 4937-49.
131. Castano, J., et al., *Specific phosphorylation of p120-catenin regulatory domain differently modulates its binding to RhoA.* Mol Cell Biol, 2007. **27**(5): p. 1745-57.
132. Valls, G., et al., *Upon Wnt stimulation, Rac1 activation requires Rac1 and Vav2 binding to p120-catenin.* J Cell Sci, 2012. **125**(Pt 22): p. 5288-301.
133. Chen, X., et al., *p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions.* J Cell Biol, 2003. **163**(3): p. 547-57.
134. Franz, C.M. and A.J. Ridley, *p120 catenin associates with microtubules: inverse relationship between microtubule binding and Rho GTPase regulation.* J Biol Chem, 2004. **279**(8): p. 6588-94.
135. Daniel, J.M. and A.B. Reynolds, *The catenin p120(ctn) interacts with Kaiso, a novel BTB/POZ domain zinc finger transcription factor.* Mol Cell Biol, 1999. **19**(5): p. 3614-23.
136. Hosking, C.R., et al., *The transcriptional repressor Glis2 is a novel binding partner for p120 catenin.* Mol Biol Cell, 2007. **18**(5): p. 1918-27.
137. Lee, M., et al., *p120-catenin regulates REST and CoREST, and modulates mouse embryonic stem cell differentiation.* J Cell Sci, 2014. **127**(Pt 18): p. 4037-51.
138. Spring, C.M., et al., *The catenin p120ctn inhibits Kaiso-mediated transcriptional repression of the beta-catenin/TCF target gene matrilysin.* Exp Cell Res, 2005. **305**(2): p. 253-65.
139. Kaulfuss, S., et al., *Leupaxin acts as a mediator in prostate carcinoma progression through deregulation of p120catenin expression.* Oncogene, 2009. **28**(45): p. 3971-82.
140. Tlatli, R. and M. El Ayeb, *MMP inhibitors and cancer treatment trials, limitations and hopes for the future.* Arch Inst Pasteur Tunis, 2013. **90**(1-4): p. 3-21.
141. Gross, J. and C.M. Lapiere, *Collagenolytic activity in amphibian tissues: a tissue culture assay.* Proc Natl Acad Sci U S A, 1962. **48**: p. 1014-22.
142. Gearing, A.J., et al., *Matrix metalloproteinases and processing of pro-TNF-alpha.* J Leukoc Biol, 1995. **57**(5): p. 774-7.
143. Fingleton, B., et al., *Matrilysin [MMP-7] expression selects for cells with reduced sensitivity to apoptosis.* Neoplasia, 2001. **3**(6): p. 459-68.
144. Sounni, N.E., et al., *MT1-MMP expression promotes tumor growth and angiogenesis through an up-regulation of vascular endothelial growth factor expression.* FASEB J, 2002. **16**(6): p. 555-64.

145. Montgomery, A.M., R.A. Reisfeld, and D.A. Cheresh, *Integrin alpha v beta 3 rescues melanoma cells from apoptosis in three-dimensional dermal collagen.* Proc Natl Acad Sci U S A, 1994. **91**(19): p. 8856-60.
146. Sabeh, F., et al., *Tumor cell traffic through the extracellular matrix is controlled by the membrane-anchored collagenase MT1-MMP.* J Cell Biol, 2004. **167**(4): p. 769-81.
147. P, O.c., et al., *Signaling pathways required for matrix metalloproteinase-9 induction by betacellulin in head-and-neck squamous carcinoma cells.* Int J Cancer, 2004. **111**(2): p. 174-83.
148. Stanciu, A.E., et al., *Serum Level of Matrix Metalloproteinase-9 in Patients with Head and Neck Squamous Cell Carcinoma.* Clin Lab, 2016. **62**(8): p. 1569-1574.
149. Liao, Y.H., et al., *Epidermal growth factor-induced ANGPTL4 enhances anoikis resistance and tumour metastasis in head and neck squamous cell carcinoma.* Oncogene, 2016.
150. Shen, C.J., et al., *Oleic acid-induced ANGPTL4 enhances head and neck squamous cell carcinoma anoikis resistance and metastasis via up-regulation of fibronectin.* Cancer Lett, 2017. **386**: p. 110-122.
151. Lallemant, B., et al., *Clinical relevance of nine transcriptional molecular markers for the diagnosis of head and neck squamous cell carcinoma in tissue and saliva rinse.* BMC Cancer, 2009. **9**: p. 370.
152. Yu, J.S., et al., *Saliva protein biomarkers to detect oral squamous cell carcinoma in a high-risk population in Taiwan.* Proc Natl Acad Sci U S A, 2016. **113**(41): p. 11549-11554.
153. Tester, A.M., et al., *LPS responsiveness and neutrophil chemotaxis in vivo require PMN MMP-8 activity.* PLoS One, 2007. **2**(3): p. e312.
154. Gearing, A.J., et al., *Processing of tumour necrosis factor-alpha precursor by metalloproteinases.* Nature, 1994. **370**(6490): p. 555-7.
155. Boire, A., et al., *PAR1 is a matrix metalloprotease-1 receptor that promotes invasion and tumorigenesis of breast cancer cells.* Cell, 2005. **120**(3): p. 303-13.
156. Rosenthal, E.L. and L.M. Matrisian, *Matrix metalloproteases in head and neck cancer.* Head Neck, 2006. **28**(7): p. 639-48.
157. Fanjul-Fernandez, M., et al., *Matrix metalloproteinases: evolution, gene regulation and functional analysis in mouse models.* Biochim Biophys Acta, 2010. **1803**(1): p. 3-19.
158. Davis, G.E., et al., *Matrix metalloproteinase-1 and -9 activation by plasmin regulates a novel endothelial cell-mediated mechanism of collagen gel contraction and capillary tube regression in three-dimensional collagen matrices.* J Cell Sci, 2001. **114**(Pt 5): p. 917-30.

159. Werb, Z., *ECM and cell surface proteolysis: regulating cellular ecology.* Cell, 1997. **91**(4): p. 439-42.
160. Saunders, W.B., K.J. Bayless, and G.E. Davis, *MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices.* J Cell Sci, 2005. **118**(Pt 10): p. 2325-40.
161. Wilhelm, S.M., et al., *Human fibroblast collagenase: glycosylation and tissue-specific levels of enzyme synthesis.* Proc Natl Acad Sci U S A, 1986. **83**(11): p. 3756-60.
162. Guo, H., et al., *EMMPRIN (CD147), an inducer of matrix metalloproteinase synthesis, also binds interstitial collagenase to the tumor cell surface.* Cancer Res, 2000. **60**(4): p. 888-91.
163. Guo, H., et al., *Stimulation of matrix metalloproteinase production by recombinant extracellular matrix metalloproteinase inducer from transfected Chinese hamster ovary cells.* J Biol Chem, 1997. **272**(1): p. 24-7.
164. Burnet, M., *Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications.* Br Med J, 1957. **1**(5023): p. 841-7.
165. Grulich, A.E., et al., *Incidence of cancers in people with HIV/AIDS compared with immunosuppressed transplant recipients: a meta-analysis.* Lancet, 2007. **370**(9581): p. 59-67.
166. Pries, R., S. Nitsch, and B. Wollenberg, *Role of cytokines in head and neck squamous cell carcinoma.* Expert Rev Anticancer Ther, 2006. **6**(9): p. 1195-203.
167. Meissner, M., et al., *Defects in the human leukocyte antigen class I antigen processing machinery in head and neck squamous cell carcinoma: association with clinical outcome.* Clin Cancer Res, 2005. **11**(7): p. 2552-60.
168. Sparano, A., et al., *Modulation of Th1 and Th2 cytokine profiles and their association with advanced head and neck squamous cell carcinoma.* Otolaryngol Head Neck Surg, 2004. **131**(5): p. 573-6.
169. Sun, W., et al., *Functionally distinct subsets of CD4(+) regulatory T cells in patients with laryngeal squamous cell carcinoma are indicative of immune deregulation and disease progression.* Oncol Rep, 2015. **33**(1): p. 354-62.
170. Huang, B., et al., *Gr-1+CD115+ immature myeloid suppressor cells mediate the development of tumor-induced T regulatory cells and T-cell anergy in tumor-bearing host.* Cancer Res, 2006. **66**(2): p. 1123-31.
171. Gabrilovich, D.I. and S. Nagaraj, *Myeloid-derived suppressor cells as regulators of the immune system.* Nat Rev Immunol, 2009. **9**(3): p. 162-74.

172. Jordan, K.R., et al., *Immunosuppressive myeloid-derived suppressor cells are increased in splenocytes from cancer patients.* Cancer Immunol Immunother, 2017. **66**(4): p. 503-513.
173. Chikamatsu, K., et al., *Immunosuppressive activity of CD14+ HLA-DR- cells in squamous cell carcinoma of the head and neck.* Cancer Sci, 2012. **103**(6): p. 976-83.
174. Chikamatsu, K., et al., *Immunoregulatory properties of CD44+ cancer stem-like cells in squamous cell carcinoma of the head and neck.* Head Neck, 2011. **33**(2): p. 208-15.
175. Horinaka, A., et al., *Invariant NKT cells are resistant to circulating CD15+ myeloid-derived suppressor cells in patients with head and neck cancer.* Cancer Sci, 2016. **107**(3): p. 207-16.
176. Vasquez-Dunddel, D., et al., *STAT3 regulates arginase-I in myeloid-derived suppressor cells from cancer patients.* J Clin Invest, 2013. **123**(4): p. 1580-9.
177. Young, M.R., M. Newby, and H.T. Wepsic, *Hematopoiesis and suppressor bone marrow cells in mice bearing large metastatic Lewis lung carcinoma tumors.* Cancer Res, 1987. **47**(1): p. 100-5.
178. Buessow, S.C., R.D. Paul, and D.M. Lopez, *Influence of mammary tumor progression on phenotype and function of spleen and in situ lymphocytes in mice.* J Natl Cancer Inst, 1984. **73**(1): p. 249-55.
179. Seung, L.P., et al., *Synergy between T-cell immunity and inhibition of paracrine stimulation causes tumor rejection.* Proc Natl Acad Sci U S A, 1995. **92**(14): p. 6254-8.
180. Wu, A., et al., *Glioma cancer stem cells induce immunosuppressive macrophages/microglia.* Neuro Oncol, 2010. **12**(11): p. 1113-25.
181. Domenis, R., et al., *Systemic T Cells Immunosuppression of Glioma Stem Cell-Derived Exosomes Is Mediated by Monocytic Myeloid-Derived Suppressor Cells.* PLoS One, 2017. **12**(1): p. e0169932.
182. Yu, J., et al., *Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer.* J Immunol, 2013. **190**(7): p. 3783-97.
183. Solovjov, D.A., E. Pluskota, and E.F. Plow, *Distinct roles for the alpha and beta subunits in the functions of integrin alphaMbeta2.* J Biol Chem, 2005. **280**(2): p. 1336-45.
184. Bronte, V., et al., *Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards.* Nat Commun, 2016. **7**: p. 12150.
185. Najjar, Y.G., et al., *Myeloid-Derived Suppressor Cell Subset Accumulation in Renal Cell Carcinoma Parenchyma Is Associated with Intratumoral*

Expression of IL1beta, IL8, CXCL5, and Mip-1alpha. Clin Cancer Res, 2016.
186. Stanojevic, I., et al., *A subpopulation that may correspond to granulocytic myeloid-derived suppressor cells reflects the clinical stage and progression of cutaneous melanoma.* Int Immunol, 2016. **28**(2): p. 87-97.
187. Shi, H., et al., *Recruited monocytic myeloid-derived suppressor cells promote the arrest of tumor cells in the premetastatic niche through an IL-1beta-mediated increase in E-selectin expression.* Int J Cancer, 2017. **140**(6): p. 1370-1383.
188. Karakasheva, T.A., et al., *CD38-Expressing Myeloid-Derived Suppressor Cells Promote Tumor Growth in a Murine Model of Esophageal Cancer.* Cancer Res, 2015. **75**(19): p. 4074-85.
189. Gong, L., et al., *Tumor necrosis factor links chronic obstructive pulmonary disease and K-ras mutant lung cancer through induction of an immunosuppressive pro-tumor microenvironment.* Oncoimmunology, 2016. **5**(10): p. e1229724.
190. Gallina, G., et al., *Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells.* J Clin Invest, 2006. **116**(10): p. 2777-90.
191. Kryczek, I., et al., *B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma.* J Exp Med, 2006. **203**(4): p. 871-81.
192. Reeves, J.P., et al., *Anti-Leu3a induces combining site-related anti-idiotypic antibody without inducing anti-HIV activity.* AIDS Res Hum Retroviruses, 1991. **7**(1): p. 55-63.
193. Reys, L.L., *[Allocation of medical resources to intensive care units].* Acta Med Port, 1991. **4**(1): p. 23-6.
194. Ba, H., et al., *Transmembrane tumor necrosis factor-alpha promotes the recruitment of MDSCs to tumor tissue by upregulating CXCR4 expression via TNFR2.* Int Immunopharmacol, 2017. **44**: p. 143-152.
195. Zhang, H., et al., *CXCL2/MIF-CXCR2 signaling promotes the recruitment of myeloid-derived suppressor cells and is correlated with prognosis in bladder cancer.* Oncogene, 2016.
196. Chang, A.L., et al., *CCL2 Produced by the Glioma Microenvironment Is Essential for the Recruitment of Regulatory T Cells and Myeloid-Derived Suppressor Cells.* Cancer Res, 2016. **76**(19): p. 5671-5682.
197. Chiu, D.K., et al., *Hypoxia induces myeloid-derived suppressor cell recruitment to hepatocellular carcinoma through chemokine (C-C motif) ligand 26.* Hepatology, 2016. **64**(3): p. 797-813.
198. Inamoto, S., et al., *Loss of SMAD4 Promotes Colorectal Cancer Progression by Accumulation of Myeloid-Derived Suppressor Cells*

through the CCL15-CCR1 Chemokine Axis. Clin Cancer Res, 2016. **22**(2): p. 492-501.
199. Alfaro, C., et al., *Tumor-Produced Interleukin-8 Attracts Human Myeloid-Derived Suppressor Cells and Elicits Extrusion of Neutrophil Extracellular Traps (NETs).* Clin Cancer Res, 2016. **22**(15): p. 3924-36.
200. Vrakas, C.N., et al., *The Measure of DAMPs and a role for S100A8 in recruiting suppressor cells in breast cancer lung metastasis.* Immunol Invest, 2015. **44**(2): p. 174-88.
201. Ilkovitch, D., R. Carrio, and D.M. Lopez, *uPA and uPA-receptor are involved in cancer-associated myeloid-derived suppressor cell accumulation.* Anticancer Res, 2012. **32**(10): p. 4263-70.
202. Zhang, L., et al., *Cigarette Smoke Mediates Nuclear to Cytoplasmic Trafficking of Transcriptional Inhibitor Kaiso through MUC1 and P120-Catenin.* Am J Pathol, 2016. **186**(12): p. 3146-3159.
203. Lehman, H.L., et al., *p120-catenin down-regulation and epidermal growth factor receptor overexpression results in a transformed epithelium that mimics esophageal squamous cell carcinoma.* Am J Pathol, 2015. **185**(1): p. 240-51.
204. Bronte, V., et al., *Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells.* Blood, 2000. **96**(12): p. 3838-46.
205. Serafini, P., et al., *High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells.* Cancer Res, 2004. **64**(17): p. 6337-43.
206. Sauer, H., M. Wartenberg, and J. Hescheler, *Reactive oxygen species as intracellular messengers during cell growth and differentiation.* Cell Physiol Biochem, 2001. **11**(4): p. 173-86.
207. Dolgorukov, M.I., *[Late results of the surgical treatment of cancer of the proximal portion of the stomach].* Klin Khir, 1978(5): p. 21-4.
208. Yang, R., et al., *CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells.* Cancer Res, 2006. **66**(13): p. 6807-15.
209. Pan, P.Y., et al., *Immune stimulatory receptor CD40 is required for T-cell suppression and T regulatory cell activation mediated by myeloid-derived suppressor cells in cancer.* Cancer Res, 2010. **70**(1): p. 99-108.
210. Umemura, N., et al., *Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics.* J Leukoc Biol, 2008. **83**(5): p. 1136-44.

211. Haile, L.A., et al., *CD49d is a new marker for distinct myeloid-derived suppressor cell subpopulations in mice.* J Immunol, 2010. **185**(1): p. 203-10.
212. Youn, J.I., et al., *Subsets of myeloid-derived suppressor cells in tumor-bearing mice.* J Immunol, 2008. **181**(8): p. 5791-802.
213. Zhang, G., et al., *A novel subset of B7-H3+CD14+HLA-DR-/low myeloid-derived suppressor cells are associated with progression of human NSCLC.* Oncoimmunology, 2015. **4**(2): p. e977164.
214. Bygrave, A.E., et al., *Spontaneous autoimmunity in 129 and C57BL/6 mice-implications for autoimmunity described in gene-targeted mice.* PLoS Biol, 2004. **2**(8): p. E243.
215. Colin S Poon, K.M.S., *Overview of the diagnosis and staging of head and neck cancer*, in *UpToDate*, M.E. Ross, Editor.: UpToDate, Waltham, MA. (Accessed on January 26, 2017.).
216. Ferlay, J., et al., *Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008.* Int J Cancer, 2010. **127**(12): p. 2893-917.
217. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer Statistics, 2017.* CA Cancer J Clin, 2017. **67**(1): p. 7-30.
218. Lehman, H.L. and D.B. Stairs, *Single and Multiple Gene Manipulations in Mouse Models of Human Cancer.* Cancer Growth Metastasis, 2015. **8**(Suppl 1): p. 1-15.
219. Opitz, O.G., et al., *A mouse model of human oral-esophageal cancer.* J Clin Invest, 2002. **110**(6): p. 761-9.
220. Lefebvre, C., et al., *Mutational Profile of Metastatic Breast Cancers: A Retrospective Analysis.* PLoS Med, 2016. **13**(12): p. e1002201.
221. Piboonniyom, S.O., et al., *Abrogation of the retinoblastoma tumor suppressor checkpoint during keratinocyte immortalization is not sufficient for induction of centrosome-mediated genomic instability.* Cancer Res, 2003. **63**(2): p. 476-83.
222. Andl, C.D., et al., *Epidermal growth factor receptor mediates increased cell proliferation, migration, and aggregation in esophageal keratinocytes in vitro and in vivo.* J Biol Chem, 2003. **278**(3): p. 1824-30.
223. Kalabis, J., et al., *Isolation and characterization of mouse and human esophageal epithelial cells in 3D organotypic culture.* Nat Protoc, 2012. **7**(2): p. 235-46.
224. Toth, M., A. Sohail, and R. Fridman, *Assessment of gelatinases (MMP-2 and MMP-9) by gelatin zymography.* Methods Mol Biol, 2012. **878**: p. 121-35.
225. Ishiyama, N., et al., *Dynamic and static interactions between p120 catenin and E-cadherin regulate the stability of cell-cell adhesion.* Cell, 2010. **141**(1): p. 117-28.

226. Hao, Y., et al., *Gain of interaction with IRS1 by p110alpha-helical domain mutants is crucial for their oncogenic functions.* Cancer Cell, 2013. **23**(5): p. 583-93.
227. Mori, S., et al., *Anchorage-independent cell growth signature identifies tumors with metastatic potential.* Oncogene, 2009. **28**(31): p. 2796-805.
228. Du, L., et al., *Overexpression of PIK3CA in murine head and neck epithelium drives tumor invasion and metastasis through PDK1 and enhanced TGFbeta signaling.* Oncogene, 2016. **35**(35): p. 4641-52.
229. Estilo, C.L., et al., *The role of novel oncogenes squamous cell carcinoma-related oncogene and phosphatidylinositol 3-kinase p110alpha in squamous cell carcinoma of the oral tongue.* Clin Cancer Res, 2003. **9**(6): p. 2300-6.
230. Soto, E., et al., *p120 catenin induces opposing effects on tumor cell growth depending on E-cadherin expression.* J Cell Biol, 2008. **183**(4): p. 737-49.
231. Schackmann, R.C., et al., *Cytosolic p120-catenin regulates growth of metastatic lobular carcinoma through Rock1-mediated anoikis resistance.* J Clin Invest, 2011. **121**(8): p. 3176-88.
232. Gymnopoulos, M., M.A. Elsliger, and P.K. Vogt, *Rare cancer-specific mutations in PIK3CA show gain of function.* Proc Natl Acad Sci U S A, 2007. **104**(13): p. 5569-74.
233. Henken, F.E., et al., *PIK3CA-mediated PI3-kinase signalling is essential for HPV-induced transformation in vitro.* Mol Cancer, 2011. **10**: p. 71.
234. Zhong, C., et al., *P120ctn may participate in epithelial-mesenchymal transition in OSCC.* Indian J Cancer, 2016. **53**(1): p. 20-4.
235. Hart, J.R., et al., *Essential role of Stat3 in PI3K-induced oncogenic transformation.* Proc Natl Acad Sci U S A, 2011. **108**(32): p. 13247-52.
236. Kaptein, A., V. Paillard, and M. Saunders, *Dominant negative stat3 mutant inhibits interleukin-6-induced Jak-STAT signal transduction.* J Biol Chem, 1996. **271**(11): p. 5961-4.
237. Wheeler, S.E., et al., *Epidermal growth factor receptor variant III mediates head and neck cancer cell invasion via STAT3 activation.* Oncogene, 2010. **29**(37): p. 5135-45.
238. Ekblad, L., et al., *Cell-line-specific stimulation of tumor cell aggressiveness by wound healing factors - a central role for STAT3.* BMC Cancer, 2013. **13**: p. 33.
239. Thumkeo, D., S. Watanabe, and S. Narumiya, *Physiological roles of Rho and Rho effectors in mammals.* Eur J Cell Biol, 2013. **92**(10-11): p. 303-15.
240. Braun, U., et al., *Purification of the 22 kDa protein substrate of botulinum ADP-ribosyltransferase C3 from porcine brain cytosol and its*

characterization as a GTP-binding protein highly homologous to the rho gene product. FEBS Lett, 1989. **243**(1): p. 70-6.
241. Taulet, N., et al., *N-cadherin/p120 catenin association at cell-cell contacts occurs in cholesterol-rich membrane domains and is required for RhoA activation and myogenesis.* J Biol Chem, 2009. **284**(34): p. 23137-45.
242. Bonelli, M.A., et al., *Inhibition of PI3K Pathway Reduces Invasiveness and Epithelial-to-Mesenchymal Transition in Squamous Lung Cancer Cell Lines Harboring PIK3CA Gene Alterations.* Mol Cancer Ther, 2015. **14**(8): p. 1916-27.
243. Liu, J., et al., *PI3K/Akt-dependent phosphorylation of GSK3beta and activation of RhoA regulate Wnt5a-induced gastric cancer cell migration.* Cell Signal, 2013. **25**(2): p. 447-56.
244. Cao, X., et al., *A phosphorylation switch controls the spatiotemporal activation of Rho GTPases in directional cell migration.* Nat Commun, 2015. **6**: p. 7721.
245. Franke, T.F., et al., *Direct regulation of the Akt proto-oncogene product by phosphatidylinositol-3,4-bisphosphate.* Science, 1997. **275**(5300): p. 665-8.
246. Brazil, D.P., J. Park, and B.A. Hemmings, *PKB binding proteins. Getting in on the Akt.* Cell, 2002. **111**(3): p. 293-303.
247. Leaner, V.D., et al., *Inhibition of AP-1 transcriptional activity blocks the migration, invasion, and experimental metastasis of murine osteosarcoma.* Am J Pathol, 2009. **174**(1): p. 265-75.
248. Tanaka, T., et al., *Selective inhibition of nuclear factor-kappaB by nuclear factor-kappaB essential modulator-binding domain peptide suppresses the metastasis of highly metastatic oral squamous cell carcinoma.* Cancer Sci, 2012. **103**(3): p. 455-63.
249. Okawa, T., et al., *The functional interplay between EGFR overexpression, hTERT activation, and p53 mutation in esophageal epithelial cells with activation of stromal fibroblasts induces tumor development, invasion, and differentiation.* Genes Dev, 2007. **21**(21): p. 2788-803.
250. Unger, M. and V.M. Weaver, *The tissue microenvironment as an epigenetic tumor modifier.* Methods Mol Biol, 2003. **223**: p. 315-47.
251. Talvinen, K., et al., *Altered expression of p120catenin predicts poor outcome in invasive breast cancer.* J Cancer Res Clin Oncol, 2010. **136**(9): p. 1377-87.
252. Fei, Y., et al., *Expression and clinical significance of p120 catenin mRNA and protein in pancreatic carcinoma.* Bosn J Basic Med Sci, 2009. **9**(3): p. 191-7.

253. Christensen, E., et al., *Liquid Biopsy Analysis of FGFR3 and PIK3CA Hotspot Mutations for Disease Surveillance in Bladder Cancer.* Eur Urol, 2017.
254. Song, Z., X. Yu, and Y. Zhang, *Mutation and prognostic analyses of PIK3CA in patients with completely resected lung adenocarcinoma.* Cancer Med, 2016. **5**(10): p. 2694-2700.
255. Cacheux, W., et al., *Mutational analysis of anal cancers demonstrates frequent PIK3CA mutations associated with poor outcome after salvage abdominoperineal resection.* Br J Cancer, 2016. **114**(12): p. 1387-94.
256. Mei, Z.B., et al., *Prognostic role of tumor PIK3CA mutation in colorectal cancer: a systematic review and meta-analysis.* Ann Oncol, 2016. **27**(10): p. 1836-48.
257. Wu, S., et al., *PIK3CA mutation is associated with poor survival among patients with metastatic colorectal cancer following anti-EGFR monoclonal antibody therapy: a meta-analysis.* J Cancer Res Clin Oncol, 2013. **139**(5): p. 891-900.
258. Ye, H., et al., *[Expression of E-cadherin and P(120ctn) in nasopharyngeal carcinoma and their relationship].* Lin Chung Er Bi Yan Hou Tou Jing Wai Ke Za Zhi, 2009. **23**(14): p. 646-8.
259. Ma, L.W., et al., *Phosphorylated p120-catenin expression has predictive value for oral cancer progression.* J Clin Pathol, 2012. **65**(4): p. 315-9.
260. Sasaya, K., et al., *Concomitant loss of p120-catenin and beta-catenin membrane expression and oral carcinoma progression with E-cadherin reduction.* PLoS One, 2013. **8**(8): p. e69777.
261. Brauswetter, D., et al., *Copy number gain of PIK3CA and MET is associated with poor prognosis in head and neck squamous cell carcinoma.* Virchows Arch, 2016. **468**(5): p. 579-87.
262. O'Donnell, J.J., 3rd, et al., *Loss of p120 catenin upregulates transcription of pro-inflammatory adhesion molecules in human endothelial cells.* Microvasc Res, 2011. **82**(2): p. 105-12.
263. Stott-Miller, M., et al., *Tumor and salivary matrix metalloproteinase levels are strong diagnostic markers of oral squamous cell carcinoma.* Cancer Epidemiol Biomarkers Prev, 2011. **20**(12): p. 2628-36.
264. Debidda, M., et al., *A role of STAT3 in Rho GTPase-regulated cell migration and proliferation.* J Biol Chem, 2005. **280**(17): p. 17275-85.
265. Arulanandam, R., et al., *Activated Rac1 requires gp130 for Stat3 activation, cell proliferation and migration.* Exp Cell Res, 2010. **316**(5): p. 875-86.
266. Li, T.F., et al., *p120-catenin participates in the progress of gastric cancer through regulating the Rac1 and Pak1 signaling pathway.* Oncol Rep, 2015. **34**(5): p. 2357-64.

267. Liu, Y., et al., *Ablation of p120-catenin enhances invasion and metastasis of human lung cancer cells.* Cancer Sci, 2009. **100**(3): p. 441-8.
268. Wu, C.Y., et al., *PI3K regulation of RAC1 is required for KRAS-induced pancreatic tumorigenesis in mice.* Gastroenterology, 2014. **147**(6): p. 1405-16 e7.
269. Cantrell, D.A., *Phosphoinositide 3-kinase signalling pathways.* J Cell Sci, 2001. **114**(Pt 8): p. 1439-45.
270. Wallin, J.J., et al., *Active PI3K pathway causes an invasive phenotype which can be reversed or promoted by blocking the pathway at divergent nodes.* PLoS One, 2012. **7**(5): p. e36402.
271. Juvin, V., et al., *Signaling via class IA Phosphoinositide 3-kinases (PI3K) in human, breast-derived cell lines.* PLoS One, 2013. **8**(10): p. e75045.
272. Schackmann, R.C., et al., *p120-catenin in cancer - mechanisms, models and opportunities for intervention.* J Cell Sci, 2013. **126**(Pt 16): p. 3515-25.
273. Airola, K., et al., *Expression of collagenases-1 and -3 and their inhibitors TIMP-1 and -3 correlates with the level of invasion in malignant melanomas.* Br J Cancer, 1999. **80**(5-6): p. 733-43.
274. Murray, G.I., et al., *Matrix metalloproteinase-1 is associated with poor prognosis in colorectal cancer.* Nat Med, 1996. **2**(4): p. 461-2.
275. Murray, G.I., et al., *Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer.* J Pathol, 1998. **185**(3): p. 256-61.
276. Inoue, T., et al., *Matrix metalloproteinase-1 expression is a prognostic factor for patients with advanced gastric cancer.* Int J Mol Med, 1999. **4**(1): p. 73-7.
277. Nakopoulou, L., et al., *Matrix metalloproteinase-1 and -3 in breast cancer: correlation with progesterone receptors and other clinicopathologic features.* Hum Pathol, 1999. **30**(4): p. 436-42.
278. Nishioka, Y., et al., *A single nucleotide polymorphism in the matrix metalloproteinase-1 promoter in endometrial carcinomas.* Jpn J Cancer Res, 2000. **91**(6): p. 612-5.
279. Korem, S., M.B. Resnick, and Z. Kraiem, *Similar and divergent patterns in the regulation of matrix metalloproteinase-1 (MMP-1) and tissue inhibitor of MMP-1 gene expression in benign and malignant human thyroid cells.* J Clin Endocrinol Metab, 1999. **84**(9): p. 3322-7.
280. Liu, M., et al., *MMP1 promotes tumor growth and metastasis in esophageal squamous cell carcinoma.* Cancer Lett, 2016. **377**(1): p. 97-104.
281. Bergmann, C., et al., *T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease.* Clin Cancer Res, 2008. **14**(12): p. 3706-15.

282. Galdiero, M.R., et al., *Tumor associated macrophages and neutrophils in cancer.* Immunobiology, 2013. **218**(11): p. 1402-10.
283. Hutchinson, J.A., et al., *Cutting Edge: Immunological consequences and trafficking of human regulatory macrophages administered to renal transplant recipients.* J Immunol, 2011. **187**(5): p. 2072-8.
284. Garcia, M.R., et al., *Monocytic suppressive cells mediate cardiovascular transplantation tolerance in mice.* J Clin Invest, 2010. **120**(7): p. 2486-96.
285. Karen Hardinger, C.C.M. *Pharmacology of cyclosporine and tacrolimus*. 2016 3-29-17]; Available from: https://www.uptodate.com/contents/pharmacology-of-cyclosporine-and-tacrolimus?source=search_result&search=cyclosporine%20toxicity&selectedTitle=6~148.
286. Fan, H.Z., et al., *Passive transfer of lipopolysaccharide-derived myeloid-derived suppressor cells inhibits asthma-related airway inflammation.* Eur Rev Med Pharmacol Sci, 2015. **19**(21): p. 4171-81.
287. Arora, M., et al., *TLR4/MyD88-induced CD11b+Gr-1 int F4/80+ non-migratory myeloid cells suppress Th2 effector function in the lung.* Mucosal Immunol, 2010. **3**(6): p. 578-93.
288. Braun-Fahrlander, C., et al., *Environmental exposure to endotoxin and its relation to asthma in school-age children.* N Engl J Med, 2002. **347**(12): p. 869-77.
289. Ozao-Choy, J., et al., *The novel role of tyrosine kinase inhibitor in the reversal of immune suppression and modulation of tumor microenvironment for immune-based cancer therapies.* Cancer Res, 2009. **69**(6): p. 2514-22.
290. Choong, N.W., et al., *Phase II study of sunitinib malate in head and neck squamous cell carcinoma.* Invest New Drugs, 2010. **28**(5): p. 677-83.
291. Tong, C.C., et al., *Phase II trial of concurrent sunitinib and image-guided radiotherapy for oligometastases.* PLoS One, 2012. **7**(6): p. e36979.
292. Davis, R.J., et al., *Anti-PD-L1 Efficacy Can Be Enhanced by Inhibition of Myeloid-Derived Suppressor Cells with a Selective Inhibitor of PI3Kdelta/gamma.* Cancer Res, 2017. **77**(10): p. 2607-2619.
293. Elkin, M. and I. Vlodavsky, *Tail vein assay of cancer metastasis.* Curr Protoc Cell Biol, 2001. **Chapter 19**: p. Unit 19 2.
294. Hernandez, L., et al., *In vivo assay for tumor cell invasion.* Methods Mol Biol, 2009. **571**: p. 227-38.
295. Tran, N.L., et al., *N-Cadherin expression in human prostate carcinoma cell lines. An epithelial-mesenchymal transformation mediating adhesion withStromal cells.* Am J Pathol, 1999. **155**(3): p. 787-98.

296. Liu, Y., et al., *P120-catenin isoforms 1A and 3A differently affect invasion and proliferation of lung cancer cells.* Exp Cell Res, 2009. **315**(5): p. 890-8.
297. Corsino, P.E., et al., *Mammary tumors initiated by constitutive Cdk2 activation contain an invasive basal-like component.* Neoplasia, 2008. **10**(11): p. 1240-52.
298. Zheng, S., et al., *PIK3CA promotes proliferation and motility but is unassociated with lymph node metastasis or prognosis in esophageal squamous cell carcinoma.* Hum Pathol, 2016. **53**: p. 121-9.
299. Bhagirath, D., et al., *Mutant PIK3CA Induces EMT in a Cell Type Specific Manner.* PLoS One, 2016. **11**(12): p. e0167064.
300. Arjumand, W., et al., *Phosphatidyl inositol-3 kinase (PIK3CA) E545K mutation confers cisplatin resistance and a migratory phenotype in cervical cancer cells.* Oncotarget, 2016. **7**(50): p. 82424-82439.
301. Zhao, S., et al., *The E545K mutation of PIK3CA promotes gallbladder carcinoma progression through enhanced binding to EGFR.* J Exp Clin Cancer Res, 2016. **35**(1): p. 97.
302. Gualtero, D.F. and A. Suarez Castillo, *Biomarkers in saliva for the detection of oral squamous cell carcinoma and their potential use for early diagnosis: a systematic review.* Acta Odontol Scand, 2016. **74**(3): p. 170-7.
303. Coussens, L.M., B. Fingleton, and L.M. Matrisian, *Matrix metalloproteinase inhibitors and cancer: trials and tribulations.* Science, 2002. **295**(5564): p. 2387-92.
304. Bergers, G., et al., *Effects of angiogenesis inhibitors on multistage carcinogenesis in mice.* Science, 1999. **284**(5415): p. 808-12.
305. Vermorken, J.B., et al., *Platinum-based chemotherapy plus cetuximab in head and neck cancer.* N Engl J Med, 2008. **359**(11): p. 1116-27.
306. Ellerbroek, S.M., et al., *Phosphatidylinositol 3-kinase activity in epidermal growth factor-stimulated matrix metalloproteinase-9 production and cell surface association.* Cancer Res, 2001. **61**(5): p. 1855-61.
307. Thant, A.A., et al., *Fibronectin activates matrix metalloproteinase-9 secretion via the MEK1-MAPK and the PI3K-Akt pathways in ovarian cancer cells.* Clin Exp Metastasis, 2000. **18**(5): p. 423-8.
308. Zahradka, P., et al., *Activation of MMP-2 in response to vascular injury is mediated by phosphatidylinositol 3-kinase-dependent expression of MT1-MMP.* Am J Physiol Heart Circ Physiol, 2004. **287**(6): p. H2861-70.
309. Ispanovic, E. and T.L. Haas, *JNK and PI3K differentially regulate MMP-2 and MT1-MMP mRNA and protein in response to actin cytoskeleton reorganization in endothelial cells.* Am J Physiol Cell Physiol, 2006. **291**(4): p. C579-88.

310. Clancy, C., *The effective health care program: evidence-based medicine joins the consumer age.* MedGenMed, 2006. **8**(1): p. 56.
311. Litherland, G.J., et al., *Synergistic collagenase expression and cartilage collagenolysis are phosphatidylinositol 3-kinase/Akt signaling-dependent.* J Biol Chem, 2008. **283**(21): p. 14221-9.
312. Ispanovic, E., D. Serio, and T.L. Haas, *Cdc42 and RhoA have opposing roles in regulating membrane type 1-matrix metalloproteinase localization and matrix metalloproteinase-2 activation.* Am J Physiol Cell Physiol, 2008. **295**(3): p. C600-10.
313. Zhang, W., et al., *Fibroblast-derived MT1-MMP promotes tumor progression in vitro and in vivo.* BMC Cancer, 2006. **6**: p. 52.
314. Xie, Z., et al., *3-phosphoinositide-dependent protein kinase-1 (PDK1) promotes invasion and activation of matrix metalloproteinases.* BMC Cancer, 2006. **6**: p. 77.
315. Garrett, J.P., et al., *Regulation of endothelial barrier function by p120-cateninVE-cadherin interaction.* Mol Biol Cell, 2017. **28**(1): p. 85-97.
316. Wang, X., et al., *Perfluorooctane sulfonate triggers tight junction "opening" in brain endothelial cells via phosphatidylinositol 3-kinase.* Biochem Biophys Res Commun, 2011. **410**(2): p. 258-63.
317. Cain, R.J., B. Vanhaesebroeck, and A.J. Ridley, *The PI3K p110alpha isoform regulates endothelial adherens junctions via Pyk2 and Rac1.* J Cell Biol, 2010. **188**(6): p. 863-76.
318. Luks, V.L., et al., *Lymphatic and other vascular malformative/overgrowth disorders are caused by somatic mutations in PIK3CA.* J Pediatr, 2015. **166**(4): p. 1048-54 e1-5.
319. Wang, Z.G., et al., *bFGF Protects Against Blood-Brain Barrier Damage Through Junction Protein Regulation via PI3K-Akt-Rac1 Pathway Following Traumatic Brain Injury.* Mol Neurobiol, 2016. **53**(10): p. 7298-7311.
320. Rubio, M.E., et al., *Assembly of the N-cadherin complex during synapse formation involves uncoupling of p120-catenin and association with presenilin 1.* Mol Cell Neurosci, 2005. **30**(4): p. 611-23.
321. Lee, C.C., C.C. Huang, and K.S. Hsu, *Insulin promotes dendritic spine and synapse formation by the PI3K/Akt/mTOR and Rac1 signaling pathways.* Neuropharmacology, 2011. **61**(4): p. 867-79.
322. Levitt, P. and D.B. Campbell, *The genetic and neurobiologic compass points toward common signaling dysfunctions in autism spectrum disorders.* J Clin Invest, 2009. **119**(4): p. 747-54.
323. Auerbach, B.D., E.K. Osterweil, and M.F. Bear, *Mutations causing syndromic autism define an axis of synaptic pathophysiology.* Nature, 2011. **480**(7375): p. 63-8.

324. Tonnies, E. and E. Trushina, *Oxidative Stress, Synaptic Dysfunction, and Alzheimer's Disease.* J Alzheimers Dis, 2017.
325. Zaltieri, M., et al., *alpha-synuclein and synapsin III cooperatively regulate synaptic function in dopamine neurons.* J Cell Sci, 2015. **128**(13): p. 2231-43.
326. Michaluk, P. and L. Kaczmarek, *Matrix metalloproteinase-9 in glutamate-dependent adult brain function and dysfunction.* Cell Death Differ, 2007. **14**(7): p. 1255-8.
327. Zeng, G.Q., et al., *High MMP-1, MMP-2, and MMP-9 protein levels in osteoarthritis.* Genet Mol Res, 2015. **14**(4): p. 14811-22.
328. Kurz, B., et al., *Pathomechanisms of cartilage destruction by mechanical injury.* Ann Anat, 2005. **187**(5-6): p. 473-85.
329. Rogers, N.K., et al., *Extra-cellular matrix proteins induce matrix metalloproteinase-1 (MMP-1) activity and increase airway smooth muscle contraction in asthma.* PLoS One, 2014. **9**(2): p. e90565.
330. Naveed, S.U., et al., *MMP-1 Activation Contributes to Airway Smooth Muscle Growth and Asthma Severity.* Am J Respir Crit Care Med, 2016.
331. Hsu, A.C., et al., *Targeting PI3K-p110alpha Suppresses Influenza Virus Infection in Chronic Obstructive Pulmonary Disease.* Am J Respir Crit Care Med, 2015. **191**(9): p. 1012-23.
332. Hu, R., et al., *MicroRNA-10a controls airway smooth muscle cell proliferation via direct targeting of the PI3 kinase pathway.* FASEB J, 2014. **28**(5): p. 2347-57.
333. Alexander, K., et al., *Levels of Matrix Metalloproteinases in Arthroplasty Patients and Their Correlation With Inflammatory and Thrombotic Activation Processes.* Clin Appl Thromb Hemost, 2016. **22**(5): p. 441-6.
334. Abd-Allah, S.H., et al., *Influence of Matrix metalloproteinase 1 and 3 genetic variations on susceptibility and severity of juvenile idiopathic arthritis.* IUBMB Life, 2015. **67**(12): p. 934-42.
335. Sauerschnig, M., et al., *Diverse expression of selected cytokines and proteinases in synovial fluid obtained from osteoarthritic and healthy human knee joints.* Eur J Med Res, 2014. **19**: p. 65.
336. Perez-Moreno, M., et al., *Loss of p120 catenin and links to mitotic alterations, inflammation, and skin cancer.* Proc Natl Acad Sci U S A, 2008. **105**(40): p. 15399-404.
337. Goren, I., et al., *A transgenic mouse model of inducible macrophage depletion: effects of diphtheria toxin-driven lysozyme M-specific cell lineage ablation on wound inflammatory, angiogenic, and contractive processes.* Am J Pathol, 2009. **175**(1): p. 132-47.
338. Weisser, S.B., N. van Rooijen, and L.M. Sly, *Depletion and reconstitution of macrophages in mice.* J Vis Exp, 2012(66): p. 4105.

339. Hochweller, K., et al., *A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells.* Eur J Immunol, 2008. **38**(10): p. 2776-83.
340. Satoskar, A.R., et al., *NK cell-deficient mice develop a Th1-like response but fail to mount an efficient antigen-specific IgG2a antibody response.* J Immunol, 1999. **163**(10): p. 5298-302.
341. Dianda, L., et al., *T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment.* Am J Pathol, 1997. **150**(1): p. 91-7.
342. Gaur, A., et al., *Amelioration of autoimmune encephalomyelitis by myelin basic protein synthetic peptide-induced anergy.* Science, 1992. **258**(5087): p. 1491-4.
343. Zhang, X., et al., *A Comparative Study of Fibronectin Cleavage by MMP-1, -3, -13, and -14.* Cartilage, 2012. **3**(3): p. 267-77.
344. Abdulghani, J. and W.S. El-Deiry, *TRAIL receptor signaling and therapeutics.* Expert Opin Ther Targets, 2010. **14**(10): p. 1091-108.
345. Cho, C., et al., *The fibronectin III-1 domain activates a PI3-Kinase/Akt signaling pathway leading to alphavbeta5 integrin activation and TRAIL resistance in human lung cancer cells.* BMC Cancer, 2016. **16**: p. 574.
346. Barilla, M.L. and S.E. Carsons, *Fibronectin fragments and their role in inflammatory arthritis.* Semin Arthritis Rheum, 2000. **29**(4): p. 252-65.
347. Rossnagl, S., et al., *EDA-Fibronectin Originating from Osteoblasts Inhibits the Immune Response against Cancer.* PLoS Biol, 2016. **14**(9): p. e1002562.
348. Ip, Y.T. and R.J. Davis, *Signal transduction by the c-Jun N-terminal kinase (JNK)--from inflammation to development.* Curr Opin Cell Biol, 1998. **10**(2): p. 205-19.
349. Huang, Z., D.P. Yan, and B.X. Ge, *JNK regulates cell migration through promotion of tyrosine phosphorylation of paxillin.* Cell Signal, 2008. **20**(11): p. 2002-12.
350. Zhu, G., et al., *An EGFR/PI3K/AKT axis promotes accumulation of the Rac1-GEF Tiam1 that is critical in EGFR-driven tumorigenesis.* Oncogene, 2015. **34**(49): p. 5971-82.
351. *Search Result: Transcription Factors Regulating Gene MMP1*. [cited 2017 3-29-17]; Available from: http://www.sabiosciences.com/chipqpcrsearch.php?species_id=0&nfactor=n&ninfo=n&ngene=n&B2=Search&src=genecard&factor=Over+200+TF&gene=MMP1.

Appendix

ELSEVIER LICENSE
TERMS AND CONDITIONS

Mar 27, 2017

This Agreement between Michal A Kidacki ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4073090766314
License date	Mar 20, 2017
Licensed Content Publisher	Elsevier
Licensed Content Publication	Current Opinion in Cell Biology
Licensed Content Title	Regulation of Rho GTPases by p120-catenin
Licensed Content Author	Panos Z Anastasiadis,Albert B Reynolds
Licensed Content Date	1 October 2001
Licensed Content Volume	13
Licensed Content Issue	5
Licensed Content Pages	7
Start Page	604
End Page	610
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Original figure numbers	fig.2
Title of your thesis/dissertation	Cooperation of p120-catenin Downregulation and PIK3CA Mutations in Progression of Head and Neck Squamous Cell Carcinoma
Expected completion date	Apr 2017
Estimated size (number of pages)	150
Elsevier VAT number	GB 494 6272 12
Requestor Location	Michal A Kidacki 500 University Dr HERSHEY, PA 17033 United States Attn: Michal A Kidacki
Publisher Tax ID	98-0397604
Total	**0.00 USD**
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms

and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world English rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.

Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.

17. **For journal authors:** the following clauses are applicable in addition to the above:

Preprints:

A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).

Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).

If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.

Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.

Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.

Policies for sharing publishing journal articles differ for subscription and gold open access articles:

Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.

Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.

Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.

Please refer to Elsevier's posting policy for further information.

18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation**: If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include

permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. Other Conditions:

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

ELSEVIER LICENSE
TERMS AND CONDITIONS

Mar 27, 2017

This Agreement between Michal A Kidacki ("You") and Elsevier ("Elsevier") consists of your license details and the terms and conditions provided by Elsevier and Copyright Clearance Center.

License Number	4073090947757
License date	Mar 20, 2017
Licensed Content Publisher	Elsevier
Licensed Content Publication	Biochimica et Biophysica Acta (BBA) - Molecular Cell Research
Licensed Content Title	Phosphorylation and isoform use in p120-catenin during development and tumorigenesis
Licensed Content Author	Ji Yeon Hong,Il-Hoan Oh,Pierre D. McCrea
Licensed Content Date	January 2016
Licensed Content Volume	1863
Licensed Content Issue	1
Licensed Content Pages	13
Start Page	102
End Page	114
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Original figure numbers	fig. 2.
Title of your thesis/dissertation	Cooperation of p120-catenin Downregulation and PIK3CA Mutations in Progression of Head and Neck Squamous Cell Carcinoma
Expected completion date	Apr 2017
Estimated size (number of pages)	150
Elsevier VAT number	GB 494 6272 12
Requestor Location	Michal A Kidacki 500 University Dr HERSHEY, PA 17033 United States Attn: Michal A Kidacki
Publisher Tax ID	98-0397604
Total	**0.00 USD**
Terms and Conditions	

INTRODUCTION

1. The publisher for this copyrighted material is Elsevier. By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the Billing and Payment terms and conditions established by Copyright Clearance Center, Inc. ("CCC"), at the time that you opened your Rightslink account and that are available at any time at http://myaccount.copyright.com).

GENERAL TERMS

2. Elsevier hereby grants you permission to reproduce the aforementioned material subject to the terms and conditions indicated.
3. Acknowledgement: If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source, permission must also be sought from that source. If such permission is not obtained then that material may not be included in your publication/copies. Suitable acknowledgement to the source must be made, either as a footnote or in a reference list at the end of your publication, as follows:
"Reprinted from Publication title, Vol /edition number, Author(s), Title of article / title of chapter, Pages No., Copyright (Year), with permission from Elsevier [OR APPLICABLE SOCIETY COPYRIGHT OWNER]." Also Lancet special credit - "Reprinted from The Lancet, Vol. number, Author(s), Title of article, Pages No., Copyright (Year), with permission from Elsevier."
4. Reproduction of this material is confined to the purpose and/or media for which permission is hereby given.
5. Altering/Modifying Material: Not Permitted. However figures and illustrations may be altered/adapted minimally to serve your work. Any other abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of Elsevier Ltd. (Please contact Elsevier at permissions@elsevier.com). No modifications can be made to any Lancet figures/tables and they must be reproduced in full.
6. If the permission fee for the requested use of our material is waived in this instance, please be advised that your future requests for Elsevier materials may attract a fee.
7. Reservation of Rights: Publisher reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.
8. License Contingent Upon Payment: While you may exercise the rights licensed immediately upon issuance of the license at the end of the licensing process for the transaction, provided that you have disclosed complete and accurate details of your proposed use, no license is finally effective unless and until full payment is received from you (either by publisher or by CCC) as provided in CCC's Billing and Payment terms and conditions. If full payment is not received on a timely basis, then any license preliminarily granted shall be deemed automatically revoked and shall be void as if never granted. Further, in the event that you breach any of these terms and conditions or any of CCC's Billing and Payment terms and conditions, the license is automatically revoked and shall be void as if never granted. Use of materials as described in a revoked license, as well as any use of the materials beyond the scope of an unrevoked license, may constitute copyright infringement and publisher reserves the right to take any and all action to protect its copyright in the materials.
9. Warranties: Publisher makes no representations or warranties with respect to the licensed material.
10. Indemnity: You hereby indemnify and agree to hold harmless publisher and CCC, and their respective officers, directors, employees and agents, from and against any and all claims arising out of your use of the licensed material other than as specifically authorized pursuant to this license.
11. No Transfer of License: This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without publisher's written permission.
12. No Amendment Except in Writing: This license may not be amended except in a writing signed by both parties (or, in the case of publisher, by CCC on publisher's behalf).
13. Objection to Contrary Terms: Publisher hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and publisher (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control.
14. Revocation: Elsevier or Copyright Clearance Center may deny the permissions described in this License at their sole discretion, for any reason or no reason, with a full refund payable to you. Notice of such denial will be made using the contact information provided by you. Failure to receive such notice will not alter or invalidate the denial. In no event will Elsevier or Copyright Clearance Center be responsible or liable for any costs, expenses or damage incurred by you as a result of a denial of your permission request, other than a refund of the amount(s) paid by you to Elsevier and/or Copyright Clearance Center for denied permissions.

LIMITED LICENSE

The following terms and conditions apply only to specific license types:
15. **Translation**: This permission is granted for non-exclusive world **English** rights only unless your license was granted for translation rights. If you licensed translation rights you may only translate this content into the languages you requested. A professional translator must perform all translations and reproduce the content word for word preserving the integrity of the article.
16. **Posting licensed content on any Website**: The following terms and conditions apply as follows: Licensing material from an Elsevier journal: All content posted to the web site must maintain the copyright information line on the bottom of each image; A hyper-text must be included to the Homepage of the journal from which you are licensing at http://www.sciencedirect.com/science/journal/xxxxx or the Elsevier homepage for books at http://www.elsevier.com; Central

Storage: This license does not include permission for a scanned version of the material to be stored in a central repository such as that provided by Heron/XanEdu.
Licensing material from an Elsevier book: A hyper-text link must be included to the Elsevier homepage at http://www.elsevier.com . All content posted to the web site must maintain the copyright information line on the bottom of each image.

Posting licensed content on Electronic reserve: In addition to the above the following clauses are applicable: The web site must be password-protected and made available only to bona fide students registered on a relevant course. This permission is granted for 1 year only. You may obtain a new license for future website posting.
17. **For journal authors:** the following clauses are applicable in addition to the above:
Preprints:
A preprint is an author's own write-up of research results and analysis, it has not been peer-reviewed, nor has it had any other value added to it by a publisher (such as formatting, copyright, technical enhancement etc.).
Authors can share their preprints anywhere at any time. Preprints should not be added to or enhanced in any way in order to appear more like, or to substitute for, the final versions of articles however authors can update their preprints on arXiv or RePEc with their Accepted Author Manuscript (see below).
If accepted for publication, we encourage authors to link from the preprint to their formal publication via its DOI. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help users to find, access, cite and use the best available version. Please note that Cell Press, The Lancet and some society-owned have different preprint policies. Information on these policies is available on the journal homepage.
Accepted Author Manuscripts: An accepted author manuscript is the manuscript of an article that has been accepted for publication and which typically includes author-incorporated changes suggested during submission, peer review and editor-author communications.
Authors can share their accepted author manuscript:

- immediately
  - via their non-commercial person homepage or blog
  - by updating a preprint in arXiv or RePEc with the accepted manuscript
  - via their research institute or institutional repository for internal institutional uses or as part of an invitation-only research collaboration work-group
  - directly by providing copies to their students or to research collaborators for their personal use
  - for private scholarly sharing as part of an invitation-only work group on commercial sites with which Elsevier has an agreement
- After the embargo period
  - via non-commercial hosting platforms such as their institutional repository
  - via commercial sites with which Elsevier has an agreement

In all cases accepted manuscripts should:

- link to the formal publication via its DOI
- bear a CC-BY-NC-ND license - this is easy to do
- if aggregated with other manuscripts, for example in a repository or other site, be shared in alignment with our hosting policy not be added to or enhanced in any way to appear more like, or to substitute for, the published journal article.

Published journal article (JPA): A published journal article (PJA) is the definitive final record of published research that appears or will appear in the journal and embodies all value-adding publishing activities including peer review co-ordination, copy-editing, formatting, (if relevant) pagination and online enrichment.
Policies for sharing publishing journal articles differ for subscription and gold open access articles:
Subscription Articles: If you are an author, please share a link to your article rather than the full-text. Millions of researchers have access to the formal publications on ScienceDirect, and so links will help your users to find, access, cite, and use the best available version.
Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.
If you are affiliated with a library that subscribes to ScienceDirect you have additional private sharing rights for others' research accessed under that agreement. This includes use for classroom teaching and internal training at the institution (including use in course packs and courseware programs), and inclusion of the article for grant funding purposes.
Gold Open Access Articles: May be shared according to the author-selected end-user license and should contain a CrossMark logo, the end user license, and a DOI link to the formal publication on ScienceDirect.
Please refer to Elsevier's posting policy for further information.
18. **For book authors** the following clauses are applicable in addition to the above: Authors are permitted to place a brief summary of their work online only. You are not allowed to download and post the published electronic version of your chapter, nor may you scan the printed edition to create an electronic version. **Posting to a repository:** Authors are permitted to post a summary of their chapter only in their institution's repository.

19. **Thesis/Dissertation:** If your license is for use in a thesis/dissertation your thesis may be submitted to your institution in either print or electronic form. Should your thesis be published commercially, please reapply for permission. These requirements include permission for the Library and Archives of Canada to supply single copies, on demand, of the complete thesis and include permission for Proquest/UMI to supply single copies, on demand, of the complete thesis. Should your thesis be published commercially, please reapply for permission. Theses and dissertations which contain embedded PJAs as part of the formal submission can be posted publicly by the awarding institution with DOI links back to the formal publications on ScienceDirect.

Elsevier Open Access Terms and Conditions

You can publish open access with Elsevier in hundreds of open access journals or in nearly 2000 established subscription journals that support open access publishing. Permitted third party re-use of these open access articles is defined by the author's choice of Creative Commons user license. See our open access license policy for more information.

Terms & Conditions applicable to all Open Access articles published with Elsevier:

Any reuse of the article must not represent the author as endorsing the adaptation of the article nor should the article be modified in such a way as to damage the author's honour or reputation. If any changes have been made, such changes must be clearly indicated.

The author(s) must be appropriately credited and we ask that you include the end user license and a DOI link to the formal publication on ScienceDirect.

If any part of the material to be used (for example, figures) has appeared in our publication with credit or acknowledgement to another source it is the responsibility of the user to ensure their reuse complies with the terms and conditions determined by the rights holder.

Additional Terms & Conditions applicable to each Creative Commons user license:

CC BY: The CC-BY license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article and to make commercial use of the Article (including reuse and/or resale of the Article by commercial entities), provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by/4.0.

CC BY NC SA: The CC BY-NC-SA license allows users to copy, to create extracts, abstracts and new works from the Article, to alter and revise the Article, provided this is not done for commercial purposes, and that the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, indicates if changes were made and the licensor is not represented as endorsing the use made of the work. Further, any new works must be made available on the same conditions. The full details of the license are available at http://creativecommons.org/licenses/by-nc-sa/4.0.

CC BY NC ND: The CC BY-NC-ND license allows users to copy and distribute the Article, provided this is not done for commercial purposes and further does not permit distribution of the Article if it is changed or edited in any way, and provided the user gives appropriate credit (with a link to the formal publication through the relevant DOI), provides a link to the license, and that the licensor is not represented as endorsing the use made of the work. The full details of the license are available at http://creativecommons.org/licenses/by-nc-nd/4.0. Any commercial reuse of Open Access articles published with a CC BY NC SA or CC BY NC ND license requires permission from Elsevier and will be subject to a fee.

Commercial reuse includes:

- Associating advertising with the full text of the Article
- Charging fees for document delivery or access
- Article aggregation
- Systematic distribution via e-mail lists or share buttons

Posting or linking by commercial companies for use by customers of those companies.

20. **Other Conditions:**

v1.9

Questions? customercare@copyright.com or +1-855-239-3415 (toll free in the US) or +1-978-646-2777.

Vita
Michal A. Kidacki

Education

2019 (in progress)	Doctor of Medicine (M.D.) The Pennsylvania State University College of Medicine: Hershey, PA
2017	Doctor of Philosophy (Ph.D.) in Biomedical Sciences The Pennsylvania State University College of Medicine: Hershey, PA
2009	Bachelor of Arts (B.A.) in Biology; Summa cum laude Rutgers University of New Jersey: Newark, NJ

Publications

Kidacki M, Lehman HL, Warrick JI, Stairs DB. p120-Catenin Downregulation and PIK3CA Mutations Cooperate to Induce Invasion through MMP1 in Head and Neck Squamous Cell Carcinoma. Molecular Cancer Research. I*n revision.*

Kidacki M, Lehman HL, Warrick JI, Stairs DB. (2015). Signaling Pathways Supporting Tumor Invasion in Head and Neck Squamous Cell Carcinoma. J Clin Exp Pathol, 5(227), 2161-0681.

Selected Abstracts

Kidacki M, Lehman HL, Warrick JI, Stairs DB. Role of p120ctn and PIK3CA in the migration of Head and Neck Squamous Cell Carcinoma. Oral presentation. 2016 Experimental Biology. April 2-6 San Diego, CA

Selected Awards/Honors

2015 MD/PhD Daniel Notterman Award
2016 MD/PhD Daniel Notterman Award
2016 MD/PhD Program Travel Award

Service

2012-2014 Co-chair of M.D./Ph.D. Program Recruiting Committee
2014-2016 Chair of the M.D./Ph.D. Program Seminar Committee
2014-2016 Student Representative to M.D./Ph.D. Program Steering Committee